RESEARCH REPORTS
Biological

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ABSTRACT

The human body displays central circadian rhythms of activity. Recent findings suggest that peripheral tissues, such as bone, possess their own circadian clocks. Studies have shown that osteocalcin protein levels oscillate over a 24-hour period, yet the specific skeletal sites involved and its transcriptional profile remain unknown. The current study aimed to test the hypothesis that peripheral circadian mechanisms regulate transcription driven by the osteocalcin promoter. Transgenic mice harboring the human osteocalcin promoter linked to a luciferase reporter gene were used. Mice of both genders and various ages were analyzed non-invasively at sequential times throughout 24-hour periods. Statistical analyses of luminescent signal intensity of osteogenic activity from multiple skeletal sites indicated a periodicity of ~ 24 hrs. The maxillomandibular complex displayed the most robust oscillatory pattern. These findings have implications for dental treatments in orthodontics and maxillofacial surgery, as well as for the mechanisms underlying bone remodeling in the maxillomandibular complex.

KEY WORDS: maxillomandibular complex, bioluminescence, circadian rhythm, osteocalcin, transgenic mice.

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Circadian Rhythm of Osteocalcin in the Maxillomandibular Complex

INTRODUCTION

The human body displays cyclic patterns of gene expression, hormone secretion, and behavioral activity, reflecting the 24-hour light/dark cycle. Recent studies indicate that tissues peripheral to the central nervous system contain their own, independent circadian clocks (Lowrey and Takahashi, 2004). The mRNAs encoding proteins associated with the circadian clock have been detected in peripheral tissues such as adipose tissue, liver, skeletal muscle, and testis (Zylka *et al.*, 1998; Panda *et al.*, 2002; Storch *et al.*, 2002; Ando *et al.*, 2005; Ptitsyn *et al.*, 2006; Zvonic *et al.*, 2006). Indeed, there is now *in vitro* evidence that peripheral "clocks" are present even within cultured cell lines, including bone-marrow-derived mesenchymal stem cells (Balsalobre *et al.*, 1998).

The circadian apparatus represents a self-contained transcriptional/translational feedback loop associated with a 24-hour oscillatory expression profile (Griffin *et al.*, 1999; Shearman *et al.*, 2000). Using transcriptomics (*i.e.*, expression profiling) and RT-PCR methods, we recently demonstrated the oscillatory expression profile of the mRNAs encoding the core components of the circadian apparatus in murine calvarial bone (Zvonic *et al.*, 2007). Other investigators have reported similar observations in the murine femur (Fu *et al.*, 2005). Recent circadian studies have tried to assess the effects of orthodontic and orthopedic forces applied to bone remodeling of the maxillomandibular complex. These studies showed a correlation between forces applied at rest times and accelerated bone remodeling (Lou *et al.*, 2000; Zheng *et al.*, 2003). Moreover, local and systemic osteocalcin expression has been shown to increase significantly when orthopedic force was applied to the mandible for 24 hrs a day *vs*. 12 hrs of daytime (Ye *et al.*, 2001).

A significant body of literature examining serum protein biomarkers supports a role for circadian mechanisms in bone metabolism (Gundberg *et al.*, 1985; Bollen *et al.*, 1995; Heshmati *et al.*, 1998; Srivastava *et al.*, 2001; Shao *et al.*, 2003; Fu *et al.*, 2005; Patel and Elefteriou, 2007). In human and preclinical animal studies, serum levels of proteins associated with bone metabolism (osteocalcin and alkaline phosphatase) all oscillated in a cosinor manner over a 24-hour period (Gundberg *et al.*, 1985; Bollen *et al.*, 1995; Heshmati *et al.*, 1998; Srivastava *et al.*, 2001; Shao *et al.*, 2003). However, the contributions of transcriptional mechanisms to the circadian control of serum protein biomarkers of bone metabolism remain relatively unexplored (Fu *et al.*, 2005; Zvonic *et al.*, 2007). To date, there is no specific model or any published study that investigated the physiological circadian rhythms related to bone remodeling and osteocalcin expression in the maxillomandibular complex.

In the current manuscript, we extend our transcriptomic observations (Zvonic *et al.*, 2007) by exploring the hypothesis that circadian mechanisms contribute to transcriptional regulation of the bone-specific gene, osteocalcin. We used a human osteocalcin promoter/luciferase reporter (hOC-Luc) transgenic murine model, previously validated *in vivo* for serial non-invasive bioluminescence imaging (Iris *et al.*, 2003).

MATERIALS & METHODS

Animal Studies

Transgenic Mouse Model

FVB/N transgenic mice were generated to harbor the luciferase gene under the control of the human osteocalcin promoter, as previously described (Clemens *et al.*, 1997). These mice express luciferase in osteogenic tissues. Wild-type FVB/N mice were used as controls. The Hebrew University Institutional Animal Care and Use Committee approved all the procedures used in this study and agreed that their care was consistent with the United States National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*.

Time Series Analysis and *in vivo* Bioluminescence Imaging

Intact mice of both genders, transgenic for the human osteocalcin promoter/luciferase reporter gene, were used at ages 1, 3, and 5 mos and 1.5 yrs (N = 5 for each gender and time-point). Mice were trained for a 12-hour light/dark cycle for a minimum of 2 wks prior to each study. Each animal was sequentially analyzed at times 0, 6, 12, 18, and 24 hrs of the light/dark cycle throughout a single 24-hour period.

In vivo bioluminescence imaging was performed as previously described (Iris *et al.*, 2003). Briefly, following anesthetization, each animal was given an intraperitoneal injection of beetle luciferin (Promega Corp., Madison, WI, USA) in phosphate-buffered saline (PBS) at 126 mg/kg body weight, and placed in a light-tight chamber. Photon emission was then integrated over a period of 2 min and recorded as pseudocolor images (Contag *et al.*, 1997; Iris *et al.*, 2003).

Quantitative analysis of luciferase expression was performed with the MetaImaging series 4.6 software (Molecular Devices, Downingtown, PA, USA), with a constant measurement field for all time-points. Results are presented in integrated luciferase units.

Seven skeletal sites were analyzed: calvaria, tail, maxillomandibular complex, carpals, and tarsals. (See detailed description of methods in the APPENDIX, under 'Animal Studies'.)

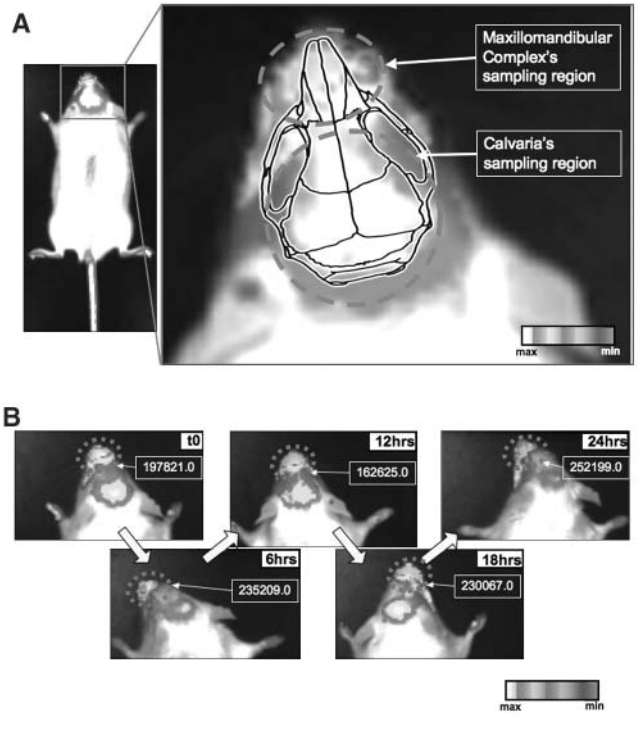

Figure 1. (A) *In vivo* bioluminescence imaging was conducted based on region sampling. A sketch of mouse skull anatomy was overlaid on a magnified area of an image acquired by a non-invasive bioluminescence imaging device. Data acquisition was based on region sampling, as can be displayed for maxillomandibular and calvarial regions. A similar approach was used for the analysis of other skeletal organs. **(B)** *In vivo* bioluminescence imaging of transgenic mouse at 5 time-points within a 24-hour cycle. Intact mice of both genders for the human osteocalcin promoter/luciferase reporter gene were used at ages 1, 3, and 5 mos and 1.5 yrs. N = 5 for each gender and time-point. Each animal was sequentially analyzed at T = 0, 6, 12, 18, and 24 hrs of the light/dark cycle throughout one 24-hour period. Boxed numbers represent signal intensity measured in Integrated Light Units (ILUs) for each sampling region.

Computational Methods

Computational methods included: phase assignment, spectral analysis, Fisher's g test, permuted time test (Pt test), autocorrelation, and data analysis pipeline. A detailed description of each is presented in the APPENDIX.

We determined the phase of expression for each individual animal by cosine-based analyses using multiple algorithms. The profiles from individual animals could be subdivided into 3 distinct phase cohorts, based on the acrophase (zenith) of expression: The phase cohort 1, with an acrophase of Zeitgeber Time (ZT) 6h, accounted for 68 % of the profiles (n = 186), while phases 2 and 3, with acrophases of ZT12h and ZT18h, included 20% (n = 54) and 12% (n = 33) of the profiles, respectively. (See detailed description of methods in the APPENDIX.) Zeitgeber Time 0 (ZT0) corresponds to the point during the day when the animal is first exposed to daylight, while ZT12 corresponds to the time of day when the animal is first exposed to darkness.

RESULTS

Osteocalcin promoter/luciferase transgenic mice were monitored individually for osteocalcin expression, based on *in vivo* bioluminescence, for a period of 24 hrs (a representative animal is displayed in Fig. 1). Based on previous experiments, which included the dissection of tissues followed by luciferase assay and RT-PCR, we were able to conclude that the OC-Luc transgenic mice showed osteogenic activity within the maxillomandibular complex, calvaria, tail, carpals, and tarsals (data not shown). This finding correlated with our bioluminescence imaging results (Fig. 1, Appendix Fig. 1). Because luciferase has a half-life of ~ 3 hrs in mammalian cells (Thompson *et al.*, 1991), it was feasible to monitor circadian mechanisms dynamically in transgenic mice. Analysis of our data showed no significant ascending trend in luminescent signal, and thus no significant luciferase accumulation between time-points.

Quantified results revealed a distinct oscillatory pattern of expression in the maxillomandibular complex (a representative group of 5-month-old female transgenic mice is presented in Fig. 2). For statistical purposes, the timeline of the raw bioluminescence data from the maxillomandibular complex for the entire group of animals in Phase cohort 1 was concatenated, sorted, and normalized for amplitude relative to ZT0 for each animal (Fig. 3A). Each sequential group of 5 datapoints corresponds to the bioluminescence signal collected from an individual animal. Analysis determined that neither age nor gender significantly

influenced the phase assignment (Fig. 3A). Overall, the maxillomandibular complex displayed the most robust oscillatory pattern. Statistical analyses indicated a periodicity of ~ 24 hrs in each of the individual skeletal sites. The magnitude of the luciferase amplitude and oscillation varied among skeletal sites in the following order: maxillomandibular complex > tarsals > carpals > calvaria > tail.

To estimate spectral density, we applied the Discrete Fourier Transform procedure (see APPENDIX), which yielded a periodogram, converting raw data from the time domain into the frequency domain, thereby highlighting the dominant rhythm. The periodograms displayed by the maxillomandibular complex of the Phase cohort 1 animals are given in Fig. 3B. The highest peak in this periodogram corresponds to the circadian frequency, *i.e.*, one complete cycle *per* one day of observation. The height of this dominant peak relative to all other peaks can be used to estimate the signal-to-noise ratio, and hence statistical confidence in the detection of circadian periodicity. In the current study, the phase-synchronized cohorts displayed a 24-hour circadian profile, as determined by a panel of 3 independent statistical tests. After scaling and smoothing procedures, less than 10% of the individual profiles did not achieve statistical significance for circadian oscillation, consistent with the conclusion reached by direct visual inspection of the raw data. The expression profiles for the remaining skeletal sites in the Phase cohort 1 animals were similar (Appendix Fig. 3). We also analyzed the signals as a function of age and gender. The majority of the results failed to support a significant difference in circadian rhythm dependent upon bone location, gender, or age (Fig. 4); the only exceptions occurred between male and female 18-month cohorts for calvaria and right carpals. These data are consistent with our previously published studies regarding the skeletal growth and development of rodents (Iris *et al.*, 2003).

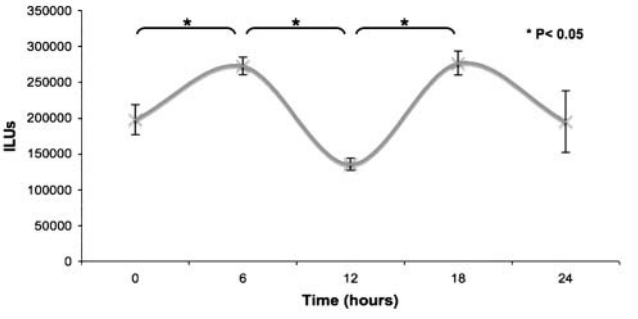

Figure 2. Quantified *in vivo* bioluminescence imaging of transgenic mice at 5 time-points within a 24-hour cycle. Chart shows a representative group of 5-month-old females. Results display a circadian rhythm at the transcription level of osteocalcin within the maxillomandibular complex. Note: * indicates a p value < 0.05 by paired *t* test statistical examination.

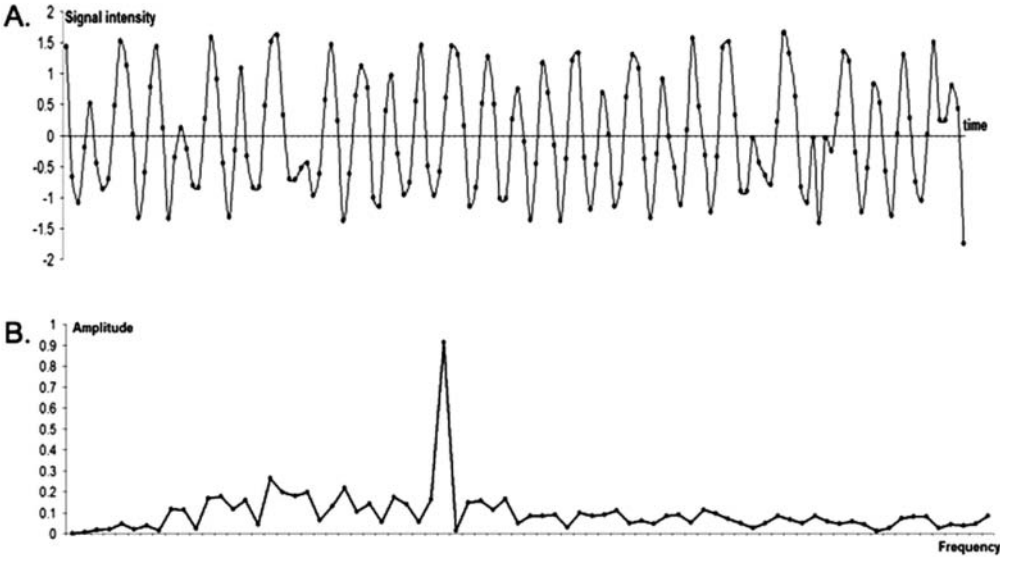

Figure 3. Concatemer of data for individual data sites (maxillomandibular complex). Circadian oscillation of osteocalcin promoter visualized *via* a luciferase reporter in the maxillomandibular complex of transgenic mice *in vivo*. Anesthetized mice (n = 23-28) were examined in a bioimaging device for light emission at six-hour intervals over a 24-hour period immediately following injection with luciferin. **(A)** Data from the individual animals have been concatenated in the order of emission amplitude and frequency profile. The Y axis shows the signal intensity relative to the standard deviation of intensity (z-score). **(B)** The periodogram is presented based on a common scale of magnitude. Harmonics contributing to the timeline expression profile (A) are displayed along the X axis (units are numbers of complete periods in the timeline of profile A). The Y axis shows the amplitude of signal intensity (z-score). The highest peak corresponds to the most prominent frequency component with complete period in 24 hrs, *i.e.,* circadian oscillation. Relative heights of the circadian and other peaks in periodogam also illustrate the signal-to-noise ratio (circadian *vs.* stochastic variation).

DISCUSSION

In this study, using non-invasive *in vivo* bioluminescence imaging in a luciferase transgenic murine model, we tested the hypothesis that circadian mechanisms regulate transcription driven by the human osteocalcin promoter. Both genders of mice were examined at 4 different ages. Analysis of the data obtained supports the conclusion that the expression of the human osteocalcin promoter in skeletal tissues is regulated in an oscillatory manner. The luciferase reporter activity in both female and male mice between the ages of 1 and 18 mos displayed a circadian expression pattern. These results extend our previously reported calvarial bone transcriptome findings regarding the murine osteocalcin mRNA expression profile (Zvonic *et al.*, 2007).

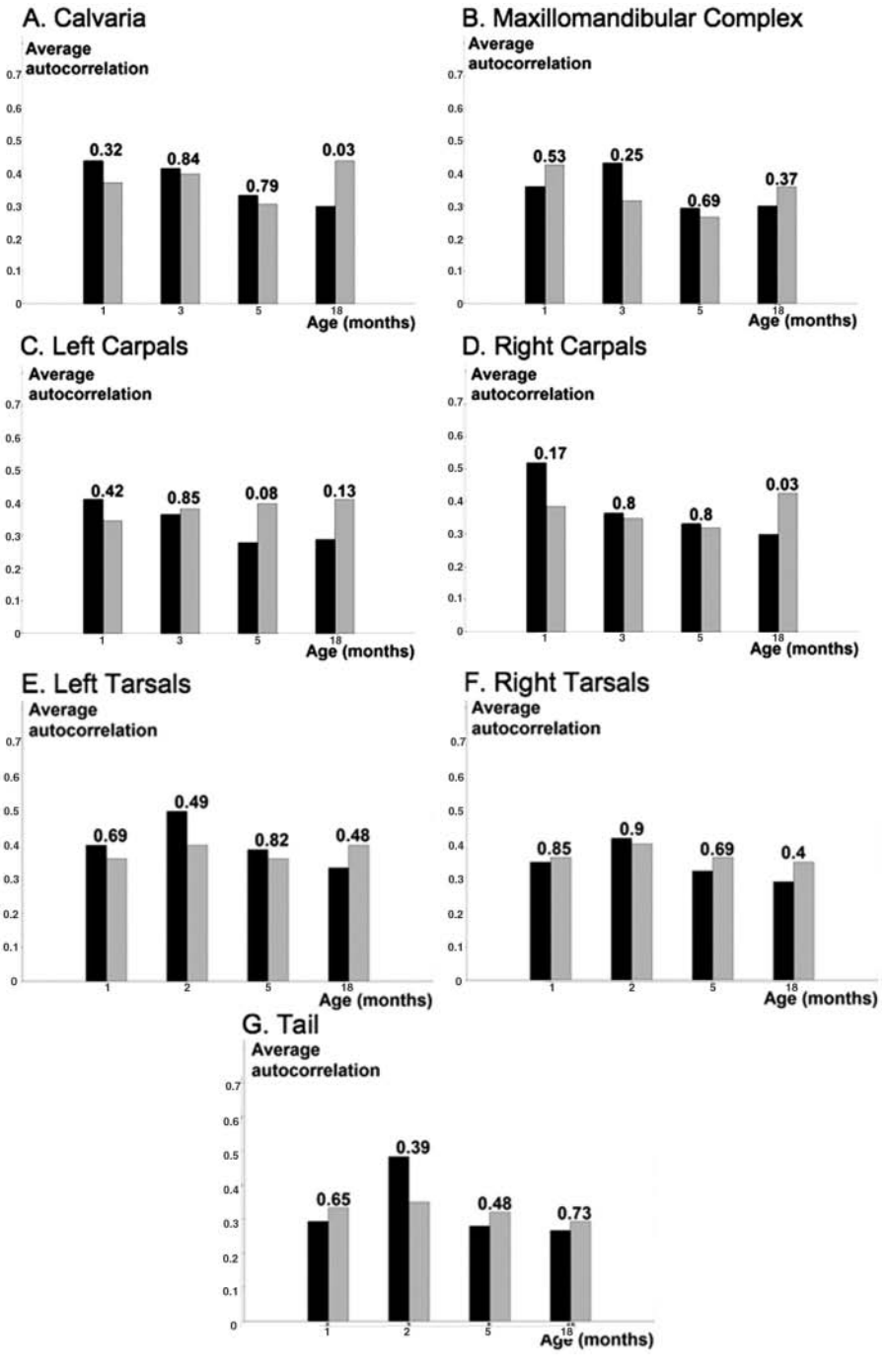

Figure 4. Circadian autocorrelation as a function of gender and age. Average circadian autocorrelation by sex and age. Each age category of 1, 3, 5, and 18 mos in consecutive order is presented by a pair of bars for male (black) and female (gray) mice. N = 5 for each gender and time-point. Significance (p-value) of difference in average autocorrelation between genders is presented on top of every age column. This study shows no significant difference in circadian rhythm dependent on the bone location, gender, or age of mice. In each panel, the Y axis shows autocorrelation with 24-hour (circadian) lag. **A**, calvaria; **B**, maxillomandibular complex; **C**, left carpals; **D**, right carpals; **E**, left tarsals; **F**, right tarsals; **G**, tail.

A major challenge for analysis of biological periodicity is the low sampling rate. The anesthetic procedure precluded a higher frequency of data collection points in the current bioluminescence study. To address this statistical challenge, we performed a phase classification of the expression profiles before testing for periodicity. In addition, for the analysis of low-frequency sampled time series, it is essential to consider all expression profiles in separate groups classified by phase. In our data, we observed one phase cohort with peak expression (acrophase) correlating with ZT6 that included ~ 2/3 of all single-animal profiles (186). However, it remains possible that some of these profiles may have been misclassified due to stochastic noise. This distribution of individual animals to each phase cohort was consistent for each bone site examined. In general, all bones had a strong prevalence for assignment to the ZT6 phase cohort. Summarizing these observations, we conclude that, in the majority of animals, the osteocalcin promoter operates in a circadian manner. While most of the animals followed the same pattern, the phase appeared to be shifted by 6 or 12 hrs in a subpopulation of animals. The mechanism accounting for the phase differences among animal cohorts remains to be determined.

It is of interest that the most pronounced circadian oscillations were evident in the maxillomandibular complex. One explanation for this outcome could be attributed to the murine incisors, which are open-rooted and therefore grow continuously throughout the lifetime of the animal (Foster *et al.*, 1983).

Night time corresponds to the peak serum levels of osteocalcin in humans, which has been recorded as 4 a.m. in multiple studies (Sokoll *et al.*, 1998; Caillot-Augusseau *et al.*, 2000). Because the mouse is a nocturnal animal, this time-point is equivalent to ZT6-12 and is consistent with the maximal luciferase activity detected in the current study. Furthermore, the peak expression for the native murine osteocalcin mRNA occurred at ZT 0-4. This correlates, in a temporally appropriate manner, with the human osteocalcin promoter-driven luciferase reporter protein in the transgenic model.

This finding could have implications with respect to dental treatment. For example, it has been suggested by orthodontists that both orthopedic and orthodontic scale forces should be routinely applied during the night-time hours, due to the fact that bone remodeling

is accelerated at resting periods (Igarashi *et al.*, 1998; Miyoshi *et al.*, 2001; Yamada *et al.*, 2002). Our results could provide support for this approach.

Also, one of the key factors for successful orthodontic treatment relates to patient compliance in the management of the various appliances and adjuncts that are used to move teeth. It is proposed that instructing persons to wear these removable orthodontic appliances and adjuncts only during a circadian temporal therapeutic window, *i.e.*, the night-time period, will both achieve a higher level of compliance and offer an improved biological effect. By so doing, the overall treatment outcome will be enhanced both clinically and temporally. Another aspect is the use of distraction osteogenesis (Ilizarov *et al.*, 1980) for maxillofacial and orthosurgical cases. The timing and magnitude of force applied are crucial for the success of treatment. To achieve the anticipated results, the person must cooperate with the activation regime, while the clinician must avoid premature consolidation of the treatment. This report links circadian mechanisms to the control of the human osteocalcin promoter in a physiological model. Future *in vitro* studies will be required to determine the *cis*-elements responsible for this phenomenon. Nevertheless, we conclude that circadian transcriptional mechanisms should be considered as contributory factors underlying common skeletal biological processes in general and in the maxillomandibular region in particular (Simmons *et al.*, 1990; Levi and Schibler, 2007). Conventional maxillofacial surgery and orthodontic treatment procedures involve bone regeneration and force application, respectively. Understanding the circadian mechanism that underlies metabolic processes in peripheral bone could enable us to harness this effect for optimization of currently available dental procedures. We predict that our model will enable us to establish the circadian temporal therapeutic window of bone in the maxillomandibular complex, to tailor efficient clinical protocols.

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