

Ph.D. Thesis

The involvement of TGF- $\beta$ /smad2 signaling pathway in  
*Aggregatibacter actinomycetemcomitans*-induced apoptosis  
of human gingival epithelial cells

(TGF- $\beta$ /smad2 シグナル伝達経路と *Aggregatibacter*  
*actinomycetemcomitans* が誘導するヒト歯肉上皮細胞における  
アポトーシスとの関連性)

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## **Chapter 1 Preface**

Periodontitis is the most prevalent infectious disease caused by periodontopathic bacteria. In periodontal tissue, the gingival junctional epithelium is located at a strategically important interface at the base of the gingival sulcus and plays a pivotal role for defense against bacteria challenge. Therefore, junctional epithelium is closely involved in the initiation and progression of periodontitis [1], [2]. Junctional epithelium is sustained by the vigorous proliferation of progenitor cells and rapid apoptosis of the superficial cells. Apoptosis of superficial cells at junctional epithelium is a highly conserved and regulated process for eliminating aged, damaged or infected cells. This process maintains tissue homeostasis. However, it is known that the enhanced apoptosis in skin decreases barrier function and evokes the inflammation. In periodontal tissue, periodontopathic bacteria increased apoptosis positive cells in gingival epithelial cells [3], [4], [5], and many apoptotic cells were detected in the gingival epithelium of patients with periodontitis [1], [6]. Thus, it is plausible that periodontopathic bacteria-induced apoptosis in gingival junctional epithelium is involved in the onset and progression of periodontitis [1], [2], [7], [8]. It is important to understand the mechanism how periodontopathic bacteria induce apoptosis in human gingival epithelial cells, and to develop novel preventive therapies for periodontitis.

Thus, the present study aimed to reveal the molecular mechanism how periodontopathic bacteria induce apoptosis on human gingival epithelial cells.

## **Chapter 2 The effect of periodontopathic bacteria on the phosphorylation of smad2 in gingival epithelial cells.**

### **1. Introduction**

In order to examine the effect of periodontopathic bacteria on the phosphorylation of smad2 in human gingival epithelial cells, representative periodontopathic bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Prevotella intermedia*, and control bacteria, *Escheria coli*, were employed as stimulants.

Smad2 is a well-known downstream signaling molecule of TGF- $\beta$  receptors (TGF- $\beta$ Rs), and plays a key role in TGF- $\beta$ -mediated apoptosis in various cell types [9-13]. Smad2 signaling pathway works on TGF- $\beta$ 1-induced apoptosis in gastric and prostate epithelial cells [14]. Binding of active TGF- $\beta$ 1 to TGF- $\beta$  receptor type II (TGF- $\beta$  RII) leads to the phosphorylation and recruitment of TGF- $\beta$  RI. This heteromeric receptor complex phosphorylates Smad2 and 3, which bind to Smad4 and translocate into the nucleus to enhance transcription of Mf-specific genes such as  $\alpha$ -SMA by cooperating with DNA transcription factors [15]. Recently, it was demonstrated that smad2 overexpression reduces the proliferation of the junctional epithelium [16]. In addition, importantly, it has been reported that an increase in the number of apoptotic-positive cells was observed in the gingival junctional epithelium in smad2 transgenic mice [17].

Taken together, I hypothesized that bacteria enhance the phosphorylation of smad2 in

human gingival epithelial cells to induce apoptosis, and lead to the onset and progression of periodontitis. In this chapter, the phosphorylation of smad2 in human gingival epithelial cells stimulated by periodontopathic bacteria was examined.

## **2. Materials and methods**

### *2.1. Cell culture*

Healthy gingival tissues, which had been surgically dissected through the process of wisdom tooth extraction and were going to be discarded, were collected with patients' informed consent. Primary human gingival epithelial cells (HGECs) were isolated as previously described with minor modification [18], [19], [20]. Briefly, gingival tissues were treated with dispase overnight at 4 °C and then divided into the epithelium and connective tissue. The epithelium was treated with 0.025% trypsin and 0.01% EDTA for 15 min, minced, and treated with a trypsin inhibitor. The HGECs suspension was centrifuged at  $120 \times g$  for 5 min, and the pellet was suspended in Humedia-KB2 medium containing 10 µg/ml insulin, 5 µg/ml transferrin, 10 µM 2-mercaptoethanol, 10 µM 2-aminoethanol, 10 µM sodium selenite, 50 µg/ml bovine pituitary extract, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were seeded in 60-mm plastic tissue culture plates coated with type I collagen, and incubated in 5% CO<sub>2</sub>/95% air at 37 °C. When the cells reached subconfluence, they were harvested and subcultured.

OBA9 cells, a Simian virus-40 (SV40) antigen-immortalized human gingival epithelial cell line, were kindly gifted by Professor Shinya Murakami (Osaka University)[21]. OBA9 cells were seeded at a density of  $2.0 \times 10^4$  cells per well in 6-well plastic culture plates. The cells were cultured with Humedia-KB2 medium containing 10 mg/ml insulin, 0.1 mg/ml hEGF, 0.67 mg/ml Hydrocortisone

Hemisuccinate, 50 mg/ml gentamycin, 50 mg/ml amphotericin B, and 2 ml BPE (medium A). Before the addition of bacteria, these cells were incubated in medium without growth factors (medium B) for 3 h.

## 2.2. Bacterial strains

*Porphyromonas gingivalis* strain W83 (*Pg*), *Aggregatibacter actinomycetemcomitans* strain Y4 (*Aa*), *Fusobacterium nucleatum* ATCC 49256 (*Fn*), *Prevotella intermedia* ATCC 25611 (*Pi*) and *Escheria coli* HB101 (*EC*) were purchased from American Type Culture Collection (Manassas, VA). *Pg*, *Fn*, and *Pi* were cultured on sheep agar plate using the Anaeropack system (Mitsubishi Gas Chemical, Tokyo, Japan) at 37 °C. *Aa* was cultured in Todd-Hewitt broth supplemented with 1% yeast extract (TSBY; Difco Laboratories, Detroit, MI) in humidified 5% CO<sub>2</sub> atmosphere at 37 °C. *EC* was cultured in Luria-Bertani (LB) broth containing ampicillin (100 µg/ml) with 1% TSBY in humidified 5% CO<sub>2</sub> atmosphere at 37 °C. After 2 days incubation, bacteria were inoculated in 40 ml of trypticase soy broth supplemented with 1% yeast extract, 40 µl hemin (50 mg/ml) and 40 µl menadion (5 mg/ml) using the Anaeropack system at 37 °C.

## 2.3. Preparation of bacteria

After cultivation, whole cells were harvested by centrifugation and washed three times in phosphate-buffered saline (PBS, pH 7.4), and then fixed with 1% formalin at

4 °C for 12 h. Periodontopathic bacteria were used at  $10^7$  cells per well (MOI : 10) in the following experiments in the present study, as this density was referred to previous studies [22], [23].

#### *2.4. Western blotting*

Cells were lysed in 150 µl of sodium dodecyl sulphate (SDS) sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromphenol blue). The cell lysates were subjected to ultrasonic treatment for 10 seconds, on ice. The cell lysate were processed by SDS-PAGE and transferred to a nitrocellulose (NC) membrane (Bio-Rad Laboratories, Hercules, CA). After blocking with 5% skim milk in Tris Buffered Saline with 0.1 % Tween<sup>®</sup> 20, pH 7.6 (TBST) for 1 h, the membrane was washed and incubated with the primary antibody, rabbit anti-human phosphorylated smad2 (Ser465/467) antibody (Cell Signaling Technology, Beverly, MA), and mouse anti-human total smad2/3 antibody (BD Transduction laboratories, San Jose, CA). The membrane was washed three times, and incubated with HRP-conjugated sheep anti-rabbit or anti-mouse IgG antibody (R&D systems, Minneapolis, MN, USA) for 1 h at room temperature. ECL Prime Western blotting detection reagents (BioRad) was used to detect the positive bands by manufactured manual.

### **3. Results**

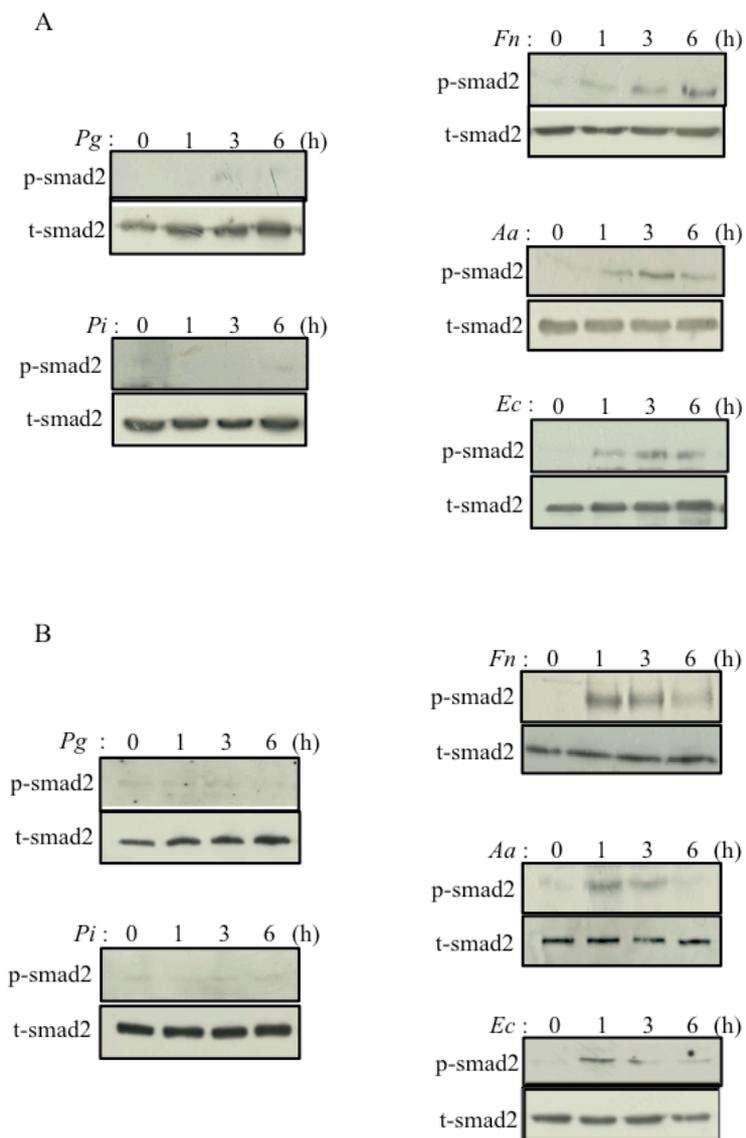
#### *3.1. The phosphorylation of smad2 in gingival epithelial cells stimulated with periodontopathic bacteria*

Smad2 phosphorylation was apparently enhanced both peaking at 3 h and then gradually decreasing in HGECs stimulated with *Aa*, *Fn*, and *EC*, but was not enhanced in HGECs stimulated with *Pg* and *Pi* (Fig1. A). In addition, smad2 phosphorylation in OBA9 cells exposed to each bacteria for the indicated periods was similar to HGECs, although smad2 phosphorylation was peaked at 1 h and then gradually decreased (Fig1. B). These results indicate that smad2 phosphorylation in gingival epithelial cells can be up-regulated with specific species in periodontopathic bacteria.

#### **4. Discussion**

The present study showed that while the phosphorylation of smad2 in both HGECs and OBA9 cells stimulated with *Aa*, *Fn* and *EC* were obviously up-regulated. These results may be dependent on bacterial species and bacterial components. *Pg* or *Pi* in optimal conditions might up-regulate smad2 phosphorylation in gingival epithelial cells. On the other hands, it may be possible that *Aa*, *Fn* and *EC* have similar factors to stimulate smad2 signaling. Although further investigation is necessary, these findings suggest that *Aa*-, *Fn*- and *EC*-elicited phosphorylation of smad2 is involved in apoptosis of gingival epithelial cells and this apoptosis may initiate periodontitis.

# Fig1



*Fig1. The phosphorylation of smad2 in gingival epithelial cells stimulated with periodontopathic bacteria*

(A and B) HGECs and OBA9 cells were exposed to *Pg*, *Aa*, *Fn*, *Pi*, and *EC* for the indicated periods. Phosphorylated (p) and total (t) smad2 levels were analyzed by Western blotting.

## **Chapter 3 The involvement of TGF- $\beta$ R/smad2 signaling pathway in *Aa*-induced apoptosis of gingival epithelial cells**

### **1. Introduction**

As described chapter 2, smad2 phosphorylation in gingival epithelial cells stimulated with *Aa*, *Fn* and *EC* was up-regulated, suggesting that the enhancement of smad2 phosphorylation caused by these bacteria is contribute to apoptosis in gingival epithelial cells. Nevertheless, it is still to be elucidated the relationship between bacteria-enhanced smad2 phosphorylation and apoptosis in gingival epithelial cells.

*Aa*, a gram-negative facultative capnophilic anaerobe, can ferment many sugars, including glucose, fructose, and maltose. *Aa* has numerous virulence factors from this bacteria that affected the gingival epithelium and triggered the onset of periodontitis, including lipopolysaccharide (LPS), leukotoxin, cytolethal-distending toxin (CDT), collagenase, and outer membrane proteins [24-28]. *Aa* is also recognized as the etiological pathogen for aggressive periodontitis and severe adult periodontitis, while being involved in other medical diseases such as thyroid and brain abscess, urinary tract infections and sub-acute bacterial endocarditis. This bacteria is commonly linked localized aggressive periodontitis which is characterized by severe and rapid destruction of the periodontal tissue [16]. Furthermore, previous reports suggest that *Aa* induces apoptosis in gingival epithelial cells [4], [29]. Accordingly, this chapter aimed to examine the involvement of smad2 signaling pathway in *Aa*-induced apoptosis of gingival epithelial cells, and reveal its molecular mechanism.

## **2. Materials and methods**

### *2.1. Cell and Cell culture*

OBA9 cells were cultured in medium A as previously described. Then, medium was changed into medium B for stimulation as previously described in chapter II, section 2.1. The cells were pretreated for 30 min with or without SB431542 (TGF- $\beta$ RI inhibitor, R&D Systems), and then treated with Omp29 for the indicated periods. Regarding chemical reagents dissolved in dimethylsulfoxide, an appropriate concentration of dimethylsulfoxide was added as a solvent control.

### *2.2. Preparation of Aa*

*Aa* was prepared as previously described in chapter 2, section 2.2 and 2.3.

### *2.3. Western blotting*

Western blotting was performed as previously described in chapter 2, section 2.4. The primary antibodies were rabbit anti-human cleaved caspase-3 antibody (CST) and mouse anti  $\beta$ -actin antibody (CST), rabbit anti-human phosphorylated smad2 (Ser465/467) antibody, (CST) and mouse anti-human total smad2/3 antibody (BD Transduction). The membrane was washed three times, and incubated with a HRP-conjugated sheep anti-rabbit or anti-mouse IgG antibody (R&D) for 1 h at room temperature. ECL Prime Western blotting detection reagents (BioRad) was used to detect the positive bands by manufactured manual.

#### *2.4. Immunoprecipitation*

Recombinant human TGF- $\beta$ 1 was obtained from R&D. The TGF- $\beta$ RI protein in the cell lysate was immunoprecipitated with rabbit anti-TGF- $\beta$ RI polyclonal antibody (Abcam, Cambridge, MA) that was pre-bound to the Crosslink Magnetic IP and Co-IP Kit (Thermo Scientific, Rockford, IL). After extensive washing with the lysis buffer, proteins captured by anti-TGF- $\beta$ RI antibody-coated beads were separated by SDS-PAGE and subjected to Western blotting with rabbit anti-phosphoserine antibody (BD transductions) or with rabbit anti-TGF- $\beta$ RI polyclonal antibody (CST). After the membrane was reacted with HRP-conjugated sheep anti-rabbit IgG (R&D systems), positive bands were detected as described above.

#### *2.5. Apoptosis Assay.*

TdT-mediated dUTP nick end labeling (TUNEL) staining for apoptotic cells was performed using a Dead-End fluorometric TUNEL system (Promega, Madison, WI). Nuclei were stained by DAPI. Fluorescence signals were detected with a deconvolution microscope system (BZ-8000, Keyence, Tokyo, Japan). DAPI and TUNEL staining positive cells were enumerated by using National Institutes of Health ImageJ software.

#### *2.6. Small interfering RNA (siRNA)*

Validated TGF- $\beta$ RI siRNA (identification nos. HSS110697), smad2 siRNA

(identification nos., HSS106249, HSS106251 and HSS180969) and negative-control siRNA (12935-3000) were obtained from Invitrogen (Van Allen Way Carlsbad, CA). OBA9 cells were seeded at a density of  $2.0 \times 10^4$  cells per well in 6-well plastic culture plates and cultured in medium B for 48 h at 37°C. A total of 30 nM of TGF- $\beta$ RI siRNA, smad2 siRNA and negative control siRNA were transfected into the cells using lipofectamine RNAiMAX reagent (Invitrogen), according to the manufacturer's instruction manual.

### *2.7. Real time PCR assay*

Total RNA from each culture was extracted with ISOGEN<sup>®</sup> (Wako Pure Chemical, Osaka, Japan) and quantified by spectrometry at 260 and 280 nm. First standard cDNA synthesis was performed with 1  $\mu$ g of total RNA extract in a total volume of 20  $\mu$ l (Roche, Tokyo, Japan). Real-time PCR was performed with a Lightcycler system using SYBR green (Roche). The mRNA expression of human TGF- $\beta$  type I receptor and mRNA expression knocked down by the siRNAs were quantified by real-time PCR. The PCR was carried out in two steps with a lightcycler system using SYBR green (Roche Diagnostics, Mannheim, Germany). The sense primers and antisense primers used to detect the mRNA of human TGF- $\beta$ RI and GAPDH are listed in Table 1.

### *2.8. Statistical analysis*

Comparisons between groups were analyzed with the Student's *t*-test.

**Table 1. Sense primers and antisense primers for real time PCR**

Target gene		Primer sequence
<i>TGF-<math>\beta</math>RI</i>	Forward	5'-TGG TCTTGCCCATCTTCACA-3'
	Reverse	5'-ATTGCATAGATGTCAGCACG-3'
<i>GAPDH</i>	Forward	5'-AACGTGTCAGTGGTGGACCTG-3'
	Reverse	5'-AGTGGGTGTCGCTGTTGAAGT-3'

### 3. Results

#### 3.1. *Aa* activates the TGF- $\beta$ type I receptor.

In general, in response to the binding of TGF- $\beta$ 1 to TGF- $\beta$ Rs, the interaction between TGF- $\beta$ RII serine kinase and TGF- $\beta$ RI elicits the phosphorylation of serine (187, 189, 191) in GS domain of TGF- $\beta$ RI, which, in turn, induce smad2 signaling [15]. Therefore, the ability of *Aa* to activate TGF- $\beta$ Rs was measured in phosphorylation-serine immunoblots of TGF- $\beta$ RI immunoprecipitated from OBA9 cells treated with *Aa*. The OBA9 cells stimulated with *Aa* for 1 h were markedly elevated the phosphorylation of serine in TGF- $\beta$ RI, approximately 6 fold comparing to basal levels (Fig2 A and B). In control experiments, TGF- $\beta$ 1 (10 ng/ml) for 1 h also increased the phosphorylation of serine in TGF- $\beta$ RI in OBA9 cells, approximately 7 fold comparing to basal levels (Fig2 B). These results suggest that *Aa* activates TGF- $\beta$ RI in OBA9 cells.

#### 3.2. *Aa* enhances the phosphorylation of smad2 via the TGF- $\beta$ type I receptor.

In order to clarify whether *Aa*-enhanced smad2 phosphorylation is involved in the activation of TGF- $\beta$ RI, SB431542 (TGF- $\beta$ RI chemical inhibitor) was used and siRNA for TGF- $\beta$ RI were transfected. SB431542 obviously attenuated the *Aa*-enhanced phosphorylation of smad2 in HGECs and OBA9 cells (Fig3. A and B). I confirmed that siRNA transfection for TGF- $\beta$ RI decreased TGF- $\beta$ RI expression at both mRNA and protein levels (Fig3. C and D). Knockdown by siRNA for TGF- $\beta$ RI apparently attenuated the *Aa*-enhanced phosphorylation of smad2 (Fig3. E). These results indicate that *Aa* up-regulated the phosphorylation of smad2 through TGF- $\beta$ RI in OBA9 cells.

### 3.3. *Aa* induces apoptosis through the TGF- $\beta$ type I receptor/smad2 cascade.

Previous studies have shown that *Aa* induces apoptosis in gingival epithelial cells [4], [29]. To confirm this premise, OBA9 cells were exposed to *Aa* for the indicated periods. *Aa* increased the protein level of cleaved caspase-3 in a time dependent manner and a maximal effect was observed at 12 h (Fig4. A). Furthermore, as shown in figure 4 C and D, *Aa* increased the number of apoptotic cells. *Aa*-increased cleaved caspase-3 level was apparently attenuated by SB431542 and TGF- $\beta$ RI siRNA transfection, respectively (Fig4. B and C). TUNEL staining showed that the blockade of TGF- $\beta$ RI by these inhibitors significantly reduced the number of apoptotic cells increased by *Aa* (Fig4. D and E). Transfection of smad2 siRNA remarkably decreased smad2 expression of protein level (Fig4. F). Smad2 siRNA transfection significantly attenuated *Aa*-induced cleaved caspase-3 expression (Fig4. G). TUNEL staining showed that smad2 siRNA transfection decreased the *Aa*-increased apoptotic cells (Fig4. H).

#### 4. Discussion

In the present study, I revealed that *Aa*-induced apoptosis of gingival epithelial cells was mediated via TGF- $\beta$ RI/smad2 signaling pathway. The results obtained in figure1 may imply that *Fn* or *EC* also induces apoptosis via TGF- $\beta$ RI/smad2 cascade in gingival epithelial cells.

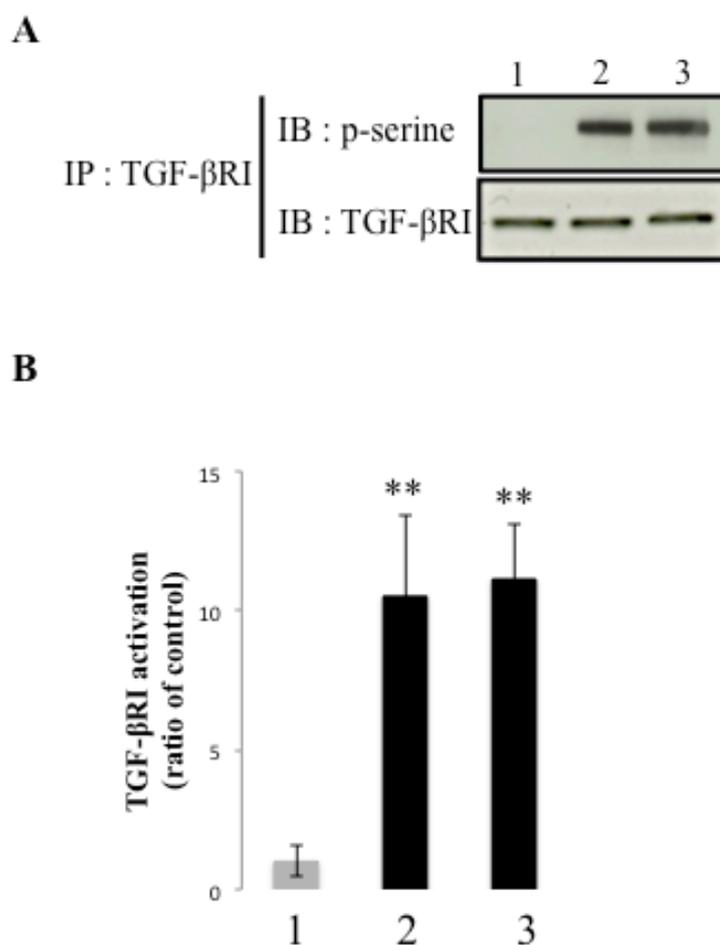
The most important result of the present study is that smad2 was phosphorylated via TGF- $\beta$ RI in OBA9 cells by the *Aa* stimulation. A previous study demonstrated that TGF- $\beta$ 1 produced by gastric epithelial cells in response to bacterial infection acted in an autocrine or paracrine manner [30]; therefore, TGF- $\beta$  may be secreted or released from gingival epithelial cells stimulated with *Aa* and bind to TGF- $\beta$ Rs to phosphorylate smad2. There is extensive crosstalk between integrins and TGF- $\beta$ R signaling. For example, previous studies reported that  $\alpha$ 3 $\beta$ 1 and TGF- $\beta$ R mediated the phosphorylation of smad2 [31], [32]. Meanwhile, other studies described interplay between bacteria and integrin [33-35]. These findings suggest that smad2 may be phosphorylated by an *Aa*-integrin-TGF $\beta$ -RI transactivation pathway in gingival epithelial cells. This mechanism needs to be elucidated in more detail.

The turnover of the gastrointestinal epithelium is rapid and requires an appropriate balance between the proliferation of progenitor cells and loss of mature cells. Previous study has shown that the activation of TGF- $\beta$ R/smad2 cascade is crucially involved in apoptosis of superficial gastric epithelial cells, which suggests that TGF- $\beta$ /smad2 signaling may play an important role in homeostasis in the gingival epithelium [14]. In

periodontal tissue, it is considered that apoptosis of superficial mature cells plays a crucial defense mechanism. Accordingly, these findings suggest that the acceleration of TGF- $\beta$ R/smad2 signaling by periodontopathic bacteria may disrupt the homeostasis in the gingival epithelium, which result in initiating the onset of periodontitis.

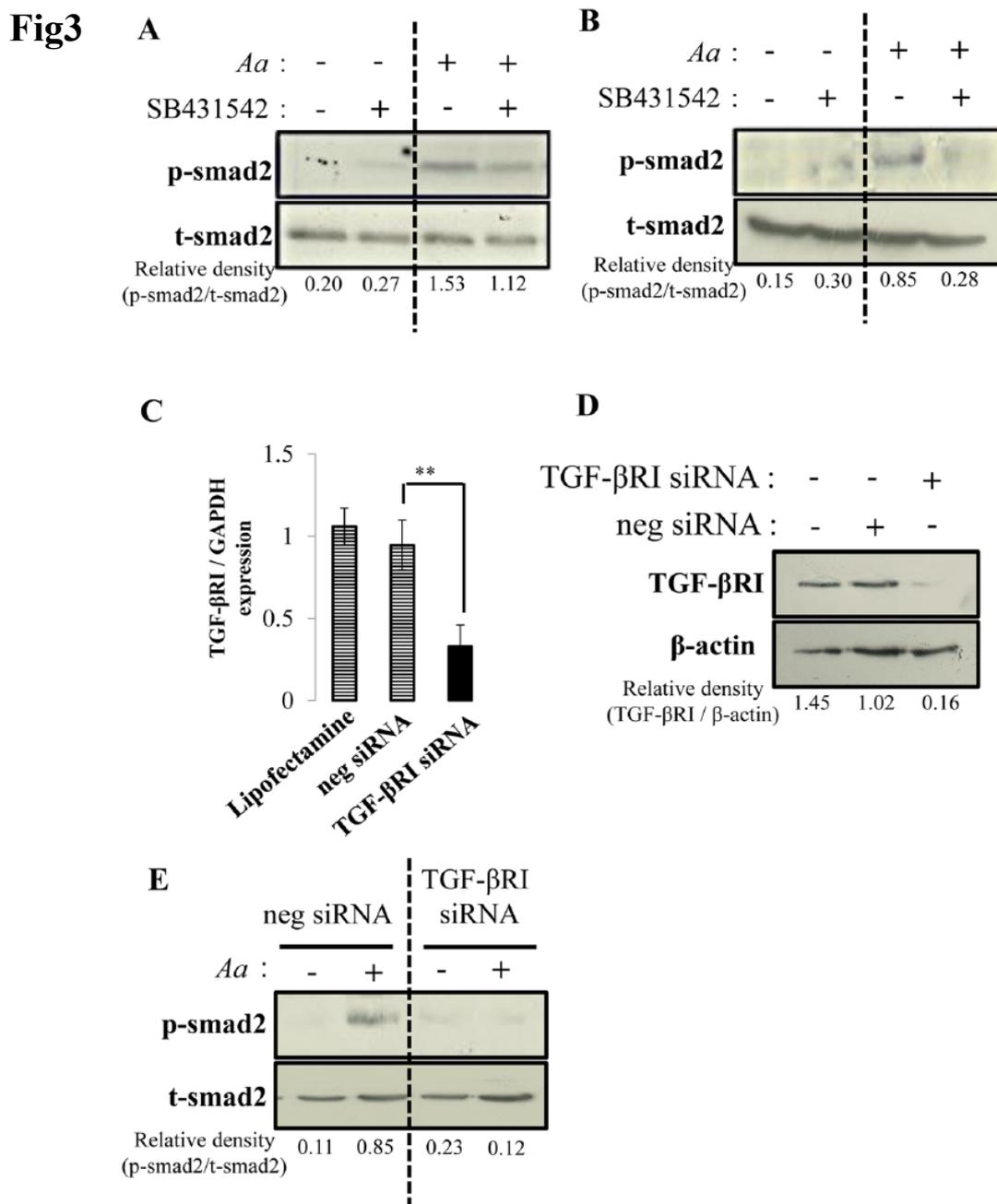
Necrotic cells were also detected in the gingival junctional epithelium in patients with periodontitis. The number of apoptotic cells in gingival epithelial cells increased by *Aa* (Fig4). Since TUNEL staining can detect necrotic cells, it is thought that *Aa* also increased necrotic cells in gingival epithelial cells. The increase of apoptotic and necrotic cells caused by periodontopathic bacteria may be contribute to exacerbate the destruction of gingival epithelium.

**Fig2**



*Fig2. Aa activates the TGF-β type I receptor.*

(A) Representative Western blotting of *Aa*- and TGF-β1-stimulated phosphorylation of serine in TGF-βRI and also of total TGF-βRI after the immunoprecipitation (IP) of TGF-βRI. lane 1, basal; lane 2, *Aa*; lane 3, 10 ng/ml TGF-β1. (B) Densitometric analysis of the *Aa*- or TGF-β1-stimulated phosphorylation of serine in TGF-βRI. OBA9 cells were incubated for 1 h with *Aa* or 10 ng/ml TGF-β1 as a control. TGF-βRI was immunoprecipitated from cell lysates and Results are expressed as a percentage above basal levels in relative densitometric units normalized to total TGF-βRI levels. Values represent the mean ± S.D. of three cultures. \*\*  $p < 0.01$  values differ significantly (*t*-test).

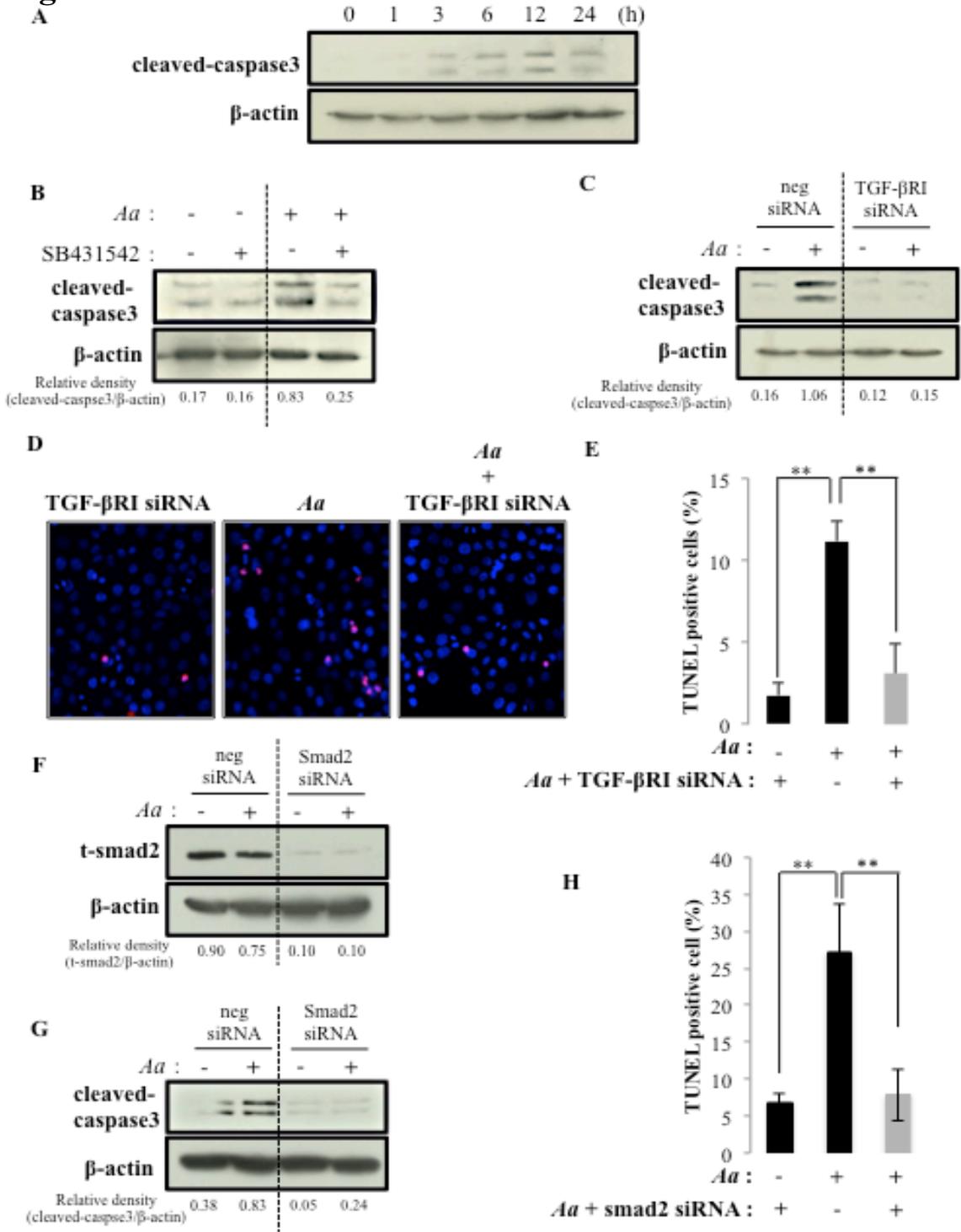


*Fig3. Aa enhances the phosphorylation of smad2 via the TGF-β type I receptor.*

HGECs (A) and OBA9 cells (B) were pretreated with or without SB431542 (10 μM) for 30 min and were then exposed to *Aa* for 1 h. Phosphorylated (p) and total (t) smad2 levels were analyzed by Western blotting, respectively. (C and D) OBA9 cells, having been transfected with the negative control (neg) or TGF-βRI siRNA, were cultured for 48 h in medium B. (C) mRNA levels of TGF-βRI and GAPDH in the cell were analyzed through real-time PCR. (D) Protein levels of TGF-βRI and β-actin were analyzed by Western blotting. (E) OBA9 cells, having been transfected with the negative control or TGF-βRI siRNA, were cultured for 48 hours in medium B and were then exposed to *Aa*

for 1 h. Phosphorylated (p) and total (t) smad2 levels were analyzed by Western blotting, respectively. Band densities were quantified through the densitometric scanning of each band by using National Institutes of Health Image J software. Values represent the mean  $\pm$  S.D. of the three cultures.  $**p < 0.01$  values differ significantly (*t*-test). Similar results were obtained from three experiments.

**Fig4**



*Fig4. Aa induces apoptosis through the TGF- $\beta$  type I receptor/smad2 cascade.*

(A) OBA9 cells were exposed to *Aa* for the indicated periods. Cleaved caspase-3 levels and  $\beta$ -actin were analyzed through Western blotting. (B) OBA9 cells were pretreated with or without SB431542 (10  $\mu$ M) for 30min and were then exposed to *Aa* for 12 h. OBA9 cells, having been transfected with the negative control or TGF- $\beta$ RI siRNA (C) or smad2 siRNA (F), were cultured for 48 h in medium B and were then exposed to *Aa* for 12 h. (C and G) Cleaved caspase-3 levels and  $\beta$ -actin were analyzed by Western blotting, respectively. Band densities were quantified through the densitometric scanning of each band using the National Institutes of Health Image J software. (D, E, H) OBA9 cells, having been transfected with the negative control or TGF- $\beta$ RI siRNA (D, E) or smad2 siRNA (H), were cultured for 48 h in medium B and were then exposed to *Aa* for 24 h. TUNEL-positive apoptotic cells (red) are shown under each set of conditions, and the graph shows the percentage of TUNEL-positive cells. Values represent the mean  $\pm$  S.D. of the three cultures.  $**p < 0.01$  values differ significantly (*t*-test). Similar results were obtained from three experiments.

## Chapter 4 Effects of Omp29 on apoptosis in OBA9 cells

### 1. Introduction

In gingival epithelial cells stimulated with *Aa*, apoptosis occurred via TGF- $\beta$ RI/smad2 cascade. The next question is how *Aa* activates TGF- $\beta$ R/smad2 signaling in OBA9 cells.

TGF- $\beta$  precursor contains 390 amino acids with an N-terminal signal peptide of 29 amino acids required for secretion from a cell, a 249 amino acids pro-region (latency associated peptide or LAP), and a 112 amino acids C-terminal region that becomes the active TGF- $\beta$ 1 upon activation. Both LAP and TGF- $\beta$ 1 exist as homodimers in circulation, but the disulfide linked homodimers of LAP and TGF- $\beta$ 1 remain non-covalently associated, forming the small latent TGF- $\beta$ 1 complex (SLC, 100 kD). The large latent TGF- $\beta$ 1 Complex (LLC, 235-260 kD) contains a third component, the latent TGF- $\beta$  binding protein (LTBP), which is linked to LAP by a single disulfide bond. The LTBP does not confer latency, but for efficient secretion of the complex to extracellular sites. Free active TGF- $\beta$ 1 can be released (activated) by many factors including enzymes and low or high pH. Association of LAP-TGF- $\beta$  with LTBP has important consequences on TGF- $\beta$  localization within the extracellular matrix (ECM) such as fibronectin and fibrillin, and subsequent activation by cells.

Outer membrane protein 29 kDa (Omp29) is a crucial virulence factor of *Aa*. Serum of patients with periodontitis contains IgG antibodies which recognize the major outer membrane proteins of *Aa*, indicating that Omp29 can be associated with *Aa*-related

periodontitis [26], [36]. In addition, it is reported that Omp29 increases inflammatory cytokines such as IL-8 and IL-6, and is associated with bacterial invasion into gingival epithelial cells by up-regulation of F-actin rearrangement. Most gram-negative bacteria have outer membrane protein A (OmpA)-like proteins that are homologous to the OmpA protein in *Ec*, and Komatsuzawa et al. revealed that Omp29 belongs to the *EC* OmpA family [37]. Intriguingly, as shown in figure1, both *Aa* and *Ec* enhanced the phosphorylation of smad2 in gingival epithelial cells. Therefore, herein, recombinant Omp29 were synthesized as a stimulant in the following experiments.

I hypothesized that smad2 phosphorylation by *Aa* stimulation is engaged in activated TGF- $\beta$  in OBA9 cells. In this chapter, I investigated whether and how Omp29 activates TGF- $\beta$ R/smad2 cascade in gingival epithelial cells.

## **2. Materials and methods**

### *2.1. Cell culture*

OBA9 cells were cultured in medium A as previously described. Then, medium was changed into medium B for stimulation as previously described in chapter 2, section 2.1. The cells were pretreated for 30 min with or without anti TGF- $\beta$ 1 antibody (R&D) and anti TGF- $\beta$  type II receptor antibody (R&D), and then stimulated with Omp29 for the indicated periods.

### *2.2. Purification of Omp29*

Omp29 was purified using a disk preparative electrophoresis apparatus (NA-1800 type; Nippon Eido, Tokyo, Japan) according to the manufacturer's protocol. Omp29 were purified following the method previously published [38]. Sarcosyl-insoluble Omps were finally solubilized with 1% SDS in 10 ml sodium phosphate buffer (pH 6.8). Omps were resolved on a 12% polyacrylamide gel by electrophoresis with a constant voltage (80 V). After the blue dye reached the bottom of the gel, sequential volumes of every 10 drops (200  $\mu$ L) were collected with a fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden). A small portion of each fraction (total ca. 100 fractions) was electrophoresed on a 12% polyacrylamide gel and then stained with Coomassie brilliant blue. Fractions showing a single band of Omp29 with the Coomassie brilliant blue stain were collected and concentrated with Centriprep (Millipore, Bedford, MA, USA) to obtain a final concentration of 1 mg/ml as a stock solution.

### *2.3. Western blotting*

Western blotting was performed as previously described in chapter 2, section 2.4. The primary antibodies were rabbit anti-human cleaved caspase-3 antibody, rabbit anti-human phosphorylated smad2 (Ser465/467) antibody, mouse anti-human  $\beta$ -actin antibody, and mouse anti-human total smad2/3 antibody.

### *2.4. Immunoprecipitation*

Immunoprecipitation was performed as previously described in chapter 3, section 2.4.

### *2.5. Apoptosis Assay.*

Apoptosis assay was performed as previously described in chapter 3, section 2.6.

### *2.6. siRNA.*

Knockdown of the TGF- $\beta$ RI siRNA and smad2 siRNA were performed as previously described in chapter 3, section 2.7.

### *2.7. Real time PCR assay*

Real time PCR assay was performed as previously described in chapter 3, section 2.5. The sense primers and antisense primers used to detect the mRNA of human TGF- $\beta$ 1 and GAPDH are listed in Table 22.

### *2.8. Enzyme-linked immunosorbent assay (ELISA)*

OBA9 cells were cultured at a density of  $1 \times 10^4$  cells per well in 96-well plates, with each well containing 100  $\mu$ L of medium. After becoming confluent, cells were stimulated with Omp29 for the various periods. The supernatants were carefully collected and stored at  $-80^\circ\text{C}$  prior to ELISA analysis. The concentration of active TGF- $\beta$ 1 was measured with a LEGEND MAX Free Active TGF- $\beta$ 1 ELISA Kit (BioLegend) according to the manufacturer's instructions. Briefly, standards and 100  $\mu$ l of supernatants were loaded into the wells, and the absorbencies were measured at 450 nm. All samples were tested in triplicate.

### *2.9. Statistical analysis*

Comparisons between groups were analyzed with the Student's *t*-test.

**Table 2. Sense primers and antisense primers for real time PCR**

Target gene		Primer sequence
<i>TGF-β1</i>	Forward	5'-GGACACCAACTATTGCTTCAG-3'
	Reverse	5'-TCCAGGCTCCAAATGTAGG-3'
<i>GAPDH</i>	Forward	5'-AACGTGTCAGTGGTGGACCTG-3'
	Reverse	5'-AGTGGGTGTCGCTGTTGAAGT-3'

### 3. Results

#### 3.1. *Omp29 potentiates the phosphorylation of smad2 via the TGF- $\beta$ type I receptor.*

To check whether Omp29 induces apoptosis via TGF- $\beta$ RI/smad2 signaling pathway on OBA9 cells in a similar manner of *Aa*, OBA9 cells were stimulated with Omp29 (1.0  $\mu$ g/ml) for the indicated periods. Smad2 phosphorylation in OBA9 cells stimulated with Omp29 was markedly potentiated both peaking at 1 h and then gradually decreasing (Fig5 A). OBA9 cells stimulated with Omp29 for 1 h were markedly elevated the phosphorylation of serine in TGF- $\beta$ RI, approximately 11 fold high comparing to basal levels (Fig5 B and C). Furthermore, SB431542 and siRNA transfection for TGF- $\beta$ RI obviously repressed the Omp29-enhanced phosphorylation of smad2 in OBA9 cells (Fig5 D-F). These findings indicate that Omp29 elevates the phosphorylation of smad2 via the TGF- $\beta$  type I receptor in OBA9 cells.

#### 3.2. *Omp29 induces apoptosis through smad2/TGF- $\beta$ type I receptor.*

In order to confirm whether Omp29 induces apoptosis in OBA9 cells, the cells were exposed to Omp29 for the indicated periods. Omp29 predictably increased the protein level of cleaved caspase-3 in a time-dependent manner and a maximal effect was observed at 6 and 12 h (Fig6. A). Furthermore, as shown in figure 6 C and F, TUNEL staining showed that Omp29 increased the number of apoptotic cells. Knockdown by TGF- $\beta$ RI siRNA transfection obviously diminished the protein levels of cleaved caspase-3 (Fig6. B). Consistent with these results, TUNEL staining showed that TGF- $\beta$ RI siRNA transfection significantly suppressed the Omp29-induced apoptotic

cells (Fig6. C). Next, to explore the relationship between smad2 and Omp29-induced apoptosis in OBA9 cells, smad2 siRNA were transfected. Smad2 siRNA clearly diminished smad2 expression of protein levels (Fig6. D). Transfection for smad2 siRNA apparently relieved that Omp29 induced the activation of cleaved caspase-3. Consistent with this result, TUNEL staining showed that smad2 siRNA transfection significantly decreased the Omp29-induced apoptotic cells (Fig6. E and F). These results demonstrate that Omp29 induces apoptosis by activating TGF- $\beta$ R/smad2 signaling pathway in OBA9 cells.

### *3.3.Omp29 increases extracellular production of active TGF- $\beta$ 1.*

Since the molecular structure of Omp29 and TGF- $\beta$ 1 are different, it can be predicted that Omp29 indirectly activates TGF- $\beta$  receptor in OBA9 cells. A previous study demonstrated that TGF- $\beta$ 1 was produced by gastric epithelial cells in response to bacterial infection and acted in an autocrine or paracrine manner [30]. There are many clinical reports that TGF- $\beta$ 1 levels in gingival crevicular fluids (GCF) of patients with gingivitis and moderate periodontitis were much higher than that of healthy subjects [39-42]. Herein, to access the secretion of active TGF- $\beta$ 1 from OBA9 cells stimulated with Omp29, I examined the mRNA levels of TGF- $\beta$ 1 in OBA9 cells stimulated with Omp29 for the indicated periods. Unexpectedly, there was no significant difference in TGF- $\beta$ 1 mRNA levels between untreated and treated with Omp29 (Fig7. A). This result indicates that Omp29 dose not affect the production of precursor TGF- $\beta$ 1 from OBA9 cell at transcriptional levels. Therefore, it is considered that Omp29 affects latent

TGF- $\beta$ 1 derived from OBA9 cells. Accordingly, to test whether Omp29 increases active TGF- $\beta$ 1 in the supernatant, active TGF- $\beta$ 1 in the supernatant after Omp29 stimulation was measured by using ELISA. Omp29 stimulation significantly increased active TGF- $\beta$ 1 in the supernatant (Fig7. B). To perform the inhibitor assay, the neutralizing anti-TGF- $\beta$ 1 antibody and anti-TGF- $\beta$  type II receptor were employed. Optimal dose of these antibodies were examined by using TGF- $\beta$ 1 as a positive control of smad2 phosphorylation (Fig7. C and E). Based on these results, the blockade of TGF- $\beta$  signaling pathway by these neutralization antibodies remarkably inhibited the phosphorylation of smad2 induced by Omp29 (Fig7. D and F). These findings suggest that Omp29 increases extracellular production of active TGF- $\beta$ 1, and then enhances TGF- $\beta$ R/smad2 cascade in gingival epithelial cells.

#### 4. Discussion

In this chapter, it was demonstrated that Omp29 increased the production of active TGF- $\beta$ 1 from OBA9 cells, followed by activation of TGF- $\beta$ R/smad2 cascade in OBA9 cells. From these findings, Omp29-induced apoptosis of gingival epithelial cells are likely involved in TGF- $\beta$ 1-dependent smad2 signaling pathway.

There are accumulating evidences that  $\alpha$ v integrins is the key activator of TGF- $\beta$ . It is thought that some integrins allows strict spatiotemporal regulation of TGF- $\beta$  activity. Evidences point to a crucial role for cell contraction in the activation of TGF- $\beta$  via integrins  $\alpha$ v $\beta$ 5 and  $\alpha$ v $\beta$ 6[43], [44]. Intriguingly, Omp29 is associated with the invasion of *Aa* into gingival epithelial cells by up-regulating F-actin rearrangement via the FAK signaling pathway [45]. Therefore, it may be plausible that Omp29-mediated actin rearrangement induces cell contraction, which, in turn, result in the activation of TGF- $\beta$ . Notably, transfection for FAK siRNA apparently relieved the Omp29-induced the phosphorylation of smad2 in OBA9 cells (Appendix Fig. A and B). Hence, these findings may support that Omp29-induced actin rearrangement via FAK signaling pathway in OBA9 cells is involved in the activation of TGF- $\beta$ 1. If various bacterial factors-induced actin rearrangement via integrin is involved in the activation of TGF- $\beta$ , this may be a novel suggestion of the relationship between bacteria and TGF- $\beta$  signaling pathway. At the same time, it has been proposed that non-integrin-mediated mechanisms have another possibility to activate latent-TGF- $\beta$ . For example, the serine protease plasmin, the MMP-2 and MMP-9 are related to cleave LAP which result in

activation of TGF- $\beta$  [46]. Further studies will be required to investigate these agendas.

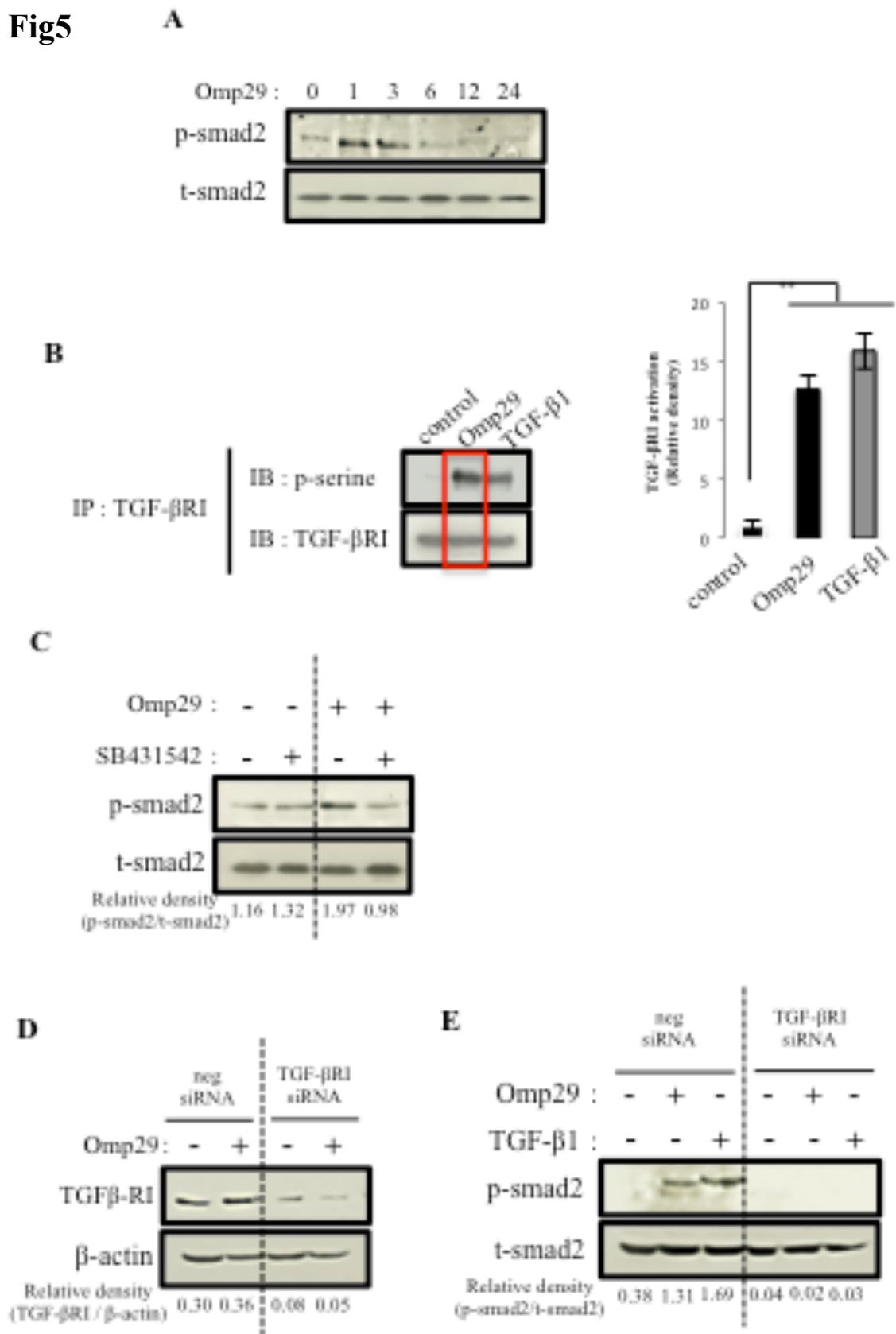
10 ng/ml recombinant TGF- $\beta$ 1 can mediate mitochondria-dependent apoptosis via smad2/caspase-3 cascade in OBA9 cells (data not shown). This data strongly supports our hypothesis that TGF- $\beta$ 1 released by bacterial stimulation induced apoptosis in OBA9 cells. Previous clinical studies have shown that TGF- $\beta$ 1 levels in GCF of patients with moderate periodontitis is  $12.12 \pm 8.80$  ng/ml [47]. This TGF- $\beta$ 1 levels was much higher than that of healthy subject, suggesting that TGF- $\beta$ 1 levels in GCF are involved in the progress of periodontal disease [39], [40], [41], [42]. Increase of TGF- $\beta$ 1 in GCF of patients with periodontitis may affect the apoptosis in junctional epithelium.

In preliminary study, I have checked low concentration (1 ng/ml) of TGF- $\beta$ 1 could induce apoptosis via TGF- $\beta$  receptor. However, the concentration of active TGF- $\beta$ 1 in the supernatant after Omp29 stimulation was approximately 40 pg/ml (Fig7. B). It is still unclear whether TGF- $\beta$ 1 at 40 pg/ml induces smad2 activation and apoptosis in OBA9 cells or not. Active TGF- $\beta$ 1 may be rapidly metabolized or disassembled in the supernatant.

Besides the apoptotic event like this study, TGF- $\beta$ 1 signaling is also well known to induce epithelial-mesenchymal transition (EMT) in epithelial cells [48] [49]. Epithelial cells undergoing EMT caused by TGF- $\beta$ 1 change to an elongated morphology, and down-regulate the expression of the E-cadherin epithelial marker and increase the expression of N-cadherin, mesenchymal marker. Interestingly, previous studies have reported that bacteria or bacteria components induced not apoptosis but EMT in

epithelial cells such as intrahepatic biliary epithelial cells [50, 51]. The results obtained from the present study may imply the mechanism how bacteria induce EMT, followed by fibrosis.

**Fig5**

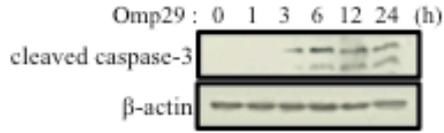


*Fig5. Omp29 elevates the phosphorylation of smad2 via TGF- $\beta$  type I receptor.*

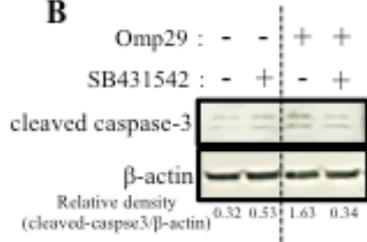
(A) OBA9 cells were exposed to Omp29 for the indicated periods. Phosphorylated (p) and total (t) smad2 levels were analyzed by Western blotting. (B) Representative Western blotting of Omp29- and TGF- $\beta$ 1-stimulated phosphorylation of serine in TGF- $\beta$ RI and also of total TGF- $\beta$ RI after the immunoprecipitation (IP) of TGF- $\beta$ RI. lane 1, basal; lane 2, Omp29; lane 3, 10 ng/ml TGF- $\beta$ 1. Densitometric analysis of the Omp29- or TGF- $\beta$ 1-stimulated phosphorylation of serine in TGF- $\beta$ RI. OBA9 cells were incubated for 1 h with Omp29 or 10 ng/ml TGF- $\beta$ 1 as a control. TGF- $\beta$ RI was immunoprecipitated from cell lysates and subjected to Western blotting analysis of the phosphorylation of serine. Blots were reprobed for total TGF- $\beta$ RI levels. Results are expressed as a percentage above basal levels in relative densitometric units normalized to total TGF- $\beta$ RI levels. Values represent the mean  $\pm$  S.D. of three cultures. \*\*  $p < 0.01$  values differ significantly ( $t$ -test). (C) OBA9 cells were pretreated with or without SB431542 (10  $\mu$ M) for 30 min and were then exposed to Omp29 for 1 h. Phosphorylated (p) and total (t) smad2 levels were analyzed by Western blotting, (D) OBA9 cells, having been transfected with the negative control or TGF- $\beta$ RI siRNA, were cultured for 48 hours in medium B. Protein level of TGF- $\beta$ RI and  $\beta$ -actin were analyzed by Western blotting. (E) OBA9 cells, having been transfected with the negative control or TGF- $\beta$ RI siRNA, were cultured for 48 hours in medium B and were then exposed to Omp29 for 1 h and TGF- $\beta$ 1 for 15 min. Phosphorylated (p) and total (t) smad2 levels were analyzed by Western blotting, respectively. Band densities were quantified through the densitometric scanning of each band by using National Institutes of Health Image J software. Similar results were obtained from three experiments.

**Fig6**

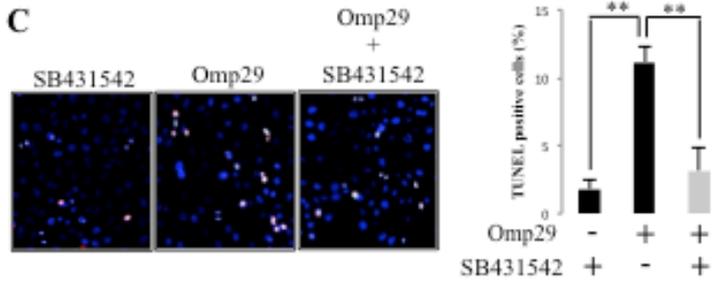
**A**



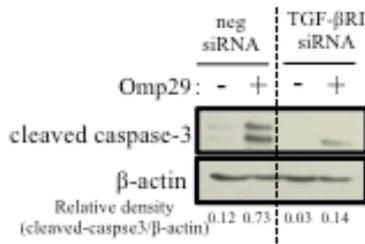
**B**



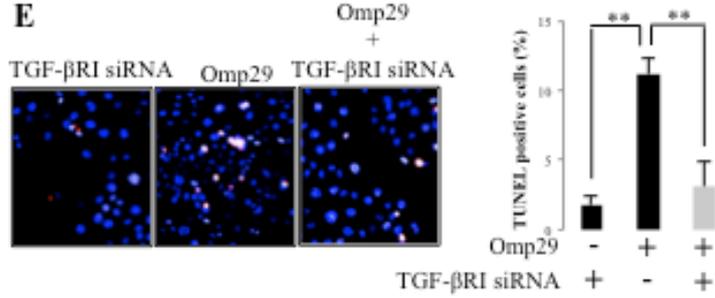
**C**



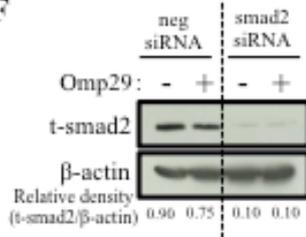
**D**



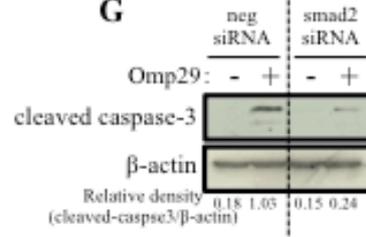
**E**



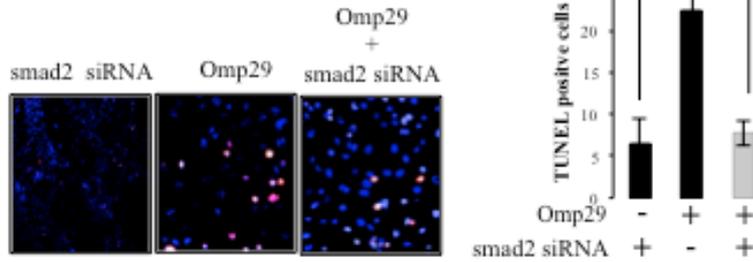
**F**



**G**



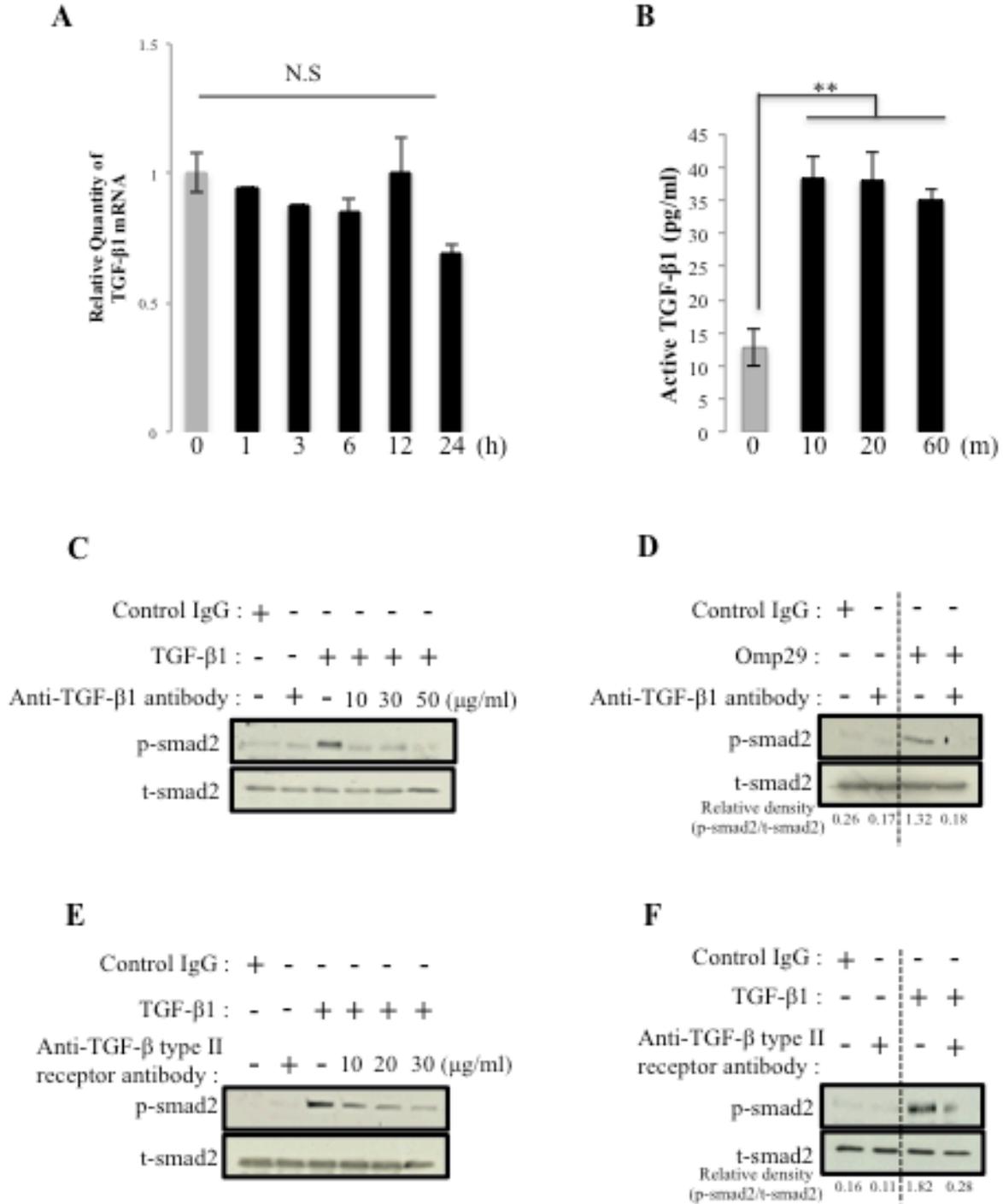
**H**



*Fig6. Omp29 induces apoptosis through TGF- $\beta$  type I receptor.*

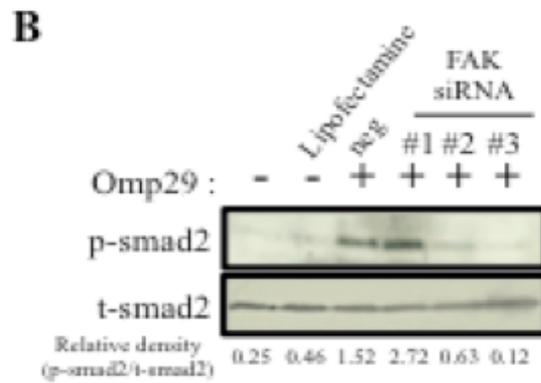
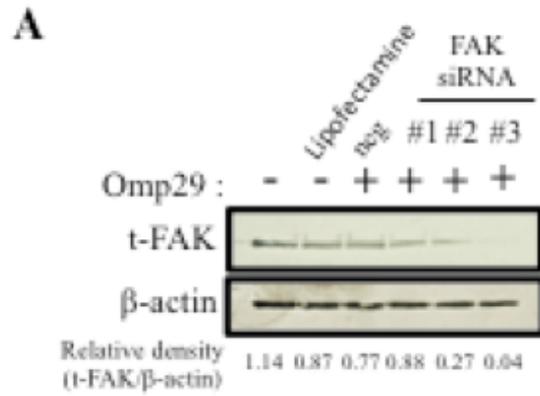
(A) OBA9 cells were exposed to Omp29 for the indicated periods. Cleaved caspase-3 levels and  $\beta$ -actin were analyzed by Western blotting. (B) OBA9 cells were pretreated with or without SB431542 (10  $\mu$ M) for 30min and were then exposed to Omp29 for 12 h. Cleaved caspase-3 levels and  $\beta$ -actin were analyzed by Western blotting. Band densities were quantified through the densitometric scanning of each band using the National Institutes of Health Image J software. (C) TUNEL-positive apoptotic cells (red) are shown under each set of conditions, and the graph shows the percentage of TUNEL-positive cells. Values represent the mean  $\pm$  S.D. of the three cultures. \*\*  $p < 0.01$  values differ significantly ( $t$ -test). Similar results were obtained from three experiments. (D-H) OBA9 cells, having been transfected with the negative control or TGF- $\beta$ RI siRNA (D) or smad2 siRNA (F and G), were cultured for 48 h in medium B and were then exposed to Omp29 for 12 h. Cleaved caspase-3 levels (D), total (t) smad2 (F and G) and  $\beta$ -actin were analyzed by Western blotting. Band densities were quantified through the densitometric scanning of each band using the National Institutes of Health Image J software. (E and H) OBA9 cells, having been transfected with the negative control or TGF- $\beta$ RI siRNA (E) or smad2 siRNA (H), were cultured for 48 h in medium B and were then exposed to Omp29 for 24 h. TUNEL-positive apoptotic cells (red) are shown under each set of conditions, and the graph shows the percentage of TUNEL-positive cells. Values represent the mean  $\pm$  S.D. of the three cultures. \*\*  $p < 0.01$  values differ significantly ( $t$ -test). Similar results were obtained from three experiments.

**Fig7**



*Fig7. Omp29 increases extracellular production of active TGF-β1*

(A) The effect of Omp29 on mRNA expression of TGF-β1 in OBA9 cells. The cells were exposed to Omp29 for the indicated periods. The mRNA levels of TGF-β1 and GAPDH in the cell were analyzed through real-time PCR. Values represent the mean ± S.D. of the three cultures. \*\*  $p < 0.01$  values differ significantly (*t*-test). Similar results were obtained from three experiments. (B) OBA9 cells were exposed to Omp29 for the indicated periods. Measurement of the concentration of active TGF-β1 was performed by ELISA according to the manufacturer's instructions. Data represents the results from three individual experiments ( $n = 3$ ). \*\*  $p < 0.01$ . Values differ significantly (*t*-test). (C-F) OBA9 cells were pretreated with or without anti-TGF-β1 antibody (C and D) or anti-TGF-β type II receptor (E and F) for 30min and were then exposed to TGF-β1 (10 ng/ml) for 15 min (C and E) and Omp29 for 1 h (D and F). Phosphorylated (p) and total (t) smad2 levels were analyzed through Western blotting, respectively. Band densities were quantified through the densitometric scanning of each band by using National Institutes of Health Image J software. Values represent the mean ± S.D. of the three cultures.



*Appendix Fig. The invasion of Omp29 into OBA9 cells involves in Omp29-augmented smad2 phosphorylation.*

(A and B) OBA9 cells, having been transfected with the negative control or FAK siRNA, were cultured for 48 h in medium B and were then exposed to Omp29 for 1 h. Total (t) FAK levels and β-actin (A), Phosphorylated (p) and total (t) smad2 levels (B) were analyzed by Western blotting. Band densities were quantified through the densitometric scanning of each band using the National Institutes of Health Image J software.

## Chapter 5 Summary & Conclusion

The present study aimed to clarify the molecular mechanism how periodontopathic bacteria induce apoptosis on human gingival epithelial cells. The following results were obtained.

1. While the phosphorylation of smad2 in HGECs and OBA9 cells stimulated with *Aa*, *Fn* and *EC* were up-regulated, was not enhanced in these cells with *Pg* and *Pi*.
2. Apoptosis of OBA9 cells stimulated with *Aa* was induced via TGF- $\beta$ R1/smad2 signaling pathway.
3. Omp29 increases active TGF- $\beta$ 1 in the extracellular and the augmentation of TGF- $\beta$ R/smad2 cascade induce apoptosis in OBA9 cells.

I demonstrated, for the first time, that apoptosis of gingival epithelial cells stimulated with *Aa* is mediated the TGF- $\beta$ R1-smad2 signaling pathway, and TGF- $\beta$ 1 in the extracellular is involved in this apoptosis. These findings provided a novel insight into bacterial infection system and introduced novel molecular targets in the search for a therapeutic chemical compound that can protect OBA9 cells from bacteria-induced apoptosis. These results may lead to the development of novel preventive or therapeutic interventions tot periodontitis. Further studies are warranted to investigate this in more detail. If bacteria widely play a role in TGF- $\beta$ R/smad2 signaling in various cells, the results of the present study may lead to better understanding of bacterial infectious diseases.

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