



The Triple Functions of D2 Silencing in Treatment of Periapical Disease

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Abstract

Introduction: Dental caries is the most widespread chronic infectious disease. Inflammation in pulp tissues caused by dental caries will lead to periapical granulomas, bone erosion, loss of the tooth, and severe pain. Despite numerous efforts in recent studies to develop effective treatments for dental caries, the need for a potent therapy is still urgent. **Methods:** In this study, we applied a gene-based therapy approach by administering recombinant adeno-associated virus (AAV)-mediated *Atp6v0d2* (d2) RNA interference knockdown of d2 gene expression to prevent periapical bone loss and suppress periapical inflammation simultaneously. **Results:** The results showed that d2 depletion is simultaneously capable of reducing bone resorption with 75% protection through reducing osteoclasts, enhancing bone formation by increasing osteon expression, and inhibiting inflammation by decreasing T-cell infiltration. Notably, AAV-mediated gene therapy of d2 knockdown significantly reduced proinflammatory cytokine expression, including tumor necrosis factor α , interferon- γ , interleukin-1 α , and interleukin 6 levels in periapical diseases caused by bacterial infection. Quantitative real-time polymerase chain reaction revealed that d2 knockdown reduced osteoclast-specific functional genes (ie, *Acp5* and *Ctsk*) and increased osteoblast marker genes (ie, *Osx* and *Opg*) in periapical tissues. **Conclusions:** Collectively, our results showed that AAV-mediated d2 depletion in the periapical lesion area can prevent the progression of endodontic disease and bone erosion while significantly reducing the inflammatory over-response. These findings show that the depletion of d2 simultaneously reduces bone resorption, enhances bone formation, and inhibits inflammation caused by periapical diseases and provide significant insights into the potential effectiveness of AAV-sh-d2-mediated d2 silencing gene therapy as a major endodontic treatment. (*J Endod* 2017;43:272–278)

Key Words

Bacteria, bone loss, caries treatment, endodontics, gene therapy, inflammation

Dental caries is one of the most widespread infectious diseases worldwide, and it may lead to endodontic disease, with damaging periapical inflammation, bone erosion, tooth loss, and severe pain (1). An endodontic-periodontal lesion often results in numerous pathogenesis with varying severity and complexity (2, 3). Currently, there is no good treatment to diminish inflammation in endodontic disease. Thus, there is a great need for a novel osteoclast (OC) inhibitor that is highly specific and capable of reducing bone loss and an anti-inflammatory agent that will mitigate the damaging effects of endodontic disease.

It is known that OCs function as primary bone resorption polykaryons (4). Adenosine triphosphatase (ATPase), H⁺ transporting (5, 6), lysosomal 38 kDa, V0 subunit D2, 1 of the 2 related isoforms for this subunit, has been shown to be expressed in various tissues, exclusively in activated OCs (7). We found that d2 is a critical protein of the osteoclast-specific proton pump that mediates extracellular acidification in bone remodeling or bone resorption (7). Also, it was recently shown that knockout of d2 enhances bone formation (8). Furthermore, d2 is a potential osteoimmune gene (9). Interestingly, d2 is highly expressed in dendritic cells, and some speculate that d2 may be a constituent of dendritic cell–specialized vacuolar systems that facilitate antigen-presenting cell function (9, 10). Thus, we pinpointed d2 as a triple regulator and an ideal target of therapeutics for endodontic disease because it is capable of simultaneously reducing bone resorption, enhancing bone formation, and inhibiting inflammation.

Silencing of target genes by adeno-associated virus (AAV) has been proven to be safe and well tolerated in clinical settings (11, 12), which is also consistent with our previous findings (13). In addition, recent studies have reported AAV therapeutic potentials are effective with various doses for long-term treatment (14). AAV can specifically insert a chosen target gene with a high success rate into the genome with nonpathogenic and long-term gene expression. It has also been proven successful in local knockdown, allowing for a promising localized and specific knockdown of single or multiple genes *in vivo* by AAV-mediated vectors (15). Furthermore, our previous

Significance

Atp6v0d2 silencing is proposed to be a novel and effective gene therapy approach in the treatment of endodontic and periapical disease that displays triple function and simultaneously reduces bone resorption, enhances bone formation, and inhibits inflammation caused by periapical diseases.

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investigations showed that lentivirus is a potent gene transfer (16) through which we had successfully applied AAV gene therapy on the periodontitis mouse model (17, 18).

In our current study, we applied a gene-based therapy approach by administering recombinant AAV-mediated Atp6v0d2 (d2) RNA interference. We propose AAV-sh-d2–mediated d2 silencing as a novel and effective triple functional gene therapy in the treatment of endodontic and periapical disease.

Materials and Methods

For complete materials and methods, please see the [Supplemental Materials and Methods section](#) ([Supplemental Materials and Methods section](#) is available online at www.jendodon.com).

Animals

Wild-type BALB/cJ mice 8 weeks old were purchased from Jackson Laboratory, Bar Harbor, ME. The animals were maintained in an animal facility in the School of Medicine, University of Alabama at Birmingham, Birmingham, AL. All animal protocols were approved by the National Institutes of Health and the Institutional Animal Care and Use Committee of the School of Medicine, University of Alabama at Birmingham. Protocol number 131209236 was approved for this study.

Exposure of Pulp and Infection with Bacterial and AAV Vectors

The periapical disease model was established as previously described (13, 19). Mice were anesthetized, and the pulp tissue of the first molars of the mandible were exposed using a ¼ round carbide burr (Osada Electric, Los Angeles, CA) under a surgical microscope. Exposed pulp tissues were then infected with a mixture of 4 common human endodontic pathogens, *Streptococcus intermedius* (American Type Culture Collection [ATCC] 27335, Manassas, VA), *Fusobacterium nucleatum* (ATCC 25586), *Prevotella intermedia* (ATCC 25611), and *Peptostreptococcus micros* (ATCC 33270). The mice were anesthetized again on day 1 and day 3 after the initial pulpal infection, and approximately 3 μ L (2×10^9 packaged genomic particles in phosphate-buffered saline [PBS]) of either AAV-sh-d2 ($n = 15$) or AAV-sh-luc-YFP ($n = 15$) were injected through the apical foramen and into the apical periodontium. In the negative control group (normal), the teeth ($n = 15$) were not treated with the viral vectors.

Sample Harvest, Preparation, and Analysis

CO₂ inhalation was applied to euthanize the animals on day 42 after the first infection. The jaw tissue from the left side was applied to micro-computed tomographic (μ CT) testing and measurement analysis of bone loss. Jaw samples from the right side were prepared for histologic analysis. For RNA and protein extraction, the periapical bone tissues and first molar root were extracted together in a block specimen using a surgical microscope. For RNA extraction, the samples were rinsed with cold $1 \times$ PBS, weighed (3–5 mg/tissue), and immediately put into RNeasy Lysis Buffer (Qiagen, Valencia, CA) overnight at 4°C and then stored at –80°C for further use. For the enzyme-linked immunosorbent assay (ELISA), the samples were rinsed in cold PBS, weighed (3–5 mg/tissue), and immediately frozen at –80°C until use. A detailed description of the radiographic procedures, histologic and immunohistochemistry analysis, ELISA, and real-time quantitative reverse transcriptase polymerase chain reaction was listed in the [Supplemental Materials and Methods section](#) ([Supplemental Materials and Methods section](#) is available online at www.jendodon.com).

Results

AAV-sh-D2 Simultaneously Targeted d2 and Efficiently Knocked Down the Expression of d2

Western blot analysis was applied to confirm AAV transduction, and we found that the expression of d2 in OCs treated with AAV-sh-d2 decreased significantly compared with OCs treated with AAV-sh-luc-YFP (Fig. 1A and B). Fluorescence of cultured cells showed that transduction of OCs with AAV-sh-d2 or AAV-sh-luc-YFP was successful (Fig. 1C, 1st column). Tartrate-resistant acid phosphatase (TRAP) staining showed that TRAP-positive OCs decreased significantly in the AAV-sh-d2 group (Fig. 1C, 2nd column). Compared with the control group, AAV-mediated knockdown of d2 inhibited bone resorption (Fig. 1C, 3rd and 4th columns). The quantification data showed that AAV-sh-d2 can inhibit bone resorption mediated by OCs *in vitro* (Fig. 1D). We further used a fluorescence microscope image to reveal the expression of an enhanced green fluorescent protein in periapical tissues to confirm d2 silencing *in vivo* (Fig. 1E). We also performed immunohistochemistry staining and quantitative polymerase chain reaction analysis to detect d2 expression *in vivo*, and the results of these experiments confirmed that the protein and messenger RNA expression level of d2 was significantly reduced in the AAV-sh-d2 group (Fig. 1F–H). Altogether, we showed that AAV-sh-d2 can inhibit d2 expression efficiently both *in vitro* and *in vivo*.

Knockdown of d2 Prevents Periapical Bone Resorption Caused by Infection

We used a model of periapical lesion induction to determine the efficacy of AAV-shRNA-d2 in oral tissues as described in previous studies (13, 17). The remaining bone tissue surrounding the distal root of the mandibular first molar was analyzed by x-ray imaging and μ CT imaging (Fig. 2A). The infected AAV-sh-luc-YFP–treated group showed significant higher bone resorption than that of the normal group, whereas AAV-sh-d2 effectively prevented periapical bone resorption as shown (Fig. 2A). Quantitative analysis was performed of the bone volume (BV) to the tissue volume (TV) of each sample group in which the percentage of BV/TV was found to be lowest in mice with AAV-sh-luc-YFP treatment. Interestingly, the AAV-sh-d2–treated group only showed a slight reduction in BV to TV compared with wild-type (Fig. 2B). In addition, hematoxylin-eosin (H&E) stain showed that monocyte infiltration increased in the AAV-sh-luc-YFP group, and the remaining bone area (*black arrows*) in the lesion area of AAV-sh-d2 was much larger than that presented in the YFP group (Fig. 2C and D).

Depletion of d2 Impaired Bone Resorption and Enhanced Bone Formation

TRAP stain showed that TRAP-positive OCs decreased largely in the AAV-sh-d2 group (Fig. 3A and B). Compared with the AAV-sh-luc-YFP group, knockdown of d2 largely reduced bone resorption, indicating that AAV-sh-d2 affected the major function of OCs (Fig. 2A). A previous study also reported mice deficient of d2 may exhibit increased bone formation (8); thus, we sought to confirm this feature in our knockdown model with alkaline phosphatase (ALP) staining. Our results showed the number of ALP-positive osteoblasts increased significantly in the AAV-sh-d2 group (Fig. 3C and D). Osterix (Osx) was reported to be a crucial element in promoting osteoblast differentiation and normal bone growth (20). Increased Osx expression was detected through immunohistochemistry staining in the AAV-sh-d2 group compared with the normal and AAV-sh-luc-YFP groups (Fig. 3E and F).

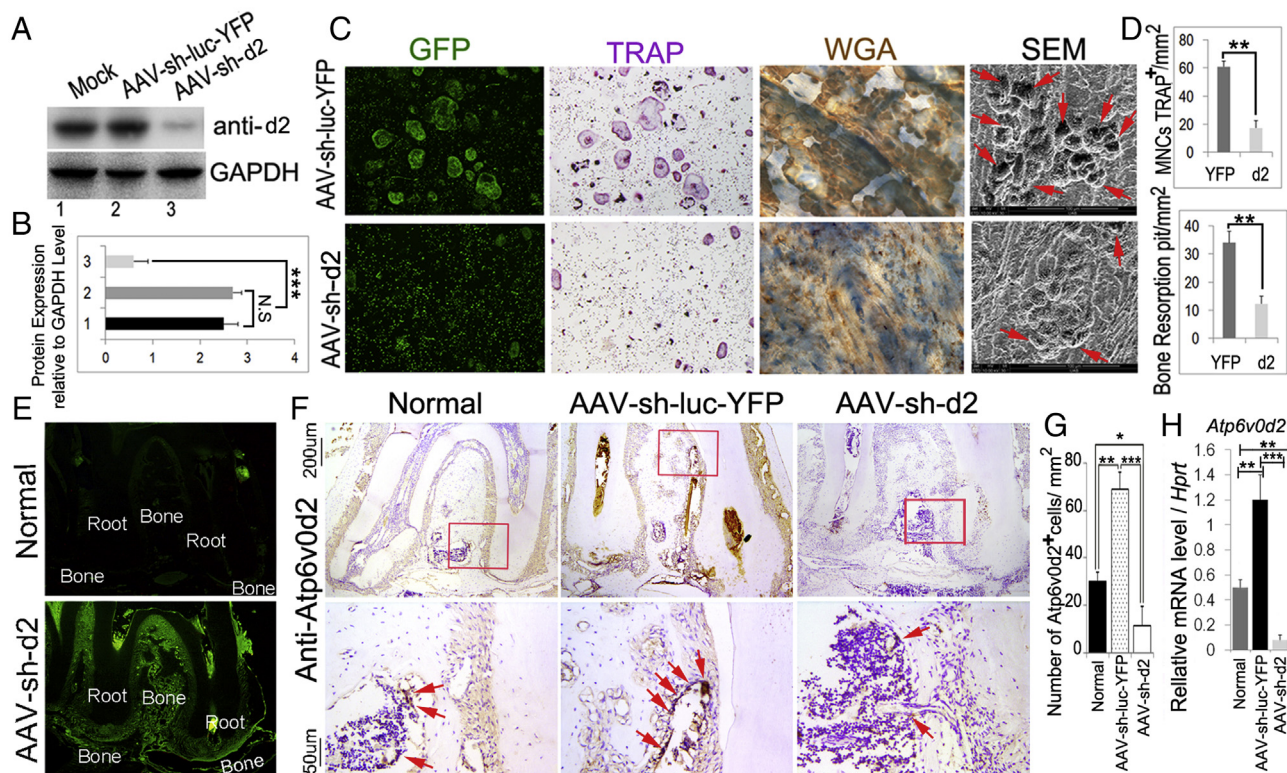


Figure 1. AAV-mediated *Atp6v0d2* knockdown effectively transduced OCs and periapical tissue and decreased the d2 expression *in vitro* and *in vivo*. (A) Western blot of d2 expression in OCs transduced with no vector, AAV-sh-luc-YFP, or AAV-sh-d2. (B) Quantification analysis showed that AAV-sh-d2 significantly reduced the expression of d2 compared with the AAV-sh-luc-YFP group. (C) Fluorescence (1st column) and TRAP stain (2nd column) of the AAV-sh-luc-YFP and AAV-sh-d2 groups. Wheat germ agglutinin (WGA) stain (3rd column) and scanning electron microscopy (4th column) of bone resorption of the AAV-sh-luc-YFP and AAV-sh-d2 groups. The red arrows indicate bone resorption pits on bone slices. (D) Quantification of TRAP stain and scanning electron microscopy. (E) Fluorescence microscopic image revealing local delivery of AAV-sh-d2 resulted in an enhanced green fluorescent protein expression in periapical tissue. (F) Immunohistochemical staining was used to verify the effectiveness of AAV-sh-d2 knockdown d2 in periapical tissues. The red arrows indicate positive staining of d2 in the periapical lesion area. (G) Quantification of d2-positive cells per tissue area (cells/mm²) in normal control, AAV-sh-luc-YFP-treated, and AAV-sh-d2-treated mice. (H) Quantitative real-time polymerase chain reaction result of *Atp6v0d2* expression in periapical tissues of uninfected mice (normal), AAV-sh-luc-YFP-treated, and AAV-sh-d2-treated mice. Expression was normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (*Hprt*). **P* < .05. ***P* < .01. ****P* < .001. *n* = 3, repeated 3 times.

Knockdown of d2 Decreased T-cell Infiltration in the Periapical Lesion Area

To detect monocyte infiltration as well as bone morphology, we performed H&E staining and immunofluorescence staining. H&E staining showed that the monocytes infiltrated greatly in the AAV-sh-luc-YFP group compared with the AAV-sh-d2 group (Fig. 2C, red arrows). Immunofluorescence staining of CD3-positive T cells was applied in infected mice that were treated with AAV-sh-d2 and compared with AAV-sh-luc-YFP and uninfected normal mice (Fig. 4A). CD3-positive cells in the periapical area were subsequently quantified, and a significant decrease of T cells was present in the AAV-sh-d2 group compared with that of the AAV-sh-luc-YFP-treated group (Fig. 4B-D).

The Expression of OC Marker Genes and Cytokines in Lesions Were Decreased because of the Deficiency of d2

The effect of d2 silencing on the levels of tumor necrosis factor α (TNF- α), interferon- γ (IFN- γ), interleukin (IL)-1 α , and IL-6 was evaluated by ELISA (13, 17). All of the detected cytokines in the AAV-sh-d2-treated group were greatly reduced compared with the AAV-sh-luc-YFP group (Fig. 4E). Messenger RNA expression levels showed that when compared with normal, OC marker genes (such as *Acp5* and *Ctsk*)

were increased in the AAV-sh-luc-YFP group yet reduced in the AAV-sh-d2-treated group. *Opg* increased significantly in the AAV-sh-d2 group (Fig. 4F). Interestingly, *Osx* also increased in AAV-sh-d2 slightly, but there was no significant difference compared with the AAV-sh-Luc-YFP-treated group.

Discussion

AAV-mediated d2 Knockdown Reduced the Bone Resorption of the Periapical Disease as a Triple Regulator

As a subunit of *Atp6i* (5, 6), d2 should have a similar function during bone resorption. The d2 subunit is considered to be directly responsible for regulation of the dissociative mechanism of the vacuolar type H + -ATPase (V-ATPase) (21, 22). X-ray and μ CT analyses revealed that knockdown of d2 could efficiently inhibit bone destruction in the mouse model of periapical disease. The data also indicated that AAV-sh-d2 could rescue bone resorption and restore the bone tissue surrounding the root of the tooth. This might be related to the impairment of d2 and further malfunction of OCs. Biochemical analysis has revealed d2 stabilizes V-ATPase complex assembly and increases pump activity, promoting bone resorption (23–25). It is plausible for us to propose that the bone protection effect might be related to the malfunction of OCs after knocking down of d2. A

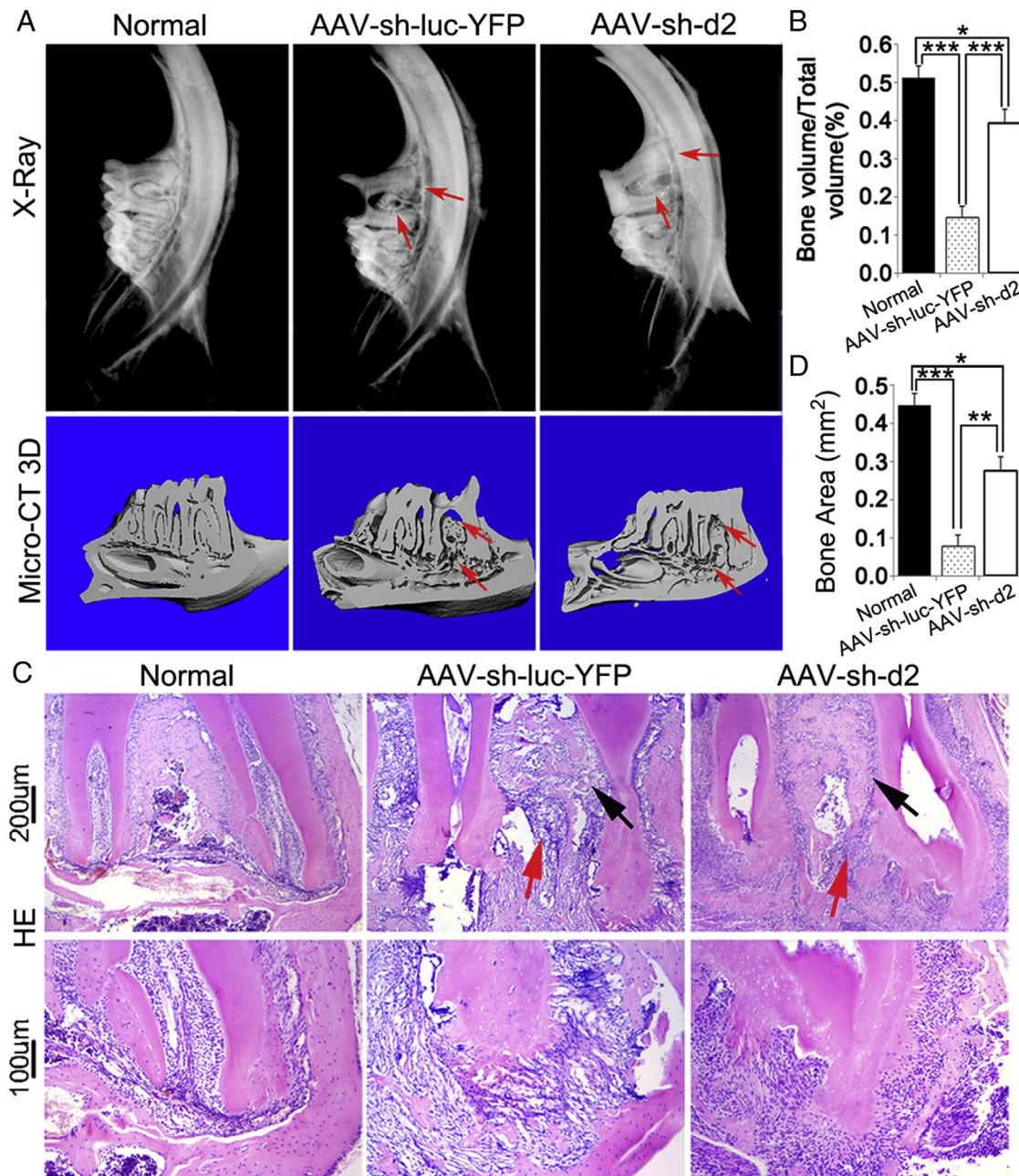


Figure 2. *Atp6v0d2* knockdown reduced bone resorption in the bacteria-stimulated periapical lesion area. (A) Representative figures of X-ray and μ CT analysis from the normal group, the AAV-sh-luc-YFP group, and the AAV-sh-*Atp6v0d2* treatment group. The red arrows indicate the bone resorption area in the X-ray and μ CT images. (B) Quantification of BV/TV from the unaffected control, AAV-sh-luc-YFP-treated, and AAV-sh-d2-treated mice. (C) Representative H&E staining of sections from uninfected mice (normal) or bacterial-infected mice treated with AAV-sh-d2 or AAV-sh-luc-YFP. The inflammatory monocyte infiltration decreased in the AAV-sh-d2 group. The black arrows indicate the bone loss area. The red arrows indicate the infiltrated monocytes. (D) Quantification of the remaining bone area from the unaffected control, AAV-sh-luc-YFP-treated, and AAV-sh-d2-treated mice. * $P < .05$. ** $P < .01$. *** $P < .001$. $n = 3$, repeated 3 times.

previous study reported d2-deficient mice exhibit not only impaired OC fusion but also increased bone formation (8). Therefore, we sought to further examine the effect of d2 ablation on bone formation. An increased number of ALP-positive osteoblasts and enhanced expression of *Osx* were detected in AAV-sh-d2 group. H&E staining showed that monocytes infiltration in the AAV-sh-d2-treated group also decreased significantly, potentially because of its ability to inhibit osteoclast fusion as well as decrease severe inflammatory response in the periapical lesions.

Mechanism Underlined the Regulation of Decreased Immune Cell Infiltration in Periapical Lesions

Monocyte infiltration was inhibited in the AAV-sh-d2 knockdown group because of the decreased number of CD3⁺ T cells in periapical lesions. Weitzmann et al (26) showed that activated T cells could induce osteoclastogenesis via receptor activator of nuclear factor kappa-B ligand-dependent and -independent mechanisms under inflammatory conditions. The previous study showed that OCs depleted of d2 have normal cell differentiation and maturation *in vitro* (7). Interestingly

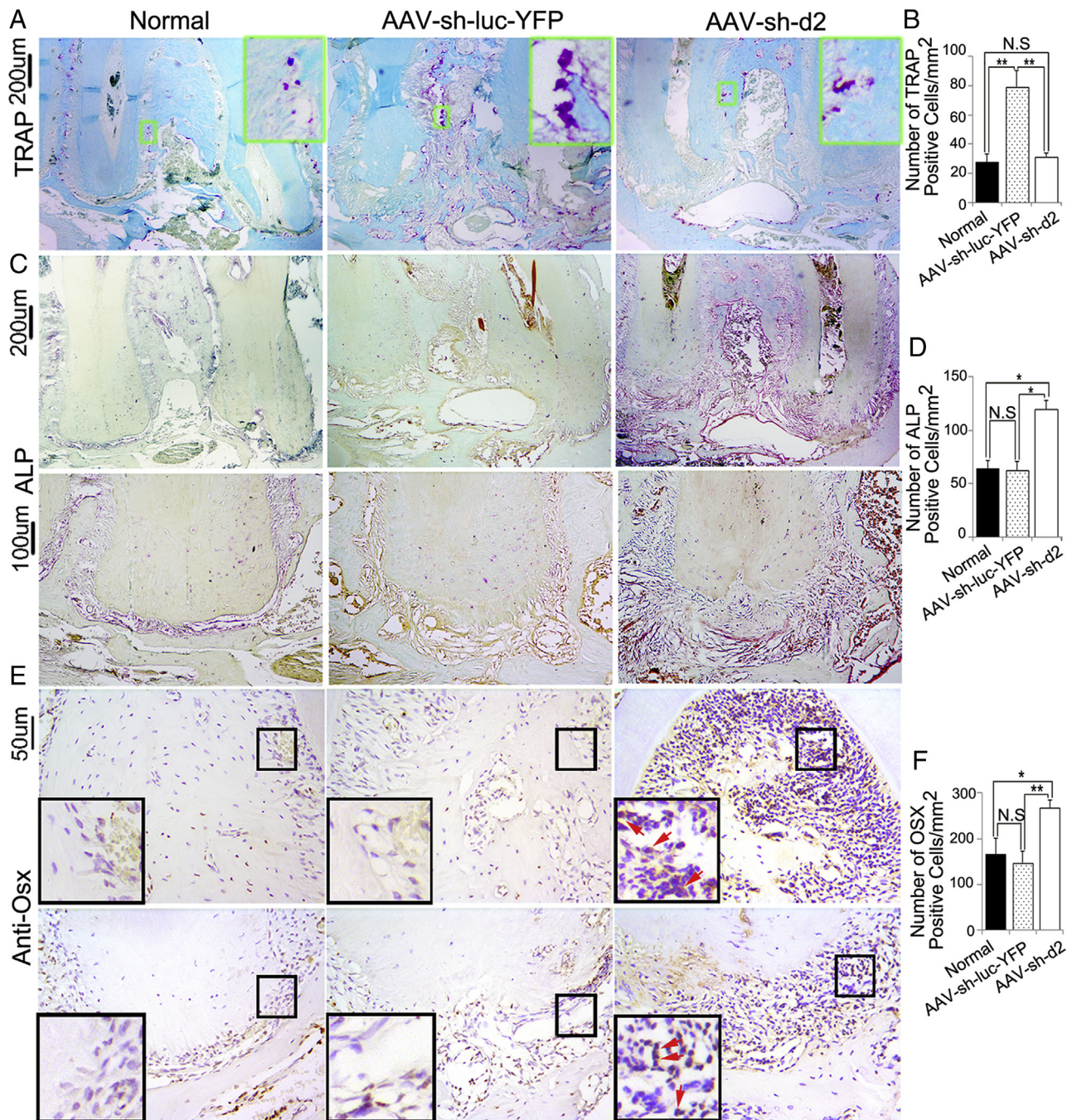


Figure 3. AAV-mediated d2 knockdown decreased active OC, enhanced bone formation, and increased OSX expression in periapical tissues. (A) TRAP staining was used to indicate post-transfection in AAV-sh-d2 knockdown in periapical tissues with a decreased number of OCs. (B) Quantification of OC TRAP-positive cells per tissue area (cells/mm²) in unaffected control, AAV-sh-luc-YFP-treated, and AAV-sh-d2-treated mice. (C) ALP staining was used to indicate AAV-sh-d2 knockdown in periapical tissues significantly induced osteoblast formation. (D) Quantification of ALP-positive cells per tissue area (cells/mm²) in unaffected control, AAV-sh-luc-YFP-treated, and AAV-sh-d2-treated mice. (E) AAV-mediated d2 knockdown increased the *Osx* expression, osteoblast marker gene, in periapical tissues. The red arrows indicate positive staining for *Osx* expression. (F) Quantification of *Osx*-positive cells per tissue area (cells/mm²) in the unaffected control, AAV-sh-luc-YFP-treated, and AAV-sh-d2-treated mice. **P* < .05. ***P* < .01. *n* = 3, repeated 3 times.

in our current *in vivo* study, it is not only that d2 expression level was reduced but also that TRAP-positive cell numbers also decreased significantly. Inflammatory signals mediated by multiple types of immune cells and cytokines have a significant influence over OC differentiation and function (27). Recent studies have shown that OCs themselves express a number of immune receptors and are regulated similarly to macro-

phages and dendritic cells, closely related cells in the innate immune system (28, 29). d2 knockdown also reduced the expression of IL-6, which is secreted by osteoblasts in response to bone resorption and is important for OC differentiation (30). AAV-sh-d2 significantly decreased the expression level of the classic proinflammatory mediators TNF- α and IFN- γ (31). These findings signify a great therapeutic potential of AAV-

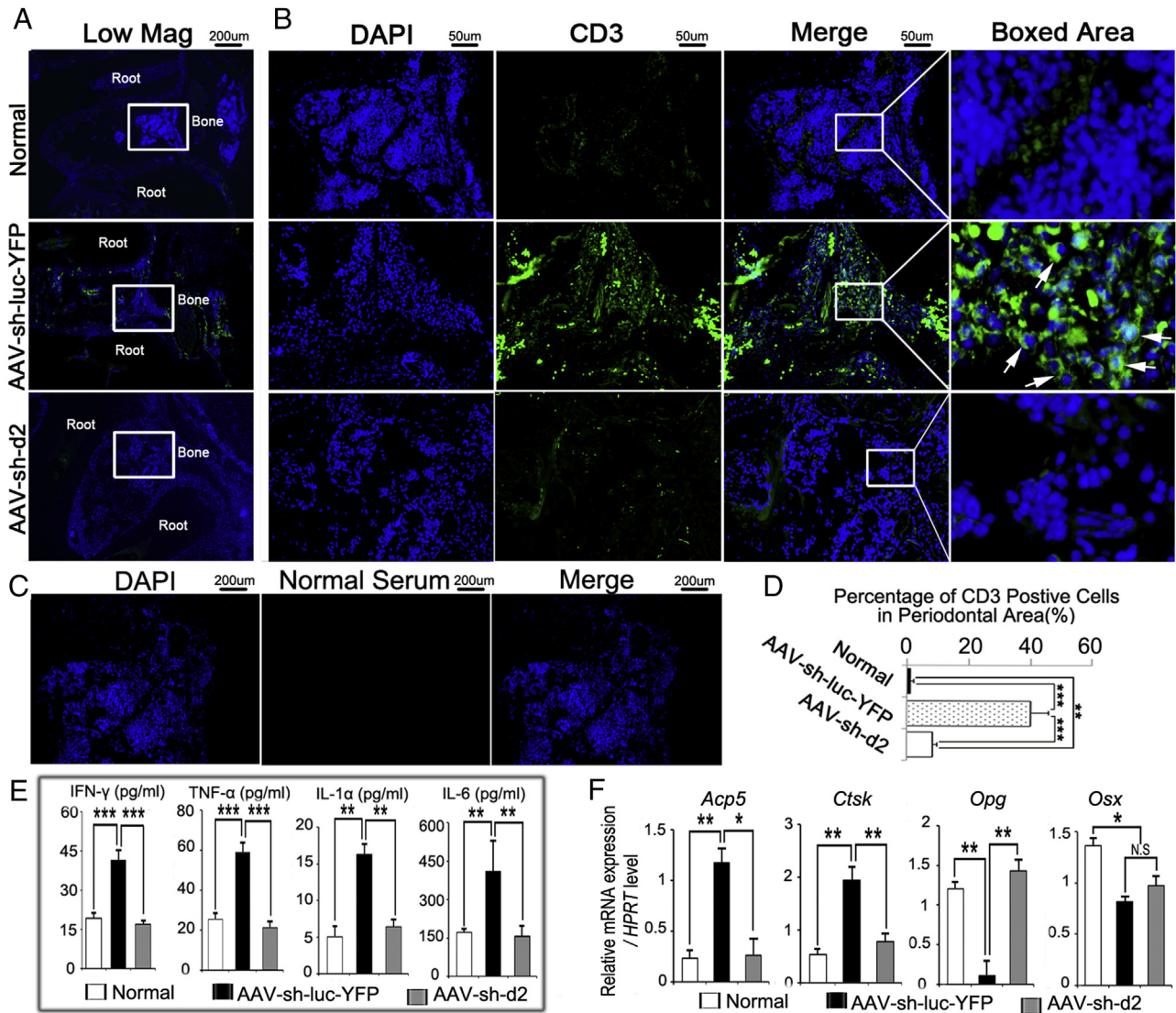


Figure 4. AAV-sh-Atp6v0d2 decreased the number of CD3⁺ T cells and the expression of OC marker genes and cytokines in periapical tissues. (A) Immunofluorescence staining of CD3-positive T cells in periapical tissues. Cell nuclei were labeled using 4', 6-diamidino-2-phenylindole DNA stain (blue). (B) AAV-mediated Atp6v0d2 knockdown decreased the number of CD3⁺ cell in periapical tissues. Columns 1 to 3 of B were the magnified boxed areas in A (Bone area). Column 4 of B was the magnified boxed area in column 3 of B. The white arrows indicate positive staining for CD3 (green), a pan T-cell marker. (C) The negative control for anti-CD3 immunofluorescence staining using normal serum instead of primary antibody. (D) Quantification of CD3⁺ cells in the periapical area (%). Statistical analysis indicates that in the AAV-sh-Atp6v0d2 treatment group, the number of CD3⁺ cell reduced significantly. (E) TNF-α, IFN-γ, IL-1α, and IL-6 levels in the periapical tissues were detected by ELISA. (F) Quantitative real-time polymerase chain reaction of OC-specific functional genes (ie, *Acp5* and *Ctsk*) and osteoblast marker genes (such as *Osx* and *Opg*) in periapical tissues of uninfected mice (normal) or bacterial-infected mice treated with AAV-sh-luc-YFP or with AAV-sh-d2. Expression levels were normalized to *Hprt*. **P* < .05. ***P* < .01. *n* = 3, repeated 3 times.

based gene therapy in alleviating severe bone loss and inflammatory over-response that often associate with endodontic disease.

d2 Is a Promising Target for Apical Periodontitis Treatment

This study uniquely combined the emerging concept of osteoimmunology and the approach of AAV-mediated gene therapy to develop a novel gene therapy approach to target d2 in oral infectious diseases for the first time. d2 knockdown through local administration of AAV-shRNA-d2 features the preclinical stage of AAV-based gene therapy application. Some studies tried to treat periodontal and endodontic diseases with antiresorptive drugs such as bisphosphonates, which interfere with osteoclastic bone resorption (32). However, the inhibition

effect of these drugs on OC activity is often accompanied by the suppression of bone formation, leading to “frozen bone,” which decreases bone quality and increases skeletal fragility (33). Furthermore, many drugs affect V-ATPase function nonspecifically with a wide ranging effect on multiple cells and tissues. For example, bafilomycin, an inhibitor of V-ATPase, affects neurons, gastric, and kidney cells in addition to inhibiting OC function (34, 35). Thus, AAV-sh-d2-mediated d2 silencing is proposed to be a more localized and specific approach and has the potential to dramatically improve the oral health of those struggling with endodontic disease while mitigating the effects of these local infections on various systemic diseases including diabetes, arthritis, postmenopausal osteoporosis, and cardiovascular disease by inhibiting the secondary inflammation involved in those diseases.

In conclusion, we used local gene knockdown based on the AAV system to investigate the potential role and silencing results of d2 in periapical disease using a polymicrobial-induced periapical mouse model. These results indicate that *Atp6v0d2* knockdown reduces messenger RNA and protein expression of genes important for osteoclastic bone resorption and inflammation and enhances the expression of markers important for osteoblast differentiation in periapical tissues. Hence, our study should provide significant insights into the potential effectiveness of AAV-sh-d2-mediated d2 silencing as a novel gene therapy approach in the treatment of endodontic and periapical disease.

Acknowledgments

Jie Pan and Jue Wang contributed equally to this study.

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The authors deny any conflicts of interest related to this study.

Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (doi:10.1016/j.joen.2016.07.014).

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