Effect of Bacterial Biofilm on the Osteogenic Differentiation of Stem Cells of Apical Papilla

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Abstract

Introduction: Although clinical success in regenerative endodontics is substantially high, histological success is limited to finding bone/cementum-like tissue instead of dentin within the canal space. The aims of this study were to investigate (1) the effect of bacterial biofilm on osteogenic gene expression in stem cells of the apical papilla (SCAP) and (2) the effect of bacterial antigens on the functional differentiation of SCAP into a mineralizing phenotype. **Methods:** Using an *ex vivo* organotypic root canal model and an American Association of Endontists-recommended regenerative endodontic procedures, we evaluated SCAP differentiation in the presence and absence of an *Enterococcus faecalis* biofilm. Gene expression analysis for dentinogenic and osteoblastic markers was performed with real-time polymerase chain reaction. The effect of *E. faecalis* antigens on SCAP differentiation into mineralizing cells *in vitro* was evaluated with 2 functional assays: Alizarin Red and alkaline phosphatase activity assays. **Results:** After regenerative endodontic procedures, residual bacteria continued to sustain within the root canal system. SCAP in the presence of *E. faecalis* biofilm significantly downregulated dentinogenic genes such as dentin sialophosphoprotein and upregulated osteoblastic genes such as bone sialoprotein, osteocalcin, distal-less homeobox 5, and runt-related transcription factor 2. *E. faecalis* antigens significantly inhibited SCAP differentiation into a mineralizing phenotype when alizarin red staining and alkaline phosphatase assays were used *in vitro*. **Conclusions:** Current disinfection protocols were ineffective in eliminating bacteria from root tips and the levels of the residual bacterial biofilm, and its by-products, were able to significantly alter osteogenic-differentiation of SCAP. *(J Endod 2017;43:916–922)*

Key Words

Bacterial antigens, biofilm, dentinogenic, osteoblastic, regenerative endodontic procedures, stem cells of apical papilla

Significance

The present study demonstrates that residual biofilm promotes osteoblastic versus dentinogenic gene expression of stem cells of apical papilla and that bacterial antigens significantly inhibit osteogenic differentiation of stem cells of apical papilla. Adequate detoxification of canal system is therefore warranted in regenerative endodontic procedures.

Clinical success with regenerative endodontic procedures (REPs), based on case reports, case series, and randomized clinical trials, appears to be substantially high. Clinical success has been defined as healing of apical periodontitis, gain in root width and length for an immature tooth, and possibly restoration of nerve function (1). These criteria for success address patient-centered as well as clinician-centered outcomes (1). Success as measured by scientist-centered outcomes, such as histology, however, is limited to noninfected cases. Histologic success would be described as regenerating lost or damaged cells of the pulp-dentin complex as well as regeneration of tissues of the pulp-dentin complex, such as fibrous connective tissue, vasculature, innervation, and dentin-like tissue.

Currently, there are few clinical studies that have examined the histology of pulp and dentin tissue after REPs (2–4), although REPs performed in teeth with vital pulps, in either human (5) or animal studies (6–8), show promising results. For example, Shimizu et al (5) reported that an REP-treated tooth with a diagnosis of irreversible pulpitis exhibited flattened odontoblast-like cells lining the predentin. In contrast, the available literature implicates bacteria in altered histologic outcomes. Nearly all histologic analyses of soft tissues after REP demonstrate regeneration of fibrous connective tissue as well as vasculature within the root canal space (2–4), but the majority of hard tissue histologic analyses, barring one report (5), indicate osteoid- or bone-like and/or cementoid- or cementum-like tissue formation in the regenerated section of these teeth (2–4), thus, dentin formation commonly is not observed. Importantly, the majority of these cases had a preoperative diagnosis of pulpal necrosis with either symptomatic apical periodontitis or acute/chronic apical abscess. These diagnoses presumably are associated with a long-standing infection and biofilm in the canal spaces of these teeth. In support of this, several studies in animals have demonstrated that infected necrotic pulps treated with REP resulted in bone and cementum-like hard tissue deposition instead of dentin (9–11).

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Although these studies suggest that bacteria alter the histologic outcomes of REP treatment, there is a large gap in knowledge because this has not been directly tested nor have mechanisms been identified; however, lipopolysaccharide (LPS) from *Porphyromonas gingivalis* reduces the levels of alkaline phosphatase (ALP) and bone sialoprotein (BSP) in dental pulp stem cells (DPSCs) (12, 13) in experiments *in vitro*. Thus, it is possible that residual bacteria and/or their antigens remain in the root canal system and affect the osteogenic potential of stem cells. Moreover, this possibly is highly relevant because REPs are a stem cell–based therapy and most REPs involve little to no mechanical instrumentation. To investigate this possibility, the present study evaluated (1) the effect of a bacterial biofilm on dentinogenic and osteoblastic gene expression in stem cells of the apical papilla (SCAP) in a root tip model and (2) the effect of bacterial antigens on the functional differentiation of SCAP into a mineralizing phenotype in tissue culture.

Methods

A 2 × 2 experimental design was used to determine the effect of a biofilm on SCAP differentiation. Four groups (n = 6/group) of SCAP cells were placed in an organotype root canal model and exposed to either control or *Enterococcus faecalis* biofilm and then treated with either vehicle or osteogenic growth factors. Subsequently, transcription of a subset of genes was measured by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

Growth and Maintenance of *E. faecalis* Cultures

E. faecalis strain ATCC 19433 was grown on brain heart infusion agar (BHI; Sigma, St Louis, MO) for 24 hours to select a colony to inoculate BHI broth medium. The purity of cultures was determined by light microscopy.

Organotype Root Canal Model

Human root tips were prepared as published previously (14). To summarize, 24 extracted human teeth were collected under an approved institutional review board protocol. Gingival and periodontal tissue was removed with a scalpel. These teeth were disinfected with sodium azide (15 mM) followed by 6% sodium hypochlorite (NaOCl). Root tips were then cut (7 mm), and a standardized lumen size of 1.3 mm was created using a #3 Peeso reamer (Dentsply Maillefer, Ballaigues, Switzerland); the roots were soaked in 17% ethylenediaminetetraacetic acid (EDTA) to remove inorganic debris and in 6% NaOCl to remove organic debris. All teeth were coated on the outer surface with nail varnish to prevent bacterial colonization on the outer surface of the root tips. After this, all root tips were gas sterilized.

Biofilm Formation and REP Disinfection

Biofilm formation using *E. faecalis* cultures was done via the protocols of previously published work (15). Sterile BHI media or *E. faecalis* cells (40 μL of 10^9 cells/mL BHI) were loaded into the lumens of the prepared root tips. Sterile BHI or *E. faecalis* in BHI media was replaced every second day for 3 weeks to establish a monospecies biofilm in the bacterial samples. Thereafter, all root tips were treated with a modified American Association of Endodontists (AAE) REP protocol. In brief, all samples were irrigated with 10 mL of 1.5% NaOCl, followed by 5 mL of full-strength sodium thiosulfate and a final flush with 10 mL of 17% EDTA; the inclusion of sodium thiosulfate irrigation is not part of the AAE protocol, but the inactivation of NaOCl is critical in interpreting data because residual NaOCl has detrimental effects on dentin and SCAP (14). All root tips were then dried with sterile paper points and each lumen was loaded with 0.1 mg/mL triple antibiotic paste (TAP; Champs pharmacy, San Antonio, TX) consisting of 1:1:1 ciprofloxacin:metronidazole:minocycline on a w/w basis. An additional 1 mL of TAP (0.1 mg/mL) was added to the wells containing root tips to fully submerge the root tips in TAP.

Although the AAE-recommended guidelines recommend the clinical use of TAP, double antibiotic paste (DAP), or calcium hydroxide ($Ca(OH)_2$), we only tested the antimicrobial efficacy of TAP because of its broader antimicrobial spectrum due to minocycline compared with DAP as well as the antimicrobial inefficacy of $Ca(OH)_2$ against *E. faecalis* (16). Moreover, TAP is the intracanal medicament used most widely in regenerative endodontic case reports and series (1). After incubation with TAP for 14 days, all root tips were irrigated with 10 mL of 17% EDTA and 10 mL of sterile saline followed by drying with paper points. To confirm the presence of bacteria after this REP-based disinfection, sterile Peeso reamers were used to collect dentin shavings from control and *E. faecalis* root tips. These samples were resuspended in sterile phosphate-buffered saline and inoculated onto BHI agar plates.

SCAP and Matrigel Loading

The SCAP cell line RP89 (17) at a density of 2 × 10^4 cells/mL along with Matrigel matrix scaffold (Corning, Corning, NY) in a 1:1 v/v ratio (15 μL each) was loaded into the lumens of the root tips as described previously (14). After formation of a gel-like consistency, enough media with or without osteogenic differentiation supplements was added to submerge the root tips in 24-well plates. A mixture of β-glycerophosphate (10 mM; Sigma) and ascorbic acid (50 μg/mL; Sigma) was used for the osteogenic supplements (18). Media with and without supplements was replaced every 4 days for a 4-week period.

Molecular Analysis

After 4 weeks, total RNA was isolated from SCAP from all root tips. To summarize in brief, RNA lysis buffer was used to wash out SCAP from the lumens of root tips. Thereafter, total RNA was isolated with the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Isolated RNA was then subjected to cDNA synthesis with Superscript III First Strand Synthesis kit (Invitrogen, CA). Next, cDNA was preamplified with the Taqman Custom PreAmp Pool (ThermoFisher Scientific, Waltham, MA) and TaqMan PreAmp Master Mix (Thermo Fisher Scientific). Real-time qRT-PCRs were conducted with the preamplified products using the Taqman Fast Advanced Master Mix (ThermoFisher Scientific) and a customized Taqman gene expression array. This array contained primers that amplified cDNA for a subset of dentinogenic/osteoblastic genes (DSSP [accession no. NM_014208.3], ALP [accession no. AH005272.2], BSP [accession no. J05213.1], osteocalcin [OCN; accession no. M34013.1], distal-less homeobox-5 [DLX-5; accession no. NM_005221.5], and runt-related transcription factor 2 [RUNX2; accession no. NM_001024630.3]). The amplification was conducted on an ABI 7500 Fast Real-Time PCR System (ThermoFisher Scientific). We determined the relative expression of each target gene messenger RNA with the comparative delta-delta cycle threshold method (ΔΔCt), after normalization to the 18S endogenous control, using the control group as the calibrator as reported previously (19).

The next experiment determined the effect of *E. faecalis* antigens on SCAP differentiation. Again, with the use of 4 groups (n = 10–12/group), a 2 × 2 experimental design was used to determine the effect of heat-killed *E. faecalis* (HKEF) on SCAP responses to an osteogenic growth factor cocktail, with mineralization and ALP activity as the outcome measures.

Preparation of HKEF

E. faecalis was grown in BHI broth and a concentration of 10^9 cells/mL was then heated to 80°C for 30 minutes to kill the bacteria as previously described (20). Aliquots of the HKEF were plated onto BHI agar plates to confirm there were no viable bacteria after the heat treatment.

Alizarin Red Assay

The RP89 SCAP cell line at 1×10^4 cells/mL density was cultured in 6-well plates with and without osteogenic media. A mixture of β-glycerophosphate (10 mM; Sigma) and ascorbic acid (50 μg/mL; Sigma) was used for the osteogenic supplements. In addition, one half of the wells were treated with 10^7 cells/mL of HKEF. Both osteogenic media and HKEF were replaced every third day for 4 weeks. Alizarin Red staining and quantification was then performed per the manufacturer's instructions with the Osteogenic Quantitation kit (EMD Millipore, Temecula, CA). Qualitative imaging of Alizarin Red staining was done by bright-field microscopy at 10× magnification using an EVOs-FL inverted microscope (Life Technologies, Waltham, MA). For quantification, in brief, Alizarin Red–stained monolayer of cells and mineral deposits was extracted using 10% acetic acid, centrifuged, and supernatant was used to measure optical density at 405. The optical density of known Alizarin Red concentrations was used to determine the concentration of Alizarin Red/mineral deposits in our test samples.

ALP Activity

The samples for this assay were prepared as described previously for the Alizarin Red assay. After incubation of SCAP with osteogenic media (or vehicle) and HKEF (or media) for 2 weeks, ALP levels were measured from SCAP with the ALP Detection kit (Sigma) per the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± standard error of mean. All *ex vivo* experiments consisted of $n = 6$ different root tips/samples for statistical analyses and all *in vitro* experiments were repeated 4 times with $n = 10$–12/group. Statistical analyses were performed with a 2-way analysis of variance with Tukey's and Bonferroni for correction. $P < .05$ was considered significant.

Results

An *E. faecalis* Biofilm Alters Gene Expression of SCAP Undergoing Osteogenic Differentiation

To evaluate the effects of an active *E. faecalis* biofilm on gene expression by SCAP, we turned to an organotypic *ex vivo* model. Standardized human root segments were either left sterile or allowed to form an *E. faecalis* biofilm. To replicate a common protocol for REPs, all root tips were then treated with an AAE-recommended protocol. After this disinfection process, root segment lumens were enlarged with Peeso #4 and dentin shavings were collected and plated onto BHI agar plates. All of the plates inoculated with dentin shavings collected from *E. faecalis*–infected root segments showed bacterial colony formation by 24 hours of incubation (Fig. 1*B*), whereas plates from the uninfected control group did not (Fig. 1*A*). Thus, despite the disinfection procedure, *E. faecalis*–infected root tips still harbored viable bacteria.

To determine whether the residual bacteria could alter osteogenic gene expression, SCAP were placed into the infected/uninfected root tips and the expression of several osteogenic/dentinogenic genes was evaluated 4 weeks later. As expected, SCAP cultured onto uninfected dentin without biofilms demonstrated significant upregulation of osteogenic/dentinogenic genes such as dentin sialophosphoprotein (DSPP) and ALP (*$P < .05$, ****<0.0001; Fig. 2*A* and *B*); however, the expression of these genes was significantly reduced in SCAP grown in the presence of *E. faecalis* biofilm. Conversely, the expression of BSP, OCN, DLX-5, and RUNX2 (*$P < .05$, **<0.01, ***<0.001; Fig. 2*C*–*F*) remained unchanged in SCAP grown in osteogenic/dentinogenic media in the absence of a biofilm but were significantly upregulated when SCAP were grown in the presence of the *E. faecalis* biofilm.

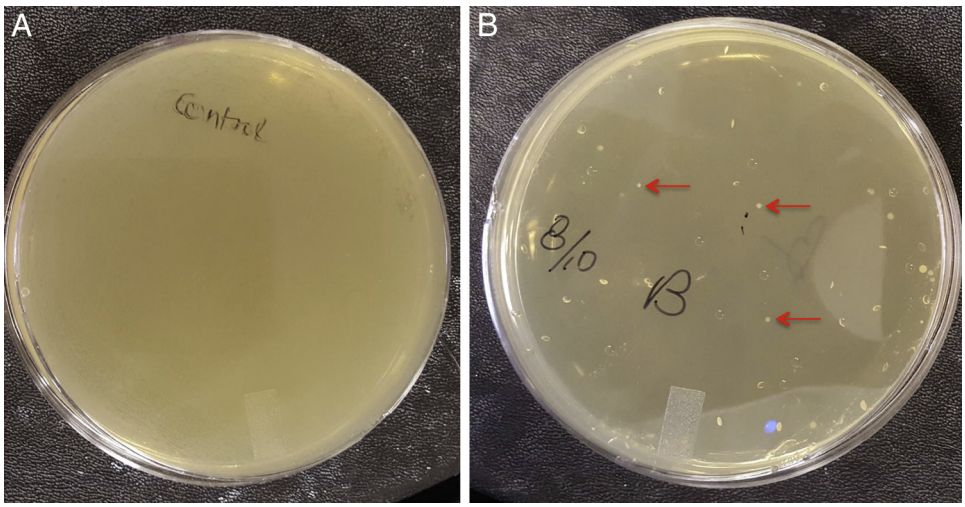

Figure 1. Effect of REP disinfection protocol on *E. faecalis* biofilm. Standardized root segments were incubated with either *E. faecalis* culture (infected) or sterile BHI broth (control). After 3 weeks of biofilm formation, root segments were disinfected with 1.5% NaOCl, 0.1 mg/mL TAP, and 17% EDTA (REP disinfection protocol). (*A*) Representative image of BHI agar plate (of 6) streaked with dentin shavings collected from control root-tips post-REP. (*B*) Representative image of BHI agar plate (of 6) streaked with dentin shavings collected from infected root-tips post-REP. Several *E. faecalis* colonies are marked by red arrows. BHI, brain heart infusion agar; EDTA, ethylenediaminetetraacetic acid; NaOCl, sodium hypochlorite; REP, regenerative endodontic procedure; TAP, triple antibiotic paste.

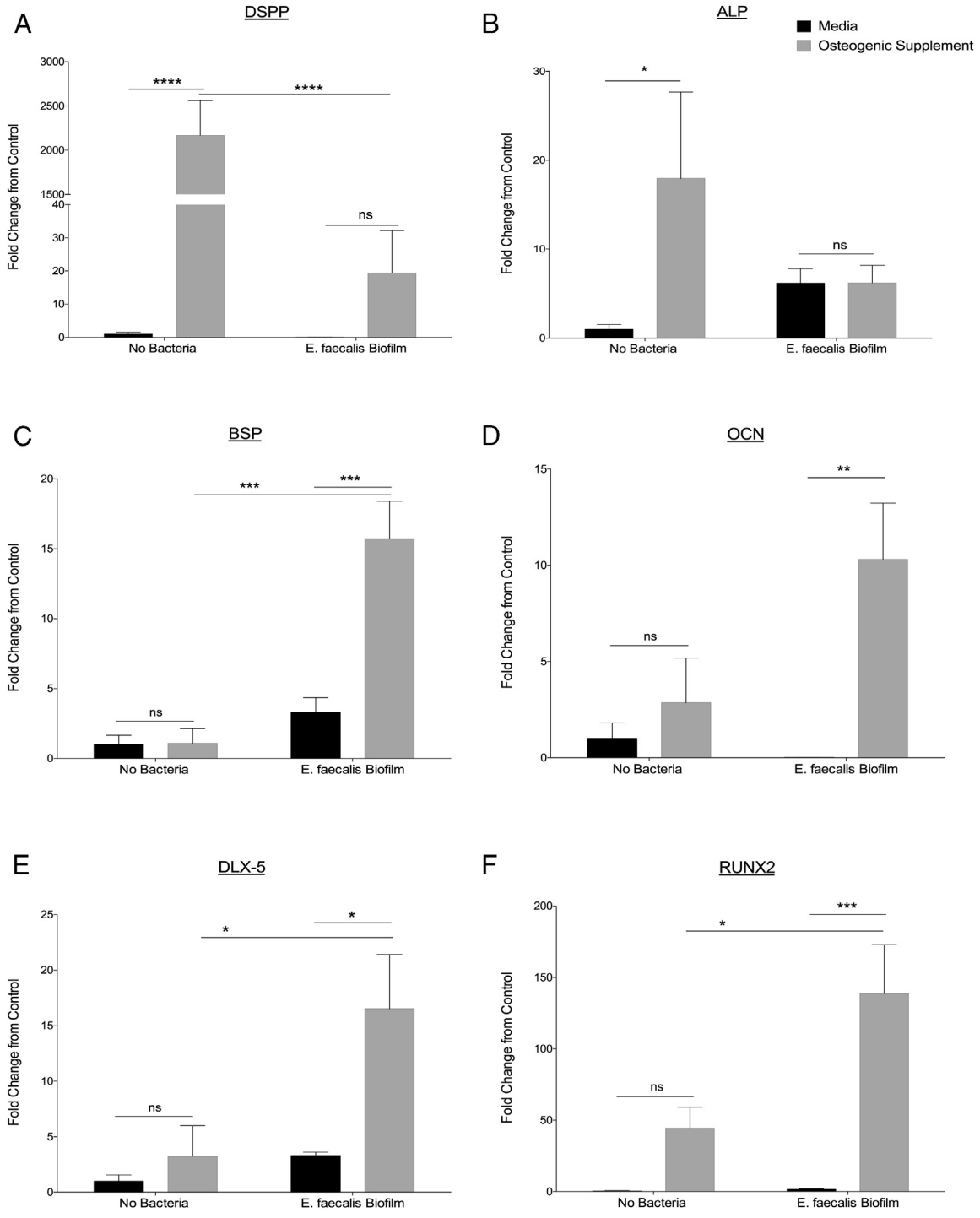

Figure 2. Effect of residual *E. faecalis* biofilm on dentinogenic and osteoblastic gene expression by SCAP. Standardized root segments were incubated with either *E. faecalis* or sterile BHI broth. After 3 weeks of biofilm formation, root segments were disinfected with 1.5% NaOCl, 0.1 mg/mL TAP, and 17% EDTA (REP disinfection protocol). The SCAP, at a density of 2×10^4 cells/mL with Matrigel in a 1:1 ratio, were then loaded into each of the root segments with (*C-F*) or without (*A* and *B*) osteogenic media. Four weeks later, mRNA was isolated from the samples and real-time qRT-PCR analyses were performed with a custom array. The levels of mRNA for each of the 6 genes tested were normalized to the corresponding uninfected control with media alone. The error bars are standard error of the mean for 6 different samples. The asterisks mark significant differences (*$P < .05$, **<0.01, ***<0.001, ****<0.0001) when the indicated samples are compared. ALP, alkaline phosphatase; BHI, brain heart infusion agar; BSP, bone sialoprotein; DLX-5, distal-less homeobox-5; DSPP, dentin sialophosphoprotein; EDTA, ethylenediaminetetraacetic acid; mRNA, messenger RNA; NaOCl, sodium hypochlorite; ns, not statistically significant; OCN, osteocalcin; REP, regenerative endodontic procedure; real-time qRT-PCR, real-time polymerase chain reaction; RUNX2, runt-related transcription factor 2; TAP, triple antibiotic paste; SCAP, stem cells of the apical papilla.

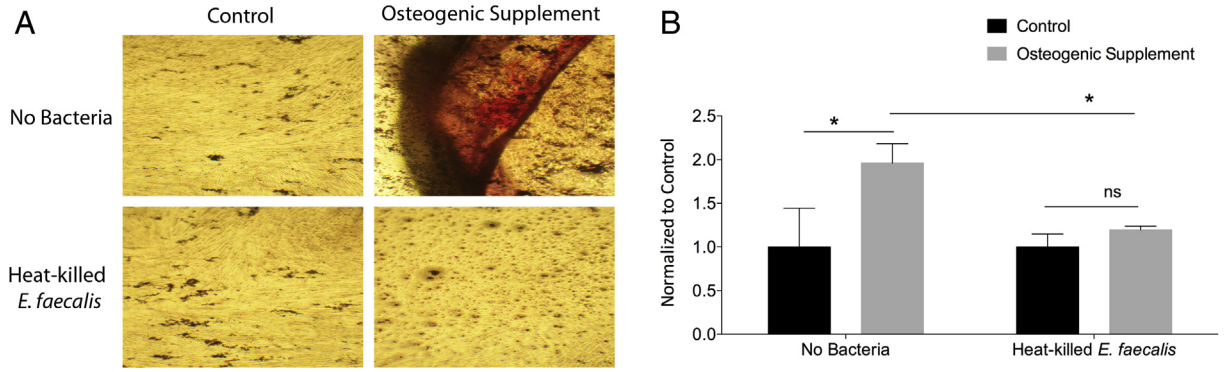

Figure 3. Effect of *E. faecalis* antigens on SCAP differentiation into a mineralizing phenotype using Alizarin Red staining assay. SCAP at a density of 1×10^4 cells/mL were plated in 6-well plates and exposed to control or heat-killed *E. faecalis* and then treated with either vehicle or osteogenic media. After 4 weeks, the samples were assayed for calcium deposits by Alizarin Red staining. (*A*) Representative images of Alizarin Red staining of the indicated samples visualized by bright-field microscopy. (*B*) Another set of samples was assessed for Alizarin Red staining using an Osteogenic Quantitation kit. The level of stain in each sample was normalized to the corresponding no bacteria control. The error bars are standard error of the mean for four experiments. The asterisks mark significant differences (*$P < .05$) when the indicated samples are compared. ns, not statistically significant; SCAP, stem cells of the apical papilla.

E. faecalis Antigens Reduce SCAP Differentiation into a Mineralizing Phenotype

We next evaluated the hypothesis that bacterial antigens could modulate SCAP differentiation into a mineralizing phenotype. To evaluate this possibility, SCAP were exposed to HKEF for 4 weeks under *in vitro* conditions either with or without treatment with osteogenic media. Mineral deposits using the Alizarin Red assay were assessed qualitatively as well as quantitatively. The Alizarin Red assays, both qualitative (Fig. 3*A*) and quantitative (*$P < .05$; Fig. 3*B*), demonstrated that HKEF antigens significantly reduced the ability of SCAP to form mineral deposits, compared with control.

Because ALP is a key enzyme involved in increasing local concentrations of inorganic phosphate essential for mineralization, we also examined, in a separate set of experiments, the activity of ALP in SCAP undergoing osteogenic differentiation. SCAP were incubated with HKEF for 2 weeks under *in vitro* conditions with and without osteogenic media before we measured the levels of ALP released from SCAP. The results show that in presence of osteogenic media, ALP activity was significantly increased in SCAP (*$P < .05$, ***<0.001; Fig. 4), as expected; however, this effect was abolished completely in SCAP exposed to antigens derived from *E. faecalis* (Fig. 4). Thus, both outcomes show that *E. faecalis* antigens can limit the ability of SCAP to differentiate into a mineralized cell type.

Discussion

Unlike acute infections, chronic infections often persist despite strong host defenses. This is true, for example, for teeth infected with a biofilm. It is well accepted that eradicating all bacterial species from root canal spaces is nearly impossible because of the innate anatomy of teeth and the deep penetration of bacteria or their byproducts into dentinal tubules. Although lowering the number of bacteria via chemo-mechanical debridement may be sufficient in healing apical periodontitis in a tooth receiving traditional nonsurgical root canal treatment, the presence of a biofilm in a tooth receiving REP poses unique challenges because of the requirement for proper stem cell proliferation and differentiation. Because REPs are a stem cell–based therapy, it is critical to understand the effects of the microenvironment on the fate of stem cells.

Numerous nonoral studies clearly demonstrate that the presence of a biofilm hinders several aspects of stem cell–mediated healing. In cutaneous nonhealing wounds, >50% of the samples were found to have a biofilm (21). In another study, biofilms were associated with nonosseous unions of long bone fractures (22). Because stem cells are critical players in wound healing by differentiating into desired phenotypes, it is conceivable that a resistant biofilm may hinder differentiation processes and/or the survival of stem cells. In fact, studies evaluating this phenomenon have demonstrated that biofilms cause reduced migratory and differentiation capacities of bone marrow stem cells (23). Moreover, bacterial molecules or metabolites such as LPS or sodium butyrate can alter the differentiation of periodontal ligament stem cells and dental follicle stem cells (12, 13). In addition, DPSCs subjected to high concentrations of LPS from *P. gingivalis* have reduced levels of ALP and BSP gene expression (24). However, there has been no evaluation of the effects of a persistent biofilm or its antigens on dentinogenic/osteogenic differentiation of SCAP.

The first part of this study evaluated the effects of a bacterial biofilm on the dentinogenic/osteoblastic differentiation potential of SCAP. Using a clinically simulative organotypic model, we demonstrated that the

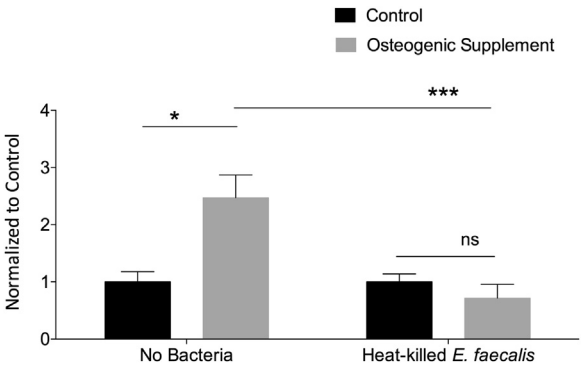

Figure 4. Effect of *E. faecalis* antigens on SCAP differentiation into a mineralizing phenotype using an alkaline phosphatase assay. SCAP at a density of 1×10^4 cells/mL were plated in 6-well plates and exposed to control or heat-killed *E. faecalis* and then treated with either vehicle or osteogenic media. After 2 weeks, the samples were assayed for alkaline phosphatase activity. The activity in each sample was normalized to the corresponding no bacteria control. The error bars are standard error of the mean for 4 experiments. The asterisks mark significant differences (*$P < .05$, ***<0.001) when the indicated samples are compared. SCAP, stem cells of the apical papilla.

presence of an *E. faecalis* biofilm alters the gene expression profile of SCAP. We analyzed a combination of dentinogenic and osteoblastic genes and, for the first time, showed that SCAP grown in osteogenic/dentinogenic conditions are unable to maintain increased levels of dentinogenic genes (eg, DSPP) in the presence of an *E. faecalis* biofilm. Conversely, osteoblastic genes (eg, BSP, OCN, DLX5, and RUNX2) were significantly upregulated in the presence of an *E. faecalis* biofilm. Although several of these genes overlap in expression in dentin and bone, the preponderance of the literature points to the expression of DSPP as largely indicative of odontoblast or odontoblast-like cells (18, 25) and upregulation of genes such as BSP, OCN, DLX5, and RUNX2 as markers for cells of an osteoblastic phenotype (26). Moreover, expression of ALP, an early marker in hydroxyapatite formation (26), also was downregulated in the presence of a biofilm. Although these results represent gene expression at a given time point and *in vivo* studies evaluating further time points are warranted, at present, these data support the hypothesis that a residual biofilm significantly alters SCAP differentiation, resulting in a marked progression toward an osteoblastic phenotype.

Although gene expression profiles are strong indicators of the potential function of stem cells, functional studies are imperative. To this end, we performed 2 assays evaluating the functional differentiation of SCAP into a mineralizing cell type. Heat-killed bacteria were used to evaluate the direct effect of bacterial molecules on the differentiation of SCAP *in vitro*. The results indicate that *E. faecalis* antigens significantly alter the differentiation of SCAP into a mineralizing phenotype. Calcium deposits, as measured by Alizarin Red staining, as well as ALP activity, which is used by cells to make inorganic phosphate, were abolished completely when SCAP differentiation was initiated in the presence of *E. faecalis* antigens. Appropriate concentrations of both calcium and phosphate ions are required for hydroxyapatite crystal formation and elongation.

Taken together, the results of these 2 assays strongly suggest that cellular components of bacteria can alter at least 2 pathways involved in the process of mineralization. Interestingly, various bacterial species have different effects on mesenchymal stem cell differentiation. For example, studies evaluating the effects of gram-negative and gram-positive bacterial antigens demonstrate that although LPS promotes proliferation and shifts differentiation of adipose-derived mesenchymal stem cells into an osteogenic phenotype, lipoteichoic acid has no such effects. Moreover, LPS from *P. gingivalis* has been shown to increase ALP activity in periodontal ligament stem cells and dental follicle stem cells but decrease activity in DPSCs. Collectively, all of our results demonstrate that bacterial biofilms and their antigens significantly alter the differentiation and therefore the functional properties of stem cells. Clearly there is an important, but complex, interaction between bacterial species and host stem cells that is not just species-specific but also stem cell-specific.

Interestingly, SCAP treated with sterile osteogenic media showed a significant upregulation of DSPP, with no statistically significant change in BSP, OCN, DLX5, and RUNX2. Comparing these findings with the functional data suggests that increasing the levels of DSPP alone is capable of promoting biomineralization even under less than significant expression of other osteogenic genes. These results are not surprising, as previous studies have demonstrated that exogenous application of dentin sialoprotein causes dental papilla mesenchymal cells to differentiate into a mineralizing phenotype via an integrin binding receptor, integrin $\beta 6$ (27). In addition, dentin sialoprotein and dentin phosphoprotein have been shown to be essential for the maintenance of transforming-growth factor-β activity in dentin (28).

Our study also highlights a key consideration when evaluating regenerative endodontic procedures. The data with *ex vivo* experiments using an organotypic-infected root canal model clearly demonstrate that despite ample irrigation with 1.5% NaOCl and 17% EDTA as well as incubation of root segments with 0.1 mg/mL TAP, root segments infected with *E. faecalis* continued to harbor viable bacteria. Although *E. faecalis* is not a prime candidate for primary infections in immature teeth, it is indicated as one of the most resistant endodontic pathogens and was therefore used in this study. Although the approach tested here did not use a polymicrobial infection, which is more representative of an infected necrotic tooth, the inability of the disinfectant procedure to eliminate *E. faecalis* clearly demonstrates the suboptimal efficacy of the current protocol. Moreover, the combined effect of a residual polymicrobial infection may likely have a more profound effect on stem cell differentiation compared with a single-species infection. In addition, although our study used sodium thiosulfate to inactivate any residual NaOCl, the lack of this step in the clinic may further alter the stem cell differentiation potential.

A critical characteristic of the current regenerative protocol is to promote the endogenous release of bioactive molecules from dentin matrix (29). The cell-cell interaction, cell-extracellular matrix molecule interaction, as well as cell-growth factor interaction together provide for an ideal environment for stem cells to thrive and function. EDTA used as a final irrigant has been shown to condition dentin, thereby facilitating the release of these key molecules (14, 29). Although the majority of studies evaluating the effects of EDTA on the endogenous release of bioactive molecules use an uninfected *ex vivo* model, no study has evaluated the EDTA-mediated release potential in an infected model. A residual biofilm may significantly diminish the bioavailability or bioactivity of the endogenously present signaling molecules. This, in addition to the direct effects of bacterial molecules on stem cells as shown here, can significantly modulate the fate of stem cell differentiation. Future studies evaluating these effects are warranted.

Recent work evaluating the efficacy of lower concentrations of irrigants and intracanal medicaments demonstrate that although 1.5% NaOCl can kill all of the bacteria in a 3-week-old *E. faecalis* biofilm *ex vivo* (16), a 0.1 mg/mL concentration of the DAP and full-strength $Ca(OH)_2$ were much less effective at reducing bacterial viability (16). In contrast, moderately greater concentrations of TAP or DAP (1 mg/mL or 10 mg/mL) have shown increasing antibacterial efficacy against biofilms (16, 30). Moreover, a study evaluating the antibacterial efficacy of residual intracanal medicaments demonstrates that both TAP and DAP at 1 mg/mL have comparable antibacterial activity as a 1000 mg/mL concentration (paste-like consistency), which represents full-strength TAP/DAP (31). In addition, at 1 mg/mL, both TAP and DAP have residual antibacterial effects up to 7 and 14 days, respectively (31). Although concentrations of TAP and DAP greater than 0.1 mg/mL have significantly more antibacterial efficacy, previous work evaluating various medicament concentrations on stem cell survival suggests significant cell death at concentrations beyond 1 mg/mL (32–34). Moreover, with respect to cell attachment, significantly lower numbers of apical papilla cells were seen to be attached to dentin surfaces at a full-strength TAP concentration compared with 0.39 μg/mL and 100 μg/mL (35).

Although disinfection may be the most critical aspect of any endodontic procedure, a biological balance between disinfection and stem cell survival is imperative in REPs. To this end, *in vitro* as well as *ex vivo* studies using 1 mg/mL TAP or DAP demonstrate >60% survival of SCAP (32, 33). Other intracanal medicaments such as $Ca(OH)_2$ also have demonstrated nondetrimental effects on stem cell survival and cell attachment (32–34). Collectively, these studies emphasize the need for additional studies to provide evidence-based REP guidelines for optimal disinfection and stem cell differentiation.

In conclusion, the current study demonstrates that SCAP differentiation is shifted towards an osteoblastic phenotype in the presence of a bacterial biofilm, as seen by gene expression studies. In addition, bacterial antigens may affect adversely the mineralization of tissues. Further studies using a polymicrobial infected model and alternative strategies of disinfection, such as using a 1 mg/mL concentration of TAP or DAP or root canal detoxification, are warranted.

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

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