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RAPID COMMUNICATION

MACF1 deficiency suppresses tooth mineralization through IGF1 mediated crosstalk between odontoblasts and ameloblasts

Tooth mineralization is a ubiquitous and tightly regulated process involving complicated interactions between dental epithelium and mesenchyme. Key molecules in tooth mineralization remain poorly identified. Microtubule actin cross-linking factor 1 (MACF1) is a spectraplakin protein that plays pivotal roles in the brain, muscle, lung, and bone developmental process.[1–3] To study the specific functions of MACF1 in bone formation, we established *Macf1* conditional knockout mice using the Cre-LoxP system driven by *Osxterix* promoter (*Osx*-Cre;*Macf1*$^{f/f}$).[2] Not surprisingly, *Osx*-Cre;*Macf1*$^{f/f}$ mice displayed the phenotypes of delayed ossification and decreased bone mass. Moreover, the *Osx*-Cre;*Macf1*$^{f/f}$ mice unexpectedly showed a white and opaque appearance of incisors, contrary to the normal yellow-brown and transparent incisors. Since *Osxterix* is expressed in dental mesenchyme during tooth development, the abnormal tooth appearance might imply a new function of MACF1 in odontoblasts, or even ameloblasts. Therefore, the present study aimed to investigate the role of MACF1 during tooth development.

In order to investigate the function of MACF1 in tooth formation, we first observed the tooth morphology of *Osx*-Cre;*Macf1*$^{f/f}$ mice. *Osx*-Cre;*Macf1*$^{f/f}$ mice showed obvious white, opaque, blunt, and worn changes in incisors. The molars of *Osx*-Cre;*Macf1*$^{f/f}$ mice were also less transparent than that of control mice (Fig. 1A). Micro-CT scanning showed reduced mineral density in both dentin and enamel of 2-month-old *Osx*-Cre;*Macf1*$^{f/f}$ mice (Fig. 1B). SEM analysis showed the rough dentin surface and disorderly arranged dentin tubules with uneven size in incisors of six-month-old *Osx*-Cre;*Macf1*$^{f/f}$ mice. Besides, the enamel of molars in conditional knockout mice also had a rough surface with a large number of pits and holes, and the enamel column crystals were disorderly arranged (Fig. 1C). Overall, these results demonstrate that the absence of MACF1 caused tooth damage with decreased quality and density of mineralization and microstructural degeneration in both dentin and enamel.

HE staining found that tooth development was disturbed in *Osx*-Cre;*Macf1*$^{f/f}$ mice (Fig. 1D). In these mice, the differentiation of odontoblasts from dental papilla cells (DPCs) delayed in E18.5 and these were few polarized cells in P0. The *Macf1* deficient odontoblasts and ameloblasts kept polarized but their arrangement became looser and more disordered in P5 tooth germ. The ameloblasts even had an inconsistent degree of polarization in the P14 stage. Both incisors and molars had an obvious thinner dentin in *Osx*-Cre;*Macf1*$^{f/f}$ mice (from P5 to P14 stage) (Fig. 1D; Fig. S1A). The blank area of decalcified enamel was also smaller (Fig. S1A). Toluidine blue staining confirmed the dentin became thinner and the pulp cavity was larger in six-month-old *Osx*-Cre;*Macf1*$^{f/f}$ mice (Fig. S1B). Thus, the deletion of *Macf1* delayed the differentiation of odontoblasts and ameloblasts, as well as the formation of dentin and enamel, which caused the abnormal appearance and morphology of both dentin and enamel in *Osx*-Cre;*Macf1*$^{f/f}$ mice.

To further study the role of MACF1 in tooth formation at a cellular level, we first detected the expression of MACF1 in developmental teeth of wide-type mice by immunohistochemistry. The results showed that MACF1 was highly expressed in both odontoblasts and ameloblasts (Fig. S2A). qPCR and Western blot results showed that the expression of MACF1 was significantly decreased in tooth germs of *Osx*-Cre;*Macf1*$^{f/f}$ mice (Fig. S1B, C). The immunohistochemistry results showed that MACF1 was specifically deleted in the odontoblast cell layer but was still expressed in the

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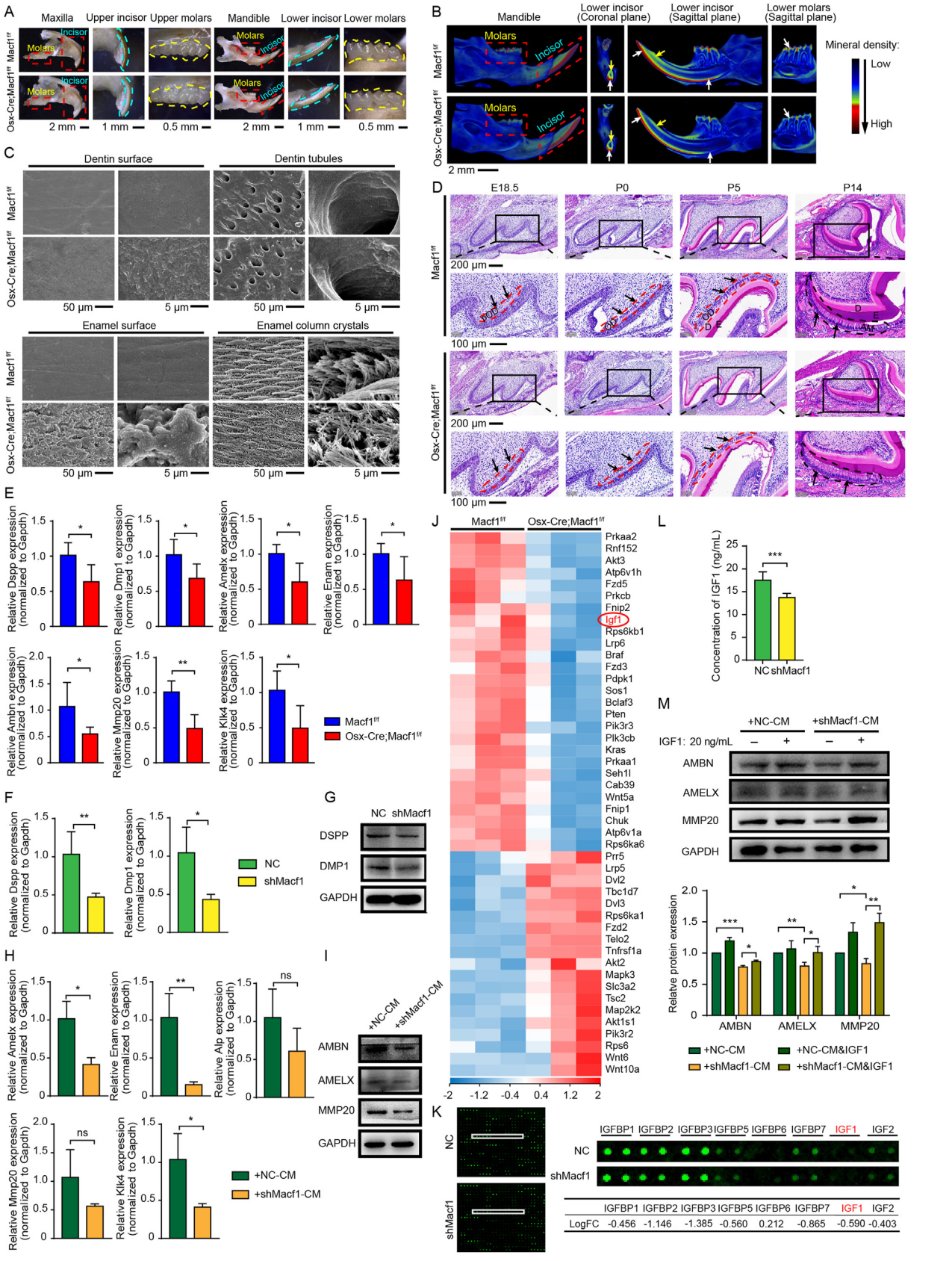

Figure 1 MACF1 deficiency in odontoblasts suppresses tooth mineralization through IGF1 mediated crosstalk between odontoblasts and ameloblasts. **(A)** Photos of incisors and molars of two-month-old *Macf1*$^{f/f}$ and *Osx*-Cre;*Macf1*$^{f/f}$ mice. **(B)** Representative micro-CT images of molars and incisors from two-month-old *Macf1*$^{f/f}$ and *Osx*-Cre;*Macf1*$^{f/f}$ mice. Yellow arrow, dentin mineral

ameloblast cell layer of *Osx*-Cre;*Macf1*$^{f/f}$ mice (Fig. S1D). In order to detect the role of MACF1 during the differentiation of odontoblasts, we isolated DPCs from mice at P2 and cultured them with osteogenic medium to induce odontoblastic differentiation. The mRNA expression of *Macf1* presented in a time-dependent way consistent with odontoblast differentiation marker genes such as *Dmp1*, *Dspp*, *Col1a1*, *Alp*, and *Ocn* (Fig. S1E). These findings suggest that MACF1 may positively regulate the differentiation of odontoblasts.

We also found the reduced mRNA levels of two dentin markers (*Dspp* and *Dmp1*) in the tooth germs of *Osx*-Cre;*Macf1*$^{f/f}$ mice at P2 (Fig. 1E). In order to ascertain whether MACF1 regulated the differentiation of odontoblasts, we constructed a stable *Macf1* knockdown MDPC-23 odontoblast-like cell line by transducing *Macf1* shRNA with lentiviral vectors (shMacf1). The mRNA and protein expression of MACF1 were effectively reduced (Fig. S3A, B). Moreover, the expression of *Dspp* and *Dmp1* was significantly downregulated after shMacf1 transfection (Fig. 1F, G). After seven-day osteogenic induction, the *Macf1*-knockdown MDPC-23 showed decreased ALP activity than the control (Fig. S3C). Also, the *in vivo* data was obtained from primary DPCs of *Osx*-Cre;*Macf1*$^{f/f}$ mice, and as predicted, showed the same results (Fig. S3D—H). These results demonstrate that MACF1 deficiency inhibited the differentiation of odontoblasts.

Although the decreased MACF1 expression was not found in ameloblast, we still detected the reduced mRNA levels of five enamel markers (*Amelx*, *Enam*, *Ambn*, *Mmp20*, and *Klk4*) in the tooth germs of *Osx*-Cre;*Macf1*$^{f/f}$ mice at P2 (Fig. 1E). We speculated that the enamel dysplasia and the altered differentiation of ameloblast in *Osx*-Cre;*Macf1*$^{f/f}$ mice might be an effect of the defected crosstalk between ameloblasts and odontoblast. Therefore, we collected the supernatant medium of MDPC-23 during the mineralization to induce the differentiation of LS8 ameloblast-like cells. After induction, the expression levels of AMELX, ENAM, AMBN, ALP, MMP20, and KLK4 at day 2 and ALP staining at day 7 were significantly inhibited (Fig. 4H, I; Fig. S4A). Similar results were observed in LS8 cultured with the supernatant medium of primary DPCs from *Osx*-Cre;*Macf1*$^{f/f}$ mice (Fig. S4B—D). These results indicate that MACF1 deficiency in odontoblasts inhibited its interaction with ameloblasts, which caused the defection in ameloblasts differentiation and enamel formation.

To investigate the potential mechanism of MACF1 regulating tooth formation, the differential gene expression profiles of tooth germ from *Macf1*$^{f/f}$ mice and *Osx*-Cre;*Macf1*$^{f/f}$ mice were established by transcriptome sequencing. We found that *Igf1* and related molecules in the mTOR pathway were most prominent (Fig. 1J). Previous research has demonstrated the important roles of the mTOR signaling pathway in tooth formation.[4] IGF1 was reported to inhibit the apoptosis of ameloblasts and promote the secretion of enamel matrix.[5] It can be inferred that deletion of *Macf1* in odontoblasts may inhibit the expression of IGF1 and its downstream mTOR pathway during tooth formation. To verify the paracrine IGF1 from odontoblasts mediates the regulation of ameloblast differentiation, a cytokine antibody chip was used to detect the protein profile in the supernatant medium of *Macf1* knockdown MDPC-23 cells. We found that the expression of IGF1 protein and its downstream proteins were consistent with transcriptome sequencing results (Fig. 1K). ELISA analysis further confirmed the decreased IGF1 concentration in the supernatant medium of *Macf1* knockdown MDPC-23 cells (Fig. 1L). We verified that IGF1 deficiency in the mineralization condition medium of MDPC-23 cells inhibited the amelogenesis of LS8 cells (Fig. S5). The effect of reduced secretion of IGF1 by odontoblast was demonstrated by rescue experiment. The exogenous IGF1 protein can effectively recover the ameloblast differentiation with increased expression of marker genes (Fig. 1M). These results prove that MACF1 in odontoblasts affects ameloblast differentiation by regulating the IGF1 paracrine from odontoblasts, thereby regulating the formation of tooth enamel.

Thus, our data demonstrated that deletion of *Macf1* in odontoblasts causes abnormal morphology and deteriorated microarchitecture in both dentin and enamel. MACF1 deficiency in odontoblasts not only affected the odontoblast differentiation but also the ameloblast differentiation via

density; white arrow, enamel mineral density. **(C)** Representative dentin (upper panel) and enamel (lower panel) SEM images of six-month-old *Macf1*$^{f/f}$ and *Osx*-Cre;*Macf1*$^{f/f}$ mice. **(D)** HE staining of the first upper molar tooth germs from *Macf1*$^{f/f}$ and *Osx*-Cre;*Macf1*$^{f/f}$ mice at E18.5, P0, P5, and P14. pOD, preodontoblast; OD, odontoblast; AM, ameloblast; D, dentin; E, enamel. **(E)** The mRNA expression of odontoblast and ameloblast differentiation marker genes in molar tooth germs of *Macf1*$^{f/f}$ and *Osx*-Cre;*Macf1*$^{f/f}$ mice at P2. **(F)** The mRNA expression of odontoblast differentiation marker genes in MDPC-23 transfected with or without *Macf1* shRNA was measured by qPCR. **(G)** The protein expression of dentin differentiation marker genes in MDPC-23 transfected with or without *Macf1* shRNA was measured by Western blot. After osteogenic induction by mineralization condition medium of MDPC-23 transfected with or without *Macf1* shRNA for 2 days, the mRNA and protein expression of ameloblast differentiation marker genes in LS8 were measured by qPCR **(H)** and Western blot **(I)**. **(J)** Differentially expressed genes in mTOR signaling pathway between the tooth germs of *Macf1*$^{f/f}$ mice and *Osx*-Cre;*Macf1*$^{f/f}$ mice. **(K)** Antibody arrays were used to measure the secretion of proteins by the MDPC-23 cell line transfected with or without *Macf1* shRNA during differentiation. The arrays were scanned and quantified. **(L)** IGF1 concentration in the medium supernatant of MDPC-23 verified by ELISA. **(M)** Western blot and quantification of AMBN, AMELX, and MMP20 level of two-day maturation-induced LS8 cultured with odontoblast medium supernatant treated with or without IGF1. NC, the group infected with lentivirus containing the nonsense shRNA sequence (negative control); shMacf1, the group infected with lentivirus containing *Macf1* shRNA; +NC-CM, culture LS8 with mineralization condition medium of negative control MDPC-23; +shMacf1-CM, culture LS8 with mineralization condition medium of MDPC-23 infected with lentivirus containing *Macf1* shRNA. The data were presented as mean ± SD. Unpaired student's *t*-tests were used to compare data between two groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

reduced secretion of IGF1 by odontoblasts. Our study reveals a new role and mechanism of MACF1 in tooth development.

Ethics declaration

All animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, and all experimental procedures were approved by the Institutional Experimental Animal Committee of Northwestern Polytechnical University (Xi'an, China).

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.101103.

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