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Short communication

Expression of circadian core clock genes in fibroblasts of human gingiva and periodontal ligament is modulated by L-Mimosine and hypoxia in monolayer and spheroid cultures

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ABSTRACT

Objective: The circadian clock is involved in a plethora of physiological processes including bone formation and tooth development. While expression of circadian core clock genes was observed in various tissues, their role in the periodontium is unclear. We hypothesized that periodontal cells express circadian core clock genes and that their levels are modulated by hypoxia mimetic agents and hypoxia.
Material and methods: Fibroblasts of human gingiva (GF) and periodontal ligament (PDLF) in monolayer and spheroid cultures were treated with the hypoxia mimetic agent L-Mimosine (L-MIM) or hypoxia. Reverse transcription and quantitative PCR were performed to assess the impact on mRNA levels of the circadian core clock genes *Clock, Bmal1, Cry1, Cry2, Per1, Per2,* and *Per3.*
Results: GF and PDLF expressed *Clock, Bmal1, Cry1, Cry2, Per1, Per2,* and *Per3* in monolayer and spheroid cultures. In monolayer cultures, L-MIM significantly reduced *Clock, Cry2,* and *Per3* mRNA expression in GF and *Clock*, *Cry1*, *Cry2*, *Per1*, and *Per3* in PDLF. Hypoxia significantly reduced *Clock, Cry2,* and *Per3* in GF and *Cry1, Cry2,* and *Per3* in PDLF. In spheroid cultures, L-MIM significantly decreased *Clock, Cry1, Cry2,* and *Per3* in GF and PDLF. Hypoxia significantly decreased *Cry2* and *Per3* in GF and *Clock* and *Per3* in PDLF.
Conclusions: GF and PDLF express circadian core clock genes. The hypoxia mimetic agent L-MIM and hypoxic conditions can decrease the expression of *Clock*, *Cry1-2* and *Per1 and Per3*. The specific response depends on cell type and culture model. Future studies will show how this effect contributes to periodontal health and disease.

1. Introduction

The circadian clock regulates a plethora of physiological processes. This complex network consists of a "central clock" located in the hypothalamic suprachiasmatic nucleus and "peripheral clocks" in peripheral tissues (Weaver, 1998). Due to the light responsiveness of the "central clock" it can be entrained *via* environmental stimuli of light-dark cycles, thereby providing an internal timer for biological processes.

There are numerous other stimuli that act as cues for the central clock. The exact mechanisms of interaction through which the central clock transmits information to peripheral clocks is unclear (Dibner, Schibler, & Albrecht, 2010; Mohawk, Green, & Takahashi, 2012). The function of the mammalian "central clock" and the "peripheral clocks" depends on a well-orchestrated expression of a set of circadian core clock genes in transcriptional–translational feedback loops which has already been described in several publications (Mohawk et al., 2012; Papagerakis et al., 2014). Clock, Bmal1, Cryptochrome (Cry1, Cry2), and Period (Per1–Per3) are the key players during this cycling mechanism (Mohawk et al., 2012).

There are indications for the involvement of circadian clock mechanisms in the oral tissue during development (Zheng et al., 2011; Zheng et al., 2014). The circadian clock was proposed to modulate the activity of ameloblasts and odontoblasts during tooth development (Athanassiou-Papaefthymiou et al., 2011; Zheng et al., 2014). Furthermore, genes including *osteocalcin,* which are essential for bone formation, have been shown to be under control of the circadian clock (Gafni et al., 2009), suggesting "peripheral clocks" in oral tissue (Papagerakis et al., 2014).

While the role of the molecular clocks in tooth development has been proposed, the role of the core clock genes in the periodontal tissue is currently unclear (Zheng et al., 2014, 2011). Since

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molecular clocks regulate a broad spectrum of cell biological processes, it is possible that they are also involved in oral tissue regeneration as proposed for other tissues (Al Mheid et al., 2014; Plikus et al., 2013; Chatterjee, Yin, Nam, Li, & Ma, 2015; Chauhan, Lorenzen, Herzel, & Bernard, 2011; Karpowicz, Zhang, Hogenesch, Emery, & Perrimon, 2013; Sukumaran, Jusko, Dubois, & Almon, 2011).

A central cue in regeneration is hypoxia. In a defect site, hypoxic conditions stimulate angiogenesis involving the transcription factor Hypoxia Inducible Factor (HIF)-1α, leading to a highly controlled release of signaling factors like Vascular Endothelial Growth Factor (Vegf) (Fraisl, Aragonés, & Carmeliet, 2009; Rabinowitz, 2013). There is evidence that HIF-1α directly influences the circadian clock (Bozek et al., 2009; Okabe et al., 2014) and regulates downstream gene expression (Ghorbel, Coulson, & Murphy, 2003; Takahata et al., 1998). The knowledge that a compromised response to hypoxia hinders healing lead to the development of hypoxia-based strategies which target this pathway *via* hypoxia mimetic agents (Agis, Hueber, Pour Sadeghian, Pensch, & Gruber, 2014; Fraisl et al., 2009; Kuchler et al., 2015; Rabinowitz, 2013; Vinzenz et al., 2015). Understanding the role of the molecular clock in the periodontal tissue will help to optimize existing therapeutic strategies and develop novel approaches.

Here we evaluated if fibroblasts of human gingiva (GF) and periodontal ligament (PDLF) express circadian core clock genes and how their expression levels are modulated by the hypoxia mimetic agent L-Mimosine (L-MIM) and hypoxia in monolayer and spheroid cell cultures.

2. Material and methods

2.1. Monolayer cultures of fibroblasts of the gingiva and the periodontal ligament

Human GF and PDLF were isolated following a previously established protocol (Agis, Watzek, & Gruber, 2012). The protocol was approved by the ethics committee of the Medical University of Vienna and informed consent was obtained (631/2007). GF and PDLF were prepared from extracted third molars with no previous history of dental inflammation. GF were prepared from the soft tissue of the gingiva attached to the tooth neck and PDLF were prepared from the soft tissue attached to the tooth root. GF and PDLF were expanded and cultivated in α-minimal essential medium (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS, PAA Laboratories, Linz, Austria) and antibiotics (Gibco, Invitrogen Corporation) at 37 °C, 5% CO_2, and 95% humidity. For the experiments GF and PDLF were used up to passage 7. The cells were plated at a density of 50,000 cells/cm^2. On the second day, cells were treated with L-MIM at 1 mM or hypoxia for 24 h. Untreated cells served as normoxic control. On the third day, cells were subjected to RNA isolation.

2.2. Spheroid cultures of fibroblasts of the gingiva and the periodontal ligament

3D agarose molds were made using 3D Petri Dishes® (Microtissues Inc., Providence, RI, USA). Dishes for spheroids were covered with agarose to produce molds with 35 recesses. These molds were soaked in α-minimal essential medium supplemented with 10% fetal calf serum and antibiotics. Then the molds were transferred to 24 well plates. To form spheroids, 75 μl GF and PDLF cell suspensions of 7,300,000 cells/mL were applied to the agarose 3D molds following the protocol of the manufacturer. Cells were incubated overnight as described above. The next day, spheroids were incubated with L-MIM at 1 mM or hypoxia for 24 h. Untreated cells were used as normoxic control. Then, spheroids were harvested and subjected to RNA isolation and quantitative PCR.

2.3. RNA isolation, cDNA synthesis, and quantitative polymerase chain reaction

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) followed by cDNA synthesis with the high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). Then, cDNA was amplified with TaqMan Real-Time PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) using primers and probes for *Clock, Bmal1 Cry1, Cry2, Per1, Per2,* and *Per3*. Quantification of gene expression levels was performed by calculating expression levels relative to *Gapdh* by using the comparative cycle threshold (ΔΔCt) method. For further details on the primers and probes see Table 1.

2.4. Statistical analysis

The results are presented as mean + standard deviation. Data were compared using the Kruskal Wallis test and Mann-Whitney test. Significance level was assigned at $p < 0.05$.

3. Results

3.1. L-Mimosine and hypoxia modulate core clock genes in 2D monolayer cultures of fibroblasts of the gingiva and periodontal ligament

All seven circadian core clock genes, *Clock*, *Bmal1*, *Cry1, Cry2, Per1, Per2,* and *Per3* were expressed in GF and PDLF under normoxic conditions in monolayer cultures. In GF mRNA expression levels relative to *Gapdh* were as follows: *Clock* (0.0008 ± 0.0007), *Bmal1* (0.0002 ± 0.0002), *Cry1* (0.0009 ± 0.0007), *Cry2* (0.0003 ± 0.0001), *Per1* (0.0006 ± 0.0002), *Per2* (0.0001 ± 0.0000), and *Per3* (0.0003 ± 0.0002). In PDLF mRNA expression relative to *Gapdh* was as follows: *Clock* (0.0004 ± 0.0002), *Bmal1* (0.00004 ± 0.00003), *Cry1* (0.0001 ± 0.0001), *Cry2* (0.0006 ± 0.0005), *Per1* (0.0002 ± 0.0002), *Per2* (0.0001 ± 0.0001), and *Per3* (0.0005 ± 0.0003).

Table 1
All TaqMan assays that were used for qPCR (Applied Biosystems).

Gene symbol	Gene name	Assay ID
Clock/CLOCK	clock circadian regulator	Hs00231857_m1
Bmal1/ARNTL	aryl hydrocarbon receptor nuclear translocator-like	Hs00154147_m1
Cry1/CRY1	cryptochrome 1	Hs00172734_m1
Cry2/CRY2	cryptochrome 2	Hs00323654_m1
Per1/PER1	period circadian clock 1	Hs01092603_m1
Per2/PER2	period circadian clock 2	Hs00256143_m1
Per3/PER3	period circadian clock 3	Hs00213466_m1
Gapdh/GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs02758991_g1