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Original Article

Melatonin ameliorates inflammation-induced developmental defects of enamel by upregulating regulator of G protein signaling 2

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Abstract *Background/purpose:* Developmental defects of enamel (DDE) is a dental disease with a high prevalence and no effective means of prevention. One of the major causes of DDE is infection, but the pathogenesis is still unclear. Melatonin is known for its anti-inflammatory and mineralization-promoting activities. However, the effects of melatonin on inflammation-induced DDE remain unknown. Here, we investigated the pathogenesis and potential therapeutic targets of inflammation-induced DDE.

Materials and methods: First, the effect of lipopolysaccharide-induced inflammation in pregnant mice on the enamel mineralization of the offspring was detected by 3D X-ray microscope analysis, immunohistochemical assays, and quantitative real-time polymerase chain reaction (qRT-PCR). Then, the ameloblastic differentiation ability of ameloblast lineage cells (ALCs) in macrophage conditioned medium (CM) was detected. Subsequently, ameloblastic

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mineralization after melatonin administration was studied both in vivo and in vitro. The underlying mechanism of melatonin was investigated by RNA sequencing and small interfering RNA transfection.
Results: Enamel mineralization was decreased in the inflammatory environment both in vivo and in vitro. Furthermore, melatonin treatment ameliorated these defects. RNA sequencing analysis revealed that regulator of G protein signaling 2 (*Rgs2*) was downregulated in the inflammation group, whereas it was upregulated after the addition of melatonin. Further studies showed that *Rgs2* knockdown resulted in decreased ameloblastic mineralization in ALCs. After *Rgs2* knockdown of ALCs in M1-CM with melatonin, the effect of melatonin-mediated attenuation of DDE was greatly reduced.
Conclusion: Our results demonstrate that melatonin ameliorates inflammation-induced DDE by upregulating RGS2, suggesting that RGS2 is a potential therapeutic target for inflammation-induced DDE.

Introduction

Enamel formation is a sequential and delicate process involving proliferation and differentiation of dental epithelial cells, secretion of enamel matrix, mineralization, and maturation. Biological imbalances during this process can lead to abnormalities known as developmental defects of enamel (DDE).[1] DDE is usually accompanied with early childhood caries, aesthetic concerns in anterior teeth, dentin hypersensitivity and negative psychological impact.[2] In recent years, the prevalence of DDE has remained high, even up to 89.9 % in some regions and age groups.[3] Significantly, clinical studies have provided proof of a strong correlation between DDE and systemic infection, as well as local infections such as periapical infection and decays of predecessors.[4–6] When infection occurs, pathogenic microorganisms can act directly on immune cells, thereby affecting ameloblasts through the inflammatory microenvironment.[7] Macrophages play an important role in response to environmental challenges and are in charge of the recognition and clearance of exogenous pathogens in microbe-mediated infectious diseases. Unstimulated macrophages (M0) modulate the inflammatory microenvironment by being activated into different phenotypes (M1/M2).[8] M1 macrophages are classically considered to be proinflammatory while M2 macrophages are anti-inflammatory.[9] However, little is known about how macrophages influence enamel formation and the biological behavior of ameloblasts in the inflammatory microenvironment.

Melatonin (MLT) is a neurohormone involved in a wide range of physiological processes. In our previous studies, we found melatonin contributed to ameloblastic mineralization in ameloblast lineage cells (ALCs).[10,11] Additionally, melatonin was indicated to exhibit an anti-inflammatory effect by inhibiting the expression of pro-inflammatory cytokines.[12] It has been reported that melatonin can also reverse tumor necrosis factor-alpha (TNF-α)/interleukin-1 beta (IL-1β)-inhibited osteogenesis under an inflammatory state.[13–15] Based on the above, we speculated that melatonin may promote enamel formation in an inflammatory microenvironment by decreasing pro-inflammatory cytokines of immune cells and/or exerting direct effects on ameloblasts. Among those previous studies, most focused on the anti-inflammatory effect of melatonin on immune cells. The direct application of melatonin in enamel formation during an inflammatory response is poorly studied.

Many extracellular stimuli elicit physiological responses in target cells by activating receptors that couple to heterotrimeric G-proteins. Regulator of G protein signaling 2 (RGS2) is a member of the RGS superfamily that modulates GTP-binding protein-coupled receptor (GPCR) signaling by shortening the duration and amplitude of GPCR-mediated responses.[16] RGS2 expression was upregulated during osteogenesis, and knockdown of RGS2 expression resulted in decreased osteogenic differentiation, suggesting that RGS2 is normally a positive osteogenic regulator.[17]

In this study, using a pregnant mouse model combined with in vitro cell culture experiments, we elucidated that enamel formation and mineralization are disturbed under inflammatory conditions. Furthermore, we found that RGS2 is pivotal in mediating the therapeutic role of melatonin in inflammation-induced DDE, providing new insights for targeted DDE therapy.

Materials and methods

Chemicals and reagents

Lipopolysaccharide (LPS, *Escherichia coli* LPS, serotype 0111: B4), melatonin, dexamethasone, ascorbic acid, β-glycerophosphate, Alizarin red S and cetylpyridinium chloride were from Sigma—Aldrich, St. Louis, MO, USA. Lipofectamine 2000 was from Invitrogen, Waltham, MA, USA. Antibodies against β-Actin, amelogenin (AMELX), ameloblastin (AMBN), tuftelin (TUFT) and HRP-linked anti-rabbit IgG were from Abcam, Waltham, MA, USA. The antibody against odontogenic ameloblast-associated protein (ODAM) was from Affinity, Changzhou, China. *Rgs2* siRNA and negative control siRNA (siControl) were synthesized by GenePharma, Shanghai, China.

Animals

All experiments were approved by the Animal Welfare Committee of Shanghai Ninth People's Hospital. BALB/c mice were purchased from Shanghai Animal Experiment Center (Shanghai, China). Eight-to 10-week-old female mice were mated with male mice. The day the vaginal plug was detected was designated as gestational day (E0.5). Then the pregnant mice were divided into three groups and each group consisted of ten mice. In the MLT + LPS group, mice were intraperitoneally injected with LPS (125 μg/kg, dissolved in saline) and melatonin (10 mg/kg, dissolved in 0.5 % ethanol: saline) 30 min later. In the LPS group, mice were intraperitoneally injected with LPS (125 μg/kg) and melatonin vehicle (0.5 % ethanol: saline) 30 min later. In the vehicle group, mice were intraperitoneally injected with LPS vehicle (saline) and melatonin vehicle (0.5 % ethanol: saline) 30 min later.

3D x-ray microscope (3D XRM) analysis

Samples were tested by 3D XRM (Zeiss Xradia 510 Versa, Zeiss, Oberkochen, Germany). The data were imported into Dragonfly (Comet Technologies Canada, Montreal, Canada). A 3D thickness heatmap of the enamel was generated through the thickness mesh. The density was read from the scanning data calibrated by two standard phantoms.

Immunohistochemical staining

Mandibles were dissected, fixed in 4 % paraformaldehyde, dehydrated, embedded in paraffin, and serially sectioned. After deparaffinization and rehydration, the slices were treated by autoclaving in citric acid buffer. The samples were incubated for 1 h at room temperature with primary antibodies, followed by incubation with secondary antibody. The color reaction was performed with diaminobenzidine.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Sigma–Aldrich). First-strand cDNA was synthesized using Hieff® 1st Strand cDNA Synthesis SuperMix for qPCR (YEASEN, Shanghai, China). qRT-PCR was performed using Hieff® qPCR SYBR Green Master Mix (YEASEN). The PCR primer sequences were as follows: *β-Actin* forward 5′-GGAGATGGCCACTGC CGCAT-3′ and reverse 5′- GCAGCTCAGTAACAGTCCGCCTA-3′; *Ambn* forward 5′-GGCATGGCTAGTTTGAGCCT-3′ and reverse 5′-GGCAAAGAATATTCATACTGCTGGG-3′; *Tuft* forward 5′-TGGAGGCTGAGAACTTAGAGATG-3′ and reverse 5′-GGATGA-GAGGCATAGGCTTGG-3′; *Amelx* forward 5′-GTCACCTCTG-CATCCCATG-3′ and reverse 5′-TTCCCGCTTGGTCTTGTC-3′; *Odam* forward 5′-ATCAATTTGGATTCGCACCACC-3′ and reverse 5′-AGTTGGATCTATCCCAGAAGTGA-3′; and *Rgs2* forward 5′-GACCCGTTTGAGCTACTTCTTG-3′ and reverse 5′-CCGTGGTGATCTGTGGCTTTTTAC-3′. The relative gene expression was calculated using equation $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct\ (mRNA) - Ct\ (\beta\text{-Actin})$. The RNA-sequencing (RNA-Seq) was performed by Majorbio technology (Shanghai, China) according to the published procedure.

Cell culture

ALCs and RAW264.7 were provided by the Shanghai Key Laboratory of Stomatology and were cultured in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum at 37 °C. Odontogenic medium (OM) contained 100 nM dexamethasone, 50 mM ascorbic acid, and 10 mM β-glycerophosphate.

Alizarin red S staining and alkaline phosphatase (ALP) staining

ALCs were stained with 1 % Alizarin red S. For quantitative analysis, the Alizarin red stain was dissolved in 10 % (w/v) cetylpyridinium chloride prior to absorbance measurements. ALP staining was carried with an ALP Staining Kit (Beyotime, Shanghai, China).

Western blot analysis

Total protein was extracted from cells using RIPA lysis buffer (YEASEN). Proteins were electroblotted onto polyvinylidene difluoride membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The membranes were then blocked with 5 % skim milk and incubated with primary antibodies overnight at 4 °C. The membranes were then incubation with the secondary antibody and visualized with super-sensitive luminescent liquid (YEASEN).

Statistical analysis

All experiments were performed in triplicate and data were expressed as the mean ± SEM. The student's t-test was used for comparison between the two groups. $P < 0.05$ were considered statistically significant.

Results

Lipopolysaccharide-induced inflammation impairs enamel formation and mineralization both in vivo and in vitro

To investigate the effect of the inflammatory environment on enamel formation of tooth germs, pregnant mice were treated with or without exposure to LPS (Fig. 1A). The enamel thickness and density of the mandibular first molars in the LPS groups were lower than those in the vehicle group (Fig. 1B and C). In addition, the molar cusps were rough and blunt in the LPS group (Fig. 1D). The LPS group had significantly decreased mRNA expression of key enamel matrix proteins (EMPs) including *Ambn*, *Tuft*, *Amelx*, and *Odam* (Fig. 1E). Furthermore, the LPS group had lower expression of AMELX and ODAM in the ameloblast layer (Fig. 1F). Then, we utilized RAW264.7-conditioned medium (CM) to study the interactions between ameloblasts and macrophages in the inflammatory environment (Fig. 2A). Alizarin red S staining, ALP staining and EMPs expression levels revealed that there was no significant difference in the ameloblastic differentiation ability of ALCs between the OM group and M0-CM group (Fig. 2B–E). When

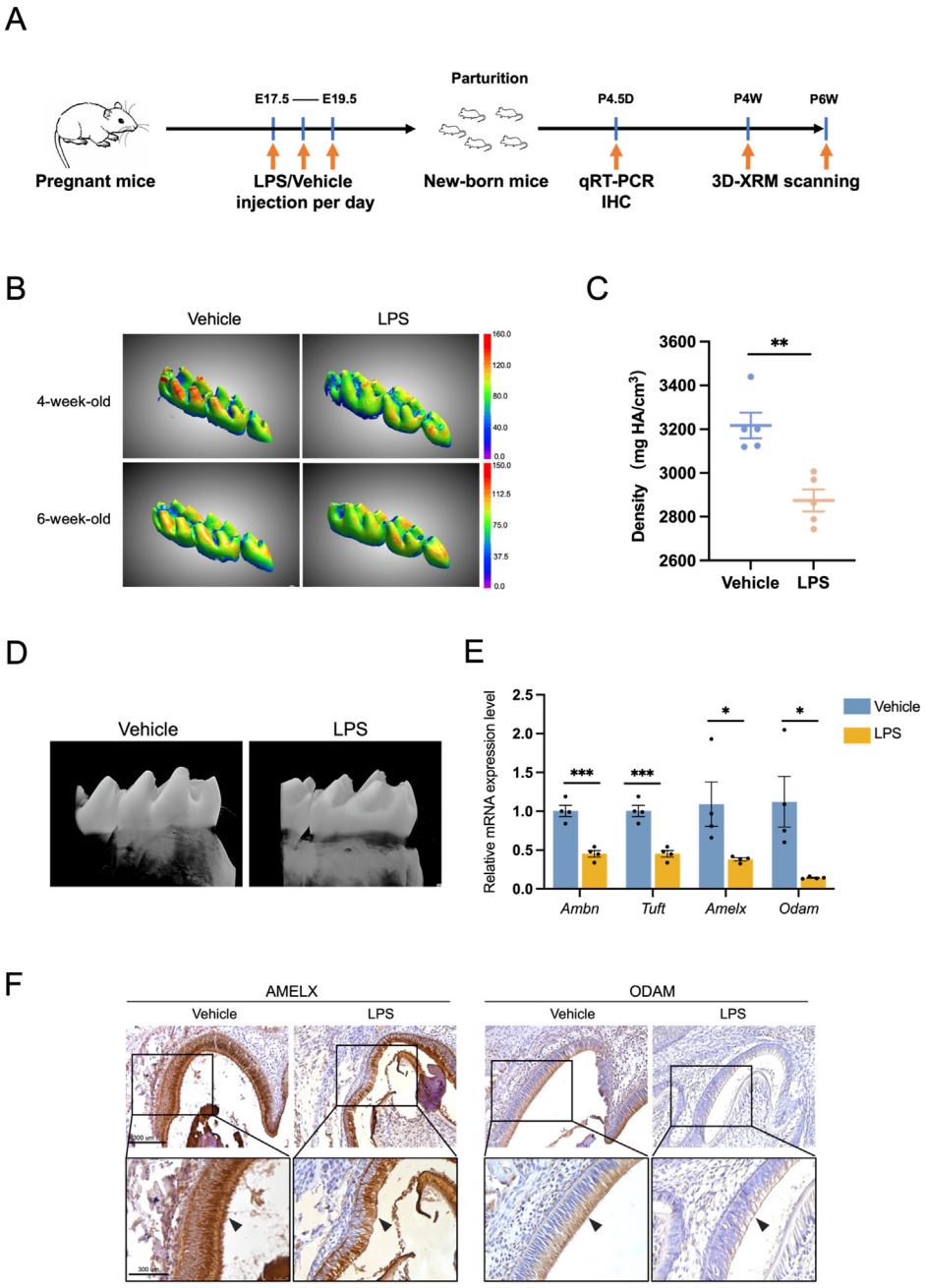

Figure 1 The effect of LPS-induced inflammation in pregnant mice on enamel mineralization in the offspring. A. Schematic representation of the experimental design for the LPS-induced inflammation model. B. 3D XRM reconstruction of molar enamel thickness in neonatal mice. Purple to red colors indicate the thickness of tissues from low to high. C. Density of mandibular first molar enamel in 4-week-old neonatal mice (n = 5). D. 3D XRM reconstruction of mandibular first molar enamel in 4-week-old neonatal mice. E. The relative mRNA levels *Ambn*, *Tuft*, *Amelx* and *Odam* in mandibular first molar germs of P4.5 neonatal mice (n = 4). F. Immunohistochemical staining of AMELX and ODAM in mandibular first molar germs of P4.5 neonatal mice. Arrows indicate ameloblasts layer. Scale bar = 300 μm ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Abbreviations: LPS, lipopolysaccharide; qRT-PCR, quantitative real-time polymerase chain reaction; IHC, immunohistochemical staining; 3D XRM, 3D X-ray microscope; *Amelx*, amelogenin; *Ambn*, ameloblastin; *Tuft*, tuftelin; *Odam*, odontogenic ameloblast-associated.

comparing the ameloblastic mineralization between M0-CM and M1-CM, ALCs treated with M1-CM showed less staining of mineralized nodules and ALP and less expression of EMPs (Fig. 2B—E). In conclusion, LPS-induced inflammation impairs enamel mineralization in vivo and the ameloblastic differentiation capacity of ALCs in vitro.

Melatonin treatment ameliorates inflammation-induced developmental defects of enamel

To investigate the effects of melatonin on DDE induced by LPS treatment, melatonin was injected into pregnant mice. The enamel surface in the LPS + MLT group tended to be

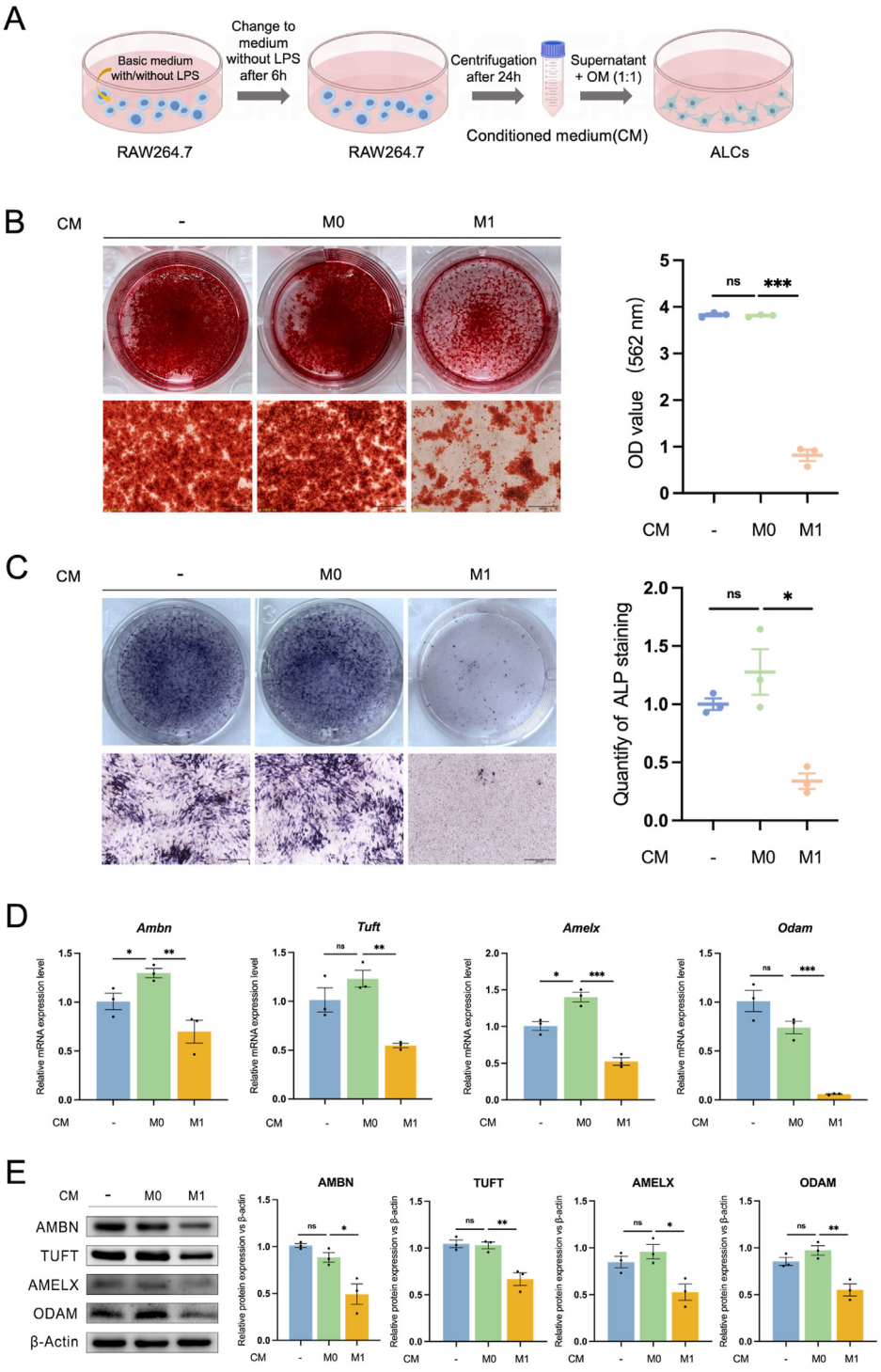

Figure 2 M1-CM impairs the ameloblastic mineralization of ALCs. A. Schematic diagram of obtaining M0/M1-CM. B. Alizarin red S staining of ALCs after 28 days of odontogenic induction. Scale bar = 200 μm. C. ALP staining of ALCs at day 7 of odontogenic induction. Scale bar = 200 μm. D. The relative mRNA levels of *Ambn*, *Tuft*, *Amelx* and *Odam* in ALCs after 7 days of odontogenic induction. E. Expression levels of AMBN, TUFT, AMELX and ODAM were determined by Western blot analysis at day 7 of odontogenic differentiation. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ and $P > 0.05$ (no significant difference, ns). Abbreviations: LPS, lipopolysaccharide; OM, odontogenic medium; ALCs, ameloblast lineage cells; CM, conditioned medium; OD, optical density; ALP, alkaline phosphatase; *Amelx*, amelogenin; *Ambn*, ameloblastin; *Tuft*, tuftelin; *Odam*, odontogenic ameloblast-associated.

thicker, smoother and completer and had a much higher density than that of the LPS group (Fig. 3A–C). Melatonin also increased the expression of EMPs in the inflammatory environment, confirming its positive regulatory role (Fig. 3D and E). Taken together, these significant changes in enamel formation demonstrated that melatonin treatment

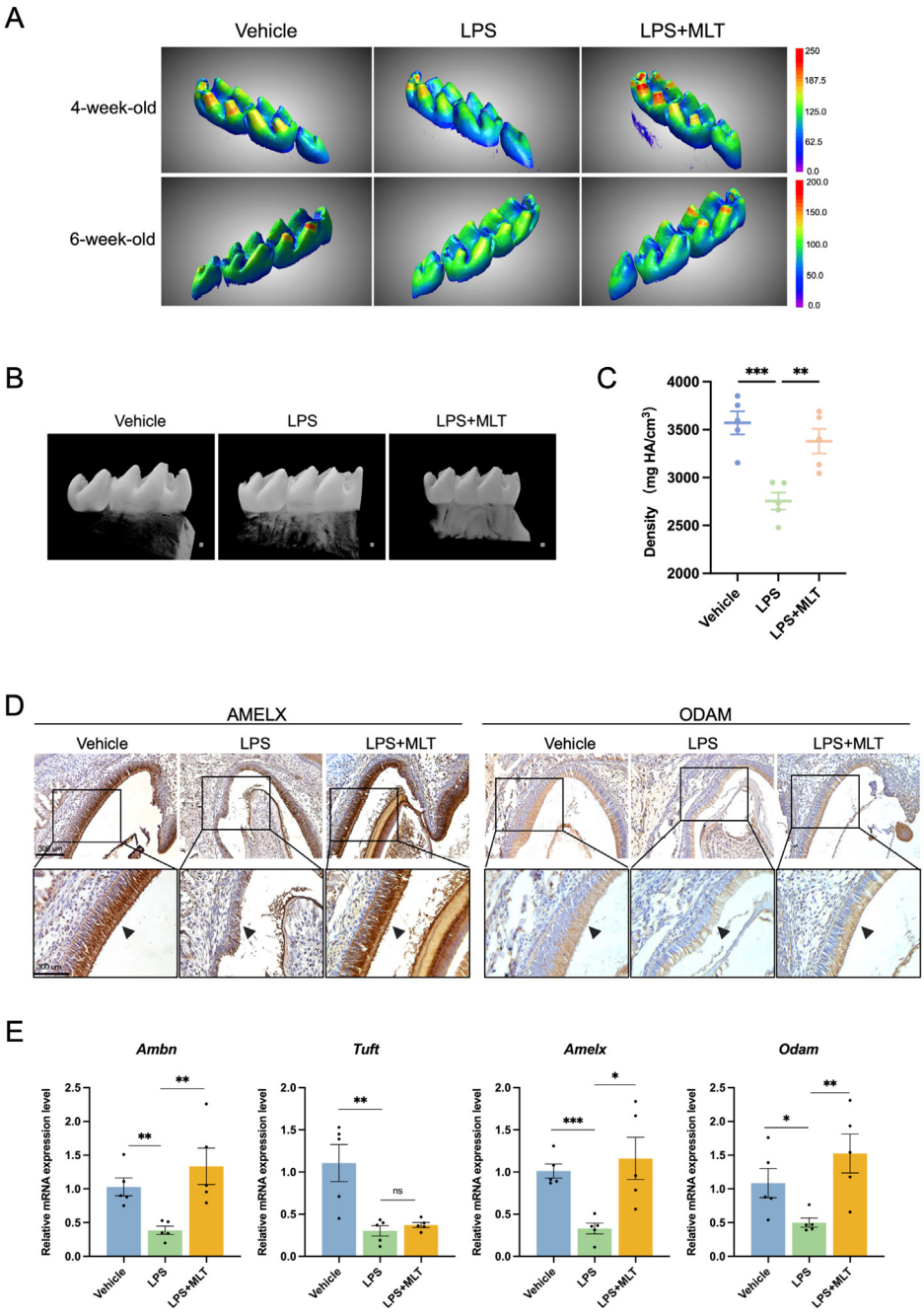

Figure 3 Melatonin ameliorates LPS-induced DDE in neonatal mice. A. 3D XRM reconstruction of molar enamel in neonatal mice. Purple to red colors indicate tissue thickness from low to high. B. 3D XRM reconstruction of mandibular first molar enamel in 4-week-old neonatal mice. C. Density of mandibular first molar enamel in 4-week-old neonatal mice (n = 5). D. Immunohistochemical staining of AMELX and ODAM in mandibular first molar germs of P4.5 neonatal mice. Arrows indicate ameloblasts layer. Scale bar = 300 μm. E. The relative mRNA levels of *Ambn*, *Tuft*, *Amelx* and *Odam* in the mandibular first molar germs of P4.5 neonatal mice (n = 5). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ and $P > 0.05$ (no significant difference, ns). Abbreviations: LPS, lipopolysaccharide; MLT, melatonin; *Amelx*, amelogenin; *Ambn*, ameloblastin; *Tuft*, tuftelin; *Odam*, odontogenic ameloblast-associated.

ameliorated LPS-induced DDE in vivo. To determine effects of different doses of melatonin on inflammation-induced ameloblastic mineralization defects in vitro, melatonin was added to M1-CM. It became evident that the positive rates of ALP and Alizarin red S and the expression of EMPs were higher after the addition of melatonin at concentrations of 0.01 μM, 0.1 μM and 1 μM (Fig. 4A—D). In general, melatonin ameliorated M1-CM-induced ameloblastic mineralization defects in vitro, and 0.1 μM melatonin showed the optimal mineralization promoting effect.

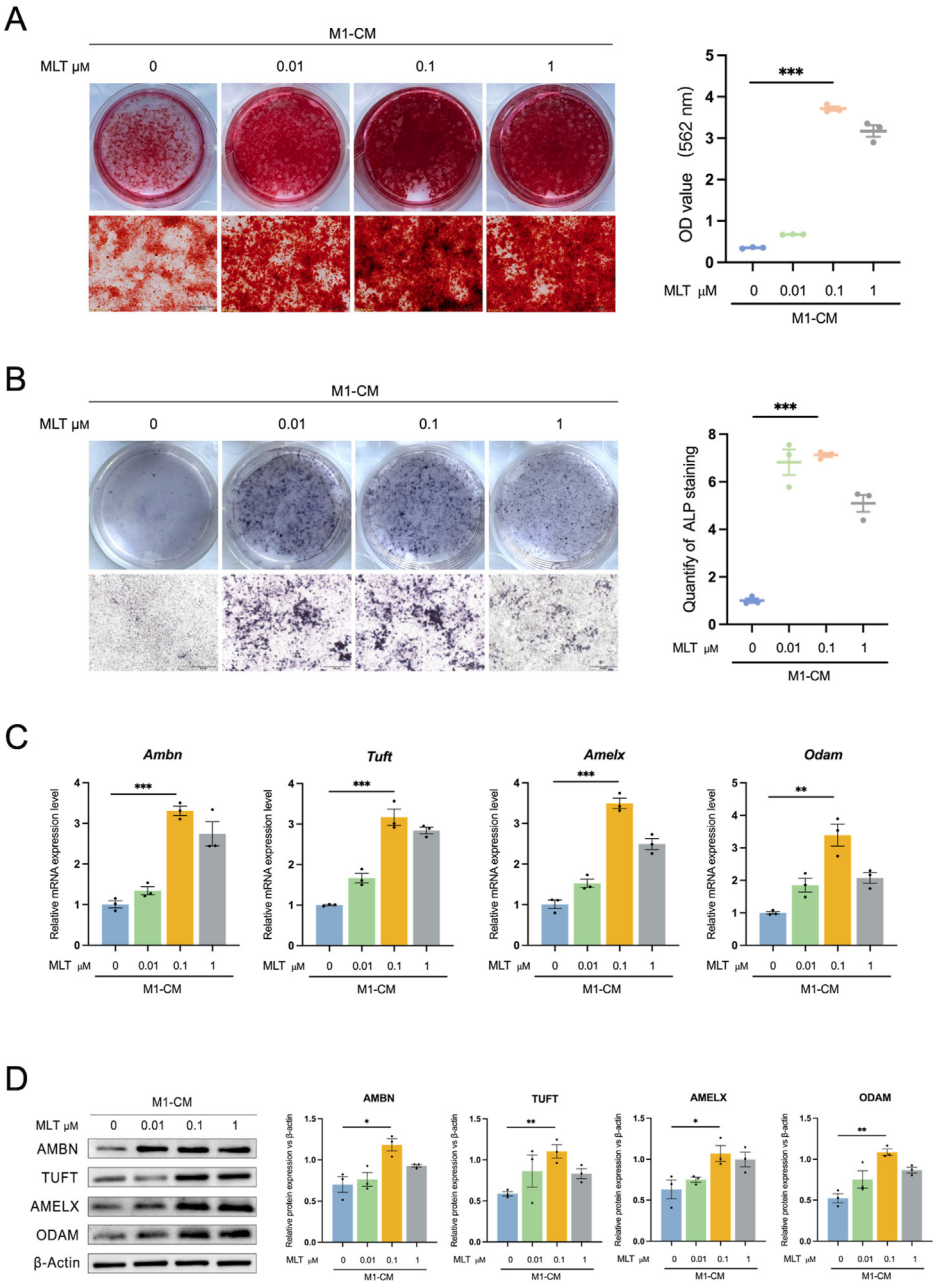

Figure 4 Melatonin improves M1-CM-induced impaired ameloblastic differentiation ability of ALCs. A. Alizarin red S staining for matrix mineralization of ALCs after 28 days of odontogenic induction in M1-CM with different concentrations of melatonin and quantification of matrix mineralization. Scale bar = 200 μm. B. ALP staining and quantification of ALCs after 7 days of odontogenic induction. Scale bar = 200 μm. C. The relative mRNA levels of *Ambn*, *Tuft*, *Amelx* and *Odam* in ALCs after 7 days of odontogenic induction. D. The expression levels of AMBN, TUFT, AMELX and ODAM were determined by Western blot analysis at day 7 of odontogenic differentiation. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Abbreviations: CM, conditioned medium; MLT, melatonin; OD, optical density; ALP, alkaline phosphatase; *Amelx*, amelogenin; *Ambn*, ameloblastin; *Tuft*, tuftelin; *Odam*, odontogenic ameloblast-associated.

Regulator of G protein signaling 2 is downregulated in M1 macrophages-conditioned medium but upregulated after melatonin administration

We sequenced the transcriptome of the M0-CM group, M1-CM group and M1-CM + MLT group to elucidate the molecular mechanism. A total of 1598 downregulated differentially expressed genes (DEGs) and 244 upregulated DEGs were identified, and there were 40 overlapping genes between them (Fig. 5A and B). Gene Ontology (GO) analysis revealed that genes related to extracellular matrix, inflammatory response, bone development, and G protein-coupled receptor binding were enriched (Fig. 5C). Among these 40 genes, *Rgs2* attracted our attention because (1)

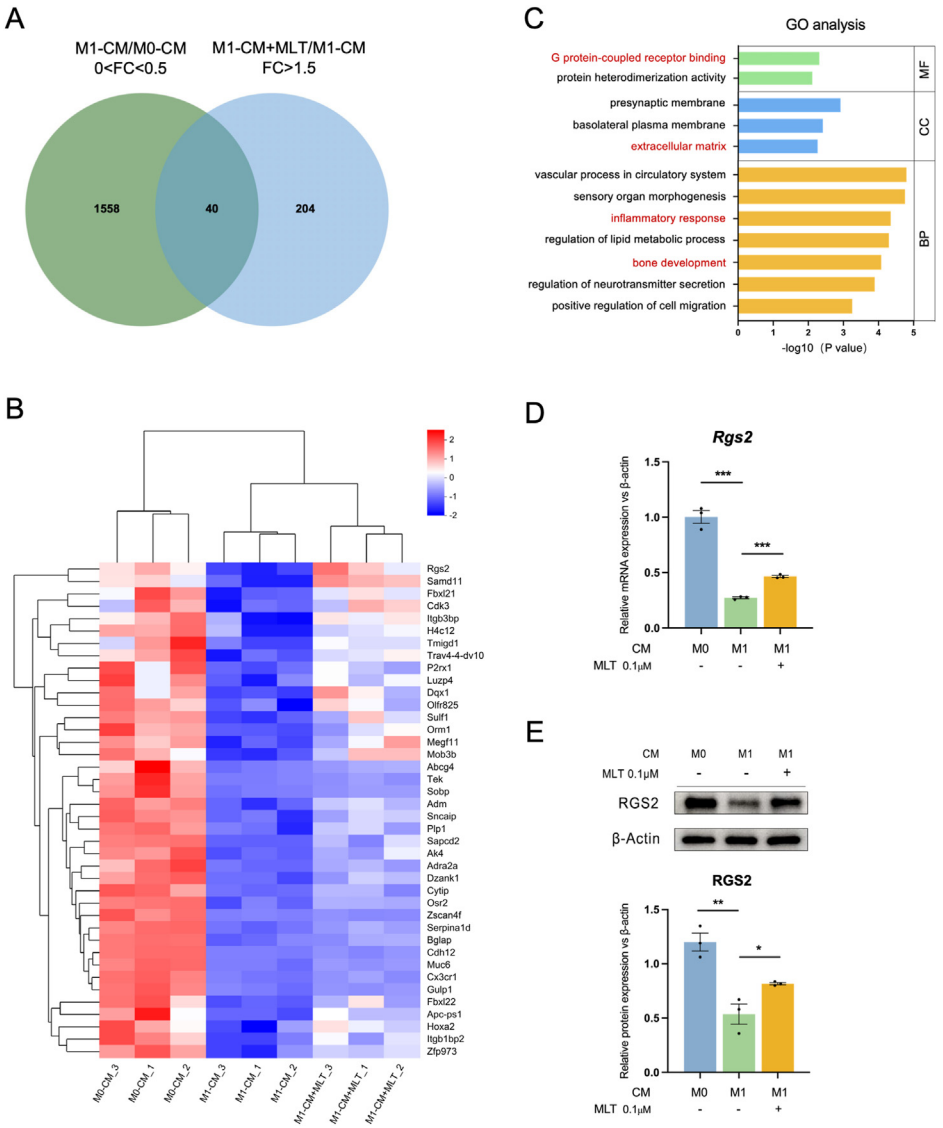

Figure 5 RGS2 is downregulated in ALCs cultured in M1-CM, whereas it is upregulated after melatonin administration. A. Venn diagram: unique and overlapping DEGs of M1-CM/M0-CM FC $<$ 0.5 and M1-CM + MLT/M1-CM FC $>$ 1.5 ($P < 0.05$). B. Heatmap of these overlapping DEGs. Red indicates upregulation and blue indicates downregulation. C. GO analysis of these overlapping DEGs. D. The relative mRNA expression level of *Rgs2* in ALCs in the M0-CM group, M1-CM group and M1-CM + MLT group. E. The relative protein level of RGS2 in ALCs in the M0-CM group, M1-CM group and M1-CM + MLT group. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Abbreviations: CM, conditioned medium; MLT, melatonin; FC, fold change; GO, Gene Ontology; CC, cell components; BP, biological processes; MF, molecular functions; *Rgs2*, regulator of G protein signaling 2.

Rgs2 was one of the most up- and down-regulated genes in these three groups; (2) RGS2 is an important protein in tissue development.[18,19] qRT-PCR and Western blot analysis proved that the changes in the expression level of RGS2 were consistent with the RNA-seq (Fig. 5D and E). Compared with the M0-CM group, its expression obviously decreased in the M1-CM group and increased with the addition of melatonin. Therefore, we speculated that RGS2 might be one of the most important factors in melatonin-mediated attenuation of impaired ameloblastic mineralization in the inflammatory environment and further analysis was conducted.

Melatonin ameliorates the impaired ameloblastic differentiation of ameloblasts in an inflammatory environment via upregulation of regulator of G protein signaling 2

To investigate the function of RGS2 in ameloblastic mineralization, *Rgs2* siRNA was transiently transfected into ALCs to silence endogenous *Rgs2* expression (Fig. 6A and B). Importantly, extracellular matrix mineralization was severely inhibited in *Rgs2* knockdown ALCs (Fig. 6C). Meanwhile, the staining intensity of ALP and the expression of EPMs were markedly decreased in *Rgs2* knockdown ALCs

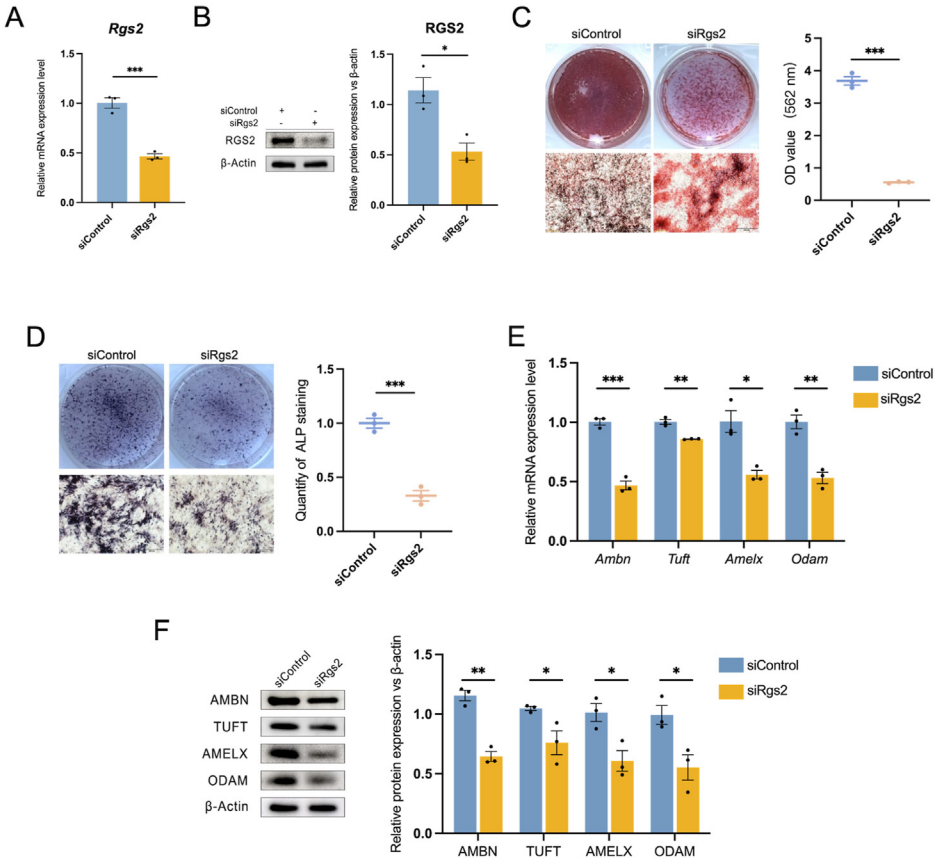

Figure 6 RGS2 acts as an important positive regulator of ameloblastic differentiation of ALCs. A. The efficiency of siRgs2 was measured by qRT-PCR after 48 h. B. The efficiency of siRgs2 was detected by Western blot analysis after 72 h. Protein expression levels were normalized to β-Actin. C. Alizarin red S staining and quantification of matrix mineralization of ALCs after 28 days of odontogenic induction in the siControl group and siRgs2 group. Scale bar = 200 μm. D. ALP staining and quantification of ALCs after 7 days of odontogenic induction in the siControl group and siRgs2 group. Scale bar = 200 μm. E. The relative mRNA levels of *Ambn*, *Tuft*, *Amelx* and *Odam* in ALCs after 7 days of odontogenic induction in the siControl group and the siRgs2 group. F. Expression levels of AMBN, TUFT, AMELX and ODAM were determined by Western blot analysis after 7 days of odontogenic induction in the siControl group and siRgs2 group. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Abbreviations: *Rgs2*, regulator of G protein signaling 2; OD, optical density; ALP, alkaline phosphatase; *Amelx*, amelogenin; *Ambn*, ameloblastin; *Tuft*, tuftelin; *Odam*, odontogenic ameloblast-associated.

(Fig. 6D–F). In general, these results indicated that downregulation of RGS2 inhibited the ameloblastic differentiation of ALCs and that RGS2 acted as a principal regulator of ameloblastic differentiation in enamel mineralization. Considering all the above, we hypothesized that the expression of RGS2 is critical for melatonin to ameliorate the impaired ameloblastic differentiation of ALCs in an inflammatory environment. To verify that the ameloblastic differentiation-promoting ability of melatonin in M1-CM was via upregulation of RGS2, we knocked down *Rgs2* in ALCs treated with M1-CM and melatonin. After siRgs2 transfection, the EMPs and the positive rate of ALP and Alizarin red S in ALCs with melatonin decreased, approaching the state of ALCs in M1-CM without melatonin (Fig. 7A–D). These results indicated that melatonin ameliorated the impaired ameloblastic differentiation of ALCs in an inflammatory environment in a RGS2-dependent manner.

Discussion

DDE can affect both primary and permanent teeth and trigger a series of short-to long-term adverse effects. Unlike bone, enamel tissue turns to be acellular upon completion of mineralization and therefore does not remodel. Based on this point, the study of the pathological mechanisms of DDE is particularly important for its prevention and treatment. Previous studies have extensively investigated inherited DDE and dental fluorosis, while there are only a few scattered reports on inflammation-induced DDE.[20–22]

Duman et al. studied the effect of LPS during pregnancy on rats, and Papp et al. applied LPS to tooth germ cultures.[23,24] However, there are some problems with these studies, such as the lack of investigation of the molecular mechanisms or the simplicity of pathological model construction. We established both an in vivo model of

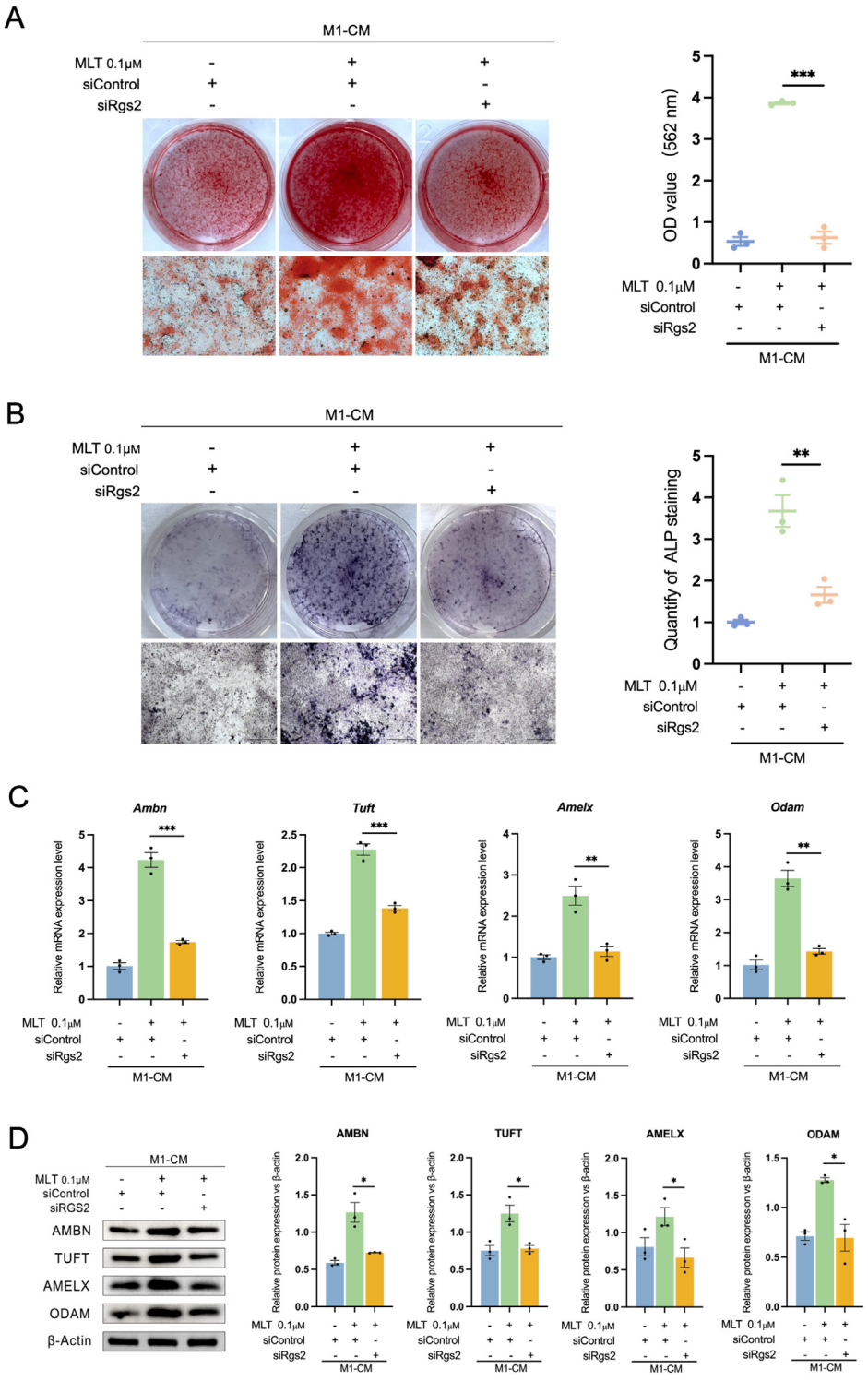

Figure 7 Melatonin attenuates inflammation-induced impaired ameloblastic differentiation of ALCs by upregulating RGS2. A. Alizarin red S staining in siRgs2/siControl transfected ALCs after 28 days of odontogenic induction in M1-CM with/without melatonin. Scale bar = 200 μm. B. ALP staining in ALCs transfected with siRgs2/siControl after 7 days of odontogenic induction. Scale bar = 200 μm. C. The relative mRNA levels of *Ambn*, *Tuft*, *Amelx* and *Odam* in ALCs transfected with siRgs2/siControl after 7 days of odontogenic induction. D. Expression levels of AMBN, TUFT, AMELX and ODAM determined by Western blot analysis in ALCs transfected with siRgs2/siControl after 7 days of odontogenic induction. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Abbreviations: CM, conditioned medium; MLT, melatonin; *Rgs2*, regulator of G protein signaling 2; OD, optical density; ALP, alkaline phosphatase; *Amelx*, amelogenin; *Ambn*, ameloblastin; *Tuft*, tuftelin; *Odam*, odontogenic ameloblast-associated.

inflammation induced by injection of LPS into pregnant mice and an in vitro model of ALCs in macrophage-CM to study the effects of inflammation on enamel development. When the periphery of the dental germ is affected by infection, such as bacterial invasion, macrophages respond to foreign substances, and regulate physiological and pathological processes.[25] M1 macrophages synthesize and secrete a large number of proinflammatory cytokines including TNF-α, IL-1β, IL-6,[26] while the communication of ameloblasts with macrophages and cytokines during enamel formation has not been reported. In this study, we found greater wear on the enamel surface, reduced enamel density, and reduced EMPs in the inflammation group in vivo, and decreased staining intensity of Alizarin red S and ALP staining in vitro. These all point to the fact that the M1 macrophages-mediated inflammatory environment is detrimental to ameloblastic differentiation and enamel mineralization, providing experimental evidence for inflammation-induced DDE.

Melatonin regulates numerous physiological parameters, such as control of circadian rhythms, modulation of differentiation and growth, antioxidative protection, and immune modulation.[27–32] In the field of dentistry, research on melatonin has focused on alleviating periodontal damage in periodontitis.[33] With regard to oral hard tissue studies, including our previous studies, the role of melatonin in mineralization has only been observed under physiological conditions and not in an inflammatory environment.[11,34] However, the state of ameloblasts is very different under physiological and pathological conditions, so in this study, by adding melatonin to in vivo and in vitro models of inflammation, we found that melatonin had a mitigating effect on inflammation-induced impaired enamel development and mineralization.

GPCRs are the largest superfamily of membrane proteins that control plenty of cellular signaling pathways and regulate key biological functions by coupling to G proteins to transduce extracellular signals into the cell.[35] The RGS protein family serves a critical role in the regulation of G-protein-mediated pathways by controlling its strength and duration.[36] RGS proteins are also critical modulators of inflammatory responses and can inhibit pro-inflammatory responses.[37] Deficiency or resistance of RGS proteins leads to elevated levels of pro-inflammatory cytokines.[38,39] When we compared the genes down-regulated in the M1-CM group and up-regulated in the M1-CM + MLT group in RNA-seq, the expression changes of *Rgs2* were prominent. We then explored the role of RGS2 in ameloblastic differentiation and found that decreased expression of RGS2 impeded ameloblastic differentiation of ALCs. Subsequently, to determine whether RGS2 plays a critical role in melatonin-mediated mitigation of impaired enamel mineralization in the inflammatory environment, we knocked down *Rgs2* in ALCs cultured with melatonin-containing M1-CM. The ability of melatonin to attenuate impaired enamel mineralization in the inflammatory environment is greatly diminished after the transfection of siRgs2. As a result, we come to the conclusion that melatonin attenuates impaired mineralization of ALCs in the inflammatory environment by upregulating the expression of RGS2. However, we did not investigate the role of RGS2 in vivo and focused only on the effect of melatonin on ALCs rather than RAW264.7 cells in vitro to explore the related molecular mechanisms. These issues will be further investigated in future studies.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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