A potential role of p75NTR in the regulation of circadian rhythm and incremental growth lines during tooth development

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Objective: Tooth morphogenesis and the formation of hard tissues have been reported to be closely related to circadian rhythms. This study investigates the spatiotemporal expression and relationship of p75NTR with core clock genes, mineralization-related or odontogenesis-related genes, and aims to derive the potential role of p75NTR in regulating circadian rhythm and incrementality growth line formation during tooth development.

Materials and methods: The dynamic morphology of the rat dental germ was observed at seven stages (E14.5 d, E16.5 d, E18.5 d, P.N. 4 d, P.N. 7 d, P.N. 10 d, and P.N. 15 d). Next, the expressions of p75NTR and other target factors were traced. The ectomesenchymal stem cells (EMSCs) were isolated from the E18.5d rat dental germs and synchronized using 50% of fetal bovine serum. Then, they were cultured in light/light (L.L.), dark/dark (D.D.), and light/dark (L.D.) conditions for 48 h. The total RNA was collected every 4 h, and the circadian rhythm dynamics of target factors were observed. To reveal the mechanism further, p75NTR was down-regulated in p75NTRExIII−/− mice and up-regulated in immortalized mouse dental apical papilla progenitor cells. The change tendencies of other target factors were also detected.

Results: The clock genes Bmal1, Clock, Per1, and Per2 were all expressed in tooth germs before the formation of dental hard tissues and demonstrated a regular oscillating expression pattern in EMSCs from dental germs. Their expression was affected by the L.D. stimulus, and most of them were promoted by D.D. conditions. p75NTR presented a similar expression pattern and a positive or negative relationship with most clock genes, mineralization-related and odontogenesis-related factors, such as brain and muscle ARNT-like protein-1 (Bmal1), runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), MSH-like 1 (MSX1), dentin matrix acidic phosphoprotein 1 (Dmp1), and dentin sialophosphoprotein (Dspp). Moreover, the arrangement, morphology, and even boundary in pre-odontoblast/pre-ameloblast layers were disordered in the p75NTRExIII−/− mice.
Conclusion: Circadian rhythm was found to affect tooth development. p75NTR might play a crucial role in regulating clock genes in the mineralization and formation of the dental hard tissues. p75NTR is actively involved in the odontoblast-ameloblast junction and cell polarity establishment during tooth morphogenesis.

KEYWORDS
p75 neurotrophin receptor, circadian rhythm, mineralization, cell polarity, tooth development

Introduction

Most physiological and behavioral processes, such as hormone secretion, metabolism, growth, sleep, among others, are governed by circadian rhythms, which are managed by internal biological clocks (Richards and Gumz, 2013; Neumann et al., 2019). The suprachiasmatic nucleus (SCN), located in the brain’s anterior hypothalamus, is generally considered the “master clock” controlling the circadian rhythms (Weaver, 1998). Moreover, peripheral clocks have been discovered in several body tissues and found to be regulated by the SCN via a transcriptional-translational feedback loop (TTFL) (Okawa et al., 2020; Allada and Bass, 2021). Importantly, circadian locomotor output cycles kaput (Clock) and brain and muscle ARNT-like protein-1 (Bmal1) serve as positive feedback signals, form heterodimers, and activate the transcription of period (Per) and cryptochrome (Cry) genes by binding to a cis-regulatory enhancer sequence known as the E-box element on the target gene promoter (Gekakis et al., 1998; Shearman et al., 2000). After reaching a certain concentration, Per and Cry proteins are phosphorylated and translocated into the nucleus to inhibit the transcriptional activation of Clock/Bmal1 by competitively binding to the E-box element (Brown et al., 2012). This negative TTFL acts as a core circadian regulator to maintain the 24-h rhythm.

Clock genes play an essential role in tooth development (Nirvani et al., 2017; Papakyrikos et al., 2020). Clock, Bmal1, Per, and Cry are expressed in dental tissues, especially in dental hard tissues during tooth development (Zheng et al., 2011; Lacruz et al., 2012). The phenomenon of regular incremental growth lines (e.g., daily Retzius’s lines in enamel, von Ebner’s lines in dentine) implies that the formation of dental hard tissues characterizes the circadian rhythm and is tightly controlled by the time (Antoine et al., 2009; Lacruz et al., 2012). The collagen production in dentin follows a 12 hour-pattern, with twice as much collagen secreted during the 12 h of daylight than the 12 h of nighttime (Lopez Franco et al., 2006). Previous studies have demonstrated that tooth morphogenesis and hard tissue formation have been closely related to circadian rhythms (Lacruz et al., 2012). However, how the core clock genes affect tooth development is still unclear.

p75 neurotrophin receptor (p75NTR) demonstrated a strong expression in epithelial-mesenchymal interaction, dental papilla, and dental follicle during tooth development, and positively regulated the mineralization in ectomesenchymal stem cells (EMSCs) (Wen et al., 2012; Li et al., 2017). Further research revealed that the incisors’ daily mineralization speed and the incremental growth line width were significantly lower in p75NTR knockout mice than in wild-type mice (Wang et al., 2020; Zhao et al., 2020). There is also evidence that p75NTR regulates tooth morphogenesis and mineralization along with the circadian rhythm and incremental growth line formation during tooth development. p75NTR, a member of the tumor necrosis factor receptor superfamily, has been reported to manage a wide range of biological functions via multiple intracellular signaling pathways. Recently, p75NTR was reported to be controlled by Clock/Bmal1 by bonding to the E-box element and was considered a clock gene–regulating oscillatory component of circadian rhythms (Baeza-Raja et al., 2013). Therefore, p75NTR might act as a clock-controlled gene in tooth development and regulate periodic mineralization during incremental growth line formation.

The aims of this study are to investigate the spatiotemporal expression and relationship of p75NTR with core clock genes, mineralization-related or odontogenesis-related genes, and further to reveal the potential role of p75NTR in regulating the circadian rhythms and incremental growth line formation during tooth development via the in vivo experiment of p75NTR+/− knockout mice and in vitro experiment of EMSCs cultured under light/light (L.L.), dark/dark (D.D.), and light/dark (L.D.) conditions, which would contribute to illustrate the circadian rhythm and biomineralization in tooth development.

Materials and methods

Experimental animals

Sprague–Dawley (S.D.) rats were provided by the Chongqing Key Laboratory of Oral Diseases and Biomedical Sciences, Chongqing Medical University. p75NTR knockout mice used in this study were gifted by the Jackson Laboratory (#:031,162). In 1992, it was reported that these mutant mice have a targeted deletion of exon III of the p75NTR (p75NTR+/−) and could not express functional full-length p75NTR (Lee et al., 1992). The presence of a vaginal plug is considered embryonic day 0.5 (E 0.5 d), and the day of littermate birth is regarded as post-natal day 1 (PN1 d). All procedures were approved by the Medical Ethics Committee of the Chongqing Medical University.
FIGURE 1
The images of H.E. staining and immunohistochemistry staining for prenatal rat dental germs. H.E. staining demonstrated that the rat dental germs entered the early cap stage at E14.5 d, the cap stage and early bell stage at E16.5 d, and the bell stage at E18.5 d. The separation between the pre-odontoblast and pre-ameloblast layers occurred in all E18.5 d species. Immunohistochemistry staining revealed that Clock, Per1, and Coll were detected in the epithelial-mesenchymal interaction area, dental follicle, and dental papilla at E14.5 d, and became stronger at E16.5 d when p75NTR, Bmal1, and ALP were detected. All the factors were expressed at E18.5 d, but Cry1 showed the weakest expression. All experiments were repeated three times independently. op: oral epithelium; dp: dental papilla; iee: inner enamel epithelium; oee: outer enamel epithelium; sr: stellate reticulum. The scale bar represents 50 μm, respectively.
FIGURE 2
The images of H.E. staining and immunohistochemistry staining for post-natal rat dental germs. H.E. staining revealed that the morphogenesis of molar cusps was completed, and hard tissues (enamel and dentine) began to form at PN4 d. The hard tissues gradually thickened at PN7 d. The tooth roots began to shape at PN10 d and PN15 d. The separation continued between the inner enamel epithelium and enamel in all the post-natal species. Immunohistochemistry staining showed that all the factors were detected and distributed in odontoblast and ameloblast layers, and then in dental papilla in the post-natal species. All experiments were repeated three times independently. dpc: dental papilla cells; od: odontoblast; am: ameloblast; e: enamel; d: dentin. The scale bar represents 500 and 50 μm, respectively.
H.E. and immunohistochemistry staining

The first molars were dissected from the E14.5 d, E16.5 d, E18.5 d, P.N.4 d, P.N.7 d, P.N.10 d, and P.N.15 d rats (Figures 1, 2) and the E16.5d p75NTR+/- and p75NTR+/+ mice (Figure 4), fixed in 4% paraformaldehyde, decalcified with 10% EDTA, and embedded in paraffin. The 6-µm sections of tissue specimens were obtained for H.E. and immunohistochemistry staining. The primary antibodies were used in this study as follows: rabbit anti-rat p75NTR (1:1,000; Abcam, Cambridge, MA, United States, ab3517, monoclonal), rabbit anti-rat BMAL1 (1:1,000; Abcam, Cambridge, MA, United States, ab230822, monoclonal), rabbit anti-rat CLOCK (1:1,000; Abcam, Cambridge, MA, United States, ab3517, monoclonal), rabbit anti-rat PER1 (1:500; Bioss, Beijing, China, bs-2350R, polyclonal), rabbit anti-rat CRY1 (1:500; Bioss, Beijing, China bs-11441R, polyclonal), rabbit anti-rat ALP (1:500; Bioss, Beijing, China, bs-2928R, polyclonal), rabbit anti-rat COL1 (1:500; Bioss, Beijing, China, bs-0578R, polyclonal), rabbit anti-rat CRY2 (1:500; Bioss, Beijing, China, bs-2578R, polyclonal), rabbit anti-rat BMAL2 (1:1,500; Bioss, Beijing, China bs-0578R, polyclonal), rabbit anti-rat Rhl (1:500; Bioss, Beijing, China bs-2350R, polyclonal), rabbit anti-rat p75NTR (1:500; Bioss, Beijing, China, bs-2928R, polyclonal), and rabbit anti-rat P2X3 (1:1,500; Bioss, Beijing, China bs-0578R, polyclonal). These specimens were treated with the DAB Visualization Kit (ZSGB-BIO, Beijing, China) according to the manufacturer’s protocols, followed by visualization under phase-contrast microscopy.

Isolation and culture of EMSCs from dental germs

The first molars of the upper and lower jaws were dissected from seven to ten embryos of E18.5d rats. The minced tissue was mixed with 1% trypsin/1 mM of the EDTA solution (Sigma, St. Louis, MO, United States) at 37°C for 10 min and neutralized with Dulbecco’s modified Eagle’s medium/Ham’s F12 (DMEM/F12) (Sigma, St. Louis, MO, United States) containing 10% of FBS (Gibco Waltham, MA United States). Next, the suspension was centrifuged at 800 rpm for 5 min. The cell pellet was resuspended in DMEM/F12 supplemented with 10% FBS and antibiotics (100 μg/ml of penicillin and 100 μg/ml of streptomycin) at a density of 2 * 10^6/ml and then cultured at 37°C in a 5% CO2 humidified incubator. The culture medium was changed every 2 days and cells were passaged when the cell density was fused to 70%~80%. E18.5 d rat EMSCs at passage three were used in the following experiment.

Clock synchronization experiment of EMSCs under three conditions

E18.5 d rat EMSCs were seeded into 6-well plates at a density of 1 × 10^4 cells per well and incubated in the routine medium as mentioned above until confluency. They were synchronized using 50% of the horse serum (Gibco Waltham, MA, United States), and total RNA was collected every 4 hours over a circadian cycle at the following zeitgeber time (Z.T.) after synchronization: ZT0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48. The cells were exposed to the serum for 2 h and then incubated under three conditions, namely L.L.-cycle, D.D.-cycle, and 12-h L.D.-cycle (12 h dark and 12 h light), until sample collection.

Genotype identification of p75NTR-knockout mice

All mice were housed under specific pathogen-free conditions (22°C, 12/12-h L.D., 50%~55% humidity) in the Chongqing Key Laboratory for Oral Diseases and Biomedical Sciences. The p75NTR knockout (p75NTR−/−) and wild-type (p75NTR+/+) littermates used in this research were obtained from mating between heterozygous (p75NTR+/−) females and males. The tail DNA was extracted and used to determine their genotypes by PCR analysis (Zhang et al., 2015).

Wild-type mice feeding under D.D. And 12-h D.L. conditions

p75NTR+/− mice were fed and mated under D.D.-cycle and D.L.-cycle (natural day and night), respectively, at PN7 d, four wild-type mice were killed at 7:30 a.m. and 7:30 p.m. The maxilla and mandible of five mice were separated under sterile conditions, and the first molars of mice were removed under the anatomical microscope and then divided into enamel organs and dental papilla. All experiments were repeated at least three times independently. Total RNA was extracted from the isolated dental papilla with the steady pure universal RNA Extraction Kit II and subsequently analyzed quantitatively by RT qPCR.

p75NTR over-expression plasmid constructs and transfection

The coding region of p75NTR was amplified from the cDNAs of mice and then cloned into vector GV492. The plasmid GV492-p75NTR was co-transfected with lentivirus helper plasmids (Helper 1.0 and Helper 2.0) into HEK-293T cells using Lipofectamine 2000 (Invitrogen, United States) according to the manufacturer’s protocol, the negative control carried corresponding fluorescent markers and contain resistant genes, but do not express other target genes. Supernatants containing the virus were collected 72 h following transfection and then infected the immortalized mouse dental apical papilla progenitor cells. iSCAP (Using the previously characterized reversible immortalization system, which expresses
SV40 T antigen flanked with Cre/loxP sites, Wang et al. (2014) demonstrated that the mouse SCAPs can be effectively immortalized with an enhanced proliferative activity was gifted by Chongqing Key Laboratory of Oral Diseases and Biomedical Sciences. The cells were selected with 2 μg/ml of puromycin 48 h later. p75NTR over-expression iSCAP and negative control iSCAP were seeded into 6-well plates at a density of 1 × 10^5 cells per well and incubated in the routine medium until confluence. All experiments were repeated at least three times independently.

### Quantitative RT qPCR

According to the manufacturer’s specifications, the total RNA was extracted using Steady Pure Universal RNA Extraction Kit II. The total RNA was determined using nanodrop spectroscopy before cDNA synthesis using Evo M-MLV Mix Kit with gDNA Clean for qPCR, with Oligo dT at 37°C for 15 min in a 20-μL reaction. Real-time RT-PCR was conducted on 0.01 μg per well cDNA samples with SYBR Green PCR SuperMix (Biorad) using the CFX Connect™ Real-Time PCR Detection System (BioRad) under the following cycling conditions: 95°C for 3 min; 40 cycles of 95°C for 5 s; and 60°C for 30 s. Results were normalized relative to a housekeeping gene’s GAPDH expression. Primers were designed against the following genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), low-affinity neurotrophin receptor p75NTR (p75NTR), MAGE family member D1 (Mage-D1), aryl hydrocarbon receptor nuclear translocator-like (ARNTL or Bmal1), clock circadian regulator (Clock), period circadian clock 1 (Per1), period circadian clock 2 (Per2), runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), collagen type I (Col1), Msh Homeobox 1 (Msh1), distal-less homeobox 1 (Dlx1), dentin matrix acidic phosphoprotein 1 (DMP1) and dentin sialophosphoprotein (Dapp) (Tables 1, 2 enlist the primer sequences used in this study). The specificity of all primers was tested by BLAST, and the melting curve of the RT-PCR result again proved its specificity.

### Statistical analyses

Following the determination of normal distribution by F-test, normally distributed data were analyzed by t test and non-normally distributed data were analyzed by Mann-Whitney test. All the data are expressed as the means ± standard deviations (S.D.). Statistical significance was assessed using the Prism 8.0 software (GraphPad Software, San Diego, CA, United States). p-values < 0.05 were considered statistically significant. All experiments were repeated thrice.

### Results

#### Dynamic histological observation of rat tooth development

H.E. staining demonstrated that the rat dental germs entered the early cap stage at E14.5 d, the cap stage and early bell stage at E16.5 d, and the bell stage at E18.5 d (Figure 1 and Supplementary Figure S1). In the post-natal species, the morphogenesis of molar cusps was completed, and hard tissues (enamel and dentine) began to be detected at PN4 d (Figure 2 and Supplementary Figure S2). Next, the hard tissues gradually thickened at PN7 d. The tooth roots began to shape at PN10 d and PN15 d. Interestingly, the inner enamel epithelium was easy to separate from odontoblast layers in the E18.5 d species during tissue sectioning, indicating that the adhesion between odontoblasts and ameloblasts would weaken at E18.5 d. This separation between the inner enamel epithelium and enamel continued to occur in all the species in the following four post-natal stages.

Immunohistochemistry staining illustrated that Clock, Per1, and Col1 were expressed in the epithelial-mesenchymal interaction area, dental follicle, and dental papilla at E14.5 d (Figure 1 and Supplementary Figure S1). The other p75NTR, Bmal1, Cry1, and ALP factors were either little or no expressed. At E16.5 d, the expressions of Clock, Per1, and Col1 became stronger, and the expressions of p75NTR, Bmal1, and ALP were detected. All factors were expressed at E18.5 d, but Cry1

### TABLE 1 Rat oligonucleotide primers used in this study.

| Genes | Upstream (5’–3’) | Downstream (5’–3’) | Product Size (bp) |
|-------|-----------------|-------------------|------------------|
| GAPDH | AAGTTCAAGCGCACGTCAAG | ACGCCAGTAGACTCCAG | 140 |
| p75NTR | CCGCTCTTTATTTTGTGC | GCGCCCTGTTTATTTTGTGC | 105 |
| Bmal1 | CGTCACTCATACGCAAAC | CGTCACTCATACGCAAAC | 100 |
| Clock | AAAGTGCTCTTGTGATGGAAG | TGTGGCTATGCTGGCTT | 89 |
| Per1 | TTGCTGTATGCTGCTTGTG | TGGCGGACACACTCCTCATACT | 113 |
| Runx2 | CTTCTGGCGCTTCACCA | CTTCTGGCGCTTCACCA | 116 |
| Dlx1 | CAGCCCTACATCAGTTCCG | CTTCTGGCGCTTCACCA | 116 |
showed the weakest expression. In the post-natal species (Figure 2 and Supplementary Figure S2), all the factors were detected and distributed in the odontoblast and ameloblast layers, and further in the dental papilla. Of the eight factors detected in this assay, Bmal1 showed the strongest expression, Per1, Clock, and p75NTR demonstrated a moderate expression, ALP and ColI had an increased expression, and Cry1 had the weakest expression throughout.

In vitro observation of the circadian rhythm dynamics in rat EMSCs

Quantitative RT-PCR analyses demonstrated the temporal expression profiles of p75NTR, clock, mineralization-related and odontogenesis-related genes in E18.5 d EMSCs collected every 4 h during the ZT 0–ZT48 h following serum synchronization (Figure 3). The relative expression levels of p75NTR mRNA were significantly higher at ZT4, ZT24, and ZT36–44 and were lower at ZT16, ZT32, and ZT48 in the L.L. condition. The peak times of p75NTR mRNA expression in the D.D. and L.D. conditions were similar to that in the L.L. condition. Still, the amplitudes in the D.D. condition considerably promoted the mRNA expression of p75NTR, Bmal1, and Clock, indicating a circadian rhythm oscillation in rat EMSCs. However, four peak times were observed in the L.D. condition: ZT0, ZT24, ZT32, and ZT44. The peak times of odontogenesis-related factor Dlx1 revealed the irregular oscillating expressions, namely ZT4, ZT20, ZT32, and ZT40 in the D.D. condition; and ZT0, ZT24, ZT32, and ZT40 in the L.L. condition; ZT8, ZT16, ZT28, ZT40, and ZT40 in the D.D. condition; and ZT0, ZT24, and ZT44 in the L.D. condition.

There are two variables in this experiment: light stimulus and time. The D.D. condition considerably promoted the mRNA expression of Bmal1 and Runx2, then p75NTR. In contrast, the L.L. condition significantly promoted the mRNA expression of Per1. Interestingly, a peak was found at ZT28 in all the six detected factors under the D.D. condition, but this peak became a V-shape (as indicated by the red arrows in Figure 3) under the L.L. condition when the cells were exposed to the second 12-h light stimulus. The results suggested that light stimulus affected the mRNA expression oscillation of clock genes in rat EMSCs.

The mineralization-related factor Runx2 revealed the three mRNA expression peaks at ZT4, ZT28, and ZT48 in both L.L. and D.D. conditions, which were similar to that of Bmal1 and Clock, indicating a circadian rhythm oscillation in rat EMSCs. However, four peak times were observed in the L.D. condition: ZT0, ZT24, ZT32, and ZT44. The peak times of odontogenesis-related factor Dlx1 showed irregular oscillating expressions, namely ZT4, ZT20, ZT32, and ZT40 in the L.L. condition; ZT8, ZT16, ZT28, ZT40, and ZT40 in the D.D. condition; and ZT0, ZT24, and ZT44 in the L.D. condition.

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FIGURE 3

The in vitro observation of circadian rhythm dynamics in rat E18.5 d EMSCs. Quantitative RT-PCR analyses depicted that p75NTR mRNA expression patterns were similar in three conditions, higher at about ZT4, ZT24, and ZT36–44 and lower at about ZT16, ZT32, and ZT48. However, the patterns had highest amplitudes, and a supramaximal peak was detected at ZT4 in the D.D. condition. Of the three clock genes, Bmal1 and Clock showed a similar mRNA expression pattern to p75NTR in three conditions. Per1 was similar in the L.L. condition, but four, not three, peak times presented in the L.D. and D.D. conditions. The mineralization-related factors Runx2 also showed a similar mRNA expression pattern to p75NTR in L.L. and D.D. conditions, but four peak times were observed in the L.D. condition: ZT0, ZT24, ZT32, and ZT44. The odontogenesis-related factor Dlx1 showed irregular oscillating expressions: ZT4, ZT20, ZT32, and ZT40 in the L.L. condition; ZT8, ZT16, ZT28, ZT40, and ZT40 in the D.D. condition; and ZT0, ZT24, and ZT44 in the L.D. condition. Besides, the peak at ZT28 in all the six detected factors in D.D. condition, but it was replaced by a V-shape (as indicated by the red arrows) in the L.D. condition when the cells were exposed to the second 12-h light stimulus. All experiments were repeated three times independently. Data are expressed as mean ± S.D. n = 13 per group.
Identification of \( p75NTR \) knockout mice and the histological observation of dental germs

The genotypes of mice were determined by RT-PCR (Figure 4A). Of the six detected littermates, three with two bands of 280 bp and 345 bp were identified as heterozygous mice, two with one band of 345 bp were identified as wild-type mice, and one with one band of 280 bp was identified as knockout mice (A). H.E. staining revealed that the pre-odontoblast and pre-ameloblast layers tightly adhered to each other in the E16.5 d mouse dental germ (B). The long columnar pre-odontoblasts and short columnar pre-ameloblasts were regularly arranged with apparent polarity and boundary in the wild-type mice. In contrast, the regular shape, polarity, and boundary disappeared in the knockout mice. (C) Immunohistochemical staining was performed on the wild-type mice and \( p75NTR \) knockout mice. The results showed that \( p75NTR \) was almost not expressed in the tooth germs of the knockout mice. dp: dental papilla; po: pre-odontoblast; pa: pre-ameloblast; sr: stellate reticulum. The scale bar represents 50 and 20 \( \mu \)m, respectively. Wild-type mice, \( n = 3 \); \( p75NTR \) knockout mice, \( n = 2 \).

In vivo observation of circadian rhythm dynamics in the dental papilla of the model mice

The results of PN7 d wild-type mice indicated that the gene expression of dental papilla was not only related to D.D. and L.D. conditions, but also related to sampling time (Figure 5). \( p75NTR \) mRNA presented a significant difference between D.D. and L.D. conditions (\( p < 0.05 \)). However, the change tendencies at two sampling times were reversed. \( p75NTR \) mRNA expression in the L.D. condition was significantly higher at 7:30 a.m. and significantly lower at 7:30 p.m. than in the D.D. condition (\( p < 0.05 \)). The results demonstrated that \( p75NTR \) mRNA expression fluctuated in a day and was significantly affected by light stimulus, further confirming that \( p75NTR \) might be involved in regulating the circadian rhythms of dental papilla. The expression pattern of \( Mage-D1 \) was similar to that of \( p75NTR \). Unlike \( p75NTR \) and Mage-D1, all the four clock genes were significantly more expressed in the D.D. condition than in the L.D. condition (\( p < 0.05 \)). Except for \( Bmal1 \), which showed the same big change at both two sampling times of 7:30 a.m. and 7:30 p.m., the changes of Clock, Per1, and Per2 were small at 7:30 a.m. and much larger at 7:30 p.m. The results demonstrated that the clock gene expression in dental papilla fluctuated in a day and greatly increased under the D.D. condition.
The mineralization-related factors of Runx2, ALP, and Col1 revealed the same tendency that their mRNA expressions under the D.D. condition were significantly higher than that under the L.D. condition \((p < 0.05)\), but the change of Runx2 between 7:30 a.m. and 7:30 p.m. in the D.D. condition \((p < 0.05)\). The expression pattern of Msx1 was similar to that of p75NTR. Most factors (Bmal1, Clock, Per1, Per2, Runx2, ALP, Col1, and Dlx1) showed the same change tendency at the two sampling times: mRNA expression was significantly higher in the D.D. condition than that in the L.D. condition \((p < 0.05)\). The change amplitudes of Clock, Per1, Per2, Runx2, and Dlx1 were significantly larger at the sampling time of 7:30 p.m. Contrary to p75NTR, Msx1 mRNA expression in the L.D. condition was significantly lower at 7:30 a.m. and significantly higher at 7:30 p.m. compared with the D.D. condition \((p < 0.05)\). Dmp1 and Dspp mRNA expression in the D.D. condition was significantly lower than that in L.D. condition at both sampling times \((p < 0.05)\). All experiments were repeated at least three times independently. Data are expressed as mean ± S.D. L. D. Condition group, \(n = 11\); D. D. Condition group, \(n = 5\).

**FIGURE 5**

The *in vivo* observation of circadian rhythm dynamics in PN7 d dental germs of wild-type mice. p75NTR mRNA showed a significant difference between D.D. and L.D. conditions \((p < 0.05)\), but the change tendencies at two sampling times were opposite: p75NTR mRNA expression in the L.D. condition was significantly higher at 7:30 a.m. and significantly lower at 7:30 p.m. in the D.D. condition \((p < 0.05)\). The expression pattern of Msx1 was similar to that of p75NTR. Most factors (Bmal1, Clock, Per1, Per2, Runx2, ALP, Col1, and Dlx1) showed the same change tendency at the two sampling times: mRNA expression was significantly higher in the D.D. condition than that in the L.D. condition \((p < 0.05)\). The change amplitudes of Clock, Per1, Per2, Runx2, and Dlx1 were significantly larger at the sampling time of 7:30 p.m. Contrary to p75NTR, Msx1 mRNA expression in the L.D. condition was significantly lower at 7:30 a.m. and significantly higher at 7:30 p.m. compared with the D.D. condition \((p < 0.05)\). Dmp1 and Dspp mRNA expression in the D.D. condition was significantly lower than that in L.D. condition at both sampling times \((p < 0.05)\). All experiments were repeated at least three times independently. Data are expressed as mean ± S.D. L. D. Condition group, \(n = 11\); D. D. Condition group, \(n = 5\).
Msx1 in the circadian rhythm of tooth development. Most of the mineralization-related and odontogenesis-related factors detected in this study revealed similar expression patterns to core clock genes, implying that the circadian rhythm would greatly affect the formation of dental hard tissues. These might be the possible reason for the formation of incremental growth lines, such as daily Retzius’s lines in enamel and von Ebner’s lines in dentine.

**Effect of p75NTR knockout or over-expression on tooth circadian rhythm and mineralization**

The effect of p75NTR knockout was determined by the *in vivo* experiment of the model mice. Figure 6 shows the mRNA expression difference between knockout and wild-type mice. As expected, p75NTR mRNA in the knockout mice was significantly lower than in the wild-type mice (p < 0.05). However, the mRNA expression of Mage-D1 was not significantly reduced in the knockout mice (p > 0.05). Interestingly, of the four core clock genes detected, Bmal1 demonstrated the exact noticeable change and tendency as p75NTR, which indicated that Bmal1 mRNA expression might be closely related to p75NTR. The other three clock genes depicted the reverse tendency. Per1 mRNA was statistically higher in the knockout mice (p > 0.05). Clock and Per2 mRNA were slightly higher in the knockout mice but showed no significant difference (p > 0.05). Of the three mineralization-related factors, Runx2 mRNA expression in the knockout mice was significantly higher than in the wild-type mice (p < 0.05). In comparison, ALP and Col1 mRNA expression in the knockout mice was significantly lower than in the wild-type mice. Dlx1 showed no significant difference among the four odontogenesis-related factors (p > 0.05). Msx1, Dmp1, and Dspp were significantly lower in the knockout mice than in the wild-type mice (p < 0.05).

The effect of p75NTR over-expression was determined by the *in vitro* experiment of iSCAP. Figure 7 shows that p75NTR mRNA expression was considerably higher in the over-expression group than in the control group (p < 0.05). Seven factors presented a significant change (p < 0.05): six positive relationships (Mage-D1, Bmal1, Clock, Runx2, Col1, and Msx1) and one negative relationship (Dspp) with p75NTR. The other five factors (Per1, Per2, ALP, Dlx1, and Dmp1) showed no significant change (p > 0.05). Compared with the results presented above, the change tendency of Bmal1, Per1, Per2, Col1, Msx1, and Dlx1 with p75NTR knockout and over-expression were consistent in the *in vivo* model mice and *in vitro* cell experiment. However, the change tendency of Runx2 and Dspp was reversed. The Mage-D1 and Clock showed no significant difference in the model mice experiment but exhibited a positive relationship with p75NTR over-expression in the cells experiment.
Discussion

Teeth are an essential model for gaining insights into the general processes of biomineralization (Jussila and Thesleff, 2012; Bakhit et al., 2018; Pagella et al., 2020). It comprises three mineralized tissues, namely dentine, cementum, and enamel, of which the biomineralization is specific for each dental hard tissue. Moreover, they are unique in the body, involving specific proteins, such as collagen and non-collagenic matrix proteins, not found elsewhere. Therefore, the mechanism of tooth biomineralization is still unclear. The evidence exhibited regular incremental growth lines in all three dental hard tissues mentioned above, indicating that tooth biomineralization was controlled by time and is a characteristic of circadian rhythm (Zheng et al., 2011; He et al., 2019; Papakyrikos et al., 2020). In this study, the clock genes were detected to be expressed in dental germs during tooth development, and p75NTR was found to play an essential role in regulating the circadian rhythm during the formation of dental hard tissues. In order to reveal the mechanism of p75NTR in regulating clock genes in the mineralization and formation of the dental hard tissues, we used four models in this study. The model of rats was used to observe the dynamic morphogenesis of the first molar and the expression of p75NTR and clock factor protein. The model of EMSCs were used to explore the possible oscillation relationship between p75NTR and core clock factors. The p75NTR knockout mouse is a model for downregulation of p75NTR expression, and overexpression of p75NTR in iSCAP cells is a model for upregulation of p75NTR expression.

The tooth morphogenesis is triggered by the sequential and reciprocal interactions between ectomesenchyme generated from the cranial neural crest and dental epithelium (Jussila and Thesleff, 2012; Bakhit et al., 2018). The dynamic histological observation of this study revealed that the rat molar germs entered the early cap stage at E14.5 d, the cap stage and early bell stage at E16.5 d, and the bell stage at E18.5 d. The morphogenesis of molar cusps was completed at PN4 d. These results were consistent with that of previous studies (Taniguchi et al., 1999; Yang et al., 2020). Interestingly, the separations between pre-odontoblast and pre-ameloblast layers were found to occur in all the E18.5 d rat first molar, implying a significant decrease in the cell adhesion between odontoblasts and ameloblasts at this stage. This finding indicated that the cell crosstalk of epithelial-mesenchymal interactions might primarily occur during the early tooth morphogenesis. In the post-natal rat first molar, the dental hard tissues began to be detected at PN4 d, and the inner enamel epithelium was also found easy to separate from enamel. It might be the structure of dentine tubules that enhanced the adhesion of odontoblasts to dentine.
The clock genes of *Bmal1*, *Clock*, *Per1*, and *Cry1* were detected in this study. Dynamic histological observation demonstrated that *Clock* and *Per1* were apparently expressed in the rat dental germs at E14.5 d, *Bmal1*, and *Cry1* began to be represented at E16.5 d. Although *Cry1* was weakly expressed throughout, the other three displayed an increased expression in the following rat first molar germs. The expression’s initial times were largely consistent with the report by Zheng et al. [12] that *Bmal1*, *Clock*, *Per1*, and *Per2* were not detected at E14 d or 15 d and began to be expressed in the mouse dental germs at E17 d. The expression distributions were completely consistent with the previous studies (Polly, 2015; Allada and Bass, 2021). The expressions of *Bmal1*, *Clock*, *Per1*, and *Cry1* were initially detected in the pre-odontoblast and pre-ameloblast layers and then detected in the dental papilla cells, stratum intermedium, and stellate reticulum. These results indicated that the clock genes might participate in the complex epithelial-mesenchymal and stellate reticulum. These results indicated that the clock genes might participate in the complex epithelial-mesenchymal crosstalk networks and mainly regulate dental hard tissue genes might participate in the complex epithelial-mesenchymal and stellate reticulum. These results indicated that the clock genes might participate in the complex epithelial-mesenchymal crosstalk networks and mainly regulate dental hard tissue genes that the circadian clock and the rhythm was then detected in the dental papilla cells, stratum intermedium, detected in the pre-odontoblast and pre-ameloblast layers and expression of the circadian rhythm was determined in the dental papilla cells, stratum intermedium, and stellate reticulum. These results indicated that the clock genes might participate in the complex epithelial-mesenchymal crosstalk networks and mainly regulate dental hard tissue genes formation during tooth development (Tao et al., 2016; Pincha et al., 2022).

*p75NTR*, a well-conserved transmembrane neurotrophin receptor, was shown to play a critical role in tooth morphogenesis and mineralization in the previous studies by this study’s researchers (Wen et al., 2012; Li et al., 2017; Wang et al., 2020; Zhao et al., 2020). Baeza-Raja et al. (2013) reported that *p75NTR* expression oscillated via the direct binding of *Clock/ Bmal1* to noncanonical E-box elements present in the *p75NTR* promoter. *p75NTR* might be a novel clock gene regulating oscillatory components of circadian rhythms. Therefore, *p75NTR* was selected and investigated in this study to determine its potential role in the circadian rhythm and incremental growth line formation during tooth development. The dynamic expression of *p75NTR* was similar to that of clock genes during the development of rat dental germs, indicating its relationship with the circadian rhythm. Moreover, *p75NTR* knockout mice exhibited the disorder of pre-odontoblast/pre-ameloblast arrangement and morphology shape and even the disappearance of boundaries between pre-odontoblast and pre-ameloblast layers. There is another possibility that these phenotypes are delayed because *p75NTR* is knocked out. In general, this finding indicated that *p75NTR* might regulate cell polarity during tooth development. Cell polarity is critical in cellular processes ranging from cell migration to asymmetric cell division and axon and dendrite specification (Pirolti et al., 2019). *p75NTR* was reported to directly interact with the polarity protein Par-3 and recruited to regulate the axon-glial junction, forming a complex that points to a critical role in establishing the cell polarity for myelination (Chan et al., 2006). As a transmembrane receptor, *p75NTR* might participate in the odontoblast-ameloblast junction and cell polarity establishment during tooth morphogenesis.

The ablation of SCN was reported to result in a disrupted patterning of rat’s incremental lines in dentine and supported the involvement of the circadian clock in tooth development (Ohtsuka-Isoya et al., 2001). The opinion was confirmed by the report that *Bmal1* ‘−/−’ mice showed the fainter daily lines in dentine than *Bmal1*+/+ and *Bmal1*+/− mice (He et al., 2019). The phenomenon of incremental lines might be related to the disorganization of pre-odontoblast/pre-ameloblast polarity and the arrangement found in *p75NTR*−/− mice of this study. *p75NTR* was speculated to play a crucial role in regulating clock genes during the formation of dental hard tissues. The recent research certainly supported this speculation that the incisors’ daily mineralization speed and incremental growth line width were significantly lower in *p75NTR*−/− mice than in *p75NTR*+/+ mice (Wang et al., 2020; Zhao et al., 2020). Importantly, further studies are needed to reveal the signaling networks under this process.

Previous studies reported the detection of clock genes in dental germs and depicted a regular oscillation expression pattern, indicating that biological clocks affect tooth development (Nirvani et al., 2017; Papakyrikos et al., 2020; Huang et al., 2021). The 48-h circadian rhythm dynamics in rat ESMCs showed that the clock genes *Bmal1*, *Clock*, and *Per1* presented a regular oscillation in mRNA expression. A similar oscillation was observed in *p75NTR* and *Runx2* mRNA expression but not in *Dlx1* mRNA expression, indicating that *p75NTR* and mineralization-related factor *Runx2* might be involved in the circadian rhythm of tooth development. Previous studies support this finding that clock genes (*Bmal1*, *Clock*, *Per1*, and *Per2*) and two markers of ameloblast differentiation (amelogenin and kallikrein-related peptidase 4) have regular oscillations in ameloblasts (Zheng et al., 2013; Huang et al., 2021). Moreover, this oscillation was affected by L.D. stimulus. The D.D. condition significantly increased the mRNA expression of *Bmal1*, *p75NTR*, and *Runx2* in this study. The light stimulus disturbed the oscillation peaks of all the detected factors. These results were confirmed by the *in vivo* experiment in this study. The clock genes (*Bmal1*, *Clock*, *Per1*, and *Per2*), mineralization-related factors (*Runx2*, *ALP*, and *Col1*), and odontogenesis-related (*Dlx1*) in dental germs presented a similar tendency that the mRNA expression was considerably higher in the D.D. condition than in L.D. Moreover, this change was much more prominent in most of them at sampling times of 7:30 p.m. These results indicated that the D.D. condition promoted the dental mineralization, which was consistent with the previous reports that the mineralization of dental hard tissues increased in the night during the day (Lacruz et al., 2012; Satou et al., 2019).

The data in this study further confirm that circadian rhythms are involved in tooth development. However, the molecular mechanisms of the effects of clock genes in tooth development and incremental growth lines are unclear. *p75NTR* was reported to directly bond to the *Clock/Bmal1* heterodimer via the E-box element and participated in regulating circadian rhythms (Baeza-Raja et al., 2013). In a recent study by Zhao et al. (2020) the calcein fluorescence assay showed that the distance between the calcein fluorescence bands was significantly lower than that in wild-type and heterozygous mice, indicating that...
**p75NTR** would regulate the daily mineralization speed and incremental growth line width during tooth development. It was also proposed that **p75NTR** might participate in regulating circadian rhythm during dental incremental line formation and **Mage-D1** might play an underlying role in this process. In this study, **Mage-D1** expression was positively correlated with **p75NTR** except that **Mage-D1** was not significantly reduced with **p75NTR** knockout, implying that **Mage-D1** is not the only bridge factor for **p75NTR** to regulate tooth periodic development and rhythmic mineralization. To further reveal its effects and mechanisms in circadian rhythms during tooth development, **p75NTR** knockout or over-expression was performed in this study. **Bmal1** and **Mmx1** showed a positive relationship with **p75NTR** in both in vivo model mice and in vitro cell experiments. **Per1**, **Per2**, and **Dicer1** showed no significant change when **p75NTR** was up- and down-expressed. In the cell experiment, the **Mage-D1** and **Clock** was positively related with **p75NTR** but uncorrelated with **p75NTR** in the model mice. Interestingly, **Runx2** was positively related to **p75NTR** in the cell experiment and negatively related in the model mice. This contradictory conclusion is attributed to the diverse biological functions of **p75NTR** and complex signal regulation mechanism. Because of the formation of bone tissue, another process of mineral deposition in vivo, our previous research showed that the mineralized development of mouse femur was inhibited after **p75NTR** knock (Zhao et al., 2020). However, Wang et al. (2020) found that loss of **p75NTR** upregulated **Runx2** expression, thereby promoting BMSCs mineralization in vitro. Thus, the signaling network related to the regulation of biological mineralization by **p75NTR** is more complex than initially understood, and further research is still needed in the future. In contrast, **ALP**, **Dmp1**, and **Dsgp** were negatively related to **p75NTR** in the cell experiment and in model mice. These reverse tendencies might be the different cell niches between in vitro cell experiments and in vivo model mice. The data in this study indicated that **p75NTR** might participate in regulating circadian rhythms during tooth development via clock genes **Bmal1** and **Clock**, especially in mineralization and dental hard tissue formation. This is consistent with the previous studies that the clock gene **Bmal1** was involved in the up-regulation of mineralization in mouse bone marrow stromal cells (Chen et al., 2012; Samsa et al., 2016). Fu et al. (2005) and Min et al. (2016) also reported that **Bmal1** promoted the expression of mineralization-related factors **Runx2** and **OCN**, which was inhibited by **Per1/Per2** and **Cry1/Cry2**. Therefore, it was speculated that the core circadian regulator of negative TTFL was involved in cell mineralization, and **p75NTR** might be crucial in this process.

**Data availability statement**

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

**Ethics statement**

The animal study was reviewed and approved by The Medical Ethics Committee of the Chongqing Medical University. Written informed consent was obtained from the owners for the participation of their animals in this study.

**Author contributions**

All authors contributed to the study concepts and design. HY, BX, XY, and CL carried out in vivo experiments on **p75NTR** knockout mice. HY, BX, ML, and YZ carried out in vitro experiments on ectomesenchymal stem cells. HY, CL, and XZ collected the data. HY, BX, MZ, and XW performed the analysis. HY, BX, MZ, and XW drafted the manuscript. All authors reviewed and approved the manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.981311/full#supplementary-material
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