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ORIGINAL ARTICLE

Compressive force regulates cementoblast migration via downregulation of autophagy

Yuhui Yang[1] | **Yiping Huang**[1] | **Hao Liu**[1] | **Yunfei Zheng**[1] | **Lingfei Jia**[2] | **Weiran Li**[1]

[1] Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China

[2] Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, China

Correspondence
Weiran Li, Department of Orthodontics, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Avenue, Haidian District, Beijing 100081, People's Republic of China.
Email: weiranli@bjmu.edu.cn

Yuhui Yang and Yiping Huang contributed equally to this study.

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Abstract

Background: Migration of cementoblasts to resorption lacunae is the foundation for repairing root resorption during orthodontic tooth movement. Previous studies reported that autophagy was activated by compression in periodontal ligament cells. The aim of this study was to investigate the migration of cementoblasts and determine whether autophagy is involved in the regulation of cementoblast migration under compressive force.

Methods: Flow cytometry was employed to examine the apoptosis of murine cementoblasts (OCCM-30) at different compression times (0, 6, 12, and 24 hours) and magnitudes (0, 1.0, 1.5, and 2.0 g/cm^2). Cell proliferation was examined using the CCK-8 method. Wound healing migration assays and transwell migration assays were performed to compare the migration of cementoblasts. Chloroquine (CQ) and rapamycin were used to inhibit and activate autophagy, respectively. The level of autophagy was determined using western blotting and immunofluorescence staining. The expression of matrix metalloproteinases (MMPs) was assessed using quantitative reverse transcription polymerase chain reaction (qRT-PCR), western blot analysis, and enzyme-linked immunosorbent assay (ELISA).

Results: Cell apoptosis and proliferation did not significantly change in OCCM-30 cells under mechanical compression at magnitude of 1.5 g/cm^2 for 12 hours. However, the migration of cementoblasts was significantly inhibited after the application of compressive force. MMP2, MMP9, and MMP13 mRNA expression was decreased, and MMP9 and MMP13 protein expression and secretion level were also decreased. Further, autophagic activity was inhibited in cementoblasts under compressive force. Treatment with chloroquine reduced the cellular migration, and rapamycin partially relieved the inhibition of cementoblast migration induced by the compressive force. MMP9 and MMP13 mRNA expression, protein expression, and secretion levels showed a similar trend.

Conclusion: Migration of OCCM-30 cells was inhibited under compressive force partially dependent on the inhibition of MMPs, which was mediated by downregulation of autophagy. The findings provide new insights into the role of

autophagy in biological behaviors of cementoblasts under compressive force and a potential therapeutic strategy for reducing external root resorption.

KEYWORDS
cell biology, cementum, matrix metalloproteinases, orthodontics, wound healing

1 | INTRODUCTION

External root resorption is one of the most common complications in orthodontic treatment, which affects the long-term stability of teeth.[1,2] Compressive force directly induces external root resorption, which occurs as a result of abnormal cementum remodeling during orthodontic tooth movement.[3,4] Cementoblasts are sensitive to compressive force and play a crucial role in the regulation of cementum resorption and repair.[5–8] Therefore, a better understanding of how cementoblasts perform under compressive force is essential for developing new approaches to reduce root resorption during orthodontic treatment.

Mechanical compression controls various cell behaviors that are crucial for tissue repair, including migration, growth, differentiation, and apoptosis.[9–11] During orthodontic tooth movement, the ability of cementoblasts to migrate to the site of resorption lacunae is the foundation for adhesion, proliferation, and differentiation to repair root resorption.[5] To achieve the remarkable repair capability, determining how to promote effective migration of cementoblasts into the resorption lacunae is important. However, the migration of cementoblasts under compressive force has not yet been reported. Compression is transmitted to the cytoskeleton of individual cells from the extracellular matrix (ECM) mediated by a specialized anchoring complex.[12] MMPs facilitate the migration of cells by degrading ECM and generating tunnels in which the cells move during various physiological and pathological processes, such as wound healing and bone remodeling.[13–16] Cementum as a part of periodontium, develops from ectomesenchymal cells and is similar in structure to bone, thus we selected several MMPs, including gelatinases MMP2, MMP9, and collagenase MMP13, which are generally found in mesenchymal tissue and associated with mineralized tissue to evaluate ECM remodeling of cementum.[17–19] Thus, in the present study, the migration and MMPs expression of cementoblasts under compressive stress were investigated.

Autophagy is an evolutionarily conserved process that degrades damaged or dysfunctional proteins and organelles in cells [20] and occurs at a basal level when stimulated by multiple environmental stresses.[21] Autophagy has been analyzed in some mechanosensitive cells such as endothelial cells and osteoblasts, which have strong ability to adapt to their physical environment.[22] In addition, autophagy is involved in various physiological and pathological processes.[23,24] In a recent study, autophagy was activated by compressive force in periodontal ligament cells (PDLCs).[25] However, during orthodontic tooth movement, the change of autophagic activity in the mechanosensitive cementoblasts is largely unknown, and whether the change of autophagy regulates cell migration has not been evaluated. Therefore, in the present study, the migration of cementoblasts under compressive force was investigated and the role of autophagy in regulating cell migration upon mechanical stimulation was explored. The findings could aid in understanding the process of cementum remodeling and provide novel insight into strategies for reducing external root resorption.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

Chloroquine (CQ) and rapamycin were purchased from ApexBio (Shanghai, China). GM6001 were purchased from MedChem Express (Monmouth Junction, NJ). The chemical activator and inhibitor were carefully dissolved in dimethyl sulfoxide (DMSO) or phosphate buffered saline (PBS) to a specific concentration. The following primary antibodies were used: MMP9 (Abcam, Cambridge, UK), MMP13 (Proteintech, Wuhan, Hubei, China), Beclin1 (Abcam), microtubule associated protein one light chain three (LC3, Cell Signaling Technology, Beverly, MA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Zhongshan Golden Bridge, Beijing, China).

2.2 | Cell culture and compression application

An immortalized murine cementoblast cell line, OCCM-30, was kindly provided by Dr. Martha J. Somerman (National Institutes of Health, Bethesda, MD). The

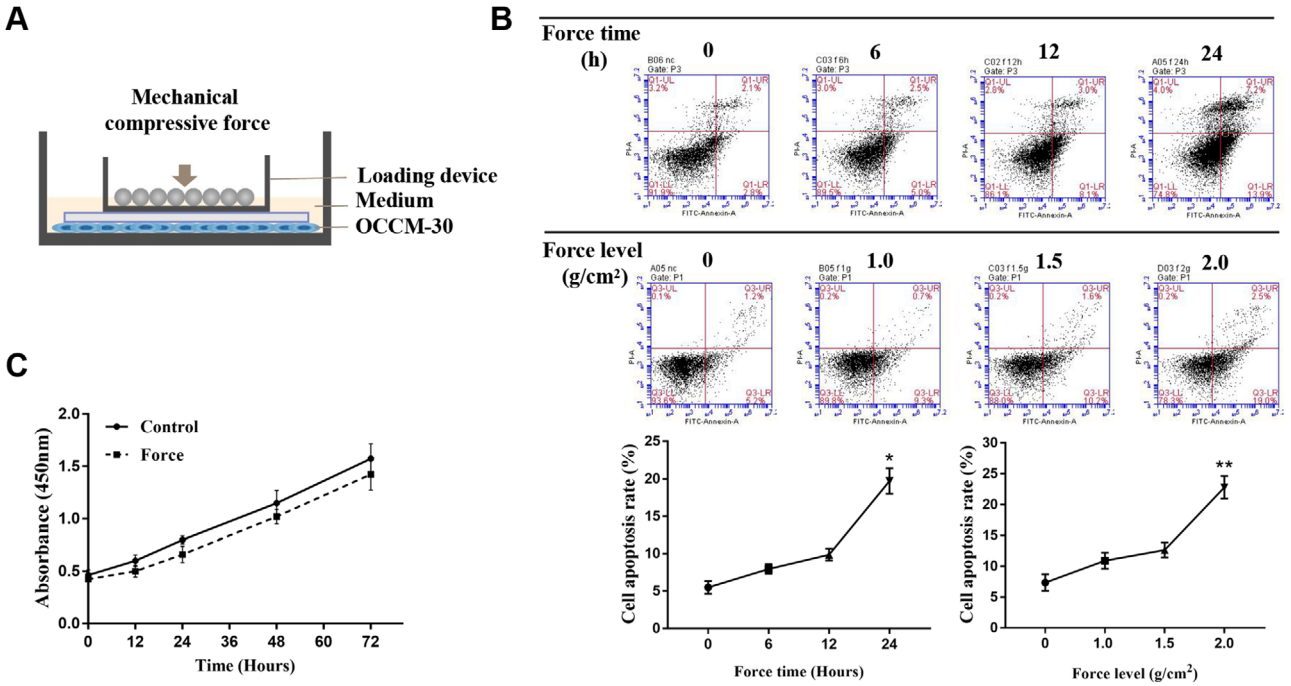

FIGURE 1 Viability of OCCM-30 cells under mechanical compressive force. (**A**) Schematic diagram showing the application of compressive force. (**B**) OCCM-30 cells were subjected to mechanical compression at 1.5 g/cm^2 for different times (0, 6, 12, and 24 hours) and at different magnitude (0, 1.0, 1.5, and 2.0 g/cm^2) for 12 hours. The ratio of apoptotic cells was measured by Annexin V-FITC and PI staining. (**C**) CCK-8 assay showing the proliferation curve of OCCM-30 cells with and without mechanical compressive force. The cells were exposed to compressive force for 12 hours, incubated for 12 to 72 hours, and the absorbance was measured at 450 nm. Data are shown as mean ± SD ($^*P < 0.05$; $^{**}P < 0.01$)

isolation and immortalization of these cells have been previously described.[26] The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere consisting of 5% CO_2 in the air. OCCM-30 cells were seeded into six-well or 12-well plates. After reaching 90% confluence, cells were compressed continuously under the uniform compressive force (Figure 1a). 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. OCCM-30 cells were subjected to 1.0, 1.5, or 2.0 g/cm^2 of compressive force for 6, 12, or 24 hours.

2.3 | Cell apoptosis assay

Cell apoptosis was assessed by using flow cytometry assay (BD, Franklin Lakes, NJ). The apoptosis rate was evaluated using the Annexin V-FITC/PI Apoptosis Detection kit (Dojindo, Kumamoto, Japan) according to the instructions from the manufacturer. In brief, cells were washed twice in PBS and resuspended in 500 μL binding buffer. Then, these cells were stained with 5 μL Annexin V-FITC and 5 μL PI for 15 minutes at room temperature in the dark. Cells were analyzed by flow cytometry within 1 hour.

2.4 | Cell proliferation assay

Cell proliferation was measured using a Cell Counting Kit (CCK-8, Dojindo). Briefly, after exposure to compressive force for 12 hours, the cells were seeded in a 96-well plate at a density of 4×10^3 cells/well. Cell viability was analyzed at 0, 12, 24, 48, and 72 hours. At the indicated time points, 10 μL CCK-8 solution was added to each well and the cells were incubated at 37°C for 2 hours. Then, absorbance was measured at 450 nm using a microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT). Each treatment consisted of six replicated wells and was repeated at least three times.

2.5 | Transwell migration assay

Cells were washed twice with PBS and re-suspended in the serum free medium and adjusted cell density to 2×10^5.

TABLE 1 Primers for quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Gene	Forward primer	Reverse primer
MMP2	CGATGTCGCCCCTAAAACAG	GCATGGTCTCGATGGTGTTC
MMP9	GGAGACGCCACGCATTTCA	CTTACGGCCTGAGGGTCTTG
MMP13	TTTATTGTTGCTGCCCATGAG	AGTTTCTCCTCGGAGACTGGT
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Then, total of 100 μL cells suspension were added to the upper compartment of 24-well Transwell culture chamber with a pore size of 8 μm (Corning, Corning, NY). The lower compartment was filled with 600 μL of complete medium containing 15% fetal bovine serum. After incubation 48 hours at 37°C, cells which did not migrate were removed from the upper face of filters by cotton swabs. Migrated cells were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet (Solarbio, Beijing, China) for 10 minutes and counted microscopically.

2.6 | Wound healing migration assay

Wound healing migration assays were performed as previously described.[27] OCCM-30 cells were resuspended and seeded into a six-well plate (4×10^6 cells/well). After reaching 90% confluence, the cells were exposed to compressive force for 12 hours or treated with GM6001, CQ or rapamycin for 24 hours. After applying compressive force to the OCCM-30 cells, the glass cover was removed carefully. Next, a linear scratch was made using a 200 μL pipette tip. After washing with PBS three times, the cells were incubated with serum-free DMEM for the specific times. Images were taken at 0, 24, and 48 hours at 10× magnification and the wound size was measured in three wells per group. The images acquired were further analyzed by comparing the percent of wound closure between the reference point at 0 hour and the end of incubation in the same field using ImageJ software.

2.7 | Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The cells were harvested and total RNA was isolated at the indicated time points using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The mRNA was reverse-transcribed into complementary DNA (cDNA) using a cDNA Transcription Kit (Takara, Tokyo, Japan). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted using a SYBR Green Q-PCR Master Mix on an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) under the following conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for mRNAs. Relative mRNA expression level was calculated using the delta-delta CT method as previously described.[28] The primer sequences of MMP2, MMP9, MMP13, and GAPDH are listed in Table 1.

2.8 | Western blot analysis

Cells were harvested, washed, and lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA). The protein concentration was determined using a BCA protein assay kit (Solarbio). The proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). After blocking with 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), membranes were incubated at 4°C overnight with primary antibodies against MMP9, Beclin1, and LC3 at 1:1000 dilution, and against GAPDH at 1:5000 dilution. After washing, secondary antibodies (1:10,000; Zhongshan Golden Bridge) were applied at room temperature for 1 hour. Protein bands were visualized using a chemiluminescence kit (Applygen, Beijing, China) and the band intensities were quantified with ImageJ software.

2.9 | Enzyme-linked immunosorbent assay (ELISA)

To measure the concentration of MMP9 and MMP13 secreted from the OCCM-30 cells, the supernatant was assessed using ELISA. Cells were planted in 12-well plates and cultured as described above. The culture-conditioned medium was collected, centrifuged, and then the supernatant was collected and immediately analyzed. The concentration of MMP9 and MMP13 in the culture media was determined by quantitative ELISA (Jin En Lai Biotechnology, Beijing, China). The assays were performed according to the manufacturer's instructions.

2.10 | Immunofluorescence staining

Immunofluorescence staining was performed as previously described [29]. OCCM-30 cells grown on glass coverslips were subjected to compressive force for 0, 4, 8, and 12 hours. The cells were washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 3% BSA. Then, cells were incubated with primary antibody (1:200) against the autophagy marker LC3 at 4°C overnight and incubated in the specified secondary antibodies for 1 hour. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). Images were captured using a confocal imaging system (Carl Zeiss, Jena, Germany).

2.11 | Statistical analysis

Statistical analysis was performed using SPSS software (IBM Corp., Armonk, NY). Differences between the treated cells and control were analyzed using Student's *t*-test. In cases of multiple comparisons, one-way analysis of variance (ANOVA) followed by the Tukey post hoc test was used. All data are expressed as mean ± standard deviation (SD) of independent experiments. A two-tailed *P*-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Effects of compressive force on the apoptosis and proliferation of OCCM-30 cells

To determine the optimal magnitude and treatment time of compression force, the cell apoptosis was quantified using annexin V and PI double staining kit. The cementoblasts were exposed to compression at 1.5 g/cm^2 for 6, 12, and 24 hours. There was no difference of cell apoptosis at six and 12 hours compared with the control group. However, the apoptosis was increased significantly after 24 hours of compression. Then, the cementoblasts were subjected to compressive force at 1.0, 1.5, and 2.0 g/cm^2 for 12 hours. The ratio of apoptotic cells at 2.0 g/cm^2 force was significantly higher, whereas it was similar among 1.0 and 1.5 g/cm^2 force and control groups (Figure 1b). Therefore, the compressive force was adjusted to 1.5 g/cm^2 maintained for 12 hours in subsequent experiments to mimic the in vivo system.

To evaluate the effect of compressive force on cementoblast proliferation, the CCK-8 assay was performed after stimulation with compression at 1.5 g/cm^2 for 12 hours. The proliferation of OCCM-30 cells was not significantly affected at 0, 12, 24, 48, or 72 hours after exposed to compressive force, although a decreasing trend was observed in cells exposed to compressive force (Figure 1c).

3.2 | Migration of OCCM-30 cells was inhibited by compressive force

To investigate the migration of cementoblasts under compressive force, OCCM-30 cells were treated with compressive force at 1.5 g/cm^2 for 12 hours and then the migration was determined by transwell assay and wound healing assay. The results showed that after mechanical stimulation, cell migration significantly decreased compared with the control (Figure 2a-b).

The expression levels of MMP2, MMP9, and MMP13 in OCCM-30 cells were determined. The qRT-PCR analysis results showed MMP2, MMP9, and MMP13 mRNA expression was decreased under compressive force compared with the control, and MMP9 and MMP13 expression was decreased more significantly (Figure 2c). The Western blot analysis also showed decreased protein expression of MMP9 and MMP13 under compression (Figure 2d). Further, the secretion levels of MMP9 and MMP13 from OCCM-30 cells were determined by ELISA. The concentration of both MMP9 and MMP13 in supernatant was decreased after stimulation of compression in OCCM-30 cells (Figure 2e).

To confirm the role of MMPs in cell migration, we treated cementoblasts with GM6001 (5 μM), a broad-spectrum MMP inhibitor. The results showed that the migration of cementoblasts was suppressed after treatment with GM6001, suggesting that MMPs are essential for cementoblast migration (Figure 2f–g).

3.3 | Autophagy was inhibited in OCCM-30 cells under compressive force

To determine the autophagic activity in OCCM-30 cells after exposure to compressive force, two biological markers of autophagy, LC3II/I and Beclin1, were examined using Western blot analysis. OCCM-30 cells were subjected to 1.5 g/cm^2 of compressive force for 0, 6, and 12 hours. The results showed the Beclin1 expression level and the LC3II/I ratio started to decrease at 6 hours, and the autophagic flux was significantly inhibited at 12 hours of compressive force (Figure 3a). In addition, immunofluorescence staining showed the formation of autophagosomes, detected as LC3 punctate dots, was significantly decreased after exposure to compressive force; the decrease began at 4 hours and reached the lowest level at 12 hours (Figure 3b).

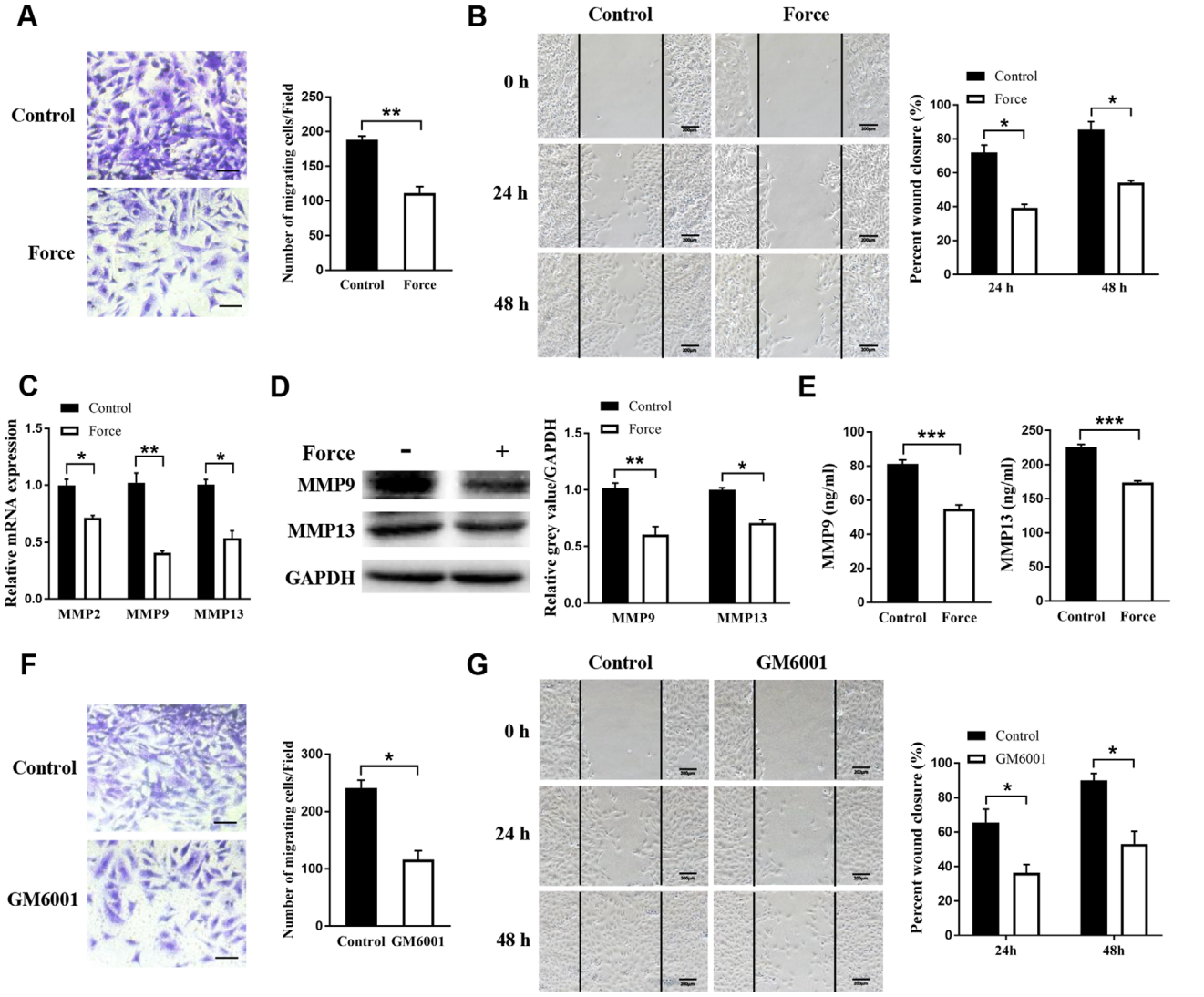

FIGURE 2 Migration of OCCM-30 cells was inhibited under compressive force. (**A**) Transwell migration assay. Representative images of migrated OCCM-30 cells in control and force groups. Cell migration was monitored by microscopy at 48 hours following cell inoculation in the chamber. Scale bars = 100 μm. (**B**) Wound-healing migration assay. Microscopy was used to record the surface of the wound immediately after scratching (0 hour) and migrating for 24 and 48 hours. (**C**) Quantification of MMP2, MMP9, and MMP13 mRNA expression measured using qRT-PCR in OCCM-30 cells or cells under compressive force. GAPDH mRNA was used for normalization. (**D**) Western blot analysis (left) and quantification (right) of MMP9 and MMP13 protein expression and the internal control GAPDH in the cells under compression or control. (**E**) Cell supernatant was collected for measuring MMP9 and MMP13 by quantitative ELISA. (**F**) Transwell migration assay of cells treated with or without 5 μM GM6001 for 24 hours. Scale bars = 100 μm. (**G**) Wound healing assay of cells treated with or without GM6001. Data are shown as mean ± SD ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$)

3.4 | Autophagy was indispensable for migration of OCCM-30 cells

Next, the role of autophagy in OCCM-30 cell migration was investigated. CQ (10 μM), was used to inhibit autophagic flux, which impairs fusion of the autophagosome to the lysosome and leads to accumulation of ineffective autophagosomes.[30] Western bolt analysis confirmed that treatment with autophagy inhibitor CQ decreased Beclin1, but increased LC3II/I ratio as a result of autophagosomes accumulation because of the inhibition of the autophagy effective stage (Figure 4a). After inhibition of autophagy, cell migration was determined. The results of the transwell and wound healing assays showed that CQ significantly decreased OCCM-30 cell migration (Figure 4b-c). Further, the mRNA expression levels of MMP2, MMP9, and MMP13 were significantly decreased in OCCM-30 cells with CQ treatment (Figure 4d). Additionally, CQ treatment significantly downregulated MMP9 and MMP13 protein expression and their concentration in supernatant (Figure 4e-f).

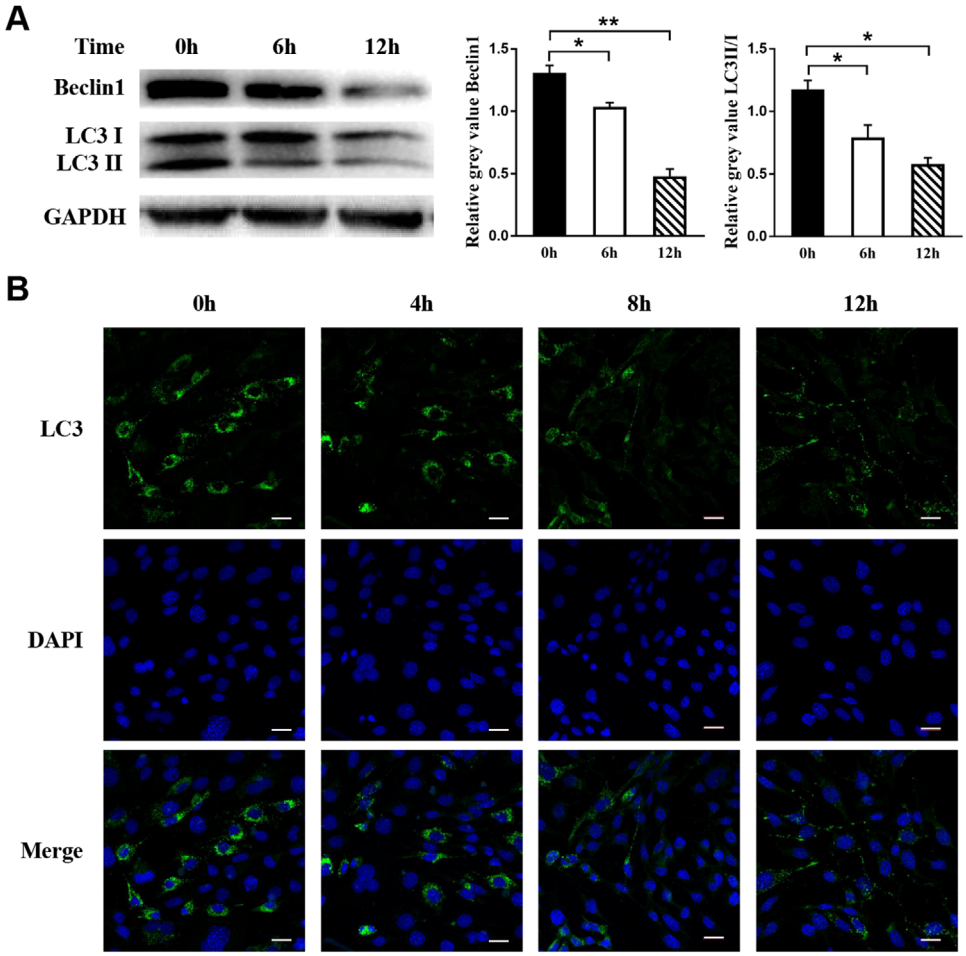

FIGURE 3 Compressive force inhibited autophagy in OCCM-30 cells. (**A**) Western blot analysis (left) and quantification (right) of protein expression of Beclin1, LC3II/I, and the internal control GAPDH under compressive force. OCCM-30 cells were exposed to mechanical compression for 0, 6, and 12 hours. Results are presented as mean ± SD ($^{*}P < 0.05$; $^{**}P < 0.01$). (**B**) Confocal microscopy of LC3 with DAPI counterstaining in OCCM-30 cells under compressive force at 0, 4, 8, and 12 hours. Scale bars = 50 μm

These results showed cell migration was decreased when autophagy was inhibited.

3.5 | Compressive force inhibited migration by regulating autophagy

To further determine whether compressive force inhibits cell migration via autophagy, rapamycin (5 μg/mL), a mammalian target of rapamycin (mTOR) targeting inhibitor, was used to activate autophagy when the OCCM-30 cells were exposed to compressive force. Rapamycin promoted the autophagy flux successfully, as indicated by increased Beclin1 and LC3II/I (Figure 5a). Cells were pre-treated with rapamycin for 24 hours, and then subjected to compressive force. Compared with the cells under compression, rapamycin treatment partially relieved cell migration as revealed by the transwell and wound healing assays (Figure 5b-c). In addition, the MMP2, MMP9, and MMP13 mRNA expression was decreased under compressive force, and this inhibition was reversed when the cells were treated with rapamycin (Figure 5d). Similar trend was showed in MMP9 and MMP13 protein expression and secretion levels (Figure 5e-f). Altogether, these results showed that compressive force inhibited migration via downregulating autophagy in OCCM-30 cells.

4 | DISCUSSION

In the present study, migration of cementoblasts was inhibited after exposure to compressive force, as shown by transwell migration assays and wound healing assays. To avoid pathological damage of cementoblasts, the compression force was set at 1.5 g/cm^2 for 12 hours. The cell apoptosis and proliferation assays confirmed the absence of significantly increased apoptosis and decreased proliferation under compression. During orthodontic tooth movement, cementoblast movement and matrix remodeling are dynamic in the microenvironment of cementum.[5,31]

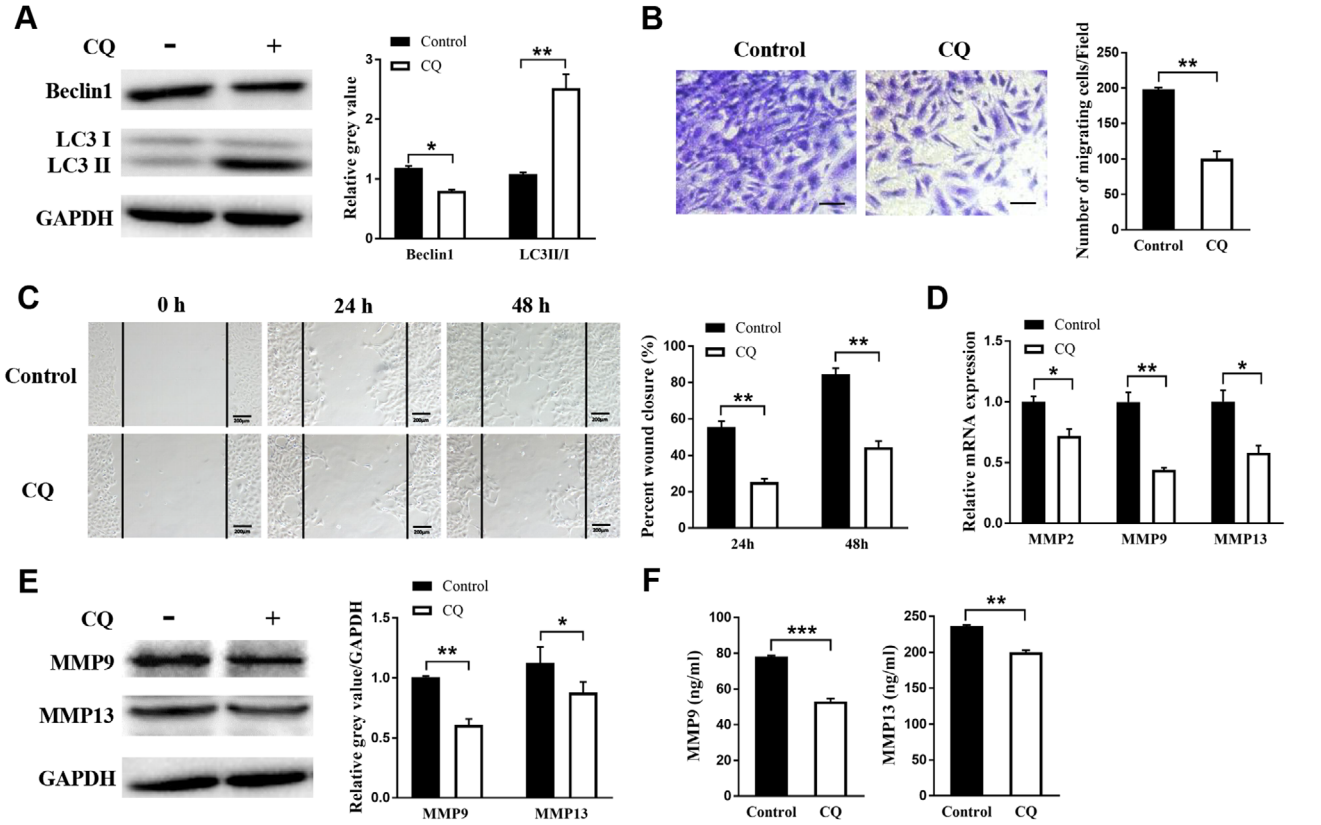

FIGURE 4 Autophagy was necessary for migration of OCCM-30 cells. (**A**) Western blot analysis (left) and quantification (right) of Beclin1, LC3II/I, and GAPDH protein expression in the cells treated with 10 μM CQ for 24 hours and control. (**B**) Transwell migration assay of cells treated with or without 10 μM CQ for 24 hours. Scale bars = 100 μm. (**C**) Wound healing assays of cells treated with or without CQ. (**D**) Relative MMP2, MMP9, and MMP13 mRNA expression in the control and CQ-treated cells was measured using qRT-PCR. GAPDH was used for normalization. (**E**) Western blot analysis (left) and quantification (right) of MMP9, MMP13, and GAPDH protein expression in the cells treated with CQ and control. (**F**) Secretion levels of MMP9 and MMP13 in cells treated with or without CQ were measured using quantitative ELISA. Data are shown as mean ± SD ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$)

Cementoblasts need to migrate to the resorption lacunae and secrete acellular and cellular cementum to repair the wound. In a previous study, mechanical compression was shown to facilitate brain cancer cell migration.[32] However, the biological behavior, especially the migration of cementoblasts, was inhibited under compressive force, indicating the function of cementoblasts to repair resorption lacunae is inhibited under compression, leading to the suppression of cementum repair. ECM is one of the most important components of the cellular microenvironment and guides cells to their destinations.[33] MMPs play an important role in ECM degradation.[34,35] Gelatinases MMP2 and MMP9 are widely expressed in the early dental mesenchyme where they play a role in the dental development, and strongly associated with bone development and periodontal tissue remodeling.[17,19] Collagenase MMP13 is expressed in differentiated phenotypes of osteoblastic lineage and important for degradation of the collagenous matrix of mineralized tissue.[36] In our present study, MMP9 and MMP13 expression and secretion were decreased under compressive force, and blocking MMPs inhibited cementoblast migration, indicating compressive force may regulate cementoblast migration by inhibiting the expression of MMPs. In vivo, activation of MMPs is controlled by endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs). TIMPs not only directly inhibit MMPs but also form complexes with MMPs to control activation of MMPs.[37,38] Further studies should also be carried out to explore the MMPs activation and their interaction with TIMPs in vivo.

Autophagy was significantly decreased in cementoblasts under compressive force. Autophagy is a response initiated by environmental stress and ensures cell survival or triggers cell death dependent on the stress threshold.[39] Increasing evidence indicates the autophagic response to stress proceeds in two phases.[40] The first phase is a rapid increase in autophagy flux after exposure to a stress condition, followed by a delayed reaction at the transcriptional level.[40] Reportedly, autophagy in PDLCs was significantly increased under compressive force before decreasing over time in the first phase.[25] In the present study, the autophagy of OCCM-30 cells induced by

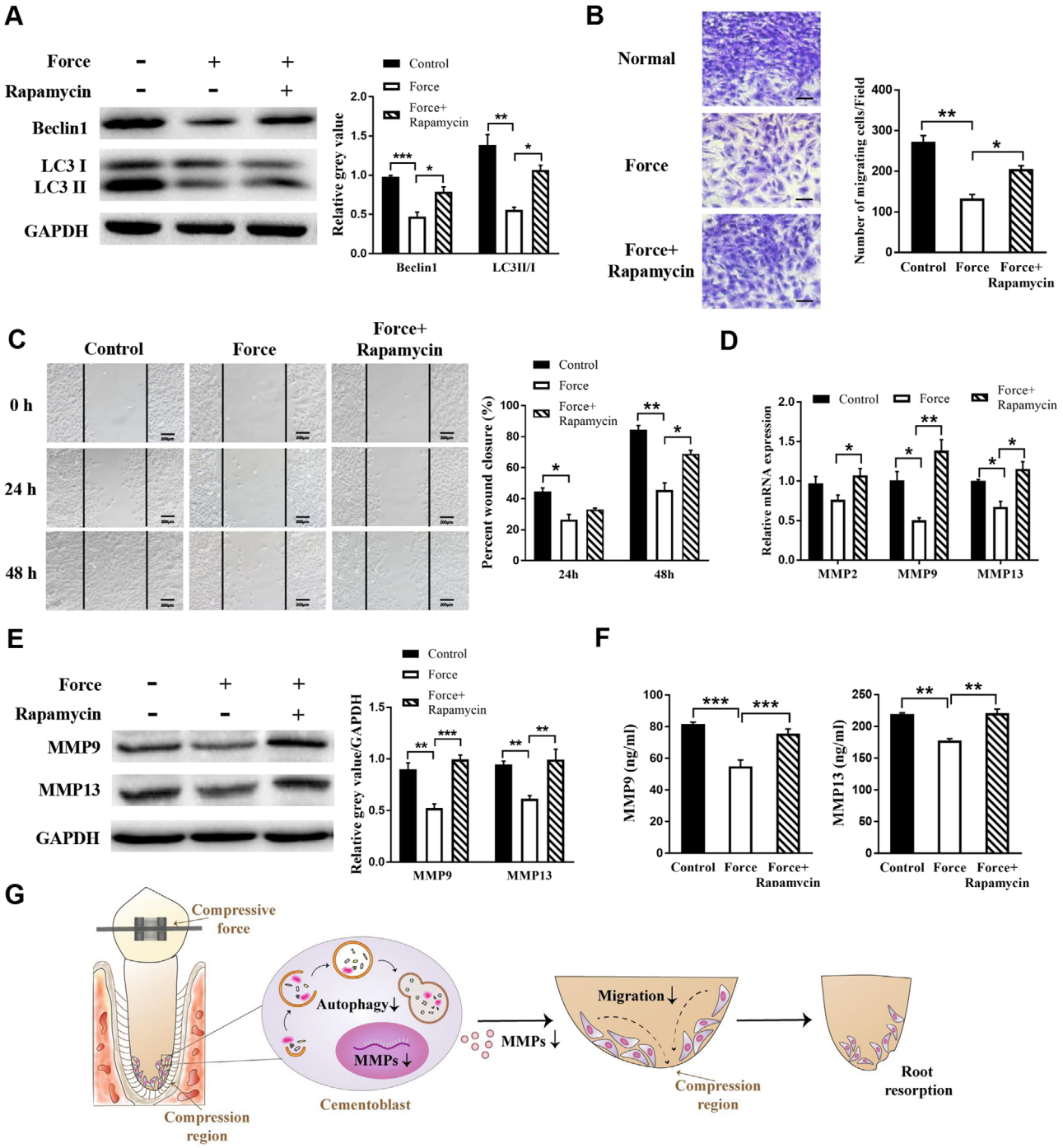

FIGURE 5 Autophagy activation relieved the inhibition of migration of cells exposed to compressive force. (**A**) Western blot analysis (left) and quantification (right) of Beclin1, LC3II/I, and GAPDH protein expression in cells subjected to compressive force with or without 5 μg/mL rapamycin for 24 hours. (**B**) Transwell migration assay in the control, compression, and compression with rapamycin cells. Scale bars = 100 μm. (**C**) Wound healing assay in the control, compression, and compression with rapamycin cells. (**D**) Relative MMP2, MMP9, and MMP13 mRNA expression in cells exposed to compression with or without rapamycin was measured using qRT-PCR. GAPDH was used for normalization. (**E**) Western blot analysis of MMP9 and MMP13 protein expression and the internal control GAPDH in the control, compression, and compression with rapamycin cells. Histograms show the quantification of band intensities. (**F**) Secretion levels of MMP9 and MMP13 in cells subjected to compression with or without rapamycin were measured using quantitative ELISA. Data are shown as mean ± SD ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (**G**) Schematics showing compressive force inhibited the migration of cementoblasts via the regulation of autophagy. Under orthodontic compressive force, autophagic flux was decreased in cementoblasts accompanied by a decrease of MMP expression and secretion into ECM, resulting in the inhibition of cell migration

compressive force at different time points was investigated. Beclin1 and LC3 were selected as autophagy markers to monitor the autophagy process. The decreased Beclin1 expression and LC3II/I ratio in cementoblasts under compression indicated the autophagy process was inhibited. Immunofluorescence staining also showed that LC3-positive autophagosomes were relatively abundant at the resting state and decreased under compressive force. However, a significant increase in autophagy under compression was not observed. The different autophagic response to mechanical compressive force in cementoblasts and PDLCs may be because PDLCs could adapt to compression more quickly and cementoblasts are less active. Thus, external root resorption is less active than alveolar bone resorption under compressive force during orthodontic tooth movement.

Compressive force inhibited cementoblast migration via downregulating autophagy. The role of autophagy in regulating cell migration remains controversial. In a previous study, autophagy induced by Toll-like receptor three or four activation facilitated migration and invasion of lung cancer cells.[41] Autophagy was involved in the regulatory mechanisms of the migration of endothelial progenitor cells (EPCs) inhibited by metformin.[42] In addition, EPC migration was decreased by autophagy inhibition under hypoxia.[43] In our present study, we demonstrated that inhibition of autophagy caused by CQ significantly decreased cementoblast migration and MMPs' expression and secretion. The basic level of autophagy was relatively active in OCCM-30 cells; decrease of autophagic activity resulted in inhibition of OCCM-30 cell migration, indicating autophagy flux is essential to maintain the biological behavior of cementoblasts. Further, the inhibition of OCCM-30 cell migration induced by the compressive force was partially relieved after activation of autophagy by rapamycin. Thus, autophagy played a protective role in maintaining the cementoblast biological process and function, and compressive force inhibited OCCM-30 cell migration by regulating autophagy. Activation of autophagy may facilitate cementoblast migration to promote its self-repairing capability under compression and subsequently improve cementum repair and prevent root resorption during orthodontic tooth movement. However, the migration and autophagy of cementoblasts were only investigated in vitro in this study and the application of mechanical compressive force in vitro does not mimic exactly the biological conditions during orthodontic tooth movement in vivo. Further study is needed to investigate the protective role of autophagy in cementoblast migration in vivo and suggest a potential therapeutic strategy for reducing external root resorption.

In conclusion, the present study results revealed that the migration of cementoblasts was decreased under compressive force partially dependent on the inhibition of MMPs, and autophagy was inhibited and mediated the migration of cementoblasts on mechanical compression (Figure 5g). These findings aid in understanding the pathogenesis of external root resorption induced by compressive force and autophagy activation could be a potential therapeutic strategy for external root resorption.

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AUTHOR CONTRIBUTIONS

Yuhui Yang performed the experiment, interpreted the results, and prepared the manuscript. Yiping Huang designed the study, performed the experiment, analyzed and discussed the findings, and edited the manuscript. Hao Liu, Yunfei Zheng, and Lingfei Jia contributed to the conception and design of the study and interpretation of data. Weiran Li conceived the idea for the project, supervised the project, and critically revised the manuscript.

ORCID

Weiran Li https://orcid.org/0000-0002-8651-3282

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