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Molecular and circadian controls of ameloblasts

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Stage-specific expression of ameloblast-specific genes is controlled by differential expression of transcription factors. In addition, ameloblasts follow daily rhythms in their main activities (i.e. enamel protein secretion and enamel mineralization). This time-related control is orchestrated by oscillations of clock proteins involved in the regulation of circadian rhythms. Our aim was to identify the potential links between daily rhythms and developmental controls of ameloblast differentiation. The effects of the transcription factors distal-less homeobox 3 (*Dlx3*) and runt-related transcription factor 2 (*Runx2*), and the clock gene nuclear receptor subfamily 1, group D, member 1 (*Nr1d1*), on secretory and maturation ameloblasts [using stage-specific markers amelogenin (*Amelx*), enamelin (*Enam*), and kallikrein-related peptidase 4 (*Klk4*)] were evaluated in the HAT-7 ameloblast cell line. *Amelx* and *Enam* steady-state mRNA expression levels were down-regulated in *Runx2* over-expressing cells and up-regulated in *Dlx3* over-expressing cells. In contrast, *Klk4* mRNA was up-regulated by both *Dlx3* and *Runx2*. Furthermore, a temporal and spatial relationship between clock genes and ameloblast differentiation markers was detected. Of interest, clock genes not only affected rhythmic expression of ameloblast-specific genes but also influenced the expression of *Runx2*. Multiscale mathematical modeling is being explored to further understand the temporal and developmental controls of ameloblast differentiation. Our study provides novel insights into the regulatory mechanisms sustaining ameloblast differentiation.

Dental enamel is formed mainly during two distinct developmental stages (1, 2): the secretory stage (at the end of which the full thickness of enamel is completed), and the maturation stage (during which residual organic material is removed and the tissue is eventually occluded by hydroxyapatite crystals). Ameloblasts, the cells responsible for making enamel, are specialized epithelial cells with distinct morphological features that change during ameloblast differentiation (2). Secretory and maturation ameloblasts are characterized by restricted expression of enamel stage-specific genes and by stage-specific functions (3–8). However, the control of gene expression in ameloblasts, resulting in specialized functions that direct enamel secretion and maturation, is unclear (9–13).

Mineralized tissue development results from a complex temporo-spatial expression of adhesion molecules and growth and transcription factors. Different mineralized tissues share common signaling pathways. Runt-related transcription factor 2 (*Runx2*) and distal-less homeobox 3 (*Dlx3*) are both key regulatory transcription factors that control bone formation (14). *Runx2* is also expressed by maturation-stage ameloblasts during enamel formation and *Runx2* mutations result in enamel abnormalities (15). Similarly to *Runx2*, *Dlx3* is strongly expressed in ameloblasts (16) and *Dlx3* mutations are linked to amelogenesis imperfecta (AI) (17). This study aimed to elucidate the transcriptional targets of *Runx2* and *Dlx3* during amelogenesis.

In addition to the stage-specific regulation by transcription factors, it has long been suggested that gene expression and dental tissue formation are under circadian control both in rodents and in humans. Previous studies demonstrated that the formation of incremental lines in rat dentin reflect a circadian rhythm in the synthesis and secretion of collagen (18). Similarly to rat dentin, human enamel is formed by appositional growth, leaving growth marks on the enamel surface every 24 h during the secretory stage (19). In addition, the secretion of amelogenin (AMELX) clearly shows daily oscillations (20). At a later stage of development – the maturation stage – ameloblasts oscillate between smooth-ended and

ruffle-ended morphologies every 8 h in rat and express a different set of proteins at each part of their cycle (2). Therefore, ameloblast differentiation is directly correlated with cyclical gene expression and specialized cell functions. However, no direct evidence for a 'dental' circadian clock exists. It is also unclear if genes expressed in ameloblasts are under circadian control and how circadian control affects ameloblast differentiation and enamel formation. This is the first study that aims to elucidate how clock genes regulate formation and maturation of mineralized tissues and how stage-specific regulation is linked to daily circadian controls.

Material and methods

Cell culture and study of circadian effects

The ameloblast-like cell line, HAT-7 (21), was cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM)/F12 (1:1, volume by volume) containing L-glutamine, 15 mM HEPES, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cells were passaged just before confluence and plated in six-well plates. For measuring the circadian effects of clock genes, HAT-7 cells were allowed to reach 80% confluence and then the medium was supplemented with 0.1 mM forskolin. Forskolin is known to induce cell cycle synchronization of cultured cells (22). Total RNA was harvested every 4 h for 28 h using TRIzol (Invitrogen). Two micrograms of RNA were reverse transcribed with TaqMan reverse transcription reagents (Applied Biosystems, Branchbury, NJ, USA), following the manufacturer's recommendations. cDNA was then quantified and used for real-time quantitative RT-PCR (qRT-PCR). qRT-PCR was conducted using SYBR Green (Invitrogen) and specific primers (Table 1) for beta-actin (*Actb*), *Amelx*, enamelin (*Enam*), ameloblastin (*Ambn*), matrix metalloproteinase 20 (*Mmp20*), kallikrein-related peptidase 4 (*Klk4*), aryl hydrocarbon receptor nuclear translocator-like (*Bmlα*1), nuclear receptor subfamily 1, group D, member 1 (*Nr1d1*), *Dlx3*, and *Runx2*.

Transfection and real-time qRT-PCR

HAT-7 cells were cultured in DMEM/F12 (1:1, volume by volume) containing L-glutamine, 15 mM HEPES, and 10% fetal bovine serum (Invitrogen), then passaged and plated in six-well plates. The cells were then transfected at 80% confluence with 2 mg of Nr1d1 or Dlx3 (gifts of Dr. Maria M. Morasso, Developmental Skin Biology Section, NIAMS-NIH, DC, USA) or Runx2 (a gift of Dr. Renny Franceschi, University of Michigan, Ann Arbor, MI, USA) or control (empty pCDNA) expression vectors using Lipofectamine LTX and Plus Reagent (Invitrogen). Total RNA was isolated 24 or 48 h later from HAT-7 cells using TRIzol (Invitrogen), and 2 μg of RNA was reversely transcribed with following the manufacturer's recommendations (Applied Biosystems). The resulting cDNA was then amplified by qRT-PCR. RT-PCR amplifications were performed at 95°C for 30 s, at 60°C for 30 s, and at 72°C for 30 s using specific primers (Table 1). The relative expression levels for each gene were calculated based on the expression levels of *Actb* and the differences are presented in graphs using the $2^{-\Delta\Delta C_T}$ method. *P*-values were calculated using two-sample *t*-test. RT-PCR products were also subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and mRNA expression was confirmed by direct sequencing.

Table 1
RT-PCR primers

Gene name	Sequence 5′–3′
Ambn	Forward: GTCCAGAAGGCTCTCCACTG
	Reverse: GTCATTGGGGAAAGCAAGAA
Amelx	Forward: TACCACCTCATCCTGGAAGC
	Reverse: CTGTTGAGACAGCACAGGGA
Dlx3	Forward: ACCCAGTGTCGGTGAAAGAG
	Reverse: GCCAGATACTGGGCTTTCTG
Enam	Forward: GATGCCCATGTGGCCTCCACCA
	Reverse: GCCAAATGGTGGGAATGGCTGA
Klk4	Forward: ACAAGGGCTCGTGTCTATGG
	Reverse: GTCTCAGGTTCCCTCAGCAG
Mmp20	Forward: AGCTCGTCCTTTGATGCAGT
	Reverse: TGGACATTAGCTGGGGAAAG
Nr1d1	Forward: CTTCCGTGACCTTTCTCAGCA
	Reverse: TGTGCGGCTCAGGAACATCAC
Runx2	Forward: CCGTCCATCCACTCTACC
	Reverse: TGCCTGGCTCTTCTTACTG
Actb	Forward: AAGTACCCCATTGAACACGG
	Reverse: ATCACAATGCCAGTGGTACG
*Bmlα*1	Forward: CCAAGAAAGTATGGACACAGACAAA
	Reverse: GCATTCTTGATCCTTCCTTGGT

Detailed sequence information is provided here for all the primers used in this study. The following gene symbols are used in this table: beta-actin (*Actb*), amelogenin (*Amelx*), enamelin (*Enam*), ameloblastin (*Ambn*), matrix metalloproteinase 20 (*Mmp20*), kallikrein-related peptidase 4 (*Klk4*), aryl hydrocarbon receptor nuclear translocator-like (*Bmlα*1), nuclear receptor subfamily 1, group D, member 1 (*Nr1d1*), distal-less homeobox 3 (*Dlx3*), and runt-related transcription factor 2 (*Runx2*).

Results

Effects of *Runx2* and *Dlx3* on ameloblast-specific gene expression

HAT-7 cells were transfected with expression vector containing *Runx2* and the changes in mRNA expression levels for stage-specific ameloblast genes (i.e. *Amelx*, *Enam*, and *Klk4*) were evaluated by qRT-PCR. Our data showed that *Runx2* down-regulates *Enam* mRNA levels (Fig. 1A) and *Amelx* mRNA levels (data not shown) and up-regulates *Klk4* mRNA levels (Fig. 1A). HAT-7 cells were also transfected with the expression vector containing *Dlx3*. The ameloblast-specific mRNA *Amelx* and *Enam* (markers of secretory ameloblasts) and *Klk4* (a marker of maturation-stage ameloblasts) were all up-regulated upon the over-expression of *Dlx3* in HAT-7 cells (Fig. 1B). Cells transfected with a control vector (pCDNA) showed no significant changes.

Effects of *Nr1d1* on ameloblast-specific gene expression

Cell cycle synchronized HAT-7 cells were used to evaluate the levels of expression of clock genes in ameloblasts at regular daily intervals. Several clock genes were

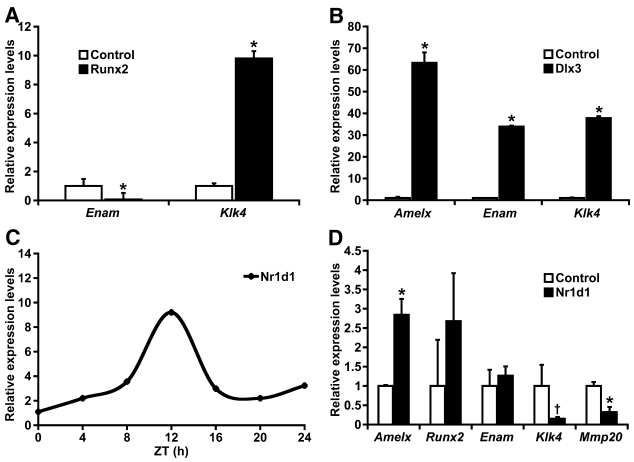

Fig. 1. (A) Effects of runt-related transcription factor 2 (*Runx2*) on HAT-7 ameloblasts. Over-expression of *Runx2* resulted in the down-regulation of enamelin (*Enam*) ($P < 0.05$) and in the up-regulation of kallikrein-related peptidase 4 (*Klk4*) ($P < 0.05$). Experiments were performed in triplicate. (B) Effects of distal-less homeobox 3 (*Dlx3*) on HAT-7 ameloblasts. Over-expression of *Dlx3* encoding full-length protein (1–287 amino acids) resulted in the up-regulation of amelogenin (*Amelx*), *Enam*, and *Klk4* ($P < 0.05$ for all three genes). All data were evaluated 24 h after transfection. (C) Circadian oscillations at the RNA level were found for clock genes in ameloblasts after cell cycle synchronization using forskolin. The results are shown here for nuclear receptor subfamily 1, group D, member 1 (*Nr1d1*), which showed a surge in expression 12 h after cell cycle synchronization ZT, Zeitgeist. (D) Transfection of HAT-7 cells with *Nr1d1* resulted in statistically significant up-regulation of *Amelx* ($P < 0.05$) and down-regulation of *Klk4* ($P < 0.056$) and *Mmp20* ($P < 0.05$). In addition, the levels of *Runx2* mRNA were also up-regulated 24 h after transfection of HAT-7 cells with *Nr1d1* (not statistically significant). In contrast, steady-state mRNA levels of *Enam* remained unchanged upon the over-expression of *Nr1d1*. *Statistically significant changes; †Very close to being statistically significant changes.

detected and found to oscillate at the RNA level. One of the most regularly oscillated clock mRNA in ameloblasts was *Nr1d1* (Fig. 1C). We then decided to evaluate if over-expression of *Nr1d1* changes the expression of mRNA levels of ameloblast-specific genes. Over-expression of *Nr1d1* resulted in the up-regulation of *Amelx* and in the down-regulation of *Mmp20* and *Klk4* mRNAs (Fig. 1D). In contrast, the expression of *Enam* (Fig. 1D) and *Ambn* (not shown) mRNAs was unchanged upon the over-expression of *Nr1d1*. Furthermore, we also evaluated changes in the levels of expression of *Runx2*, a key regulator of ameloblast-specific genes (Fig. 1D).

Multilevel and time-dependent control of ameloblast-specific gene expression and cell functions

We are also analyzing how the stage-specific regulation and circadian control networks govern ameloblast differentiation and enamel formation using a multiscale modeling approach. In our computational approach, cells are modeled as discrete entities that respond to intracellular and extracellular signals, which are modeled continuously with differential equations. Key circadian clock genes involved in amelogenesis are being integrated into a Boolean gene network. In their simplest form, Boolean models are interaction networks where each biochemical species is represented as a node in one of two possible states: expressed ('on' or 1) or non-expressed ('off' or 0) (23). Transfer functions between states are derived from biochemical interactions using logical operators (e.g. AND, OR, and NOT). The response to signals from the intracellular gene network determines whether each cell differentiates, proliferates or dies, and therefore directly influences the cellular and the extracellular tissue scales. The spatial distribution of cells is computed using a continuous macroscopic tissue model based on the viscous liquid theory of tissue dynamics. Finally, the number and spatial configuration of cells are used to activate tissue signals, which in turn were input into the Boolean model (Fig. 2). This combination of discrete and continuous modeling of several steps of amelogenesis will be used to analyze key cellular events (such as ameloblast extension of differentiation) and to predict the most important regulatory networks necessary for enamel formation. This multiscale modeling approach provides a powerful tool for addressing questions of how cells interact with each other and their environment, and how these interactions, in turn, affect gene expression.

Discussion

Our laboratory focuses on the study of gene expression during ameloblast differentiation. Enamel formation depends largely on a complex temporo-spatial expression of adhesion molecules and growth and transcription factors, described in early tooth development (24), which continues during cell differentiation and enamel formation. During the first stage of amelogenesis, secretory ameloblasts delineate the enamel space, and *Amelx*, *Enam*, and *Ambn* proteins are secreted and assembled to form an extracellular framework (25). Ameloblasts then transport calcium and phosphate ions into this framework, forming hydroxyapatite crystallites (4). During enamel formation, the organic materials in the matrix are

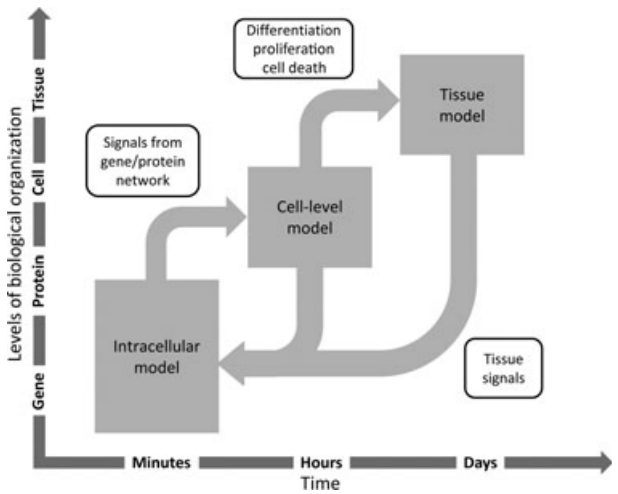

Fig. 2. A multiscale Boolean model is being designed to predict the complex interactions between circadian controls and stage-specific regulators such as runt-related transcription factor 2 (*Runx2*) and distal-less homeobox 3 (*Dlx3*) that control gene expression in ameloblasts and ultimately orchestrate ameloblast differentiation and enamel formation. A schematic view is presented of the multiscale nature of our model composed of four different levels. At the genetic level we integrate the main genes involved in the regulation of amelogenesis within a Boolean network, which results in regulatory signals that control differentiation. The response to these signals occurs at the cellular level, determining whether each cell progresses through differentiation or dies. Given this information, at the macroscopic model the new spatial distribution of the cells is computed at the tissue level. The number and spatial configuration of cells determine the activation of the regulatory signals, which in turn input to the genetic level. Clock genes induce daily oscillations of key transcription factors, which, in the model, activate stage-specific ameloblast genes at the genetic level.

degraded by two proteases – *Mmp20* and *Klk4* – leaving behind a fluid-filled porous tissue where secondary crystal growth and mineral accretion can occur to go on to produce the final mature enamel. Defects in the formation of enamel are seen in patients with AI, and mutations in *AMELX*, *ENAM*, *KLK4*, and *MMP20* have been implicated in the etiology of AI (26). Of all these genes, expression of *ENAM* is exclusive to secretory ameloblasts (27). *Amelx* and *Mmp20* are expressed in secretory ameloblasts as well as in odontoblasts (5), whereas *Klk4* is mainly expressed in the maturation-stage ameloblasts (28).

The aim of this study was to test the hypothesis that *Runx2* and *Dlx3* are involved in ameloblast stage-specific gene regulation. We found that *Runx2* down-regulates *Enam* and up-regulates *Klk4*. This is consistent with the developmental expression patterns of *Enam* and *Klk4*. The expression of *Runx2* is initiated at the end of the secretory stage when *Enam* expression is suppressed. *Runx2* expression continues during the maturation stage when *Klk4* is exclusively expressed. Therefore, we propose that *Runx2* is a key regulator of ameloblast differentiation with a role which involves suppressing genes expressed in the secretory stage, such as *Amelx* and *Enam* and up-regulating genes of the maturation stage, such as *Klk4*. Further *in vivo* studies are needed to confirm these preliminary indications and to identify any partners of *Runx2* that may be involved in the down-regulation or up-regulation of ameloblast genes studied here.

Dlx3 is another major player in amelogenesis. *Dlx3* is strongly expressed by ameloblasts (16) and *Dlx3* mutations result in AI (17). In this study, we showed that *Dlx3* up-regulates the expression of *Amelx*, *Enam*, and *Klk4*. These findings are consistent with suggested roles of *Dlx3* during both the secretory and the maturation stages of amelogenesis. Our data are also in accordance with previous studies that reported *Amelx* regulation by *Dlx2* based on gel-shift assays and promoter *Dlx2*-binding site predictions (29). It is possible that synergistic and/or competitive relationships between DLX proteins take place during amelogenesis. More studies are needed to clarify the precise roles of the *Dlx* family of transcription factors in amelogenesis. Nevertheless, our data support a key role of *Dlx3* in ameloblast differentiation.

Circadian rhythms are self-sustained endogenous oscillations that occur over a 24-h period. They correspond to the environmental light–dark cycles of an organism but persist even after the light–dark stimulus has been removed. These biological rhythms are involved in most physiological processes. Although there is a site in the suprachiasmatic nucleus of the brain that is considered as the 'master clock', peripheral clocks have been found in several tissues in the body. The relationship between these two types of circadian biological clocks is, as yet, unclear (30). Several genes have been identified as core maintainers of the circadian rhythm. The main mammalian genes include Circadian Locomotor Output Cycles Kaput (*Clock*), Brain and Muscle Aryl Hydrocarbon Receptor Nuclear Translocation (*ARNT*)-like (*Bmal1*), Period 1 (*Per1*), Period 2 (*Per2*), Period 3 (*Per3*), Cryptochromes (*Cry1*) and *Cry2*. The genes *Nr1d1*, *Nr1d2*, RAR-Related Orphan Receptor Alpha (*Rorα*), and Albumin D-binding protein (*Dbp*) also play a key role in modifying the expression of the main clock genes (31). Transcription of these 'clock genes' oscillates over a 24-h period and their output signals induce rhythms of target gene expression that create patterns in physiological processes. Inducing a rhythm involves the binding of a clock gene transcription factor to the promoter region of a clock-controlled gene (32).

We have recently shown that clock genes and clock proteins are expressed during ameloblast differentiation (20, 33). This present study further supports the concept that clock genes are expressed in ameloblasts and that their expression oscillates during 24-h intervals. Furthermore, we presented evidence showing that clock genes regulate several ameloblast stage-specific genes, supporting the idea that clock genes are key regulators of ameloblast differentiation. These data are consistent with our previous discoveries reporting that the amounts of AMELX secreted vary during different daily intervals (20). In addition, we showed that the over-expression of *Nr1d1* results in the up-regulation of *Runx2* mRNA, a key transcription factor strongly expressed in maturation ameloblasts. Our data also showed that *Runx2* regulates the expression of *Enam* and *Klk4* mRNAs. We therefore hypothesize that clock genes may regulate the daily

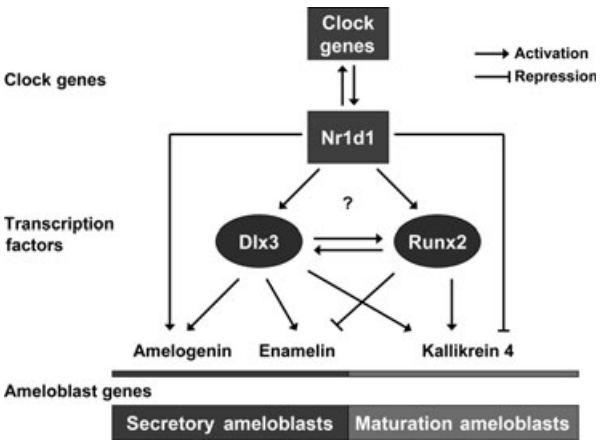

Fig. 3. This cartoon summarizes our findings reported here. Gene regulation in ameloblasts is orchestrated by stage-specific controls, as shown for amelogenin (*Amelx*), enamelin (*Enam*), and kallikrein-related peptidase 4 (*Klk4*) regulation by runt-related transcription factor 2 (*Runx2*) and distal-less homeobox 3 (*Dlx3*). Gene expression in ameloblasts is also subject to circadian controls, as shown for *Amelx*, matrix metalloproteinase 20 (*Mmp20*), *Klk4*, and *Runx2* regulation by nuclear receptor subfamily 1, group D, member 1 (*Nr1d1*). Accordingly, we postulate that circadian control of ameloblast genes can be direct but also indirect (e.g. through *Runx2*). It is currently unknown if *Dlx3* is also subject to circadian regulation. It is also unknown if *Runx2* and/or *Dlx3* can regulate the expression of clock genes in ameloblasts in a negative-feedback loop. Another complexity is that clock genes regulate each other, resulting in complex network interactions. We are currently analyzing these networks and their effects on ameloblast differentiation and enamel formation using mathematical modeling.

variations of gene expression in ameloblasts either directly, by regulating their transcriptional rates, or indirectly, by regulating the expression of key transcription factors (*Runx2* in our case) that regulate the expression of ameloblast genes. More studies are needed to understand the precise roles of clock genes in enamel formation. Nevertheless, we suggest that in addition to the stage-specific controls, amelogenesis is subject to very precise, rhythmic daily controls of gene-expression levels and cell activity (Fig. 3).

In conclusion, our study offers novel insights into the role of clock genes in ameloblast differentiation and explores the potential links between circadian control and stage-specific regulation of ameloblast genes. Our hypothesis, that an ameloblast peripheral clock regulates enamel formation orchestrating the expression of ameloblast-specific genes, is further strengthened. Direct links between changes in the expression of clock genes and dental diseases remain to be confirmed using *in vivo* models. Nevertheless, this initial study, using an ameloblast cell line, lays the foundation for more research in the chronobiology of tooth development and diseases.

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Conflicts of interest – The authors declare no conflicts of interest.

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