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ORIGINAL ARTICLE

Circadian rhythms and gene expression during mouse molar tooth development

Minou Nirvani[a], Cuong Khuu[a], Tor Paaske Utheim[a,b,c,d], Henriette Stavik Hollingen[a], Simon Furre Amundsen[a], Lars Peter Sand[a] and Amer Sehic[a]

[a]Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway; [b]Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway; [c]Department of Ophthalmology, Drammen Hospital, Vestre Viken Hospital Trust, Drammen, Norway; [d]Faculty of Health Sciences, University College of South East Norway, Kongsberg, Norway

ABSTRACT

Objectives: Incremental markings in dental enamel suggest that the circadian clock may influence the molecular underpinnings orchestrating enamel formation. The aim of this study was to investigate whether the genes and microRNAs (miRNAs) oscillate in a circadian pattern during tooth and enamel development.

Material and methods: Comparative gene and miRNA expression profiling of the first mandibular molar tooth germ isolated at different time-points during the light and night period was performed using microarrays and validated using real-time RT-PCR. Bioinformatic analysis was carried out using Ingenuity Pathway Analysis (IPA), and TargetScan software was used in order to identify computationally predicted miRNA–mRNA target relationships.

Results: In total, 439 genes and 32 miRNAs exhibited significantly different ($p < 0.05$) levels of expression in the light phase compared with the night phase tooth germs. Genes involved in enamel formation, i.e. *Amelx, Ambn, Amtn,* and *Odam*, oscillated in a circadian pattern. Furthermore, the circadian clock genes, in particular *Clock* and *Bmal1,* oscillated in mouse molar tooth germ during 24-h intervals. The expression of *Clock* and *Bmal1* was inversely correlated with the expression of miR-182 and miR-141, respectively.

Conclusions: MiRNAs, including miR-182 and miR-141, are involved in the control of peripheral circadian rhythms in the developing tooth by regulating the expression of genes coding for circadian transcription factors such as CLOCK and BMAL1. Regulation of circadian rhythms may be important for enamel phenotype, and the morphology of dental enamel may vary between individuals due to differences in circadian profiles.

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Introduction

Circadian rhythms are involved in most physiological processes and are generated within an organism by endogenous biological clocks driven by cyclic events [1]. Although there is a site in the suprachiasmatic nucleus of the brain that is considered as the 'master clock' for the circadian rhythms, peripheral clocks have been found in several tissues in the body. The master clock is responsive to light and can be entrained by light/dark cycles. Several peripheral genes, called clock genes, have been identified as key maintainers of the circadian rhythm. The main mammalian clock genes are Circadian locomoter output cycles kaput (Clock), Aryl hydrocarbon receptor nuclear translocator-like (Arntl or Bmal1), Cryptochrome 1 and 2 (Cry1 and Cry2), Period1 (Per1), Period2 (Per2), and Period 3 (Per3). Transcription of these clock genes oscillates over a 24-h period and they transmit output signals that drive rhythms of gene expression in central and peripheral tissues. The mechanism by which clock genes drive circadian gene expression is by binding to the promoter region of a clock-controlled gene [2]. It has been suggested that about 10–20% of the organ specific genes may be under circadian control [3].

Dental enamel is the hardest and most mineralized tissue in the vertebrate body, formed by ameloblast cells. We have recently investigated and described the occurrence and periodicity of enamel incremental markings in mouse molars in an attempt to draw attention to some key questions about the rhythm in the activity of the secreting ameloblasts during formation of mouse molar enamel [4]. Because the circadian clock regulates a broad range of physiological processes [5–8], including that of other mineralized tissues influencing bone homeostasis and growth [9,10], the periodicity observed in enamel suggests that the molecular underpinnings orchestrating enamel development may be influenced by the circadian clock. However, the association between the circadian clock and enamel development remains poorly defined. Circadian clock genes (*Clock, Bmal1, Per1,* and *Per2*) are expressed in mouse molars, suggesting that these genes may be involved in the regulation of ameloblast and odontoblast functions, such as enamel and dentine matrix secretion and mineralization [11]. Moreover, intravenous injections of 3H-methionine administered at different times of the day visualized daily variations of radiolabeled proteins secreted by ameloblasts [12–14]. Using an ameloblast cell line, other

CONTACT Amer Sehic amer.sehic@odont.uio.no Department of Oral Biology, University of Oslo, Box 1052 Blindern, 0316 Oslo, Norway

researchers have explored the potential links between circadian control and stage-specific regulation of ameloblast genes [15]. Another recent study has demonstrated that circadian clock genes in an ameloblast cell line and amelogenin gene (*Amelx*) in 2-d postnatal mouse molars oscillate in a circadian pattern [16].

Taken together, these studies strongly support the assertion that the circadian clock genes modulate enamel development and that amelogenesis is subject to diurnal rhythms in gene-expression levels and cell activity during development of mouse molar enamel. Nevertheless, circadian rhythms and expression of other genes in the tooth germ, including microRNAs (miRNAs) have not been investigated. MiRNAs constitute a class of non-coding RNAs that regulate gene expression at a posttranscriptional level [17] and are considered as important regulatory molecules during foetal development [18]. The recent report brought new insights into possible roles of miRNAs in modulating circadian clocks, revealing that the interplay between different types of RNAs and proteins can exert modulation of circadian-clock period and entrainment [19]. Therefore, in the present study, we carried out comparative gene-expression profiling of the mouse molar tooth germ to investigate whether the miRNAs and mRNAs oscillate in a circadian pattern. Furthermore, we wanted to study the correlation between the expression of specific miRNA and corresponding target mRNA.

Materials and methods

Experimental animals

Timed-pregnant CD-1 mice were kept according to the regulations of the Norwegian Gene Technology Act of 1994. All animals were housed in the vivarium with 21 °C and a relative humidity of 65%, and in normal light-dark cycles with the light phase starting at 06:00 h and the dark phase at 1800 h. Prior to experimental use, animals were given standard laboratory fodder and water *ad libitum*. The first right mandibular molar tooth germ was isolated from mouse pups at postnatal day 1 (PN1). Starting at 08:00 h five pups were euthanized every 4 h until 04:00 h the day after, and the right mandibular molar tooth germs were immediately dissected free from the jaw. During the night period, the pups were euthanized under dim red illumination within 3 min from entering the vivarium room, and the tooth germs were immediately dissected free from the jaw. The pups were sacrificed by decapitation and their heads were immediately immersed in RNAlater (Ambion, Austin, TX). The right mandibular molar tooth germ was dissected out while immersed in RNAlater diluted 1:4 with phosphate-buffered saline and subsequently transferred into undiluted RNAlater.

Isolation of RNA

Fractions of total RNA, and RNA-fractions enriched with respect to miRNAs, were isolated according to the protocol of the manufacturer from the mandibular molar tooth germs using the Qiagen RNA Mini-kit and Qiagen miRNAeasy Mini-kit, respectively (Qiagen, Hilden, Germany). This yielded RNA fractions exhibiting a ratio of OD_{260}/OD_{280} and OD_{260}/OD_{230} of at least 1.8 and 2.0, respectively. RNA was isolated from five phenotypical pups at every time point. The quality of RNA in solutions was assessed using Agilent-Bioanalyzer (Agilent, Palo Alto, CA). All solutions used had RIN (RNA integrity number) values higher than 8.5. Concentrations of solutions of purified RNA were assayed using Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA).

Microarray analysis of mRNAs

Mouse OneArray microarrays (Phalanx Biotech Group, Palo Alto, CA) used in this study included a series of control spots to monitor the quality of sample processing, hybridization efficiency, and the specificity of hybridization. Microarray analysis was carried out in triplicates for every time point; each of three microarrays was hybridised with cDNA prepared from 1 μg of RNA derived from each of the resulting solutions of RNA. cDNA from tooth germs dissected during the light period (08:00 h, 12:00 h, and 16:00 h) was Cy5 labelled while cDNA from tooth germs dissected during the night period (20:00 h, 24:00 h, and 04:00 h) was Cy3 labelled.

Preparation of cDNA, Cy3- and Cy5-labelling and hybridization were carried out using the Genisphere 3DNA Array 900 detection kit as described by the manufacturer (Genisphere, Hatfield, PA). Hybridization of labelled samples of RNA was carried out at 58 °C for 18h in a SlideBooster 400 Hybridization Station (Advalytix, Munich, Germany). The microarrays were scanned in a Packard Bioscience Scanarray Lite microarray scanner (Perkin-Elmer, Waltham, MA). The Cy3 and Cy5 fluorescence signals were quantified by using the Scanarray Express v.3.0 program (Perkin-Elmer, Waltham, MA). The resulting fluorescence data (contained in .csv-files) was analysed using the Spotfire v.9.0 microarray analysis program (Spotfire, Somerville, MA). The microarray methods were otherwise as described previously [20].

Microarray analysis of miRNAs

Mouse and Rat miRNA OneArray microarrays (Phalanx Biotech Group, Palo Alto, CA) were used. The array probe set included 728 unique miRNA sequences common to mice and rats. The arrays also included 135 control spots derived from 25 unique sequences, which were drawn from a variety of organisms to act as positive and negative controls. All sequences had been printed in triplicates. The miRNA-enriched fractions were labelled using Kreatech ULS labelling of miRNA (Kreatech, Amsterdam, The Netherlands). Each slide was hybridized with samples containing 1 μg of RNA. Hybridization of labelled samples of RNA was carried out at 37 °C for 16h in a SlideBooster 400 Hybridization Station (Advalytix, Munich, Germany). Microarray analysis was carried out in duplicates with samples from molar tooth germs dissected during the light period (08:00 h and 16:00 h) and the night period (20:00 h and 04:00 h). The microarray methods used were otherwise as described above.

Real-time RT-PCR assays

Fractions of total RNA, and RNA-fractions enriched with respect to miRNAs, were isolated from five molar tooth germs at 08:00 h (light phase) and 20:00 h (night phase) and used for RT-PCR assays. Quantitative real-time PCR was carried out using TaqMan MicroRNA Assay (mmu-miR-141-3p, mmu-miR-145a-5p, mmu-miR-182-5p, and mmu-miR-207) and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Expression of selected genes (*Ambn, Amelx, Enam, Bmal1, Clock, Per1,* and *Per2*) was assayed by real-time quantitative RT-PCR using primers designed with Primer3. We used *Rpl27* as a housekeeping gene as it is relatively stably expressed during tooth development [20]. cDNA was synthesized by oligo-dT priming using a First Strand Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany). Real-time PCR assays were carried out with a qPCR core kit (Eurogentec, Seraing, Belgium). Every analysis was carried out in technical triplicates. Statistical evaluation of the significance of differences between measured cycle threshold (Ct) values was carried out using the REST 2005 program [21]. All RT-PCR assays were carried out using the Stratagene Mx 3005P PCR instrument (Stratagene, LaJolla, CA).

Statistical analysis of microarray data

Statistical analysis of mRNA microarray data was carried out on data derived from sets of triplicate slides, which were combined into a single data-file. Genes exhibiting net fluorescence values of less than 300 in both channels were excluded in further analysis. False discovery rate (FDR) of Benjamini et al. [22] was used to correct selection of genes for false positives ($p < 0.05$). The ANOVA facility of the Spotfire program was used to select genes, which exhibited statistically significant differences in levels of expression ($p < 0.05$) in the tooth germs isolated at different time points. Statistical analysis of miRNA microarray data was carried out on data derived from sets of duplicate slides, which were combined into a single data-file. MiRNAs exhibiting net fluorescence values of less than 500 in both channels from two out of three replicate spots were excluded in further analysis. The statistical analysis of microarray data was otherwise as described previously [20].

Bioinformatic analysis

Bioinformatic analysis using Ingenuity Pathway Analysis (IPA) was carried out in an attempt to provide molecular and cellular functional interpretations of genes exhibiting significantly different expression ($p < 0.05$) in the tooth germs isolated in the light phase compared with the tooth germs isolated in the night phase. Bioinformatic analysis of miRNA expression data is relatively unexplored compared with bioinformatic analysis of mRNA expression data. In this study, we used TargetScan Release 6.2 software (http://www.targetscan.org) in order to identify computationally predicted mRNA targets for miRNAs, which were differentially expressed in tooth germs isolated in the light phase compared with the tooth germs isolated in the night phase.

Results

Microarray analysis of differentially expressed mRNAs and miRNAs

Using the ANOVA function within the Spotfire program, we screened the entire data-set for genes exhibiting significantly different ($p < 0.05$) levels of expression in the light-phase tooth germs compared with the night phase tooth germs, resulting in 439 genes (Figure 1). Among the differentially expressed genes, we identified 228 and 211 genes exhibiting lower and higher levels of expression in the light phase compared with the night phase tooth germ, respectively (Figure 1). Interestingly, all 228 genes in the light phase showed decreased levels of expression at all three time-points investigated (i.e. 08:00 h, 12:00 h, and 16:00 h). Only 2 h after entering the night phase at 18:00 h, the expression was increased and remained high during the night phase until 04:00 h. A similar, but opposite expression pattern was observed for the 211 genes exhibiting high levels of expression in the light phase. When entered into the night phase, the expression decreased. Moreover, the 439 mRNAs exhibiting significantly different levels of expression were arranged by self-organizing maps (SOM) (Figure 2). Based on the pattern of expression of these genes, and in order to isolate groups of genes that may be functionally related to each other, the expression data were organized into five clusters (Figure 2). The 228 genes that exhibited lower levels of expression in the light phase were clustered into three groups (SOM 1 with 39 genes, SOM 2 with 153 genes, and SOM 3 with 36 genes) (Figure 2). The 211 genes that exhibited higher levels of expression in the light phase were clustered into two groups (SOM 1 with 79 genes and SOM 2 with 132 genes) (Figure 2). Interestingly, two of the genes coding for enamel matrix proteins, *Amelx* and *Ambn*, were down-regulated during the night phase and were identified in the same cluster. Furthermore, two clock genes, *Clock* and *Bmal1*, were up-regulated during the night phase and based on the pattern of expression they were clustered together (Figure 2).

Differentially expressed ($p < 0.05$) miRNAs in the light phase (08:00 h and 16:00 h) compared with the night phase (20:00 h and 04:00 h) were identified using ANOVA. Microarray analysis of miRNAs suggested that 32 miRNAs exhibited significantly different levels of expression in the light phase compared with the night-phase tooth germs. These 32 miRNAs were hierarchically clustered (Figure 3), and based on the pattern of expression they were divided into two groups. The 12 miRNAs (group 1) and 20 miRNAs (group 2) exhibited higher and lower levels of expression in the night phase compared with the light-phase tooth germs, respectively (Figure 3). The expression levels were consistent at both time-points in the light (08:00 h and 16:00 h) and night phase (20:00 h and 04:00 h).

Validation of microarray analysis by real-time RT-PCR

To validate microarray data, seven genes (*Ambn, Amelx, Enam, Bmal1, Clock, Per1, and Per2*) were selected to have

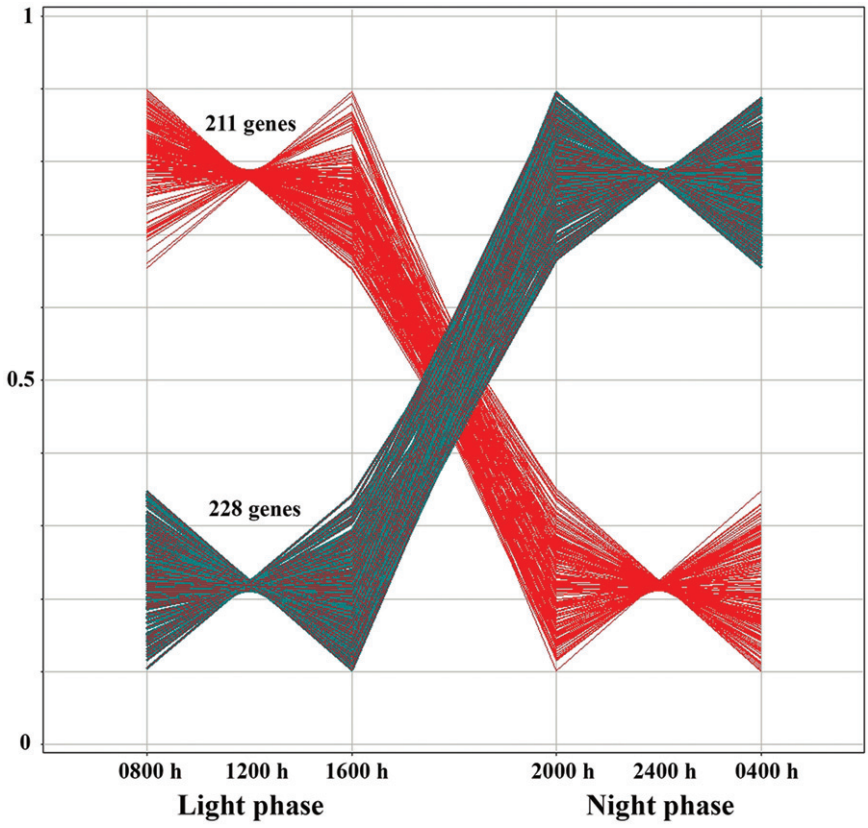

Figure 1. Genes (mRNAs) exhibiting significantly different expression ($p < 0.05$) in the tooth germs isolated in the light phase compared with the night phase. Total RNA fractions were isolated from tooth germs from five phenotypical pups at every time point as described in Materials and methods section. Expression profiling of mRNAs was carried out using microarrays, and differentially expressed ($p<0.05$) mRNAs were identified using the ANOVA function in the Spotfire program. Microarray analysis was carried out in triplicates for every time point.

their expression assayed also by real-time RT-PCR. These genes were selected because their patterns of expression were different between the light-phase and night-phase tooth germs across all time-points investigated. In addition, we wanted to focus our attention on clock genes and genes coding for enamel matrix proteins. Results obtained using real-time RT-PCR showed good correlation with results obtained with microarrays (Figure 4(A)). Three of the selected mRNAs coding for the enamel matrix proteins exhibited lower levels of expression in the night phase, i.e. *Ambn* (coding for ameloblastin), *Amelx* (coding for amelogenin), and *Enam* (coding for enamelin). The differences in gene expression were significant ($p < 0.05$) for *Amelx* and *Ambn* (Figure 4(A)). Four of the selected genes coding for clock proteins were up-regulated in the night phase, i.e. *Bmal1, Clock, Per1, and Per2.* Among the four selected clock genes, the changes in levels of expression between the light and the night phase were significant for *Clock*, *Bmal1*, and *Per2*, and most marked for *Clock* and *Bmal1* (Figure 4(A)).

Levels of selected miRNAs (miR-141, miR-145, miR-182, and miR-207) in the light- and night-phase tooth germs were also measured by quantitative real-time RT-PCR. These miRNAs were selected because their patterns of expression were different between the light-phase and night-phase tooth germs across all time-points investigated. Furthermore, the computational prediction using TargetScan suggested that miR-182 targets *Clock* and that miR-141 targets *Bmal1*, therefore, we wanted to validate their expression. The RT-PCR results showed good agreement with microarray results (Figure 4(B)). Expression of miR-141 and miR-182 was down-regulated in the night-phase tooth germs, while the expression levels of miR-145 and miR-207 were higher in the night phase compared with the light-phase tooth germs (Figure 4(B)).

Cellular and molecular functions significantly associated with genes exhibiting different levels of expression in the light-phase compared with the night-phase tooth germs

IPA identified every gene that exhibited different levels ($p < 0.05$) of expression in the light phase compared with the night-phase tooth germs. Bioinformatic analysis of these genes provides a guide to molecular and cellular functions, which likely are down-regulated and up-regulated in the light-phase tooth germ. Results demonstrated that down-regulated genes in the light-phase tooth germ constituted an important part of a functional network involved in 'cell-cycle progression', 'transcription of DNA', 'transactivation of RNA', and 'tissue morphology' (Table 1). On the contrary, up-regulated genes in the light phase were associated with functions involved in 'cell cycle', 'gene expression', 'cellular development', and 'cell signalling'. The remaining cellular and

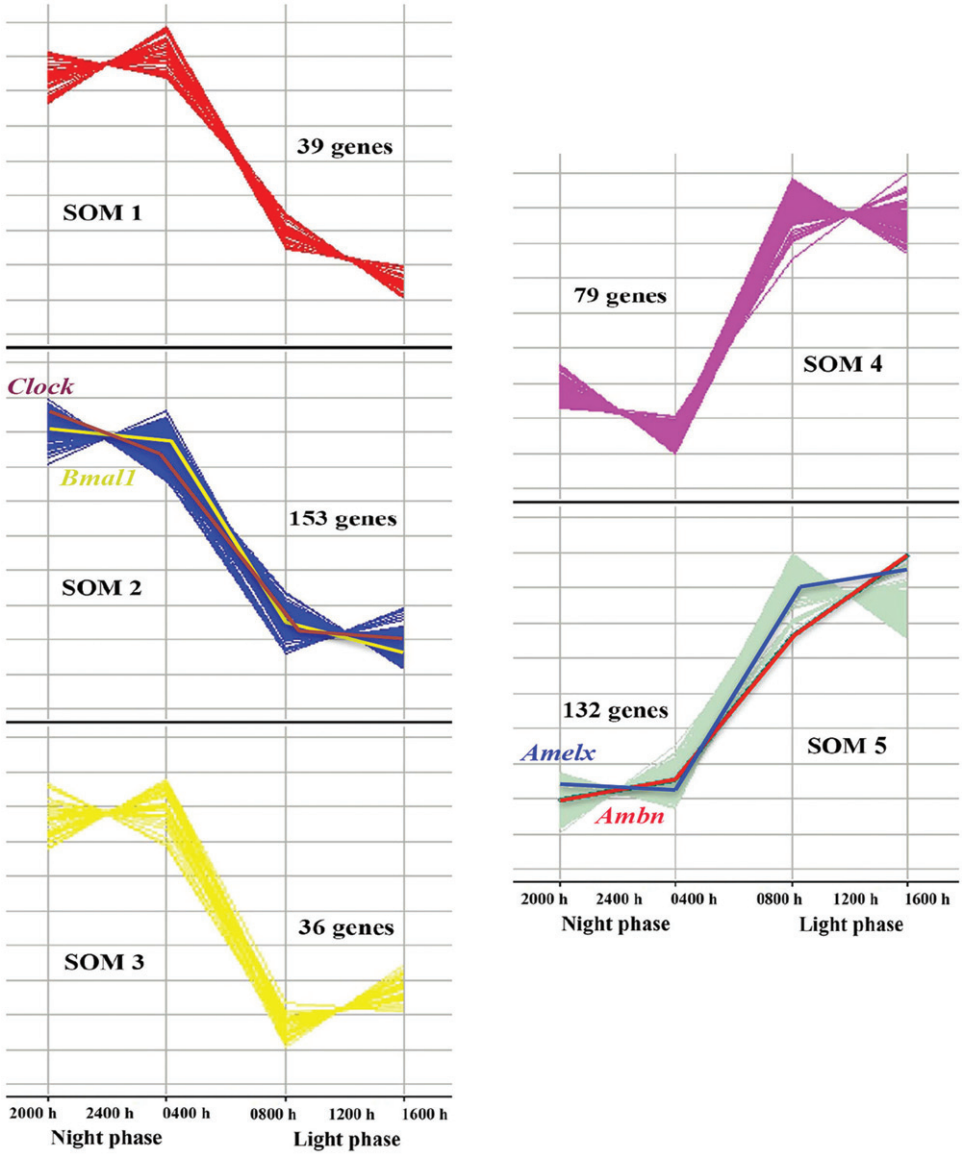

Figure 2. Self-organizing maps (SOM) clustering of differentially expressed genes. Differences in gene expression in tooth germs isolated in the light and night phase are visualized by SOM clustering. In order to isolate a population of genes that may be functionally related to each other, the expression data were organized into 5 clusters. The results show that 228 genes exhibiting lower levels of expression in the light phase compared with the night phase were clustered into three groups (SOM 1, 2, and 3). The remaining 211 genes that exhibited higher levels of expression in the light phase compared with the night phase were clustered into two groups (SOM 1 and 2). Genes coding for enamel matrix proteins, *Amelx* and *Ambn*, were down-regulated during the night phase and were identified in the same cluster. Clock genes, *Clock* and *Bmal1*, were up-regulated during the night phase and were also presented in the same cluster.

molecular functional categories, with less than 20 associated genes, are presented in Table 1.

Differentially expressed miRNAs and their predicted mRNA targets

In this study, we have identified potential circadian miRNA–mRNA target pairs in the mouse molar tooth germ using microarray-based expression profiling of both miRNAs and mRNAs. Using a total of 728 probes recognizing rat and mouse miRNA sequences, we identified 32 miRNAs that were expressed in a circadian manner in molar tooth germs (Figure 3). Here, we defined a circadian miRNA–mRNA target pair as one in which both elements showed rhythmic expression and for which TargetScan had established the sequence-based target relationship computationally. Based on these criteria, 30 significant miRNA–mRNA target relationships were identified between a total of 32 miRNAs and 439 genes. Two differentially expressed miRNAs (miR-466h and miR-805) did not have any predicted targets that were differentially expressed in our data (Tables 2 and 3).

In general, our results revealed that the expression of cycling miRNAs in the molar tooth germ is often correlated, either positively or negatively, with the circadian expression profile of their corresponding mRNA targets. Notably, tooth germ levels of seven up-regulated miRNAs in the night phase were inversely correlated with the expression of their respective target mRNAs (Table 2), i.e. miRNAs being up-regulated, whereas their targets are down-regulated. In contrast, expression of nine up-regulated miRNAs was positively correlated with their corresponding mRNA targets, i.e. both miRNAs and mRNA targets being up-regulated. Six miRNAs

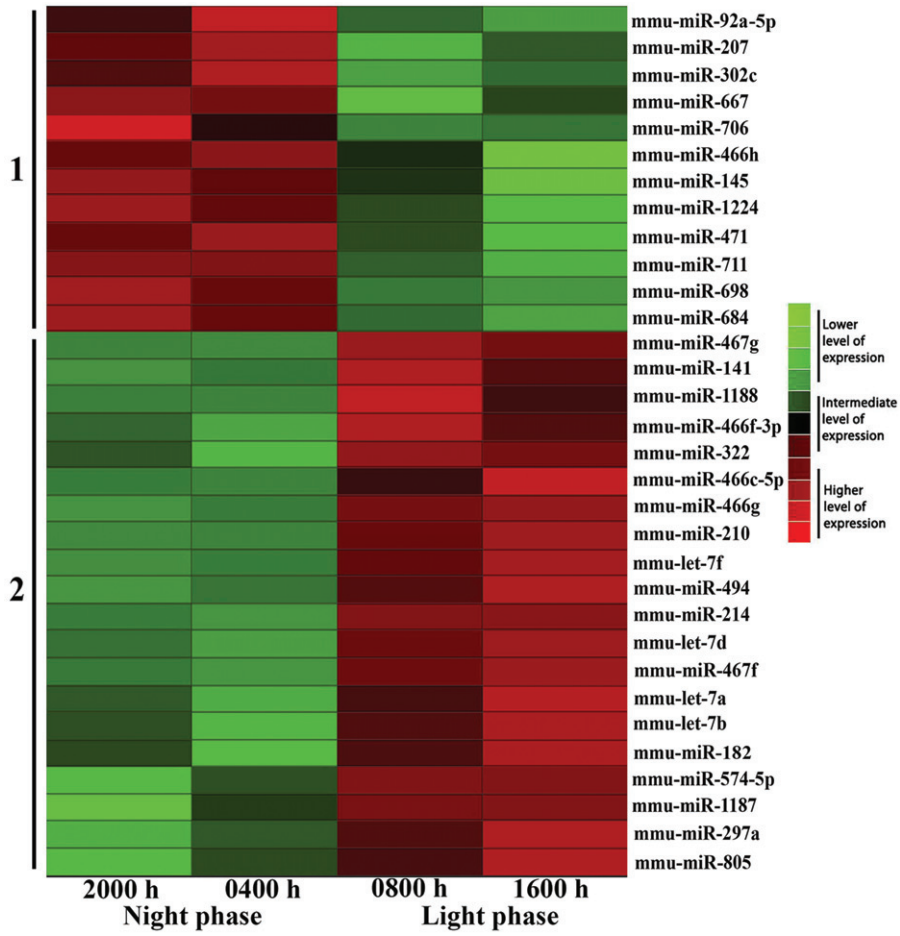

Figure 3. Heat map resulting from hierarchical clustering of the 32 miRNAs exhibiting different expression ($p < 0.05$) in the tooth germs isolated in the light phase compared with the night phase. Expression of miRNAs in tooth germs isolated in the light and night phase was measured using microarray analysis as described in Materials and Methods section. Based on the pattern of expression generated by the heat map (graphical representation of data where the values taken by a variable in a two-dimensional map are represented as colours), the miRNAs were divided into two groups (groups 1 and 2, as indicated on the left-hand side). The colours indicate relative fluorescence intensities as shown on the right-hand side. The heat map was generated using Spotfire software.

(miR-92a-5p, miR-207, miR-302c, miR-1224, miR-711, and miR-684) had target relationships with both up-regulated and down-regulated mRNAs in the night phase (Table 2). Interestingly, one of the genes coding for enamel matrix proteins, *Ambn*, was inversely correlated with the expression of its respective miRNAs, miR-302c, and miR-706, whereas the expression of *Odam* was positively correlated with the expression of miR-302c and miR-698.

On the contrary, tooth germ levels of 19 down-regulated miRNAs in the night phase were inversely correlated with the expression of their respective target mRNAs (Table 3), i.e. miRNAs being down-regulated, whereas their targets are up-regulated. In contrast, expression of 14 down-regulated miRNAs was positively correlated with their corresponding mRNA targets, i.e. both miRNAs and mRNA targets being down-regulated. In total, 14 down-regulated miRNAs exhibited target relationship with both up-regulated and down-regulated mRNAs in the night phase (Table 3). Expression of clock genes, *Clock* and *Bmal1*, was inversely correlated with the expression of miR-182 and miR-141, respectively. Moreover, expression of *Amelx* and *Ambn* was positively correlated with the expression of miR-466g and miR-466f-3p, respectively. Two other genes known to be involved in enamel formation, *Amtn* and *Odam*, were inversely correlated with their respective miRNAs (Table 3).

Discussion

Although a system of evenly spaced incremental lines, especially evident in the outer and cervical enamel of humans and other primates has been thoroughly studied for many years [23], and is highly indicative of a physiological rhythm in enamel formation, the expression of clock genes has not been investigated in dental tissues until recently. A study published by Zheng et al. [11] in 2011 represents the first step towards understanding the role of clock genes in formation of enamel and dentin. Their results demonstrated that main clock genes (*Bmal1, Clock, Per1*, and *Per2*) known to control circadian rhythms are expressed in teeth. Protein analysis further demonstrated that all four proteins were strongly expressed in the nucleus of matrix secreting cells, i.e. ameloblasts, odontoblasts, and osteoblasts, whereas only PER1 exhibited high levels of expression in dental pulp cells [11]. Based on these findings, it was suggested that the clock genes might be involved in the regulation of ameloblast and odontoblast functions, such as enamel and dentin matrix

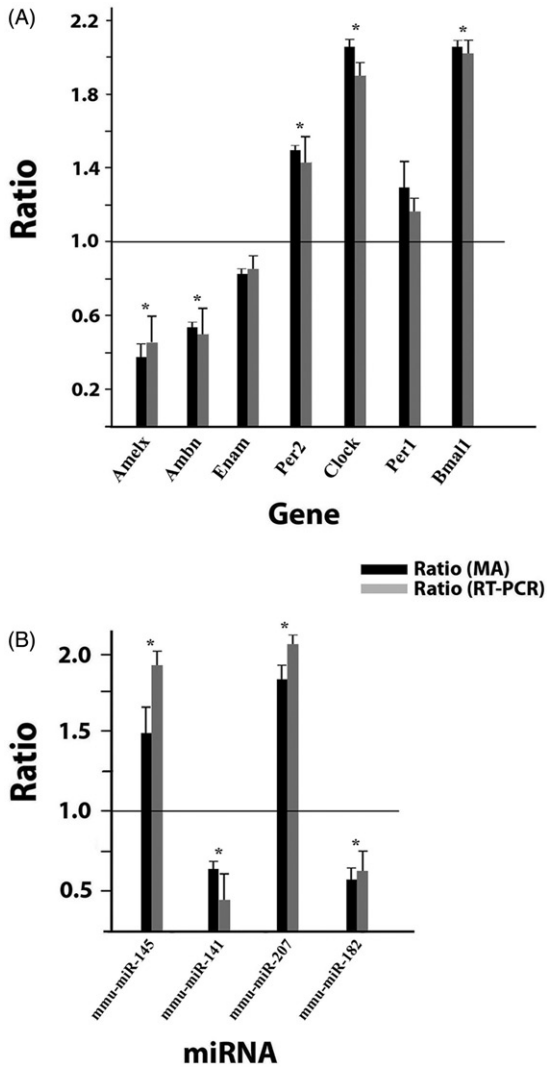

Figure 4. Validation of microarray data using RT-PCR. Fractions of total RNA, and RNA-fractions enriched with respect to miRNAs, were isolated from five molar tooth germs at 08:00 h (light phase) and 20:00 h (night phase) and used for RT-PCR assays. Gene expression was measured with microarrays and with real-time RT-PCR on seven selected genes and four selected miRNAs. Plotted data represents mean ratios of level of expression at 20:00 h (night phase)/ 08:00 h (light phase), with standard deviations indicated. Experimental details are otherwise given in Materials and methods section. The results for mRNAs (A) and miRNAs (B) are presented in the figure.

Table 1. Top 10 cellular and molecular functions significantly associated with down-regulated and up-regulated genes in the light-phase tooth germ.

	p Value	No. of genes
Associations with *down-regulated* genes in the light phase		
Cell cycle progression	3.37E − 04	31
Transcription of DNA	1.33E − 03	29
Transactivation of RNA	3.26E − 02	26
Tissue morphology	3.53E − 03	26
Cell-to-cell signaling and interaction	2.21E − 03	19
Cellular growth and proliferation	3.72E − 02	17
Activation of DNA endogenous promotor	1.26E − 02	16
Cellular assembly and organization	1.45E − 02	10
DNA repair	2.24E − 02	6
Cellular function and maintenance	1.33E − 03	5
Associations with up-*regulated* genes in the light phase		
Cell cycle	3.38E − 03	28
Gene expression	1.91E − 03	23
Cellular development	2.46E − 02	22
Cell signaling	1.64E − 03	22
Cellular function and maintenance	3.53E − 03	18
Binding of DNA	1.21E − 02	16
Protein synthesis	4.36E − 02	13
Transcription of DNA	2.29E − 02	11
Cell cycle progression	1.42E − 02	7
Cell death	1.24E − 03	4

The genes were identified using the ANOVA function in the Spotfire program, and subjected to bioinformatic analysis using IPA. The plotted data represent associations between genes and cellular/molecular functional categories. Experimental details are given in Materials and methods section.

Table 2. mRNA Target predictions for miRNAs being up-regulated in the night phase.

Up-regulated miRNAs in the night phase	Up-regulated mRNA targets in the night phase	Down-regulated mRNA targets in the night phase
mmu-miR-92a-5p	*Tpcn1, Serpinf1, Muc5ac*	*Mis18a, Adam19, Npnt, Myh3*
mmu-miR-207	*Rogdi, Pex26*	*Ddx21*
mmu-miR-302c	*Lama3, Odam*	*Ambn*
mmu-miR-667	*Pex26*	
mmu-miR-706		*Ambn, Trip13, Smpx, Cdc25c, Rbp1, Apip, Csrp3, Med4*
mmu-miR-466h		
mmu-miR-145	*Fam20a, Ror1, Papss2*	
mmu-miR-1224	*Papss2, Pex26, Fkbp9*	*Cryz, Psmc1*
mmu-miR-471		
mmu-miR-711	*Xnpep2, Pex26, C7orf50*	*Grb10, Myl1, Tubgcp2, Snrnp25, Myd88, Ddx21*
mmu-miR-698	*Odam*	
mmu-miR-684	*Fkbp9*	*Aldh8a1, Med4, Psmb7*

Predicted mRNA targets for miRNAs exhibiting higher levels of expression (Figure 3, group 1) in the night phase compared with the light phase tooth germs were identified using TargetScan. Each miRNA can target several mRNAs. Genes coding for enamel matrix proteins are highlighted. The predicted targets found among mRNAs that were differentially expressed are presented in the table.

protein secretion and biomineralization. Thereafter, *in vitro* studies have confirmed that clock genes (*Bmal1, Clock, Per1,* and *Per2*) oscillate in LS8 and HAT-7 ameloblast cell lines at regular circadian 24-h intervals and that clock genes modulate expression of AMELX protein [15,16,24]. Nevertheless, the association between the circadian clock and enamel development remains elusive. There is only one study showing that *Amelx* expression is down-regulated during the night period in developing mouse molars, whereas the expression levels of other genes involved in enamel development (*Lamp1, Car2*, and *Slc4a4*) increased during the dark period [16].

Circadian rhythms and expression of other genes in the tooth germ, including miRNAs, have so far not been studied. We have investigated some aspects of the possible association between the circadian clock and tooth and enamel development using microarray-based expression profiling of both mRNAs and miRNAs. To the best of our knowledge, this is the first study bringing new insights into possible roles of miRNAs in modulating circadian clocks during tooth and enamel development. The most striking finding emerging from our results is that numerous genes and miRNAs exhibit significantly different levels of expression in mouse molar tooth germ during the dark period compared with the light period (Figures 1–3). The other interesting finding is that among differentially expressed genes we found genes involved in enamel formation, i.e. *Ambn, Amelx, Amtn*, *Enam,* and *Odam*. Validation of microarray data using RT-PCR suggested good correlation between the two methods as judged from results obtained with seven genes and four miRNAs (Figure 4).

Table 3. mRNA Target predictions for miRNAs being down-regulated in the night phase.

Down-regulated miRNAs in the night phase	Up-regulated mRNA targets in the night phase	Down-regulated mRNA targets in the night phase
mmu-miR-467g	*Clk1, Fam20a, Pex26, Pcdh7, Hist1h2ad*	*Tacstd, Mybpc1, Aurka, Cdc25c, Mis18a, Ccnb2, Htra1, Myh3, Nup93*
mmu-miR-141	*Bmal1*	
mmu-miR-1188	*Fam20a, Pex26, Fkbp9*	*Myl1, Tead2, Gemin5*
mmu-miR-466f-3p	*Amtn, Lum, Steap2, C1r*	*Ambn, Incenp, Aurka, Ccnb2, Pus1, Nup93*
mmu-miR-322	*Ctnna2, Lnpep, Clu*	*Aurka*
mmu-miR-466c-5p	*Lamc2, Clu*	
mmu-miR-466g	*Rbm8a, Npc2, Tnnt*	*Amelx, Incenp, Mad2l1, Ddx39b, Cryz, Tead2*
mmu-miR-210	*Tpcn1, Ror1*	
mmu-let-7f	*Pex26, C7orf50*	*Trip13, Pleckho1, Tgm2, Rbp1, Med4*
mmu-miR-494	*Ctnna2, Papss2, Arsb, Fkbp9*	*Erh, Tgm2, Mrpl13*
mmu-miR-214	*Mrc2, Ctnna2, Papss2, Muc5ac, Ltpb3, Fkbp9, Myoz1*	*Gart, Pcbp4, Tgm2, Rbp1, Ddx21, Pin4, Tmem48*
mmu-let-7d	*Pex26, C7orf50*	*Trip13, Pleckho1, Tgm2, Rbp1, Med4*
mmu-miR-467f	*Clk1, C1r, Pcdh7, Steap2*	*Cks2, Tacstd, Incenp, Aurka, Pds5b, Pus1, Med4, Hist1h2ad*
mmu-let-7a	*Igf2, Fam20a, Pex26, Stxbp2, Odam, C7orf50*	*Trip13, Pleckho1, Cdc25c, Parp1, Tgm2, Rbp1, Med4*
mmu-let-7b	*Igf2, Fam20a, Pex26, Stxbp2, Odam, C7orf50*	*Trip13, Pleckho1, Cdc25c, Parp1, Tgm2, Rbp1, Med4*
mmu-miR-182	*Clock*	
mmu-miR-574-5p	*Cacna1, Plac8*	*Erh, Mrpl13*
mmu-miR-1187	*Papss2, Pex26, Lum*	*Gemin5, Med4*
mmu-miR-297a	*Papss2, Pex26*	
mmu-miR-805		

Predicted mRNA targets for miRNAs exhibiting lower levels of expression (Figure 3, group 2) in the night phase compared with the light-phase tooth germs were identified using TargetScan. Each miRNA can target several mRNAs. Genes coding for enamel matrix proteins and clock protein are highlighted. The predicted targets found among mRNAs that were differentially expressed are presented in the table.

The present study using mouse molar tooth germ further supports the concept that expression of genes coding for enamel matrix proteins oscillates during 24-h intervals. We detected oscillations in the expression of *Ambn*, *Amelx,* and *Enam* in developing mouse molars; however, the differences in expression for *Enam* were not significant, suggesting that enamelin may not be under circadian control (Figure 4A). The expression of *Ambn* and *Amelx* decreased markedly during the dark period relative to the light period; however, their expression was not arrested during the sampled timepoints in the night period, but instead oscillated (Figures 2 and 4A). This is in accordance with previous study demonstrating that *Amelx* expression is down-regulated during the night period in developing mouse molars [16], and *in situ* hybridization data showing changes in *Amelx* expression in whole mouse incisors [12]. Two other genes involved in enamel development, *Amtn* and *Odam*, exhibited higher levels of expression in the night compared with the light period (Tables 2 and 3). This is the first study to demonstrate that other genes than *Amelx*, i.e. *Ambn, Amtn*, and *Odam*, oscillate in a circadian pattern during development of mouse molar enamel. Presently, the precise molecular understanding of the oscillations of these genes during normal enamel development is still unclear. It is now well known that *Amelx is* involved in both development of normal enamel and in several pathological conditions, which collectively are referred to as amelogenesis imperfecta [25]. It is, therefore, reasonable to speculate that the circadian clock has significant impact in enamel development; however, more investigation using animal models defective in clock-related genes and the influence on enamel matrix proteins is warranted.

Our results also suggest that the expression of genes associated with other functions in the developing tooth such as insulin-like growth factor 2 (*Igf2*), clusterin (*Clu*), and troponin T (*Tnnt*) are up-regulated during the night period (Table 3), when *Amelx* and *Ambn* are down-regulated. Previously, it has been shown that IGF2 is expressed during tooth development and has been connected to epigenetic regulation of the genes, whereas CLU functions during secretory odontogenesis and the early eruptive phase [26,27]. *Tnnt* gene is also expressed in mouse molar tooth germ and is associated with actin cytoskeleton signalling [28]. These results suggest that ameloblasts may segregate some of these functions according to the time of the day, with a possible crucial dependence on production of enamel matrix proteins or reduction during the fabrication cycles. Furthermore, as shown in this study, several other non-enamel specific gene products and associated molecular and cellular functions changes between the night and light period, suggesting that some functions may exhibit different activities according to the circadian rhythm. Bioinformatic analysis suggested highly significant associations between differentially expressed genes and cellular functions relating to gene expression, cell cycle, and cellular development (Table 1).

MiRNAs provide a convenient and efficient pathway for regulation of gene expression at a posttranscriptional level and exert their effects by base pairing with the target by binding to specific mRNAs for cleavage or translational regulation [17]. In general, the expression of the specific miRNA is, therefore, inversely correlated with the expression of corresponding target mRNA. However, it has previously been shown that altered cellular levels of miRNAs may also increase the expression levels of mRNAs [20,29]. By interacting with E-box units situated in gene regulatory regions, the complex formed by CLOCK and BMAL1 proteins governs the gene expression [30,31]. In the present study, we have shown that the circadian clock genes, in particular *Clock* and *Bmal1*, oscillate in mouse molar tooth germ during 24-h intervals (Figures 2 and 4A). Computational prediction using TargetScan suggested that miR-182 targets *Clock* and that miR-141 targets *Bmal1*. Accordingly, our results show that the expression of *Clock* and *Bmal1* is inversely correlated with the expression of miR-182 and miR-141, respectively.

These findings, therefore, suggest that miRNAs, including miR-182 and miR-141, are involved in the control of peripheral circadian rhythms in the developing tooth by regulating the expression of genes coding for circadian transcription factors such as CLOCK and BMAL1. This is in accordance with previous *in vitro* study providing evidence for that *Clock* is a direct target of miR-182 [32]. However, so far there are no studies that have further explored *Bmal1* and miR-141 and validated their computationally predicted sequence-based target relationship. It is noteworthy that there may be additional miRNAs targeting *Clock* and *Bmal1*. Each miRNA may have multiple target genes, and each gene can be targeted by multiple miRNAs. Therefore, it is possible that miRNAs other than miR-182 and miR-141 can target *Clock* and *Bmal1* genes. Because the seed pairing does not have to match perfectly and allows wobbles, as shown in let-7:lin41 and miR-196:HoxB8 pairings [33,34], there may be more clock-relevant miRNAs that cannot be detected by the current prediction algorithms. However, even if other miRNAs contribute to the circadian rhythm during tooth development, our data indicate that *Clock* and *Bmal1* expression is modulated by miRNA interaction with the 3'UTR of *Clock* and *Bmal1* genes.

Moreover, the expression of genes known to be important for enamel formation, i.e. *Ambn, Amelx, Amtn*, *Enam,* and *Odam,* was both inversely and positively correlated with the expression of their respective miRNAs (Tables 2 and 3). The biological significance of these relationships in regard to enamel secretion and biomineralization needs to be further investigated. Although not perfect, computational algorithms allow for the identification of putative miRNA–mRNA targets. However, these target pairs have to be validated and approaches using miRNA mimics and inhibitors may be used for this purpose. That is, if a gene is a true target of a given miRNA, its miRNA mimic will decrease the target gene expression level, while a miRNA inhibitor will increase the target gene expression level. At present, ongoing research in our group further investigates some of these aspects using ameloblast cell lines.

There are many available databases listing predicted mRNA targets for a given miRNA, most of them using different algorithms, e.g. miRANDA, TargetScan, PicTar, DIANA-microT, PITA, and RNA22 [35]. Because the databases use different algorithm to predict mRNA targets for a given miRNA, mRNAs may be high ranked as a true targets in one database while given a low ranking in another database. In this study, we have used TargetScan in order to identify computationally predicted mRNA targets for miRNAs, which were differentially expressed in tooth germs isolated in the light phase compared with the tooth germs isolated in the night phase. TargetScan uses seed match, complementarity, and position contribution [36]. However, it has to be pointed that selecting and using only one database has its limitations; therefore, a combinatorial use of more databases with different algorithms would be favourable.

Collectively, the present study gains new knowledge about the circadian role of miRNAs during enamel development. The hypothesis, that a peripheral clock in ameloblasts regulates enamel development by directing the expression of ameloblast-specific genes, is further strengthened. However, additional studies are required to understand the precise roles of miRNAs and clock genes in enamel formation. Such knowledge will lay the foundation for a better comprehension of abnormalities in enamel and differences among the population. The time spent each day by ameloblasts to accomplish singular functions like secretion and maturation are crucial for the final morphology of dental enamel. It may be speculated that enamel may vary between individuals due to differences in circadian profiles. Therefore, regulation of circadian rhythms may be important for enamel phenotype, predisposing teeth to disease or protecting them against it. The relationships between changes in the expression of clock genes, miRNAs, genes coding for enamel matrix proteins, and dental diseases should further be verified using *in vivo* models. Nevertheless, this initial study, using mouse molar tooth germs, lays the foundation for more research, which may be important for diagnosis and treatment of diseases associated with enamel formation.

Disclosure statement

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References

[1] Richards J, Gumz ML. Mechanism of the circadian clock in physiology. Am J Physiol Regul Integr Comp Physiol. 2013;304:R1053–R1064.
[2] Kondratov RV, Shamanna RK, Kondratova AA, et al. Dual role of the CLOCK/BMAL1 circadian complex in transcriptional regulation. FASEB J. 2006;20:530–532.
[3] Kumaki Y, Ukai-Tadenuma M, Uno KD, et al. Analysis and synthesis of high-amplitude Cis-elements in the mammalian circadian clock. Proc Natl Acad Sci USA. 2008;105:14946–14951.
[4] Sehic A, Nirvani M, Risnes S. Incremental lines in mouse molar enamel. Arch Oral Biol. 2013;58:1443–1449.
[5] DeBruyne JP, Weaver DR, Reppert SM. Peripheral circadian oscillators require CLOCK. Curr Biol. 2007;17:R538–R539.
[6] King DP, Takahashi JS. Molecular genetics of circadian rhythms in mammals. Annu Rev Neurosci. 2000;23:713–742.
[7] Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature. 2002;418:935–941.
[8] Schibler U, Sassone-Corsi P. A web of circadian pacemakers. Cell. 2002;111:919–922.
[9] Fu L, Patel MS, Bradley A, et al. The molecular clock mediates leptin-regulated bone formation. Cell. 2005;122:803–815.
[10] Zvonic S, Ptitsyn AA, Kilroy G, et al. Circadian oscillation of gene expression in murine calvarial bone. J Bone Miner Res. 2007;22:357–365.
[11] Zheng L, Papagerakis S, Schnell SD, et al. Expression of clock proteins in developing tooth. Gene Expr Patterns. 2011;11:202–206.
[12] Xu Y, Zhou YL, Luo W, et al. NF-Y and CCAAT/enhancer-binding protein alpha synergistically activate the mouse amelogenin gene. J Biol Chem. 2006;281:16090–16098.
[13] Smith CE, Nanci A. Secretory activity as a function of the development and maturation of ameloblasts. Connect Tissue Res. 1989;22:147–156.
[14] Simmer JP, Papagerakis P, Smith CE, et al. Regulation of dental enamel shape and hardness. J Dent Res. 2010;89:1024–1038.

[15] Athanassiou-Papaefthymiou M, Kim D, Harbron L, et al. Molecular and circadian controls of ameloblasts. Eur J Oral Sci. 2011;119(Suppl 1):35–40.
[16] Lacruz RS, Hacia JG, Bromage TG, et al. The circadian clock modulates enamel development. J Biol Rhythms. 2012;27:237–245.
[17] Ambros V. The functions of animal microRNAs. Nature. 2004;431:350–355.
[18] Asli NS, Pitulescu ME, Kessel M. MicroRNAs in organogenesis and disease. Curr Mol Med. 2008;8:698–710.
[19] Chen R, D'Alessandro M, Lee C. miRNAs are required for generating a time delay critical for the circadian oscillator. Curr Biol. 2013;23:1959–1968.
[20] Sehic A, Risnes S, Khuu C, et al. Effects of *in vivo* transfection with anti-miR-214 on gene expression in murine molar tooth germ. Physiol Genomics. 2011;43:488–498.
[21] 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.
[22] Benjamini Y, Drai D, Elmer G, et al. Controlling the false discovery rate in behavior genetics research. Behav Brain Res. 2001;125:279–284.
[23] Risnes S. Growth tracks in dental enamel. J Hum Evol. 1998;35:331–350.
[24] Zheng L, Seon YJ, Mourao MA, et al. Circadian rhythms regulate amelogenesis. Bone. 2013;55:158–165.
[25] Wright JT. The molecular etiologies and associated phenotypes of amelogenesis imperfecta. Am J Med Genet A. 2006;140:2547–2555.
[26] Khan QE, Sehic A, Khuu C, et al. Expression of Clu and Tgfb1 during murine tooth development: effects of *in-vivo* transfection with anti-miR-214. Eur J Oral Sci. 2013;121:303–312.
[27] Khan QE, Sehic A, Skalleberg N, et al. Expression of delta-like 1 homologue and insulin-like growth factor 2 through epigenetic regulation of the genes during development of mouse molar. Eur J Oral Sci. 2012;120:292–302.
[28] Sehic A, Khuu C, Risnes S, et al. Differential gene expression profiling of the molar tooth germ in peroxisome proliferator-activated receptor-alpha (PPAR-alpha) knockout mouse and in wild-type mouse: molar tooth phenotype of PPAR-alpha knockout mouse. Eur J Oral Sci. 2009;117:93–104.
[29] Krutzfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs *in vivo* with 'antagomirs'. Nature. 2005;438:685–689.
[30] Ueda HR, Hayashi S, Chen W, et al. System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat Genet. 2005;37:187–192.
[31] Gekakis N, Staknis D, Nguyen HB, et al. Role of the CLOCK protein in the mammalian circadian mechanism. Science. 1998;280:1564–1569.
[32] Saus E, Soria V, Escaramis G, et al. Genetic variants and abnormal processing of pre-miR-182, a circadian clock modulator, in major depression patients with late insomnia. Hum Mol Genet. 2010;19:4017–4025.
[33] Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. Science. 2004;304:594–596.
[34] Reinhart BJ, Slack FJ, Basson M, et al. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. Nature. 2000;403:901–906.
[35] Ekimler S, Sahin K. Computational Methods for MicroRNA Target Prediction. Genes (Basel). 2014;5:671–683.
[36] Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120:15–20.