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mmu-miR-1963 negatively regulates the ameloblast differentiation of LS8 cell line by directly targeting Smoc2 3'UTR

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ABSTRACT

RUNX2 is a key regulator of osteogenic differentiation and odontoblastic differentiation. *RUNX2* mutations could cause Cleidocranial dysplasia (CCD; OMIM119600), which is featured by abnormal development of bone and teeth. By using microRNA array, we identified a large number of microRNAs that showed different expression between wild-type *Runx2* group and mutant groups. The aim of this study is to find out the effect of mmu-miR-1963, which was downregulated in all mutant *Runx2* groups, on the ameloblast differentiation of LS8 cells. qPCR and Western Blot results showed the suppressive effect of mmu-miR-1963 on ameloblast differentiation of LS8 cell line. We further confirmed *Smoc2* as one direct target of mmu-miR-1963. For the first time, we showed that mmu-miR-1963 could regulate the ameloblast differentiation of LS8 by targeting *Smoc2*. This study suggests the suppressive role of mmu-miR-1963 on ameloblast differentiation of LS8 via directly targeting the 3'UTR of *Smoc2*. We also demonstrated that *Smoc2* itself could promote the ameloblast differentiation of LS8 for the first time. Our results indicate a novel explanation to the enamel hypoplasia phenotype in part of CCD patients.

1. Introduction

Runt-related transcription factor 2 (RUNX2;OMIM # 600211) is a member of runt family and located on chromosome 6p21 [1,2]. As transcriptional factors, runt family plays an important role in a variety of biological processes, such as cell differentiation and organ development, especially in chondrogenesis and osteogenesis [3,4]. Mutant *RUNX2* is the main cause of Cleidocranial dysplasia (CCD; OMIM119600), which is a dysplasia inherited as an autosomal dominant trait and it has a prevalence of 1 in 100,000 as reported [5]. CCD is mainly characterized by skeletal dysplasia including delayed closure of the anterior fontanelle, abnormal clavicles, supernumerary teeth, enamel hypoplasia and some other skeletal abnormalities [6,7].

Concerning to tooth development, heterozygous mutations in RUNX2 could cause a variety of tooth phenotypes, such as supernumerary teeth, enamel hypoplasia and delayed eruption of permanent dentition [6,7]. Runx2 showed a unique expression pattern very early in mesenchyme. Then it is downregulated in fully differentiated odontoblasts and also expressed in ectodermally derived ameloblasts which form enamel mineralized tissue of tooth crown [8,9]. The expression profile indicates that Runx2 plays a particular role in tooth development, especially in tooth morphology and formation of tooth mineralized tissues. Previous studies have shown that loss function of Runx2 in mouse embryos could cause failure of ameloblast and odontoblast differentiation [8,10,11]. Because of the deficient differentiation, Runx2 heterozygous mice showed misshaped tooth and lack of normal dentin and enamel matrices [12]. Besides, Runx2 could regulate tooth development via FGFs, especially Fgf3, which might be a target gene of Runx2 during the tooth development process [8,11]. However, the reason why mutant *RUNX2* could cause diversified phenotypes in tooth enamel development is still unclear.

microRNAs (miRNAs) are endogenous, small non-coding RNAs (~22 nucleotides) that take part in cellular process, organ development and tumorigenesis. miRNAs function as post-transcriptional mediators by binding to 3'-UTR region of target mRNAs [13–16]. It has been predicted that 1/3 of the protein coding genes are regulated by miRNAs [17]. In tooth developmental process, miRNAs play an important role which is illustrated by multiple incisors, misshaped molar

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cusps and lack of enamel in *Dicer1* knockout mice [18]. Several studies made further investigation into the effect of miRNAs on ameloblast differentiation. Cao et al. identified the Pitx2: miR-200c/141: noggin pathway was important in epithelial cells differentiation and tooth development both in vivo and in vitro[19]. Fan et al. found that miR-224 could regulate enamel mineralization by targeting *SLC4A4* and *CFTR*. The enamel Ca/P ratio and microhardness of mice incisors were obviously reduced with miR-224 agomir treatment [20]. Our previous study showed that mutant *Runx2* decreased the ameloblast differentiation of LS8 cells. We further identified the effect of the miR-185-5p-*Dlx2* axis on the ameloblast differentiation process [21]. We also found that mmu-miR-1963 was significantly downregulated in LS8 cell line by mutant Runx2 in the same screening system.

In this study, for the first time, we identified mmu-miR-1963 as a mediator in the ameloblast differentiation process of LS8 cell line. Firstly, the expression profile of mmu-miR-1963 were examined by real-time PCR, then we transfected LS8 cells with mmu-miR-1963 to explore ameloblast differentiation change caused by overexpression of mmu-miR-1963. Bioinformatical analysis and dual-luciferase reporter assay helped us to identify *Smoc2* was a target gene of mmu-miR-1963 in the amelobalst differentiation process. This study not only unveils the role of mmu-miR-1963 in ameloblast differentiation of LS8 cells through targeting *Smoc2,* but also suggests a novel explanation of the enamel hypoplasia phenotype in CCD patients.

2. Materials and methods

2.1. Cell culture

LS8 cells (kindly provided by Dr. Malcolm L. Snead in USC, Los Angeles, CA) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Islang, NY, USA), containg 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum(FBS) (Gibco, Grand Island, NY, USA) at 37 temperature in 5% CO_2 humidified atmosphere. A supplement of 20 mg/ml retinoic acid (RA, U.S. Pharmacopeia) and 10^{-7} M dexamethasone (DEX, Sigma) were used to induce LS8 cells maturation.

2.2. Bioinformatics methods

A search for predicted target mRNAs was performed using three databases: microRNA.org (http://www.microrna.org), TargetScan (http://www.targetscan.org) and miRDB (www.mirdb.org/).

2.3. Quantitative RT-PCR

Total RNA was isolated with TRIzols reagent (Invitrogen Life Technologies, Grand Island, NY, USA) and 2 μg of RNA was reverse-transcribed into cDNA using the Superscript first-strand synthesis system (Invitrogen Life Technologies) according to manufacturer's instruction. Real-time PCR reactions were conducted in a 20-μl reaction mixture (containing cDNA and SYBR green master mix) using an ABI 7500 real-time PCR system (Life Technologies Corporation). The mRNA expressions were normalized to β-actin and calculated using the $2^{-\Delta\Delta Ct}$ method. The forward and reverse primers for the amplification of mouse genes were as follows (5'–3'):

Smoc2 (F: ATGAGTGGCTCCTTCGATCGCAAGCT; R: TCAGTACACA GCCCCAGGGTTGGGC)

Amelx (F: GATGGCTGCACCACCAAATC; R: CTGAAGGGTG TGACTCGGG)

Klk4 (F: CCGGATCATACAAGGCCAGG; R: CCCACGATGTA GGACTCCTGT)

Mmp20 (F: GGCGAGATGGTGGCAAGAG; R: CTGGGAAGAG GCGGTAGTT)

β-actin (F: CTAAGGCCAACCGTGAAAAG; R: ACCAGAGGCATA CAGGGACA).

2.4. Quantitative analysis of miRNA expression

miRNA was isolated with miRcute miRNA isolation kit (TIANGEN BIOTECH, China). miRNA was reverse-transcribed into cDNA with miRcute Plus miRNA First-Strand cDNA Synthesis Kit (TIANGEN BIOTECH, China). miRNA real-time PCR was performed by using the miRcute Plus miRNA qPCR Detection Kit (TIANGEN BIOTECH, China). The mmu-miR-1963 expression was normalized to murine U6 and calculated using the $2^{-\Delta\Delta Ct}$ method. The primers for the real-time PCR are as follows (5'–3'):

mmu-miR-1963(F: GGACGAGATCATGAGGCCT; R: GTCGGTGTC GTGGAGTCG)

U6 (F: GCTTCGGCAGCACATATACTAAAAT; R: CGCTTCACGAATT TGCGTGTCAT)

2.5. Western blot analysis

Proteins were prepared by using RIPA containing protease inhibitor. Each sample, which contained equal amounts of protein, was subjected to SDS-PAGE. Then the protein was transferred to a PVDF membrane (Millipore, USA). The membrane was blocked with 5% BSA in Tris-buffered saline for 1 h and then incubated with the primary antibody against SMOC2, AMELX, KLK4 and MMP20 (Abcam, USA) overnight at 4 °C. Then with the fluorescent secondary antibodies (Rockland, USA) for 1 h at room temperature. Protein bands were visualized on an Odyssey infrared imaging system (Odyssey LI-COR Biosciences, Lincoln, NE).

2.6. Overexpression and inhibition of mmu-miR-1963

The cells were transiently transfected with a mmu-miR-1963 mimic oligonucleotide (mimic) or a hairpin inhibitor of mmu-miR-1963 (inhibitor). Control oligonucleotides (miR-NC or inhibitor NC) were used as negative controls. The small RNA mimic and hairpin inhibitor were obtained from Genepharma (Suzhou, China). Transfection was performed using Lipo2000 (Life Technologies Corporation, Grand Island, NY, USA) according to the manufacturer's instructions. 6 h after transfection, the culture medium was replaced with fresh proliferation medium or amelogenic inducing medium.

2.7. Luciferase reporter assay

The Smoc2 3'-UTR WT luciferase reporter construct was produced by subcloning the Smoc2 3'UTR PCR fragments downstream of the luciferase gene in the psi-CHECK2.0 vector (Promega, Madison, Wisconsin, USA) after digestion with the *Xba*I and *Not*I enzyme. The mutant Smoc2 3'UTR (Smoc2 3'UTR-Mut) was generated from Smoc2-3'UTR-WT using single-site mutation kit (Promega, Madison, WI). The sequence of Smoc2-3'UTR-WT and Smoc2-3'UTR-Mut was confirmed by DNA sequencing. Smoc2-3'UTR-WT or Smoc2-3'UTR-Mut, with 50 nM mmu-miR-1963 mimics or NC, were co-transfected into 293 T cells using the Lipo2000 (Life Technologies Corporation, Grand Island, NY, USA). Cells were harvested at 24 h after transfection and assayed for Renilla and Firefly luciferase activity using the Dual-Lucy Assay Kit (Vigorous Biotechnology, Beijing, China).

2.8. Knockdown and overexpression of Smoc2

The cells were transiently transfected with siRNA of Smoc2. Control oligonucleotides (NC) were used as negative controls. The siRNA and NC were obtained from Genepharma (Suzhou, China). Transfection was performed using Lipo2000 (Life Technologies Corporation, Grand Island, NY, USA) according to the manufacturer's instructions. 6 h after transfection, the culture medium was replaced with fresh proliferation medium or amelogenic inducing medium. Complementary DNAs for the mouse *Smoc2* gene were obtained by an RT-PCR technique using the

Prime-Script™ PT reagent kit (TaKaRa) and subcloned to the Smoc2 3'-UTR WT plamid. Plasmid DNA was transfected into cells using Lipo2000.

2.9. Statistical analysis

All data was representative of each assay repeated independently at least three times. Statistical significance was determined using the Student's t-test with Graph Pad Prism software (Graph Pad Soft-ware, La Jolla, CA, USA). A P-value less than 0.05 was considered statistically significant, and the level of significance was indicated as follows: *, $P < 0.05$; **, $P < 0.01$.

3. Results

3.1. Expression profile of mmu-miR-1963 and Smoc2 during the amelogenic differentiation of LS8 cells

The endogenous level of mmu-miR-1963 in LS8 cells was decreased gradually during the amelogenic differentiation. The expression of mmu-miR-1963 was more than 50% at 48 h compared with the un-differentiated group (Fig. 1A). We examined the predicted target genes of mmu-miR-1963 on three different miRNA target prediction databases. All of these databases indicated that mmu-miR-1963 could target the 3'UTR of *Smoc2* (Fig. 1C). The expression pattern of *Smoc2* in LS8 cells was opposite to mmu-miR-1963. At 48 h, the level of *Smoc2* mRNA was more than twice than the undifferentiated group (Fig. 1B). The expression of SMOC2 protein showed similar pattern with that of *Smoc2* mRNA (Fig. 1D).

3.2. Negative effects of mmu-miR-1963 on the amelogenic differentiation of LS8 cells

We transfected LS8 with miR-NC and mmu-miR-1963 mimics to over-express mmu-miR-1963. The qPCR results of mmu-miR-1963 showed that mmu-miR-1963 was elevated about 2.5-fold in the mimic group compared with the NC group after 48-hours transfection in the LS8 cells without amelogenic differentiation induce (Fig. 2A). Along with the elevated mmu-miR-1963, the expression levels of *Amelx, Klk4* and *Mmp20* were downregulated both in amelogenic differentiated group (the 48 h group in the figure) and undifferentiated group (the 0 h group in the figure) (Fig. 2B, D and F). Western Blot results showed the similar changes pattern of AMELX, KLK4 and MMP20 (Fig. 2 C, E and G). These results indicate that overexpression of mmu-miR-1963 in LS8 inhibits the amelogenic differentiation.

3.3. mmu-miR-1963 inhibits expression of Smoc2 by directly binding to the 3'UTR

To verify the prediction that mmu-mir-1963 could bind to the 3'UTR of Smoc2, we firstly checked the expression level of Smoc2 in cells that were transfected with NC or mmu-miR-1963 mimics. The qPCR results showed that overexpression of mmu-miR-1963 could inhibit the expression of *Smoc2* mRNA, especially in the amelogenic inducing group (Fig. 3A). The similar inhibition effect was examined in protein level of SMOC2. Furthermore, expression level of SMOC2 protein was upregulated when we transfected cells with mmu-miR-1963 inhibitors (Fig. 3B). These results indicate that mmu-miR-1963 could regulate the expression of Smoc2 in both mRNA and protein level.

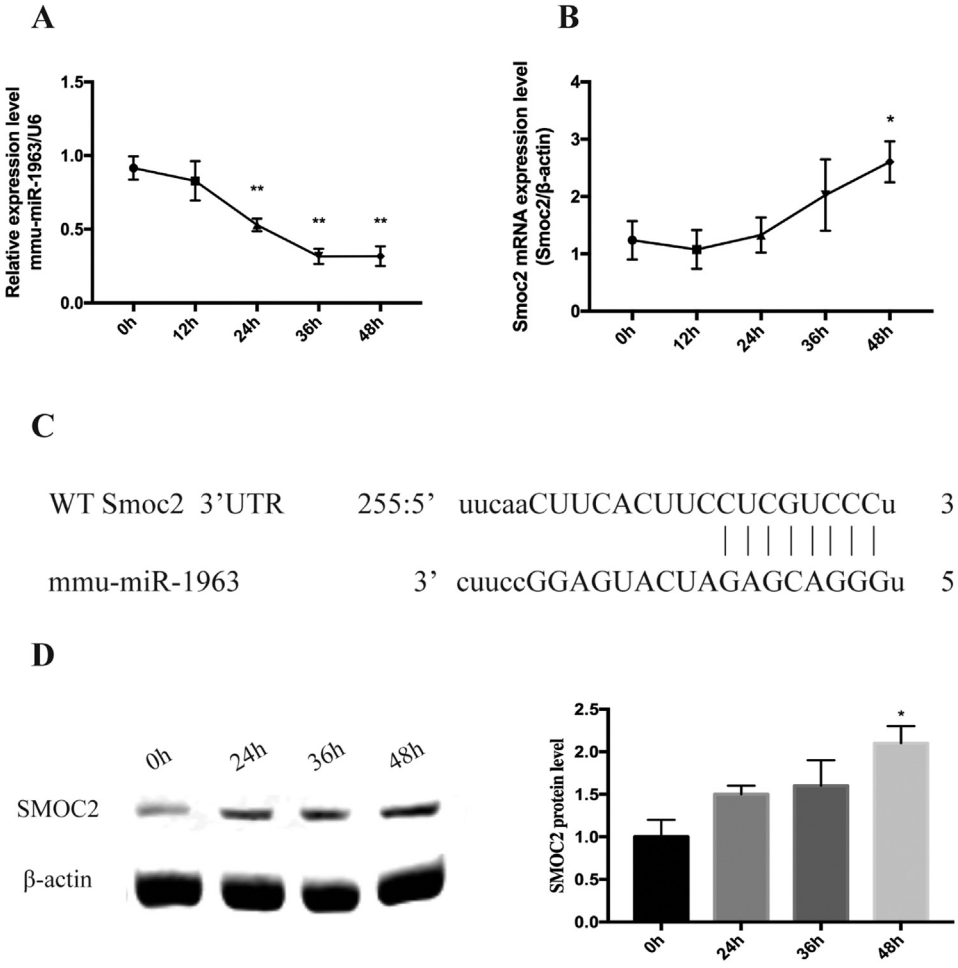

Fig. 1. Changes in the expression levels of mmu-miR-1963 and Smoc2 during the amelogenic differentiation of LS8 cells. A. The endogenous expression of mmu-miR-1963 was examined by qRT-PCR at 0, 12, 24, 36 and 48 h after induction of amelogenesis. (**$p < 0.01$). **B.** Expression level of Smoc2 mRNA was measured by qRT-PCR (*$p < 0.05$). **C.** Bioinformatics analyse of the complementarity of the mmu-miR-1963 seed sequence to the 3'UTR of Smoc2. **D.** Expression level of Smoc2 protein was examined by Western Blot. (*$p < 0.05$).

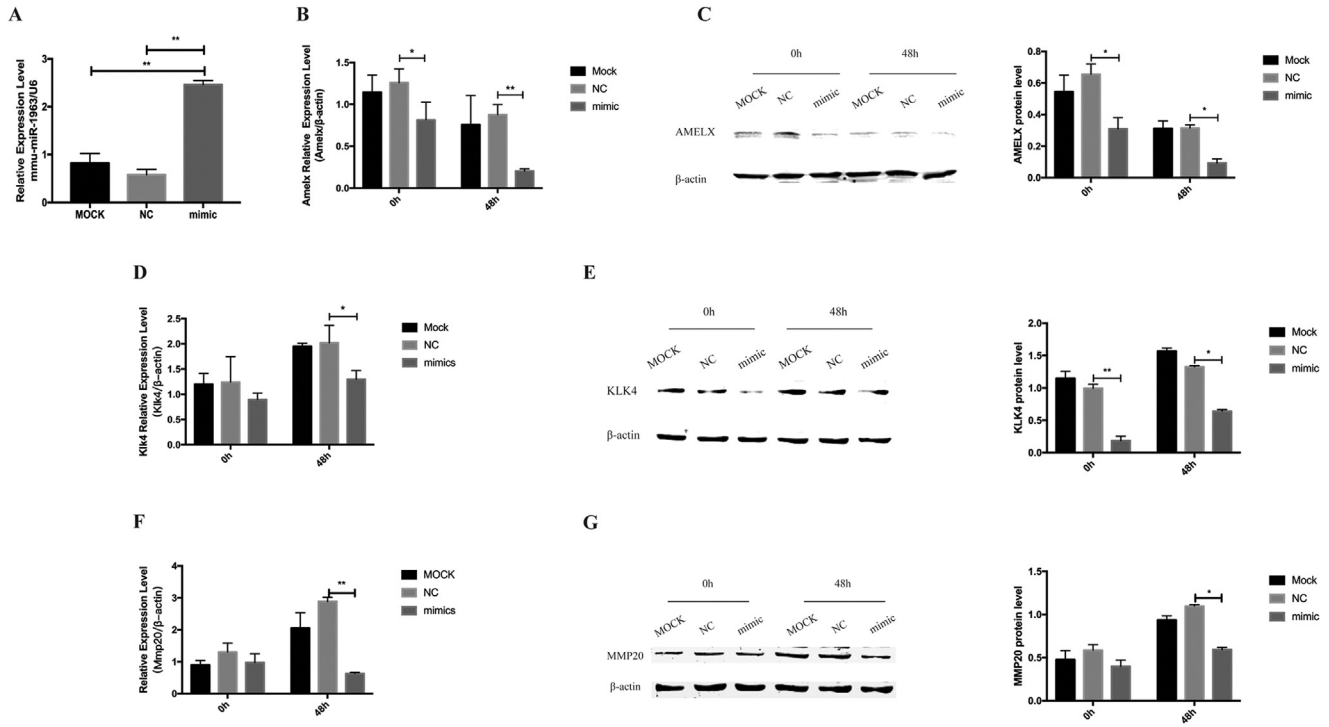

Fig. 2. Effects of mmu-miR-1963 on the amelogenic differentiation of LS8 cells. A. Expression of mmu-miR-1963 in LS8 cells transfected with mimics was measured by qRT-PCR. (**p < 0.01). **B. D. F.** Changes at mRNA level of Amelx, Klk4 and Mmp20 were measured by qRT-PCR. (*p < 0.05, **p < 0.01). **C. E. G.** Changes at protein level of AMELX, KLK4 and MMP20 were accessed by western blot.

To confirm the direct association between mmu-miR-1963 and the 3'UTR of Smoc2, we conducted the dual-luciferase reporter assay. The predicted binding site and mutations generated in seed region was shown in Fig. 3C. The relative luciferase activity of Smoc2-3'UTR-WT was decreased by about 60% in mmu-miR-1963 mimics co-transfected 293 T cells than the NC co-transfected cells. However, there was no change of relative luciferase activity in Smoc2-3'UTR-Mut co-transfected groups (Fig. 3D).

3.4. Smoc2 promotes the amelogenic differentiation of LS8

To reveal the function of Smoc2 on amelogenic differentiation, we transfected LS8 with Smoc2 overexpression plasmids. Results of real-time PCR and western blot showed that overexpression of Smoc2 could

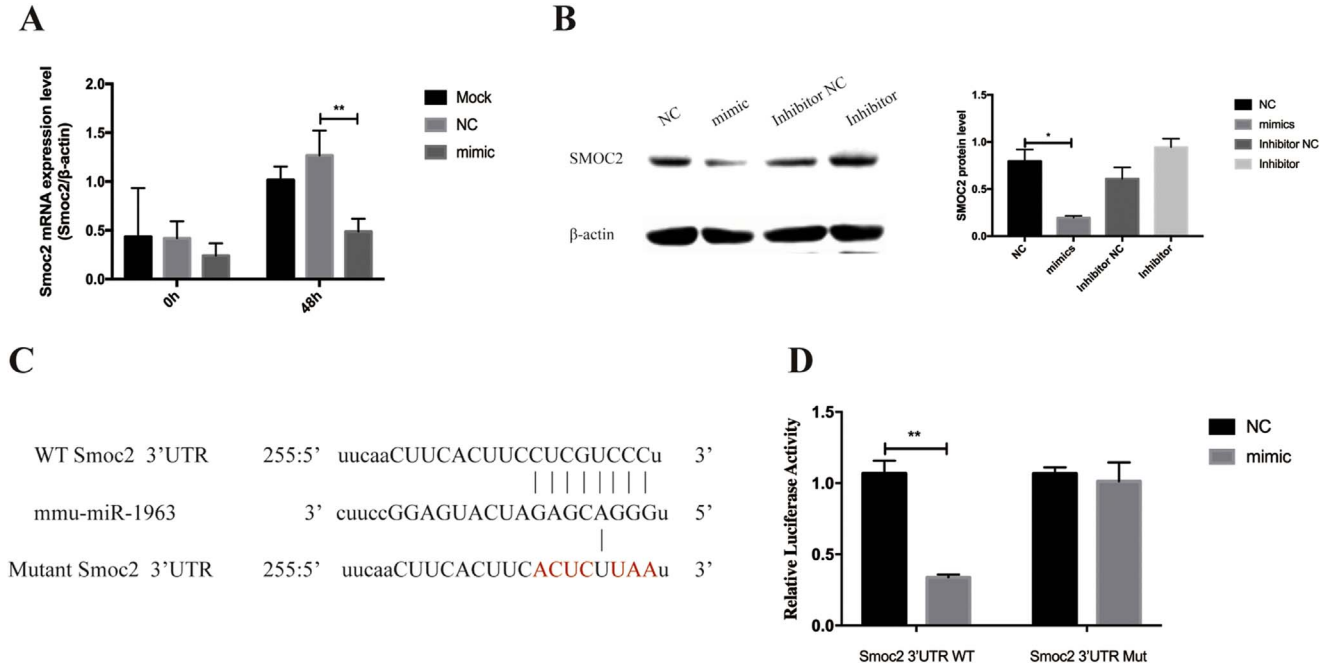

Fig. 3. Direct link between mmu-miR-1963 and Smoc2. A. The expression of Smoc2 in LS8 cells transfected with mmu-miR-1963 mimic at 0 h and 48 h after the amelogenic induction was measured by qRT-PCR. (**p < 0.01). **B.** Western blot of SMOC2 in LS8 cells transfected with mmu-miR-1963 mimic or the inhibitor 48 h after the amelogenic induction. **C.** The sequence of 3'UTR-binding site in Smoc2 was mutated. **D.** The relative luciferase activities were decreased by overexpression of mmu-miR-1963.

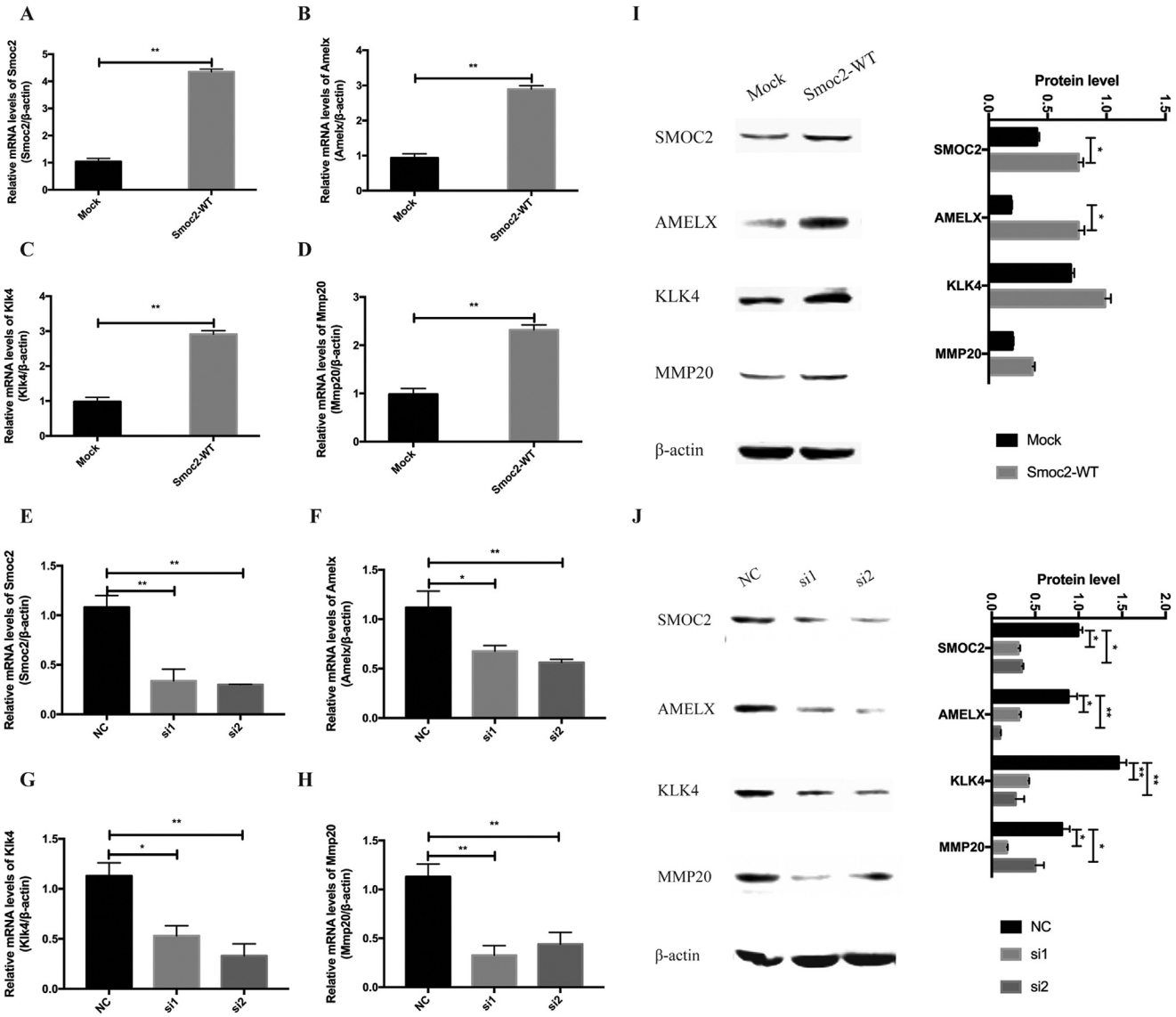

Fig. 4. Effects of Smoc2 on the amelogenic differentiation of LS8 cells. A-D. Overexpression of Smoc2 upregulated the mRNA level of *Amelx, Klk4* and *Mmp20*. **E-H.** The expression levels of *Amelx, Klk4* and *Mmp20* were inhibited by si-Smoc2. **I.** The protein levels of Amelx, Klk4 and Mmp20 were upregulated by Smoc2-WT plasmids. **J.** The protein level of Amelx, Klk4 and Mmp20 was repressed by si-Smoc2.

upregulate the expression level of *Amelx, Klk4* and *Mmp20* after 48 h of amelogenic induction (Fig. 4A-D, I). Furthermore, knockdown of Smoc2 by siRNAs decreased the protein level of these markers (Fig. 4E-H, J). These results demonstrate that Smoc2 could promote the amelogenic differentiation of LS8.

4. Discussion

Tooth enamel formation initiated from the ameloblast differentiation of cells under the predentin. Runx2 is essential for the enamel formation and it is illustrated by the lack of enamel in incisors of mutant Runx2 mice [8]. Ambn is an extracellular matrix protein and may play a role in enamel crystal formation. Runx2 could regulate its expression by directly binding to the promoter region. [22]. Furthermore, our previous studies found that mutant Runx2 could inhibit the ameloblast differentiation of LS8 via mmu-miR-185-5p-Dlx2 axis [21]. These results suggest that Runx2 could be involved in the enamel formation with directly or indirectly regulation of target gene expression.

Previous studies on the function of miRNAs in ameloblast differentiation revealed that miRNAs play important roles in tooth development [18,23–25]. Cao et al. identified the Pitx2: miR-200c: noggin pathway as a mediator in the dental stem cell differentiation process [19]. Sharp et al. showed that miR-200a could specify the fate of dental epithelial cells by repressing β-catenin. They further identified *Zeb* as the target gene of miR-200a in regulating tooth morphology [26]. In a recent study, Le et al. found a novel miRNA that produced from amelogenin exon4 could regulate the ameloblast differentiation of LS8 by targeting Runx2 [27].

In this study, we illustrated the role of mmu-miR-1963, which was downregulated in all three-type mutant Runx2 groups (Supplemental Fig. 1), in the ameloblast differentiation process of LS8 cell line. We reveal that mmu-miR-1963 could inhibit ameloblast differentiation of LS8 cells with and without amelogenic induce. We further verified that mmu-miR-1963 regulates the ameloblast differentiation of LS8 via Smoc2. Smoc2 was first isolated by Vannahme et al. in 2003 [28]. They found that Smoc2 was a member of BM-40 family which was reported to participate in the regulation of cell-matrix interactions, in particular influencing bone mineralization, wound repair and angiogenesis [29]. The expression of Smoc2 was found at all stages of developing tooth germ. Surprisingly, SMOC2 mutation was identified in patients with

oligodontia [30]. Patients carried the mutation showed oligodontia, microdontia and thistle shaped permanent molars [31]. Therefore, it's obvious that SMOC2 takes part in tooth development. However, the function of SMOC2 on tooth development has yet to be fully investigated. In this study, we found that overexpression of Smoc2 could upregulate the expression levels of amelogenic markers, such as Amelx, Klk4 and Mmp20 in LS8 cells, and knockdown of Smoc2 by siRNAs inhibited the expression levels of these markers. These results suggested that Smoc2 played an important role in the amelogenic differentiation of LS8 cells. However, our study was limited by the in vitro system, further in vivo studies are needed to carry out for revealing the detail mechanisms that how Smoc2 regulates amelogenic differentiation and other tooth phenotypes.

To our knowledge, for the first time, we report that mmu-miR-1963 inhibits the ameloblast differentiation of LS8 cells via post-trancriptional regulation of Smoc2. Furthermore, the function of how Smoc2 influences tooth development are newly interpreted as well. However, the molecular mechanisms still need further exploration. Taken together, our findings provide new explanations on the regulation of amelogenesis differentiation.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2017.12.008.

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