© 2019. Published by The Company of Biologists Ltd | Journal of Cell Science (2020) 133, jcs236588. doi:10.1242/jcs.236588

RESEARCH ARTICLE

SPECIAL ISSUE: CELL BIOLOGY OF THE IMMUNE SYSTEM

Epidermal growth factor receptor signaling suppresses αvβ6 integrin and promotes periodontal inflammation and bone loss

Jiarui Bi[1,*], Leeni Koivisto[1,*], Jiayin Dai[2], Deshu Zhuang[1,2], Guoqiao Jiang[1], Milla Larjava[1], Ya Shen[1], Liangjia Bi[2], Fang Liu[3], Markus Haapasalo[1], Lari Häkkinen[1] and Hannu Larjava[1,‡]

ABSTRACT

In periodontal disease (PD), bacterial biofilms cause gingival inflammation, leading to bone loss. In healthy individuals, αvβ6 integrin in junctional epithelium maintains anti-inflammatory transforming growth factor-β1 (TGF-β1) signaling, whereas its expression is lost in individuals with PD. Bacterial biofilms suppress β6 integrin expression in cultured gingival epithelial cells (GECs) by attenuating TGF-β1 signaling, leading to an enhanced pro-inflammatory response. In the present study, we show that GEC exposure to biofilms induced activation of mitogen-activated protein kinases and epidermal growth factor receptor (EGFR). Inhibition of EGFR and ERK stunted both the biofilm-induced *ITGB6* suppression and *IL1B* stimulation. Furthermore, biofilm induced the expression of endogenous EGFR ligands that suppressed *ITGB6* and stimulated *IL1B* expression, indicating that the effects of the biofilm were mediated by autocrine EGFR signaling. Biofilm and EGFR ligands induced inhibitory phosphorylation of the TGF-β1 signaling mediator Smad3 at S208. Overexpression of a phosphorylation-defective mutant of Smad3 (S208A) reduced the β6 integrin suppression. Furthermore, inhibition of EGFR signaling significantly reduced bone loss and inflammation in an experimental PD model. Thus, EGFR inhibition may provide a target for clinical therapies to prevent inflammation and bone loss in PD.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: αvβ6 integrin, Biofilm, Inflammation, Epidermal growth factor receptor, Periodontal disease

INTRODUCTION

Periodontal diseases (PDs) are inflammatory conditions that affect the supporting structures of the teeth, resulting in destruction of alveolar bone and loss of the periodontal attachment (Kinane et al., 2017). The prevalence of PDs is high, with 10 to 15% of adult population worldwide suffering from the severe forms of the disease (Petersen and Ogawa, 2005; Petersen and Ogawa, 2012). These diseases also contribute to systemic inflammation and thus to systemic diseases, including cardiovascular disease, cerebrovascular disease, Alzheimer's disease, rheumatoid arthritis and cancer (Cullinan and Seymour, 2013; Dominy et al., 2019). During the PD process, junctional epithelium, which seals the gingiva to the tooth enamel in the healthy periodontal condition, transforms into pocket epithelium that proliferates into the connective tissue, allowing infiltration of bacteria and their virulence factors into the periodontal and other tissues (Larjava et al., 2011). Multispecies bacterial biofilms then accumulate deeper between the tooth and the pocket epithelium causing further periodontal inflammation, tissue degradation and alveolar bone loss. Thus, the function of junctional epithelial cells, as the first line of defence, may play a key role in PD. However, the molecular pathways in the host pocket and junctional epithelial cells that could regulate the 'inflammatory' host response in PD still remain largely elusive.

In the inflammatory infiltrate, the outcome of the cytokine response is regulated by the balance of the pro-inflammatory and anti-inflammatory cytokine levels (Dutzan et al., 2009). Transforming growth factor-β1 (TGF-β1), together with interleukin-10 (IL-10), serves as a major balancing anti-inflammatory cytokine in PD (Dutzan et al., 2009). The central anti-inflammatory surveillance role of TGF-β1 has been evidenced in studies with TGF-β1-knockout mice, which die soon after birth from massive infiltration of lymphocytes and macrophages into many organs (Shull et al., 1992; Kulkarni et al., 1993).

Integrin αvβ6 is an exclusively epithelial receptor with limited expression in adult tissues (Koivisto et al., 2018). It plays a major role in activating latent TGF-β1 bound to extracellular matrix or from the cell surface of immune cells by binding to the TGF-β1 latency-associated peptide, and thus has an essential role in innate anti-inflammatory surveillance *in vivo* (Annes et al., 2003; Taylor, 2009; Wang et al., 2012). We have shown previously that αvβ6 integrin is highly expressed in the junctional epithelium where it colocalizes with TGF-β1 (Ghannad et al., 2008). However, in human PD, αvβ6 integrin is strongly diminished in the pocket epithelium (Ghannad et al., 2008; Haapasalmi et al., 1995). Patients with mutations in the *ITGB6* gene, which encodes the rate-limiting subunit for αvβ6 integrin heterodimer formation, have been reported to suffer from severe PD (Ansar et al., 2016). Furthermore, β6 integrin-null mice not only spontaneously develop PD but exhibit severe inflammation in the skin and lungs (Bi et al., 2019; Ghannad et al., 2008; Huang et al., 1996), emphasizing the critical importance of the αvβ6 integrin-mediated localized activation of TGF-β1 in the anti-inflammatory surveillance *in vivo*. Recently, we have also shown that the presence of αvβ6 integrin in the junctional epithelium may provide protection against periodontal inflammation through positive regulation of the AIM2 inflammasome and the anti-inflammatory IL-10 expression (Bi et al., 2019). Therefore, αvβ6 integrin may play a critical role in the maintenance of periodontal health.

The human *ITGB6* promoter contains a binding site for TGF-β1 signaling mediator Smad3 (Xu et al., 2015). Consequently,

[1]Faculty of Dentistry, Department of Oral Biological and Medical Sciences, University of British Columbia, Vancouver, BC V6T 1Z3, Canada. [2]Department of Stomatology, The Fourth Affiliated Hospital, Harbin Medical University, Harbin, 150001, China. [3]Center for Advanced Biotechnology and Medicine, Susan Lehman Cullman Laboratory for Cancer Research, Ernest Mario School of Pharmacy, Rutgers Cancer Institute of New Jersey, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA.
*These authors contributed equally to this work

‡Author for correspondence (larjava@dentistry.ubc.ca)

J.B., 0000-0003-3068-2391; J.D., 0000-0003-2641-831X; D.Z., 0000-0001-9009-4415; M.L., 0000-0002-5265-3009

Received 15 July 2019; Accepted 5 November 2019

Journal of Cell Science (2020) 133, jcs236588. doi:10.1242/jcs.236588

endogenous TGF-β1 is required for sustained *ITGB6* expression in gingival epithelial cells (GECs) and exogenously added TGF-β1 strongly upregulates its expression, indicating that there is a mutual positive feedback loop between these two molecules (Ghannad et al., 2008; Bi et al., 2017). However, signaling pathways that suppress *ITGB6* expression in epithelial cells are not well understood. Other cytokines associated with PD, such as IL-1β, tumor necrosis factor-α and IL-6, lipopolysaccharides from the common periodontal pathogens *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* and synthetic bacterial components, including triacylated lipopeptide Pam3CSK4, flagellin, iE-DAP (a peptidoglycan dipeptide) and lipoteichoic acid, all failed to suppress *ITGB6* expression in these cells (Ghannad et al., 2008; Bi et al., 2017). However, extracts of mature, multispecies oral bacterial biofilms could specifically and dose dependently suppress the β6 integrin mRNA and protein expression in GECs, and promote the expression of inflammatory cytokines, such as IL-1β, potentially by interfering with the anti-inflammatory TGF-β1 signaling (Bi et al., 2017). FSL-1, a synthetic diacylated lipopeptide derived from *Mycoplasma salivarium* (a common component of oral microflora) downregulated *ITGB6* expression in a similar manner (Bi et al., 2017). Moreover, suppression of β6 integrin expression alone by RNA interference stimulated IL-1β expression in these cells, suggesting a shared regulatory pathway (Bi et al., 2017).

In the present study, we explored the cellular signaling pathways contributing to the bacterial biofilm- and FSL-1-induced *ITGB6* downregulation in GECs. We show that the suppression of β6 integrin and the upregulation of inflammatory cytokine expression were mediated by epidermal growth factor receptor (EGFR) signaling and required extracellular signal-regulated kinase 1 and 2 (ERK1/2, also known as MAPK3 and MAPK1, respectively; hereafter denoted ERK) activity. This downregulation was mediated by EGFR- and ERK-dependent phosphorylation, and inhibition of the TGF-β1 signaling mediator Smad3, which in turn attenuates TGF-β1-mediated maintenance of β6 integrin expression. We also show that blocking EGFR activity reduces alveolar bone loss and the inflammatory response in a ligature-induced experimental PD mouse model, supporting the functional relevance of EGFR in the etiology of PD *in vivo*.

RESULTS

Oral biofilm extract induces activation of cellular signaling in gingival epithelial cells

Mitogen-activated protein kinases (MAPKs), including ERK, p38 MAPK family proteins (hereafter denoted p38) and c-Jun N-terminal kinases (JNKs), regulate a variety of cellular processes in epithelial cells, such as cell proliferation, adhesion, differentiation and migration (Huang et al., 2004b; Lee et al., 2016; Ihermann-Hella et al., 2014). Many oral bacteria activate MAPK pathways (Chung and Dale, 2004; Hasegawa et al., 2007). We investigated, therefore, whether MAPKs are activated by the biofilm extract in the GECs. Cells exposed to biofilm extract showed time-dependent, transient phosphorylation of ERK, p38 and JNK1 (also known as

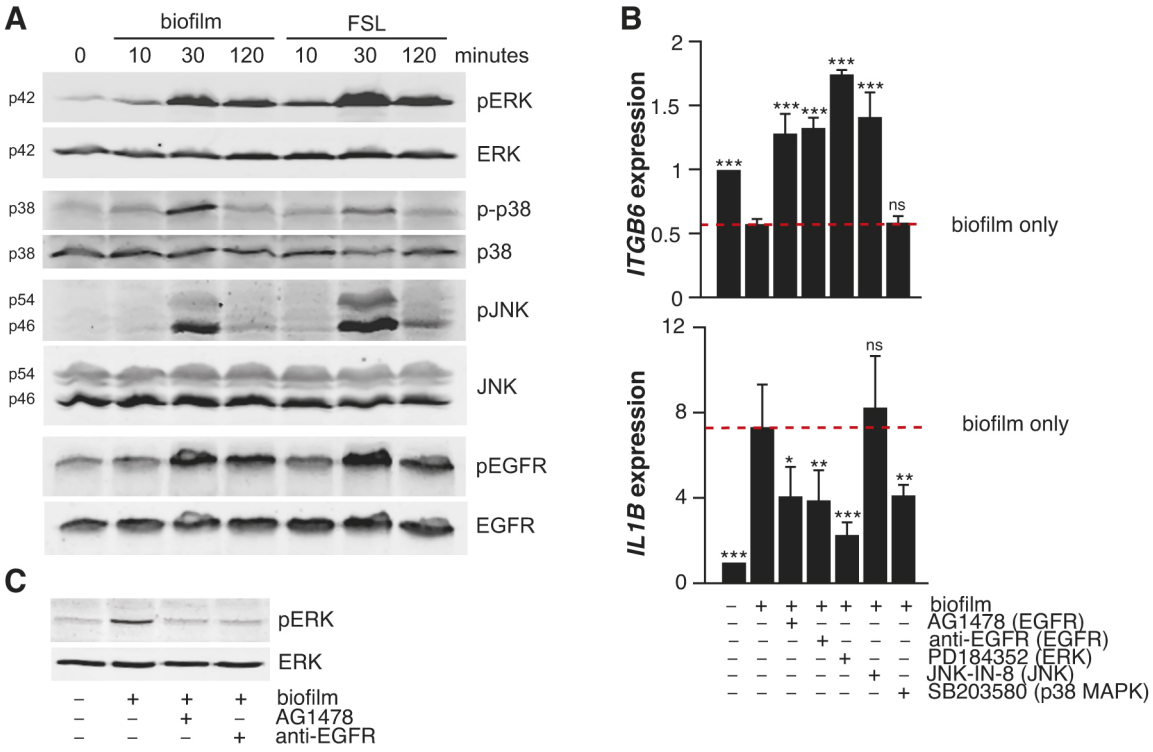

Fig. 1. Activation and involvement of cell signaling in biofilm-induced β6 integrin and IL-1β regulation in GECs. (A) GECs were treated with biofilm extract (60 µg protein/ml) or FSL-1 (100 ng/ml) for 0–120 min and analyzed for ERK, p38, JNK and EGFR phosphorylation (denoted by a 'p' prefix; activation) relative to the respective total protein expression by western blotting. (B) GECs were incubated in the presence or absence of cellular signaling inhibitors (2 µM PD184352, 1 µM JNK-IN-8, 20 µM SB203580, 300 nM tyrphostin AG1478 or 2 µg/ml anti-EGFR antibody) for 2 h, followed by addition of biofilm extract (60 µg protein/ml) for 32 h and analysis of *ITGB6* and *IL1B* expression by RT-qPCR. Results are mean±s.e.m. of 3–7 experiments. **P*<0.05; ***P*<0.01; ****P*<0.001 relative to biofilm only treatment. Signaling pathways targeted are in parentheses after the inhibitor names. C, GECs were incubated in the presence or absence of EGFR inhibitors for 2 h, followed by addition of biofilm extract for 30 min. Phospho-ERK relative to total ERK expression was determined by western blotting. Blots in A and C are representative of three experiments.

Journal of Cell Science (2020) 133, jcs236588. doi:10.1242/jcs.236588

MAPK8) that peaked at ∼30 min of exposure (Fig. 1A), indicating activation of these three protein kinases families. Since MAPKs are commonly activated downstream of EGFR in epithelial cells (Shi et al., 2016), we also tested EGFR activation by the biofilm extract. This also showed activation-dependent Y1068 phosphorylation after a 30-min biofilm extract exposure (Fig. 1A). The synthetic bacterial lipopeptide FSL-1, which we have previously shown to regulate *ITGB6* and *IL1B* expression similarly to biofilm (Bi et al., 2017), also induced MAPK and EGFR activation (Fig. 1A), indicating a shared mechanism of activation between this lipopeptide and the biofilms.

The EGFR and MAPK pathways are involved in the biofilm-induced regulation of *ITGB6* and *IL1B* mRNA expression

Next, we tested whether the suppression of β6 integrin expression by the biofilm extract involves EGFR and MAPKs by using chemical inhibitors [AG1478 for EGFR receptor kinase, PD184352 for the ERK upstream activator MEK1 (also known as MAP2K1), JNK-IN-8 for JNK1, SB203580 for p38 MAPKs] or a function-blocking anti-EGFR antibody. We found that biofilm-induced downregulation of *ITGB6* was completely prevented by inhibition of either the ERK or JNK pathway, whereas inhibition of p38 had no effect (Fig. 1B). A chemical inhibitor of the EGFR kinase and a neutralizing antibody against EGFR ligand binding also blocked the biofilm-induced *ITGB6* suppression (Fig. 1B), indicating that *ITGB6* suppression by biofilm is MAPK- and EGFR-dependent in GECs. We then studied whether these pathways also regulate biofilm-induced *IL1B* stimulation, a key pro-inflammatory cytokine in PD. Inhibition of ERK, p38 or the EGFR pathway reduced *IL1B* upregulation by 40–70%, whereas inhibition of JNK had no effect (Fig. 1B). The results suggest that EGFR and ERK are part of the common mechanism involved in the regulation of both *ITGB6* and *IL1B* by biofilm. ERK activation by biofilm was blocked in the presence of EGFR inhibitors, suggesting that ERK is activated downstream of EGFR (Fig. 1C).

Bacterial biofilm induces endogenous EGFR ligand expression in gingival epithelial cells

To explore whether endogenously expressed EGFR ligands might play a role in EGFR–ERK-mediated regulation of *ITGB6* and *IL1B* by biofilm, we first determined whether biofilm treatment regulates the expression of the major epithelial cell EGFR ligands, including heparin-binding EGF-like growth factor (HB-EGF), TGFα, amphiregulin and epiregulin (Chu et al., 2005). Cell treatment with either biofilm extract or FSL-1 significantly induced mRNA expression of all these ligands (Fig. 2A). While the induction of *TGFA* was modest, based on the real-time quantitative PCR (RT-qPCR) Cq values, it was already highly expressed in GECs prior to their treatment with biofilm or FSL-1 (Table S2). Consequently, we tested whether HB-EGF and TGFα, the two most highly expressed EGFR ligands, could regulate *ITGB6* and *IL1B* in these cells similarly to biofilm. Both growth factors induced a similar, dose-dependent downregulation of *ITGB6* and upregulation of *IL1B* (Fig. 2B), and also suppressed the expression of β6 integrin at the protein level (Fig. 2C), suggesting that biofilm-induced autocrine EGFR signaling is involved in mediating the effects of biofilm on *ITGB6* and *IL1B*.

EGFR ligands attenuate the upregulating effect of TGF-β1 on *ITGB6* expression

Integrin β6 expression in normal epithelial cells is primarily maintained by TGF-β1–Smad signaling, whereas in cancer cells other transcription factors, such as Stat3, may also play a role (Bi et al., 2017; Ghannad et al., 2008; Sullivan et al., 2011; Xu et al., 2015). Therefore, we tested the effect of chemical inhibitors of TGF-β1 receptor kinase (SB431542), Smad3 (SIS3) and Stat3

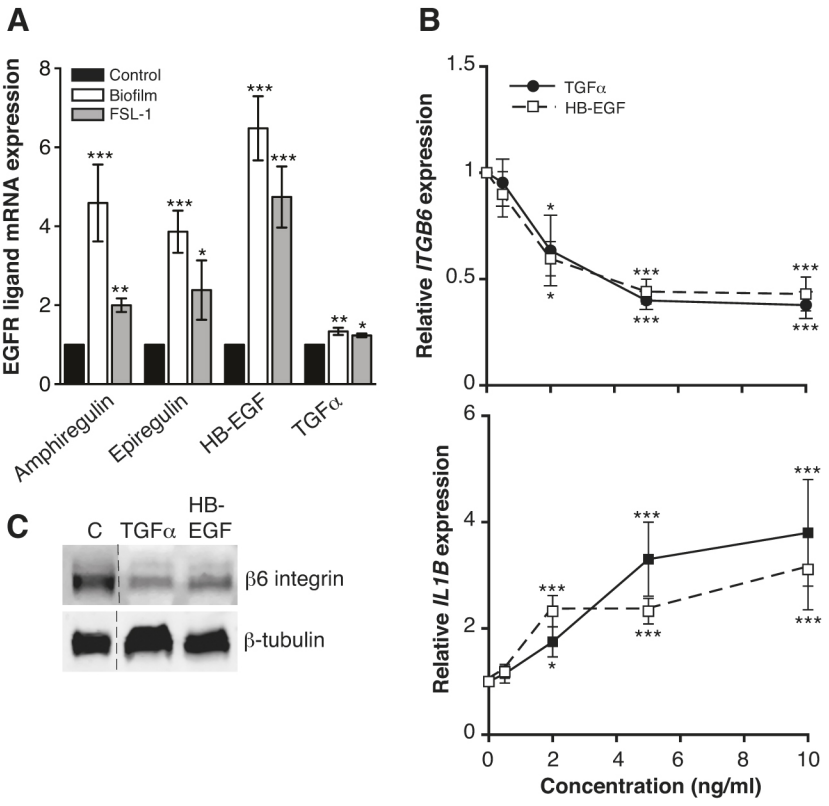

Fig. 2. EGFR ligand expression and involvement in biofilm-induced β6 integrin and IL-1β regulation in GECs. (A) GECs were treated with biofilm extract (60 µg protein/ml) or FSL-1 (100 ng/ml) or left untreated for 32 h and then analyzed for EGFR ligand mRNA expression. Results are mean±s.e.m. for n=5. *P<0.05; **P<0.01; ***P<0.001 relative to untreated cells. B, GECs were treated with TGFα or HB-EGF (0–10 ng/ml) or left untreated for 32 h and then analyzed for *ITGB6* and *IL1B* expression by RT-qPCR. Mean±s.e.m.; n=4–6; *P<0.05; ***P<0.001 relative to untreated cells. (C) GECs were treated with TGFα or HB-EGF (5 ng/ml) or left untreated (denoted C) for 48 h, followed by detection of β6 integrin levels relative to β-tubulin by western blotting. The blot in C is representative of three experiments.

Journal of Cell Science (2020) 133, jcs236588. doi:10.1242/jcs.236588

(Stattic) on the basal GEC *ITGB6* expression. Inhibition of either the TGF-β1 receptor or Smad3 similarly reduced *ITGB6* expression by ∼50%, whereas Stat3 inhibitor had no effect (Fig. 3A), suggesting that β6 integrin expression in GECs is largely dependent on TGF-β1 and Smad3. Treatment with TGF-β1 induced an ∼2–3-fold increase in *ITGB6* expression in these cells that was dose dependently reduced by the addition of HB-EGF or TGFα (Fig. 3B,C), suggesting that EGFR ligands may interfere with TGF-β1–Smad3 regulation of *ITGB6* expression.

Biofilm and EGFR ligands induce phosphorylation of Smad3 in the linker region

EGFR–ERK signaling can inhibit Smad3 activity via ERK-mediated phosphorylation of the Smad3 linker region; the main ERK target being the S208 based on the EGF dose curve, time course of phosphorylation induction and other criteria (Matsuura et al., 2005). Therefore, we investigated whether biofilm, FSL-1 and the EGFR ligand TGFα induce phosphorylation of this residue in GECs. Treatment with any of these agents induced Smad3 S208 phosphorylation that peaked at 30 min (Fig. 3D). This phosphorylation was ERK-dependent, as a MEK1 inhibitor that prevented ERK activation also blocked the biofilm- and EGFR ligand-induced Smad3 phosphorylation (Fig. 3E,F), suggesting that the inhibitory Smad3 phosphorylation is indeed involved in the EGFR–ERK-mediated suppression of *ITGB6* expression.

Adenoviral expression of Smad3 S208A mutant attenuates biofilm- and EGFR ligand-mediated β6 integrin downregulation

To confirm the essential role of Smad3 S208 phosphorylation in the biofilm- and EGFR ligand-mediated *ITGB6* downregulation, GECs were transduced with recombinant adenoviruses to overexpress

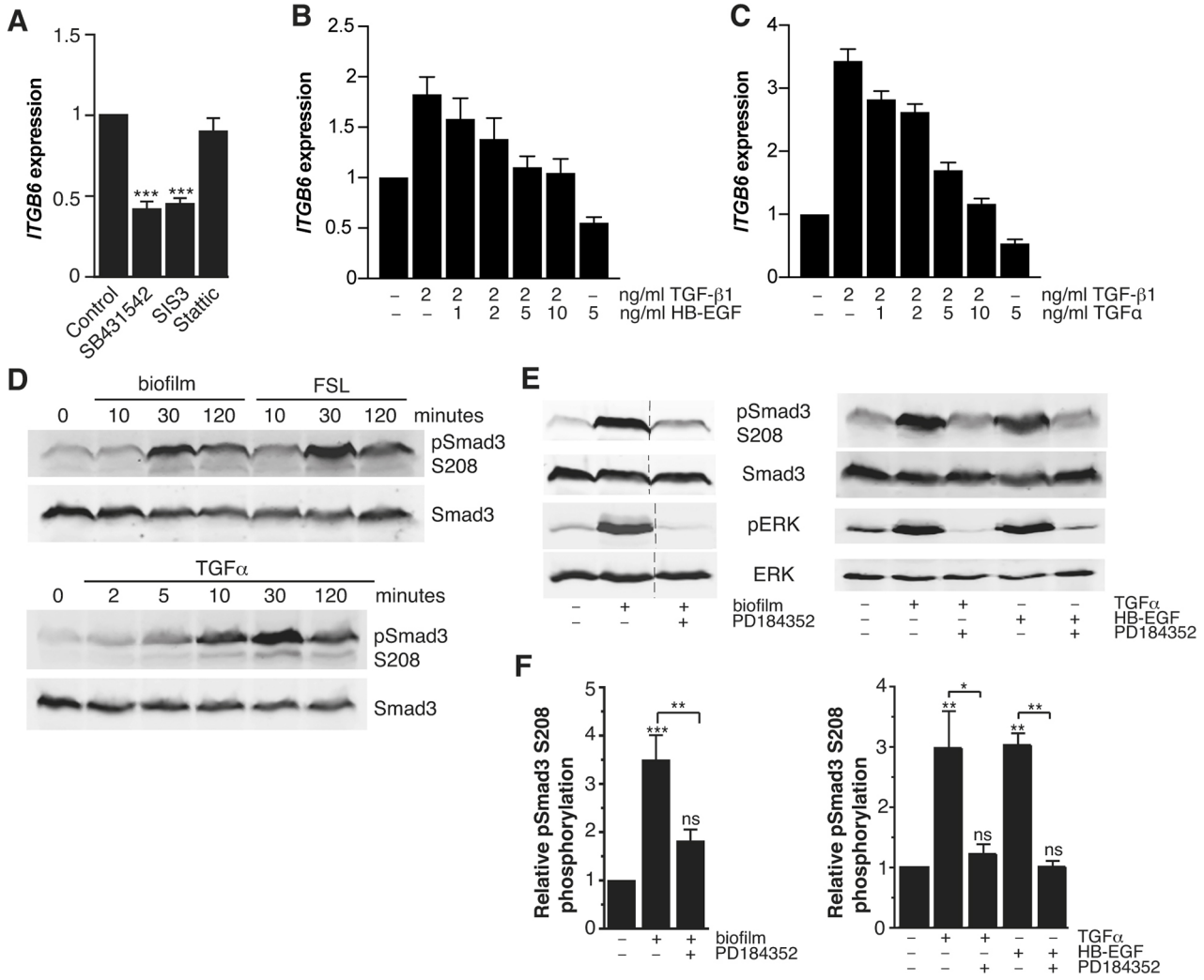

Fig. 3. EGFR ligands interfere with TGF-β1–Smad3 signaling in GECs. (A) GECs were treated with cellular signaling inhibitors (15 µM SB431542, 3 µM SIS3 or 10 µM Stattic) for 32 h and then analyzed for *ITGB6* expression by RT-qPCR. Results are mean±s.e.m. for n=6. ***P<0.001 relative to untreated cells. (B,C) GECs were treated with TGF-β1 (2 ng/ml), HB-EGF (5 ng/ml; B), TGFα (5 ng/ml; C) or with a combination of TGF-β1 and HB-EGF (0–10 ng/ml; B) or with a combination of TGF-β1 and TGFα (0–10 ng/ml; C) for 32 h and analyzed for *ITGB6* expression by RT-qPCR. Results are mean±s.e.m. for n=4–5. (D) GECs were treated with biofilm extract (60 µg protein/ml), FSL-1 (100 ng/ml) or TGFα (5 ng/ml) for 0–120 min and analyzed for Smad3 S208 phosphorylation relative to the respective total protein expression by western blotting. (E) GECs were treated for 30 min with biofilm extract, HB-EGF or TGFα in the presence or absence of PD184352 (an inhibitor of ERK activation) and analyzed for Smad3 S208 and ERK phosphorylation relative to the respective total protein expression by western blotting. (F) Quantification of Smad3 S208 phosphorylation relative to total Smad3 levels in cells treated with biofilm (n=6), HB-EGF (n=3) or TGFα (n=3) in the presence or absence of PD184352. Results are mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001 relative to untreated cells; ns, not significant.

Journal of Cell Science (2020) 133, jcs236588. doi:10.1242/jcs.236588

either the wild-type or S208A mutant of Smad3 that cannot be phosphorylated at this residue. Both the wild-type- and S208A mutant-transduced cells overexpressed Smad3 by ∼6-fold compared to cells transduced with viral particles carrying the empty vector only (Fig. 4A,B). Similarly, overexpression of either Smad3 construct resulted in an ∼3-fold induction of β6 integrin protein (Fig. 4A,B), indicating Smad3 regulation of *ITGB6* expression. Treatment of cells expressing the S208A mutant of Smad3 exhibited significantly attenuated β6 integrin downregulation by biofilm or EGFR ligand treatment compared to cells overexpressing wild-type Smad3, indicating that phosphorylation of Smad3 S208 was indeed involved in the EGFR–ERK-mediated suppression of β6 integrin expression (Fig. 4C,D).

Systemic and local EGFR inhibition reduces the severity of bone loss and inflammation in the experimental mouse periodontitis model

We have recently demonstrated aggravated bone loss and inflammation in mice in an experimental ligature PD model compared to nonligatured control animals (Bi et al., 2019); also shown in Fig. 5 (panels A and B for control; E and F for ligature) and Fig. 6 (panels A–D for control; I–L for ligature), respectively. In order to test whether this bone loss and inflammation could be alleviated by EGFR inhibition, we first screened an array of chemical EGFR inhibitors currently in clinical use or in clinical trials as cancer drugs (gefitinib, afatinib, erlotinib, lapatinib, dacomitinib and canertinib) (Roskoski, 2019) as well as the tyrphostin AG1478 that was used in the cell culture experiments, for their efficacy in inhibiting HB-EGF-induced *ITGB6* suppression in GECs. All of these inhibitors dose dependently blocked β6 integrin downregulation by HB-EGF (Fig. S1). Next, we tested selected inhibitors in the ligature model, namely local application of AG1478 directly to the silk ligature and systemic administration of gefitinib by gavage. As evidenced by the micro-computed tomography (μCT) images, both inhibitors reduced periodontal bone loss compared to the ligature only treatment (Fig. 5C,D compared to E,F, respectively; Fig. 5G,H). The bone loss at the root furcation was also significantly reduced in the gefitinib-treated animals (Fig. 5G). Histological staining of decalcified tissue sections of the mouse jaw showed high levels of inflammatory cell accumulation in the ligatured animals in the absence of EGFR inhibitors (Fig. 6I–L), whereas in the presence of either inhibitor, the inflammatory cell infiltration was significantly reduced (Fig. 6E–H,M,N). Collectively, the data show that EGFR inhibition significantly reduces both bone loss and periodontal inflammation in the experimental mouse periodontitis model, suggesting that EGFR inhibition is potentially an effective treatment for periodontitis.

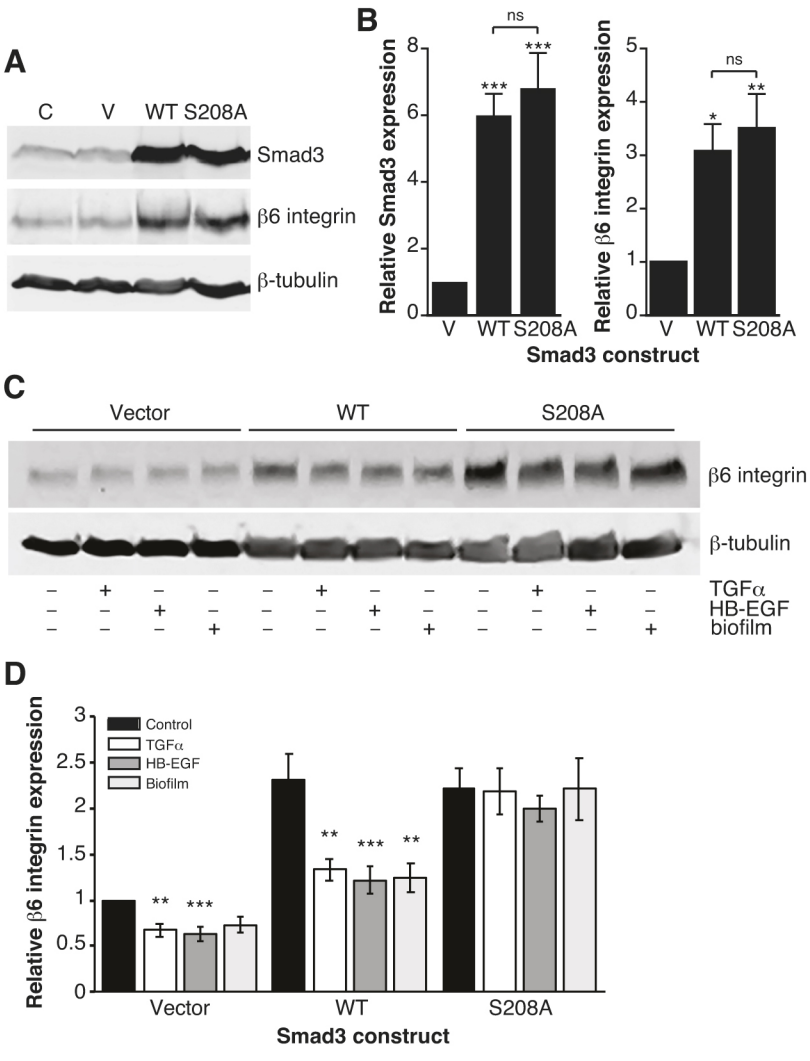

Fig. 4. Adenoviral expression of S208A-mutated Smad3 reduces *ITGB6* downregulation by biofilm and EGFR ligands in GECs. (A) GECs were transduced with recombinant adenoviruses expressing the full-length wild-type human Smad3 cDNA (WT), S208A-mutated Smad3 or with the empty virus vector (V), or left nontransduced (denoted C) for 48 h, followed by detection of Smad3 and β6 integrin levels relative to β-tubulin by western blotting. A representative image is presented. (B) The relative Smad3 and β6 integrin levels were quantified from repeated experiments. Results are mean±s.e.m. for n=8. *P<0.05; **P<0.01; ***P<0.001 relative to untreated cells; ns, non-significant difference in expression between WT- and S208A-transduced cells. (C) GECs were transduced as in A and then treated with TGFα, HB-EGF or biofilm extract for 48 h and analyzed for β6 integrin levels relative to β-tubulin by western blotting. (D) The relative β6 integrin levels were quantified from repeated experiments. Results are mean±s.e.m. for n=6. **P<0.01; ***P<0.001; significance calculated for treatment relative to each untreated control.

Journal of Cell Science (2020) 133, jcs236588. doi:10.1242/jcs.236588

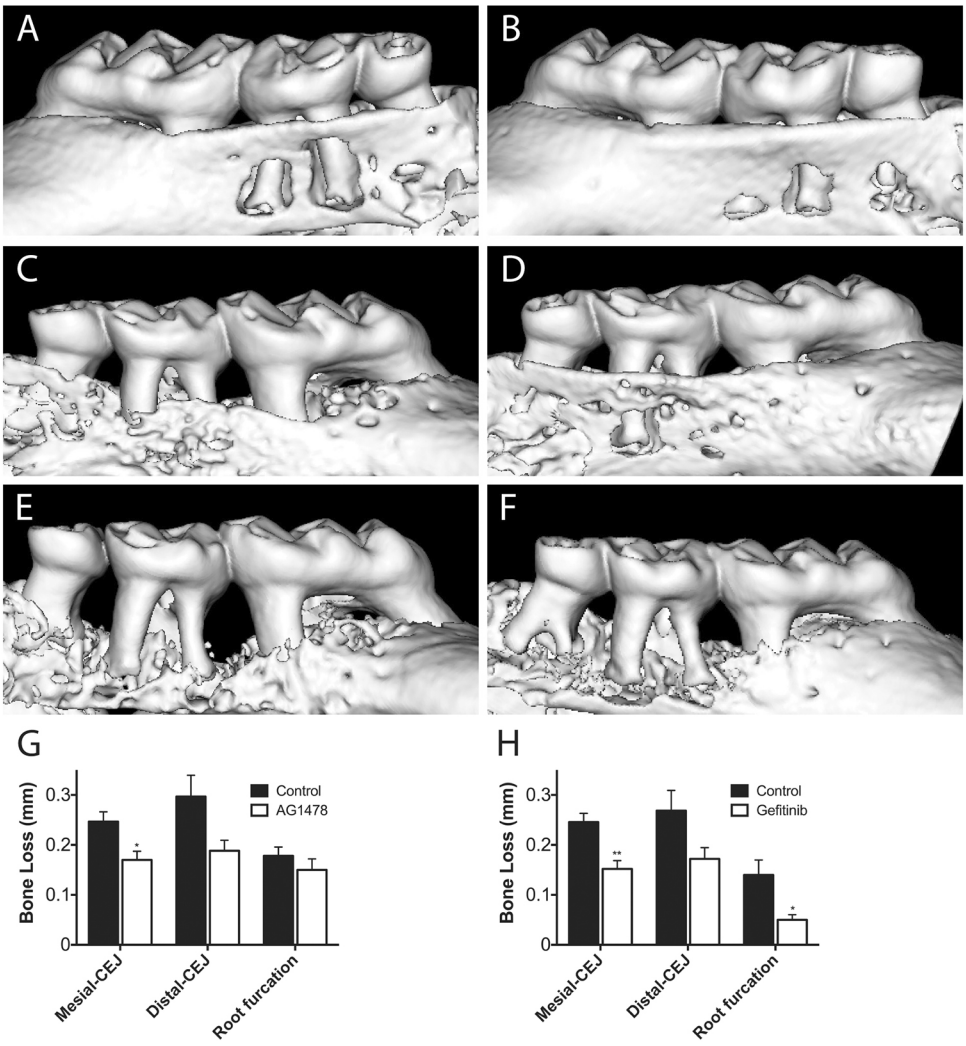

Fig. 5. EGFR inhibitors reduce ligature-induced bone loss in an experimental mouse periodontitis model. (A–F) Silk ligatures were tied around the second maxillary molar on one side of the jaw of 9-week-old mice (C–F), while the other side served as the non-ligatured control (A,B). The ligatured animals were treated with EGFR inhibitors tyrphostin AG1478 (10 µM; local treatment; C) or gefitinib (100 mg/kg body weight/dose; systemic treatment; D) or left untreated (E and F, respectively) for 2 weeks. µCT images of the maxillary molars are presented. (G,H) Quantification of the bone loss around the second molars in three locations (mesial side, distal side and root furcation) and comparisons of ligatured control and ligatured treatment (G, local treatment with AG1478; H, systemic treatment with gefitinib). Results are mean± s.e.m. for n=6 animals per group. *P<0.05; **P<0.01.

DISCUSSION

Expression of αvβ6 integrin in the junctional epithelium is essential for periodontal health (Koivisto et al., 2018). In PD, αvβ6 integrin is downregulated, but the mechanism is not well understood. Our previous studies have shown that TGF-β1–Smad3 signaling sustains the expression of this receptor in healthy junctional epithelium and that oral bacterial biofilm extracts suppress β6 integrin mRNA and protein expression in GECs by interfering with this pathway (Bi et al., 2017). The data from the present study point to substances that activate EGFR and MAPK pathways as the likely culprits for downregulation of αvβ6 integrin during initiation and progression of PD. During this process, EGFR–ERK signaling may be further sustained by the induction of autocrine EGFR ligands. It seems also likely that both the β6 integrin downregulation and the pro-inflammatory IL-1β stimulation are regulated through these same signaling pathways, as biofilm-induced cytokine responses were also prominently attenuated by blockers of EGFR and ERK signaling. While MAPK pathways have previously been indicated in the GEC immune response and in their pro-inflammatory cytokine stimulation by oral bacteria (Watanabe et al., 2001; Krisanaprakornkit et al., 2002; Huang et al., 2004a; Hasegawa et al., 2007), the critical involvement of EGFR–ERK signaling in αvβ6 integrin suppression by oral bacterial biofilm components is a novel finding. Illustration of the putative signaling pathways regulating αvβ6 integrin downregulation and inflammatory cytokine upregulation is presented in Fig. 7.

FSL-1 represents the N-terminal lipopeptide of the cell membrane lipoprotein LP44 of the common oral bacterium *M. salivarium* (Shibata et al., 2000). We have previously shown that FSL-1 suppressed *ITGB6* and upregulated *ILB1* expression similarly to the biofilm extracts in GECs, whereas various other bacterial components failed to suppress *ITGB6* expression (Bi et al., 2017; Ghannad et al., 2008). Here, we show that it also activates EGFR–MAPK signaling and induces autocrine EGFR ligand expression in a similar manner to the biofilm extract. Toll-like receptors (TLRs) play a key role in oral pathogen signaling and can transactivate EGFR (Koff et al., 2008; Ebersole et al., 2013; Shaykhiev et al., 2008). FSL-1 is typically considered an agonist for TLR2 and TLR6 (Kang et al., 2009). However, blocking the function of this receptor with specific antibodies did not diminish the ability of either FSL-1 or the biofilm extract to suppress GEC *ITGB6* expression (Bi et al., 2017), suggesting alternative recognition sites on host cell surface. Thus, the mechanism of how FSL-1 activates EGFR in GECs remained to be determined. Interestingly, the N-terminal peptide of lipoprotein p37 from another mycoplasma strain, *M. hyorhinis*, has been shown to form a cell surface complex with EGFR and annexin A2, inducing EGFR activation in cultured human gastric cancer cells (Duan et al., 2014a;

Journal of Cell Science (2020) 133, jcs236588. doi:10.1242/jcs.236588

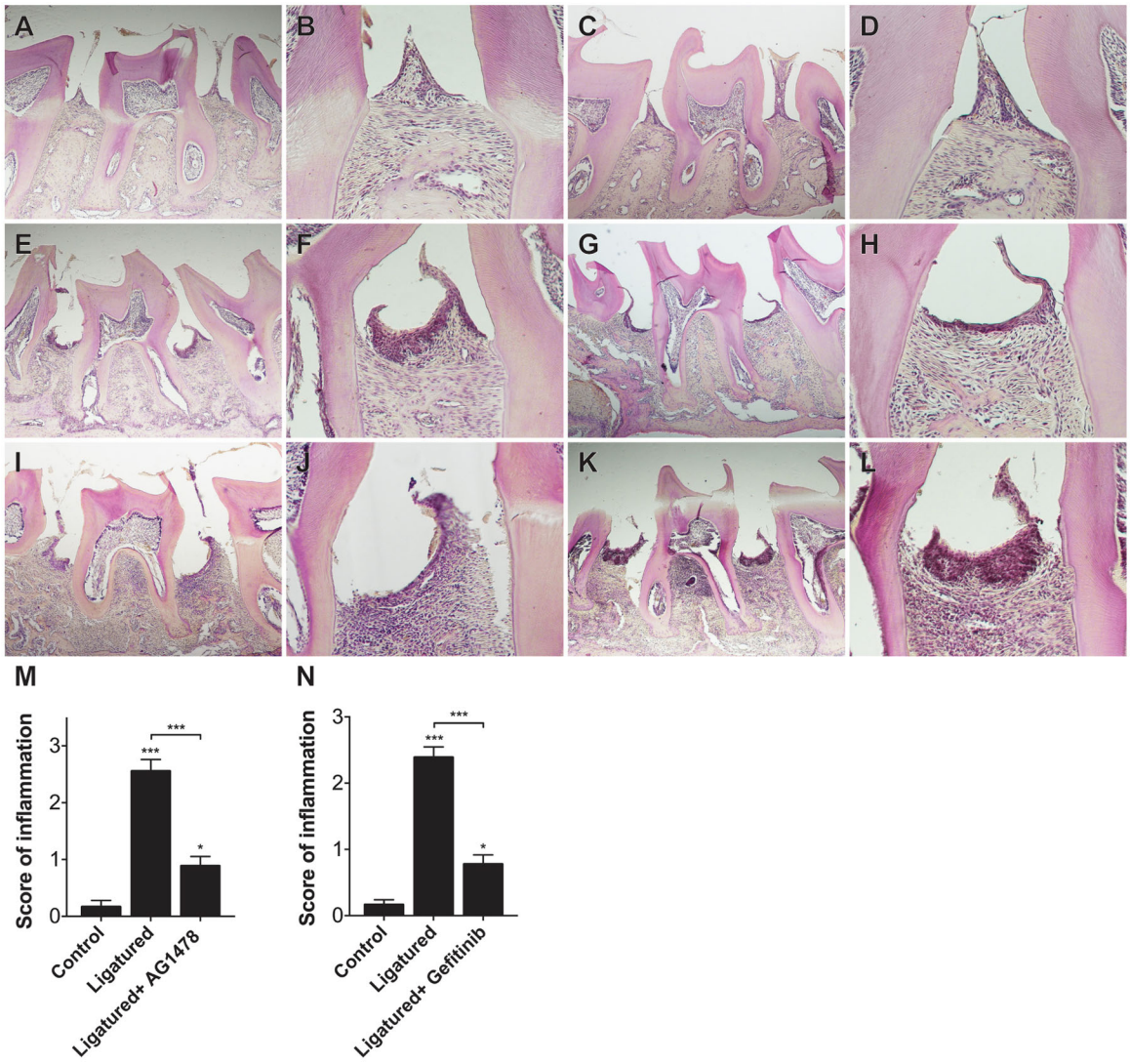

Fig. 6. EGFR inhibitors attenuate ligature-induced periodontal inflammation in mice. (A–F) Silk ligatures were tied around the second maxillary molar on one side of the jaw of 9-week-old mice (E–I), while the other side served as the non-ligatured control (A–D). The ligatured animals were treated with EGFR inhibitors tyrphostin AG1478 (10 µM; local treatment; E and F) or gefitinib (100 mg/ kg body weight/dose; systemic treatment; G and H) or left untreated (I–L, respectively) for 2 weeks. Hematoxylin-eosin-stained sections of periodontal tissues are presented. (M,N) Periodontal inflammation scores in the periodontal tissue were calculated and comparisons between non-ligatured, ligatured control and ligatured treatment (M, local treatment with AG1478; N, systemic treatment with gefitinib) were performed. Results are mean±s.e.m. for n=6 animals per group. *P<0.05; ***P<0.001.

Duan et al., 2014b), suggesting a direct interaction with EGFR is also a possibility.

Both biofilm extract and EGFR ligands were able to attenuate the stimulatory TGF-β1 effect on *ITGB6* expression in GECs (this study and Bi et al., 2017), suggesting EGFR-mediated repression of TGF-β1–Smad3 signaling. EGFR signaling inhibits Smad3 activity via ERK-mediated phosphorylation of the Smad3 linker region, with S208 being the primary ERK target site (Matsuura et al., 2005). In our study, both biofilm extract and EGFR ligands induced ERK-dependent Smad3 S208 phosphorylation, and overexpression of the phosphorylation-defective S208A mutant of Smad3 significantly attenuated the ability of biofilm or EGFR ligands to suppress β6 integrin expression, highly suggesting that ERK-mediated Smad3 S208 phosphorylation is an essential mechanism for *ITGB6* downregulation. EGFR–ERK signaling might also attenuate TGF-β1–Smad3 functions by other means, for example by ERK-mediated phosphorylation and stabilization of the Smad2/3 co-repressor TGIF (Yang et al., 2008; Liu et al., 2014). While both EGFR ligands and biofilm extract increased TGIF protein levels in GECs, this increase was not affected by EGFR or ERK inhibition (L.K., unpublished results), suggesting that this was not the primary mechanism for EGFR–ERK-mediated *ITGB6* suppression in these cells, at least *in vitro*. The role of TGIF accumulation in the suppression of αvβ6 integrin expression in PD needs to be further studied *in vivo*. It should be noted that TGF-β1 itself can also activate ERK (Zhang, 2009). However, the TGFβ receptor inhibitor SB431542 had no inhibitory effect on biofilm-induced ERK activation (L.K., unpublished results), making it unlikely that at least the initial ERK activation was mediated by TGFβR. Unraveling the crosstalk between the EGFR and TGF-β1 pathways will be important in understanding how αvβ6 integrin participates in the regulation of the inflammatory response and to identify novel targets for the treatment of PD.

Journal of Cell Science (2020) 133, jcs236588. doi:10.1242/jcs.236588

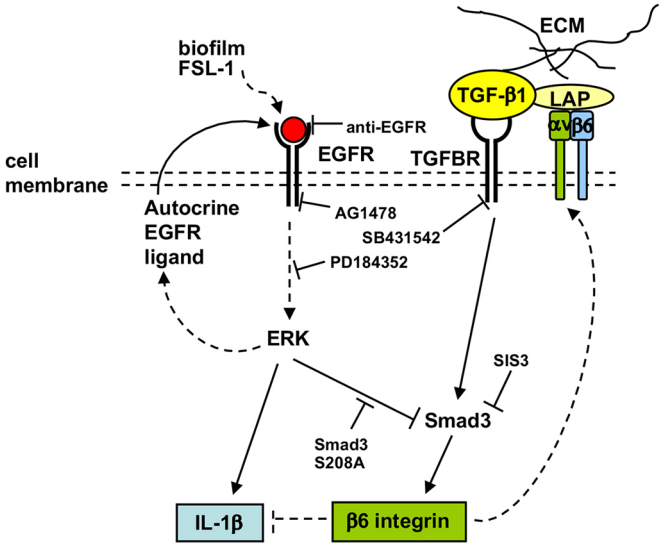

Fig. 7. Putative signaling pathways regulating αvβ6 integrin downregulation and inflammatory cytokine upregulation in the junctional epithelium by bacterial biofilms in PD. In a healthy periodontal condition, αvβ6 integrin activates extracellular matrix (ECM)-bound latent (LAP bound) TGF-β1, a major anti-inflammatory cytokine. Active TGF-β1 in turn maintains the expression of αvβ6 integrin in the junctional epithelium. During periodontitis, bacterial biofilm induces the expression and activation of endogenous EGFR ligands in the pocket epithelium and EGFR signaling. ERK-mediated inhibitory phosphorylation of Smad3 at S208 leads to attenuation of TGF-β1 signaling, downregulation of αvβ6 integrin expression and increased inflammatory cytokine (such as IL-1β) production. Inhibitors interrupting these signaling pathways that were tested in the study: SB431542 for TGF-β1 receptor; SIS3 for Smad3; AG1478 and a blocking anti-EGFR antibody for EGFR; PD184352 for MEK1 (MEK1 activates ERK) and Smad3 S208A mutation for inhibition of ERK-mediated phosphorylation of Smad3.

Epithelial tight junctions have been shown to be compromised in periodontal lesions and, consequently, translocation of harmful bacteria into gingival tissue is significantly increased in these lesions compared to healthy sites, eliciting a defensive host inflammatory response that can induce alveolar bone loss (Choi et al., 2014; Jiao et al., 2014). As shown in airway epithelia, increased epithelial permeability may result in chronic autocrine EGFR receptor activation and a wound healing response that can contribute to the tissue remodeling that is a hallmark of PD (Vermeer et al., 2003). PD may thus result from the capacity of the bacterial components to redirect host responses towards those that are chronic and detrimental to the health of tissues and cells, resulting in PD as collateral damage from a dysregulated host response (Ebersole et al., 2013). Therefore, PD may represent a survival response gone awry, and periodontal health could be restored by rebalancing the TGF-β1–EGFR equilibrium.

In addition to its anti-inflammatory functions, TGF-β1 is a negative regulator of epithelial cell proliferation (Ten Dijke et al., 2002; Bretones et al., 2015). Integrin αvβ6-mediated TGF-β1 activation limits epithelial cell growth, and the loss of this receptor may lead to epithelial hyperplasia (Ludlow et al., 2005; Xie et al., 2012). Conversely, EGFR–ERK signaling promotes epithelial cell growth and migration (Sun et al., 2015; Wang et al., 2019). Therefore, the extension of pocket epithelium both downwards and into the gingival connective tissue during the progression of human PD may be caused by an imbalance between the TGF-β1 and EGFR signaling in the pocket epithelium. Increased EGFR expression and activation has been detected in several types of lesions that exhibit aberrant epithelial cell proliferation and saw tooth-like epithelial penetration into the underlying connective tissue, such as oral leukoplakia, oral lichen planus and skin psoriasis (Wang et al., 2019; Cortes-Ramirez et al., 2014; Ribeiro et al., 2012). Interestingly, patients with psoriasis undergoing cancer treatment with EGFR inhibitors showed remarkable therapeutic improvement of their chronic psoriasis lesions, indicating aberrant EGFR activity as a contributing factor in this skin condition (Wang et al., 2019). However, EGFR expression and activation in PD is not well studied. EGFR expression is low or absent in healthy junctional epithelium, whereas some studies have reported increased expression in inflamed gingiva and in the pocket epithelium of PD compared to healthy junctional epithelium (Nordlund et al., 1991; Irwin et al., 1991). The EGFR activation status and autocrine epithelial EGFR ligand expression in the pocket epithelium will be further studied but it is likely to be responsible for the promotion of the increase in cell proliferation and apical migration of junctional epithelium that are observed in the progression of PD similar to other conditions of epithelial hyperplasia.

We have shown that EGFR inhibition provided a significant reduction in periodontal inflammation and alveolar bone loss in the experimental mouse periodontitis model. EGFR inhibition may thus provide an attractive treatment target for human PD. Several EGFR inhibitors have already been approved for human use as a treatment for epithelial cancers (Roskoski, 2019). Although their systemic administering can produce side effects (Guggina et al., 2017), their local use for restoring the αvβ6 integrin levels in the junctional epithelium could be used to treat periodontal inflammation and bone loss in patients with PD.

MATERIALS AND METHODS

Antibodies and reagents

Reagents used for cell treatment were: FSL-1 (Pam2CGDPKHPKSF; a synthetic diacylated lipopeptide derived from *Mycoplasma salivarium*; Abcam, Cambridge, MA); EGFR ligands TGFα (GenWay Biotech, San Diego, CA) and HB-EGF (R&D Systems, Minneapolis, MN) and TGF-β1 (EMD Millipore, Etobicoke, ON); and the EGFR inhibitors, function-blocking anti-EGFR antibody (#GR13L, clone 225, Calbiochem, San Diego, CA), tyrphostin AG1478 (Sigma-Aldrich Canada, Oakville, ON), gefitinib and afatinib (both Toronto Research Chemicals, Toronto, ON, Canada), erlotinib (Cayman Chemicals, Ann Arbor, MI), lapatinib ditosylate (Phoenix Pharmaceuticals, Burlingame, CA), dacomitinib and canertinib dihydrochloride (both LC Laboratories, Woburn, MA). Other cellular signaling inhibitors were: PD184352 (an inhibitor of ERK activation; Axon Medchem BV, Groningen, The Netherlands), JNK-IN-8 (a JNK inhibitor) and SB431542 (a TGF-β receptor inhibitor; both Selleckchem Chemicals, Houston, TX), SB203580 (a p38 MAPK inhibitor; New England Biolabs, Whitby, ON), SIS3 (Smad3 inhibitor; BioVision, Milpitas, CA) and Stattic (Stat3 inhibitor; R&D Systems). Primary antibodies used for western blotting were: anti-pERK1/2 (T202/Y204; #4370; 1:2000), anti-p38 MAPK (#9228; 1:1000) and anti-pEGFR (Y1068; #3777; 1:1000) from Cell Signaling Technology (Danvers, MA); anti-ERK1/2 (sc-154-G; 1:500), anti-JNK1 (sc-571; 1:1000) and anti-EGFR (sc-373746; 1:500) from Santa Cruz Biotechnology (Dallas, TX); anti-active p38 MAPK (V121A; 1:2000) and anti-active JNK (V7931; 1:5000) from Promega (Madison, WI); anti-β6 integrin (AF2389; 1:3000) from R&D Systems; anti-β-tubulin (MAB3408; 1:2000) from EMD Millipore and Smad3 (ab283790; 1:2000) from Abcam; and anti-phospho-Smad3 S208 produced as described (Matsuura et al., 2005; used at 0.15 µg/ml). All PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table S1.

Multispecies oral *in vitro* biofilms

Procedures involving human biofilm donors were reviewed and approved by the Clinical Research Ethics Board at the University of British Columbia (Protocol number H17-02478). Oral bacterial biofilms were grown, and

Journal of Cell Science (2020) 133, jcs236588. doi:10.1242/jcs.236588

biofilm extracts prepared as previously described in detail (Bi et al., 2016). Briefly, subgingival plaque from the first and second upper molars was collected from healthy volunteers in brain heart infusion broth (Difco, Detroit, MI), dispersed, seeded on collagen-coated (bovine dermal type I collagen; Cohesion, Palo Alto, CA) sterile hydroxyapatite discs (Clarkson Chromatography Products, Williamsport, PA) and incubated under anaerobic conditions (AnaeroGen; Oxoid, Cambridge, UK) at 37°C. The growth medium was changed once a week. After 21 days, the discs were rinsed once with phosphate-buffered saline (PBS; pH 7.4), and the bacteria then dispersed in PBS by pipetting, snap-frozen, ground over liquid nitrogen using a mortar and pestle and sonicated with Branson Sonifier 250 on ice. The non-solubilized bacterial debris was removed by centrifugation at 10,000 ***g*** for 10 min and the supernatant stored at −20°C for later use. The total biofilm extract protein concentration was quantified with Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules). The biofilm extract was heated for 5 min at 95°C before being used for cell treatment as previously described (Bi et al., 2017).

Cell culture

The spontaneously immortalized human gingival epithelial cell line (GEC; Mäkelä et al., 1998) was maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, Inc., Burlington, ON) supplemented with 23 mM sodium bicarbonate, 20 mM HEPES, 1% antibiotics (50 µg/ml streptomycin sulfate, 100 U/ml penicillin; Gibco) and 10% heat-inactivated fetal bovine serum (FBS; Gibco) as previously described (Bi et al., 2017).

Cell stimulation and signaling blocking experiments

GECs were seeded into cell culture dishes (5×10^5 cells/cm^2) and allowed to grow to confluency for 2 days in their complete growth medium, rinsed once with PBS and switched to FBS-free DMEM for 2 h in the presence or absence of cellular signaling inhibitors (2 µM PD184352, 1 µM JNK-IN-8, 20 µM SB203580, 300 nM AG1478, 2 µg/ml anti-EGFR, 15 µM SB431542, 3 µM SIS3 or 10 µM Stattic), prior to adding biofilm extract (60 µg/ml of protein), FSL-1 (100 ng/ml), TGFα (1–10 ng/ml), HB-EGF (1–10 ng/ml) or TGF-β1 (2 ng/ml) to the cells. The cells were further cultured for 32 h or 48 h, rinsed with PBS and used for RNA isolation and RT-qPCR or for western blotting, respectively, as described in more detail below. To investigate cellular signaling pathway activation, the cells were grown, pre-treated with inhibitors and then stimulated as above for 0–120 min, and their phosphorylated protein to total protein expression ratio was determined by western blotting and subsequent quantification.

RT-qPCR

The RNA isolation and RT-qPCR were performed as previously described (Bi et al., 2016). Briefly, total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA) and RNA concentration measured by spectrophotometry. Then, 1 µg of total RNA was reverse-transcribed into cDNA using an iScript Select cDNA synthesis kit (Bio-Rad). The cDNA was diluted to a concentration for which the threshold-cycle value was well within the range of its standard curve. 5 µl of cDNA was mixed with 10 µl of 2×iQ SYBR Green I Supermix (Bio-Rad) and 5 pmol of primers in each well of the 96-well plate. Asparagine-linked glycosylation 9 (*ALG9*), beta-2-microglobulin (*B2M*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as reference genes to conduct amplification reactions of the target genes in triplicates. Real-time PCR amplification was performed with the CFX96 system (Bio-Rad). The running program was 3 min at 95°C, followed by 45 cycles of 15 s at 94°C, 20 s at 60°C and 20 s at 72°C. The data were analyzed by CFX Manager Software Version 2.1 (Bio-Rad). PCR detection level was adjusted to ≥1.5-fold to reflect significant change. The mean Cq values lower than 33 were included in the gene expression analyses.

Western blotting

Western blotting was performed as previously described (Bi et al., 2016). The cells were washed once with PBS and lysed in 1x Laemmli sample buffer. The cell lysates were separated by SDS/PAGE and transferred onto Hybond Protran membrane (Amersham, Little Chalfont, Buckinghamshire, UK). The membranes were incubated in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h, followed by incubation with primary antibodies for overnight at +4°C. After incubation with species-appropriate IRdye-conjugated secondary antibodies (LI-COR) for 1 h, the blots were washed and scanned with LI-COR Odyssey Infrared Imaging system. The results were analyzed with Odyssey software (version 3.0).

Generation of recombinant adenoviruses and cell treatment

The recombinant adenoviruses were produced using AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA). Briefly, the LZRSΔ-IRES-GFP retroviral vectors carrying the full-length human Smad3 cDNA or its mutant form with a mutation of serine to non-phosphorylatable alanine residue at position 208 (S208A) (Matsuura et al., 2005) were digested with *Eco*RI and blunted with Klenow fragment. The sequences encoding wild-type or S208A Smad3 were then ligated into *Sal*I-digested, Klenow fragment-blunted pShuttle-CMV to generate pShuttleSmad3 and its mutant, respectively. The resulting plasmid constructs, and pShuttle as an empty vector control, were linearized with *Pme*I, dephosphorylated and then co-transformed into *Escherichia coli* BJ5183 strain with pAdEasy-1 to generate their corresponding recombinant viral plasmids. These recombinant plasmids were further amplified in *E. coli* XL10-Gold strain and verified with *Pac*I digestion. The plasmids were then transfected into human HEK293 embryonal kidney cells with Lipofectamine (Invitrogen, Carlsbad, CA, USA) to generate the respective replication-deficient human serotype 5 adenoviruses. Subsequently, these viruses were propagated and amplified in the HEK293 cells, pooled and recovered in FBS-free DMEM through four cycles of freeze thawing. Viral titers were determined by plaque forming units (pfu).

To verify adenoviral overexpression of Smad3, GECs were trypsinized, and suspended in serum-free DMEM. The cells were mixed with the recombinant adenoviruses (10 pfu/cell), gently shaken at room temperature for 30 min and then plated at 5×10^5 cells/cm^2. After a 3-hour incubation and attachment at 37°C (5% CO_2), FBS was added to 10%. After 48 h, the cells were analyzed for their Smad3 and β6 integrin levels by western blotting. In a set of experiments, the adenovirus-transduced cells were further treated with EGFR ligands and biofilm extract for 48 h and analyzed for β6 integrin expression by western blotting.

In-cell western

GECs were seeded into 96-well plates (5×10^5 cells/cm^2) in their complete growth medium for 24 h, washed once with PBS and treated with TGF-β1 (2 ng/ml) for 24 h in DMEM containing 1% FBS. The cells were then washed with PBS and incubated in FBS-free medium for 2 h in the presence or absence of EGFR inhibitors (0.003–3 µM), followed by addition of HB-EGF (5 ng/ml) for 48 h. The cells were fixed with formaldehyde fixative [PBS containing 4% (v/v) formaldehyde and 5% (w/v) sucrose] for 20 min, permeabilized in PBS containing 0.5% Triton X-100 for 5 min, blocked with Odyssey Blocking Buffer for 1 h and then incubated with anti-β6 integrin antibody for overnight at +4°C, followed a species-appropriate IRdye800-conjugated secondary antibody (LI-COR) and CellTag700 (LI-COR) staining for 1 h. The wells were then washed and scanned with LI-COR Odyssey Infrared Imaging system. The results were analyzed with Odyssey software (version 3.0) relative to the cell numbers (CellTag700) in each well.

Animal ligature model

All animal experiments were reviewed and approved by the University of British Columbia Committee on Animal Care (Protocol number A16-0034). A total of 24 mice were used for the experiment (12 mice each for local treatment group and systemic treatment group; 6 mice for treatment with drug and 6 control mice without drug in each group). To induce experimental periodontitis, silk sutures (6-0; Ethicon, San Lorenzo, PR, USA) were tied around the left second maxillary molars on 9-week-old FVB/NHsd mice as we have done previously (Bi et al., 2019). The right-side maxillary molar of the same animal was left untied as a control. For local EGFR inhibition, the silk was first soaked in ethanol with or without tyrphostin AG1478 (10 µM) (Johns et al., 2003) at +4°C for overnight. The inhibitor-infused sutures were changed every second day for 14 days. For

systemic EGFR inhibition, another set of mice were gavaged with 1% Tween-80 in PBS (pH 7.4) with or without gefitinib (100 mg/kg body weight/dose) (Zhao et al., 2013) every second day for 14 days. The silk sutures were tied a day after the first gavage and retained in place until the end of the experiment. Maxilla specimens were then collected and used for analysis of bone loss and histological assessment by micro-computed tomography (μCT) and histochemistry, respectively.

Micro-computed tomography

Maxilla specimens were fixed in 4% formaldehyde (v/v) in PBS for 24 h at +4°C and then transferred into 2% formaldehyde in PBS for μCT scanning (10 μm voxel size; Scanco CT 40; Scanco, Wayne, PA). Alveolar bone loss was evaluated from the horizonal-angulated μCT images with Microview Software (Parallax, Ilderton, ON, Canada). The distances from the cemento-enamel junction to the alveolar bone crest in mesial and distal sites of the ligatured second molar were measured and averaged.

Histological assessment of mouse jaw specimens

After μCT scanning, the maxilla specimens were decalcified in PBS containing 2% formaldehyde and 0.4 M EDTA for 5 weeks. The specimens were paraffin-embedded and sectioned (8 μm) in the mesio-distal direction followed with hematoxylin and eosin staining. A sliding visual score was used to score the level of inflammation between the first and second molars (0, no inflammation; 1, mild; 2, moderate; 3, severe). The scores from three investigators that were blind to the experimental conditions were averaged.

Statistical analysis

All cell experiments were separately repeated for at least three times. Statistical comparisons between two experimental groups were analyzed by unpaired, two-tailed Student's *t*-test. Multiple comparison tests were performed using one-way ANOVA followed by post hoc comparison with Tukey–Kramer multiple comparisons test or least significant difference test (LSD). Statistical significance was set at $P<0.05$. Statistical significance for RT-qPCR data (performed in triplicates) was calculated using log2-transformed data.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.B., L.K., F.L., M.H., H.L.; Methodology: J.B., L.K., G.J., Y.S., L.B.; Software: J.B., L.K.; Validation: J.B., L.K., J.D., D.Z., G.J., M.L., Y.S., L.B., F.L., M.H., L.H., H.L.; Formal analysis: J.B., L.K.; Investigation: J.B., L.K., J.D., D.Z., M.L.; Resources: J.B., L.B., F.L.; Data curation: J.B., L.K.; Writing - original draft: J.B., L.K.; Writing - review & editing: J.B., L.K., F.L., M.H., L.H., H.L.; Visualization: J.B., L.K.; Supervision: L.K., Y.S., H.L.; Project administration: H.L.; Funding acquisition: L.H., H.L.

Funding

This work was supported by a grant from the Canadian Institutes of Health Research (PJT-153379 and PJT-156387).

Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.236588.supplemental

References

Annes, J. P., Munger, J. S. and Rifkin, D. B. (2003). Making sense of latent TGFbeta activation. *J. Cell Sci.* **116**, 217-224. doi:10.1242/jcs.00229

Ansar, M., Jan, A., Santos-Cortez, R. L. P., Wang, X., Suliman, M., Acharya, A., Habib, R., Abbe, I., Ali, G., Lee, K. et al. (2016). Expansion of the spectrum of ITGB6-related disorders to adolescent alopecia, dentogingival abnormalities and intellectual disability. *Eur. J. Hum. Genet.* **24**, 1223-1227. doi:10.1038/ejhg.2015.260

Bi, J., Koivisto, L., Owen, G., Huang, P., Wang, Z., Shen, Y., Bi, L., Rokka, A., Haapasalo, M., Heino, J. et al. (2016). Epithelial microvesicles promote an inflammatory phenotype in fibroblasts. *J. Dent. Res.* **95**, 680-688. doi:10.1177/0022034516633172

Bi, J., Koivisto, L., Pang, A., Li, M., Jiang, G., Aurora, S., Wang, Z., Owen, G. R., Dai, J., Shen, Y. et al. (2017). Suppression of alphavbeta6 integrin expression by polymicrobial oral biofilms in gingival epithelial cells. *Sci. Rep.* **7**, 4411. doi:10.1038/s41598-017-03619-7

Bi, J., Dai, J., Koivisto, L., Larjava, M., Bi, L., Häkkinen, L. and Larjava, H. (2019). Inflammasome and cytokine expression profiling in experimental periodontitis in the integrin beta6 null mouse. *Cytokine* **114**, 135-142. doi:10.1016/j.cyto.2018.11.011

Bretones, G., Delgado, M. D. and León, J. (2015). Myc and cell cycle control. *Biochim. Biophys. Acta* **1849**, 506-516. doi:10.1016/j.bbagrm.2014.03.013

Choi, Y. S., Kim, Y. C., Ji, S. and Choi, Y. (2014). Increased bacterial invasion and differential expression of tight-junction proteins, growth factors, and growth factor receptors in periodontal lesions. *J. Periodontol.* **85**, e313-e322. doi:10.1902/jop.2014.130740

Chu, E. K., Foley, J. S., Cheng, J., Patel, A. S., Drazen, J. M. and Tschumperlin, D. J. (2005). Bronchial epithelial compression regulates epidermal growth factor receptor family ligand expression in an autocrine manner. *Am. J. Respir. Cell Mol. Biol.* **32**, 373-380. doi:10.1165/rcmb.2004-0266OC

Chung, W. O. and Dale, B. A. (2004). Innate immune response of oral and foreskin keratinocytes: utilization of different signaling pathways by various bacterial species. *Infect. Immun.* **72**, 352-358. doi:10.1128/IAI.72.1.352-358.2004

Cortes-Ramirez, D. A., Rodriguez-Tojo, M. J., Coca-Meneses, J. C., Marichalar-Mendia, X. and Aguirre-Urizar, J. M. (2014). Epidermal growth factor receptor expression in different subtypes of oral lichenoid disease. *Med. Oral Patol Oral Cir. Bucal* **19**, e451-e458. doi:10.4317/medoral.19452

Cullinan, M. P. and Seymour, G. J. (2013). Periodontal disease and systemic illness: will the evidence ever be enough? *Periodontol 2000* **62**, 271-286. doi:10.1111/prd.12007

Dominy, S. S., Lynch, C., Ermini, Benedyk, M., Marczyk, A., Konradi, A., Nguyen, M., Haditsch, U., Raha, D., Griffin, C. F. et al. (2019). Porphyromonas gingivalis in Alzheimer's disease brains: evidence for disease causation and treatment with small-molecule inhibitors. *Sci. Adv.* **5**, eaau3333. doi:10.1126/sciadv.aau3333

Duan, H., Chen, L., Qu, L., Yang, H., Song, S. W., Han, Y., Ye, M., Chen, W., He, X. and Shou, C. (2014a). Mycoplasma hyorhinis infection promotes NF-kappaB-dependent migration of gastric cancer cells. *Cancer Res.* **74**, 5782-5794. doi:10.1158/0008-5472.CAN-14-0650

Duan, H., Qu, L. and Shou, C. (2014b). Activation of EGFR-PI3K-AKT signaling is required for Mycoplasma hyorhinis-promoted gastric cancer cell migration. *Cancer Cell Int* **14**, 135. doi:10.1186/s12935-014-0135-3

Dutzan, N., Gamonal, J., Silva, A., Sanz, M. and Vernal, R. (2009). Over-expression of forkhead box P3 and its association with receptor activator of nuclear factor-kappa B ligand, interleukin (IL) –17, IL-10 and transforming growth factor-beta during the progression of chronic periodontitis. *J. Clin. Periodontol.* **36**, 396-403. doi:10.1111/j.1600-051X.2009.01390.x

Ebersole, J. L., Dawson, D. R., III, Morford, L. A., Peyyala, R., Miller, C. S. and Gonzaléz, O. A. (2013). Periodontal disease immunology: 'double indemnity' in protecting the host. *Periodontol 2000* **62**, 163-202. doi:10.1111/prd.12005

Ghannad, F., Nica, D., Fulle, M. I. G., Grenier, D., Putnins, E. E., Johnston, S., Eslami, A., Koivisto, L., Jiang, G., Mckee, M. D. et al. (2008). Absence of alphavbeta6 integrin is linked to initiation and progression of periodontal disease. *Am. J. Pathol.* **172**, 1271-1286. doi:10.2353/ajpath.2008.071068

Guggina, L. M., Choi, A. W. and Choi, J. N. (2017). EGFR Inhibitors and cutaneous complications: a practical approach to management. *Oncol. Ther.* **5**, 135-148. doi:10.1007/s40487-017-0050-6

Haapasalmi, K., Makela, M., Oksala, O., Heino, J., Yamada, K. M., Uitto, V. J. and Larjava, H. (1995). Expression of epithelial adhesion proteins and integrins in chronic inflammation. *Am. J. Pathol.* **147**, 193-206.

Hasegawa, Y., Mans, J. J., Mao, S., Lopez, M. C., Baker, H. V., Handfield, M. and Lamont, R. J. (2007). Gingival epithelial cell transcriptional responses to commensal and opportunistic oral microbial species. *Infect. Immun.* **75**, 2540-2547. doi:10.1128/IAI.01957-06

Huang, X. Z., Wu, J. F. and Cass, D. (1996). Inactivation of the integrin beta 6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin. *J. Cell Biol.* **133**, 921-928. doi:10.1083/jcb.133.4.921

Huang, C., Jacobson, K. and Schaller, M. D. (2004a). MAP kinases and cell migration. *J. Cell Sci.* **117**, 4619-4628. doi:10.1242/jcs.01481

Huang, G. T.-J., Zhang, H. B., Dang, H. N. and Haake, S. K. (2004b). Differential regulation of cytokine genes in gingival epithelial cells challenged by Fusobacterium nucleatum and Porphyromonas gingivalis. *Microb. Pathog.* **37**, 303-312. doi:10.1016/j.micpath.2004.10.003

Ihermann-Hella, A., Lume, M., Miinalainen, I. J., Pirttiniemi, A., Gui, Y., Peränen, J., Charron, J., Saarma, M., Costantini, F. and Kuure, S. (2014). Mitogen-activated protein kinase (MAPK) pathway regulates branching by remodeling epithelial cell adhesion. *PLoS Genet.* **10**, e1004193. doi:10.1371/journal.pgen.1004193

Irwin, C. R., Schor, S. L. and Ferguson, M. W. J. (1991). Expression of EGF-receptors on epithelial and stromal cells of normal and inflamed gingiva. *J. Periodontal Res.* **26**, 388-394. doi:10.1111/j.1600-0765.1991.tb01727.x

Jiao, Y., Hasegawa, M. and Inohara, N. (2014). Emerging roles of immunostimulatory oral bacteria in periodontitis development. *Trends Microbiol.* **22**, 157-163. doi:10.1016/j.tim.2013.12.005

Johns, T. G., Luwor, R. B., Murone, C., Walker, F., Weinstock, J., Vitali, A. A., Perera, R. M., Jungbluth, A. A., Stockert, E., Old, L. J. et al. (2003). Antitumor

efficacy of cytotoxic drugs and the monoclonal antibody 806 is enhanced by the EGF receptor inhibitor AG1478. *Proc. Natl. Acad. Sci. USA* **100**, 15871-15876. doi:10.1073/pnas.2036503100

Kang, J. Y., Nan, X., Jin, M. S., Youn, S.-J., Ryu, Y. H., Mah, S., Han, S. H., Lee, H., Paik, S.-G. and Lee, J.-O. (2009). Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity* **31**, 873-884. doi:10.1016/j.immuni.2009.09.018

Kinane, D. F., Stathopoulou, P. G. and Papapanou, P. N. (2017). Periodontal diseases. *Nat Rev Dis Primers* **3**, 17038. doi:10.1038/nrdp.2017.38

Koff, J. L., Shao, M. X., Ueki, I. and Nadel, J. A. (2008). Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **294**, L1068-L1075. doi:10.1152/ajplung.00025.2008

Koivisto, L., Bi, J., Hakkinen, L. and Larjava, H. (2018). Integrin alphavbeta6: Structure, function and role in health and disease. *Int. J. Biochem. Cell Biol.* **99**, 186-196. doi:10.1016/j.biocel.2018.04.013

Krisanaprakornkit, S., Kimball, J. R. and Dale, B. A. (2002). Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappaB transcription factor family. *J. Immunol.* **168**, 316-324. doi:10.4049/jimmunol.168.1.316

Kulkarni, A. B., Huh, C. G., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M. and Karlsson, S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. USA* **90**, 770-774. doi:10.1073/pnas.90.2.770

Larjava, H., Koivisto, L., Häkkinen, L. and Heino, J. (2011). Epithelial integrins with special reference to oral epithelia. *J. Dent. Res.* **90**, 1367-1376. doi:10.1177/0022034511402207

Lee, G., Kim, H. J. and Kim, H.-M. (2016). RhoA-JNK regulates the E-cadherin junctions of human gingival epithelial cells. *J. Dent. Res.* **95**, 284-291. doi:10.1177/0022034515619375

Liu, X., Hubchak, S. C., Browne, J., A. and Schnaper, H. W. (2014). Epidermal growth factor inhibits transforming growth factor-beta-induced fibrogenic differentiation marker expression through ERK activation. *Cell. Signal.* **26**, 2276-2283. doi:10.1016/j.cellsig.2014.05.018

Ludlow, A., Yee, K. O., Lipman, R., Bronson, R., Weinreb, P., Huang, X., Sheppard, D. and Lawler, J. (2005). Characterization of integrin beta6 and thrombospondin-1 double-null mice. *J. Cell. Mol. Med.* **9**, 421-437. doi:10.1111/j.1582-4934.2005.tb00367.x

Mäkelä, M., Salo, T. and Larjava, H. (1998). MMP-9 from TNF alpha-stimulated keratinocytes binds to cell membranes and type I collagen: a cause for extended matrix degradation in inflammation? *Biochem. Biophys. Res. Commun.* **253**, 325-335. doi:10.1006/bbrc.1998.9641

Matsuura, I., Wang, G., He, D. and Liu, F. (2005). Identification and characterization of ERK MAP kinase phosphorylation sites in Smad3. *Biochemistry* **44**, 12546-12553. doi:10.1021/bi050560g

Nordlund, L., Hormia, M., Saxen, L. and Thesleff, I. (1991). Immunohistochemical localization of epidermal growth factor receptors in human gingival epithelia. *J. Periodontal Res.* **26**, 333-338. doi:10.1111/j.1600-0765.1991.tb02071.x

Petersen, P. E. and Ogawa, H. (2005). Strengthening the prevention of periodontal disease: the WHO approach. *J. Periodontol.* **76**, 2187-2193. doi:10.1902/jop.2005.76.12.2187

Petersen, P. E. and Ogawa, H. (2012). The global burden of periodontal disease: towards integration with chronic disease prevention and control. *Periodontol 2000* **60**, 15-39. doi:10.1111/j.1600-0757.2011.00425.x

Ribeiro, D. C., Gleber-Netto, F. O., Sousa, S. F., Bernardes, V. F., Guimaraes-Abreu, M. H. N. and Aguiar, M. C. F. (2012). Immunohistochemical expression of EGFR in oral leukoplakia: association with clinicopathological features and cellular proliferation. *Med. Oral Patol Oral Cir. Bucal* **17**, e739-e744. doi:10.4317/medoral.17950

Roskoski, R.Jr (2019). Small molecule inhibitors targeting the EGFR/ErbB family of protein-tyrosine kinases in human cancers. *Pharmacol. Res.* **139**, 395-411. doi:10.1016/j.phrs.2018.11.014

Shaykhiev, R., Behr, J. and Bals, R. (2008). Microbial patterns signaling via Toll-like receptors 2 and 5 contribute to epithelial repair, growth and survival. *PLoS ONE* **3**, e1393. doi:10.1371/journal.pone.0001393

Shi, T., Niepel, M., Mcdermott, J. E., Gao, Y., Nicora, C. D., Chrisler, W. B., Markillie, L. M., Petyuk, V. A., Smith, R. D., Rodland, K. D. et al. (2016). Conservation of protein abundance patterns reveals the regulatory architecture of the EGFR-MAPK pathway. *Sci. Signal.* **9**, rs6. doi:10.1126/scisignal.aaf0891

Shibata, K., Hasebe, A., Into, T., Yamada, M. and Watanabe, T. (2000). The N-terminal lipopeptide of a 44-kDa membrane-bound lipoprotein of Mycoplasma salivarium is responsible for the expression of intercellular adhesion molecule-1 on the cell surface of normal human gingival fibroblasts. *J. Immunol.* **165**, 6538-6544. doi:10.4049/jimmunol.165.11.6538

Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D. et al. (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* **359**, 693-699. doi:10.1038/359693a0

Sullivan, B. P., Kassel, K. M., Manley, S., Baker, A. K. and Luyendyk, J. P. (2011). Regulation of transforming growth factor-beta1-dependent integrin beta6 expression by p38 mitogen-activated protein kinase in bile duct epithelial cells. *J. Pharmacol. Exp. Ther.* **337**, 471-478. doi:10.1124/jpet.110.177337

Sun, Y., Liu, W.-Z., Liu, T., Feng, X., Yang, N. and Zhou, H.-F. (2015). Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *J. Recept. Signal Transduct. Res.* **35**, 600-604. doi:10.3109/10799893.2015.1030412

Taylor, A. W. (2009). Review of the activation of TGF-beta in immunity. *J. Leukoc. Biol.* **85**, 29-33. doi:10.1189/jlb.0708415

Ten Dijke, P., Goumans, M. J., Itoh, F. and Itoh, S. (2002). Regulation of cell proliferation by Smad proteins. *J. Cell. Physiol.* **191**, 1-16. doi:10.1002/jcp.10066

Vermeer, P. D., Einwalter, L. A., Moninger, T. O., Rokhlina, T., Kern, J. A., Zabner, J. and Welsh, M. J. (2003). Segregation of receptor and ligand regulates activation of epithelial growth factor receptor. *Nature* **422**, 322-326. doi:10.1038/nature01440

Wang, R., Zhu, J., Dong, X., Shi, M., Lu, C. and Heldin, C.-H. (2012). GARP regulates the bioavailability and activation of TGFbeta. *Mol. Biol. Cell* **23**, 1129-1139. doi:10.1091/mbc.e11-12-1018

Wang, S., Zhang, Z., Peng, H. and Zeng, K. (2019). Recent advances on the roles of epidermal growth factor receptor in psoriasis. *Am J Transl Res* **11**, 520-528.

Watanabe, K., Yilmaz, O., Nakhjiri, S. F., Belton, C. M. and Lamont, R. J. (2001). Association of mitogen-activated protein kinase pathways with gingival epithelial cell responses to Porphyromonas gingivalis infection. *Infect. Immun.* **69**, 6731-6737. doi:10.1128/IAI.69.11.6731-6737.2001

Xie, Y., McElwee, K. J., Owen, G., Häkkinen, L. and Larjava, H. S. (2012). Integrin beta6-deficient mice show enhanced keratinocyte proliferation and retarded hair follicle regression after depilation. *J. Invest. Dermatol.* **132**, 547-555. doi:10.1038/jid.2011.381

Xu, M., Chen, X., Yin, H., Yin, L., Liu, F., Fu, Y., Yao, J. and Deng, X. (2015). Cloning and characterization of the human integrin beta6 gene promoter. *PLoS ONE* **10**, e0121439. doi:10.1371/journal.pone.0121439

Yang, S., Nugent, M. A. and Panchenko, M. P. (2008). EGF antagonizes TGF-beta-induced tropoelastin expression in lung fibroblasts via stabilization of Smad corepressor TGIF. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **295**, L143-L151. doi:10.1152/ajplung.00289.2007

Zhang, Y. E. (2009). Non-Smad pathways in TGF-beta signaling. *Cell Res.* **19**, 128-139. doi:10.1038/cr.2008.328

Zhao, S., Kuge, Y., Zhao, Y., Takeuchi, S., Hirata, K., Takei, T., Shiga, T., Dosaka-Akita, H. and Tamaki, N. (2013). Assessment of early changes in 3H-fluorothymidine uptake after treatment with gefitinib in human tumor xenograft in comparison with Ki-67 and phospho-EGFR expression. *BMC Cancer* **13**, 525. doi:10.1186/1471-2407-13-525