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Force-Induced H_2S by PDLSCs Modifies Osteoclastic Activity during Tooth Movement

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Abstract

Hydrogen sulfide (H_2S), a gasotransmitter, has been recently linked to mesenchymal stem cell (MSC) function and bone homeostasis. Periodontal ligament stem cells (PDLSCs) are the main MSCs in PDL, which respond to mechanical force to induce physiological activities during orthodontic tooth movement (OTM). However, it is unknown whether mechanical force might induce endogenous H_2S production by PDLSCs to regulate alveolar bone homeostasis. Here, we used a mouse OTM model to demonstrate that orthodontic force-induced endogenous H_2S production in PDL tissue was associated with macrophage accumulation and osteoclastic activity in alveolar bone. Then, we showed that mechanical force application induced cystathionine β-synthase (CBS) expression and endogenous H_2S production by PDLSCs. Moreover, blocking endogenous H_2S or systemically increasing H_2S levels could decrease or enhance force-induced osteoclastic activities to control tooth movement. We further revealed how force-induced H_2S production by PDLSCs contributed to the secretion of monocyte chemoattractant protein-1 (MCP-1) and the expression of receptor activator of nuclear factor–κB ligand/osteoprotegerin (RANKL/OPG) system by PDLSCs. The secretion and expression of these factors controlled macrophage migration and osteoclast differentiation. This study demonstrated that PDLSCs produced H_2S to respond to and transduce force signals. Force-induced gasotransmitter H_2S production in PDLSCs therefore regulated osteoclastic activities in alveolar bone and controlled the OTM process through the MCP-1 secretion and RANKL/OPG system.

Keywords: hydrogen sulfide, mechanical force, osteoclast, bone remodeling, orthodontic tooth movement, mesenchymal stem cells

Introduction

Hydrogen sulfide (H_2S), an inorganic molecule, used to be defined as a colorless and poisonous gas that imparted the characteristic foul odor of rotten eggs (Beauchamp et al. 1984). Recently, H_2S has been found to be a gasotransmitter that regulates multiple signaling pathways in mammalian cells (Gadalla and Snyder 2010; Vandiver and Snyder 2012). Mesenchymal stem cells (MSCs) secrete several autocrine and paracrine factors to regulate tissue homeostasis. Recent studies show that MSCs can also produce H_2S and that endogenous H_2S regulates the self-renewal and osteogenic differentiation of MSCs to ensure bone homeostasis (Liu et al. 2014; Yang et al. 2016). However, it is still unclear whether endogenous H_2S produced by MSCs are paracrine factors interacting with other cells to modulate bone remodeling.

Mechanical force plays an important role in tissue development, homeostasis, and human disease (Serluca et al. 2002; Magnusson et al. 2010; Liaw and Zimmermann 2016). Mechanical stimulation can regulate MSC proliferation and differentiation (Luu et al. 2009; Ruan et al. 2015), as well as regulate signaling pathways to communicate with the tissue microenvironment (Thompson et al. 2012). In addition, force can promote extracellular matrix homeostasis and the secretion of various mediating factors (Krishnan and Davidovitch 2009; Humphrey et al. 2014). However, how force stimulations transduce into cellular information is still unclear. Recently, H_2S has been reported as a new gasotransmitter in various pathways (Gadalla and Snyder 2010) and to be involved in MSC function and bone homeostasis (Liu et al. 2014; Yang et al. 2016). Therefore, we hypothesized that mechanical force may contribute to bone remodeling by modulating MSC-produced H_2S.

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A supplemental appendix to this article is available online.

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Orthodontic tooth movement (OTM) is a unique bone-remodeling process mediated by mechanical force. Two sides (the pressure and tension sides) in the periodontal space are generated by root movement (Meikle 2006). The periodontal ligament (PDL) displays compression and disorganization, and it induces bone resorption on the pressure side (toward the force direction), whereas the stretching of PDL fiber bundles stimulates bone deposition on the tension side (opposite to force direction) (Krishnan and Davidovitch 2006). PDL stem/progenitor cells are the main MSCs that differentiate into cell components and generate the PDL complex structure (Seo et al. 2004; Liu et al. 2008), which are involved in PDL homeostasis. Recent reports demonstrated that human periodontal ligament stem cells (PDLSCs) express 2 H_2S-generating enzymes, cystathionine β-synthase (CBS) and cystathionine-γ-lyase, and produce H_2S to regulate PDLSC proliferation and differentiation (Su et al. 2015). PDLSCs are force-sensitive and force-responsive cells in the initial stage of OTM (Reitan 1985). However, it is unknown whether force can promote H_2S production by PDLSCs. In addition, how the force-induced endogenous H_2S production contributes to alveolar bone remodeling during OTM remains to be clarified.

In this study, we demonstrated the force-induced H_2S production by PDLSCs. We also showed that mechanical force controlled alveolar bone remodeling and the OTM process by inducing endogenous H_2S production by PDLSCs.

Materials and Methods

Animals and Orthodontic Force Application and Treatment

The study used 7- to 8-wk-old male C57BL/6 mice (Weitong Lihua Experimental Animal Center, Beijing, China) weighing 20 to 25 g each. Experimental protocols were approved by the Animal Use and Care Committee of Peking University (LA2013-92).

Mice were anesthetized with pentobarbital sodium (100 mg/kg, injected intraperitoneally) prior to surgery. Mechanical force was applied following a previously described method (Cao et al. 2014). Briefly, a nickel-titanium coil spring (0.2 mm in wire size, 1 mm in diameter, and 1 mm in length; Smart Technology) was bonded between the maxillary right first molar and maxillary incisors by flowable restorative resin (3M ESPE). Approximately 30 g of force was applied (Taddei et al. 2012). The contralateral first molar, which was barely padded with resin, was used as the control. The occlusal view of the maxilla was recorded by a stereomicroscope (SWZ1000; Nikon). OTM distance was measured by a modified method as previously described (Appendix Materials and Methods) (Cao et al. 2014).

Measurement of H_2S in Periodontal Tissues, Serum, and Supernatant

Equal amounts of minced periodontal tissues were homogenized in 80 μL saline on ice. After centrifugation, H_2S in the supernatant from tissue homogenates and in serum was measured with a mouse H_2S enzyme-linked immunosorbent assay (ELISA) kit (TSZ ELISA) following the manufacturer's instructions. H_2S in the cultured supernatant from human PDLSCs was measured with a human H_2S ELISA kit (TSZ ELISA) according to the manufacturer's instructions.

Immunofluorescence Staining

Immunofluorescence staining was performed as previously described (Kou et al. 2011). Tissue sections and cell slides were double-stained with antibodies of anti-CBS (1:50, ab135626; Abcam) and anti-CD90 (1:200, ab225; Abcam) or anti-CD90 (1:100, 328106; Biolegend) to detect the change in CBS expression. Then, sections were incubated with fluorescein isothiocyanate–conjugated or tetramethylrhodamine isothiocyanate–conjugated secondary antibodies (1:200; Jackson Immuno Research Laboratories). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Confocal microscopic images were processed with LSM 5 Release 4.2 software after acquisition by a laser-scanning microscope (LSM 510; Zeiss).

Co-culture of PBMCs and PDLSCs

$CD11b^+$ peripheral blood mononuclear cells (PBMCs) were selected by the MACS system (Miltenyi Biotec) from peripheral blood. PDLSCs were seeded into 24-well plates at 2.5×10^3 cells/well 1 d prior to the addition of $CD11b^+$ PBMCs. $CD11b^+$ PBMCs were added to PDLSCs at a concentration of 2.5×10^5 cells/well. The cells were fixed in paraformaldehyde after 3 wk of co-culture in a medium containing macrophage colony-stimulating factor (M-CSF) (30 ng/mL) and soluble receptor activator of nuclear factor–κB ligand (sRANKL) (50 ng/mL). Tartrate-resistant acid phosphatase (TRAP) staining was then performed. The number of TRAP-positive multinucleated (>2 nuclei) osteoclasts in 5 visual fields at 20× magnification in each well was counted ($n = 3$). The final result came from the average of 3 tests.

PDLSCs and THP-1–Induced Macrophage Transwell Co-culture System

PDLSCs were seeded into 6-well plates at 2×10^5 cells/well and divided into 4 groups: control, sodium hydrosulfide (NaSH), force, and force + hydroxylamine (HA) group. Then, 1.5 g/cm^2 compressive force was applied for 24 h to the force and force + HA groups at 80% confluence with or without HA treatment (100 μm). The NaSH group was treated with NaSH (100 μm) for 24 h. The transwell chambers were placed in 6-well plates after removing the mechanical force instrument. THP-1 (a human monocytic cell line) cells were added to the upper chamber at 1×10^6 cells/well with phorbol myristate acetate (100 ng/L). Cells were co-cultured for 24 h. Those cells remaining in the upper surface of the transwell chambers were removed by cotton swabs before staining with crystal violet. Cells stained by crystal violet on the bottom surface served as

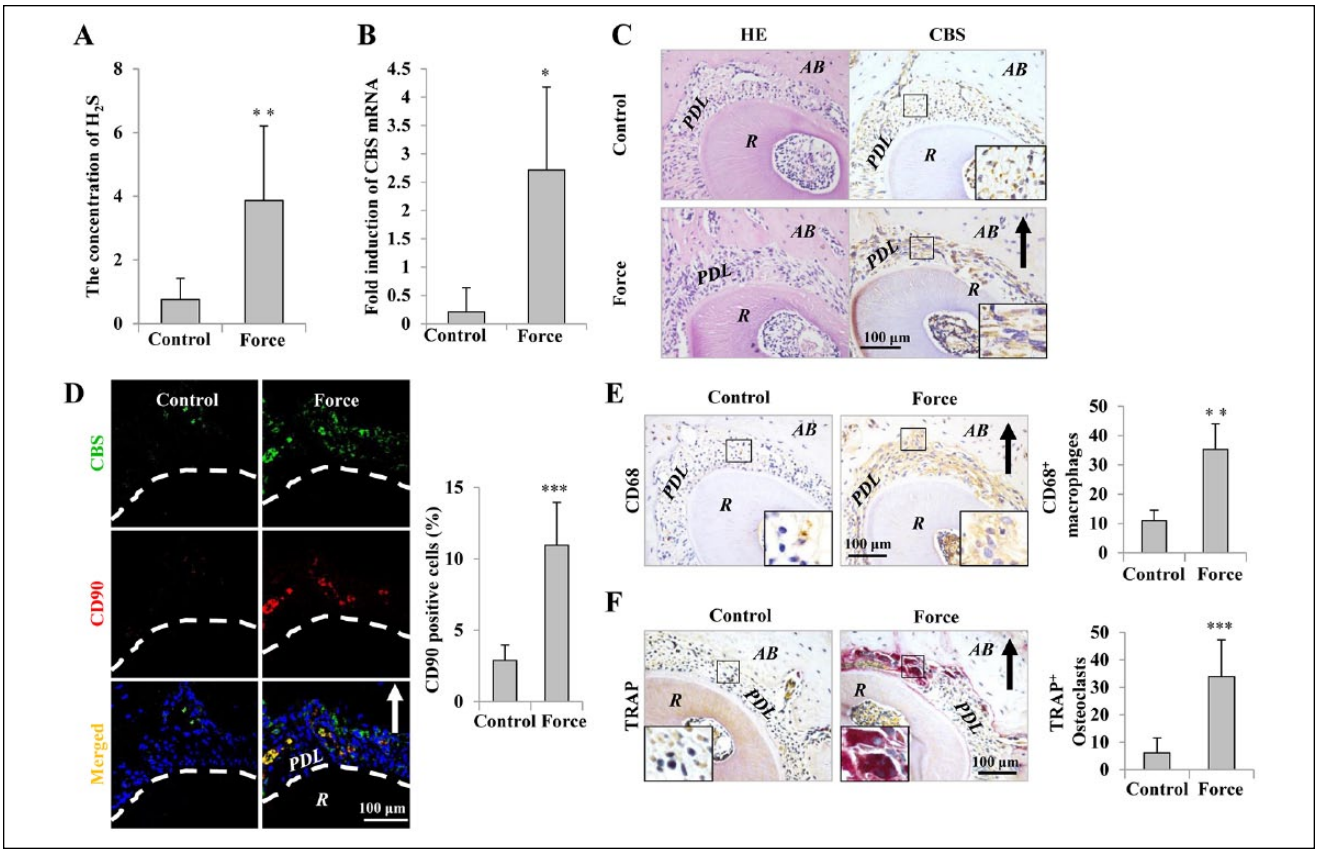

Figure 1. Endogenous hydrogen sulfide (H_2S) production and cystathionine β-synthase (CBS) expression increased in the periodontal tissue after orthodontic force application. (**A**) The concentration of H_2S in periodontal tissue homogenate increased after orthodontic force application for 7 d. n = 5–6; $^{**}P < 0.01$. (**B**) CBS messenger RNA expression in periodontal tissue homogenate of the force side was upregulated significantly after orthodontic tooth movement (OTM) compared with the control side. n = 5–6; $^{*}P < 0.05$. (**C**) Representative immunohistochemical images and hematoxylin and eosin (H&E) staining on the compression side of the distal root of the upper first molar. The CBS expression was upregulated in periodontal ligament (PDL) cells after force application. The H&E staining correspondingly showed the histomorphology of the immunostained sections. Large boxed areas show higher magnification views of the small boxes. The black arrow indicates the direction of orthodontic force. AB, alveolar bone; R, root. Scale bar: 100 μm. (**D**) Representative immunofluorescence images on the compression side of distal roots. The number of CBS-positive (green) and CD90-positive (red) double-stained PDL progenitors (merged yellow) increased after 7 d of OTM. Dashed lines indicate the outline of the roots. The white arrow indicates the direction of force application. Scale bar: 50 μm. Semi-quantification of CD90-positive periodontal ligament stem cells (PDLSCs) showed that the number of CD90-positive cells increased on the compression side of PDL after force application. (**E**, **F**) Representative immunohistochemical images and tartrate-resistant acid phosphatase (TRAP) staining on the compression side of distal roots. Both the number of CD68-positive macrophages and TRAP-positive osteoclasts increased around the PDL and alveolar bone site after force application. $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bar: 100 μm.

the migrated cells. The number of those migrated cells in 5 visual fields at 10× magnification in each chamber was counted (n = 3). The final result came from the average of 3 tests.

Methods

The following are described in detail in the Appendix:

- Measurement of OTM distance
- Injection of H_2S donor and inhibitor
- Periodontal tissue sampling and trimming for homogenate
- PDLSC culture and treatment
- Hematoxylin and eosin (H&E) staining and TRAP staining
- Immunohistochemical staining
- Quantitative real-time polymerase chain reaction (PCR)
- Western blotting

Statistical Analysis

Statistical analysis was performed by SPSS 19.0 for Windows (SPSS, Inc.). The normal distribution of the raw data was confirmed by a 1-sample Kolmogorov-Smirnov test. Comparisons between 2 groups were analyzed by independent unpaired 2-tailed Student's t tests. In addition, comparisons between more than 2 groups were analyzed using 1-way analysis of variance (ANOVA) and Tukey's multiple-comparison tests. $P < 0.05$ was considered statistically significant. All data are presented as mean ± SD.

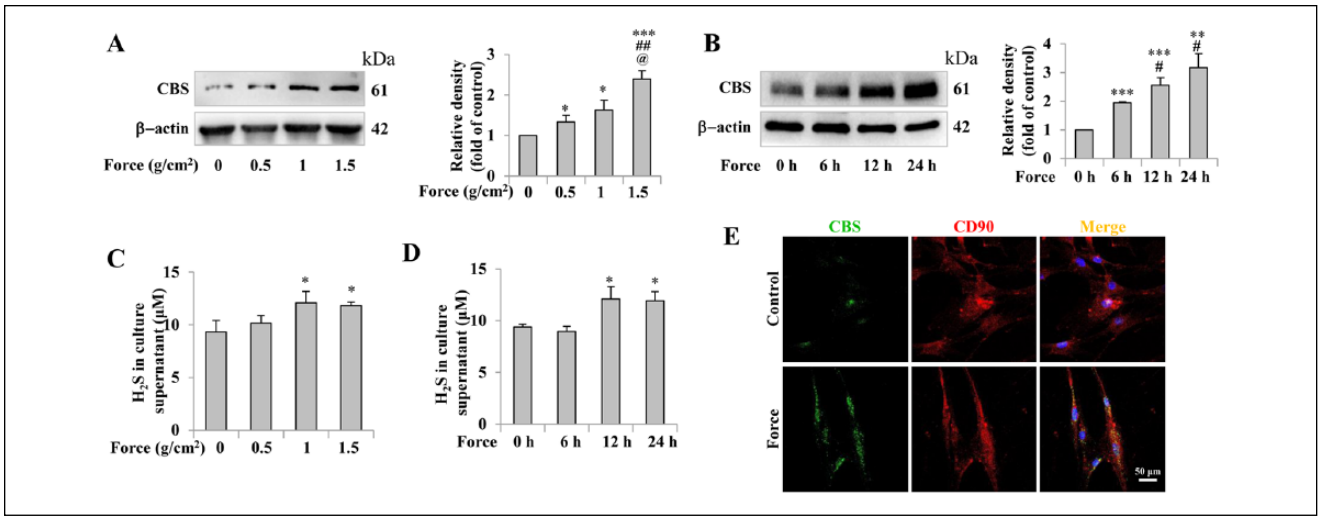

Figure 2. Mechanical force upregulated cystathionine β-synthase (CBS) expression and hydrogen sulfide (H_2S) secretion in periodontal ligament stem cells (PDLSCs). (**A, B**) Western blot results of CBS protein expression. CBS expression in PDLSCs was upregulated with increasing force-treated intensity for 24 h. $^{*}P < 0.05$, $^{***}P < 0.001$ versus 0-g/cm^2 group. $^{\#\#}P < 0.01$ versus 0.5-g/cm^2 group. $^{@}p < 0.05$ versus 1-g/cm^2 group (A). CBS expression in PDLSCs was upregulated as force-treated time prolonged at 1.5-g/cm^2 loaded force. $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0-h group. $^{\#}p < 0.05$ versus 6-h group (B). Beta-actin served as internal control for equal loading. The data were representative of 3 independent experiments. (**C, D**) The concentration of H_2S secreted by PDLSCs in the supernatant after compressive force application. The concentration of H_2S increased significantly after compression at 1 g/cm^2 and 1.5 g/cm^2 for 24 h, $n = 3$; $^{*}P < 0.05$ versus 0 g/cm^2, as well as at 12 h and 24 h after 1.5-g/cm^2 compressive force application. $n = 3$; $^{*}p < 0.05$ versus 0 h. (**E**) Representative immunofluorescence images of PDLSCs treated by compressive force for 24 h. The number of CBS (green) and CD90 (red) double-stained positive PDLSCs (merged yellow) increased. Scale bar: 50 μm.

Results

Orthodontic Force-Induced CBS Expression and H_2S Production in PDL, Associated with Increased Osteoclasts

We used a mouse OTM model to test the in vivo function of mechanical force-induced endogenous H_2S production. Compared with the control group, orthodontic force application elevated H_2S production and upregulated CBS messenger RNA (mRNA) expression in PDL tissue (Fig. 1A, B). We used immunohistochemical staining to further confirm that orthodontic force enhanced CBS expression in PDL cells (Fig. 1C). Moreover, immunohistofluorescence showed that most CBS expression co-localized with CD90, an MSC marker, in PDL tissue (Fig. 1D). CBS expression accumulated with the increased CD90-positive cells after orthodontic force application (Fig. 1D and Appendix Fig. 1A). To explore the function of increased H_2S production in PDL tissue, we showed that compared with the force-unloaded control group, the application of orthodontic force during the OTM process increased the number of CD68-positive macrophages and TRAP-positive osteoclasts on the compression side and the number of osteocalcin (OCN)–positive osteoblasts on the tension side of PDL (Fig. 1E, F and Appendix Fig. 1A, B). These results imply that orthodontic force induces H_2S production by PDLSCs during OTM, which is associated with the osteoclastic activity around the alveolar bone.

Mechanical Force Upregulated CBS Expression and Enhanced H_2S Secretion in PDLSCs

To further explore the effects of force on endogenous H_2S production by PDLSCs, we applied static compressive force to the cells. Western blot results showed that force loading upregulated CBS protein expression with increased force intensity and prolonged force treatment duration (Fig. 2A, B). Correspondingly, static compressive force enhanced H_2S secretion in the cultured supernatant (Fig. 2C, D). This finding is consistent with the change in CBS expression in PDLSCs. We then used immunofluorescence staining to demonstrate that CBS coexpressed with CD90 and to further confirm that mechanical force upregulated CBS expression in PDLSCs (Fig. 2E). These results suggested that mechanical force promotes endogenous H_2S production by PDLSCs, which may link the signal transduction of mechanical force signal with biological function.

H_2S Level Regulated Osteoclastic Activity and the OTM Process

To confirm the function of force-induced H_2S production during the OTM process, we systemically injected CBS inhibitor HA to suppress endogenous H_2S production during orthodontic force application (Fig. 3A). Decreased serum H_2S levels confirmed that the HA injection was effective (Fig. 3B). Immunohistochemical staining showed that compared with the vehicle-injected control group, blocking endogenous H_2S production during the OTM process repressed the orthodontic force-induced accumulation of CD68-positive macrophages and TRAP-positive osteoclasts on the compression side of PDL and alveolar bone (Fig. 3C–F). In addition, force-induced increase of OCN-positive osteoblasts along the alveolar bone surface on the tension site was also repressed by HA injection (Appendix Fig. 1A, B). We further showed that, in addition to repressing osteoclastic activity, blocking endogenous H_2S decreased the distance of orthodontic force-induced tooth

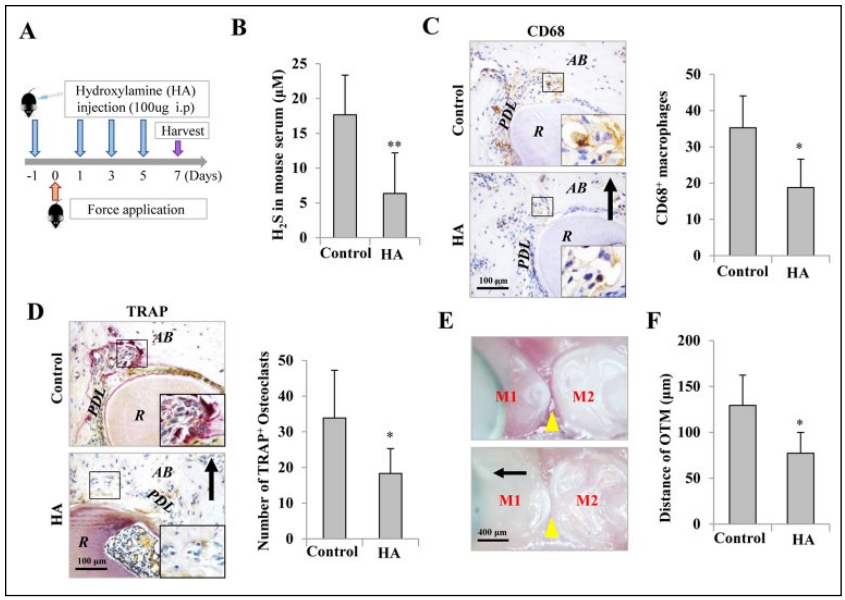

Figure 3. Inhibition of cystathionine β-synthase (CBS) by hydroxylamine (HA) injection repressed macrophage accumulation and osteoclastic activity to decrease the subsequent orthodontic tooth movement (OTM). (**A**) Schedule of the experiment. Orthodontic force was applied to mice in 2 groups for 7 d. Injection of HA was performed every other day since 1 d prior to the 7-d course of OTM. The vehicle-injected control group included 8 mice, and the HA-injected group included 7 mice. (**B**) The levels of endogenous hydrogen sulfide (H_2S) in serum decreased significantly after interval injections of HA. n = 7–8, $^{**}P < 0.01$. (**C, D**) Representative immunohistochemical images and tartrate-resistant acid phosphatase (TRAP) staining on the compression side of distal roots. HA injections significantly repressed the accumulation of CD68-positive macrophages during OTM (C). The number of TRAP-positive osteoclasts also decreased after being treated by HA injections (D). Large boxed areas show higher magnification views of the small boxes. The arrow shows the direction of orthodontic force. $^{*}P < 0.05$. Scale bar: 100 μm. (**E**) The representative images of the occlusal view of the first and second molars after 7 d of OTM in mice. M1, first molar; M2, second molar. Yellow arrows indicate the distance of OTM. Black arrows show the direction of the orthodontic force. Scale bars: 400 μm. (**F**) Statistical results of tooth movement in 2 groups. OTM distance significantly decreased in the HA-injected group after 7 d of force application compared with the vehicle-injected control group (vehicle-injected control group, n = 8; HA-injected group, n = 7). $^{*}P < 0.05$.

movement compared with the vehicle-injected control group (Fig. 3G, H). Next, we prolonged the orthodontic process to 14 d and showed that the distance of OTM and the number of $TRAP^+$ osteoclasts increased compared with that of the 7-d tooth movement (Appendix Fig. 2A–C). More important, the increased OTM distance and osteoclast activity of the 14-d group was also partially blocked by the injection of HA, which was consistent with the 7-d result (Fig. 3D–F and Appendix Fig. 2D, E). These results indicated that the time of animal experiments mostly affects the scale but not the tendency of our findings.

To further confirm the function of H_2S during OTM, we then increased H_2S levels with H_2S donor GYY4137 dichloromethane complex (Liu et al. 2014) (Fig. 4A). Increased serum H_2S levels confirmed that the GYY4137 injection was effective (Fig. 4B). Immunohistochemical staining results showed that compared with the vehicle-injected control group, increasing H_2S during the OTM process promoted the force-induced accumulation of CD68-positive macrophages and TRAP-positive osteoclasts on the compression side of PDL and alveolar bone (Fig. 4C–F). Force-induced increased number of OCN-positive osteoblasts along the tension site of the alveolar bone was further potentiated by GYY4137 injection (Appendix Fig. 1A, B). Furthermore, in addition to enhancing osteoclastic activity, upregulated H_2S levels increased the distance of OTM compared with the vehicle-injected control group (Fig. 4G, H). These results suggested an essential role for H_2S in OTM. Force-induced endogenous H_2S may affect both macrophage accumulation and osteoclast differentiation to regulate the OTM process.

H_2S Controlled the Expression of Force-Upregulated MCP-1 and the RANKL/OPG System in PDLSCs

The PDLSC-expressed chemokine monocyte chemoattractant protein-1 (MCP-1) contributes to the recruitment of inflammatory monocytes to PDL during OTM (Zeng et al. 2015). Moreover, the ratio of receptor activator of nuclear factor–κB ligand/osteoprotegerin (RANKL/ OPG) is essential to osteoclast differentiation (Lu et al. 1998; Boyce and Xing 2007). Therefore, we hypothesized that force-induced H_2S production by PDLSCs might contribute to macrophage accumulation and osteoclastic activity by regulating MCP-1 and the RANKL/OPG ratio. PDLSCs were treated with NaSH and HA to increase H_2S concentrations in the culture medium or to decrease H_2S production by PDLSCs in vitro (Fig. 5A). Western blot results showed that compared with the control group, MCP-1 expression in PDLSCs was upregulated by NaSH and downregulated by HA treatment (Fig. 5B). Moreover, mechanical force loading upregulated MCP-1 expression in PDLSCs, which was partially reversed by HA treatment (Fig. 5C). These results suggested that force-induced H_2S production contributes to MCP-1 secretion by PDLSCs, which may lead to macrophage recruitment.

We then investigated whether PDLSC-produced H_2S also affected osteoclastogenesis signaling. Western blot results showed that compared with the control group, RANKL protein expression was upregulated by NaSH and downregulated by HA treatment (Fig. 5D). By contrast, OPG protein expression was downregulated by NaSH and upregulated by HA treatment (Fig. 5D). Therefore, the RANKL/OPG ratio significantly increased in the NaSH group and decreased in the HA group (Fig. 5E). Moreover, force application on PDLSCs also upregulated RANKL protein expression and downregulated OPG expression compared with the control group (Fig. 5F). HA reversed the force-induced RANKL upregulation and OPG downregulation in PDLSCs (Fig. 5F). As a result, the RANKL/

OPG ratio increased after compression force application but was partially blocked by simultaneous treatment with HA (Fig. 5G). These results suggest that force-induced H_2S production by PDLSCs regulates osteoclast differentiation by affecting the RANKL/OPG system.

PDLSCs-Produced H_2S Controlled Force-Induced Macrophage Migration and Contributed to Osteoclast Differentiation in Co-culture Systems

We used a co-culture system of PDLSCs and macrophages to further confirm the function of PDLSC-produced H_2S. We found that NaSH or the conditional medium from force-loaded PDLSCs increased the number of migrated THP-1–induced macrophages compared with the control group (Fig. 5H, I). Moreover, the conditional medium from HA-pretreated, force-loaded PDLSCs failed to increase the migration of macrophages compared with the control and force group (Fig. 5H, I). These results suggest that force-induced H_2S production by PDLSCs promotes macrophage migration.

To test whether PDLSC-produced H_2S also contributed to osteoclastogenesis, $CD11b^+$ PBMCs were co-cultured with NaSH- or HA-pretreated PDLSCs. TRAP staining results showed that NaSH enhanced osteoclast differentiation in the co-cultured system compared with the control group, whereas HA-pretreated PDLSCs failed to induce obvious osteoclast differentiation in the co-cultured system (Fig. 5J, K). This result indicated the critical effects of PDLSC-produced H_2S on osteoclast differentiation, which may consequently regulate the OTM process.

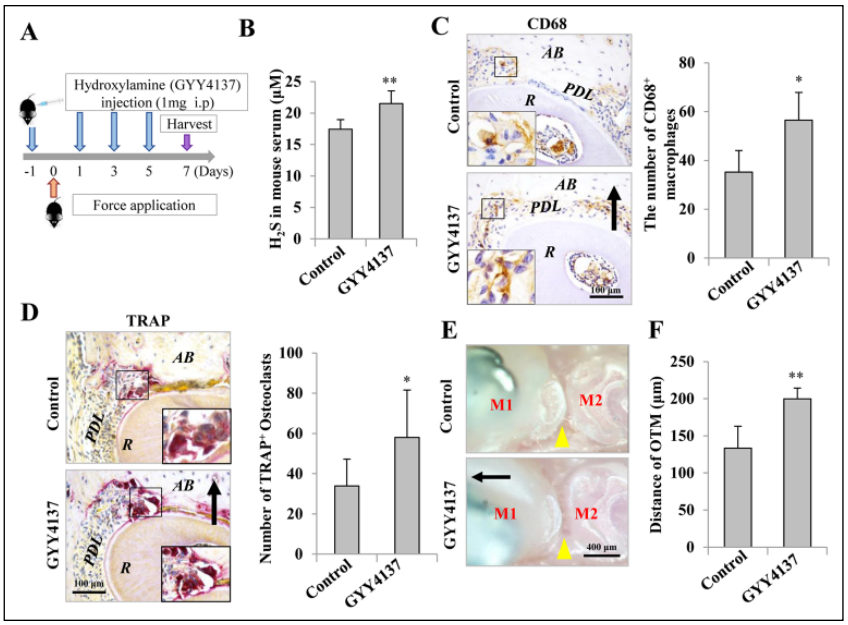

Figure 4. Upregulated hydrogen sulfide (H_2S) level by GYY4137 injections enhanced macrophage accumulation and osteoclastic activity to promote orthodontic tooth movement (OTM). (**A**) Schedule of the experiment. The procedure was the same with hydroxylamine (HA) injection. The vehicle-injected control group included 8 mice, and the GYY4137-injected group included 5 mice. (**B**) The H_2S levels in serum were significantly upregulated after interval injections of GYY4137. n = 5–8, $^{**}P < 0.01$. (**C, D**) Representative immunohistochemical images and tartrate-resistant acid phosphatase (TRAP) staining on the compression side of distal roots. GYY4137 injections significantly increased the number of CD68-positive macrophages accumulated around the periodontal ligament (PDL) and alveolar bone site during OTM compared with the vehicle-injected control group (C). The number of TRAP-positive osteoclasts also increased after being treated by GYY4137 injections (D). Large boxed areas show higher magnification views of the small boxes. The arrow shows the direction of orthodontic force. Scale bar: 100 μm. $^{*}P < 0.05$. (**E**) The representative images of the occlusal view of the first and second molars after 7 d of OTM in mice. The OTM distance was increased after being treated by GYY4137 injections. Scale bars indicate 400 μm. (**F**) Statistical results of tooth movement in 2 groups. OTM distance significantly increased in the GYY4137-injected group after 7 d of force application compared with the vehicle-injected control group (vehicle-injected control group, n = 8; GYY4137-injected group, n = 5). $^{**}P < 0.01$.

Discussion

In the present study, we provided multiple lines of evidence that mechanical force-induced endogenous H_2S production by PDLSCs modifies osteoclastic activity to regulate the OTM process. First, orthodontic force-induced endogenous H_2S production in PDL was associated with macrophage accumulation and osteoclastic activity in alveolar bone. Second, mechanical force application upregulated CBS expression and induced H_2S production by PDLSCs in vitro. Third, blockage of endogenous H_2S or systematic increases in H_2S levels resulted in downregulated or enhanced force-induced osteoclastic activity in alveolar bone to affect tooth movement. Finally, force-induced H_2S production by PDLSCs contributed to MCP-1 secretion and RANKL/OPG system expression in PDLSCs to regulate macrophage migration and osteoclast differentiation. These results suggest that force-induced endogenous H_2S production by PDLSCs promotes macrophage migration and osteoclast differentiation, which contributes to alveolar bone homeostasis during OTM.

Our study is the first to find that mechanical force modulates CBS expression and subsequent H_2S production by PDLSCs in periodontal tissue. Although the physiological effects of H_2S have received considerable interest (Kabil and Banerjee 2014; Kimura 2015), the modulation of H_2S production is still poorly understood. The regulation of CBS expression and subsequent H_2S release was previously associated with carbon monoxide in brain microcirculation (Morikawa et al. 2012) or with oxidative stress in HEK293 cells (Niu et al. 2015). However, our results showed that mechanical force induced CBS expression and H_2S production by PDLSCs, which is different from the biochemical regulatory mechanism reported by previous studies. This finding provides a new vision for the physiological regulation of endogenous H_2S production. PDLSCs present the main regulating effect on

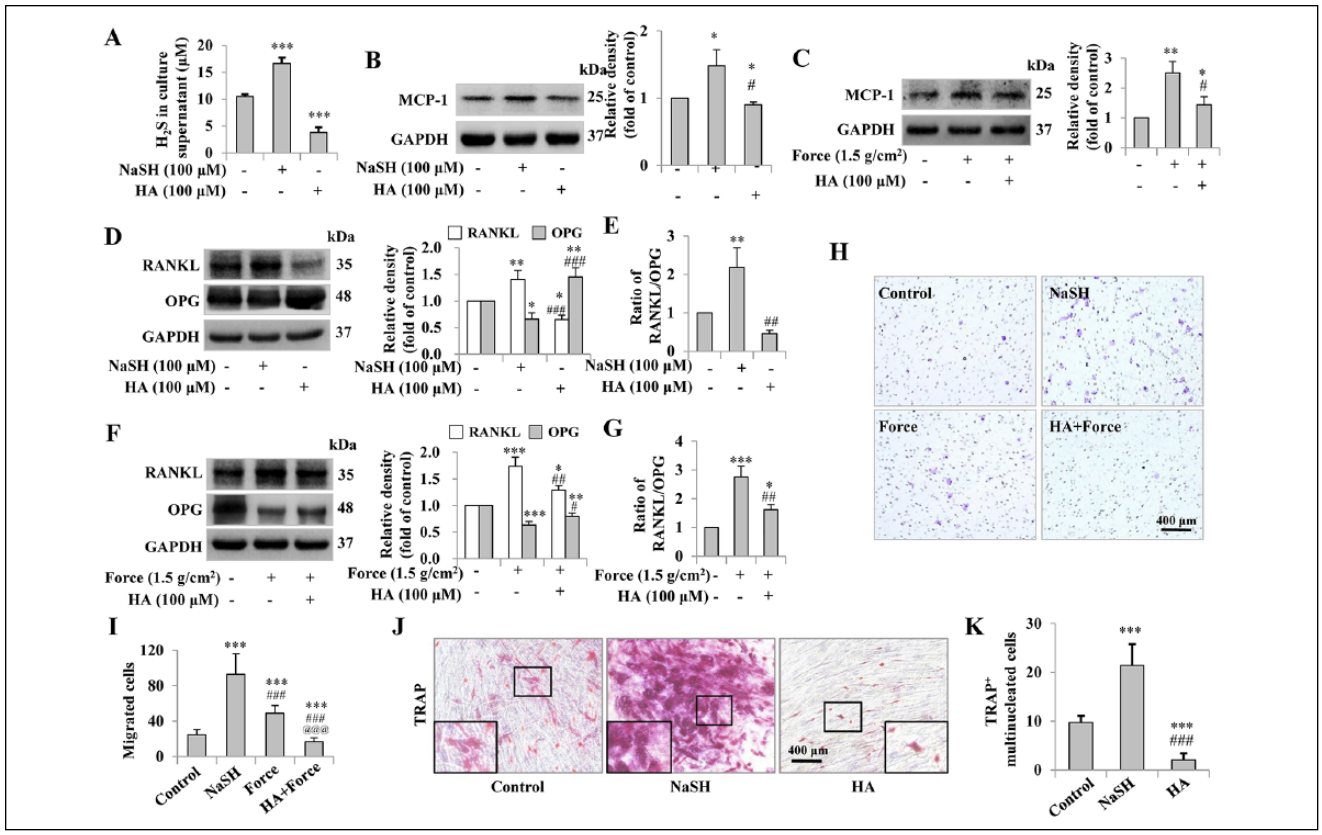

Figure 5. Hydrogen sulfide (H_2S) regulated force-induced monocyte chemoattractant protein-1 (MCP-1) expression and receptor activator of nuclear factor–κB ligand (RANKL)/osteoprotegerin (OPG) system in periodontal ligament stem cells (PDLSCs), and H_2S produced by PDLSCs enhanced macrophage migration as well as osteoclast differentiation. (**A**) The concentration of H_2S in cultured supernatant of PDLSCs was upregulated by sodium hydrosulfide (NaSH) and downregulated by hydroxylamine (HA) treatment. $^{***}P < 0.001$ versus control. (**B, C**) Western blot results of MCP-1 expression in PDLSCs. MCP-1 protein expression in PDLSCs was upregulated after being treated by NaSH while downregulated by HA treatment compared with the control group (B). MCP-1 protein expression was upregulated after mechanical force loading, which was partially reversed by HA treatment (C). GAPDH served as an internal control for equal loading. Data represent 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ versus NaSH group or force group. (**D–G**) Western blot results of RANKL and OPG expression in PDLSCs. RANKL expression was upregulated after being treated by NaSH and downregulated by HA treatment. To the contrary, OPG expression was upregulated after HA treatment and downregulated by NaSH compared with the control (D). Ratio of RANKL/OPG was upregulated after treatment by NaSH and downregulated by HA treatment (E). RANKL expression was upregulated after treatment by compressive force and partially reversed by HA treatment. OPG expression was downregulated after treatment by mechanical force and also partially reversed by HA treatment (F). Ratio of RANKL/OPG was upregulated by mechanical force and partially reversed by HA treatment (G). GAPDH served as an internal control for equal loading. Data represent 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus NaSH group or force group. (**H**) Representative images of crystal violet staining of THP-1 (a human monocytic cell line)–induced macrophage transwell test. The number of migrated cells increased after treatment by NaSH. Conditional medium from force-loaded PDLSCs also enhanced the migration of macrophages compared with the control group, whereas the conditional medium from HA-pretreated force-loaded PDLSCs failed to increase the migration of macrophages compared with the control group and force group. Scale bar: 400 μm. (**I**) The amount of migrated cells increased in the NaSH group and force-loaded conditional medium group compared with the control group. The former had a larger scale of increase. The number of migrated cells of the HA-pretreated force-loaded group was less than the other groups. $^{***}P < 0.001$ versus control; $^{\#\#\#}P < 0.001$ versus NaSH group; $^{@@@}P < 0.001$ versus force-loaded conditional medium group. (**J**) Representative images of tartrate-resistant acid phosphatase (TRAP) staining of osteoclasts in peripheral blood mononuclear cells (PBMCs) co-cultured with PDLSCs. Large boxed areas show higher magnification views of the small boxes. The osteoclast differentiation of PBMCs was significantly enhanced by NaSH and repressed by HA. Scale bar: 400 μm. (**K**) The number of TRAP-positive cells increased after treatment by NaSH and decreased by HA treatment. $^{***}P < 0.001$ versus control; $^{\#\#\#}P < 0.001$ versus NaSH group.

endogenous H_2S production, although we also found that force stimuli upregulated CBS expression in some $CD90^-$ fibroblasts because CBS activity reaches maximal during proliferation and is reduced 2- to 5-fold when cells become quiescent (Maclean et al. 2002). Although we cannot deny the participation of fibroblast-expressed CBS in OTM, our in vivo and in vitro data suggest at least that PDLSC-produced H_2S plays an important function in osteoclast differentiation, thus contributing to OTM. The role of other differentiated cells in H_2S production will be investigated further in our next study.

H_2S was recently found as an essential gasotransmitter in mammalian cells (Gadalla and Snyder 2010; Vandiver and Snyder 2012). Nitric oxide, a defined gasotransmitter, is found to increase and modulate bone remodeling during OTM

(Shirazi et al. 2002). Which role does H_2S play in OTM? In this study, we showed that force application increased endogenous H_2S production in PDL. H_2S exerted its chemoattractant effect on macrophages and induced osteoclastogenesis to control OTM. This result is consistent with those of previous reports that H_2S tends to promote RANKL-induced osteoclast differentiation in vitro (Itou et al. 2014) and induce osteoclast differentiation in vivo (Irie et al. 2009). However, it was also reported that H_2S would inhibit nicotine and lipopolysaccharide-induced osteoclast differentiation (Lee et al. 2013). This discrepancy should be attributed to the different states or micro-environments in which the osteoclasts/osteoclast progenitor cells stay before H_2S treatment. Although the signaling of H_2S in osteoclasts has been noted in previous studies, such as Ca^{2+} influx, TLR4 signaling, or antioxidant effect, the exact mechanism needs to be further clarified (Huh et al. 2006; Irie et al. 2012; Itou et al. 2014).

H_2S may also influence osteogenic cells in addition to its effects on macrophages and osteoclasts during OTM. It has been recently shown that H_2S maintains the self-renewal and osteogenic differentiation of bone marrow MSCs and PDLSCs (Liu et al. 2014; Su et al. 2015), which serve as the main source of osteoblasts in PDL during OTM. Consistently, the results in our study also revealed that H_2S could regulate osteoblast activity during OTM. The increase of endogenous H_2S enhanced both osteoclastic activity and osteogenesis, while a decrease of endogenous H_2S repressed both. Therefore, the combined effects of H_2S on both bone resorption and osteogenesis indicate that H_2S promotes alveolar bone turnover during OTM.

MSCs have both autocrine and paracrine effects on bone remodeling and regeneration (Eom et al. 2014; Li et al. 2016). Cell-specific paracrine actions have been reported between osteoblasts, osteoclasts, and osteocytes (Sims 2016). These paracrine actions control bone remodeling (Sims 2016), as well as the interactions between PDLSCs and osteoclast precursors (Bloemen et al. 2010). In this study, we found that PDLSCs produced H_2S to affect MCP-1 secretion and the RANKL/OPG ratio. This indicates that H_2S mediated autocrine activity of PDLSCs during OTM. Furthermore, our coculture experiments showed that H_2S promoted macrophage migration and osteoclast differentiation by inducing MCP-1 secretion and shifting the RANKL/OPG ratio in PDLSCs. Therefore, H_2S served as both autocrine and paracrine mediators among paradental cells.

In conclusion, force-induced endogenous H_2S by PDLSCs promotes OTM. H_2S posed its effects on osteoclastic activity in alveolar bone remodeling. To our knowledge, this study is the first to show that H_2S, a gaseous mediator, induced by force application in periodontal tissues contributes to alveolar bone remodeling and consequent OTM.

Author Contributions

F. Liu, contributed to conception, design, and data acquisition, drafted and critically revised the manuscript; F. Wen, contributed to design, data acquisition, and analysis, drafted the manuscript; D. He, contributed to design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; D. Liu, contributed to design and data analysis, critically revised the manuscript; R. Yang, contributed to conception and data analysis, critically revised the manuscript; X. Wang, contributed to conception, data analysis, and interpretation, critically revised the manuscript; Y. Yan, contributed to acquisition and analysis, critically revised the manuscript; Y. Liu, contributed to conception, critically revised the manuscript; X. Kou, contributed to conception and design, drafted and critically revised the manuscript; Y. Zhou, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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