Title

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Abstract

Oral mucosa provides the first line of defense against a diverse array of environmental and microbial irritants by forming the barrier of epithelial cells interconnected by multiprotein tight junctions (TJ), adherens junctions, desmosomes, and gap junction complexes. Grainyhead-like 2 (GRHL2), an epithelial-specific transcription factor, may play a role in the formation of the mucosal epithelial barrier, as it regulates the expression of the junction proteins. The current study investigated the role of GRHL2 in the *Porphyromonas gingivalis* (*Pg*)–induced impairment of epithelial barrier functions. Exposure of human oral keratinocytes (HOK-16B and OKF6 cells) to *Pg* or *Pg*-derived lipopolysaccharides (*Pg* LPSs) led to rapid loss of endogenous GRHL2 and the junction proteins (e.g., zonula occludens, E-cadherin, claudins, and occludin). GRHL2 directly regulated the expression levels of the junction proteins and the epithelial permeability for small molecules (e.g., dextrans and *Pg* bacteria). To explore the functional role of GRHL2 in oral mucosal barrier, we used a *Grhl2* conditional knockout (KO) mouse model, which allows for epithelial tissue-specific *Grhl2* KO in an inducible manner. *Grhl2* KO impaired the expression of the junction proteins at the junctional epithelium and increased the alveolar bone loss in the ligature-induced periodontitis model. Fluorescence in situ hybridization revealed increased epithelial penetration of oral bacteria in *Grhl2* KO mice compared with the wild-type mice. Also, blood loadings of oral bacteria (e.g., *Bacteroides, Bacillus, Firmicutes*, β-*proteobacteria*, and *Spirochetes*) were significantly elevated in *Grhl2* KO mice compared to the wild-type littermates. These data indicate that *Pg* bacteria may enhance paracellular penetration through oral mucosa in part by targeting the expression of GRHL2 in the oral epithelial cells, which then impairs the epithelial barrier by inhibition of junction protein expression, resulting in increased alveolar tissue destruction and systemic bacteremia.

Keywords: oral mucosa, periodontal diseases, tight junctions, lipopolysaccharides, cell adhesion, gene knockout techniques

Introduction

Oral epithelium provides physical, chemical, and immunological barriers against microbial challenges in the oral cavity (Fujita et al. 2018). Oral epithelial cells are generally interconnected by tight junctions (TJs), adherens junctions, desmosomes, and gap junctions, providing the epithelial barrier (Meyle et al. 1999; Boivin and Schmidt-Ott 2017). Defects in the oral epithelial barrier may increase susceptibility to microbial infection, leading to local tissue destruction and systemic diseases (Nagpal et al. 2015). Hence, understanding the mechanisms that establish the epithelial barrier is important in elucidating the pathogenesis of oral inflammatory diseases linked to bacterial infection, such as periodontal diseases. Literatures indicate that *Grainyhead-like* (*Grhl*) family genes (e.g., *Grhl1, Grhl2*, and *Grhl3*), which determine epithelial phenotype, including cell polarity, motility, and differentiation, also determine epithelial barrier (Wang and Samakovlis 2012; Mlacki et al. 2014; Aue et al. 2015; Sumigray and Lechler 2015). In particular, our laboratory has shown that GRHL2 plays a unique role in the control of epithelial cell proliferation and differentiation, as well as epithelial plasticity, through transcriptional

regulation of a diverse array of target genes, including *p63, E-cadherin (E-cad)*, and *microRNA-200* family (Mehrazarin et al. 2015; Chen et al. 2016). Thus, GRHL2 may be a determinant of

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A supplemental appendix to this article is available online.

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the oral mucosal barrier and be a target of periodontal pathogens to initiate bacterial invasion.

Periodontal disease is a bacterial infectious disease associated with a wide diversity of bacterial pathogens. It initiates with accumulation of dental plaque in the gingival sulcus, which leads to release of microbial virulence factors and toxins (e.g., lipopolysaccharide [LPS]), through oral epithelium to stimulate the inflammatory process and tissue destruction (Ji et al. 2015). Loss of epithelial integrity is linked with microbial penetration into oral epithelial and underlying connective tissues (Ji et al. 2015). Prior studies showed that periodontal pathogens, like *Porphyromonas gingivalis* (*Pg*), abrogate the epithelial barrier in part through proteolytic degradation of the junction proteins (Katz et al. 2000, 2002; Groeger et al. 2010; Groeger and Meyle 2015) or through downregulation of TJ protein expression (Choi et al. 2014). The detailed molecular mechanism by which *Pg* or other invasive oral bacteria abrogates the mucosal barrier still remains unclear.

The current study investigated the mechanisms by which periodontal pathogens evade the oral mucosal barrier to cause tissue destruction. To that end, we used cultured *Pg* bacteria to determine its effects on GRHL2 and the junction protein expression in oral epithelial cells. Our data indicated that *Pg* inoculation induced precipitous loss of GRHL2 and the junction protein expression, which would impair the mucosal barrier effects. To delineate the functional role of GRHL2 in oral mucosal barrier, we used the *Grhl2* conditional knockout (cKO) mouse model (Chen et al. 2018). *Grhl2* knockout (KO) led to reduced expression of the junction proteins in the junctional epithelium and increased alveolar bone loss in the ligature-induced periodontitis model. Interestingly, *Grhl2* KO allowed for increased penetration of oral bacteria and bacterial load in the systemic blood circulation compared to the wild-type (WT) mice. These data suggest that *Pg* bacteria may gain access through the mucosal barrier in part by targeting GRHL2, which is required for the oral mucosal barrier by maintaining the expression of the junction proteins.

Materials and Methods

Cells and Cell Culture

Primary NHKs were established from the oral epithelium of normal mucosa obtained from healthy patients using methods published elsewhere (Kang et al. 2000). NHK, HOK-16B, OKF6, and HOK-SI cells were cultured in keratinocyte growth medium (KGM) (Lonza) with 60 µM Ca^{++} (Kang et al. 2000). To overexpress GRHL2, we used a retroviral vector (LXSN-GRHL2) expressing GRHL2 according to methods described elsewhere (Chen et al. 2016). Endogenous GRHL2 was knocked down in HOK-16B using commercial GRHL2 shRNA vectors (ShGRHL2-1, -2) targeting different *Grhl2* sequences (Origene).

Fn strain (ATCC 23726) was maintained on Columbia agar supplemented with 5% sheep blood or in Columbia broth (CB; Difco) under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) at 37 °C. *Pg* (ATCC 33277) was maintained in the Columbia broth supplemented with hemin at 5 µg/mL and menadione at 1 µg/mL. Thiamphenicol at 5 µg/mL and clindamycin at 1 µg/mL (MP Biomedicals) were used for the selection and maintenance of strains possessing the *catP* and *ermB* determinants, respectively.

Generation of Mouse Periodontitis Model Using GRHL2 cKO Mice

Animal experiments were performed according to the protocol approved by the UCLA Institutional Animal Care and Use Committee. *Grhl2* cKO was achieved with intraperitoneal (IP) administration of tamoxifen (Tmx) (75 mg/kg) in 8-wk-old K14/*Grhl2* cKO mice for 7 d to induce homozygous deletion of *Grhl2* in epithelial tissues, as described in our previous study (Chen et al. 2018). To induce experimental periodontitis, ligatures were placed around the left maxillary second molars using a 5-0 silk suture (Teleflex) with a surgical knot (Abe and Hajishengallis 2013). After 21 d, the mice were sacrificed, and maxillae were collected for µCT and immunohistochemical analyses, as previously described (Williams et al. 2014; Kim et al. 2018). To assess *Pg* bacterial penetration through oral mucosa, *Grhl2* WT, heterozygous, and KO mice were orally inoculated with 1×10^9 *Pg* bacteria once every other day for 6 wk. Whole blood was collected through cardiac puncture, and genomic DNA was extracted to assess *Pg* bacterial loading in blood by PCR.

Detection of Bacteria by FISH

In situ hybridization was performed on formalin-fixed and paraffin-embedded tissue sections using AlexaFluor 488–labeled oligonucleotide probes (Alex488-POGI) specific for *Pg* 5′- CAATA CTCGTATCGCCCGTTATTC-3′ (Sunde et al. 2003). FISH was then performed according to the published methods (Rivera et al. 2013) using deparaffinized tissue sections of oral mucosal tissues with and without *Pg* inoculation. Stained slides were dried overnight and viewed using the Olympus FV101 confocal microscope.

Epithelial Permeability Assay

HOK-16B cells with or without GRHL2 knockdown or HOK-SI cells with or without GRHL2 overexpression were seeded on transwell culture inserts (Merck Millipore) and grown in KGM until confluence. The confluent monolayers were incubated for another 24 h in KGM in the absence or presence of different concentrations of *Pg* LPS (1 µg/mL and 5 µg/mL). Then, 5 µL of 10 mg/mL FITC-dextran (Sigma) was added to the upper compartment of the transwell chamber, and the fluorescence signal in the bottom compartment was determined at various time points using an Omega plate reader (BMG Labtech) at 485/520 nm. We also determined the epithelial permeability of *Pg* through the HOK-16B monolayer with or without GRHL2 knockdown in the transwell chamber system. After *Pg* (multiplicity of infection [MOI] = 100) was inoculated in the upper chamber, 20 µL medium from the bottom chamber was collected for quantitative PCR using the

specific primers for *Pg* to investigate the level of *Pg* (Oppong et al. 2013; Abe-Yutori et al. 2017).

Reagents

The following antibodies were used in this study: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), E-cad, β-cat, and occludin (Santa Cruz Biotech); p-p65 and TNF-α (Cell Signaling Technology); and CLDN-1, -3, and -4 and ZO-1 antibodies (Invitrogen). Alex488-POGI was purchased from Integrated DNA Technologies; anti-GRHL2 antibody and FITC-dextran were from Sigma-Aldrich. *Pg* LPS was purchased from InvivoGen.

Statistical Analysis

Statistical analysis was performed using the Student's *t* test (2-tailed) for the quantitative reverse transcription PCR gene expression, Western blotting, and immunostaining experiments. *P* values <0.05 were considered significant. All data are expressed as mean ± SD. The number of mice in each group was determined by phenotypic changes observed with *Grhl2* deficiency in the cell lines (Kang et al. 2009; Chen et al. 2016).

Results

Exposure of Human Oral Keratinocytes to Pg Inhibits GRHL2 and Intercellular Adhesion Molecule Expression

To investigate the role of GRHL2 in oral mucosal barrier, we first tested the effects of *Pg* inoculation on the expression of GRHL2, adherens complex (E-cad and β-catenin [β-cat]), and TJ proteins (e.g., claudins [CLDNs] and zonula occludens [ZO]). Within hours after bacterial inoculation, the levels of GRHL2, the adherens, and TJ proteins were strongly suppressed in cultured human oral keratinocytes (HOKs) (Fig. 1A). We further tested the effect of *Pg* inoculation on the junction gene expression in the presence or absence of another periodontal pathogen, *Fusobacterium nucleatum* (*Fn*) (Fig. 1B). *GRHL2, E-cad*, and *β-cat* levels as well as the *TJ* gene expression levels were significantly suppressed by *Pg* alone or in combination with *Fn*, but *Fn* inoculation alone elicited mild effects. To determine the kinetics of these protein expression changes exerted by *Pg*, we performed Western blotting within minutes after bacterial inoculation. The suppression of GRHL2 and ZO-1 by *Pg* was detectable rapidly within 15 min of bacterial inoculation, and the suppressive effect progressively increased over the 60-min period (Fig. 1C). This effect was specific to *Pg* because *Fn* inoculation did not provoke any effect on GRHL2 or junction protein levels.

To assess whether the diminution of GRHL2 expression and the junction protein upon *Pg* inoculation was linked to inflammatory signaling, we exposed cells to purified LPS from *Pg* and performed Western blotting. *Pg* LPS exposure readily induced the inflammatory signaling, as evinced by increasing tumor necrosis factor–α (TNF-α) and phosphor-p65 levels, and suppressing the levels of GRHL2 and the junction proteins (Fig. 2A, B). Interestingly, overexpression of GRHL2 ameliorated the inhibitory effects of *Pg* LPS on the expression of GRHL2 and the junction proteins in HOK-SI cells (Fig. 2C). To explore the role of GRHL2 in epithelial barrier function, we assessed the permeability of a small-molecule, fluorescein isothiocyanate (FITC)–conjugated dextran (4 kDa) across the confluent monolayer of HOK-SI cells with or without GRHL2 overexpression. With ectopic expression of GRHL2 in HOK-SI cells, the level of FITC-dextran penetration was markedly decreased, reflecting reduced epithelial permeability (Fig. 2D). Increased FITC-dextran penetration was observed through the control HOK-SI cells exposed to *Pg* LPS, while GRHL2 overexpression inhibited *Pg* LPS-induced epithelial permeability of FITC-dextran. These data indicate that GRHL2 is required for the maintenance of the epithelial barrier and that *Pg* impairs epithelial barrier by targeting GRHL2.

GRHL2 Is Required for Oral Epithelial Barrier Function

The functional role of GRHL2 in the oral epithelial barrier was further explored through GRHL2 knockdown in HOK-16B cells, in which E-cad, CLDN-1, CLDN-3, CLDN-4, OCLN, and ZO-1 were suppressed (Fig. 3A, B). On the contrary, when GRHL2 was overexpressed in normal human keratinocytes (NHKs), gene expression of junction molecules was strongly induced (Fig. 3C, Appendix Fig. 1). Immunofluorescent staining (IFS) revealed that the loss of GRHL2 in HOK-16B cells ameliorated the expression of E-cad, ZO-1, and CLDN-4 (Fig. 3D). Hence, GRHL2 appears to directly regulate the expression of the junction molecules in HOKs. Then the permeability of FITC-dextran through the monolayer of HOK-16B cells with or without GRHL2 knockdown was evaluated. The level of fluorescent signal was markedly increased through the confluent monolayer of HOK-16B cells with GRHL2 knockdown, reflecting increased epithelial permeability. Similarly, increased FITC-dextran penetration was observed through the epithelial cells after exposure to *Pg* LPS (Fig. 3E). We also compared the penetration of *Pg* across the monolayer of oral epithelial cells with or without GRHL2 knockdown by detecting the presence of *Pg* in the bottom compartment of the transwell after inoculation of *Pg* in the upper chamber. With GRHL2 knockdown in HOK-16B cells, *Pg* penetration began to increase in 6 h after *Pg* inoculation and significantly increased by 24 h (Fig. 3F). These data indicate the functional significance of GRHL2 in maintaining the epithelial barrier of oral keratinocytes against small molecules and *Pg* in vitro.

GRHL2 Is Required for the Maintenance of Oral Mucosal Barrier In Vivo

To evaluate the role of GRHL2 in oral mucosal barrier function in vivo, we generated *Grhl2* cKO mice by crossing *Grhl2* floxed (fl/fl) mice with K14-Cre^ERT mice, which allowed
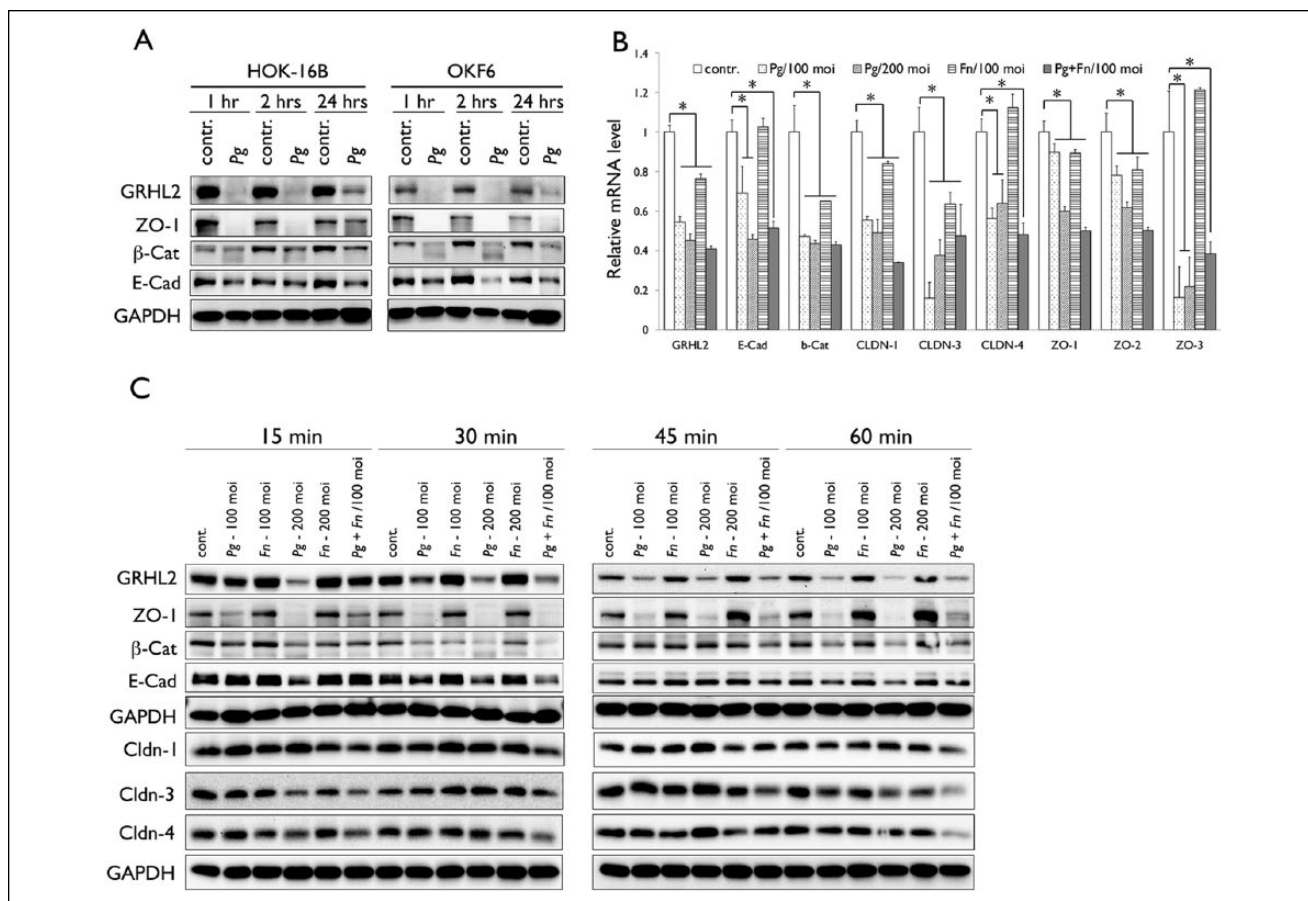
Figure 1. Cellular exposure to *Porphyromonas gingivalis* (*Pg*) suppressed the expression of Grainyhead-like 2 (GRHL2) and junction proteins in cultured oral keratinocytes. (**A**) Western blotting was performed with HOK-16B and OKF6 cells after exposure to *Pg* bacteria to assess the levels of GRHL2 and junction proteins, zonula occludens (ZO)–1, β-catenin (β-cat), and E-cadherin (E-cad). (**B**) Quantitative reverse transcription polymerase chain reaction was performed with HOK-16B exposed to *Pg* (multiplicity of infection [MOI] = 100 or 200) alone or in combination with *Fusobacterium nucleatum* (*Fn*) (MOI = 100) for 24 h to assess the messenger RNA levels of *GRHL2* and various junction molecules (e.g., *E-cad; β-cat;* claudin *(CLDN)–1, -3, -4;* and *ZO-1, -2, -3*). Error bars indicate mean/SD. *Statistically significant (*P* < 0.05) compared with control cells. (**C**) HOK-16B cells were inoculated with *Pg, Fn*, or combined *Pg/Fn* at the indicated MOI and harvested at 15, 30, 45, or 60 min after inoculation. Western blotting was performed for GRHL2, ZO-1, β-cat, E-cad, and claudins. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was probed for loading control.

inducible *Grhl2* KO in epithelial tissues through intraperitoneal (IP) administration of tamoxifen (Tmx). The resulting *Grhl2* KO mice revealed absence of GRHL2 expression and downregulation of the adhesion molecules (e.g., β-cat; CLDN-1, -3, -4; and ZO-1) in the epidermis (Fig. 4A–C). Next, we analyzed if there is difference in the oral microbial loads on the surface of oral mucosa or in the blood between *Grhl2* KO and WT mice. The relative microbial loads on the buccal mucosa or tongue were randomly distributed between the *Grhl2* WT and KO mice (Appendix Fig. 2). However, the microbial loads in the blood were consistently increased in the *Grhl2* KO mice compared to the WT controls (Fig. 4D). These data suggested the possibility that the oral mucosa in *Grhl2* KO mice was more permeable to oral microbiota compared to the WT controls.

We then determined the effect of *Grhl2* KO on alveolar bone loss in the experimental periodontitis model by placement of ligature around maxillary second molars. After 21 d of

ligature placement, we compared the level of alveolar bone loss by micro–computed tomography (μCT) of the dentoalveolar complex and found that *Grhl2* KO led to increased alveolar bone loss compared to the WT littermates (Fig. 5A, B). We also compared the blood level of *Pg* in *Grhl2* WT, hetero (+/–), and KO mice after intraoral inoculation of the bacteria by polymerase chain reaction (PCR) using primers against *Pg*-specific 16S ribosomal DNA (rDNA). Compared to *Grhl2* WT mice, those with *Grhl2* hetero or KO genotype revealed increased *Pg* content in the blood after the ligature placement (Fig. 5C). Also, the blood levels of *Bacteroides* and *Bacillus* were significantly elevated in the *Grhl2* KO mice compared with the WT mice with or without the ligature placement (Fig. 5D). Fluorescence in situ hybridization (FISH) using the *Pg*-specific probe revealed increased bacterial presence in the tongue epithelium from *Grhl2* KO or hetero (+/–) mice inoculated with *Pg* compared to WT mice (Fig. 5E). These results indicate that *Grhl2* KO damaged the oral mucosal barrier and led to increased
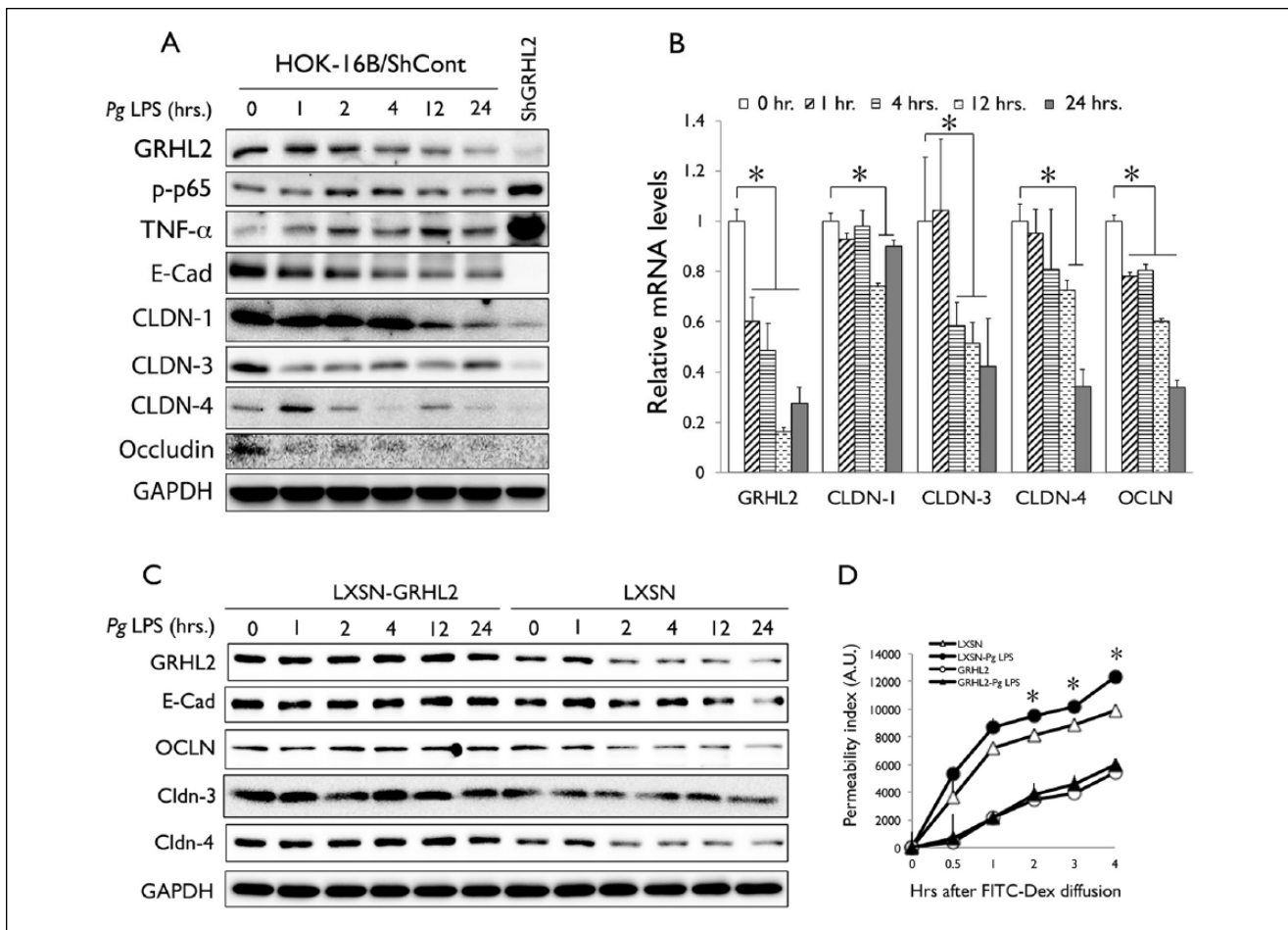
Figure 2. Grainyhead-like 2 (GRHL2) and junction protein levels were lost in oral keratinocytes exposed to *Porphyromonas gingivalis* (*Pg*) lipopolysaccharide (LPS). (**A**) HOK-16B cells were exposed to *Pg* LPS (5 μg/mL) for up to 24 h, and Western blotting was performed for GRHL2, E-cadherin (E-cad), claudin (CLDN)–1, CLDN-3, CLDN-4, and occludin (OCLN) protein levels. We also probed for p-p65 and tumor necrosis factor–α (TNF-α) in the cells to assess the level of inflammatory signaling after *Pg* LPS treatment. HOK-16B cells with *Grhl2* knockdown by ShRNA (HOK-16B/ShGRHL2) were included as the positive control in which endogenous GRHL2 was abrogated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for loading control. (**B**) Quantitative reverse transcription polymerase chain reaction was performed for gene expression levels of *Grhl2* and junction molecule genes (e.g., *CLDN-1, -3, -4* and *OCLN*) in HOK-16B exposed to *Pg* LPS (5 μg/mL) for various time points. (**C**) HOK-SI with GRHL2 overexpression (LXSN-GRHL2) and control cells transfected with empty viral vector (LXSN) were exposed to *Pg* LPS (5 μg/mL) for up to 24 h, and Western blotting was performed for GRHL2, E-cad, CLDN-3, CLDN-4, and OCLN protein levels. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for loading control. (**D**) Permeability of FITC-dextran across a confluent HOK-SI monolayer was determined using the cells with GRHL2 overexpression (LXSN-GRHL2 vs. LXSN) or exposed to *Pg* LPS (5 μg/mL) for 24 h. Error bars indicate mean/SD. *Statistically significant (*P* < 0.05) compared with the control cells.

penetration of *Pg* into the oral mucosa. Immunohistochemical staining revealed reduced protein levels of CLDN-3, ZO-1, and β-cat at the junctional epithelium in the *Grhl2* KO mice compared with the WT littermates (Fig. 5F). Collectively, these data suggest that the loss of the junction protein expression in *Grhl2* KO mice impaired the oral epithelial barrier function, leading to increased bacterial penetration in local tissues and in systemic circulation.

Discussion

The current study elucidated a novel mechanism by which microbial pathogens alter the oral mucosal barrier and enhance inflammatory bone destruction at the alveolar crest. Our data indicated that pathogenic bacteria, such as *Pg*, effectively suppressed the expression of junction proteins in part through downregulation of GRHL2. This was demonstrated in our in vitro experiments in which inoculation of cells with live *Pg* led to precipitous loss of the junction proteins along with loss of GRHL2 level. Interestingly, this suppressive effect was specific to *Pg*, while similar inoculation with *Fn* yielded mild or no effects. Loss of GRHL2 and junction protein levels was also induced by *Pg* LPS. Thus, the suppressive effect of *Pg* on GRHL2 and junction protein may in part be linked to proinflammatory effects as well as other unknown mechanisms elicited by *Pg*.
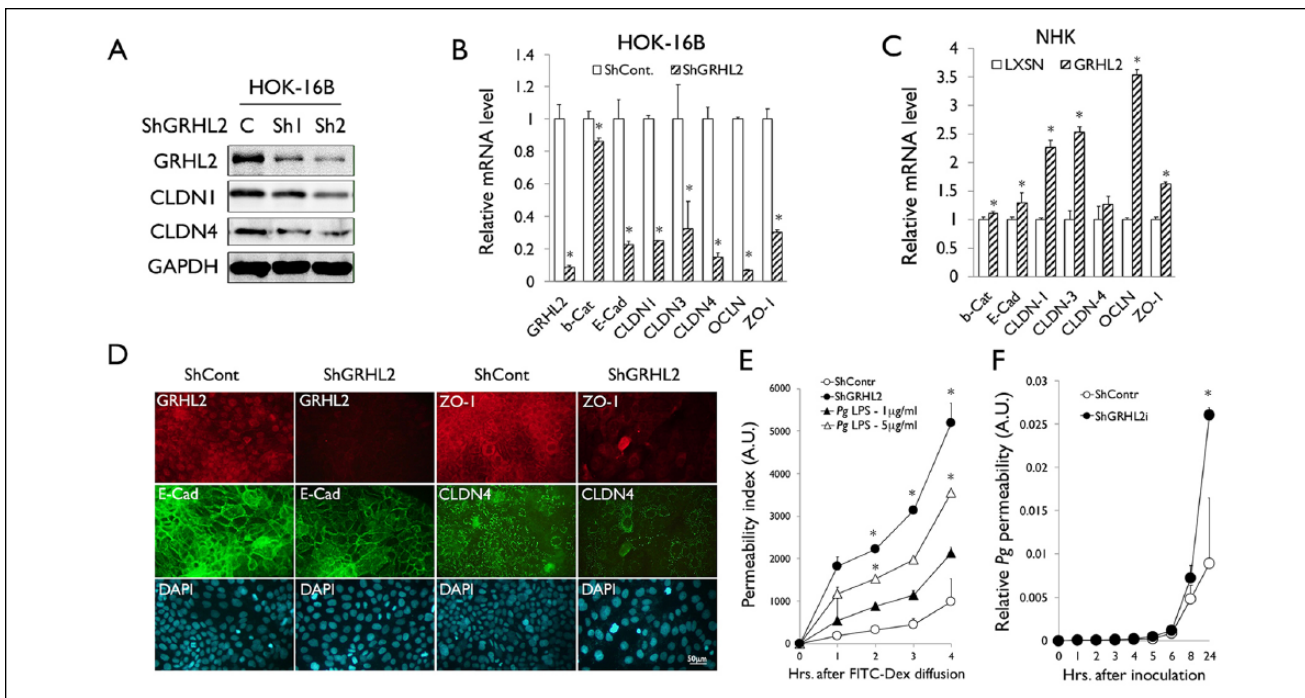
Figure 3. Grainyhead-like 2 (GRHL2) is required for the junction protein expression and the maintenance of epithelial barrier function in oral epithelial cells. (**A**) GRHL2 was knocked down in HOK-16B cells by 2 independent *Grhl2* ShRNAs (Sh1 and Sh2). Western blotting was performed to assess the level of GRHL2, claudin (CLDN)–1, and CLDN-4. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was probed for loading control. (**B**) In HOK-16B cells with GRHL2 knockdown, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to assess the level of *GRHL2, β-catenin (β-cat), E-cadherin (E-cad), CLDN-1, CLDN-3, CLDN-4, occludin (OCLN)*, and *zonula occludens (ZO)–1* messenger RNA (mRNA). (**C**) qRT-PCR was performed with total RNAs isolated from normal human keratinocyte (NHK)/GRHL2 or NHK/LXSN to assess the mRNA levels of junction molecules (*β-cat; E-cad; CLDN-1, -3, -4; OCLN*; and *ZO-1*). (**D**) Immunofluorescent staining was performed to examine GRHL2 and junction protein (E-cad, ZO-1, and CLDN-4) expression in HOK-16B cells with or without GRHL2 knockdown. (**E**) Permeability of FITC-dextran across confluent HOK-16B monolayer was determined using the cells exposed to *Porphyromonas gingivalis* (*Pg*) LPS (1 or 5 μg/mL) for 24 h or the cells with GRHL2 knockdown (ShGRHL2 vs. ShContr). Error bars indicate mean/SD. *Statistically significant ($P < 0.05$) compared with the control cells. (**F**) Permeability of *Pg* across HOK-16B cell monolayer with or without GRHL2 knockdown was determined at different time intervals after *Pg* bacterial inoculation using the transwell culture system. *Pg* bacterial content in the bottom chamber was determined by quantitative polymerase chain reaction using *Pg*-specific primer sets. Error bars indicate mean/SD. *Statistically significant ($P < 0.05$) compared with the control cells.
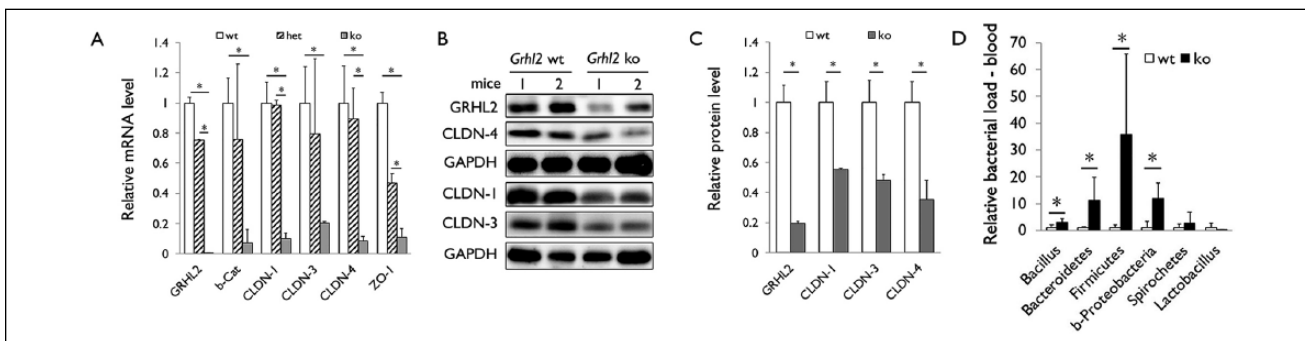

Figure 4. *Grainyhead-like 2* (*Grhl2*) knockout (KO) suppresses the gene expression of junction proteins in vivo. (**A**) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with total RNAs isolated from *Grhl2* wild-type (WT), *Grhl2* heterozygote (+/–), and *Grhl2* KO mice epidermis for junction molecules (e.g., *β-catenin; claudin [CLDN]–1, -3, -4*; and *zonula occludens [ZO]–1*). Data were derived from 3 independent experiments and qRT-PCR assays were performed in triplicates. (**B**) Western blotting was performed with epidermal tissues isolated from *Grhl2* WT and *Grhl2* KO mice for GRHL2 and the junction proteins, CLDN-1, -3, and -4. (**C**) Western blotting signals were quantitated by densitometric analysis and plotted with the mean values for *Grhl2* WT and KO mice groups. (**D**) Quantitative polymerase chain reaction was performed to assess the level of oral bacterial species, *Bacteroidetes, Firmicutes, Spirochetes*, β-*proteobacteria, Bacillus*, and *Lactobacillus*, in the whole blood harvested from *Grhl2* WT and KO mice. Bars indicate mean/SD. *Statistically significant ($P < 0.05$) compared with WT mice.
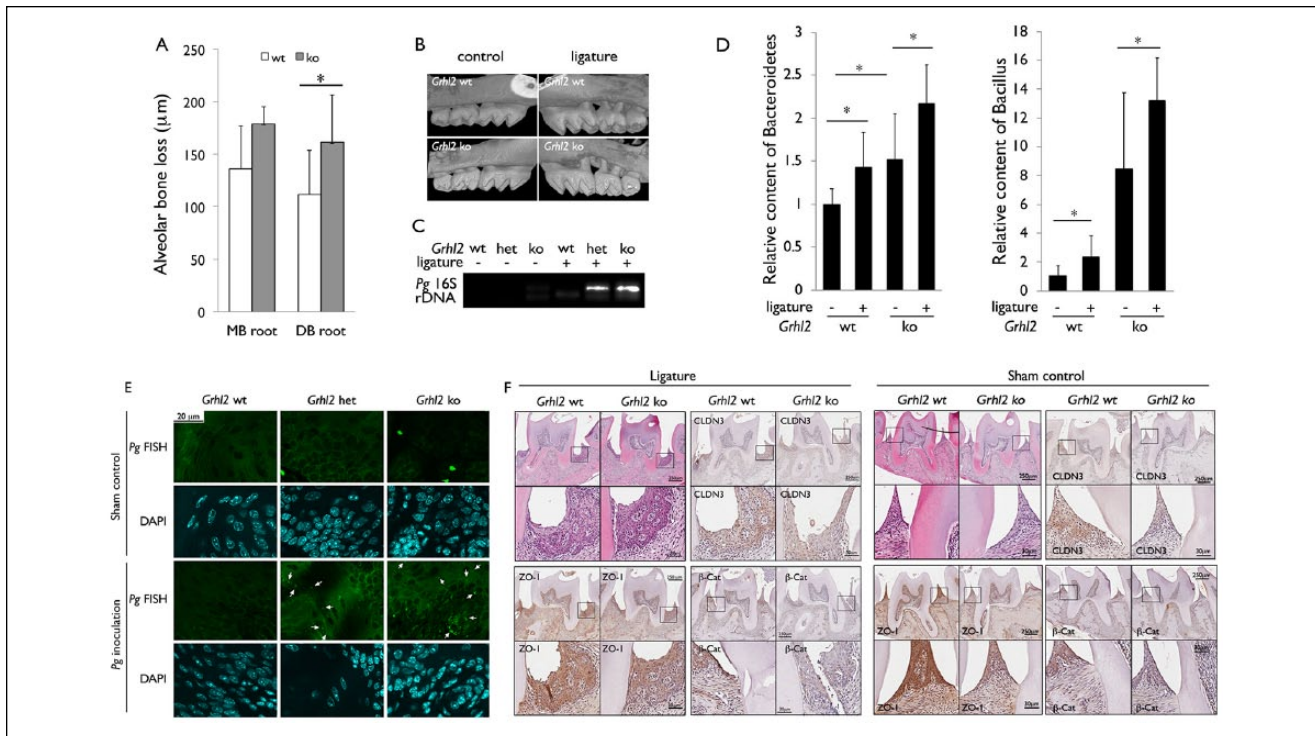
Figure 5. *Grainyhead-like 2* (*Grhl2*) knockout (KO) in oral epithelium led to increased alveolar bone loss and bacterial penetration to systemic circulation in the ligature-induced periodontitis mouse model. (**A**, **B**) Mice with ligatures placed around the maxillary second molars for 3 wk showed increased alveolar bone loss in *Grhl2* KO mice compared to *Grhl2* wild-type (WT). After 3 wk, micro–computed tomography images of maxillary molars from the mice were obtained and reconstructed. DB, distobuccal; MB, mesiobuccal. *n* = 5. (**C**) Whole blood was collected by cardiac puncture to assess the level of *Porphyromonas gingivalis* (*Pg*) bacteria in *Grhl2* WT and KO mice, which were orally inoculated with 1×10^9 live *Pg* once every 2 d for 6 wk; *n* = 3. (**D**) Genomic DNA was extracted from the whole blood collected from *Grhl2* WT and KO mice at 3 wk after the placement of the ligature, and quantitative polymerase chain reaction was performed to assess the levels of bacterial species of *Bacteroidetes* and *Bacillus*. (**E**) *Pg*-specific fluorescence in situ hybridization (FISH) was performed using tongue epithelia from *Grhl2* WT, hetero (+/–), and KO mice after *Pg* inoculation in the oral cavity. White arrows indicate the FISH signals representing interepithelial *Pg* penetration. (**F**) Representative hematoxylin and eosin staining of the maxillary second molars with the junctional epithelium was shown, alongside with the immunohistochemical staining for claudin (CLDN)–3, zonula occludens (ZO)–1, and β-catenin (β-cat) in the junctional epithelium from the *Grhl2* WT and KO mice with ligatures (or sham control) placed around the maxillary second molars for 3 wk.

The functional role of GRHL2 in the regulation of junction proteins was demonstrated in HOK-16B cells with GRHL2 knockdown, which revealed concomitant loss of gene expression of the junction molecules, as well as increased penetration of *Pg* and small molecules. This was also confirmed in our in vivo model, in which we conditionally knocked out *Grhl2* in oral epithelium (Chen et al. 2018). *Grhl2* KO led to decreased expression of CLDN-1, CLDN-3, CLDN-4, and ZO-1 and increased blood loading of oral bacteria. Consequently, the *Grhl2* KO mice showed increased alveolar bone loss compared with the WT littermates in the ligature-induced periodontitis model. Immunohistochemical assessment of the periodontal lesions confirmed decreased protein levels of ZO-1, β-cat, and CLDN-3 in the junctional epithelium of *Grhl2* KO mice compared with the WT, suggesting an impaired mucosal barrier that led to increased bacterial penetration with *Grhl2* KO. These findings are in keeping with earlier studies that showed increased bacterial penetration in gingival tissues with periodontal disease. Saglie et al. (1986) reported abundance of *Bacteroides*

and *Capnocytophaga* species in advanced periodontitis, as well as *Actinobacillus actinomycetemcomitans* in juvenile periodontitis, while healthy gingiva lacked bacterial presence in situ. A subsequent study used immunohistochemical staining of *Pg*-specific thiol proteinase protein as a marker of the bacteria to show increased intracellular *Pg* penetration in the gingival epithelial cells of patients with chronic periodontitis (Rautemaa et al. 2004). Our current study primarily deals with the role of GRHL2 in regulation of junction protein expression in light of establishing the oral mucosal barrier.

Although it is unknown whether GRHL2 alters intracellular invasion of pathogenic bacteria into epithelial cells, maintenance of intact junction protein expression by GRHL2 appears to be critical to impede paracellular bacterial invasion. Prior studies have shown that pathogenic oral bacteria have also devised other unique mechanisms to abrogate the cell adhesion complexes to promote invasion through oral mucosa. Some *Pg* strains release gingipains to trigger proteolytic degradation of epithelial cell junction complexes (e.g., occludin and E-cad),

thereby aiding in paracellular bacterial penetration through the oral mucosa (Katz et al. 2000, 2002). The functional role of the gingipains in altering the mucosal barrier was demonstrated by use of the mutant *Pg* strain defective in gingipain expression (KDP136), which led to a reduced inhibitory effect on the epithelial barrier (Groeger et al. 2010). Our current study used *Pg* strain ATCC33277, which is gingipain positive. Thus, it remains to be determined whether *Pg* abolishes GRHL2 expression in a gingipain-dependent manner or whether a *Pg* inhibitory effect on GRHL2 is additive to gingipain's proteolysis of TJ proteins to impair the mucosal barrier. Likewise, *Bacteroides fragilis*, a common colonic microflora, is known to abrogate the intestinal barrier function by proteolytic degradation of TJ proteins, like ZO-1, through release of its toxin fragilysin (Obiso et al. 1997). As such, alteration of TJ complex stability has a significant impact on the epithelial barrier functions against a microbial challenge.

Increased microbial penetration would have profound health effects that may result in local destruction of alveolar tissues and systemic pathologies. Increased periodontal bone loss was evinced in the mouse model of *Grhl2* KO, which led to significant downregulation of junction proteins (e.g., CLDN-1, CLDN-3, CLDN-4, and ZO-1), suggesting that local microbial infiltration may facilitate tissue damage. Also, our current bacteriological studies revealed increased abundance of oral microflora in systemic blood circulation in the *Grhl2* KO mice compared to the WT controls. These data indicated that the oral mucosa in *Grhl2* KO mice became more permeable to oral microbiota compared to the WT mice, although there remains the possibility that the microbial permeability in other mucosal epithelia (e.g., intestinal mucosa) may similarly be affected by *Grhl2* KO. Further experiments will test these possibilities. Beyond the local alveolar tissue destruction, increased bacteremia through epithelial barriers has broad pathological consequences in systemic diseases. The current study highlights the crucial role of GRHL2 in establishing the oral mucosal barrier in part through regulation of junction protein expression. Interestingly, our study revealed for the first time that some periodontal pathogens like *Pg* inhibit the junction protein expression by targeting GRHL2 and abrogate the epithelial barrier. Further research will elucidate the mechanism by which microbial challenges regulate GRHL2 expression and oral mucosal barrier functions, in light of its importance in preserving the local alveolar tissues and systemic health.

Author Contributions

W. Chen, M.K. Kang, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; A. Alshaikh, contributed to design, data acquisition, analysis, and interpretation, critically revised the manuscript; S. Kim, J. Kim, C. Chun, S. Mehrazarin, J. Lee, R. Lux, R.H. Kim, K.H. Shin, N.H. Park, K. Walentin, K.M. Schmidt-Ott, contributed to 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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