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RESEARCH ARTICLE

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Autophagy mediates cementoblast mineralization under compression through periostin/β-catenin axis

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Abstract

Repair of orthodontic external root resorption and periodontal tissue dysfunction induced by mechanical force remains a clinical challenge. Cementoblasts are vital in cementum mineralization, a process important for restoring damaged cementum. Despite autophagy plays a role in mineralization under various environmental stimuli, the underlying mechanism of autophagy in mediating cementoblast mineralization remains unclear. Here we verified that murine cementoblasts exhibit compromised mineralization under compressive force. Autophagy was indispensable for cementoblast mineralization, and autophagic activation markedly reversed cementoblast mineralization and prevented cementum damage in mice during tooth movement. Subsequently, messenger RNA sequencing analyses identified periostin (Postn) as a mediator of autophagy and mineralization in cementoblasts. Cementoblast mineralization was significantly inhibited following the knockdown of Postn. Furthermore, Postn silencing suppressed Wnt signaling by modulating the stability of β-catenin. Together our results highlight the role of autophagy in cementoblast mineralization via Postn/β-catenin signaling under compressive force and may provide a new strategy for the remineralization of cementum and regeneration of periodontal tissue.

KEYWORDS
autophagy, cementum, mineralization, periostin, signal transduction, ubiquitination

1 | INTRODUCTION

Cementum, a thin layer of mineralized tissue covering the root surface, connects the periodontal ligament (PDL) to the tooth. The integrity of cementum is important to dental stability and periodontal function (Bosshardt & Selvig, 1997). However, cementum damage often occurs when subjected to mechanical force, resulting in external root resorption and periodontal tissue dysfunction (Ahangari et al., 2015; Arzate et al., 2015). Cementoblasts secrete mineralized materials to form cementum tissue and thus are responsible for repairing cementum and restoring periodontal function (Ahangari et al., 2015; Bosshardt, 2005). Cementoblast functions are modulated by compressive force, but the effects of compressive force on cementoblast mineralization remain controversial (Rego et al., 2011; Zhang, Huang, et al., 2017). Investigations into how compressive force impacts cementoblast mineralization are important for the development of therapeutics to repair external root resorption and achieve integrated periodontal function following tooth movement.

Autophagy is a process of self-degradation in which dysfunctional macromolecules and organelles are degraded and recycled (Nollet et al., 2014). Although autophagy is required to maintain cellular homeostasis, insufficient or excessive autophagy induced by

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environmental stimuli can influence cell survival and differentiation (Galluzzi et al., 2008; Glick et al., 2010). Accumulating data have confirmed that autophagic vesicles are responsible for transporting mineralized materials to the extracellular matrix (ECM), highlighting the role of autophagy in the remolding of mineralized tissue (Li, Su, et al., 2018; Nollet et al., 2014). However, the effects of autophagy on mineralization in cementoblasts subjected to compression remain unclear, and the downstream mechanism that regulate autophagy-mediated changes in cementoblasts requires further exploration.

Mineralization-related signaling pathways, including the transforming growth factor beta (TGF-β), Wnt, and Hippo pathways, have attracted interest as therapeutic targets for root resorption (Koba et al., 2021; Turkkahraman et al., 2020; Wu, Ou, et al., 2019). Increasing evidence has shown that autophagy regulates many signaling pathways, including TGF-β, Wnt, and PI3K signaling (Fan et al., 2018; Li, Li, et al., 2018; Zhang, Zhang, et al., 2017). The cooperation between autophagy and mineralization-related pathways remains poorly understood. Identifying autophagy-mediated molecules or signaling pathways could develop new strategies for cementoblast mineralization.

Here, we elucidate the effects of compressive force on mineralization and autophagy in cementoblasts. The role of autophagy in cementoblast mineralization under compression was determined in vitro and in vivo. Then we investigated the downstream mechanism of autophagic effects on cementoblast mineralization. We profiled gene expression of autophagy inhibitor-treated cementoblasts to identify molecules and signaling pathways involved in cementoblast mineralization. These findings broaden understanding of the cellular and molecular events underlying mineralization in cementoblasts under compressive force and will aid in the development of new therapeutic targets for the repair of root resorption and periodontal dysfunction.

2 | MATERIALS AND METHODS

2.1 | Reagents

Chloroquine (CQ) and rapamycin (Rapa) were purchased from ApexBio. 3-methyladenine (3-MA), SKL2001, and MG132 were purchased from MedChem Express. Cycloheximide (CHX) was purchased from AZKKA. The chemical reagents were dissolved in dimethyl sulfoxide (DMSO) or phosphate-buffered saline (PBS) to a specific concentration.

2.2 | Cell culture

OCCM-30, an immortalized murine cementoblast cell line, was provided by Dr. Martha J. Somerman (National Institutes of Health). The cell proliferation medium (PM) was prepared with Dulbecco's modified Eagle medium (DMEM; Gibco), 10% fetal bovine serum (FBS; Gibco), and 1% penicillin-streptomycin (Invitrogen) in a humidified atmosphere at 37°C with 5% CO_2 as described previously (D'Errico et al., 1999). To induce cementoblast mineralization, we prepared mineralized medium (MM) with DMEM, 10% FBS, 1% penicillin-streptomycin, 10 mM sodium β-glycerophosphate (Sigma-Aldrich), and 50 μg/mL ascorbic acid (Sigma-Aldrich).

2.3 | Application of mechanical compressive force

To apply compressive force in vitro, we used a mechanical force-loading device. Briefly, a cover glass was placed over a confluent cell layer in the well, and the compressive force was adjusted by changing the number of steel balls in the plastic bottle. Based on our previously established protocols, OCCM-30 cells were subjected to compressive force at a magnitude of 1.5 g/cm^2 for 12 h (Yang et al., 2021). For experiments assessing the effects of compressive force on cementoblast mineralization, we removed the force-loading device after the application of compression and incubated cells in PM or MM for 4 or 7 days.

2.4 | Mouse root resorption model

Adult male C57BL/6 mice (22–25 g, 6–7 weeks old) were purchased from Vital River Laboratory Animal Technology for use in this study. The experimental protocols were approved by the Animal Use and Care Committee of Peking University (LA2021076). Mice were anesthetized by intraperitoneal injection of 10% chloral hydrate. The root resorption model was established in the right maxilla of each mouse, and the left side was designated the negative control site. The application of orthodontic mechanical force was performed as described previously (Liu et al., 2022). A nickel–titanium coil spring (wire size, 0.2 mm; Smart Technology) was used to connect the right maxillary first molar and maxillary incisors and provided a nearly constant force of approximately 20 g. Mice were randomly divided into different groups with five mice in each group.

2.5 | Statistical analysis

Statistical analyses were performed with SPSS 13.0 and GraphPad Prism 7 (GraphPad Software). All data are presented as means ± standard deviations and were evaluated by independent two-tailed Student's t tests or one-way analysis of variance (ANOVA). Significance was defined as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. $p > 0.05$ was considered not significant.

The following methods are described in detail in the supplemental file:

- Quantitative real-time polymerase chain reaction (qRT-PCR)
- Western blot analysis analysis
- Alkaline phosphatase (ALP) staining and activity
- Immunofluorescence staining

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- Injection of the autophagy inhibitor CQ or activator Rapa
- Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC)
- Microcomputed tomography (micro-CT) scanning
- Picrosirius red (PSR) staining
- High-throughput RNA sequencing (RNA-seq)
- Small interfering RNA (siRNA) knockdown
- Immunoprecipitation (IP)

3 | RESULTS

3.1 | Compressive force suppresses the mineralization of cementoblasts

Cementoblasts were subjected to compression loading for 12 h, and subsequently incubated in proliferative media (PM) or mineralized media (MM) (Figure 1a). Compressive force inhibited messenger RNA (mRNA) expression of OCN and OSX, which are related to cementoblast mineralization, in both the PM and MM groups (Figure 1b). Consistent with these results, Western blot analysis and ALP staining showed a decreased mineralization capacity in cementoblasts under compression loading (Figure 1c–f).

To assess changes in the mineralization of cementoblasts under orthodontic force in vivo, we established a mouse root resorption model (Figure 1g). With increasing mechanical force-loading time, distance of tooth movement gradually increased (Supporting Information: Figure S1A), and obvious resorption lacunae were observed on the compressed side of the distal buccal root after the application of force for 3 weeks (Figure 1h). IHC showed that the expression of the mineralization-related protein OCN was decreased in cementoblasts on the compressed side (Figure 1i,j).

3.2 | Autophagy is inhibited in cementoblasts under compressive force

To evaluate the effects of compressive force on the autophagy of cementoblasts, two reliable markers of autophagy, the expression of the autophagic receptor P62 and the conversion of LC3-I to LC3-II, were examined (Klionsky et al., 2021). Western blot analysis showed that compressive force increased the expression of P62 but decreased the conversion of LC3-I to LC3-II in cementoblasts (Figure 2a,b). Furthermore, immunofluorescence staining showed that the formation of autophagosome-positive LC3 was significantly decreased in cells stimulated with compressive force (Figure 2c,d), which indicates that compressive force inhibits autophagic activity in cementoblasts. In addition, IHC showed that compressive force led to significant down-regulation of LC3 in cementoblasts on the compressed side (Figure 2e,f). LC3 staining on Cap+ cells located on the surface of root confirmed that there were fewer LC3+ cementoblasts under compressive force (Figure 2g,h).

3.3 | Autophagy is required for cementoblast mineralization

To determine the role of autophagy in cementoblast mineralization, we first detected the effect of mineralization induction on cementoblast autophagy and found that expression of P62 decreased but the conversion of LC3-I to LC3-II increased with induction time (Supporting Information: Figure S2A). Then we treated cementoblasts with the autophagy activator Rapa to enhance autophagy or the autophagy inhibitor CQ to suppress autophagy (Figure 3a). The effects of Rapa (500 nM) and CQ (5 μM) on autophagy were confirmed (Supporting Information: Figure S2B). qRT-PCR analyses indicated that treatment with Rapa significantly increased the expression of genes related to cementoblast mineralization, whereas treatment with CQ decreased the expression of mineralization-related genes in both PM and MM groups (Figure 3b). Western blot analysis and immunofluorescence analyses also showed the enhanced capacity of cementoblast mineralization when cells were treated with Rapa and decreased mineralization ability when cells were treated with CQ (Figure 3c–f). ALP staining and activity confirmed these results (Figure 3g,h). To verify these findings in vivo, we injected mice intraperitoneally for 3 weeks with autophagy activator Rapa, or inhibitor CQ. The effects of injection of Rapa and CQ on autophagy were confirmed by immunofluorescence staining of LC3 (Supporting Information: Figure S2C). IHC showed that OCN expression on the surface of the root was significantly increased in the Rapa treatment group and decreased in the CQ treatment group (Figure 3i,j).

3.4 | Compression-suppressed cementoblast mineralization is partially dependent on autophagy

Next, we determined whether compressive force regulates cementoblast mineralization via autophagy. Cementoblasts were pretreated with Rapa, and then exposed to compression (Figure 4a). Compression-inhibited expression of the mineralization-related genes OSX and OCN was rescued by Rapa in both PM and MM groups (Figure 4b). Western blot analysis also revealed that the decrease in OSX and OCN protein levels under compressive force was reversed following treatment with Rapa (Figure 4c,d). To further confirm the effects of autophagy on cementoblast mineralization in vivo, we systemically injected mice with Rapa and applied force for 3 weeks. Root resorption on the compressed side of the root was observed by micro-CT. The results showed that force-induced root resorption volume of distal roots was lower in Rapa-injected mice, suggesting that root resorption was partially relieved by Rapa injection (Figure 4e,f). The compressed side of the root exhibited reduced cementoblast mineralization, as reflected by the expression of OCN; this effect was reversed following treatment with Rapa (Figure 4g,h).

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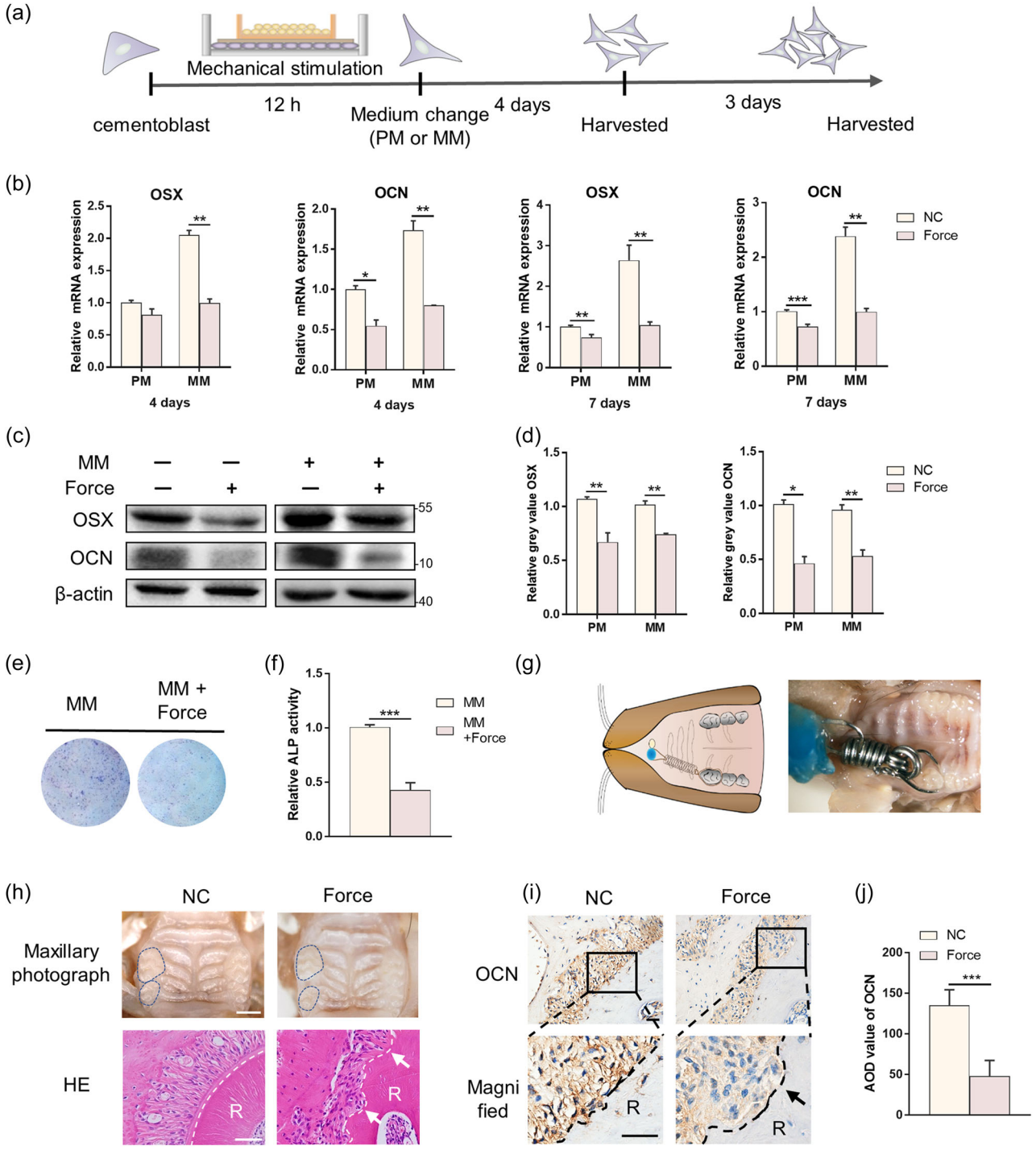

FIGURE 1 Compressive force suppresses mineralization of cementoblasts. (a) Cementoblasts were subjected to compressive force (1.5 g/cm^2, 12 h) and incubated in PM or MM for 4 or 7 days. (b) qRT-PCR analyses of mineralization-related genes (OSX and OCN) in cementoblasts. (c, d) Relative protein expression of OSX and OCN (normalized to β-actin) in cementoblasts after 7 days of induction and quantification of protein levels. (e) ALP staining of cementoblasts after 7 days of mineralization induction. (f) Quantification of ALP activity. (g) Schematic diagram and a picture of the mouse root resorption model. (h) Maxillary images and H&E staining after the application of force for 3 weeks. White arrows indicate root resorption lacunas. Scale bar, 5 mm (up), 50 μm(down). (i) Representative IHC and magnified images of OCN in cementoblasts on the compressed side of distal roots. Dashed lines mark the outline of the root. Black arrows indicate root resorption lacunas. Scale bar, 50 μm. (j) Quantification of OCN in cementoblasts on the compressed side ($n = 5$). Data are means ± SDs (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. normal control). ALP, alkaline phosphatase; H&E, hematoxylin and eosin; IHC, immunohistochemistry; MM, mineralized medium; PM, proliferation medium; qRT-PCR, quantitative real-time polymerase chain reaction.

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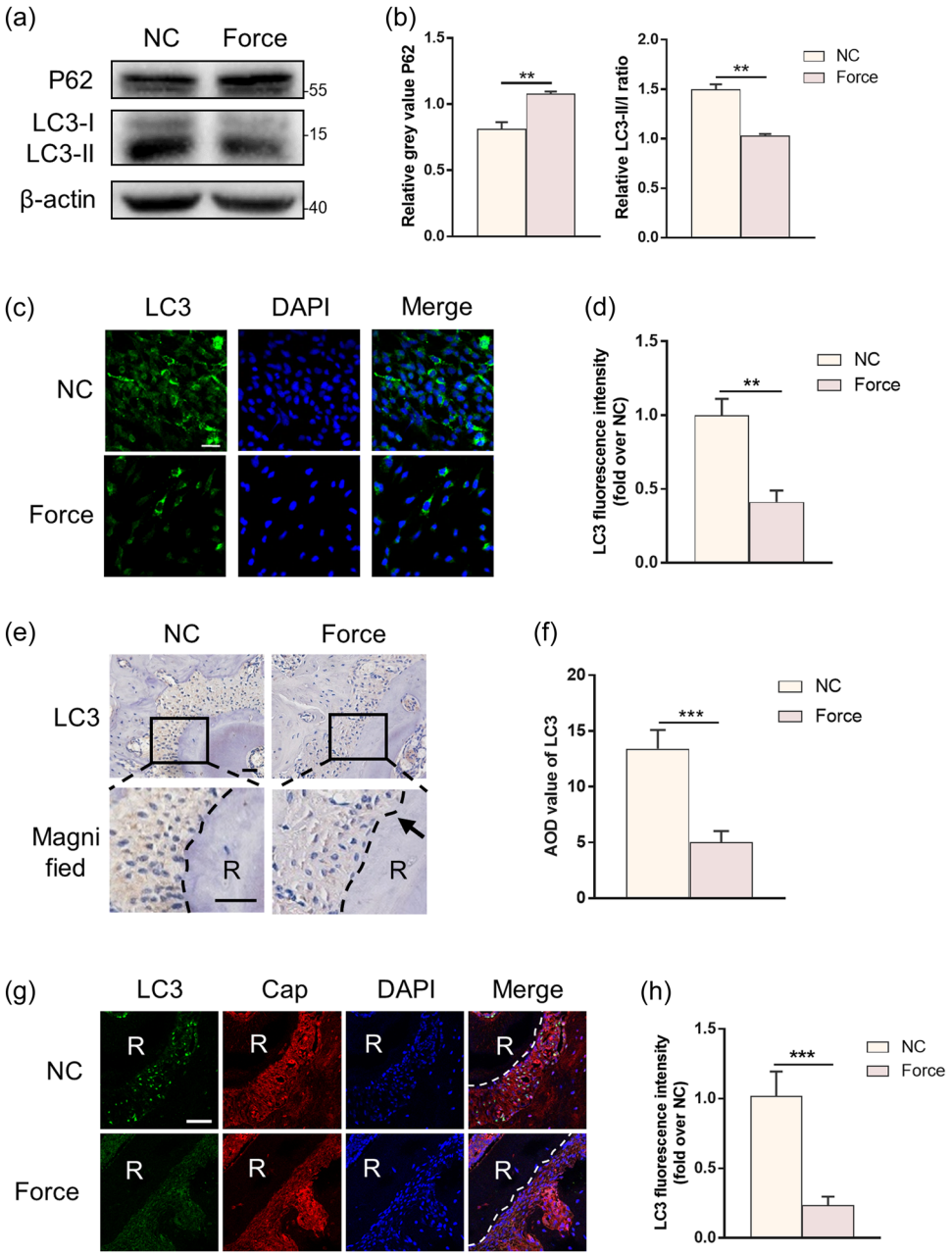

FIGURE 2 Autophagy is inhibited in cementoblasts under compression. (a, b) Relative protein levels of P62 (normalized to β-actin) and LC3-II/I in cementoblasts under compressive force and quantification of protein levels. (c) Representative images of immunofluorescence staining with LC3 (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue) in cementoblasts subjected to compression. Scale bar, 50 μm. (d) Quantification of LC3 fluorescence intensity (normalized to the control group). (e) Representative IHC and magnified images of LC3 in cementoblasts on the compressed side. Dashed lines mark the outline of the root. Scale bar, 50 μm. (f) Quantification of LC3 in cementoblasts on the compressed side ($n = 5$). (g) Representative images of immunostainings with LC3 (green), Cap (red), and DAPI (blue). Scale bar, 50 μm. (h) Quantification of LC3 fluorescence intensity (normalized to the control group) in cementoblasts on the compressed side. Data are means ± SDs (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. normal control).

Because the quality of PDL attachment is vital for restoring periodontal function after the application of force, PSR staining was used to evaluate collagen fibers attached to the cementum. PDL attachment to the compressed side of root surfaces was significantly decreased; this was partially reversed by Rapa injection (Figure 4i,j).

3.5 | Identification and validation of periostin as a key molecule associated with autophagy and mineralization of cementoblasts

To identify the molecular mechanism by which the inhibition of autophagy inhibits cementoblast mineralization, we performed

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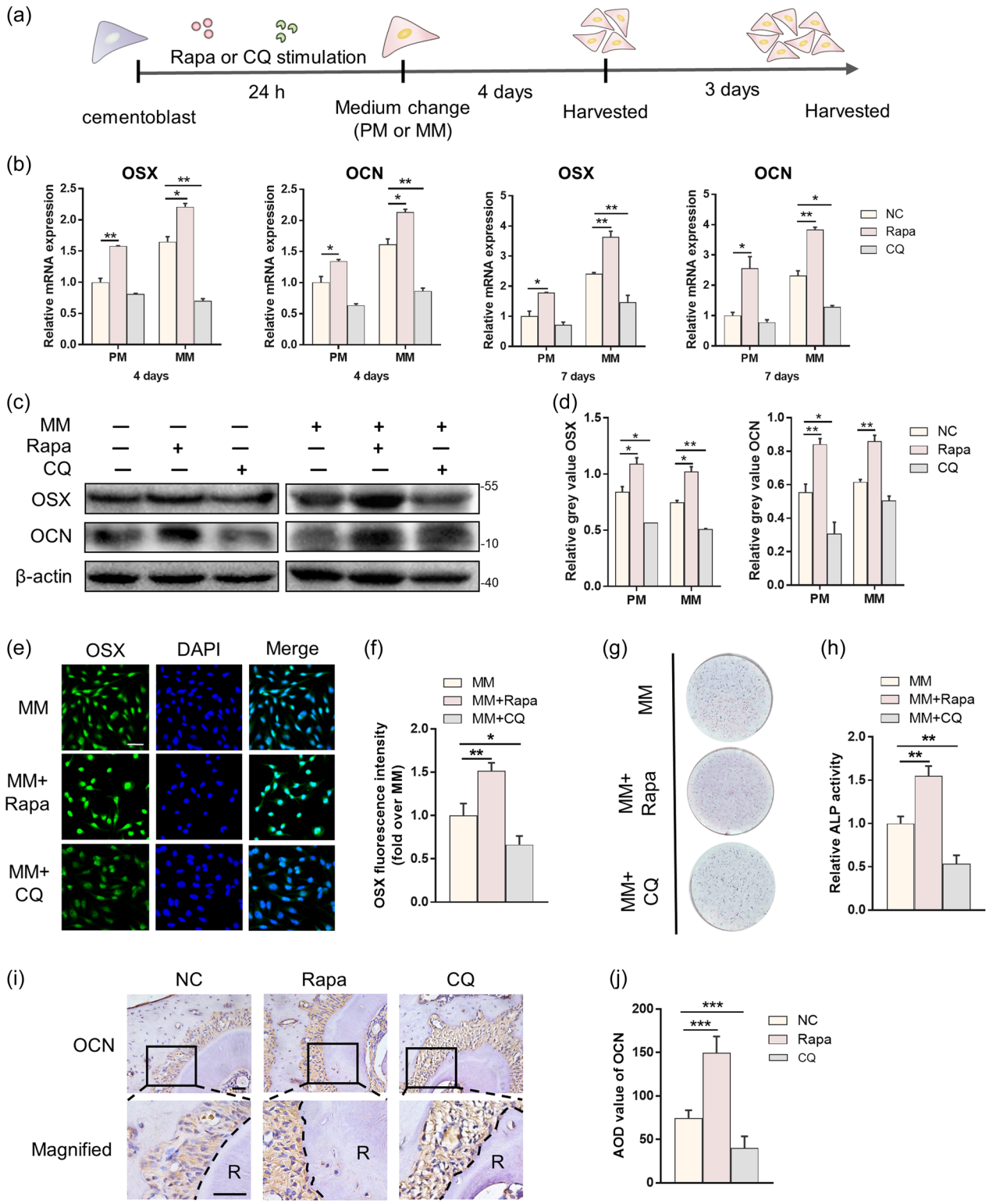

FIGURE 3 Autophagy is required for cementoblast mineralization. (a) Cementoblasts were pretreated with or without Rapa (500 nM) or CQ (5 μM) for 24 h and incubated in PM or MM for 4 or 7 days. (b) Relative expression of mineralization-related genes (OSX and OCN) in cementoblasts. (c, d) Relative protein levels of OSX and OCN (normalized to β-actin) and quantification of protein levels. (e) Representative images of immunofluorescence staining with OSX (green) and DAPI (blue) in cementoblasts pretreated with or without Rapa or CQ for 24 h and incubated in MM for 4 days. Scale bar, 50 μm. (f) Quantification of OSX fluorescence intensity (normalized to the control group). (g) ALP staining of cementoblasts after 7 days of induction. (h) Quantification of ALP activity. (i) Representative IHC staining and magnified images of OCN in cementoblasts on the mesial side of roots in mice injected with or without Rapa (3 mg/kg) or CQ (25 mg/kg) for 3 weeks. Dashed lines mark the outline of the root. Scale bar, 50 μm. (j) Quantification of OCN in cementoblasts ($n = 5$). Data are means ± SDs (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. normal control). ALP, alkaline phosphatase; CQ, chloroquine; IHC, immunohistochemistry; MM, mineralized medium; PM, proliferation medium.

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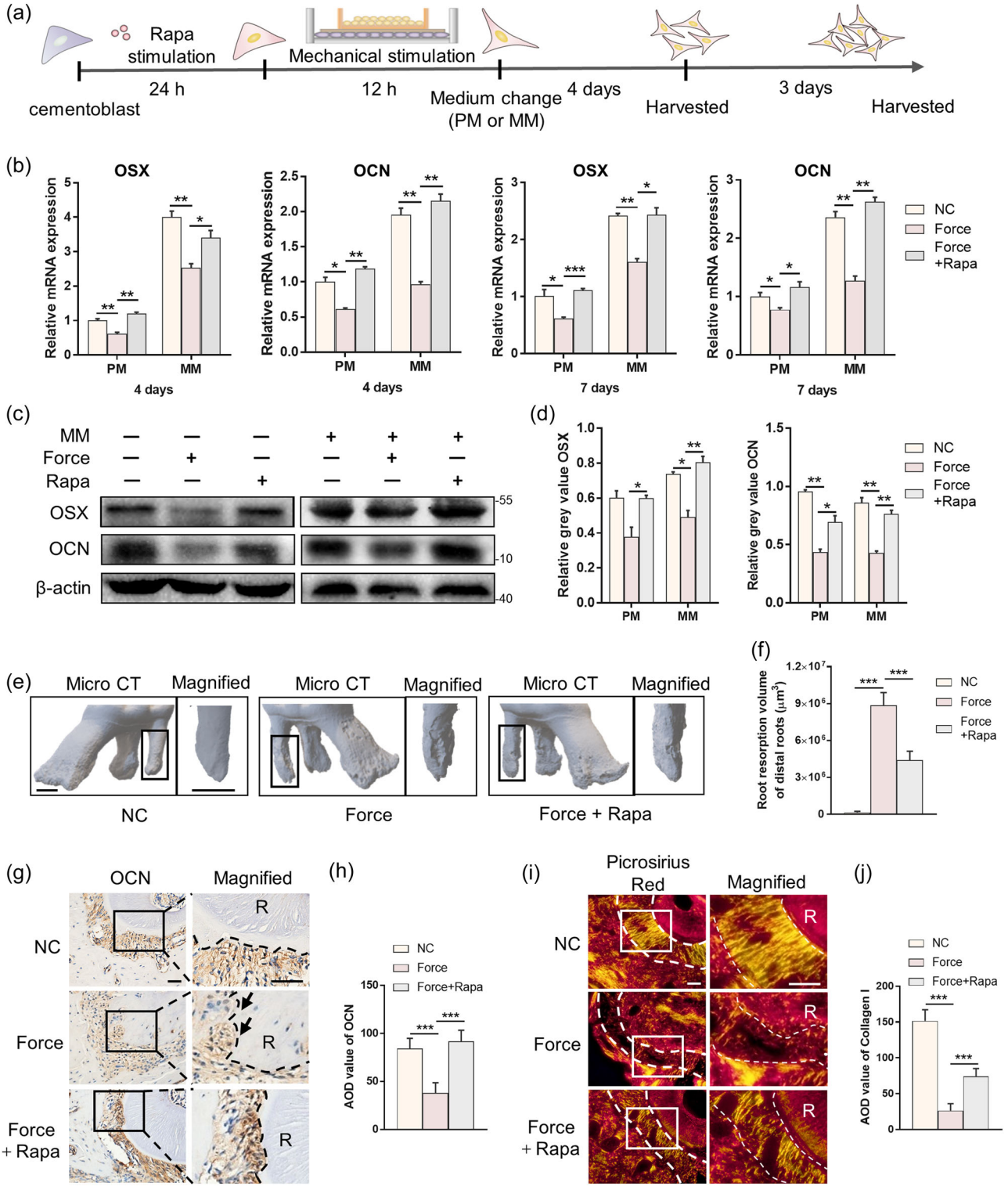

FIGURE 4 Autophagy restores compression-suppressed cementoblast mineralization. (a) Cementoblasts were pretreated with or without Rapa for 24 h, subjected to compressive force for 12 h, and then incubated in PM or MM for 4 or 7 days. (b) Relative expression of mineralization-related genes (OSX and OCN) in cementoblasts. (c, d) Relative protein levels of OSX and OCN (normalized to β-actin) and quantification of protein levels. (e) Micro-CT reconstruction images of roots and the magnified mesial side of distal roots subjected to mechanical force and injected with or without Rapa for 3 weeks. Scale bar, 500 μm. (f) Quantification of root resorption volume of distal roots by micro-CT analysis. (g) Representative IHC and magnified images of OCN in cementoblasts on the compressed side of distal roots in mice. Dashed lines mark the outline of the root. Scale bar, 50 μm. (h) Quantification of OCN in cementoblasts on the compressed side ($n = 5$). (i) Representative picrosirius red and magnified images of collagen I in PDL on the compressed side. Dashed lines mark the outline of the PDL. Scale bar, 50 μm. (j) Quantification of collagen I on the compressed side ($n = 5$). Data are means ± SDs (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. normal control). IHC, immunohistochemistry; MM, mineralized medium; micro-CT, microcomputed tomography; PDL, periodontal ligament; PM, proliferation medium.

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high-throughput RNA-seq to identify differentially expressed mRNAs of cementoblasts treated with or without CQ. The results were visualized in volcano plots (Figure 5a). A total of 73 mRNAs were significantly upregulated and 88 mRNAs were significantly downregulated in cementoblasts treated with CQ compared to the control group ($p < 0.05$ and fold change > 1.5). To further identify the mRNAs correlated with cementoblast mineralization, we subjected all differentially expressed mRNAs to Gene Ontology (GO) enrichment analyses. The GO results indicated that bone mineralization (GO:0030500), and ECM organization (GO:0030198) were enriched (Figure 5b). Among these GO terms, four mRNAs (periostin [Postn], tenascin-C [Tnc], osteomodulin [Omd], and matrix Gla protein [Mgp]) were significantly downregulated and thus may be involved in cementoblast mineralization. We treated cells with the autophagy inhibitor CQ or 3-MA (2.5 mM) and found that two mRNAs (Postn and Tnc) were significantly decreased (Figure 5c). Postn binds Tnc and fibronectin (FN) and transports them outside the cell, which is fundamental to the construction of the Tnc and FN meshwork structure for mineralized ECM organization (Duchamp de Lageneste et al., 2018). In light of this, we selected Postn for further validation and mechanistic investigation. Protein levels of Postn were significantly inhibited in CQ- and 3-MA-treated cementoblasts, as determined by Western blot analysis and immunofluorescence staining (Figure 5d–g). Furthermore, IHC analyses of CQ-injected mice showed that expression of Postn was significantly decreased on the root surface compared to the control group (Figure 5h,i).

3.6 | Knockdown of Postn inhibits cementoblast mineralization

To determine the effects of Postn on cementoblast mineralization, we infected cells with siRNAs against Postn (si Postn-1/2) or scrambled control (si normal control [NC]). Expression of Postn was potently reduced following transfection with si Postn-1 and si Postn-2, as determined by qRT-PCR and Western blot analysis (Supporting Information: Figure S3A,B). Note that after 4 days of induction, knockdown of Postn significantly decreased gene expression of BSP, OSX, OCN, and COL1 compared to si NC (Figure 6a). Western blot analysis of BSP, OSX, and OCN and immunofluorescence staining of BSP and OSX showed that cementoblast mineralization was inhibited following the knockdown of Postn (Figure 6b–e). ALP staining and activity showed similar inhibition of cementoblast mineralization following si Postn-1/2 treatment (Figure 6f,g). Collectively our data indicate that the knockdown of Postn inhibits cementoblast mineralization in vitro. Growing evidence suggests that Postn has a regulatory effect on autophagy (Qin & Cai, 2019; Thongchot et al., 2020); thus, we examined the effects of Postn on autophagy in cementoblasts. Autophagy was upregulated with the knockdown of Postn, as reflected by decreased levels of P62 and increased conversion of LC3-I to LC3-II (Supporting Information: Figure S3C).

3.7 | Knockdown of Postn regulates Wnt signaling by promoting ubiquitination of β-catenin

Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses revealed that the Wnt signaling was influenced in cementoblasts when autophagic activity was suppressed (Supporting Information: Figure S4A). qRT-PCR analysis revealed that the mRNA expression of Wnt target genes (cyclin D1 [CCND1], lymphoid enhancer binding factor 1 [LEF1], and cellular-myelocytomatosis viral oncogene [c-MYC]) decreased when cementoblasts were treated with CQ or 3-MA (Supporting Information: Figure S4B). Since Postn regulates Wnt ligands and the signal transducer of Wnt signaling, β-catenin (Bonnet et al., 2016; Han et al., 2020), we further tried to determine the role of Postn on Wnt/β-catenin signaling. Postn knockdown decreased mRNA levels of Wnt target genes, whereas β-catenin transcript levels were only slightly inhibited (Figure 7a). At the protein level, Postn knockdown significantly reduced nonphospho (active) β-catenin, as well as a slight reduction of total β-catenin (Figure 7b,c). Immunofluorescence analyses further confirmed the inhibition of nuclear accumulation of β-catenin following the knockdown of Postn (Figure 7d,e).

To further determine whether Postn regulates the stabilization of β-catenin, we detected the half-life of β-catenin with CHX (10 μM). Results showed that half-life periods of β-catenin were decreased in cementoblasts with Postn knockdown compared to control cells (Figure 7f,g). Next, we treated cells with the Wnt signaling agonist SKL2001 (30 μM), which disrupts the formation of the β-catenin destruction complex. SKL2001 significantly increased the expression of β-catenin in control cells, whereas only a slight upregulation of β-catenin was observed in si Postn cells (Figure 7h,i), which indicates that Postn may play a role in the stability of β-catenin. Ubiquitination is required for the degradation of β-catenin. Therefore, we treated cementoblasts with the proteasome inhibitor MG132 (5 μM) and found that β-catenin levels decreased after Postn knockdown; this decrease was reversed following treatment with MG132, which suggests that Postn regulates the degradation of β-catenin in a proteasome-dependent manner (Figure 7j,k). IP analyses confirmed that Postn knockdown in cementoblasts significantly increased the ubiquitination of β-catenin compared to si NC (Figure 7l). To further determine the effects of the Postn/β-catenin axis on cementoblast mineralization, we treated si Postn or si NC cementoblasts with SKL2001. Decreased expression of mineralization-related genes and proteins following knockdown of Postn were reversed by activation of β-catenin, as determined by qRT-PCR and Western blot analysis (Supporting Information: Figure S4C,D). Taken together, autophagy restores cementoblast mineralization under compressive force through Postn/β-catenin signaling axis (Figure 8).

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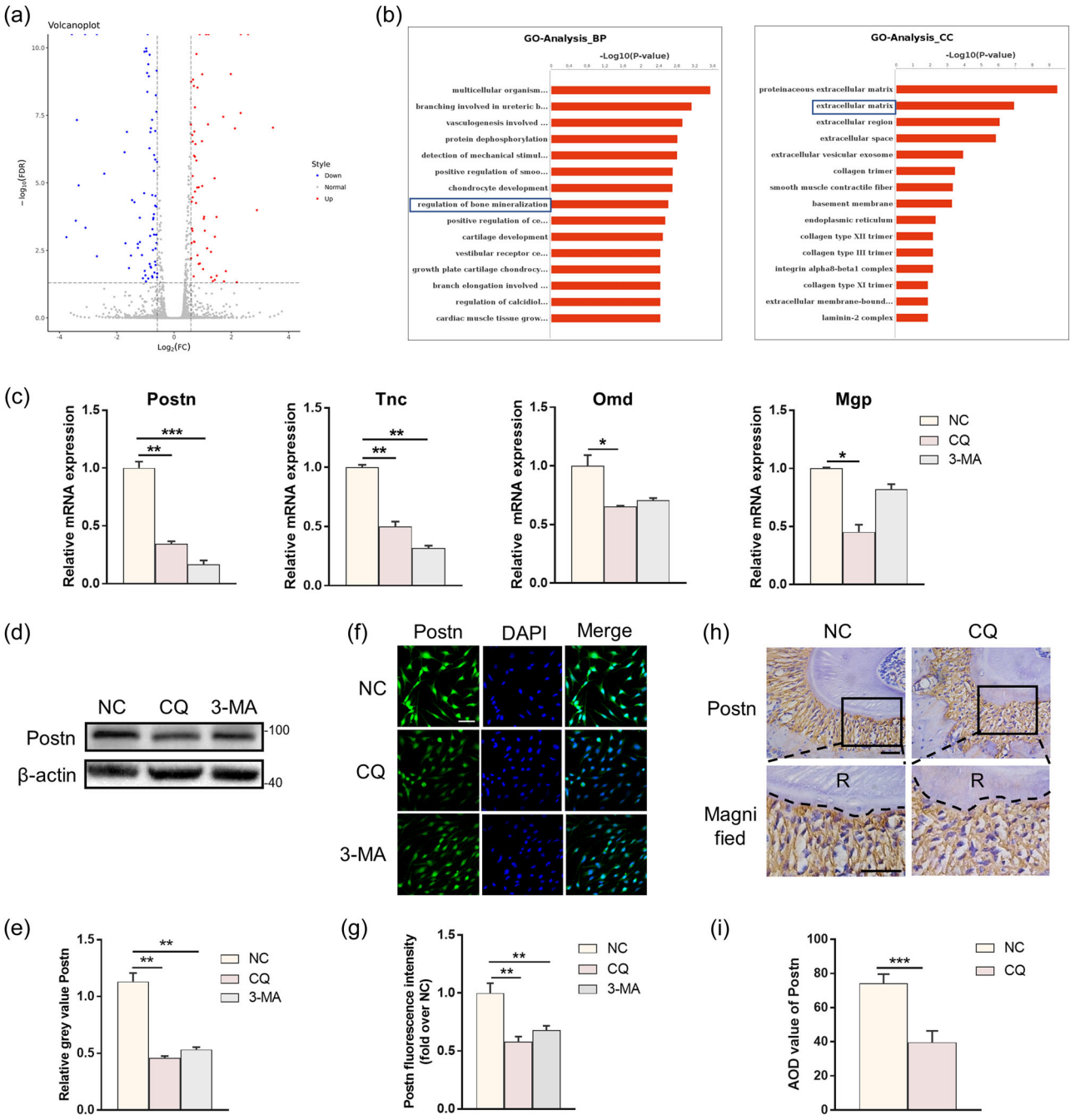

FIGURE 5 Identification and validation of periostin as a key molecule associated with autophagy and mineralization of cementoblasts. (a) Volcano plots of differentially expressed (fold change > 1.5 and adjusted $p < 0.05$) mRNAs in cementoblasts treated without or with CQ. (b) The top 15 GO enrichments among BP and CC terms. (c) qRT-PCR analyses of Postn, Tnc, Omd, and Mgp gene expression in cementoblasts treated with or without CQ, or 3-MA (2.5 mM). (d, e) Protein levels of Postn (normalized to β-actin) in cementoblasts treated with or without CQ or 3-MA. (f) Representative immunofluorescence images of Postn (green) and DAPI (blue) in cementoblasts treated as in (d). Scale bar, 50 μm. (g) Quantification of Postn fluorescence intensity (normalized to the control group). (h) Representative IHC and magnified images of Postn in cementoblasts on the mesial side of distal roots in mice injected with or without CQ for 3 weeks. Dashed lines mark the outline of the root. Scale bar, 50 μm. (i) Quantification of Postn in cementoblasts ($n = 5$). Data are means ± SDs (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. normal control). 3-MA, 3-methyladenine; BP, biological process; CC, cellular component; CQ, chloroquine; qRT-PCR, quantitative real-time polymerase chain reaction; GO, Gene Ontology; IHC, immunohistochemistry; mRNA, messenger RNA.

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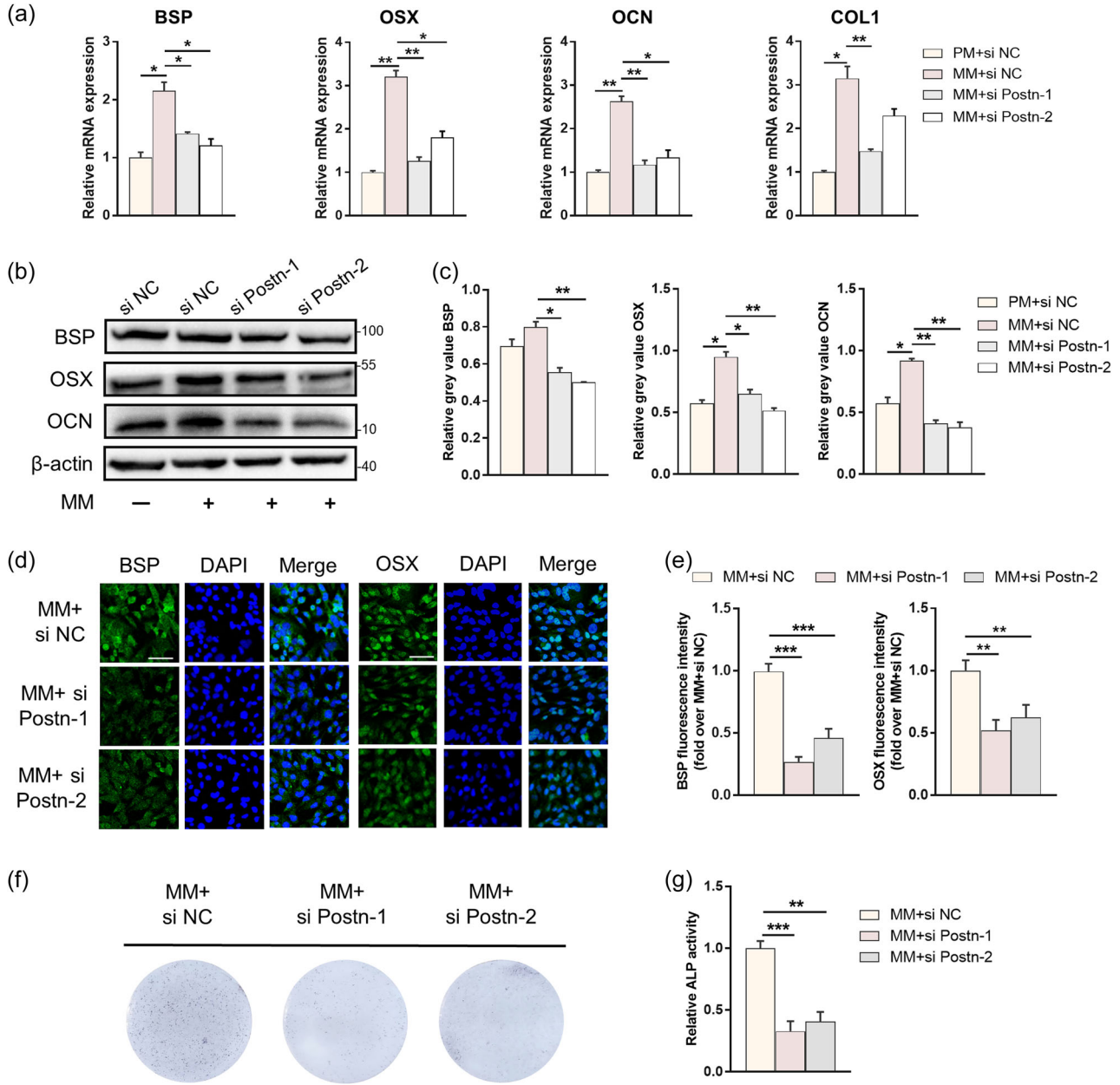

FIGURE 6 Knockdown of Postn inhibits cementoblast mineralization. (a) Cementoblasts incubated in PM or MM infected with si NC, si Postn-1, or si Postn-2 for 4 days. qRT-PCR analyses of BSP, OSX, OCN, and COL1 expression. (b, c) Relative protein levels of BSP, OSX, and OCN (normalized to β-actin) treated as in (a) and quantification. (d) Representative images of immunofluorescence (BSP or OSX, green; DAPI, blue) of cementoblasts transfected with si NC, si Postn-1, or si Postn-2. Scale bar, 50 μm. (e) Quantification of BSP or OSX fluorescence intensity (normalized to the control group). (f) ALP staining of cementoblasts transfected with si NC, si Postn-1, or si Postn-2 after 7 days of induction. (g) Quantification of ALP activity. Data are means ± SDs (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. normal control). ALP, alkaline phosphatase; MM, mineralized medium; PM, proliferation medium; qRT-PCR, quantitative real-time polymerase chain reaction.

4 | DISCUSSION

The mechanical compressive force modulates the function of cementoblasts, which is closely correlated with external root resorption and periodontal regeneration. However, the remineralization of compression-damaged cementum remains a hard challenge. Several studies have indicated that mechanical force regulates the osteogenic differentiation of PDL cells (Li et al., 2019; Manokawinchoke et al., 2019), but the effects remain controversial. In particular, the effects of compressive force on cementoblast mineralization are ambiguous. Our previous study found that compressive force inhibits cementoblast mineralization in vivo (Liu et al., 2022). Consistent with previous findings, the present study also showed an adverse effect of compressive stimulation on cementoblast mineralization.

Autophagy is a crucial survival mechanism of cells under stress (Mizushima & Komatsu, 2011). Our data demonstrated that

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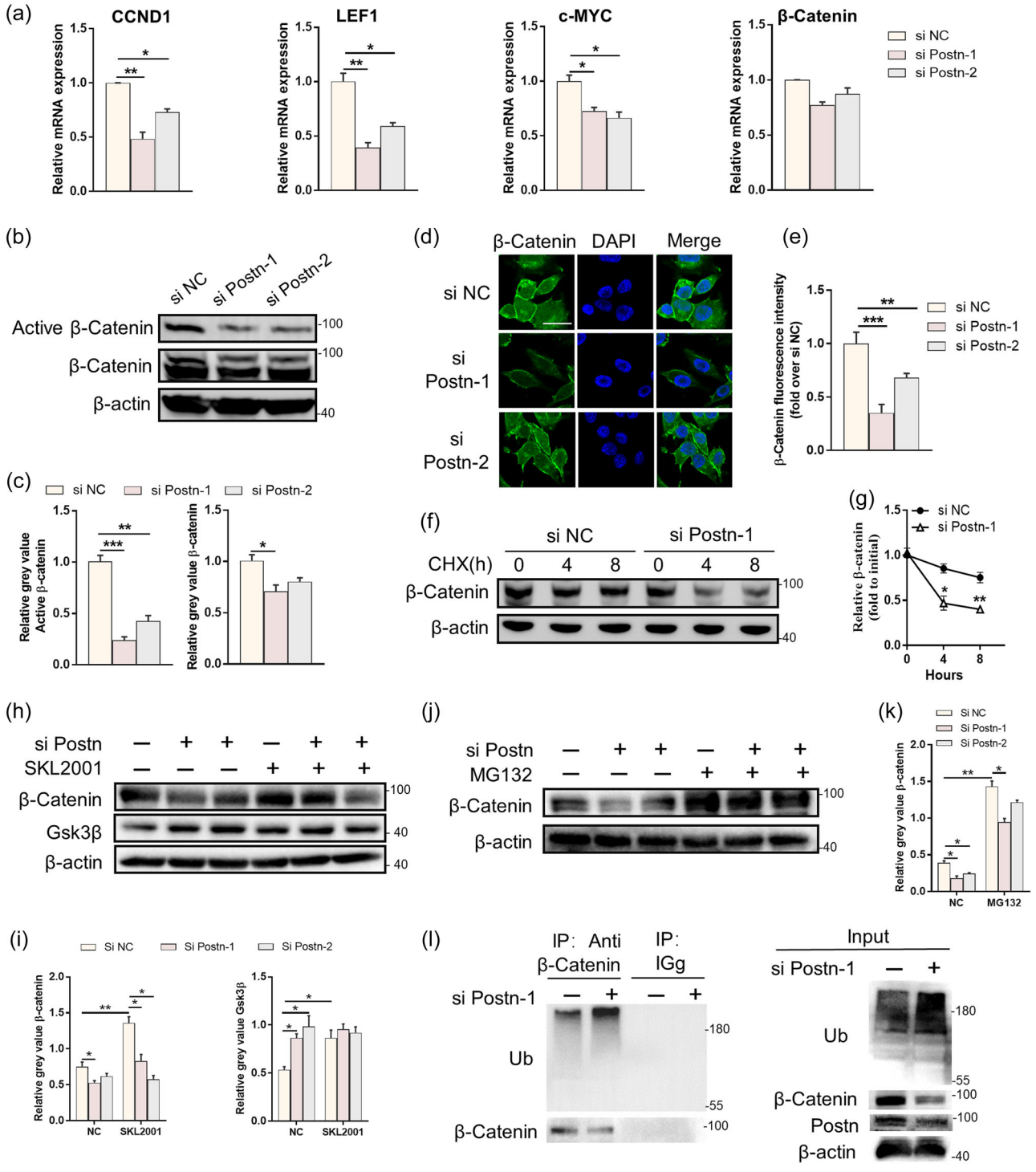

FIGURE 7 Knockdown of Postn regulates Wnt signaling by promoting ubiquitination of β-catenin. (a) qRT-PCR analyses of CCND1, LEF1, c-MYC, and β-catenin mRNA expression in cementoblasts transfected with si NC, si Postn-1, or si Postn-2. (b, c) Active β-catenin and total β-catenin (normalized to β-actin) levels in cementoblasts treated as in (a) and quantification (down). (d) Immunofluorescence analyses of β-catenin (green) in si NC, si Postn-1, or si Postn-2 cementoblasts. Scale bar, 25 μm. (e) Quantification of β-catenin fluorescence intensity (normalized to the control group). (f,g) Protein levels of β-catenin (normalized to initial) in si NC or si Postn-1 cementoblasts treated with CHX (10 μM) for the indicated time periods and quantification (right). (h, i) Protein levels of β-catenin and GSK3β (normalized to β-actin) in si NC, si Postn-1, or si Postn-2 cementoblasts treated with SKL2001 (30 μM, 24 h) and quantification (down). (j, k) Protein levels of β-catenin (normalized to β-actin) in si NC, si Postn-1, or si Postn-2 cementoblasts treated with MG132 (5 μM, 6 h) and quantification (right). (l) Lysates from cementoblasts infected with si NC or si Postn-1 were immunoprecipitated with anti-β-catenin antibody. Western blot analysis analyses of Ub-conjugated β-catenin with anti-Ub and anti-β-catenin. Input shows expression of the corresponding proteins in whole cell lysates (right). Data are means ± SDs (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. normal control). CHX, cycloheximide; MM, mineralized medium; mRNA, messenger RNA; PM, proliferation medium; qRT-PCR, quantitative real-time polymerase chain reaction; Ub, ubiquitin.

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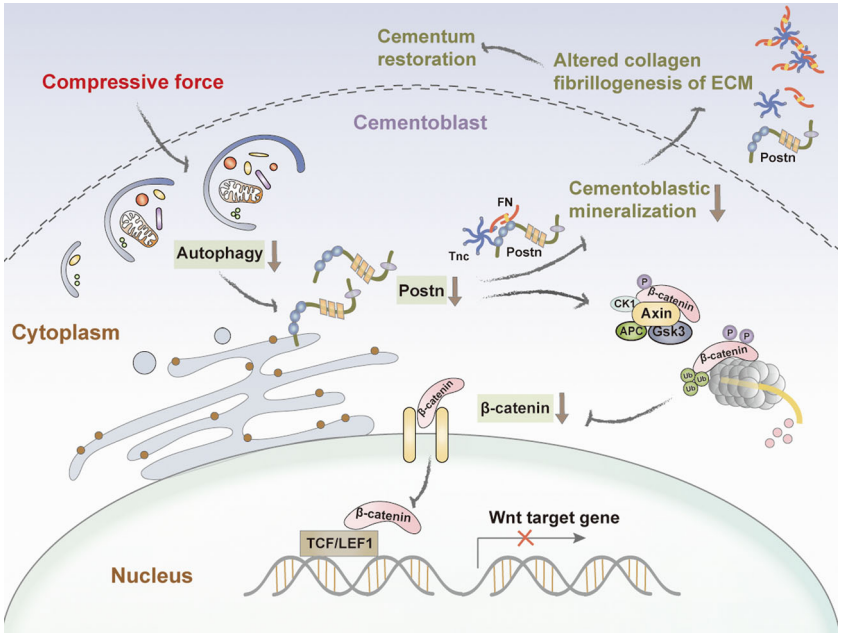

FIGURE 8 The role of autophagy in mediating cementoblast mineralization under mechanical compression. Autophagy is indispensable in mediating cementoblast mineralization, and autophagic activation is required for relieving root resorption under compressive force. Autophagy regulates cementoblast mineralization through Postn, and Postn further regulates Wnt/β-catenin signaling, partially via modulating the stability of β-catenin.

autophagy was inhibited in cementoblasts subjected to compressive force. Recently, evidence has emerged in support of the role of autophagy in promoting bone mineralization (Li, Li, et al., 2018; Nollet et al., 2014; Wu, Wang, et al., 2019). Based on insights from these studies, we investigated whether autophagy is involved in cementoblast mineralization. We found that activation of autophagy enhanced cementoblast mineralization, whereas inhibition of autophagy inhibited cementoblast mineralization. Furthermore, inhibition of cementoblast mineralization induced by compressive force was markedly reversed by autophagic activation with Rapa. It is worth noting that Rapa injection also rescued PDL attachment to the compressed root surface. Together these data demonstrate that increased autophagy helps to restrain force-induced external root resorption and restore the function of PDL.

To identify how autophagy influences cementoblast mineralization, we used transcriptional profiling to analyze gene expression in cementoblasts treated with or without the autophagy inhibitor CQ. After screening and validation, Postn was selected for further study. Postn is preferentially expressed at sites of the hard tissue–soft tissue interface of teeth, where it mediates cell-matrix interactions (Du & Li, 2017; Romanos et al., 2014). The biological functions of Postn in collagen fibrillogenesis are of great importance in ECM mineralization and periodontal functional maintenance (Norris et al., 2007; Tang et al., 2017; Yan et al., 2020). However, the effects of autophagy on Postn remain largely unknown. We found that the expression of Postn in cementoblasts was downregulated following inhibition of autophagy in vitro and in vivo, suggesting that autophagy inhibition in cementoblasts has a negative effect on the expression of Postn. Based on this, we next knocked down Postn to determine its role in cementoblast mineralization, and the results showed that the knockdown of Postn led to impaired mineralization capacity of cementoblasts. In addition, we found that Postn knockdown enhanced autophagic activity in cementoblasts, which is consistent with previous studies (Qin & Cai, 2019; Thongchot et al., 2020). Our data indicate that autophagy participates in cementoblast mineralization through Postn, and the negative feedback of Postn on autophagy is beneficial for maintaining autophagic balance in stressful conditions.

KEGG analyses revealed that the inhibition of autophagy influences Wnt signaling. The Wnt/β-catenin pathway contributes to osteogenic differentiation and bone formation (Duan & Bonewald, 2016; Shen et al., 2020). We thus investigated whether Postn regulates cementoblast mineralization under compression via Wnt/β-catenin signaling. To simulate the decrease of Postn following the inhibition of autophagy under compressive force, Postn was knocked down in cementoblasts for further verification of its role in Wnt signaling regulation. Decreased protein levels of active and total β-catenin, and nuclear accumulation of β-catenin following knockdown of Postn were observed. As a core signal transducer of the Wnt pathway, the regulation of β-catenin stabilization is complex (Clevers & Nusse, 2012; Park et al., 2020). Here our data revealed that treatment with an activator of the Wnt signaling, or proteasomal inhibitor reversed Postn knockdown-mediated β-catenin downregulation. More important, Postn knockdown promoted the ubiquitination of β-catenin in cementoblasts. These findings highlight the role of Postn in the aberrant inactivation of Wnt signaling under compression in cementoblasts.

Nevertheless, the application of mechanical compressive force on murine cementoblasts in vitro does not mimic exactly the biological conditions on the pressure side of roots during orthodontic tooth movement. In addition, further studies are needed to precisely locate the protective effects of autophagy in cementoblasts in vivo and explore a precise therapeutic strategy on cementum for reducing external root resorption.

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5 | CONCLUSION

Overall, our data provide evidence that autophagy is indispensable to the restoration of compression-suppressed cementoblast mineralization in vitro and in vivo. We further demonstrate that autophagy regulates Postn expression and that Postn modulates cementoblast mineralization partially through the ubiquitination of β-catenin. This newly discovered function of the Postn/β-catenin axis could explain how autophagy mediates cementoblast mineralization under compressive force. Our findings also identify a new therapeutic target for the restoration of cementum mineralization and periodontal tissue regeneration.

AUTHOR CONTRIBUTIONS

Yuhui Yang contributed to design, data acquisition, analysis, and interpretation, and drafted the manuscript. Hao Liu contributed to the conception, data acquisition, and interpretation, critically revised the manuscript. Ruoxi Wang and Yi Zhao contributed to data acquisition. Yunfei Zheng contributed to the interpretation of data. Yiping Huang and Weiran Li contributed to the conception, design, data analysis, supervised the project, and critically revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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