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Phenytoin Activates Smad3 Phosphorylation and Periostin Expression in Drug-induced Gingival Enlargement

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Abstract

Drug-induced gingival enlargement (DIGE) is a fibrotic condition associated with systemic administration of the anti-epileptic drug, phenytoin. We have previously demonstrated that periostin, which is transforming growth factor-beta (TGF-β) inducible gene, is upregulated in various fibrotic conditions including gingival enlargement associated with nifedipine. The objective of this study was to assess periostin expression in phenytoin-induced gingival enlargement (PIGE) tissues and to investigate the mechanisms underlying periostin expression. Human PIGE tissues were assessed using Masson’s trichrome, with cell infiltration and changes in extracellular matrix composition characterized through labeling with antibodies to periostin, phospho-SMAD 3, TGF-β, as well as the macrophage markers CD68 and RM3/1. Using human gingival fibroblasts (HGFs) in vitro, we examined the pathways through which phenytoin acts on fibroblasts. In PIGE tissues, which demonstrate altered collagen organization and increased inflammatory cell infiltration, periostin protein was increased compared with healthy tissues. p-SMAD2/3, the transcription factor associated with canonical TGF-β signaling, is localized to the nuclei in both gingival fibroblasts and oral epithelial cells in PIGE tissues, but not in healthy tissue. In vitro culture of HGFs with 15 and 30 µg/ml of phenytoin increased periostin protein levels, which correlated with p-SMAD3 phosphorylation. Inhibition of canonical TGF-β signaling with SB431542 significantly reduced phenytoin induction of SMAD3 phosphorylation and periostin expression in HGFs. Analysis of PIGE tissues showed a subset of CD68 stained macrophages were TGF-β positive and that RM1/3 regenerative macrophages were present in the tissues. Our results demonstrate that phenytoin up-regulates periostin in HGFs in a TGF-β-dependent manner.
**Introduction**

Drug-induced gingival enlargement (DIGE) is a pathological condition that can develop due to the systemic administration of the anti-seizure drug phenytoin. DIGE is often evident within 1 month of the onset of the drug regimen (Brew et al., 2000; Kaur et al., 2010). DIGE can cause severe problems with regards to speech, breathing, eating, and can cause malocclusion in the jaw. Of great clinical concern, DIGE also increases the oral bacterial load by generating plaque retention sites surrounding the tissue that cannot be removed/accessed by brushing. DIGE was classified by the American Academy of Periodontology as a plaque-mediated condition (Armitage., 1999), with current evidence suggesting that DIGE is a fibrotic lesion (Brown et al., 1991; Dill and Iacopino., 1997; Steinsvoll et al., 1999; Kataoka et al., 2000; Uzel et al., 2001).

Histologically, DIGE is characterized by the presence of an irregular and thickened epithelium, elongated rete ridges, and increased deposition of fibrous connective tissue (Heasman and Hughes, 2014). The large epithelial ridges protrude deep into the underlying connective tissue (van der Wall et al., 1985; Nery et al., 1995). In the connective tissue, increased proliferation of fibroblasts coupled with diminished apoptosis is evident, as well as excess production of ECM. The excess ECM is characterized by increased bundles of irregular collagen (Heasman and Hughes., 2014) as well as an increase in glycosaminoglycan content (Mariani et al., 1996).

Another major hallmark of DIGE is extensive infiltration of inflammatory cells, including macrophages, monocytes, and lymphocytes (Heasman and Hughes., 2014). The degree of inflammation in the connective tissue varies depending on the drug underlying the condition, with phenytoin induced enlargement considered the most fibrotic with the least inflammation.
However, phenytoin-induced gingival enlargement (PIGE) is still characterized by an extensive infiltration of macrophages, plasma cells, and lymphocytes (Heasman and Hughes, 2014). An increased number of regenerative macrophages were evident in DIGE tissues compared to inflamed tissues from subjects not treated with phenytoin (Iacopino et al., 1997). RM3/1, a marker associated with a regenerative macrophage phenotype, was higher in PIGE tissues compared to tissues from subjects not treated with phenytoin. Regenerative macrophages are associated with production of growth factors such as transforming growth factor-beta 1 (TGF-beta1), whereas inflammatory macrophages produce pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-alpha1) (Zwadlo et al., 1985; Zwadlo et al., 1986; Noble et al., 1993).

Many proteins that have been identified to contribute to fibrosis are activated in response to TGF-β, including matricellular proteins that modulate cell-matrix interactions (Bornstein and Sage, 2002). Matricellular proteins, other than connective tissue growth factor (CCN2), have not been investigated in the context of DIGE (Uzel et al., 2001). Periostin, a recently classified matricellular protein (Norris et al., 2008a), is associated with collagen-rich tissues where it regulates functional and structural properties of connective tissues (Hamilton, 2008). In recent years, increased periostin expression levels have been strongly correlated with several fibrotic conditions and regenerative responses (Elliott and Hamilton, 2011). However, the expression of periostin in PIGE has never been investigated.

Whether phenytoin directly induces gingival enlargement is still unclear and based on the literature, controversial, since other contributing factors such as gender, drug dose, and duration have yet to be fully eliminated. In this study, we assessed whether periostin is associated with PIGE and the mechanism of action of phenytoin on gingival cells and tissues.
Materials and Methods

Tissue Preparation and Immunohistochemistry

Clinically healthy gingiva was obtained with informed consent from six patients undergoing periodontal or implant therapies at the Oral Surgery Clinic at The University of Western Ontario. The use of all tissue material was in accordance with the guidelines of the University’s Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB) requiring informed consent. Tissues were fixed in 10% neutral buffered formalin (Sigma Aldrich; St. Louis, MO, USA, paraffin embedded, and 5 μm sections cut. Formalin fixed gingival tissues from eleven patients with PIGE were obtained from the oral pathology laboratory. The patient demographics is shown in table 1. Immunohistochemistry was performed as previously described (Wen et al., 2010; Zhou et al., 2010; Elliott et al., 2012). In brief, tissues were deparaffinized and immune-labeled using primary antibodies against periostin (sc49480; Santa Cruz Biotechnology; Dallas, TX, USA; 1:100), alpha-smooth muscle actin (α-SMA) (ab5694; Abcam; Cambridge, MA, USA; 1:400), RM3/1 (ab17051; Abcam) and phosphorylated-SMAD2/3 (p-SMAD2/3) (Ser 423/425) (sc-11769; Santa Cruz Biotechnology; Dallas, TX, USA; 1:100). Primary antibodies were detected using the ImmPRESS Reagent Kit Peroxidase (Vector Laboratory; Burlingame, CA, USA) and DAB reagent (Vector Laboratory) following the manufacturer’s instructions. Tissue sections omitting the primary antibody were used as a negative experimental control. All sections were counterstained with haematoxylin (Sigma Aldrich). To assess collagen density and arrangement in tissues, deparaffinized histological sections were stained using a Masson’s trichrome stain (University Hospital, London, ON, Canada). Images of tissues stained with
Masson’s trichrome were taken on a DM1000 light microscope (Leica; Concord, Ontario, Canada) and Leica Application Suite Software (version 3.8).

**Immunofluorescence**

Deparaffinized sections were also fluorescently stained. Tissues were permeabilized with 0.1% Triton X-100 (Caledon; Georgetown, ON, Canada) PBS, blocked with 10% horse serum in 0.1% Triton X-100 PBS, and incubated with CD68 (ab955; Abcam; Cambridge, MA, USA; 1:100), TGF-β (R&D Systems; Minneapolis, MN, USA; 1:100), and TNF-α (ab6671; Abcam; 1:100) primary antibodies overnight. CD68 was detected using Cy5-conjugated anti-mouse secondary antibodies (Molecular Probes; Carlsbad, CA, USA). TGF-β primary antibody was detected using FITC-conjugated anti-rabbit secondary antibody (Molecular Probes). All sections were counterstained with Hoechst 3342 dye (1:5000) for nuclei. Images were taken on Carl Zeiss Imager M2m microscope (Carl Zeiss; Jena, Germany) using Zen Pro 2012 software.

**Human Gingival Fibroblast Isolation**

Clinically healthy gingiva was obtained from 6 patients undergoing crown lengthening or implant therapies at the Oral Surgery Clinic at The University of Western Ontario under informed consent. The use of all tissue material was in accordance with the guidelines of the University’s Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB) requiring informed consent. Healthy HGFs were obtained from tissue from individuals who have no history of the drug therapy using explant cultures (Brunette et al., 1983). HGFs were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Carlsbad, CA,
USA) and 1X antibiotics and antimycotics (AA; 100 μg/ml penicillin G, 50 μg/ml gentamicin, 25 μg/ml amphotericin B), in 75 cm² tissue culture plastic flasks, at 37°C in a humidified atmosphere of 95% air 5% CO₂. Cells were removed from the growth surface with trypsin [0.25% trypsin (Gibco), 0.1% glucose, citrate-saline buffer (pH 7.8)]. Cells between passage 2 and 7 were used in experiments.

**Phenytoin treatment**

HGFs were seeded at a density of 60,000 cells per well. Prior to nifedipine treatment, HGFs were serum-starved in low-glucose DMEM overnight before treatments. For phenytoin treatment, 5,5-Diphenylhydantoin (phenytoin) (D4007; Sigma Aldrich) was reconstituted in dimethyl sulfoxide (DMSO) (Sigma Aldrich) followed by dilution in low-glucose DMEM (0.5% FBS and 1% AA) at final concentrations of 15 and 30 μg/ml. HGFs were cultured in DMEM containing 15 μg/ml of phenytoin, or 30 μg/ml of phenytoin for 30 minutes, or 24 hours. HGFs treated with DMEM containing the equal volume of DMSO without phenytoin served as control to provide baseline.

To inhibit TGF-β signaling ALK5 (TGF-β receptor I), SB431542 (10 μM) (S4317; Sigma Aldrich) was used while the equal volume of DMSO solvent served as control. Parallel cultures of HGFs were pre-treated with low-glucose DMEM (0.5% FBS and 1% AA) containing ALK5 inhibitor SB431542 at 10 μM or control DMSO 30 minutes prior to phenytoin treatment. Subsequently, HGFs were treated with DMEM containing DMSO alone, phenytoin (30 μg/ml), or both phenytoin (30 μg/ml) and SB431542 for 30 minutes, and 24 hours.
Western blot

HGFs were washed twice with PBS and harvested with RIPA buffer (Sigma Aldrich) containing protease (Roche Diagnostics GmbH; Mannheim, Germany) and phosphatase inhibitor (Calbiocam; Billerica, MA, USA) cocktails. Cell lysates were sonicated and centrifuged at 13,000 rpm for 20 minutes. Protein concentration was determined by Pierce® BCA Protein assay kit (Pierce; Waltham, MA, USA). 25 μg proteins of each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were washed with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and blocked with 5% dried milk in TBS-T. Primary antibodies for phosphorylated-SMAD3 (p-SMAD3) (ser423/425) (ab52903; Abcam), periostin (ab92460; Abcam), and GAPDH (Millipore; Billerica, MA, USA) were used. Detection was with appropriate peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch; West Grove, PA, USA), which were developed with SuperSignal Western Pico Chemiluminescence Substrate (Pierce; Waltham, MA, USA).
Results

Extracellular matrix organization is altered and periostin is increased in PIGE tissue

To assess collagen organization and cell infiltration in healthy and DIGE human gingival samples, sections were stained using Masson’s trichrome. In comparison to healthy gingival tissues (n = 6), the collagen content was qualitatively higher in gingival tissues from patients diagnosed with PIGE (n = 11) (Figure 1). In all PIGE samples, epithelial hyperplasia was evident and increased inflammatory cell infiltrate in PIGE was noted compared to healthy gingiva, although the level of inflammatory cells observed varied between patients with PIGE (Figure 1). Gingival connective tissues in healthy gingiva had low immuno-reactivity for periostin, with the protein detectable only in the basement zone and superficial papillary lamina propria. (Figure 2A). In contrast, an elevated level of periostin was observed in the connective tissues of phenytoin-induced gingival enlargement compared to healthy tissue. In PIGE, periostin immunoreactivity localized to collagen fibrils in the gingival connective tissue (Figure 2A), but periostin was not associated with regions of inflammatory cells.

Phenytoin increases periostin through TGF-β signaling in HGFs in vitro

As we have shown in a previous paper (Kim et al., 2013) that nifedipine activates TGF-β signaling to increase periostin expression, we next assessed whether a similar mechanism would exist in HGFs in the presence of phenytoin. Using western blotting, periostin protein level was assessed in cell lysates. Periostin protein increased in HGFs treated with phenytoin at either 15 or 30 µg/ml compared to control cells, at 24 h post-treatment (Figure 2B). We next assessed whether phenytoin increases periostin through TGF-β signaling. Untreated HGFs, those cultured
with phenytoin (30 µg/ml) or concurrently with phenytoin (30 µg/ml) and SB431542 (10 µM) were assessed for periostin levels using western blot at 24 h. While phenytoin (30 µg/ml) treatment alone increased periostin level in HGFs, SB431542 and phenytoin co-treatment did not alter periostin level compared to control.

**Increased p-SMAD2/3 evident in PIGE tissues**

We qualitatively examined the patterns of phosphorylated-SMAD2/3 (p-SMAD2/3) level, in pathological samples isolated from patients with PIGE. Two specific patterns of p-SMAD 2/3 were observed in the oral epithelium. Nuclear localization of p-SMAD3 through all layers of the epithelium was noted in samples with high inflammatory cell infiltration (Figure 3A, 68 yo). In samples with lower immune cell presence, p-SMAD3 localized mainly to the basal epithelial cells (Figure 3A, 56 yo). High immunoreactivity for p-SMAD2/3 was evident in the nuclei of gingival fibroblasts in all connective tissues obtained from PIGE samples (n = 11) but no labeling of p-SMAD2/3 was evident in healthy gingival tissues (n = 6) (Figure 3A). In samples with high immune cell infiltration, nuclear localization of p-SMAD3 was also evident in the nuclei of endothelial cells in the vasculature (Figure 3A, 68 yo).

**Phenytoin stimulates TGF-β signaling in HGFs in vitro**

TGF–β is a central player in fibrosis and is known to regulate periostin expression (Wen et al., 2010; Zhou et al., 2010). We assessed whether phenytoin increases periostin protein levels through canonical TGF-β signaling in HGFs. To investigate the direct effect of phenytoin on TGF-β signaling in HGFs, we assessed SMAD3 activation changes in HGFs in vitro by
observing phosphorylated-SMAD3 (p-SMAD3) using western blot. Elevated p-SMAD3 was observed in HGFs treated with 15 and 30 µg/ml phenytoin, compared to control cells, at 30 minutes post-treatment (Figure 3B). While phenytoin (30 µg/ml) treatment elevated p-SMAD3, SB431542 and PHE concurrent treatment attenuated p-SMAD3 increase. In fact, SB431542 and phenytoin co-treatment led to complete attenuation of p-SMAD3 compared to basal level (control).

**Myofibroblasts are not present in DIGE**

Prolonged persistence of myofibroblasts is a characteristic of fibrosis (Gabbiani., 2003). To evaluate whether myofibroblast differentiation is associated with DIGE, immunoreactivity for alpha-smooth muscle actin (α-SMA) was assessed in histological gingival tissue sections from healthy subjects and patients clinically diagnosed with DIGE (Figure 4). In both groups of healthy and DIGE gingival tissues, α-SMA immunoreactivity was only evident in the vasculature.

**A subset of CD68 positive macrophages in PIGE tissues express TGF-β**

To assess whether any macrophages present in PIGE tissues have a pro-regenerative polarization, tissues were double stained for CD68 and TGF-β. In the healthy tissue, we did not observe CD68 stained cells (Figure 5A, B). In PIGE tissues, many CD68 positive cells were detected, with a subset of these cells labeling positive for TGF-β (Figure 5B, arrow).
RM3/1 positive macrophages are associated with PIGE

As a subset of CD68 positive cells were associated with TGF-β expression, we labelled samples with antibodies to RM3/1, a marker of late stage inflammation and regenerative macrophages (Figure 6). Immunoreactivity for RM3/1 in healthy gingival samples was low, but in all PIGE samples, RM3/1 macrophages were present (Figure 6). The number of RM3/1 positive cells varied depending on the patient, but all PIGE samples were associated with regenerative macrophages.

Discussion
Current treatment options for the treatment of DIGE are largely limited to surgical removal of the overgrown tissue using gingival flap and scalpel gingivectomy. Such treatments persist due to our relatively poor understanding of the molecular mechanism underlying the disease (Brown et al., 1991). Although current surgical techniques are promising and often result in fairly good clinical outcomes, the recurrence rate of DIGE is high, around 34%, and the patients are then subjected to repeated surgical interventions (Ilgenli et al., 1999). Moreover, such surgical procedures are distressing and costly for patients, and whether these repeated insults to the tissue increase the rate of fibrosis is unknown. Discontinuation of the drug therapy can improve the condition but is rarely an option. Moreover, genetic, age, dose, duration of administration, and plaque are also reported to influence the development of DIGE. There have been several reports in particular highlighting an importance of plaque accumulation and gingival inflammation in DIGE. However, whether plaque is a significant factor in DIGE is controversial and reports are conflicting (Banthia et al., 2014; Sam and Sebastian., 2014). There is a fundamental lack of understanding of the molecular effects of these different drugs on gingival tissue. Development of novel preventative and therapeutic strategies to treat the disease are desperately needed, but a
clear understanding of the pathogenesis of DIGE, and the molecular mechanisms underlying the condition are required.

Periostin, a pro-fibrotic matricellular protein, has been shown to be a significant player of collagen fibrillogenesis and synthesis in healing and fibrosis, often in collagen rich tissues (Norris et al., 2007; Norris et al., 2008a; Norris et al., 2008b; Zhou et al., 2010). Consistent to these previous findings, PIGE tissues also demonstrated elevated levels of periostin in the connective tissue. The elevated periostin level in PIGE may be the inducer of ECM accumulation that leads to fibrosis. Further studies need to be done to assess whether periostin is inducing fibrotic phenotypes in PIGE. Periostin was identified as a critical modulator of myofibroblast differentiation in the granulation tissue of healing skin, with expression peaking at day 7 (Elliott et al., 2012); periostin activates adhesive signaling through β1 integrins and focal adhesion kinase leading to myofibroblast differentiation. The prerequisite for myofibroblast differentiation is known to be a stiff matrix environment and adhesive signaling (Hinz, 2009). This suggests that there is likely a difference in the stiffness of healing skin and fibrotic lesions of the gingiva. This may explain the lack of myofibroblasts in PIGE regardless of elevated periostin. Furthermore, it demonstrates that periostin does not induce adhesive signaling in HGFs as seen in dermal fibroblasts (Elliott et al., 2012).

As is the case in many types of fibrosis (Darby et al., 1990), the presence of myofibroblasts was described in gingival fibrosis, and indeed it was suggested to be central in drug-induced gingival fibromatosis (Yamasaki et al., 1987; Dill and Iacopino, 1997). However, recent research has shown an absence or low levels of myofibroblasts in fibrotic gingiva, idiopathic gingival fibromatosis (Sakamoto et al., 2002; Martelli et al., 2010), and DIGE resulting from administration of phenytoin (Sobral et al., 2010), nifedipine and amlopidine.
(Pisoschi et al., 2014). These findings were not consistent with fibrotic features associated with gingival fibromatosis, and whether myofibroblast differentiation is present in DIGE is still controversial and needs to be further investigated, although our study suggests myofibroblasts are not present. What is evident is that whether myofibroblasts are present or not, higher collagen accumulation is consistently observed in fibrotic gingiva. Human gingival fibroblasts grown in nifedipine or phenytoin independently demonstrated a reduced phagocytic capacity in vitro (McCulloch and Knowles., 1993). Subsequently it has been demonstrated that reduced intracellular calcium levels are evident in the presence of the drugs (due to inhibited passage of calcium ions across membranes or release of calcium intracellular stores), which is likely to reduce the ability of the cells to limit phagocytosis (McCulloch., 2004).

We respect to mechanisms of action of phenytoin in PIGE, it is clear that phenytoin acts through TGF-β pathways, resulting in p-SMAD2/3 nuclear translocation. We have previously shown that calcium-channel blocker nifedipine, activates TGF-β signaling to increase periostin in HGFs, although whether this is a direct effect of the drug has yet to be established (Kim et al., 2013). Animal studies have demonstrated that TGF-β signaling plays a significant role in progression of cyclosporine and phenytoin induced gingival enlargement (Al-Hamilly et al., 2016). The observation that both phenytoin and nifedipine can activate canonical TGF-β signaling in cultured cells suggests a common mechanism of action in gingival enlargement although the primary action of the drugs is different physiologically (phenytoin promotes sodium efflux Vs nifedipine which is a calcium antagonist). A recent study has suggested that phenytoin acts on HGFs through the non-selective cation channel transient receptor potential ankyrin 1 (TRPA1) (Lopez-Gonzalez et al., 2017). Interestingly, a previous study demonstrated that TRPA1 is required for TGF-β signaling (Okada et al., 2014) and its deletion reduces corneal
fibrosis. Significantly, inhibition of TRPA1 suppressed inflammation and cellular processes associated with TGF-β signaling/fibrosis. Potential inhibition of TRPA1 as a mechanism to reduce DIGE is intriguing, although problems could exist for clinical utility based on its role as an analgesic (Gupta et al., 2016).

A further finding of this study was that immunoreactivity for TGF-β co-localized with a subset of CD68 positive macrophages. It is therefore possible that TGF-β released by regenerative macrophages may also contribute to SMAD phosphorylation and periostin expression by gingival fibroblasts evident in PIGE tissues. Staining of the tissues for CD163 (RM3/1), a marker of macrophages associated with the late inflammatory phase and tissue healing, found significant numbers of these cells present in all the PIGE tissues examined. Therefore, although it appears that phenytoin can activate TGF-β signaling directly through TRPA1, the presence of regenerative macrophages suggests that there is more than one mechanism leading to TGF-β signaling and progression of the fibrotic process in PIGE. TGF-β release from macrophages would bind directly to TGF-β receptors on HGFs, leading to SMAD phosphorylation. Therefore, inhibition of TRPA1 alone may not reduce fibrotic processes.

In conclusion, we have demonstrated that PIGE is associated with TGF-β signaling and elevated levels of periostin protein. Moreover, our study shows that TGF-β signaling in PIGE could be attributed to direct activation of TGF-β likely through TRPA1 (Lopez-Gonzalez et al. 2017) on gingival fibroblasts, as well as through macrophage release of TGF-β. We suggest that strategies to reduce DIGE will have to address different sources of TGF-β within the tissue and that one inhibitor alone may not be sufficient to prevent DIGE.
Acknowledgments

We would also like to extend our appreciation to Dr. Tom Daley and Linda Jackson-Boeters (Department of Pathology) for guidance and helpful discussion. SSK is a recipient of a Canadian Institutes of Health Research Doctoral Award scholarship.

References


**Figure legends**

**Figure 1:** Altered matrix production and tissue organization in gingival tissues from patients with PIGE.

Collagen deposition in gingival tissues derived healthy subjects and DIGE patients. Masson’s trichrome stains collagen fibers in blue. Dense collagen accumulations are evident in the gingival tissues from patients experiencing DIGE. Lower magnification images are shown on the top panel whereas higher magnification images are shown on the low panel. Black arrows highlight areas of inflammatory cell infiltration.

**Figure 2:** Periostin is upregulated in gingival connective tissues of phenytoin-induced gingival enlargement compared to healthy gingival tissue.

A. Immuno-reactivity for periostin of gingival tissues from healthy subjects (n = 6), and phenytoin-induced gingival enlargement (n = 11) patients by immunohistochemistry. Representative images are shown. Sections were incubated with rabbit periostin primary antibody, which was detected with peroxidase conjugated secondary antibody and DAB. In
phenytoin-induced gingival enlargement tissues, periostin immuno-reactivity is greatly elevated in the connective tissue (Black arrows) compared to healthy tissue, where it localizes to the lamina propria (Black arrowheads). Primary delete (Negative) is shown inset B. PHE increases periostin levels via TGF-β signaling. Western blot showing periostin protein in cell lysates when HGFs were cultured with PHE (15 and 30 µg/ml) or without PHE (DMSO alone) for 1 day. HGFs treated with PHE (30 µg/ml) expressed a greater level of periostin. Western blot showing periostin is decreased in cell lysates when HGFs were cultured in combinations of PHE (30 µg/ml) and SB431542 (10 µM) for 1 day. GAPDH was used as a loading control.

**Figure 3: Increased p-SMAD2/3 nuclear translocation is evident in PIGE.** Immuno-reactivity for phosphorylated-SMAD2/3 (p-SMAD2/3) gingival tissues from healthy subjects (N=6) and PIGF (N=11) patients using immunohistochemistry. Representative images are shown. Paraffin sections were incubated with a primary antibody and detected using peroxidase-conjugated secondary antibody and DAB with haematoxylin counter-staining. Epithelium areas are shown on the top panel and the connective tissue areas are shown on the bottom panel. In some patients, pSMAD2/3 nuclear translocation was evident in the endothelial layer of blood vessels (White arrows). Black arrows highlight basal keratinocyte activation of pSMAD2/3. Primary delete (Negative) is shown inset B. Phenytoin induces canonical TGF-β signaling in HGFs. HGFs were cultured with phenytoin (15 µg/ml and 30 µg/ml) or without phenytoin (DMSO alone) for 30 minutes. SMAD3 activation was studied by assessing phosphorylated-SMAD3 (p-SMAD3) by western blot. Phenytoin treated HGFs showed greater levels of p-SMAD3. Inhibition of the TGF-β type I receptor attenuated p-SMAD3 in the presence of phenytoin. HGFs were cultured with without phenytoin (DMSO alone), phenytoin (30 µg/ml), or
both phenytoin (30 µg/mL) and SB431542 (10 µM) combined, and p-SMAD3 level was assessed using western blots in cell lysates. GAPDH was used as a loading control.

**Figure 4: Myofibroblasts are absent in the gingival tissues from patients with PIGE.**

A. Immunoreactivity for α-smooth muscle actin (α-SMA), the marker for myofibroblasts, in gingival tissues from healthy subjects (n = 6) and PIGE patients (n = 11). Sections were incubated with a primary antibody against α-SMA and detected using peroxidase-conjugate secondary antibody and DAB. All samples from both groups lacked α-SMA positive cells in the connective tissue, except in the smooth muscle of the blood vessel walls, which is the internal positive control for α-SMA. α-SMA-stained myofibroma tissue was used as the experimental positive control and primary delete (Negative) is shown inset.

**Figure 5. Co-localization of CD68 and TGF-β in PIGE tissues**

Fluorescent immunoreactivity for periostin in the gingival tissues from healthy subjects (n = 6) and PIGE patients and phenytoin (n = 11). Representative images are shown. Sections were incubated with primary antibodies against CD68 and TGF-β, which was detected using Cy5 (red) and FITC (green) conjugated secondary antibodies, respectively. Nuclei are stained with Hoechst 3342 dye (blue). Elevated CD68 positive cells was observed in the connective tissues of PIGE and a subset of these cells were positive for TGF-β (white arrows). Primary delete (Negative) is shown inset.
Figure 6. RM3/1 positive macrophages are present in PIGE.

Immunoreactivity for RM3/1 (CD163), a marker of regenerative macrophages, in gingival tissues from healthy subjects (n = 6) and PIGE patients (n = 11). Sections were incubated with a primary antibody against RM3/1 and detected using peroxidase-conjugate secondary antibody and DAB. Elevated immunoreactivity for RM3/1 was observed in all PIGE samples. Primary delete (Negative) is shown inset.

Table 1: Demographics of the patients with PIGE used in the study.

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<td>PIGH10</td>
<td>Female</td>
<td>69</td>
<td>Maxillary gingiva</td>
</tr>
<tr>
<td>PIGH11</td>
<td>Female</td>
<td>68</td>
<td>Mandibular gingiva</td>
</tr>
<tr>
<td>Patient</td>
<td>Gender</td>
<td>Age</td>
<td>Location</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>-----</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>PIGH1</td>
<td>Female</td>
<td>20</td>
<td>Left anterior maxillary gingiva</td>
</tr>
<tr>
<td>PIGH2</td>
<td>Male</td>
<td>69</td>
<td>Interdental papilla</td>
</tr>
<tr>
<td>PIGH3</td>
<td>Female</td>
<td>53</td>
<td>Left posterior mandibular gingiva</td>
</tr>
<tr>
<td>PIGH4</td>
<td>Male</td>
<td>48</td>
<td>Alveolar mucosa</td>
</tr>
<tr>
<td>PIGH5</td>
<td>Female</td>
<td>30</td>
<td>Maxillary and mandibular anterior gingiva</td>
</tr>
<tr>
<td>PIGH6</td>
<td>Female</td>
<td>56</td>
<td>Maxillary anterior buccal gingiva</td>
</tr>
<tr>
<td>PIGH7</td>
<td>Male</td>
<td>43</td>
<td>Left anterior mandibular gingiva</td>
</tr>
<tr>
<td>PIGH8</td>
<td>Female</td>
<td>65</td>
<td>Right posterior mandibular gingiva</td>
</tr>
<tr>
<td>PIGH9</td>
<td>Male</td>
<td>74</td>
<td>Left maxillary buccal alveolar mucosa</td>
</tr>
<tr>
<td>PIGH10</td>
<td>Female</td>
<td>69</td>
<td>Maxillary gingiva</td>
</tr>
<tr>
<td>PIGH11</td>
<td>Female</td>
<td>68</td>
<td>Mandibular gingiva</td>
</tr>
</tbody>
</table>
A

Healthy 68 yo, Female Mandibular Gingiva

56 yo, Female, Buccal Mucosa

B

Phenytoin (µg/ml) 0 15 30

PN

GAPDH

Phenytoin (30 µg/ml) - + +

SB431542 - - +

PN

GAPDH
Healthy 68 yo, Female, Mandibular Gingiva

68 yo, Female, Mandibular Gingiva

56 yo, Female, Buccal Mucosa

Phenytoin (30 µg/ml)

B Phenytoin (µg/ml) 0 15 30

Phenytoin (30 µg/ml) - + +

SB431542 - - +

P-SMAD3

P-SMAD3

GAPDH

GAPDH
Healthy 69 yo, Female, Maxillary Gingiva

CD68  TGF-β1  Merged + Hoescht

Negative  Negative

200 μm
HISTOLOGY AND HISTOPATHOLOGY

20 yo Female Maxillary Gingiva

Healthy

30 yo Female Maxillary Gingiva

Healthy

53 yo Female Mandibular Gingiva

Negative

69 yo Male Papilla Gingiva

100 μm