The IL-1β-p65 axis stimulates quiescent odontogenic epithelial cell rests via TGF-β signalling to promote cell proliferation of the lining epithelia in radicular cysts: A laboratory investigation

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A short running title: Cell growth in radicular cyst

KEYWORDS: IL-1β, p65, TGF-β, Smad2/3, Proliferation, Radicular cyst

Abstract

Aim: Cyst formation of the jaws is frequently accompanied by the proliferation of odontogenic epithelial cells located in the periodontal ligament (PDL), which consists of heterozygous cells and includes the most fibroblasts. The lining epithelium of radicular cyst, an odontogenic cyst of inflammatory origin, is derived from the proliferation of the remnants of the Hertwig epithelial root sheath (odontogenic epithelial cell rests of Malassez; ERMs) in the PDL. ERMs are maintained at a lower proliferative state under physiological conditions, but the regulatory mechanisms underlying the inflammation-dependent enhanced-proliferative capabilities of ERMs are not fully understood. The aim of this study was to evaluate the effects of cytokine pathway association between TGF-β signaling and IL-1β signaling on the regulation of odontogenic epithelial cell proliferation using radicular cyst pathological specimens and odontogenic epithelial cell lines.

Methodology: Immunofluorescence analyses were performed to clarify the expression levels of Smad2/3 and Ki-67 in ERMs of 8-week-old mouse molar specimens. In radicular cyst ($n = 52$) and dentigerous cysts ($n = 6$) specimens from human patients, the expression of p65 (a main subunit of NF-κB), Smad2/3 and Ki-67 were investigated using immunohistochemical analyses. Odontogenic epithelial cells and PDL fibroblastic cells were co-cultured with or without an inhibitor or siRNAs. Odontogenic epithelial cells were cultured with or without TGF-β1 and IL-1β. The proliferative capabilities and Smad2 phosphorylation levels of odontogenic epithelial cells were examined.

Results: Immunohistochemically, Smad2/3-positivity was increased, and p65-positivity and Ki-67-positivity were decreased both in ERMs and in the epithelial cells in dentigerous cysts, a non-inflammatory developmental cyst. In contrast, p65-positive cells, along with the expression of Ki-67, were increased and Smad2/3-positive cells were

decreased in the lining epithelia of radicular cysts. Co-culture experiments with odontogenic epithelial cells and PDL fibroblastic cells revealed that PDL cells-derived TGF-β1/2 and their downstream signalling suppressed odontogenic epithelial cell proliferation. Moreover, TGF-β1 stimulation induced Smad2 phosphorylation and suppressed odontogenic epithelial cell proliferation, while IL-1β stimulation reversed these phenotypes through p65 transactivation.

Conclusions: These results suggest that IL-1β-p65 signaling promotes odontogenic epithelial cell proliferation through suppressing TGF-β-Smad2 signaling, which would be involved in the pathogenesis of radicular cysts.

INTRODUCTION

Radicular cysts are the most common cysts of the jaws, accounting for approximately 55% of all odontogenic cysts (Speight et al, 2017). Histopathologically, radicular cysts have a wall composed of inflamed fibrous or granulation tissue lined by non-keratinized stratified squamous epithelium. The remnants of the Hertwig epithelial root sheath (epithelial cell rests of Malassez; ERMs) show less proliferative capabilities under physiological conditions in the periodontal ligament (PDL) (Lin et al., 2007). Meanwhile, in the inflammation with apical periodontitis, the ERMs would proliferate, and the cyst would be lined by epithelium (Speight et al., 2017).

Some proinflammatory cytokines and growth factors, including interleukin (IL)-1, IL-6, epidermal growth factor, keratinocyte growth factor, tumor necrosis factor, transforming growth factor (TGF)-α, TGF-β and insulin-like growth factor, are capable of inducing epithelial cells to proliferate, thereby developing into an apical cyst (Lin et al., 2007; Rios et al., 2023). Clinically, most cases can be successfully treated by non-surgical endodontic therapy. In contrast, tooth extraction or apicectomy with enucleation of the cystic cavity is needed in some cases. After therapy, the lining epithelium of cyst will stop proliferating under anti-/less-inflammatory conditions (Lin et al., 2007; Rios et al., 2023). Therefore, it is thought that endodontic treatments suppress inflammatory responses, leading to reduce the proliferative capabilities of epithelial lining cells of radicular cysts. Meanwhile, the underlying inflammatory-mediated mechanisms that reversibly regulate odontogenic epithelial cellular growth are unclear.

IL-1β, a proinflammatory cytokine, transactivates nuclear factor-κB (NF-κB) signalling and its activation plays pivotal roles in regulating inflammation (Diep et al., 2022; Jimi et al., 2019). NF-κB, in which p65 is a main subunit, is a transcriptional factor

that regulates gene expression, resulting in controlling cell proliferation (Jimi et al., 2019). Although the expression of IL-1β and p65 in radicular cysts and its involvement in the progression of periapical lesions were reported (Yang et al., 2018; Yu et al., 2018), the precise function of NF-κB signaling in odontogenic epithelial cellular growth during the formation of radicular cysts is largely unknown.

TGF-β1 is a polypeptide member of the transforming growth factor-β superfamily of cytokines, which is secreted by platelets and inflammatory cells. It performs diverse cellular functions including the control of cellular growth and inflammation (Alaeddini et al., 2017). In addition, TGF-β1 is important for tissue repair because it increases angiogenesis and fibroblast collagen formation (Wikesjö et al., 1998). TGF-β signaling is activated by the binding of TGF-βs (TGF-β1, TGF-β2 and TGF-β3) to TGF-β type II receptor (TGFBR2), resulting in TGFBR1 activation. The receptor complex phosphorylates receptor-regulated Smads (R-Smads; Smad2, Smad3) and then forms a complex with the common Smad (Co-Smad; Smad4) and translocates into the nucleus to regulate transcription of target genes and cell proliferation (David & Massagué, 2018).

In the aspect of cellular growth, TGF-β signaling usually inhibits cell proliferation in a cell-type-specific manner (David & Massagué, 2018) and TGF-β1 is reportedly expressed in throughout the PDL tissue, which consist of heterozygous cells and in which fibroblasts are most abundant (Fujii et al., 2010). Therefore, we hypothesized that TGF-βs secreted by PDL cells may maintain the resting state of ERMs in the steady state. We aimed to clarify the effect of TGF-βs derived from PDL cells on odontogenic epithelial cell proliferation using cell lines to mimic the *in vivo* situations.

In this study, we found that the expression of p65 was elevated in lining epithelial cells with higher proliferative capabilities in pathological specimens of radicular cyst and demonstrated the role of IL-1β-p65 signaling in regulating odontogenic epithelial cell proliferation through the suppression of TGF-β-Smad2 signaling.

MATERIALS AND METHODS

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Figure 1) (Nagendrababu et al., 2021).

Cell lines, reagents and antibodies

SF2 (rat odontogenic epithelial cells), which is derived from the cervical loop at the apical end of the lower incisors from a Sprague-Dawley rat (Arakaki et al., 2012), were cultured in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) (Nagano et al., 2022). STPLF-E (immortalized human heterozygous PDL cells) (Fujii et al., 2006) and 1-11 (a clonal human PDL progenitor/stem cell line) (Fujii et al., 2008) were maintained in α-MEM (Invitrogen) containing 10% FBS (Invitrogen).

A83-01 (an inhibitor of TGF-β receptor I ALK5: which was used for the inhibition of TGF-β signaling), recombinant human TGF-β1 (for the activation of TGF-β signalling) and recombinant human IL-1β (for the activation of IL-1β signaling) were obtained from FUJI-FILM Wako (Osaka, Japan) and used in cell culture studies. Anti-p65 (8242S) (for western blotting, immunofluorescence and immunohistochemistry), anti-Smad2/3 (8685S) (for western blotting, immunofluorescence and immunohistochemistry) and anti-Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (8828S) (for western blotting) antibodies were obtained from cell signalling technology (CST) (Beverly, MA, USA). Anti-Cytokeratin 14 (CK14) (ab7800) (for immunofluorescence) and anti-Ki-67 (ab16667) (for immunohistochemistry and immunofluorescence) antibodies were obtained from Abcam (Cambridge, UK). Anti-E-

cadherin (610181) (for immunohistochemistry) antibody was obtained from BD Biosciences (San José, CA, USA). Anti-β-actin (A5441) (for western blotting) antibody was from Sigma-Aldrich (Steinheim, Germany).

Co-culture of odontogenic epithelial cells and PDL cells

SF2 cells (5.0×10^4 cells) and PDL cells (STPLF-E or 1-11 cells) (5.0×10^5 cells) were seeded in DMEM/F12 supplemented with 10% FBS for 48 h, where the epithelial cells and PDL cells would be directly contacted. In this study, these culture conditions were defined as 'co-culture'. Before the co-culture, SF2 cells were treated with 1 μM A83-01 for 24 h and/or STPLF-E or 1-11 cells were transfected with two different human TGF-β1 and TGF-β2 siRNAs for 48 h.

Cell proliferation assay

SF2 cells were cultured with TGF-β1 and/or IL-1β in the presence of 2% FBS for 48 h, and then the proliferative capabilities were evaluated using CyQUANT NF assay (Invitrogen) according to the manufacturer's protocol as previously described (Fujii et al., 2020; Hasegawa et al., 2021). Then, the fluorescence intensity was measured using FlexStation 3 microplate reader (Molecular Devices, Tokyo, Japan) at 485 nm excitation/530 nm emission and was shown as relative fluorescence units.

Knockdown of protein expression by siRNA and quantitative RT-PCR

The effects of protein knockdown by siRNA were analyzed as previously described (Alkhatib et al., 2023; Fujii et al., 2019). Briefly, siRNAs (final concentration 20 nM) were transfected into SF2, STPLF-E and 1-11 cells using Lipofectamine RNAiMAX

(Invitrogen). The following target sequences were used: randomized control, 5'-CAGTCGCGTTTGCGACTGG-3', rat p65, 5'-AGCCATAAGACAGTCTTTACT-3', human TGF-β1 #1, 5'-CAGCATATATATGTTCTTCAA-3', human TGF-β1 #2, 5'-ACCAGAAATACAGCAACAATT-3', human TGF-β2 #1, 5'-CACTTTTGTACCATCTAATAA-3' and human TGF-β2 #2, 5'-AAGATTGAACAGCTTTCTAAT-3'. The transfected cells were then used for experiments conducted at 48 h post-transfection.

Quantitative RT-PCR was performed as described previously (Hasegawa et al., 2022). Forward and reverse primers were as follows: rat iNos, 5'-CACCTTGGAGTTCACCCAGT-3' and 5′-ACCACTCGTACTTGGGATGC-3′; rat Vimentin, 5'-AGATCGATGTGGACGTTTCC-3' and 5'-CACCTGTCTCCGGTATTCGT-3'; rat p65, 5'-AACACTGCCGAGCTCAAGAT-3' and 5'-CATCGGCTTGAGAAAAGGAG-3'; human TGF-β1, 5'-CCTCCTTGGCGTAGTAGTCCG-3' and 5'-GGGACTATCCACCTGCAAAGA-3'; human TGF-β2, 5'-CTCCATTGCTGAGACGTCAA-3' and 5'-CGCCAAGGAGGTTTACAAAA-3'; rat GAPDH, 5'-CTCATGACCACAGTCCATGC-3' and 5'-TTCAGCTCTGGGATGACCTT-3'; human GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-TGGTGAAGACGCCAGTGGA-3'.

Patients and immunohistochemical staining

The ages of radicular cyst patients ranged from 18 to 82 years (median, 49 years). The ages of dentigerous cyst patients ranged from 7 to 17 years (median, 12 years). Radicular cyst (n = 52; all of the patients were not in post-marsupialization, and 45 received

endodontic treatments and 7 did not receive any treatments) and dentigerous cyst (n = 6; all of the patients did not receive any treatments and all of the specimens did not show inflammatory responses with infiltration of immune cells) specimens from patients who underwent surgery at the Department of Oral and Maxillofacial Surgeries or the Department of Pediatric and Special Needs Dentistry, Kyushu University Hospital, Japan, from Oct 2017 to Feb 2023 were examined in this study. The study protocol was approved by the ethical review board of the Local Ethical Committee of Kyushu University, Japan (#2022-50). All specimens were fixed in 10% (v/v) formalin and embedded in paraffin blocks. Subsequently, the paraffin-embedded specimens were sliced into 4-μm-thick sections, stained with Haemotoxylin-eosin, and examined by three experienced pathologists to confirm diagnoses.

Immunohistochemical staining was performed on 4-μm-thick paraffin sections. Antigen retrieval (Dako, Carpentaria, CA, USA), elimination of the endogenous peroxidase activity (Dako) and blocking (Dako) were carried out as previously mentioned (Mikami et al., 2018; Mikami et al., 2017). Then the sections were reacted with each primary antibody (used at 1:1000 for p65, 1:500 for Smad2/3, 1:300 for E-cadherin and Ki-67) at 4°C overnight. The sections were incubated with a secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan) for 1 h at room temperature (RT). The immunoreactivity was visualized with DAB substrate solution (Histofine). Subsequently, the sections were counterstained with hematoxylin. When the total epithelial cells of a specimen showed >10% staining with a similar nucleus and/or cytoplasm staining pattern, the results were defined as p65-positive (Lessard et al., 2005). The nuclear Smad2/3 and Ki-67 expressions were considered positive when the total

epithelial cells of a section showed >50% (Karathanasi et al., 2013; Liu et al., 2020) and >5% (Martín-Hernán et al., 2022) staining, respectively.

Immunofluorescences staining

Mandibles of 8-week-old female mice were fixed for 48 h with 4% paraformaldehyde buffered by phosphate buffered saline (PBS) and were decalcified using 0.5M EDTA at 4°C for two weeks, changed every second or third day. Subsequently, the paraffin-embedded mandibles were sliced into 4-μm-thick sections and were stained for histological examination with Haemotoxylin-eosin.

SF2, STPLF-E and 1-11 cells were fixed in 4% paraformaldehyde buffered by PBS for 30 min at RT. After that, cells were permeabilized in PBS containing 0.5% (w/v) Triton X-100 and 40 mg/ml BSA (Wako) for 10 min at RT, and then blocked with 1% BSA to prevent non-specific binding for 30 min at RT.

The cells and the sections were incubated with each primary antibody (used at 1:300 for p65, CK14, Smad2/3 or Ki-67) for 24 h at RT, and then with secondary antibody (Jackson) and Hoechst 33342 (Dojindo, Kumamoto, Japan) for 3 h at RT in accordance with the manufacturer's protocols (Jackson). The samples were viewed with an All-in-one Fluorescence Microscope BZ 9000 (Keyence, Osaka, Japan) (Fujii et al., 2022).

Statistical analysis

Statistical analyses were performed using JMP Pro 16 software. Significant differences were determined using Fisher's exact test for clinicopathological analyses. For other experiments, significant differences were determined using Student's *t*-test and one-way

ANOVA with Tukey's test. A *P* value of <0.05 was considered to indicate statistical significance.

RESULTS

NF-κB signaling is activated in the proliferating lining epithelia of radicular cysts

First, we examined the activation of TGF-β signalling in ERMs. Immunofluorescence images of 8-week-old mouse molar specimens revealed Smad2/3-positive signals and less Ki-67-positive signals in ERMs, which were positive for CK14; a marker of odontogenic epithelial cells (Kawano et al., 2004) (Figure 2a), suggesting that activated TGF-β signalling might suppress ERMs proliferation under physiological conditions.

In contrast, immunohistochemical analyses revealed that the lining epithelia of radicular cysts, which are derived from ERMs (Rios et al., 2023), were frequently positive for p65 and Ki-67, while the specimens were less frequently positive for Smad2/3 (Figure 2b). In radicular cyst specimens from human patients, p65 was detected in nucleus and/or cytoplasm of epithelial cells in 44/52 (84.6%) specimens (Figure 2c; upper panel). Meanwhile, the p65-positive rate in dentigerous cysts, which are non-inflammatory developmental cysts, was significantly lower than that in radicular cysts ($p<0.01$; Fisher's exact test) (Figure 2c; upper panel). Consistent with the report of the frequent TGF-β expression in epithelial and stromal cells in dentigerous cysts (Alaeddini et al., 2017), Smad2/3 positivity was detected in 6/6 (100%) dentigerous cysts but only in 13/52 (25.0%) radicular cysts, showing a significant difference ($p<0.01$; Fisher's exact test) (Figure 2c; lower panel, Figure S1). These data indicate that the localization of p65 and Smad2/3 may depend on the presence or absence of inflammation. Importantly, in radicular cysts, Ki-67 positivity was detected in 34/44 (77.3%) of p65-positive cases, but only in 3/8 (37.5%) of p65-negative cases, showing a significant difference ($p<0.05$; Fisher's exact test) (Figure 2d). These results suggest that p65 signaling may induce the proliferative capabilities in the lining epithelia of radicular cysts. It could be difficult to

clarify the cytokine pathway association between TGF-β-Smad signalling and IL-1β-p65 signaling on the regulation of odontogenic epithelial cell proliferation only by pathological specimens, therefore, odontogenic epithelial cell lines were used to elucidate the association in the following experiments.

The proliferation capabilities of odontogenic epithelial cells are suppressed by TGF-β1 and TGF-β2 derived from PDL cells.

To evaluate the involvement of PDL cell-derived TGF-βs in regulating odontogenic epithelial cell growth, SF2 cells were co-cultured with two different human PDL fibroblastic cells, such as STPLF-E cells and 1-11 cells. The ratio of Ki-67-positivity of SF2 cells was decreased in co-culture with each PDL cells (Figure 3a, Figure S2a), suggesting that some factors form PDL cells or direct contact between PDL cells and SF2 cells would suppress the proliferative capabilities of SF2 cells. In contrast, A83-01, a TGF-β receptor I ALK5 inhibitor, did not change its ratio (Figures 3b), suggesting endogenous TGF-β signaling would be insufficient to regulate the cellular growth of SF2. Next, we co-cultured SF2 cells (beforehand with/without A83-01) and PDL cells (beforehand with/without siRNAs against TGF-β1 and TGF-β2). These PDL cells showed the lower expression of *TGF-β3* (data not shown). The co-culture experiments showed that either A83-01 stimulation or two different combinations of TGF-β1 and TGF-β2 siRNAs reversed the PDL cells-dependent growth suppression of SF2 cells (Figures 3c,d, Figures S2b-e). Moreover, a combination of A83-01 stimulation and two different combinations of TGF-β1 and TGF-β2 siRNAs fully rescued the reduction of SF2 cells proliferative capabilities in the co-culture with STPLF-E cells (Figure 3e). These data suggest that odontogenic epithelial cellular growth would be suppressed by

TGF-β1 and TGF-β2 derived from surrounding PDL cells, which may mimic the physiological conditions shown in Figure 2a.

TGF-β1-dependent growth suppression is inhibited by activation of IL-1β-p65 signaling in odontogenic epithelial cells.

To gain further mechanistic insight into the signaling association of IL-1β-p65 and TGF-β-Smad, we performed following experiments. TGF-β1 stimulation mainly induced Smad2 phosphorylation and *Vimentin* expression, a transcriptional target of TGF-β signaling, in a time dependent manner (Figures S3a,b) and reduced the cellular growth of SF2 cells even at low doses of TGF-β1 (Figure 4a). These data suggested the activation of TGF-β signaling negatively regulates odontogenic epithelial cells proliferation. IL-1β stimulation induced p65 translocation into the nucleus and the expression of *inducible Nitric oxide synthase* (*iNos*), a p65 direct target gene (Simon et al., 2015), (Figures S3c,d), suggesting that IL-1β stimulation activates NF-κB signaling in SF2 cells. Importantly, IL-1β stimulation reduced the TGF-β1-dependent phosphorylation of Smad2 through p65 transactivation (Figures 4b,c, Figure S3e). Notably, IL-1β stimulation reversed TGF-β1-dependent suppressed-cellular growth and increased-expression of *Vimentin* (Figures 4d,e, Figure S3f). Although it is well known that Vimentin is a marker of mesenchymal cells, we examined the expression of *Vimentin* as a transcriptional target of TGF-β signaling. Collectively, these results suggest that IL-1β-p65 signaling suppresses TGF-β1-dependent phenotypes of odontogenic epithelial cells, including their cell proliferation and gene expression.

DISCUSSION

Cyst formation of the jaws is frequently associated with anatomical properties, wherein odontogenic epithelial cells are surrounded by mesenchymal PDL cells under physiological conditions. In this study, we demonstrated that cytokine pathways may play a role in stimulating quiescent odontogenic cells to proliferate into epithelial lining cells of radicular cysts of the jaws using pathological specimens and cell lines.

Here, we used the pathological specimens, such as radicular cysts (an inflammatory cyst) and dentigerous cysts (a non-inflammatory developmental cyst) to explore the immunohistochemical expression of Smad2/3, p65 and Ki-67 (see Figure 2b, Figure S1). We aimed to assess the effects of cytokine pathway association between TGF-β signaling and IL-1β signaling on the regulation of odontogenic epithelial cell proliferation. Therefore, we examined the expression of downstream molecules of their signallings, such as Smad2/3, the expression of which was detected in the nucleus when TGF-βs are treated (Fink et al., 2003), p65, the expression of which was detected in the nucleus when IL-1β is treated (Simon et al., 2015), and Ki-67, which is a marker of proliferating cells.

Meanwhile, the presence or absence of inflammation seems to complicate the situation. For instance, some parts of dentigerous cysts may be inflamed and in some, long-standing, radicular cysts the inflammation can subside. In the current study, we omitted the specimens of dentigerous cysts with inflammatory conditions, which accompany with the infiltration of immune cells, and selected the specimens of non-inflammatory dentigerous cysts corresponding to clinical history. Similarly, the specimens of radicular cysts were selected as inflammatory cyst with the infiltration of immune cells and corresponding to the clinical inflammatory history. Since these

sampling might result in the patients with the current dentigerous cysts being younger than that with radicular cysts, the protein expression patterns might be affected by the differences of ages, which would be raised a limitation of this study. Therefore, further study needs to confirm that the odontogenic epithelial cell proliferation of dentigerous cysts with inflammation could be regulated by the cytokine pathways, such as IL-1β-p65 signaling and TGF-β-Smad signaling, and the association would be dependent on the patient age or not.

The experimental settings using odontogenic epithelial cells and PDL cells revealed that TGF-βs secreted by PDL cells and its downstream Smad2/3 signaling maintain the resting proliferating state of epithelial cells in the steady state, which would be reversed by inflammatory signaling, such as IL-1β-p65 signaling. SF2 cells exhibited cluster-like structures when cultured with or without PDL cells (see Figure 3), which was like to mimic *in vivo* situations, such as ERMs surrounded by PDL cells. Furthermore, the phenotypes of SF2 both in co-culture and in stimulations with TGF-β1 were similar, therefore the current co-culture system appeared to be a convenient experimental model mimicking *in vivo* situations to explore the biology of PDL tissues.

Since a previous report showed that IL-1β stimulation reduced TGF-β1 expression in PDL cells (Fujii et al., 2010), IL-1β stimulation would suppress TGF-β1 signaling at the levels of ligands and intracellular signaling. Considering the patient's dental disease with bacterial infection, (e.g., periodontitis and dental caries), it is reasonable that inflammatory IL-1β-p65 signaling may have affected the behaviors of odontogenic epithelial cells and that the reversibility of its effects would be dependent on the signaling. When odontogenic epithelial cells harbor some genetic mutations, such as

BRAF V600E mutation shown in ameloblastoma (Brown et al., 2014; Kurppa et al., 2014), their proliferative capabilities would not be reversible.

Since the lining epithelium of apical cysts may act to prevent the extent of inflammatory region to the surrounding alveolar bone (Lin et al., 2007), further study to clarify the effects of odontogenic epithelial cell proliferation on bacterial infections is needed. Furthermore, in vivo lineage tracing in mice should demonstrate that inflammatory cytokine pathways stimulate quiescent odontogenic cell rests (e.g., ERMs) into proliferating epithelial lining cells of radicular cysts. It is intriguing to speculate that conventional endodontic therapies, a routine treatment when due to caries, orient the balance of IL-1β-p65 signaling and TGF-β-Smad2 signaling in radicular cysts to transit to the suppressed cell growth of ERMs.

CONCLUSIONS

These results suggest that IL-1β-p65 signaling promotes odontogenic epithelial cell proliferation through suppressing TGF-β-Smad2 signaling, which would be involved in the pathogenesis of radicular cysts.

Supporting Information

Supplementary figure legends

FIGURE S1. NF-κB signaling is hardly activated in the odontogenic epithelial cells in dentigerous cysts.

FIGURE S2. The proliferation capabilities of odontogenic epithelial cells are suppressed by TGF-β1 and TGF-β2 derived from PDL cells.

FIGURE S3. TGF-β1-dependent growth suppression is inhibited by activation of IL-1β-p65 signaling in odontogenic epithelial cells.

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Figure Legends

FIGURE 1 PRILE 2021 flowchart illustrating the steps involved in conducting the present study.

FIGURE 2 NF-κB signaling is activated in the proliferating lining epithelia of radicular cysts.

(a) (Left panels) Hematoxylin-eosin stained images of 8-week-old mouse mandible. (Right panels) Sections of 8-week-old mouse mandible were stained with anti-CK14, anti-Smad2/3 and anti-Ki-67 antibodies and Hoechst 33342. The boxes show enlarged image of PDL of the second molar and ERMs in the PDL. Solid lines and dotted lines indicate the borders between cementum and PDL, PDL and alveolar bone (AB), respectively. An arrowhead indicates ERMs in the PDL. (b-d) Radicular cyst tissues (n = 52) and dentigerous cyst tissues without inflammation (n = 6) were stained with anti-p65, anti-Smad2/3 and anti-Ki-67 antibodies and hematoxylin. (b) Representative images of radicular cyst. The boxes indicate enlarged images. (c) Percentages of p65 (upper panel) and Smad2/3 (lower panel)-positive or -negative cases in radicular cyst tissues and dentigerous cyst tissues are shown. (d) The number of cases with p65-positive or -negative and Ki-67-positive or -negative in radicular cyst tissues was examined. Scale bars, 1 mm (a,b; upper panels), 200 μm (b; middle panels), 50 μm (a,b; lower panels). Statistical analyses were performed by Fisher's exact test. M1; first molar, M2; second molar, M3; third molar.

FIGURE 3 The proliferation capabilities of odontogenic epithelial cells are suppressed by TGF-β1 and TGF-β2 derived from PDL cells.

(a-e) After the culture, the cells were stained with anti-CK14 (Green) and anti-Ki-67 (Red) antibodies and Hoechst 33342, and then Ki-67-positive SF2 cells and Hoechst 33342-stained SF2 cells were counted, respectively. Results are expressed as the percentage of Ki-67-positive cells compared with total Hoechst 33342-stained cells. SF2 cells were specifically positive for CK14. (a) SF2 cells were co-cultured with STPLF-E cells for 48 h and counted (n = 45,297). (b) SF2 cells were cultured with or without 1 μM A83-01 for 24 h, then reseeded, cultured for 48 h and counted (n = 67,420). (c) SF2 cells were cultured with or without 1 μM A83-01 for 24 h, then reseeded with or without STPLF-E cells for 48 h and counted (n = 23,874). (d) STPLF-E cells were transfected with control or two different human TGF-β1 and TGF-β2 siRNAs. Then, STPLF-E cells were co-cultured with SF2 cells for 48 h and counted (n = 24,400). (e) SF2 cells were cultured with or without 1 μM A83-01 for 24 h, then reseeded with or without STPLF-E cells, which were already transfected with control or two different human TGF-β1 and TGF-β2 siRNAs, for 48 h and counted (n = 23,516). Results are shown as means ± s.d. of three independent experiments. Scale bars, 50 μm. **P<0.01. *P<0.05.

FIGURE 4 TGF-β1-dependent growth suppression is inhibited by activation of IL-1β-p65 signaling in odontogenic epithelial cells.

(a) SF2 cells were cultured with or without TGF-β1 at indicated concentrations in the presence of 2% FBS for 48 h, and the relative cell numbers were quantified using the CyQUANT NF cell proliferation assay. (b,c) Cell lysates were probed with indicated antibodies. pSmad2 band intensities were quantified using NIH image software and the ratio of pSmad2/β-actin was presented as fold-change compared with control cells. (b) SF2 cells were cultured with or without 10 ng/ml IL-1β for 1 h and cultured with or

without 1 ng/ml TGF-β1 for last 10 min. (c) SF2 cells were transfected with control or p65 siRNA for 48 h, then the cells were cultured with or without 10 ng/ml IL-1β for 1 h and cultured with or without 1 ng/ml TGF-β1 for last 10 min. (d,e) SF2 cells were cultured with or without 10 ng/ml TGF-β1 and/or 10 ng/ml IL-1β in the presence of 2% FBS for 48 h. (d) The relative cell numbers were quantified using the CyQUANT NF cell proliferation assay. (e) (Left panels) The cells were stained with anti-Ki-67 antibody and Hoechst 33342, and then Ki-67-positive cells and Hoechst 33342-stained cells were counted, respectively. (Right graph) Results are expressed as the percentage of Ki-67-positive cells compared with total Hoechst 33342-stained cells (n = 279,280). Scale bars, 50 μm. Results are shown as means ± s.d. of three independent experiments. $^{**}P<0.01$.

The IL-1β-p65 axis stimulates quiescent odontogenic epithelial cell rests via TGF-β signalling to promote cell proliferation of the lining epithelia in radicular cysts: A laboratory investigation

A short running title: Cell growth in radicular cyst

KEYWORDS: IL-1β, p65, TGF-β, Smad2/3, Proliferation, Radicular cyst

Abstract

Aim: Cyst formation of the jaws is frequently accompanied by the proliferation of odontogenic epithelial cells located in the periodontal ligament (PDL), which consists of heterozygous cells and includes the most fibroblasts. The lining epithelium of radicular cyst, an odontogenic cyst of inflammatory origin, is derived from the proliferation of the remnants of the Hertwig epithelial root sheath (odontogenic epithelial cell rests of Malassez; ERMs) in the PDL. ERMs are maintained at a lower proliferative state under physiological conditions, but the regulatory mechanisms underlying the inflammation-dependent enhanced-proliferative capabilities of ERMs are not fully understood. The aim of this study was to evaluate the effects of cytokine pathway association between TGF-β signaling and IL-1β signaling on the regulation of odontogenic epithelial cell proliferation using radicular cyst pathological specimens and odontogenic epithelial cell lines.

Methodology: Immunofluorescence analyses were performed to clarify the expression levels of Smad2/3 and Ki-67 in ERMs of 8-week-old mouse molar specimens. In radicular cyst ($n = 52$) and dentigerous cysts ($n = 6$) specimens from human patients, the expression of p65 (a main subunit of NF-κB), Smad2/3 and Ki-67 were investigated using immunohistochemical analyses. Odontogenic epithelial cells and PDL fibroblastic cells were co-cultured with or without an inhibitor or siRNAs. Odontogenic epithelial cells were cultured with or without TGF-β1 and IL-1β. The proliferative capabilities and Smad2 phosphorylation levels of odontogenic epithelial cells were examined.

Results: Immunohistochemically, Smad2/3-positivity was increased, and p65-positivity and Ki-67-positivity were decreased both in ERMs and in the epithelial cells in dentigerous cysts, a non-inflammatory developmental cyst. In contrast, p65-positive cells, along with the expression of Ki-67, were increased and Smad2/3-positive cells were

decreased in the lining epithelia of radicular cysts. Co-culture experiments with odontogenic epithelial cells and PDL fibroblastic cells revealed that PDL cells-derived TGF-β1/2 and their downstream signalling suppressed odontogenic epithelial cell proliferation. Moreover, TGF-β1 stimulation induced Smad2 phosphorylation and suppressed odontogenic epithelial cell proliferation, while IL-1β stimulation reversed these phenotypes through p65 transactivation.

Conclusions: These results suggest that IL-1β-p65 signaling promotes odontogenic epithelial cell proliferation through suppressing TGF-β-Smad2 signaling, which would be involved in the pathogenesis of radicular cysts.

INTRODUCTION

Radicular cysts are the most common cysts of the jaws, accounting for approximately 55% of all odontogenic cysts (Speight et al, 2017). Histopathologically, radicular cysts have a wall composed of inflamed fibrous or granulation tissue lined by non-keratinized stratified squamous epithelium. The remnants of the Hertwig epithelial root sheath (epithelial cell rests of Malassez; ERMs) show less proliferative capabilities under physiological conditions in the periodontal ligament (PDL) (Lin et al., 2007). Meanwhile, in the inflammation with apical periodontitis, the ERMs would proliferate, and the cyst would be lined by epithelium (Speight et al., 2017).

Some proinflammatory cytokines and growth factors, including interleukin (IL)-1, IL-6, epidermal growth factor, keratinocyte growth factor, tumor necrosis factor, transforming growth factor (TGF)-α, TGF-β and insulin-like growth factor, are capable of inducing epithelial cells to proliferate, thereby developing into an apical cyst (Lin et al., 2007; Rios et al., 2023). Clinically, most cases can be successfully treated by non-surgical endodontic therapy. In contrast, tooth extraction or apicectomy with enucleation of the cystic cavity is needed in some cases. After therapy, the lining epithelium of cyst will stop proliferating under anti-/less-inflammatory conditions (Lin et al., 2007; Rios et al., 2023). Therefore, it is thought that endodontic treatments suppress inflammatory responses, leading to reduce the proliferative capabilities of epithelial lining cells of radicular cysts. Meanwhile, the underlying inflammatory-mediated mechanisms that reversibly regulate odontogenic epithelial cellular growth are unclear.

IL-1β, a proinflammatory cytokine, transactivates nuclear factor-κB (NF-κB) signalling and its activation plays pivotal roles in regulating inflammation (Diep et al., 2022; Jimi et al., 2019). NF-κB, in which p65 is a main subunit, is a transcriptional factor

that regulates gene expression, resulting in controlling cell proliferation (Jimi et al., 2019). Although the expression of IL-1β and p65 in radicular cysts and its involvement in the progression of periapical lesions were reported (Yang et al., 2018; Yu et al., 2018), the precise function of NF-κB signaling in odontogenic epithelial cellular growth during the formation of radicular cysts is largely unknown.

TGF-β1 is a polypeptide member of the transforming growth factor-β superfamily of cytokines, which is secreted by platelets and inflammatory cells. It performs diverse cellular functions including the control of cellular growth and inflammation (Alaeddini et al., 2017). In addition, TGF-β1 is important for tissue repair because it increases angiogenesis and fibroblast collagen formation (Wikesjö et al., 1998). TGF-β signaling is activated by the binding of TGF-βs (TGF-β1, TGF-β2 and TGF-β3) to TGF-β type II receptor (TGFBR2), resulting in TGFBR1 activation. The receptor complex phosphorylates receptor-regulated Smads (R-Smads; Smad2, Smad3) and then forms a complex with the common Smad (Co-Smad; Smad4) and translocates into the nucleus to regulate transcription of target genes and cell proliferation (David & Massagué, 2018).

In the aspect of cellular growth, TGF-β signaling usually inhibits cell proliferation in a cell-type-specific manner (David & Massagué, 2018) and TGF-β1 is reportedly expressed in throughout the PDL tissue, which consist of heterozygous cells and in which fibroblasts are most abundant (Fujii et al., 2010). Therefore, we hypothesized that TGF-βs secreted by PDL cells may maintain the resting state of ERMs in the steady state. We aimed to clarify the effect of TGF-βs derived from PDL cells on odontogenic epithelial cell proliferation using cell lines to mimic the *in vivo* situations.

In this study, we found that the expression of p65 was elevated in lining epithelial cells with higher proliferative capabilities in pathological specimens of radicular cyst and demonstrated the role of IL-1β-p65 signaling in regulating odontogenic epithelial cell proliferation through the suppression of TGF-β-Smad2 signaling.

MATERIALS AND METHODS

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Figure 1) (Nagendrababu et al., 2021).

Cell lines, reagents and antibodies

SF2 (rat odontogenic epithelial cells), which is derived from the cervical loop at the apical end of the lower incisors from a Sprague-Dawley rat (Arakaki et al., 2012), were cultured in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) (Nagano et al., 2022). STPLF-E (immortalized human heterozygous PDL cells) (Fujii et al., 2006) and 1-11 (a clonal human PDL progenitor/stem cell line) (Fujii et al., 2008) were maintained in α-MEM (Invitrogen) containing 10% FBS (Invitrogen).

A83-01 (an inhibitor of TGF-β receptor I ALK5: which was used for the inhibition of TGF-β signaling), recombinant human TGF-β1 (for the activation of TGF-β signalling) and recombinant human IL-1β (for the activation of IL-1β signaling) were obtained from FUJI-FILM Wako (Osaka, Japan) and used in cell culture studies. Anti-p65 (8242S) (for western blotting, immunofluorescence and immunohistochemistry), anti-Smad2/3 (8685S) (for western blotting, immunofluorescence and immunohistochemistry) and anti-Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (8828S) (for western blotting) antibodies were obtained from cell signalling technology (CST) (Beverly, MA, USA). Anti-Cytokeratin 14 (CK14) (ab7800) (for immunofluorescence) and anti-Ki-67 (ab16667) (for immunohistochemistry and immunofluorescence) antibodies were obtained from Abcam (Cambridge, UK). Anti-E-

cadherin (610181) (for immunohistochemistry) antibody was obtained from BD Biosciences (San José, CA, USA). Anti-β-actin (A5441) (for western blotting) antibody was from Sigma-Aldrich (Steinheim, Germany).

Co-culture of odontogenic epithelial cells and PDL cells

SF2 cells (5.0×10^4 cells) and PDL cells (STPLF-E or 1-11 cells) (5.0×10^5 cells) were seeded in DMEM/F12 supplemented with 10% FBS for 48 h, where the epithelial cells and PDL cells would be directly contacted. In this study, these culture conditions were defined as 'co-culture'. Before the co-culture, SF2 cells were treated with 1 μM A83-01 for 24 h and/or STPLF-E or 1-11 cells were transfected with two different human TGF-β1 and TGF-β2 siRNAs for 48 h.

Cell proliferation assay

SF2 cells were cultured with TGF-β1 and/or IL-1β in the presence of 2% FBS for 48 h, and then the proliferative capabilities were evaluated using CyQUANT NF assay (Invitrogen) according to the manufacturer's protocol as previously described (Fujii et al., 2020; Hasegawa et al., 2021). Then, the fluorescence intensity was measured using FlexStation 3 microplate reader (Molecular Devices, Tokyo, Japan) at 485 nm excitation/530 nm emission and was shown as relative fluorescence units.

Knockdown of protein expression by siRNA and quantitative RT-PCR

The effects of protein knockdown by siRNA were analyzed as previously described (Alkhatib et al., 2023; Fujii et al., 2019). Briefly, siRNAs (final concentration 20 nM) were transfected into SF2, STPLF-E and 1-11 cells using Lipofectamine RNAiMAX

(Invitrogen). The following target sequences were used: randomized control, 5'-CAGTCGCGTTTGCGACTGG-3', rat p65, 5'-AGCCATAAGACAGTCTTTACT-3', human TGF-β1 #1, 5'-CAGCATATATATGTTCTTCAA-3', human TGF-β1 #2, 5'-ACCAGAAATACAGCAACAATT-3', human TGF-β2 #1, 5'-CACTTTTGTACCATCTAATAA-3' and human TGF-β2 #2, 5'-AAGATTGAACAGCTTTCTAAT-3'. The transfected cells were then used for experiments conducted at 48 h post-transfection.

Quantitative RT-PCR was performed as described previously (Hasegawa et al., 2022). Forward and reverse primers were as follows: rat iNos, 5'-CACCTTGGAGTTCACCCAGT-3' and 5′-ACCACTCGTACTTGGGATGC-3′; rat Vimentin, 5'-AGATCGATGTGGACGTTTCC-3' and 5'-CACCTGTCTCCGGTATTCGT-3'; rat p65, 5'-AACACTGCCGAGCTCAAGAT-3' and 5'-CATCGGCTTGAGAAAAGGAG-3'; human TGF-β1, 5'-CCTCCTTGGCGTAGTAGTCCG-3' and 5'-GGGACTATCCACCTGCAAAGA-3'; human TGF-β2, 5'-CTCCATTGCTGAGACGTCAA-3' and 5'-CGCCAAGGAGGTTTACAAAA-3'; rat GAPDH, 5'-CTCATGACCACAGTCCATGC-3' and 5'-TTCAGCTCTGGGATGACCTT-3'; human GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-TGGTGAAGACGCCAGTGGA-3'.

Patients and immunohistochemical staining

The ages of radicular cyst patients ranged from 18 to 82 years (median, 49 years). The ages of dentigerous cyst patients ranged from 7 to 17 years (median, 12 years). Radicular cyst (n = 52; all of the patients were not in post-marsupialization, and 45 received

endodontic treatments and 7 did not receive any treatments) and dentigerous cyst (n = 6; all of the patients did not receive any treatments and all of the specimens did not show inflammatory responses with infiltration of immune cells) specimens from patients who underwent surgery at the Department of Oral and Maxillofacial Surgeries or the Department of Pediatric and Special Needs Dentistry, Kyushu University Hospital, Japan, from Oct 2017 to Feb 2023 were examined in this study. The study protocol was approved by the ethical review board of the Local Ethical Committee of Kyushu University, Japan (#2022-50). All specimens were fixed in 10% (v/v) formalin and embedded in paraffin blocks. Subsequently, the paraffin-embedded specimens were sliced into 4-μm-thick sections, stained with Haemotoxylin-eosin, and examined by three experienced pathologists to confirm diagnoses.

Immunohistochemical staining was performed on 4-μm-thick paraffin sections. Antigen retrieval (Dako, Carpentaria, CA, USA), elimination of the endogenous peroxidase activity (Dako) and blocking (Dako) were carried out as previously mentioned (Mikami et al., 2018; Mikami et al., 2017). Then the sections were reacted with each primary antibody (used at 1:1000 for p65, 1:500 for Smad2/3, 1:300 for E-cadherin and Ki-67) at 4°C overnight. The sections were incubated with a secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan) for 1 h at room temperature (RT). The immunoreactivity was visualized with DAB substrate solution (Histofine). Subsequently, the sections were counterstained with hematoxylin. When the total epithelial cells of a specimen showed >10% staining with a similar nucleus and/or cytoplasm staining pattern, the results were defined as p65-positive (Lessard et al., 2005). The nuclear Smad2/3 and Ki-67 expressions were considered positive when the total

epithelial cells of a section showed >50% (Karathanasi et al., 2013; Liu et al., 2020) and >5% (Martín-Hernán et al., 2022) staining, respectively.

Immunofluorescences staining

Mandibles of 8-week-old female mice were fixed for 48 h with 4% paraformaldehyde buffered by phosphate buffered saline (PBS) and were decalcified using 0.5M EDTA at 4°C for two weeks, changed every second or third day. Subsequently, the paraffin-embedded mandibles were sliced into 4-μm-thick sections and were stained for histological examination with Haemotoxylin-eosin.

SF2, STPLF-E and 1-11 cells were fixed in 4% paraformaldehyde buffered by PBS for 30 min at RT. After that, cells were permeabilized in PBS containing 0.5% (w/v) Triton X-100 and 40 mg/ml BSA (Wako) for 10 min at RT, and then blocked with 1% BSA to prevent non-specific binding for 30 min at RT.

The cells and the sections were incubated with each primary antibody (used at 1:300 for p65, CK14, Smad2/3 or Ki-67) for 24 h at RT, and then with secondary antibody (Jackson) and Hoechst 33342 (Dojindo, Kumamoto, Japan) for 3 h at RT in accordance with the manufacturer's protocols (Jackson). The samples were viewed with an All-in-one Fluorescence Microscope BZ 9000 (Keyence, Osaka, Japan) (Fujii et al., 2022).

Statistical analysis

Statistical analyses were performed using JMP Pro 16 software. Significant differences were determined using Fisher's exact test for clinicopathological analyses. For other experiments, significant differences were determined using Student's *t*-test and one-way

ANOVA with Tukey's test. A *P* value of <0.05 was considered to indicate statistical significance.

RESULTS

NF-κB signaling is activated in the proliferating lining epithelia of radicular cysts

First, we examined the activation of TGF-β signalling in ERMs. Immunofluorescence images of 8-week-old mouse molar specimens revealed Smad2/3-positive signals and less Ki-67-positive signals in ERMs, which were positive for CK14; a marker of odontogenic epithelial cells (Kawano et al., 2004) (Figure 2a), suggesting that activated TGF-β signalling might suppress ERMs proliferation under physiological conditions.

In contrast, immunohistochemical analyses revealed that the lining epithelia of radicular cysts, which are derived from ERMs (Rios et al., 2023), were frequently positive for p65 and Ki-67, while the specimens were less frequently positive for Smad2/3 (Figure 2b). In radicular cyst specimens from human patients, p65 was detected in nucleus and/or cytoplasm of epithelial cells in 44/52 (84.6%) specimens (Figure 2c; upper panel). Meanwhile, the p65-positive rate in dentigerous cysts, which are non-inflammatory developmental cysts, was significantly lower than that in radicular cysts ($p<0.01$; Fisher's exact test) (Figure 2c; upper panel). Consistent with the report of the frequent TGF-β expression in epithelial and stromal cells in dentigerous cysts (Alaeddini et al., 2017), Smad2/3 positivity was detected in 6/6 (100%) dentigerous cysts but only in 13/52 (25.0%) radicular cysts, showing a significant difference ($p<0.01$; Fisher's exact test) (Figure 2c; lower panel, Figure S1). These data indicate that the localization of p65 and Smad2/3 may depend on the presence or absence of inflammation. Importantly, in radicular cysts, Ki-67 positivity was detected in 34/44 (77.3%) of p65-positive cases, but only in 3/8 (37.5%) of p65-negative cases, showing a significant difference ($p<0.05$; Fisher's exact test) (Figure 2d). These results suggest that p65 signaling may induce the proliferative capabilities in the lining epithelia of radicular cysts. It could be difficult to

clarify the cytokine pathway association between TGF-β-Smad signalling and IL-1β-p65 signaling on the regulation of odontogenic epithelial cell proliferation only by pathological specimens, therefore, odontogenic epithelial cell lines were used to elucidate the association in the following experiments.

The proliferation capabilities of odontogenic epithelial cells are suppressed by TGF-β1 and TGF-β2 derived from PDL cells.

To evaluate the involvement of PDL cell-derived TGF-βs in regulating odontogenic epithelial cell growth, SF2 cells were co-cultured with two different human PDL fibroblastic cells, such as STPLF-E cells and 1-11 cells. The ratio of Ki-67-positivity of SF2 cells was decreased in co-culture with each PDL cells (Figure 3a, Figure S2a), suggesting that some factors form PDL cells or direct contact between PDL cells and SF2 cells would suppress the proliferative capabilities of SF2 cells. In contrast, A83-01, a TGF-β receptor I ALK5 inhibitor, did not change its ratio (Figures 3b), suggesting endogenous TGF-β signaling would be insufficient to regulate the cellular growth of SF2. Next, we co-cultured SF2 cells (beforehand with/without A83-01) and PDL cells (beforehand with/without siRNAs against TGF-β1 and TGF-β2). These PDL cells showed the lower expression of *TGF-β3* (data not shown). The co-culture experiments showed that either A83-01 stimulation or two different combinations of TGF-β1 and TGF-β2 siRNAs reversed the PDL cells-dependent growth suppression of SF2 cells (Figures 3c,d, Figures S2b-e). Moreover, a combination of A83-01 stimulation and two different combinations of TGF-β1 and TGF-β2 siRNAs fully rescued the reduction of SF2 cells proliferative capabilities in the co-culture with STPLF-E cells (Figure 3e). These data suggest that odontogenic epithelial cellular growth would be suppressed by

TGF-β1 and TGF-β2 derived from surrounding PDL cells, which may mimic the physiological conditions shown in Figure 2a.

TGF-β1-dependent growth suppression is inhibited by activation of IL-1β-p65 signaling in odontogenic epithelial cells.

To gain further mechanistic insight into the signaling association of IL-1β-p65 and TGF-β-Smad, we performed following experiments. TGF-β1 stimulation mainly induced Smad2 phosphorylation and *Vimentin* expression, a transcriptional target of TGF-β signaling, in a time dependent manner (Figures S3a,b) and reduced the cellular growth of SF2 cells even at low doses of TGF-β1 (Figure 4a). These data suggested the activation of TGF-β signaling negatively regulates odontogenic epithelial cells proliferation. IL-1β stimulation induced p65 translocation into the nucleus and the expression of *inducible Nitric oxide synthase* (*iNos*), a p65 direct target gene (Simon et al., 2015), (Figures S3c,d), suggesting that IL-1β stimulation activates NF-κB signaling in SF2 cells. Importantly, IL-1β stimulation reduced the TGF-β1-dependent phosphorylation of Smad2 through p65 transactivation (Figures 4b,c, Figure S3e). Notably, IL-1β stimulation reversed TGF-β1-dependent suppressed-cellular growth and increased-expression of *Vimentin* (Figures 4d,e, Figure S3f). Although it is well known that Vimentin is a marker of mesenchymal cells, we examined the expression of *Vimentin* as a transcriptional target of TGF-β signaling. Collectively, these results suggest that IL-1β-p65 signaling suppresses TGF-β1-dependent phenotypes of odontogenic epithelial cells, including their cell proliferation and gene expression.

DISCUSSION

Cyst formation of the jaws is frequently associated with anatomical properties, wherein odontogenic epithelial cells are surrounded by mesenchymal PDL cells under physiological conditions. In this study, we demonstrated that cytokine pathways may play a role in stimulating quiescent odontogenic cells to proliferate into epithelial lining cells of radicular cysts of the jaws using pathological specimens and cell lines.

Here, we used the pathological specimens, such as radicular cysts (an inflammatory cyst) and dentigerous cysts (a non-inflammatory developmental cyst) to explore the immunohistochemical expression of Smad2/3, p65 and Ki-67 (see Figure 2b, Figure S1). We aimed to assess the effects of cytokine pathway association between TGF-β signaling and IL-1β signaling on the regulation of odontogenic epithelial cell proliferation. Therefore, we examined the expression of downstream molecules of their signallings, such as Smad2/3, the expression of which was detected in the nucleus when TGF-βs are treated (Fink et al., 2003), p65, the expression of which was detected in the nucleus when IL-1β is treated (Simon et al., 2015), and Ki-67, which is a marker of proliferating cells.

Meanwhile, the presence or absence of inflammation seems to complicate the situation. For instance, some parts of dentigerous cysts may be inflamed and in some, long-standing, radicular cysts the inflammation can subside. In the current study, we omitted the specimens of dentigerous cysts with inflammatory conditions, which accompany with the infiltration of immune cells, and selected the specimens of non-inflammatory dentigerous cysts corresponding to clinical history. Similarly, the specimens of radicular cysts were selected as inflammatory cyst with the infiltration of immune cells and corresponding to the clinical inflammatory history. Since these

sampling might result in the patients with the current dentigerous cysts being younger than that with radicular cysts, the protein expression patterns might be affected by the differences of ages, which would be raised a limitation of this study. Therefore, further study needs to confirm that the odontogenic epithelial cell proliferation of dentigerous cysts with inflammation could be regulated by the cytokine pathways, such as IL-1β-p65 signaling and TGF-β-Smad signaling, and the association would be dependent on the patient age or not.

The experimental settings using odontogenic epithelial cells and PDL cells revealed that TGF-βs secreted by PDL cells and its downstream Smad2/3 signaling maintain the resting proliferating state of epithelial cells in the steady state, which would be reversed by inflammatory signaling, such as IL-1β-p65 signaling. SF2 cells exhibited cluster-like structures when cultured with or without PDL cells (see Figure 3), which was like to mimic *in vivo* situations, such as ERMs surrounded by PDL cells. Furthermore, the phenotypes of SF2 both in co-culture and in stimulations with TGF-β1 were similar, therefore the current co-culture system appeared to be a convenient experimental model mimicking *in vivo* situations to explore the biology of PDL tissues.

Since a previous report showed that IL-1β stimulation reduced TGF-β1 expression in PDL cells (Fujii et al., 2010), IL-1β stimulation would suppress TGF-β1 signaling at the levels of ligands and intracellular signaling. Considering the patient's dental disease with bacterial infection, (e.g., periodontitis and dental caries), it is reasonable that inflammatory IL-1β-p65 signaling may have affected the behaviors of odontogenic epithelial cells and that the reversibility of its effects would be dependent on the signaling. When odontogenic epithelial cells harbor some genetic mutations, such as

BRAF V600E mutation shown in ameloblastoma (Brown et al., 2014; Kurppa et al., 2014), their proliferative capabilities would not be reversible.

Since the lining epithelium of apical cysts may act to prevent the extent of inflammatory region to the surrounding alveolar bone (Lin et al., 2007), further study to clarify the effects of odontogenic epithelial cell proliferation on bacterial infections is needed. Furthermore, in vivo lineage tracing in mice should demonstrate that inflammatory cytokine pathways stimulate quiescent odontogenic cell rests (e.g., ERMs) into proliferating epithelial lining cells of radicular cysts. It is intriguing to speculate that conventional endodontic therapies, a routine treatment when due to caries, orient the balance of IL-1β-p65 signaling and TGF-β-Smad2 signaling in radicular cysts to transit to the suppressed cell growth of ERMs.

CONCLUSIONS

These results suggest that IL-1β-p65 signaling promotes odontogenic epithelial cell proliferation through suppressing TGF-β-Smad2 signaling, which would be involved in the pathogenesis of radicular cysts.

Supporting Information

Supplementary figure legends

FIGURE S1. NF-κB signaling is hardly activated in the odontogenic epithelial cells in dentigerous cysts.

FIGURE S2. The proliferation capabilities of odontogenic epithelial cells are suppressed by TGF-β1 and TGF-β2 derived from PDL cells.

FIGURE S3. TGF-β1-dependent growth suppression is inhibited by activation of IL-1β-p65 signaling in odontogenic epithelial cells.

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Figure Legends

FIGURE 1 PRILE 2021 flowchart illustrating the steps involved in conducting the present study.

FIGURE 2 NF-κB signaling is activated in the proliferating lining epithelia of radicular cysts.

(a) (Left panels) Hematoxylin-eosin stained images of 8-week-old mouse mandible. (Right panels) Sections of 8-week-old mouse mandible were stained with anti-CK14, anti-Smad2/3 and anti-Ki-67 antibodies and Hoechst 33342. The boxes show enlarged image of PDL of the second molar and ERMs in the PDL. Solid lines and dotted lines indicate the borders between cementum and PDL, PDL and alveolar bone (AB), respectively. An arrowhead indicates ERMs in the PDL. (b-d) Radicular cyst tissues (n = 52) and dentigerous cyst tissues without inflammation (n = 6) were stained with anti-p65, anti-Smad2/3 and anti-Ki-67 antibodies and hematoxylin. (b) Representative images of radicular cyst. The boxes indicate enlarged images. (c) Percentages of p65 (upper panel) and Smad2/3 (lower panel)-positive or -negative cases in radicular cyst tissues and dentigerous cyst tissues are shown. (d) The number of cases with p65-positive or -negative and Ki-67-positive or -negative in radicular cyst tissues was examined. Scale bars, 1 mm (a,b; upper panels), 200 μm (b; middle panels), 50 μm (a,b; lower panels). Statistical analyses were performed by Fisher's exact test. M1; first molar, M2; second molar, M3; third molar.

FIGURE 3 The proliferation capabilities of odontogenic epithelial cells are suppressed by TGF-β1 and TGF-β2 derived from PDL cells.

(a-e) After the culture, the cells were stained with anti-CK14 (Green) and anti-Ki-67 (Red) antibodies and Hoechst 33342, and then Ki-67-positive SF2 cells and Hoechst 33342-stained SF2 cells were counted, respectively. Results are expressed as the percentage of Ki-67-positive cells compared with total Hoechst 33342-stained cells. SF2 cells were specifically positive for CK14. (a) SF2 cells were co-cultured with STPLF-E cells for 48 h and counted (n = 45,297). (b) SF2 cells were cultured with or without 1 μM A83-01 for 24 h, then reseeded, cultured for 48 h and counted (n = 67,420). (c) SF2 cells were cultured with or without 1 μM A83-01 for 24 h, then reseeded with or without STPLF-E cells for 48 h and counted (n = 23,874). (d) STPLF-E cells were transfected with control or two different human TGF-β1 and TGF-β2 siRNAs. Then, STPLF-E cells were co-cultured with SF2 cells for 48 h and counted (n = 24,400). (e) SF2 cells were cultured with or without 1 μM A83-01 for 24 h, then reseeded with or without STPLF-E cells, which were already transfected with control or two different human TGF-β1 and TGF-β2 siRNAs, for 48 h and counted (n = 23,516). Results are shown as means ± s.d. of three independent experiments. Scale bars, 50 μm. **P<0.01. *P<0.05.

FIGURE 4 TGF-β1-dependent growth suppression is inhibited by activation of IL-1β-p65 signaling in odontogenic epithelial cells.

(a) SF2 cells were cultured with or without TGF-β1 at indicated concentrations in the presence of 2% FBS for 48 h, and the relative cell numbers were quantified using the CyQUANT NF cell proliferation assay. (b,c) Cell lysates were probed with indicated antibodies. pSmad2 band intensities were quantified using NIH image software and the ratio of pSmad2/β-actin was presented as fold-change compared with control cells. (b) SF2 cells were cultured with or without 10 ng/ml IL-1β for 1 h and cultured with or

without 1 ng/ml TGF-β1 for last 10 min. (c) SF2 cells were transfected with control or p65 siRNA for 48 h, then the cells were cultured with or without 10 ng/ml IL-1β for 1 h and cultured with or without 1 ng/ml TGF-β1 for last 10 min. (d,e) SF2 cells were cultured with or without 10 ng/ml TGF-β1 and/or 10 ng/ml IL-1β in the presence of 2% FBS for 48 h. (d) The relative cell numbers were quantified using the CyQUANT NF cell proliferation assay. (e) (Left panels) The cells were stained with anti-Ki-67 antibody and Hoechst 33342, and then Ki-67-positive cells and Hoechst 33342-stained cells were counted, respectively. (Right graph) Results are expressed as the percentage of Ki-67-positive cells compared with total Hoechst 33342-stained cells (n = 279,280). Scale bars, 50 μm. Results are shown as means ± s.d. of three independent experiments. $^{**}P<0.01$.

PRILE 2021 Flowchart

RATIONALE/JUSTIFICATION (what the investigation contributes to the literature)

The present study provides new insights into the signaling crosstalk in odontogenic epithelial cells to control the proliferative capabilities. This study is important for the increase of the understanding of mechanisms involved in the pathogenesis of radicular cysts.

AIM/HYPOTHESIS

To investigate the role of the relationship between TGF-β signaling and NF-κB signaling in the regulation of odontogenic epithelial cell proliferation using pathological specimens and cell lines. Our hypothesis is that TGF-β signaling suppresses their proliferation under physiolosical conditions and that the activation of NF-κB signaling by inflammation inihibits TGF-β signaling and consequently induces their proliferation.

ETHICAL APPROVAL (IF APPLICABLE)

The study protocol was approved by the ethical review board of the Local Ethical Committee of Kyushu University, Japan (#2022-50).

SAMPLES

Cellular samples: SF2, STPLF-E and 1-11 were used for *in vitro* experiments, such as immunofluorescences staining, RT-PCR and western blotting.
Mouse samples: Mandibles of eight-week-old female mice were uesed for immunofluorescence staining.
Human samples: Radicular cysts and dentigerous cysts specimens were used for immunohistochemical staining.

EXPERIMENTAL AND CONTROL GROUPS, INCLUDE INDEPENDENT VARIABLES

Immunohistochemical staining: dentigerous cysts without inflammation (n=6) (control group), radicular cysts (n=52) (experimental group).
All *in vitro* experiments: SF2 cells cultured without or with solvent (n=3) (control group), SF2 cells cultured with reagents and/or with periodontal ligament cells (n=3) (experimental group).

OUTCOME(S) ASSESSED, INCLUDE DEPENDENT VARIABLES AND TYPE

The activation of TGF-β signaling and NF-κB signaling and cell proliferation.

METHOD USED TO ASSESS THE OUTCOME (S) AND WHO ASSESSED THE OUTCOME(S) (e.g. ELISA by laboratory staff)

The activation of TGF-β signaling by immunofluorescence or immunohistochemical staining of Smad2/3 and western blotting of phosphorylated Smad2/3. The activatin of NF-κB signaling by immunofluorescence or immunohistochemical staining of p65. Cell proliferation by immunofluorescence or immunohistochemical staining of Ki-67 and CyQUANT NF assay based on DNA content. All were conducted by the co-first authors.

RESULTS

Immunofluorescence and immunohistochemical analyses showed increased Smad2/3-positive cells and decreased NF-κB-p65-positive and Ki-67-positive cells both in ERMs and in the epithelial cells of non-inflammatory developmental dentigerous cysts. In contrast, p65-positive cells, along with the expression of Ki-67, were increased and Smad2/3-positive cells were decreased in the lining epithelia of radicular cysts. Co-culture experiments with odontogenic epithelial cells and periodontal ligament (PDL)-derived cells revealed that PDL cell-derived TGF-β1/2 suppressed odontogenic epithelial cell proliferation. Moreover, TGF-β1 stimulation induced Smad2 phosphorylation and suppressed odontogenic epithelial cell proliferation, while IL-1β stimulation reversed these phenotypes through p65 transactivation.

CONCLUSION(S)

IL-1β-p65 signaling promotes odontogenic epithelial cell proliferation through suppressing TGF-β-Smad2 signaling in the pathogenesis of radicular cysts.

CONFLICT OF INTEREST

No conflicts of interests were declared

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Figure 2

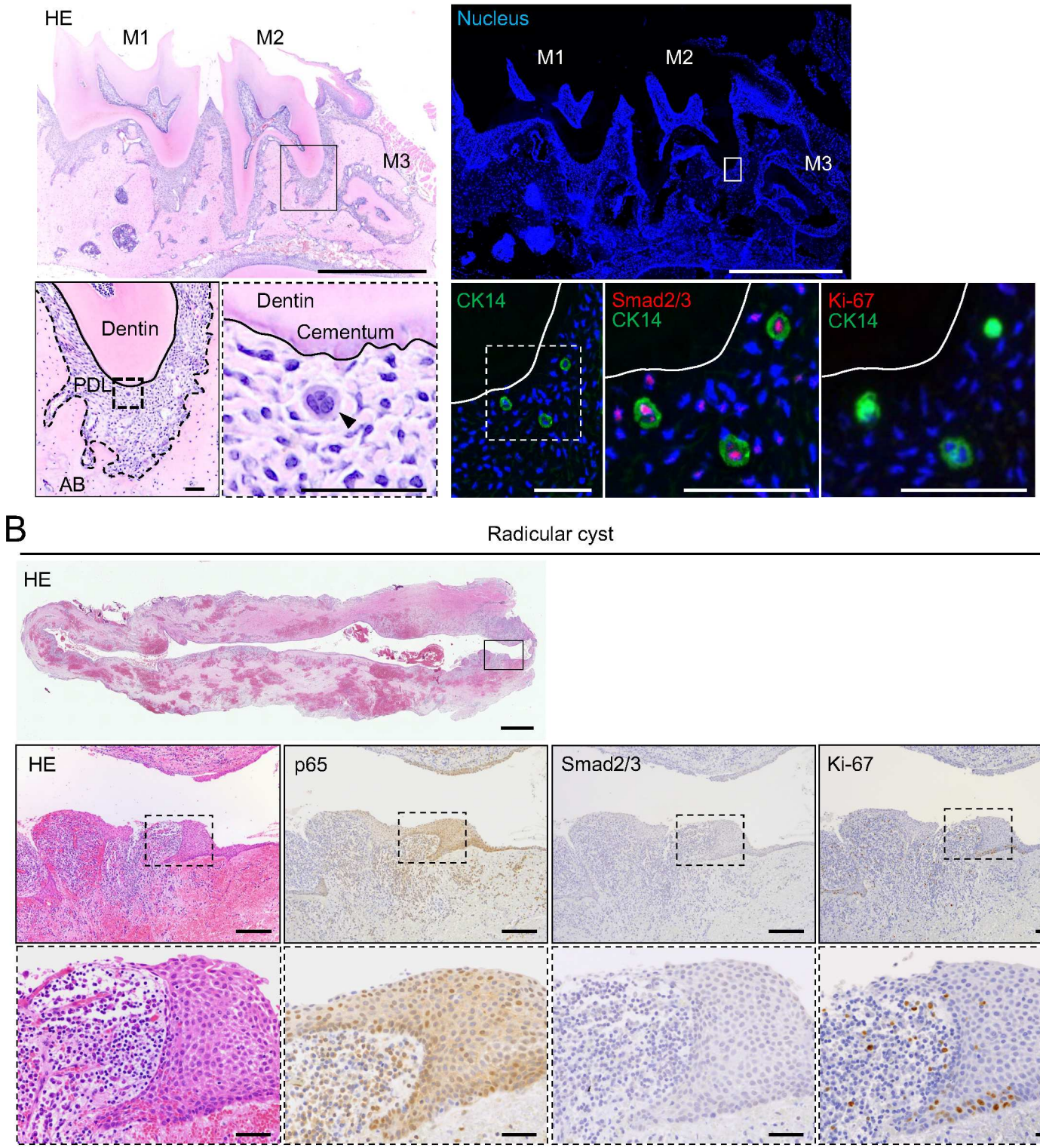

A
8-week-old mouse mandible
HE
M1
M2
M3
Nucleus
Dentin
PDL
AB
Cementum
CK14
Smad2/3
Ki-67
B
Radicular cyst
HE
p65
Smad2/3
Ki-67
C
Radicular cyst (n=52)
Dentigerous cyst (n=6)
15%
85%
83%
17%
Negative
Positive
P-value 0.0015
p65-positive cases (%)
75%
25%
100%
P-value 0.0007
Smad2/3-positive cases (%)
D
Radicular cysts
p65 expression in epithelium
Negative
Positive
Ki-67 expression in epithelium
+
-
3
34
5
10
P-value 0.0358

Figure 3

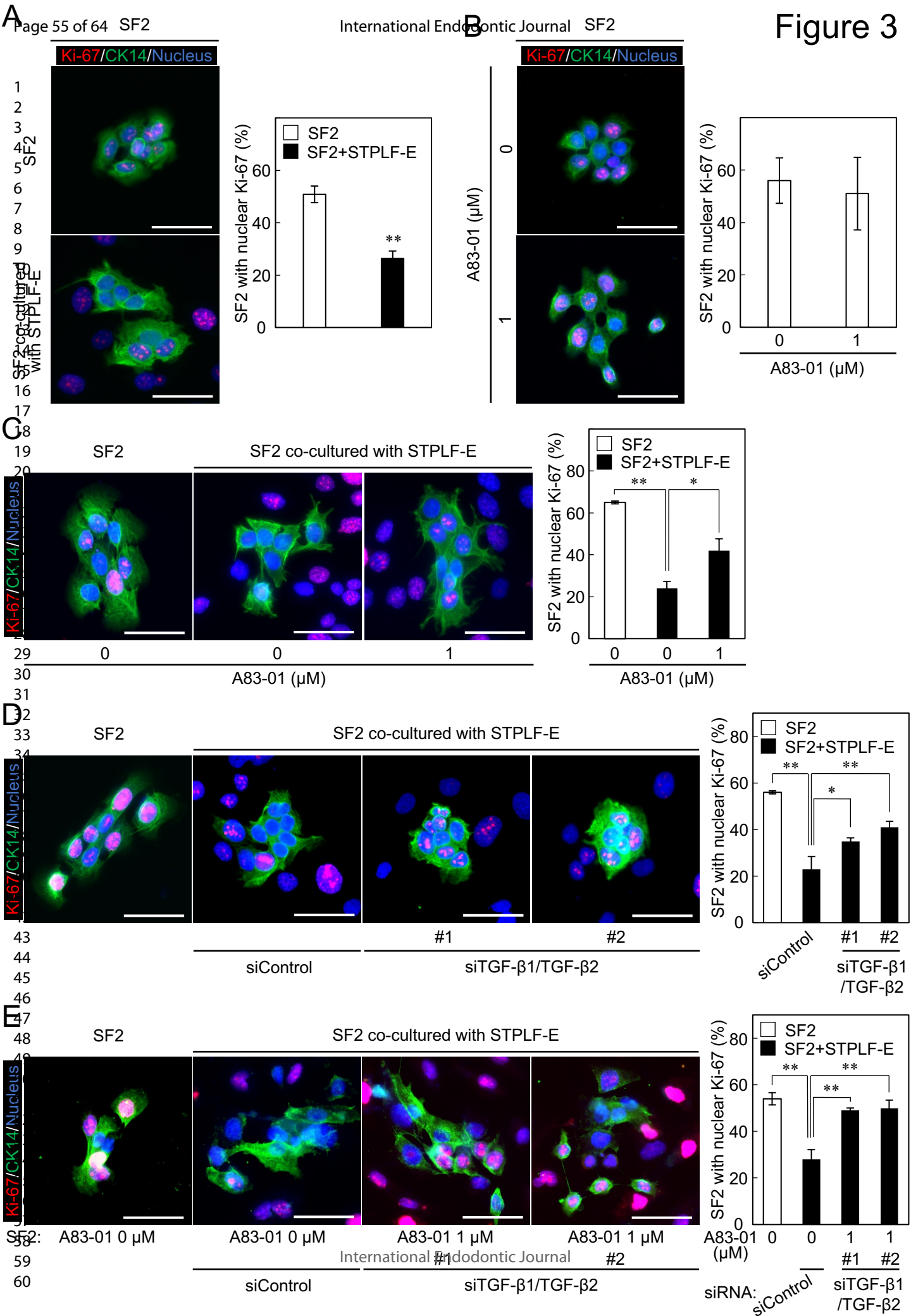
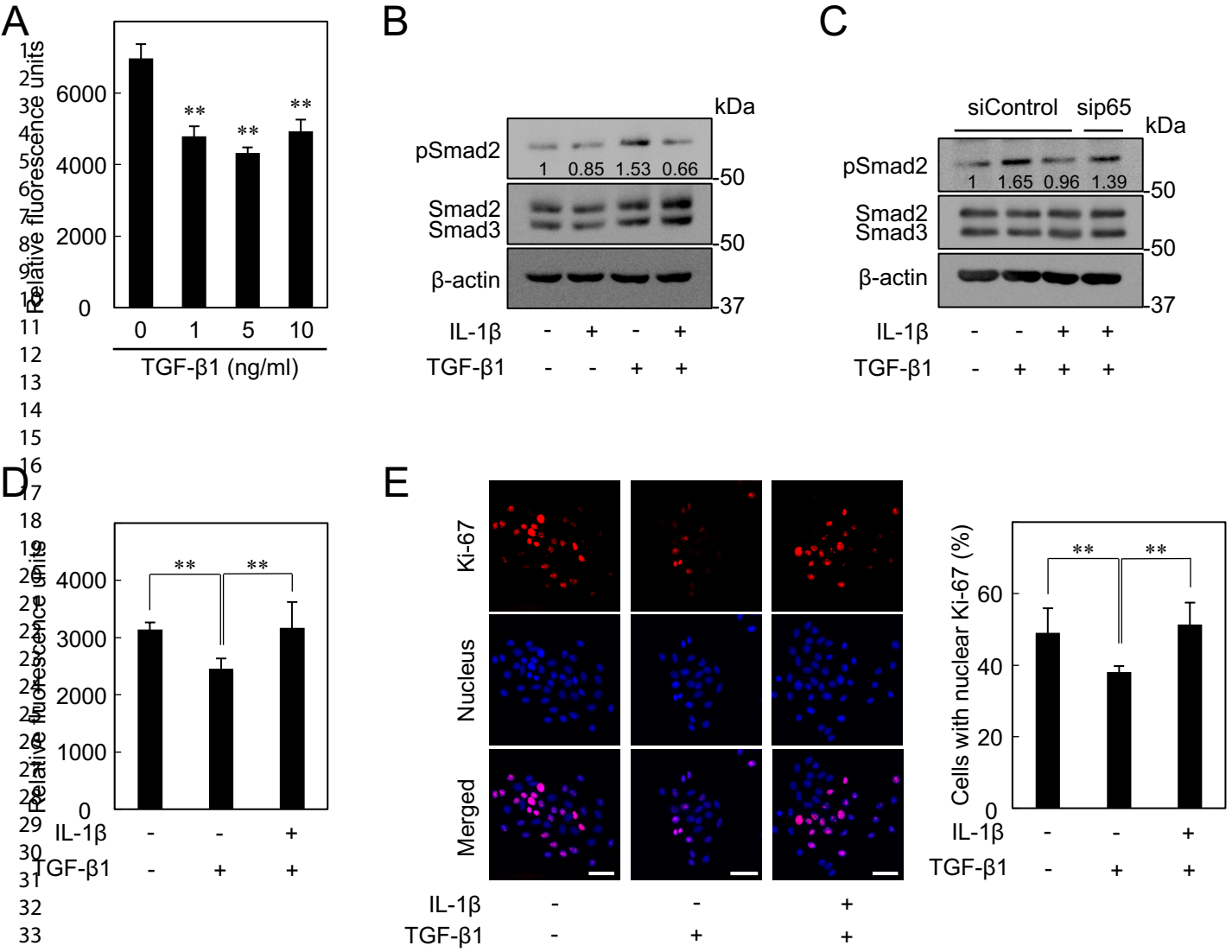

A
Ki-67/CK14/Nucleus
SF2
SF2 co-cultured with STPLF-E
SF2
SF2+STPLF-E
SF2 with nuclear Ki-67 (%)
**
B
Ki-67/CK14/Nucleus
A83-01 (μM)
0
1
SF2 with nuclear Ki-67 (%)
A83-01 (μM)
C
SF2
SF2 co-cultured with STPLF-E
Ki-67/CK14/Nucleus
0
0
1
A83-01 (μM)
SF2
SF2+STPLF-E
**
*
SF2 with nuclear Ki-67 (%)
A83-01 (μM)
D
SF2
SF2 co-cultured with STPLF-E
Ki-67/CK14/Nucleus
#1
#2
siControl
siTGF-β1/TGF-β2
SF2
SF2+STPLF-E
SF2 with nuclear Ki-67 (%)
siControl
siTGF-β1
/TGF-β2
E
SF2
SF2 co-cultured with STPLF-E
Ki-67/CK14/Nucleus
SF2: A83-01 0 μM
A83-01 0 μM
A83-01 1 μM
A83-01 1 μM
#1
#2
siControl
siTGF-β1/TGF-β2
SF2
SF2+STPLF-E
SF2 with nuclear Ki-67 (%)
A83-01 (μM)
0
0
1
1
siRNA:
siControl
siTGF-β1
/TGF-β2

Figure 4


A
Relative fluorescence units
6000
4000
2000
0
**
**
**
0
1
5
10
TGF-β1 (ng/ml)
B
kDa
pSmad2
1
0.85
1.53
0.66
50
Smad2
Smad3
50
β-actin
37
IL-1β - + - +
TGF-β1 - - + +
C
siControl
sip65
kDa
pSmad2
1
1.65
0.96
1.39
50
Smad2
Smad3
50
β-actin
37
IL-1β - - + +
TGF-β1 - + + +
D
Relative fluorescence units
4000
3000
2000
1000
0
**
**
IL-1β - - +
TGF-β1 - + +
E
Ki-67
Nucleus
Merged
IL-1β - - +
TGF-β1 - + +
Cells with nuclear Ki-67 (%)
60
40
20
0
**
**
IL-1β - - +
TGF-β1 - + +

PRILE 2021

Checklist of items to be included when reporting laboratory studies in Endodontology*

Section/ Topic	Item Number	Checklist Items	Reported on page number
Title	1a	The Title must identify the study as being laboratory-based, e.g. "laboratory investigation" or "*in vitro*," or "*ex vivo*" or another appropriate term	P1
	1b	The area/field of interest must be provided (briefly) in the Title	P1
Keywords	2a	At least two keywords related to the subject and content of the investigation must be provided	P1
Abstract	3a	The rationale/justification of what the investigation contributes to the literature and/or addresses a gap in knowledge must be provided	P2
	3b	The aim/objectives of the investigation must be provided	P2
	3c	The body of the Abstract must describe the materials and methods used in the investigation and include information on data management and statistical analysis	P2
	3d	The body of the Abstract must describe the most significant scientific results for all experimental and control groups	P2-3
	3e	The main conclusion(s) of the study must be provided	P3
Introduction	4a	A background summary of the scientific investigation with relevant information must be provided	P4-5
	4b	The aim(s), purpose(s) or hypothesis(es) of an investigation must be provided ensuring they align with the methods and results	P5
Materials and Methods	5a	A clear ethics statement and the ethical approval granted by an ethics board, such as an Institutional Review Board or Institutional Animal Care and Use Committee, must be described	P8
	5b	When harvesting cells and tissues for research, all the legal, ethical, and welfare rights of human subjects and animal donors must be respected and applicable procedures described	P6-10
	5c	The use of reference samples must be included, as well as negative and positive control samples, and the adequacy of the sample size justified	P6-10
	5d	Sufficient information about the methods/materials/supplies/samples/specimens/instruments used in the study must be provided to enable it to be replicated	P6-10
	5e	The use of categories must be defined, reliable and be described in detail	P6-10
	5f	The numbers of replicated identical samples must be described within each test group. The number of times each test was repeated must be described	P21-23
	5g	The details of all the sterilization, disinfection, and handling conditions must be provided, if relevant	Not Applicable
	5h	The process of randomization and allocation concealment, including who generated the random allocation sequence, who decided on which specimens to be included and who assigned specimens to the intervention must be provided(if applicable)	P6-10
	5i	The process of blinding the operator who is conducting the experiment (if applicable) and the examiners when assessing the results must be provided	P6-10

	5j	Information on data management and analysis including the statistical tests and software used must be provided	P10
Results	6a	The estimated effect size and its precision for all the objective (primary and secondary) for each group including controls must be provided	P11-14
	6b	Information on the loss of samples during experimentation and the reasons must be provided, if relevant	Not Applicable
	6c	All the statistical results, including all comparisons between groups must be provided	P11-14 P21-23
Discussion	7a	The relevant literature and status of the hypothesis must be described	P15-16
	7b	The true significance of the investigation must be described	P15-16
	7c	The strength(s) of the study must be described	P15-16
	7d	The limitations of the study must be described	P15-16
	7e	The implications for future research must be described	P15-16
Conclusion(s)	8a	The rationale for the conclusion(s) must be provided	P16
	8b	Explicit conclusion(s) must be provided, i.e. the main "take-away" lessons	P16
Funding and support	9a	Sources of funding and other support (such as supply of drugs, equipment) as well as the role of funders must be acknowledged and described	Title page
Conflicts of interest	10a	An explicit statement on conflicts of interest must be provided	Title page
Quality of images	11a	Details of the relevant equipment, software and settings used to acquire the image(s) must be described in the text or legend	P21-23
	11b	If an image(s) is included in the manuscript, the reason why the image(s) was acquired and why it is included must be provided in the text	Not Applicable
	11c	The circumstances (conditions) under which the image(s) were viewed and evaluated must be provided in the text	P6-10 P21-23
	11d	The resolution and any magnification of the image(s) or any modifications/ enhancements (e.g. brightness, image smoothing, staining etc.) that were carried out must be described in the text or legend	P21-23
	11e	An interpretation of the findings (meaning and implications) from the image (s) must be provided in the text	P11-14
	11f	The legend associated with each image must describe clearly what the subject is and what specific feature(s) it illustrates	P21-23
	11g	Markers/labels must be used to identify the key information in the image(s) and defined in the legend	P21-23
	11h	If relevant, the legend of each image must include an explanation whether it is pre-experiment, intra-experiment or post-experiment and, if relevant, how images over time were standardised	Not Applicable

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Supporting Information

The IL-1β-p65 axis stimulates quiescent odontogenic epithelial cell rests via TGF-β signalling to promote cell proliferation of the lining epithelia in radicular cysts: A laboratory investigation

A short running title: Cell growth in radicular cyst

Figure Legends

SUPPLEMENTARY FIGURE 1 NF-κB signaling is hardly activated in the odontogenic epithelial cells in dentigerous cysts.

Dentigerous cyst tissues without inflammation (n = 6) were stained with Hematoxylin-eosin, anti-E-cadherin, anti-p65, anti-Smad2/3 and anti-Ki-67 antibodies and hematoxylin. Representative images of dentigerous cyst. The boxes indicate enlarged images. Scale bars, 1 mm (upper left panel), 200 μm (upper right panel), 50 μm (middle and lower panels).

SUPPLEMENTARY FIGURE 2 The proliferation capabilities of odontogenic epithelial cells are suppressed by TGF-β1 and TGF-β2 derived from PDL cells.

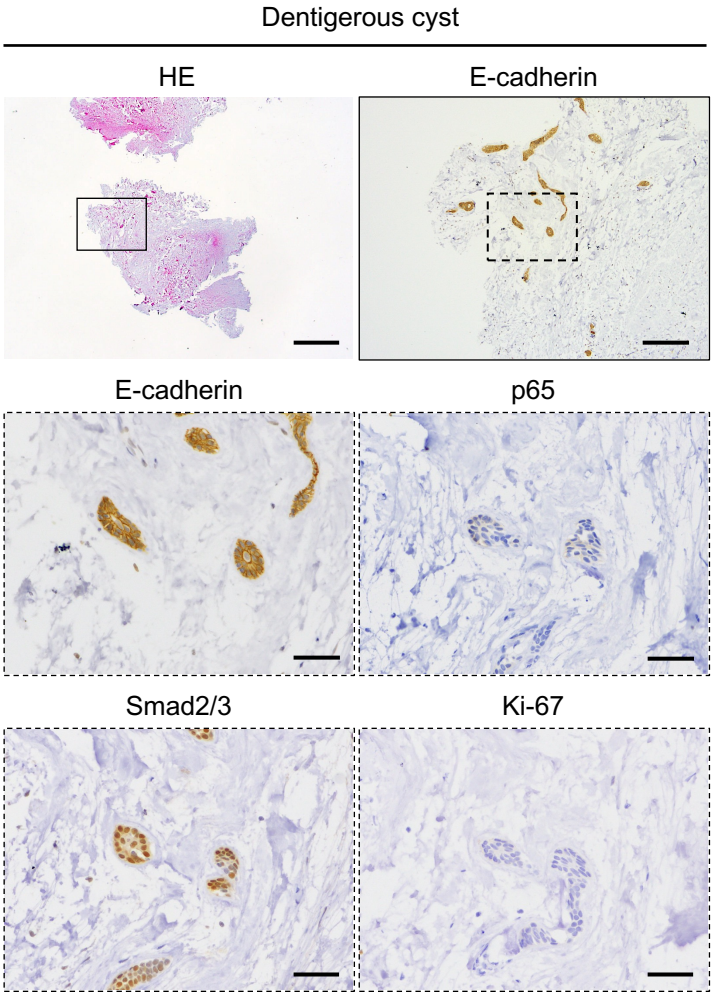
(a,d,e) After the culture, the cells were stained with anti-CK14 (Green) and anti-Ki-67 (Red) antibodies and Hoechst 33342, and then Ki-67-positive SF2 cells and Hoechst 33342-stained SF2 cells were counted, respectively. Results are expressed as the percentage of Ki-67-positive cells compared with total Hoechst 33342-stained cells. SF2 cells were specifically positive for CK14. (a) SF2 cells were co-cultured with 1-11 cells for 48 h and counted (n = 54,009). (b,c) STPLF-E cells (b) and 1-11 cells (c) were transfected with control or two different TGF-β1 and TGF-β2 siRNAs for 48 h, and *TGF-β1* and *TGF-β2* mRNA levels were measured by quantitative RT-PCR. Relative *TGF-β1* and *TGF-β2* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells, respectively. (d) SF2 cells were cultured with or without 1 μM A83-01 for 24 h, then reseeded with or without 1-11 cells for 48 h and counted (n = 10,258). (e) 1-11 cells were transfected with control or two different human TGF-β1 and TGF-β2 siRNAs. Then, 1-11 cells were co-cultured with

SF2 cells for 48 h (n = 44,537). Results are shown as means ± s.d. of three independent experiments. Scale bars, 50 μm. **P<0.01. *P<0.05.

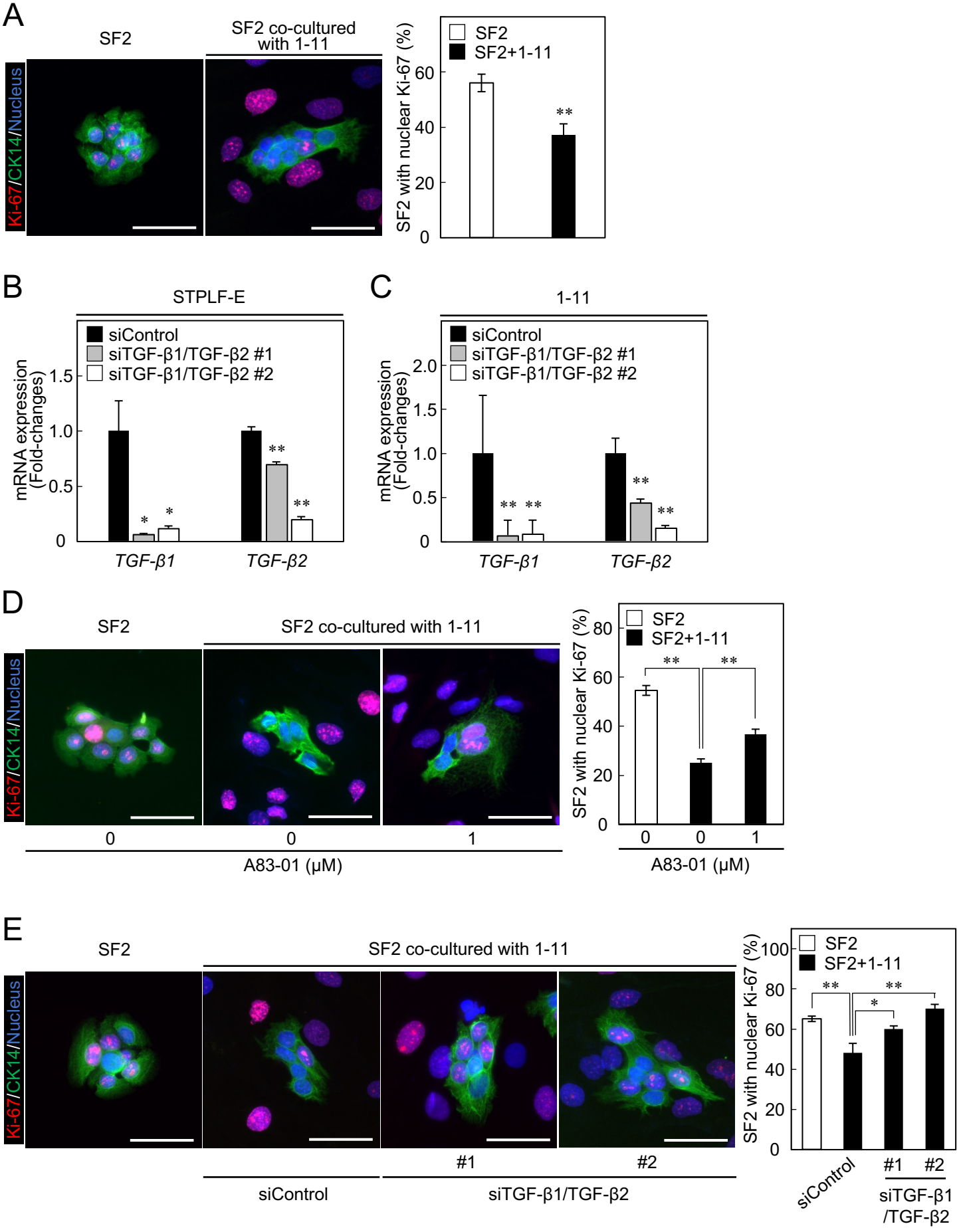
SUPPLEMENTARY FIGURE 3 TGF-β1-dependent growth suppression is inhibited by activation of IL-1β-p65 signaling in odontogenic epithelial cells.

(a,b) SF2 cells were cultured with or without 10 ng/ml TGF-β1 for indicated times. (a) Cell lysates were probed with indicated antibodies. (b) Quantitative RT-PCR for *Vimentin* mRNA in SF2 cells was performed. Relative mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control cells. (c) (Left panels) SF2 cells were cultured with or without 10 ng/ml IL-1β for indicated times, and then stained with anti-p65 antibody and Hoechst 33342. (Right graph) Cells with nuclear p65 are expressed as the percentage of p65-positively stained cells compared with total Hoechst-stained cells (n = 13,419). (d) SF2 cells were cultured with or without 10 ng/ml IL-1β for indicated times. Quantitative RT-PCR for *iNos* mRNA in SF2 cells was performed. Relative mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control cells. (e,f) (Left graph) SF2 cells were transfected with control or p65 siRNA for 48 h. (e) *p65* mRNA levels were measured by quantitative RT-PCR. Relative *p65* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells. (Right panels) Cell lysates were probed with indicated antibodies. (f) SF2 cells were cultured with or without 10 ng/ml IL-1β and/or 10 ng/ml TGF-β1 for 24 h. Quantitative RT-PCR for *Vimentin* mRNA in SF2 cells was performed. Relative mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control cells. Results are shown as means ± s.d. of three independent experiments. **P<0.01. Scale bars, 50 μm.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3