Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 578–583

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2012.01469.x

Streptococcus cristatus ArcA interferes with *Porphyromonas gingivalis* pathogenicity in mice

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Xie H, Hong J, Sharma A, Wang B-Y. Streptococcus cristatus *ArcA interferes with* Porphyromonas gingivalis *pathogenicity in mice. J Periodont Res 2012; 47: 578–583. © 2012 John Wiley & Sons A/S*

Background and Objective: Porphyromonas gingivalis has been implicated as one of the major pathogens in chronic periodontitis, an infectious disease affecting the majority of the adult population. We have previously demonstrated that a surface protein, arginine deiminase (ArcA), of *Streptococcus cristatus* represses production of *P. gingivalis* long fimbriae and interrupts the formation of *P. gingivalis* biofilms *in vitro*. Our *in vivo* studies have also shown that the distribution of *P. gingivalis* and *S. cristatus* in human subgingival plaque is negatively correlated. The objective of this study was to determine if *S. cristatus* ArcA inhibits *P. gingivalis* colonization and attenuates its subsequent pathogenesis in alveolar bone loss in the murine oral cavity.

Material and Methods: A wild-type strain of *S. cristatus* (CC5A) and its *arcA* knockout mutant (ArcAE) were used as initial colonizers in the oral cavity of BALB/cByJ mice. Colonization of *P. gingivalis* on the existing *S. cristatus* biofilms was assessed by quantitative PCR, and *P. gingivalis*-induced alveolar bone loss was measured 6 wk after *P. gingivalis* infection.

Results: The presence of *S. cristatus* CC5A, but not its *arcA* mutant, attenuated *P. gingivalis* colonization in the murine oral cavity. In addition, *P. gingivalis*-induced alveolar bone loss was significantly lower in mice initially infected with *S. cristatus* CC5A than in those infected with the *arcA* mutant.

Conclusion: This study provides direct evidence that *S. cristatus* ArcA has an inhibitory effect on *P. gingivalis* colonization, which may in turn attenuate the pathogenicity of *P. gingivalis*.

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Key words: mouse; pathogenesis; *Porphyromonas gingivalis*; *Streptococcus cristatus;* ArcA

Accepted for publication January 16, 2012

Chronic periodontitis is among the most common infectious diseases in humans and affects not only the periodontium but also systemic conditions. Associations have been demonstrated between periodontitis and several systemic diseases such as cardiovascular diseases, preterm labor and respiratory diseases (1–3). It has been generally accepted that the initiation of periodontitis depends on the existence of certain gram-negative species of bacteria, and *Porphyromonas gingivalis* is the predominant species implicated in periodontitis (4,5).

The role of *P. gingivalis* in chronic periodontitis has been studied extensively *in vitro* and *in vivo*. The majority of *P. gingivalis* clinical isolates are fimbriated, especially when isolated from deep periodontal pockets (6,7), and different FimA genotypes have been demonstrated (8–10). The major fimbriae (long fimbriae) of *P. gingivalis*, composed of polymeric FimA subunit proteins, are a well-studied virulence factor contributing to

colonization, biofilm formation, cell invasion, bone resorption and evasion of host defenses (11–19). Thus, reduction of FimA production in *P. gingivalis* has been considered as an attractive strategy for preventing *P. gingivalis* colonization and subsequent periodontal pathogenesis. We previously reported that arginine deiminase (ArcA), a surface protein of *Streptococcus cristatus*, is capable of selectively repressing FimA expression in *P. gingivalis* (20). ArcA was identified as the signaling molecule to which *P. gingivalis* responds by repressing expression of the *fimA* gene and producing the FimA protein. Our recent results also showed that the distribution of *S. cristatus* and *P. gingivalis* in dental plaque in periodontitis patients was negatively correlated (21). These studies suggested that as an early colonizer of dental plaque, *S. cristatus*, may play a beneficial role by blocking *P. gingivalis* accumulation in dental plaque in sufficient numbers to reduce periodontal inflammation.

In the present study, we investigated the role of ArcA in *P. gingivalis* colonization and its pathogenesis in alveolar bone loss using a mouse model. Utilizing a wild-type *S. cristatus* strain (CC5A) and its *arcA* knockout mutant (ArcAE) (22) as initial colonizers, we report here direct evidence that *S. cristatus* ArcA attenuates sequential *P. gingivalis* colonization and subsequent *P. gingivalis*-induced alveolar bone loss *in vivo*.

Material and methods

Bacterial strains and media

S. cristatus CC5A and its *arcA* knockout mutant, ArcAE (erythromycin-resistant) (22), were grown in trypticase peptone broth supplemented with 0.5% glucose at 37°C under aerobic conditions. *P. gingivalis* 33,277 was grown from frozen stocks in trypticase soy broth or on trypticase soy broth blood agar plates supplemented with yeast extract (1 mg/mL), hemin (5 μg/mL) and menadione (1 μg/mL) at 37°C in an anaerobic chamber (85% N_2, 10% H_2, 5% CO_2).

Infection of mice

Specific-pathogen-free BALB/cByJ male mice (Jackson Laboratory, Bar Harbor, ME, USA) were maintained in the Laboratory Animal Facility of the University at Buffalo, State University of New York. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University at Buffalo. The infection timeline and the experimental groups are listed in Table 1. Briefly, 6-wk-old mice (eight mice per group) were treated with kanamycin (Sigma-Aldrich, St Louis, MO, USA) at 1 mg/mL in water *ad libitum* for 7 d, followed by a 3-d antibiotic-free period (23). Mice were infected orally with 2×10^9 colony-forming units (CFUs) of live *S. cristatus* CC5A or its *arcA* knockout mutant (ArcAE); the bacteria were suspended in 50 μL of phosphate-buffered saline (PBS) containing 2% carboxymethyl cellulose (PBS-CMC) and administered via micropipettes, twice a day for 5 d. *P. gingivalis* 33277 cells in late-log phase were pelleted and resuspended in PBS-CMC. Inoculation of *P. gingivalis* (5×10^9 CFUs in 50 μL of PBS-CMC, administered intra-orally by micropipettes; three inoculations were given at 2-d intervals) was carried out 7 d after the last inoculation of *S. cristatus* and was repeated 2 wk after the last *P. gingivalis* inoculation. Control (sham-infected) mice received the antibiotic treatment and the intra-oral inoculation of PBS-CMC without the bacteria. Mice not allowed to eat or drink for 1 h after each inoculation of bacteria.

Bacterial quantitation

The colonization of *S. cristatus* or *P. gingivalis* in the murine oral cavities was examined at 7 or 14 d after the final inoculation of bacteria, respectively (see Table 1). Plaque samples were taken from tooth surfaces and surrounding gingival mucosa using cotton applicators and immersed in 1 mL of Tris–EDTA (TE) buffer (pH 7.5). The suspensions were dispersed by full-speed vortexing for 30 s, and the bacteria were harvested by centrifugation. The samples obtained were boiled for 20 min to release chromosomal DNA.

Table 1. Timeline and experimental groups

	Timeline						
	Days 1–7	Days 8–10	Days 11–16	Day 23	Days 23, 25, 27, 41, 43, 45	Day 41	Day 69
Experimental group	Antibiotics	Antibiotics	*Streptococcus* cristatus infection	qPCR and colony morphology	*Porphyromonas* gingivalis infection	qPCR	Assessment of bone loss
Group 1	Yes	No	CC5A	No	Yes	Yes	Yes
Group 2	Yes	No	ArcAE	No	Yes	Yes	Yes
Group 3	Yes	No	CC5A	Yes	No	No	Yes
Group 4	Yes	No	ArcAE	Yes	No	No	Yes
Group 5	Yes	No	PBS-CMC	Yes	No	Yes	Yes

ArcAE, mutant *Streptococcus cristatus*; CC5A, wild-type *Streptococcus cristatus*; PBS-CMC, phosphate-buffered saline containing 2% carboxymethyl cellulose; qPCR, quantitative PCR.

To minimize experimental error, plaque samples from the oral cavity were collected by a single investigator (JH). The sample volume was adjusted to 50 μL by addition of TE buffer before boiling to release DNA. A fixed volume of 5 μL of DNA per sample was utilized in quantitative PCR (qPCR). *P. gingivalis* and *S. cristatus* cells were enumerated using a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) with *P. gingivalis* species-specific 16S ribosomal DNA (rDNA) gene primers (TGTAGATGACTGATGGTGAAA and ACTGTTAGCAACTACCGATGT) (24) or *S. cristatus* 23S rDNA gene primers (ACTGCAATGTGGACTCAGAATTTAT and TACAGAATCTATTTAAAATACGAGGCTCT). Standards used to determine the numbers of *P. gingivalis* or *S. cristatus* cells were prepared using genomic DNAs from the wild-type strain 33,277 or CC5A (21). A fresh culture of bacteria was serially diluted in PBS and plated to enumerate the CFUs at each dilution. Chromosomal DNA was isolated from the dilutions and a qPCR assay was performed to determine cell numbers.

S. cristatus colonization in the oral cavity was confirmed by colony morphology. The colonization of *S. cristatus* in the murine oral cavities was examined 7 d after the final inoculation of the bacteria. The plaque samples were taken from tooth surfaces and surrounding gingival mucosa using cotton applicators (from three mice per group that had not been sampled for qPCR) and smeared on Mitis Salivarius (MS) agar plates. Blue colonies representing *S. cristatus* were counted after 36–48 h of incubation at 37°C in candle jars.

Alveolar bone loss measurement

To examine alveolar bone loss, the mice were killed on day 69 (see Table 1). The maxillae from the killed mice were removed, autoclaved and mechanically defleshed to remove all the soft tissue. The maxillae were then immersed overnight in 3% hydrogen peroxide and stained with 1% methylene blue. The distance between the cemento–enamel junction (CEJ) and the alveolar bone crest (ABC) were measured for a total of 14 buccal sites on the right and left maxillary molars [three sites on the first molar, two sites on the second molar and another two sites on the third molar, as described by Sharma *et al.* (25)]. Measurement was made under a dissecting microscope with an Aquinto imaging measurement system. Alveolar bone loss in each experimental group was calculated as the average 14-site total CEJ-ABC distance for each group.

Statistical analysis

Analysis of variance and the Student's *t*-test were performed to determine statistically significant differences in colonization and alveolar bone loss among different experimental groups. A p value of < 0.05 was considered significant.

Results

Colonization of *S. cristatus* in the murine oral cavity

To establish the feasibility of *S. cristatus* colonization in the murine oral cavity, wild-type *S. cristatus* CC5A and its *arcA* knockout mutant, ArcAE, were introduced into the murine oral cavities. Quantitation of colonized *S. cristatus* was carried out using qPCR, with *in vitro*-cultured *S. cristatus* CC5A as a standard control. Both the wild-type CC5A and the knockout mutant consistently colonized the murine oral cavity (a positive qPCR result was obtained from every mouse infected; data not shown). There was no significant difference in colonization efficiencies between the two groups [$(13.0 \pm 3.4) \times 10^3$ vs. $(14.2 \pm 2.9) \times 10^3$; $p > 0.05$] (Fig. 1).

Colonization of *S. cristatus* strains in the murine oral cavity was also determined by culturing the bacteria on MS agar plates; streptococcal colonies were observed in the samples retrieved from mice infected with *S. cristatus* CC5A or with its *arcA* mutant, but not from sham-infected mice. This result further confirmed the colonization of *S. cristatus* in the murine oral cavity (Fig. 2). We also confirmed that the streptococcal colonies on MS agar plates were indeed the *S. cristatus* strains, using qPCR with the *S. cristatus* 23S rDNA primers (data not shown).

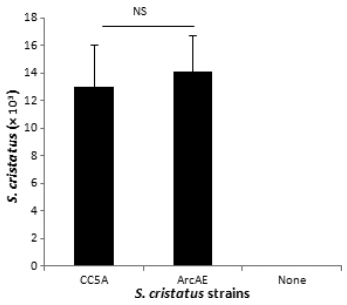

Fig. 1. Colonization of *Streptococcus cristatus* in murine oral cavities. Plaque samples were taken from tooth surfaces and the surrounding gingival mucosa 7 d after the last inoculation of *S. cristatus* into the oral cavities. DNA extracted from these plaque samples [50 μL final volume/sample in Tris–EDTA (TE) buffer] was used for quantitation of *S. cristatus* by quantitative PCR (qPCR) with primers corresponding to 23S ribosomal DNA (rDNA) as probes. Analysis of variance revealed no significant difference in colonization efficiencies between the wild-type CC5A and the knockout mutant ArcAE ($p > 0.05$). Data represent the mean ± standard deviation from five mice per group in experimental groups 3, 4 and 5. NS, No significant difference in colonization.

Streptococcus cristatus ArcA attenuated the colonization of *P. gingivalis* in the murine oral cavity

Formation of dental plaque on human enamel chips has been shown to be a sequential process with streptococci as the dominating initial colonizers (26,27). Based on our earlier observations that ArcA of *S. cristatus* inhibits the formation of *P. gingivalis* biofilms *in vitro*, we examined the role of *S. cristatus* biofilms on *P. gingivalis* colonization in the murine oral cavities. Colonization of *P. gingivalis* was determined using qPCR, with 16S rDNA primers as probes. As shown in Fig. 3A, 2 wk after *P. gingivalis* inoculation, the colonization of *P. gingivalis* in the mice colonized with wild-type *S. cristatus* CC5A was significantly less efficient than that in the mice colonized with the *arcA* mutant (ArcAE) [$(4.48 \pm 2.54) \times 10^3$ vs. $(24.93 \pm 2.08) \times 10^3$; $p = 0.0062$], indicating that ArcA

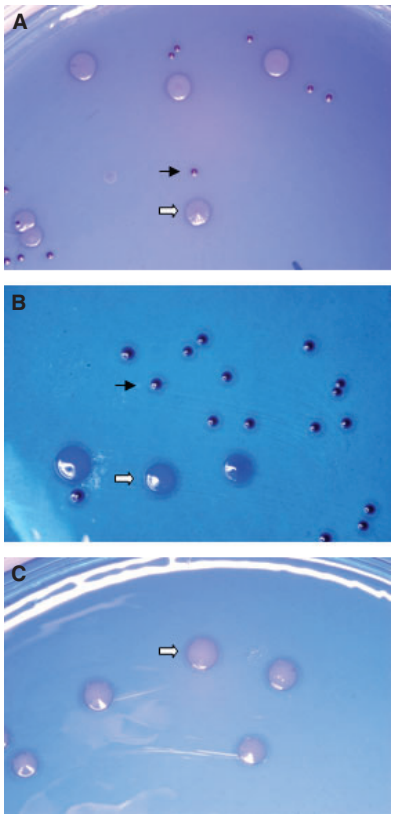

Fig. 2. Streptococcus cristatus morphology on Mitis Salivarius (MS) agar plates. The plaque samples were taken from tooth surfaces and the surrounding gingival mucosa 7 d after the last intra-oral inoculation of *S. cristatus* into the oral cavities. The bacteria on the cotton applicator were streaked onto the MS plates and incubated for 36–48 h anaerobically. The photographs are representative samples of each experimental group (three mice per group sampled from experimental groups 3, 4 and 5). (A) CC5A infected. (B) ArcAE infected. (C) Sham infected. The small blue colonies are colonized *S. cristatus* (black arrows) and the large white colonies are residual endogenous murine bacteria (white arrows).

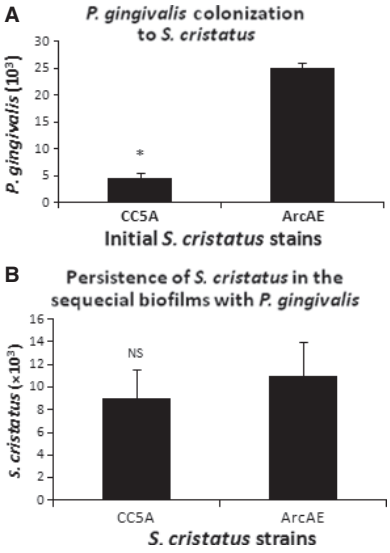

Fig. 3. Sequential *Porphyromonas gingivalis* colonization on pre-existing *Streptococcus cristatus* biofilms in the murine oral cavity. The plaque samples were taken from tooth surfaces and surrounding gingival mucosa 2 wk after inoculation of *P. gingivalis*. DNA extracted from these plaque samples [50 μL final volume/sample in Tris–EDTA (TE) buffer] was used for quantitation of *P. gingivalis* and *S. cristatus* by quantitative PCR (qPCR), with primers corresponding to 16S or 23S ribosomal DNA (rDNA) as probes. Data represent the mean ± standard deviation of eight mice per group. The between-group variance was evaluated by analysis of variance. (A) *P. gingivalis* colonization on *S. cristatus* biofilms. (B) Persistence of *S. cristatus* in the sequential biofilms with *P. gingivalis*. NS, No significant difference. *, Significant difference ($p < 0.01$) in *P. gingivalis* colonization between the two groups.

of *S. cristatus* interferes with *P. gingivalis* colonization in the murine oral cavity.

We also examined the persistence of initial *S. cristatus* colonization in the mixed *S. cristatus–P. gingivalis* biofilms. As shown in Fig. 3B, *S. cristatus* colonization persisted for 4 wk after the last *S. cristatus* inoculation and 2 wk after the sequential *P. gingivalis* inoculation. There was no statistically significant difference in quantity between the wild-type *S. cristatus* CC5A and its *arcA* knockout counterpart [$(8.94 \pm 2.65) \times 10^3$ vs. $(10.93 \pm 3.04) \times 10^3$; $p = 0.2245$].

Our results demonstrated that *S. cristatus* and *P. gingivalis* are able to co-exist in the murine oral cavity, and the expression of ArcA by *S. cristatus* inhibits *P. gingivalis* colonization.

S. cristatus ArcA prevented *P. gingivalis*-induced alveolar bone loss in mice

We next examined *P. gingivalis*-induced alveolar bone loss in the presence of *S. cristatus* CC5A or the *S. cristatus* ArcAE mutant on day 69 (6 wk after *P. gingivalis* infection). As shown in Fig. 4, no significant bone loss was observed in the mice infected only with *S. cristatus* CC5A or *S. cristatus* ArcAE, relative to the sham-infected mice (0.076 ± 0.019 mm for *S. cristatus* CC5A vs. 0.077 ± 0.028 mm for *S. cristatus* ArcAE vs. 0.073 ± 0.018 mm for sham-infected; $p > 0.05$). *P. gingivalis*-induced alveolar bone loss was significantly more prominent in the *S. cristatus* ArcAE group than in the comparable CC5A group (0.120 ± 0.037 mm for *S. cristatus* ArcAE vs. 0.092 ± 0.033 mm for *S. cristatus* CC5A; $p = 0.011$). These results indicate that ArcA serves as a potent inhibiter for *P. gingivalis* pathogenesis.

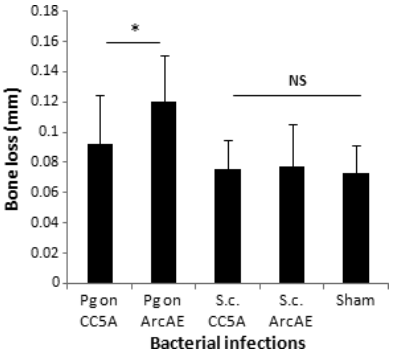

Fig. 4. Effect of *Streptococcus cristatus* ArcA on *Porphyromonas gingivalis*-induced alveolar bone loss in BALB/cByJ mice. Data represent the mean ± standard deviation of eight mice per group. Alveolar bone loss was calculated as the average of 14-site (three sites on the first molar, two sites on the second molar and two sites on the third molar, at both right and left sides of the maxilla) total cemento–enamel junction and alveolar bone crest (CEJ-ABC) distances for each group. NS, no significant difference in alveolar bone loss when compared with the sham-infected group. *, Significant difference ($p < 0.05$) in alveolar bone loss between the two *P. gingivalis*-infected groups. S.c., *S. cristatus;* Pg, *P. gingivalis*.

Discussion

Dental plaque formation is generally programmed to begin with an initial colonization by gram-positive aerobic bacteria, followed by colonization with

a succession of gram-negative anaerobic bacteria. The transition from commensal bacterial accumulation to periodontopathic plaque involves the colonization of certain species of gram-negative anaerobic bacteria such as *P. gingivalis*. Evidence is accumulating that some early colonizers of dental plaque provide a favorable environment for *P. gingivalis*, at least with regard to attachment sites and lower oxygen tension. This, in turn, facilitates retention and multiplication of *P. gingivalis*, ultimately leading to the progression of adult periodontitis (14). Although less well understood, antagonistic relationships have been reported between and among oral bacteria. *P. gingivalis*, for example, does not grow with *Streptococcus oralis* in two-species biofilms (28) and does not co-aggregate with *Streptococcus mutans* (29). We have reported earlier that *S. cristatus* represses expression of *fimA* in *P. gingivalis* and attenuates bacterial colonization *in vitro* (22,30). The present study was designed to test our hypothesis that *S. cristatus* ArcA interferes with *P. gingivalis* colonization in the murine oral cavity and attenuates the subsequent *P. gingivalis*-induced alveolar bone loss, an indicator of *P. gingivalis* pathogenesis in periodontitis.

P. gingivalis-induced alveolar bone loss in rodent models has been widely accepted as a valid model for *P. gingivalis* pathogenicity, which is critical for periodontitis. Furthermore, studies utilizing these rodent models have indicated that *P. gingivalis*-induced alveolar bone loss depends on its expression of FimA protein (17,18,31). These studies have shown that isogenic mutants lacking FimA or its associated minor proteins are less virulent and induce significantly less alveolar bone loss relative to the wild-type bacterium. As we have previously demonstrated that ArcA of *S. cristatus* represses *fimA* gene expression in *P. gingivalis in vitro* (20,22,30), in this study we intended to determine if biofilms of *S. cristatus* CC5A, relative to those of an isogenic mutant lacking ArcA, would interfere with the pathogenesis of *P. gingivalis* in the murine oral cavity.

S. cristatus is a component of earlier colonizers in human dental plaque and has been detected in the 8-h biofilms on freshly cleaned enamel chips (26). In this study, we demonstrated that *S. cristatus*, like some other oral streptococci (32–34), could colonize the rodent oral cavity. The presence of *S. cristatus* in the oral cavity could be detected as monobiofilms (Fig. 1) and also as heterotypic biofilms with *P. gingivalis* (Fig. 3B).

The objective of this study was to determine if *S. cristatus* ArcA attenuates *P. gingivalis* colonization and subsequent *P. gingivalis*-induced alveolar bone loss *in vivo*. Therefore, *P. gingivalis* infection was carried out in the oral cavity with existing biofilms of wild-type *S. cristatus* CC5A or its *arcA* knockout mutant. This experimental design allows us to test our hypothesis because the only difference between the two groups was the presence or absence of *S. cristatus* ArcA. As demonstrated in Fig. 4, the wild-type CC5A strain expressing ArcA is more potent than the ArcA-deficient mutant in blocking *P. gingivalis*-induced alveolar bone loss. As *P. gingivalis* was not recovered, 2-wk postinfection, from the group infected with *P. gingivalis* alone, in contrast to the *S. cristatus*-colonized groups where *P. gingivalis* was recovered persistently, the group infected with *P. gingivalis* alone was considered an inappropriate control for the study and was excluded. However, in light of our findings, it would be interesting to compare the group infected with *P. gingivalis* alone with the *S. cristatus* precolonized groups in future bone-loss experiments.

Interspecies interactions play important roles in the initiation and development of oral infectious diseases as these can affect the pathogenicity of a particular pathogen. Our results provide direct evidence that *S. cristatus* ArcA attenuates *P. gingivalis* biofilm formation (Fig. 3A) and subsequent pathogenesis (as indicated by *P. gingivalis*-induced alveolar bone loss) *in vivo* (Fig. 4). The presence of *S. cristatus* CC5A did not completely eliminate *P. gingivalis* colonization and *P. gingivalis*-induced alveolar bone loss in mice. However, both the quantity of *P. gingivalis* colonized and the degree of *P. gingivalis*-induced bone loss were significantly lower in the *S. cristatus* CC5A-infected group than in the corresponding *S. cristatus* ArcAE-infected mice. Further *in vivo* studies are warranted to determine if purified *S. cristatus* ArcA or a synthetic ArcA functional domain attenuates *P. gingivalis* pathogenesis, as they did in *P. gingivalis* biofilm formation *in vitro* (20,30).

Acknowledgements

This study was supported in part by grants NIH NIDCR DE017708 to BYW and NCRR 1U54RR026140 and UL1RR024975 to HX. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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