GINGIVAL FIBROBLAST AND EPITHELIAL FUNCTIONS IN PERIODONTAL DISEASE-MODULATION BY INSULIN RESISTANCE

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ABSTRACT

Ning Yu: Gingival Fibroblast and Epithelial Functions in Periodontal Disease-Modulation by Insulin Resistance
(Under the direction of Steven Offenbacher)

Periodontal disease is a biofilm-initiated inflammatory condition that affects the tooth supporting apparatus. Gingival epithelium provides an early line of defense against bacteria while gingival connective tissue fibroblast plays an integral role in tissue remodeling and host responses. Therefore, investigating the alterations of gingival fibroblast and epithelial functions in periodontal disease improves our understandings about the disease pathogenesis. In addition, both cells need energy support to survive and act, which comes from glucose utilization that is profoundly influenced by insulin response and glucose metabolism.

In the second chapter, we find that acute gingival inflammation markedly induces insulin response genes. However, these changes are not evident in chronic inflammation. This study suggests that acute gingival inflammation may induce tissue metabolism, which may contribute to the pathogenesis of periodontal disease.

In the third chapter, we demonstrate that advanced glycation end products (AGEs) inhibit gingival fibroblast migration. In addition, lipopolysaccharides (LPS) potently induce PTGS2 and MMP1 genes transcription and prior exposures to AGEs enhance the up-regulation. LPS or AGEs fail to induce FGF2, which is an anabolic growth factor. This study links AGEs with accelerated periodontium destruction and impaired gingival wound healing during diabetic periodontitis.
In the fourth chapter, we seek to unravel the role of desmosome structural molecule plakophilin-2 (PKP2) in periodontal disease. Decreased epithelial PKP2 is associated with periodontitis in gingival biopsy samples. *Porphyromonas gingivalis* (P.g) specifically degrade PKP2 protein through cysteine proteases, not serine proteases, or intracellular proteosomal or lysosomal degradation pathways. Although in vitro stimulations of P.g increase the overall PKP2 DNA methylation level, periodontitis gingival biopsies do not display differential DNA methylation patterns compared to the healthy biopsies. Gingival epithelial cells that lack PKP2 have inhibited cell proliferation, cell spreading, and impaired cell permeability. This chapter provides innovative evidence about the association between dampened desmosome molecules and periodontal disease.

In summary, this dissertation work has deepened our knowledge about the pathogenesis of periodontal disease from both tissue and cellular levels.
To my parents Yujiang Yu and Shulian Guo who have instilled the values of education in the younger me and encouraged me to chase my dreams. Mom and Dad, it is the unconditional love that you have been providing turns me into a happy and confident person. You two are the reasons why I become who I am. If I have or will have anything valuable contributing to the world, it is because of you.

To my Aunt Dr. Shujuan Guo for being an inspiration for me growing up. Thank you for helping me initiate this voyage and supporting me all the way through.

To my dearest husband and best friend Maoxuan Lin. Mao, this dissertation thesis is as much mine, as it is yours. It would not have been achieved had not been your dedication and support. Thank you for coming into my life and making me a better person.
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<th>Description</th>
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<tbody>
<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>AL</td>
<td>Attachment loss</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase C</td>
</tr>
<tr>
<td>ARVC</td>
<td>Arrhythmogenic right ventricular cardiomyopathy</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BOP</td>
<td>Bleeding on probing</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C.r</td>
<td>Campylobacter rectus</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBD</td>
<td>Catenin-binding domain</td>
</tr>
<tr>
<td>Cdt</td>
<td>Cytolethal distending toxin</td>
</tr>
<tr>
<td>CEBPA</td>
<td>CCAAT/Enhancer Binding Protein (C/EBP), Alpha</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Cox-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CRB</td>
<td>Crumbs-PALS1-PATJ complex</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DLG</td>
<td>Discs large</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNM1L</td>
<td>Dynamin-like protein</td>
</tr>
<tr>
<td>DP</td>
<td>Desmoplakin</td>
</tr>
<tr>
<td>DSC</td>
<td>Desmocollin</td>
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<td>DSG</td>
<td>Desmoglein</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>DUSP4</td>
<td>Dual specificity phosphatase 4</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetus bovine serum</td>
</tr>
<tr>
<td>FDR</td>
<td>False discover rate</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast growth factor 2</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GEC</td>
<td>Gingival epithelial cells</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporters</td>
</tr>
<tr>
<td>hBD</td>
<td>human β-defensins family</td>
</tr>
<tr>
<td>HGEPs</td>
<td>Human gingival cells</td>
</tr>
<tr>
<td>HGF</td>
<td>Human gingival fibroblast</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filaments</td>
</tr>
<tr>
<td>IFNG</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL6R</td>
<td>Interleukin 6 receptor</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate 3</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity pathway analysis</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced pluripotent stem</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>JMD</td>
<td>Juxtamembrane domain</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>LEF1</td>
<td>Lymphoid enhancer-binding factor 1</td>
</tr>
<tr>
<td>LGL</td>
<td>Lethal giant larvae</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitor factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney epithelial cells</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteases</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MUPP1</td>
<td>Multi-PDZ domain protein 1</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NLRP/NALP</td>
<td>NLR family pyrin domain containing</td>
</tr>
<tr>
<td>NLRs</td>
<td>Nod like receptors</td>
</tr>
<tr>
<td>Nod</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>P.g</td>
<td><em>Porphyromonas gingivalis</em></td>
</tr>
<tr>
<td>PALS 1</td>
<td>Proteins associated with Lin Seven 1</td>
</tr>
<tr>
<td>PAR</td>
<td>Partitioning-defective protein</td>
</tr>
<tr>
<td>PATJ</td>
<td>PALS-1 associated tight junctions protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Probing depth</td>
</tr>
<tr>
<td>PG</td>
<td>Plakoglobin</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PKP</td>
<td>Plakophilin</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
</tr>
<tr>
<td>PV</td>
<td>Pemphigus vulgaris</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosome RNA</td>
</tr>
<tr>
<td>STX4</td>
<td>Syntaxin 4</td>
</tr>
<tr>
<td>STXBP2</td>
<td>Syntaxin binding protein 2</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TCF</td>
<td>T cell factor</td>
</tr>
<tr>
<td>TER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-type MMTV integration site family member</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula occludens</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Overview of gingiva

Periodontal disease is a biofilm-initiated inflammatory condition that leads to destructions of periodontal tissue, which includes gingiva, cementum, periodontal ligament, and alveolar bone. In the oral cavity where over 700 species of microorganisms reside, the outermost layer of periodontal tissue—gingiva is exposed to constant microbial challenges (Hajishengallis, 2015). When dysbiosis occurs, the gingival-biofilm interface becomes disrupted which eventually leads to clinical symptoms of periodontal disease.

Based on distinct anatomic characteristics, three types of gingiva are defined: marginal gingiva, attached gingiva, and interdental gingiva (Newman et al., 2012). Marginal gingiva is the free end of the gingiva, encompassing the teeth like a collar. It forms the soft tissue wall for the (Newman et al., 2012). Attached gingiva is the soft tissue area between marginal gingiva and alveolar mucosa. Cementum on the cervical side of the root and ridge of alveolar bones can be found underneath the attached gingiva. Attached gingiva also provides support for the gingival tissues to withstand mechanical forces during mastication, speaking, and tooth brushing (Newman et al., 2012). Interdental gingiva refers to the gingiva that fills the interdental space (Bath-Balogh et al., 2006). The shape and size of interdental gingiva depend on the contact area of the adjacent teeth. Losing interdental gingiva may cause esthetic failure, food retention, and potentially cause periodontal disease.

Histologically, gingiva is composed of gingival epithelium and connective tissue. Gingival epithelium is characterized as stratified squamous epithelium, which from apical to basal side,
has four layers of cornieum, granulosum, spinosum and basal (Newman et al., 2012). There are three distinctive types of gingival epithelium, namely sulcular epithelium, junctional epithelium, and oral epithelium (Hatakeyama et al., 2006). Sulcular epithelium lines the gingival sulcus and interacts with bacteria intensively. Junctional epithelium, being the base of the gingival sulcus, is responsible for gingival attachment to the teeth. During the progression of periodontal disease, junctional epithelium detaches from the tooth surface, which results in clinical attachment loss (Bosshardt and Lang, 2005). Both sulcular and junctional epithelium are non-keratinized epithelium. The keratinized oral epithelium is the outer layer of the gingival epithelium ranging from marginal to attached gingiva. The main function of gingival epithelium is to form a barrier to defend against mechanical, chemical and microbial intruders.

**Gingival epithelial functions**

Gingival epithelium provides an early line of defense against bacteria in the gingival sulcus during periodontal disease-associated bacterial disturbance (Schroeder and Listgarten, 1997). Most surfaces and cavities of human organs are lined with epithelium to act as physical and chemical barriers (Peterson and Artis, 2014). Therefore, much of our knowledge of epithelial functions is learned from organs like skin, intestine, urinary bladder, and eyes. An intact epithelial sheet relies on the three characteristics: 1) epithelial cell-to-cell adhesion; 2) epithelial homeostasis: 3) mucosal inflammation.

**Epithelial cell-to-cell adhesion**

No single human cell can survive without adhering and communicating with surrounding cells or extracellular matrix. Although cell-matrix adhesions play a crucial role in the host defense and disease progress, intercellular junctions are keystones for establishing lateral cell-to-
cell adhesions. These junctional structures not only contribute to sealing the epithelium but also exert a variety of biological functions. Four types of intercellular junctions are commonly identified as tight junctions, adherens junctions, gap junctions, and desmosome junctions (Alberts, 2008). In Figure 1.1-1.3, major components of intercellular junctions are presented (Durham and Garrett, 2009; Green and Gaudry, 2000; Neunlist et al., 2013).

**Tight junctions**

Tight junctions reside on the most apical side of the epithelium and have complex protein compositions. To date, at least 40 tight junctions-associated proteins have been discovered, but only three major transmembrane proteins are well characterized, which are claudin, occludin, and junctional adhesion molecule (JAM) (Gonzalez-Mariscal et al., 2003; Schneeberger and Lynch, 2004; Yamazaki et al., 2008).

**Occludin** is the first identified transmembrane component of tight junctions with a transmembrane domain that passes through the membrane four times (Furuse et al., 1993). Within occludin, there are one N-terminus and one C-terminus facing inside and two extracellular loop domains facing outside of the cell. The long C-terminus functions as the docking sites for tight junctions-associated proteins (eg. ZO-1, ZO-2, ZO-3) (Furuse et al., 1994; Haskins et al., 1998; Itoh et al., 1999). Zonula occludens (ZO) are critical scaffolding proteins that bridge the transmembrane proteins and actin cytoskeletons. ZO also has a dual role in regulating cell growth (Bauer et al., 2010). Overexpression of occludin appears to increase numbers of tight junctions strands, with accordingly increased transepithelial electrical resistance (TER) activity, which is an indicator for tight junctions activities (McCarthy et al., 1996). Several lines of in vitro evidence have shown that disrupted occludin leads to impaired barrier functions (Bamforth et al., 1999). However, numerous studies have concluded that occludin is
not essential for tight junctions (Saitou et al., 2000; Schulzke et al., 2005). One piece of evidence is that occludin deficient mice are still viable and maintain an intact barrier in the GI tract as well as the urinary bladder. These findings have suggested that there might be alternative proteins for occludin (Schulzke et al., 2005). In endothelial cells from non-neuronal tissues, occludin is apparently missing even though the tight junctions complex persists (Hirase et al., 1997).

Additionally, occludin-deficient embryonic stem cells bear proper tight junctions (Saitou et al., 1998). These findings have suggested that occludin mainly associates with tight junctions in a regulatory fashion (Nusrat et al., 2005).

Claudin has a structure similar to that of occludin with a tetraspan transmembrane domain and a relatively shorter N-terminus and C-terminus facing cytosolically (Furuse et al., 1998; Morita et al., 1999). The C-terminus of claudin not only binds to ZO-1, ZO-2, ZO-3, but also binds to multi-PDZ domain protein 1 (MUPP1) and PALS-1 associated tight junctions protein (PATJ) (Jeansonne et al., 2003; Poliak et al., 2002). Since its discovery in 1998, claudin has become an integral transmembrane component of tight junctions (Furuse et al., 1998). When claudin is introduced into fibroblast that originally lacks tight junctions, fibroblast has newly formed tight junctions-like strands and starts aggregating. Claudin-1 deficient mice demonstrated a more severe phenotype than occludin deficient mice with much compromised epidermal barriers (Furuse et al., 2002). The interaction between ZO and claudin is also critical for tight junctions. A lack of ZO-1 significantly delays the assembly of tight junctions, suggesting that actin cytoskeletons are required to maintain the characteristics of tight junctions (McNeil et al., 2006).

JAM is another important transmembrane member of tight junctions. Unlike occludin and claudin, JAM has a single transmembrane domain and an intracellular C-terminus domain
containing PDZ binding motifs that bind to ZO-1 and MUPP-1 (Hamazaki et al., 2002). JAM participates in regulating epithelial barriers. One study showed that antibodies against JAM had no effect on changing the confluency of epithelial cells monolayers, but inhibited the reconstruction of tight junctions after being disrupted (Liu et al., 2000). Although inhibiting JAM in endothelial cells improves transendothelial migration of neutrophils, dampening JAM does not affect the transepithelial migration of neutrophils (Zemans et al., 2009).

**Adherens junctions**

Adherent junctions are positioned basally to tight junctions in epithelium and their molecular structures are relatively simple, comprising epithelial cadherin (E-cadherin), p120 catenin, β-catenin, and α-catenin (van Roy and Berx, 2008). E-cadherin belongs to a family of cadherin proteins that are calcium-dependent. E-cadherin consists of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The extracellular domain forms bonds between adjacent cells, while the cytoplasmic tail binds to p120 catenin and β-catenin to exert biological functions (van Roy and Berx, 2008). Similar to other transmembrane proteins, E-cadherin is assembled in the Golgi apparatus and later translocated to the plasma membrane. To engage in the dynamics of adherens junctions, membrane-bound E-cadherin experiences active turnovers through endocytosis and degradation (Bryant and Stow, 2004).

p120 catenin binds to the juxtamembrane domain (JMD) of E-cadherin, which contributes to stabilizing adherens junctions complex at the cell membrane (Reynolds et al., 1992). No evidence supports that p120 participates in E-cadherin trafficking to the membrane, but p120 prevents E-cadherin from endocytosis and degradation. This notion has been proved by many studies showing that loss of p120 promotes the transportation of E-cadherin to the lysosomes for proteolysis (Davis et al., 2003; Xiao et al., 2003). Therefore, it is not surprising to see p120
regulating E-cadherin expression (Ireton et al., 2002). Additionally, the interactions between p120 and microtubules assist in shifting roles of adherens junctions from cell adhesiveness to cell movement (Yanagisawa et al., 2004). β-catenin binds to E-cadherin as well, but only through the catenin-binding domain (CBD). Unlike p120, β-catenin is required for E-cadherin trafficking to the plasma membrane. The association between β-catenin and E-cadherin is essential for the transportation of E-cadherin from endoplasmic reticulum to the membrane, and protects E-cadherin from being degraded. More importantly, β-catenin interacts with α-catenin, which subsequently couples with actin filaments and some other cytoskeleton-associated molecules, including vinculin, afadin, formin, and ZO-1 (Kobielak and Fuchs, 2004; Watabe-Uchida et al., 1998). These interactions provide more pieces of evidence to prove that adherens junctions are closely associated with cell movement.

In addition to its function as part of adherens junctions, β-catenin belongs to Wnt signaling, which controls many basic physiological processes like cell proliferation, cell polarity, and tissue homeostasis (Eastman and Grosschedl, 1999). When Wnt receptors are activated, β-catenin proteins start accumulating in the cytosol and translocating to the nucleus. Inside the nucleus, β-catenin acts as a co-activator for the transcription factors family TCF/LEF, which controls the transcription of various target genes (Eastman and Grosschedl, 1999). In summary, adherens junctions play important roles in cell adhesion, cell motility, and linking the intercellular junctions to the intracellular signaling pathways.

**Gap junctions**

Gap junctions are formed by a cluster of connexin (Cx) proteins that contains a family of at least 21 members in humans (Naus and Laird, 2010). Six connexins aggregate to become a hemichannel-like connexon. Two connexons from the opposing cells contact and form a
channel-like structure of gap junctions. This organization creates a space (~2-4 nm) between the cells and this where the name of “Gap” is introduced (Maeda et al., 2009). Based on whether six connexins are from the same or different connexons, there are hetermeric or homomeric connexon channels. Connexins have distinct expression patterns among the cell or tissue types. For instance, Cx26 is highly expressed in the skin, cochlea, liver, and placenta, while Cx46 and Cx50 are predominantly found in retina cells. Moreover, Cx43 is considered to be the major gap junctions protein in cardiomyocytes (Evans and Martin, 2002). In gingival epithelium, Cx43 is abundantly expressed, while Cx32 is clearly missing and Cx26 is moderately expressed (Ye et al., 2000).

Due to an unique architecture, gap junctions allow diffusions of ions (K$^+$ and Ca$^{2+}$), second messengers (cAMP, cGMP, and IP3), and small metabolites between cells, all of which participate in relaying electrical and biochemical signals across the body (Mese et al., 2007). In addition to the cellular communications, connexins bind with ZO-1, calmodulin, and tubulin to participate in cell movement (Barker et al., 2002; Giepmans et al., 2001; Peracchia et al., 2000). Cx43 is a functional target for Wnt signaling, which links gap junctions with adherens junctions (van der Heyden et al., 1998).

**Desmosome junctions**

Desmosome junctions are widely expressed at all layer of epithelium except for stratum cornium (Getsios et al., 2004). Two cadherin proteins-desmoglein (DSG, 1-4) and desmocollin (DSC, 1-3) constitute for the extracellular components of desmosomes; three intracellular molecules-plakophilin (PKP, 1-3, and p0071-sometimes known as PKP4), plakoglobin (PG), and desmoplakin (DP) engage in bridging cadherin proteins to intermediate filaments (IF).
DSG and DSC are both glycoproteins that form strong adhesiveness between the opposing plasma membranes through their extracellular domains, in a calcium dependent manner. The interactions can be either homophilic (DSC-DSC, DSG-DSG) or heterophilic (DSG-DSC) (Chitaev and Troyanovsky, 1997; Syed et al., 2002). The role of DSG is widely studied in an autoimmune disease-pemphigus vulgaris (PV), which is caused by aberrant productions of immunoglobulins against DSG1 and DSG3 (Hashimoto et al., 1995). The phenotype of PV is characterized by basal keratinocytes dissociation in the epidermis and oral mucosa due to loss of desmosomes. Targeted disruptions of Dsg3 in mice led to the dissociations of basal cells in the oral mucosa, which mimicked the phenotype of PV (Koch et al., 1997). Functions of DSG1 are further described in bullous impetigo, in which exfoliative toxins secreted by *Staphylococcus aureus* specifically degrade DSG1, causing keratinocyte dissociations (Amagai et al., 2000). In addition to DSG, DSC1 is also a target in PV, as Dsc1 deficient mice display loss of cell-to-cell adhesions in the epidermis with defective barrier functions (Chidgey et al., 2001).

Plakoglobin is also called γ-catenin due to its homology to the catenin members. Similar to β-catenin, plakoglobin localizes with E-cadherin as well, but with much stronger binding affinities (Chitaev et al., 1996). Mice lacking plakoglobin have defective heart and skins as embryos (Bierkamp et al., 1996). Plakophilins are also important desmosome molecules, which will be discussed in a separate session. Desmoplakin is the most abundant desmosome molecule (Mueller and Franke, 1983). However, it does not bind to cadherins directly but rather bind to plakoglobin and plakophilin through its N-terminus binding sites. The C-terminus of desmoplakin directly binds to intermediate filaments. Mice lacking desmoplakin die in an early embryo stages with much compromised numbers of desmosomes (Gallicano et al., 1998). Although an epidermis-specific loss of desmoplakin in mice does not affect numbers of
desmosomes in the epidermis, desmoplakin-null keratinocytes have fewer desmosomes formed in vitro (Mueller and Franke, 1983). Actin organizations and sealing properties during the epithelial sheet formation are largely potentiated in Desmoplakin-null keratinocytes (Mueller and Franke, 1983). Therefore, ample evidence has suggested that desmoplakin is required for the assembly of functional desmosomes. In the absence of desmosome molecules, mice experience early embryo stalls due to malfunctions of the epidermis, neuroepithelium, heart and blood vessels, suggesting critical roles of desmosomes.

**In summary**, intercellular junctions play crucial roles in cell-to-cell adhesion. Additionally, these molecules exert certain fundamental physiological activities. Much evidence has suggested that all four junctional complexes tend to work together by interacting with each other directly and indirectly.

**Epithelial homeostasis**

Epithelial homeostasis represents the basic biological features of epithelial cells, encompassing cell proliferation and differentiation, cell migration, and cell death/apoptosis.

Epithelial cells are highly proliferative cells. Cell renewals usually occur at the basal layer where there is a niche for progenitor cells and stem cells to proliferate (Boers et al., 1998). Proliferative cells differentiate actively as they move to the apical side. During the proliferation and differentiation, cell polarity becomes crucial in deciding the asymmetrical distributions of molecules within different domains of the cell (Martin-Belmonte et al., 2001). There are two types of cell polarities: **apical basolateral polarity** and **planar cell polarity**. Apical basolateral polarity is used to describe the distinct distributions of organelles and molecules in the apical or basolateral plasma membranes. Epithelial sodium channels are most likely found at the apical
plasma membranes. In the lateral domain, junctional molecules are predominantly expressed. At the basal domain, integrin and hemidesmosomes are uniquely expressed to adhere epithelium to ECM. The underlying control over the apical basolateral polarity has been widely studied with three complexes being identified: 1) PAR3-PAR6-aPKC complex; 2) Crumbs-PALS1-PATJ complex (CRB); 3) LGL-Scribble-DLG complex (Martin-Belmonte et al., 2001). Planar cell polarity refers to the polarization of cells within the plane of the cell sheet. When cells divide perpendicularly to the plane, it gives rise to stratified epithelium; when cells divide parallel to the plane, it expands the epithelial sheet (Rodriguez-Boulan and Macara, 2014).

Cell migration is an integral part of epithelial homeostasis, playing a central role in tissue remodeling and disease development. Upon receiving biological or mechanical cues, cells spread on the attachment sheet and move to a designated area. Migratory activities largely rely on the cytoskeleton structures like actin, microtubules, and intermediate filaments. Both cell-to-cell and cell-to-ECM adhesions affect cell migration (Cuvelier et al., 2007; Vicente-Manzanares et al., 2005). In a large-scale gene search, Simpson KJ et al has reported three major signaling nodes (β-catenin, β-integrin, actin) are involved in migration (Simpson et al., 2008). However, enhanced cell adhesion does not necessarily increase migration. In the same study, genes that accelerated migration may also inhibit adhesion, suggesting a negative correlation. In this end, cell adhesion and cell migration interact in two directions.

Cell death refers to the cessation of cellular functions, which counteracts to cell proliferation. Highly proliferative epithelial cells require active cell death to maintain the epithelial homeostasis (Dannappel et al., 2014). There are three major types of cell death, which are necrosis, apoptosis, and autophagy (Kroemer et al., 2009). Many studies have demonstrated that pathogen components, bacterial metabolites, and inflammatory mediators promote gingival
epithelial cell death (Brozovic et al., 2006; Kang et al., 2012; Stathopoulou et al., 2009; Tsuda et al., 2010).

**Mucosal inflammation**

Although the role of epithelium in defending microorganisms has been empirically focused on its physical barrier capability, its immune aspects are also of great significance. Epithelial cells display several pattern recognition receptors (PRRs) that are either membrane-bound or cytoplasmic distributed (Peterson and Artis, 2014). Upon activations, PRRs promote a pyramid of signaling events to produce pro-inflammatory cytokines and chemokines. Pro-inflammatory cytokines like interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) promote inflammations to restrict infections. However, a prolonged inflammatory status brings damages to the tissue. Epithelial cells secrete a wide range of chemokines via PRRs to attract leukocytes to the local lesions to diminish bacterial or viral intrusions. Another immune feature for epithelial cells lies is to produce anti-microbial peptides, the expression of which also depends on PRRs (Nakatsuji and Gallo, 2012).

For epithelial cells, two major PRRs are toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (Nod) like receptors (NLRs) (Janeway and Medzhitov, 2002; Meylan et al., 2006). TLRs interact with bacterial components such as lipopolysaccharides (LPS), lipoproteins, flagellin, unmethylated CpG DNA, and etc. NLRs are cytoplasmic receptors that recognize bacterial peptidoglycan, bacterial toxins, and other pathogenic structures of microorganisms. NLR family pyrin domain containing 3 (NLRP3, also known as NALP3) contributes to IL-1β productions in gingival epithelial cells via activating the inflammasome (Bostanci et al., 2009; Yilmaz et al., 2010). Gingival epithelial cells that are challenged by P.
gingivalis produce high levels of IL-1β. Gingival epithelial cells that are challenged by A. actinomycetemcomitans induce high levels of IL-8 (Stathopoulou et al., 2010). F. nucleatum increase more IL-6 productions in gingival epithelial cells than commensal bacteria-S. gordini (Hasegawa et al., 2007). Via TLR2, P. gingivalis induce monocyte chemoattractant protein-1 (MCP-1) and IL-8, both of which are chemokines that promote neutrophils to migrate and exert phagocytosis at the site of infections (Hasegawa et al., 2007).

The best-characterized antimicrobial peptides in gingival epithelial cells are members of human β-defensins family (hBD). hBD-1 is constitutively expressed in gingival epithelial cells, while hBD-2 and hBD-3 are stimulation-dependent (Gursoy and Kononen, 2012; Krisanapakornkit et al., 1998). hBDs are differentially regulated in gingival epithelial cells by oral bacteria regardless whether they are pathogenic or commensal (Gursoy and Kononen, 2012). Gingival epithelial cells markedly produce hBD in response to the challenge of F. nucleatum and TLR2 and NLRP2 are involved in this regulation (Ji et al., 2009).

**Overview of Plakophilins**

Plakophilins are a group of armadillo family members (PKP1, PKP2, PKP3, and p0071) that are widely expressed in many tissues and organs. PKPs are located in both cytosol and nucleus. In addition to being structural scaffolds to increase desmosome plaques, PKPs regulate the intracellular signalings as well (Bass-Zubek 2009).

PKP1 is the first identified and smallest PKPs member (Heid et al., 1994). PKP1 expression is increased from basal to granular layers in stratified epithelium, suggesting its roles in keratinocyte differentiation (McMillan et al., 2003). PKP1 assists in forming desmosome junctions by recruiting partner desmosome molecules (eg. DSG1, DSC1 and DP) (Hatzfeld 2000,
Patients with autosomal recessive defects of \textit{PKP1} gene have ectodermal dysplasia and skin fragility. In these patients, deregulated desmosome sizes and numbers can be found in the epidermis (McMillan JR, 2003).

PKP2 is the best-characterized member of PKPs. Rare mutations of \textit{PKP2} gene in humans cause arrhythmogenic right ventricular cardiomyopathy (ARVC), which is an inherited heart disease that is characterized by a sudden myocardium breakdown (van Tintelen et al., 2006). PKP2 works in building cadherin-PKP-DP links in the desmosome assembly. A loss of PKP2 results in failure of DP to accumulate at the cell border. Protein kinase C alpha (PKC\textalpha) is the major functional molecule in this process. PKP2 recruits PKC\textalpha to form a complex with DP. Subsequently, PKC\textalpha phosphorylates DP, modulates its interactions with IF, and guides DP assembly into the cell border (Bass-Zubek et al., 2008). Since PKP2 is the major PKPs member in cardiomyocytes, impaired PKP2 cannot be compensated by other PKPs, resulting in dampened desmosomes. Grossmann KS et al found that PKP2 null mice exhibited an embryonic lethality due to defects of heart morphogenesis, indicating PKP2 also plays important roles in cardiac development (Grossmann et al., 2004).

Functions of PKP2 are not limited to being structural molecule of desmosomes. PKP2 is highly expressed in the basal layer of epithelium, an area that is particularly active for cell renewal. Studies have shown that PKP2 induces the phosphorylation of epidermal growth factor receptor (EGFR) to promote cell proliferation (Arimoto et al., 2014; Kazlauskas, 2014). Deficient PKP2 in keratinocytes leads to delayed migration through altered focal adhesion (Koetsier et al., 2014). An additional study has shown that PKP2 directly interacts with \textbeta-catenin to potentiate TCF/LEF1-mediated transcription of target genes (Chen et al., 2002). PKP2 also
associates with RNA polymerase III in the nucleus (Mertens et al., 2001). Since pol III regulates tRNA, rRNA 5S, and some small RNAs, PKP2 may present post-translational activities.

Unlike PKP1 and 2, PKP3 is not expressed in the nucleus and mice lacking PKP3 present relatively mild phenotypes (Sklyarova et al., 2008). Current evidence associates PKP3 with cancer development. PKP3 binds to dynamin-like protein (DNM1L) to act in vesicle trafficking within the secretory pathway in non-small cell lung carcinomas (Furukawa et al., 2005). By associating with RNA-binding proteins, PKP3 is involved in protein translation and RNA metabolisms responding to environmental stressors (Hofmann et al., 2006). p0071 is referred as PKP4 but its structure is more closed to p120 catenin. p0071 has been shown to regulate Rho signaling during cytokinesis (Wolf et al., 2006). In one study that shows the staining pattern for all PKP members in primary oropharyngeal tumors, only p0071 is associated with tumor grade, clinical parameters, and patient survivals (Papagerakis et al., 2003).

It is noteworthy that even though PKPs have been described in various diseases, the role of PKP2 in periodontal disease remains unknown.

**Alterations of junctional molecules in periodontal disease**

Gingival epithelium is constantly exposed to microorganisms in the form of gingival plaque. To date, various studies have addressed the disturbance of junctional molecules under periodontitis and the underlying mechanisms.

Gingival tissues contain many intercellular junctional proteins, which are listed in Table 1.1. The differences of junctional proteins between healthy and diseased gingival tissues are also documented. For instance, gingival biopsies from periodontal disease subjects have aberrant staining patterns for tight junctions proteins, including occludin, claudin-4 and 15, ZO-1 and 2,
and JAM-A (Choi et al., 2014; Ye et al., 2014). Fujita et al challenged rats with LPS in the gingival sulcus for 8 weeks and found that claudin-1 was significantly decreased in the junctional epithelium (Fujita et al., 2012). Numerous studies have also shown that E-cadherin is significantly reduced in periodontitis gingival biopsies than healthy controls (Arun et al., 2010; Loo et al., 2010; Nagarakanti et al., 2007). Connexin 26 and 43 are reduced in the pathological lining epithelium of periodontal pockets, indicating the alterations of gap junctions (Ye et al., 2000). In addition, DSG1 and DSC1 mRNA are down-regulated in periodontitis gingival biopsies, indicating the alteration of desmosomes (Abe et al., 2011).

While tissue data provides convincing evidence linking dampened junctional molecules to periodontitis, results from in vitro studies do not always have the same trend. Epithelial bound E-cadherin can be degraded by P. gingivalis, which is mainly due to proteases produced by gingipain (Katz et al., 2000; Katz et al., 2002). However, cytolethal distending toxin (Cdt) from A. actinomycetemcomitans increases cytoplasmic distribution of E-cadherin and overall expression of β-catenin in both gingival tissue explants and gingival epithelial cells (Damek-Poprawa et al., 2013). Proteases from P. gingivalis degrade occludin protein in epithelial cells, accompanied by decreased TER activities (Katz et al., 2002). Collectively, bacterial products regulate junctional molecules productions, leading to altered epithelial homeostasis.

**Gingival fibroblast functions**

Human gingival fibroblast (HGF) is major resident cell in gingival connective tissue. One major function of HGF is to exert immune responses. This function is mainly through activations of PRRs. In addition to TLRs and NLRs, another important PRR in HGF is receptor for advanced glycation end products (RAGE) (Lalla et al., 2000). HGF potently produces pro-
inflammatory cytokines (e.g. IL-1 and IL-6), chemokines (e.g. IL-8), and enzymes (e.g. Cox-2, MMPs) under the circumstances of periodontitis or bacterial challenges (Morton and Dongari-Bagtzoglou, 2001; Takada et al., 1991; Tamura et al., 1992; Yu et al., 2012). Although immune cells like monocytes and macrophages take a major responsibility in producing cytokines, both of them develop LPS tolerance quickly (Mages et al., 2007). LPS tolerance allows pathogens to escape from immune surveillance during sustained bacterial exposures and elongated inflammatory conditions. HGFs appear not to display LPS tolerance, which indicates that HGF may compensate for limiting bacterial effects when monocytes and macrophages are antisensitized (Ara et al., 2009).

HGF is also responsible for synthesis and degradation of ECM, a group of molecules that constitutes for the backbones of the gingival architecture. HGF produces ECM and at the same time secrets matrix metalloproteases (MMPs) to degrade ECM. Therefore, HGF is the key cell in ECM remodeling within gingival tissues. Collagen is the major ECM in gingiva and the predominant type is type I collagen (Newman et al., 2012). HGF secrets a large amount of fibronectin as well, which is a glycoprotein that mediates the adhesions between fibroblast and ECM and fibroblast and surrounding cells. Over 20 MMPs have been identified in gingiva and many of them have distinct roles in development of periodontal disease: MMP1 and MMP8 are collagenases that degrade type I, II, III, and X collagens; MMP2 and MMP9 degrade gelatins and elastin; MMP3 and MMP10 degrade pro-collagen and fibronectin (Reynolds et al., 1994).

Lastly, HGF engages in gingival wound healing processes (Hakkinen et al., 2000). Oral mucosa healing is scar free which is very distinct from skin healing. Because of this feature, many researchers believe that by investigating the uniqueness of oral mucosal healing, we may improve healings with scars as general (Mah et al., 2014). In some studies, gingival fibroblast
acquired from both mice and human gingiva demonstrates phenotypes of induced pluripotent stem (iPS) cells (Egusa et al., 2010; Wada et al., 2011).

**Alterations of gingival fibroblast in periodontal disease**

A good understanding about alterations of gingival fibroblast in periodontal disease sheds lights on the pathogenesis of periodontal disease. Whole or components of pathogens unanimously enhance productions of pro-inflammatory cytokines in HGF (Agarwal et al., 1995; Imatani et al., 2001). Topical additions of IL-1β and TNF-α result in increased transcription of IL-6, IL-11, and leukemia inhibitor factor (LIF) in HGF (Palmqvist et al., 2008). Both LPS and IL-1 induce prostaglandin E₂ (PGE₂) in HGF through increasing cyclooxygenase 2 (Cox-2) (Okamura et al., 1999; Richards and Rutherford, 1988). PGE₂ is a well-known vasodilator engaging in increasing the vascular permeability and promoting inflammation that leads to periodontium destructions (Offenbacher et al., 1993).

HGF presents altered ECM regulation during periodontal disease as well. Periodontitis connective tissues have decreased collagen fibers and MMPs when compared to healthy tissues (Seguier et al., 2001). Periodontal pathogens might be likely reasons since they make proteases that potently degrade ECM (Bachrach et al., 2004; Zhou and Windsor, 2006).

Not many studies investigate the wound healing potential of gingival fibroblast under healthy or diseased conditions. Smoking has long been regarded as a risk factor for periodontitis and impaired wound healing. Nicotine inhibits HGF migration via Rac signaling pathways (Fang Y 2005).

**Glucose metabolism, insulin resistance, and periodontal disease**
All human cells need to obtain energy in order to act and survive. Fat, protein, and glucose constitute for the major energy sources. Glucose uptake and utilization are predominantly affected by insulin. Glucose transporters (GLUT 1-4) and related insulin signaling molecules modulate glucose uptake. Inside cells, insulin regulates glucose utilization through a series of enzymatic reactions. Improper glucose metabolism leads to severe consequences and the most profound consequence is diabetes.

Diabetes is a hyperglycemic condition that affects approximately 30 millions of Americans (Centers for Disease and Prevention, 2014). As the most common form of diabetes, type 2 diabetes is characterized by insulin resistance, which refers to failures of tissues to react to a normal level of insulin. When unnecessary large amounts of glucose deposit in the tissues and organs, physiological damages occur with consequential complications. Periodontal disease is one of the diabetic complications and these two diseases share a two-way relationship (Mealey et al., 2006). Systemic inflammation is considered to be the common links between insulin resistance and periodontal disease. Major pro-inflammatory mediators are elevated in gingivitis and periodontitis subjects systematically (Page, 1991). A lot of these mediators are casually linked to altered glucose utilization and metabolism (Shoelson et al., 2006). TNF-α, IL-6, IL-1β, and increased level of oxidative stress are all known to cause insulin resistance by disrupting c-Jun N-terminal Kinase (JNK) and insulin signaling pathways (Evans et al., 2005; Hotamisligil et al., 1993; Lee et al., 2003).

Both GLUT1 and GLUT4 are expressed in gingival tissues, suggesting their potential roles in regulating glucose metabolism during periodontal disease development (Kuroki et al., 2009; Rao et al., 2012). The modulatory roles of inflammatory cytokines on glucose metabolism appear to be cell and tissue specific. TNF-α inhibits glucose uptake by decreasing GLUT4 expression in
the adipose tissue (Ruan et al., 2002). However, in noninsulin-sensitive tissues, TNF-α and IL-1β have been proved to increase glucose utilization (Shikhman et al., 2001; Yu et al., 1995). TNF-α is also known to block lipoprotein lipase production, which may as a result increase glucose utilization (Kim et al., 2001). Whether local gingival inflammation alters insulin response genes or glucose metabolism remains unknown. Therefore, we aimed to solve this puzzle in Chapter 2.
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<th>Types</th>
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<td>Tight junctions</td>
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<td>Gap junctions</td>
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<td>Desmosome junctions</td>
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Figure 1.1 Major components of tight junctions and adherens junctions.

Adapted from (Neunlist et al., 2013)
Figure 1.2 Gap junction components.

Adapted from (Durham and Garrett, 2009)
Figure 1.3 Molecular structures of desmosome junctions.

Adapted from (Green and Gaudry, 2000)
CHAPTER 2: INSULIN RESPONSE GENE EXPRESSION IN PERIODONTAL DISEASE-MODULATION OF OBESITY

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Abstract

Background: Bacterial infections are known to alter glucose metabolism within tissues via mechanisms of inflammation. We conducted this study to examine whether insulin response genes are differentially expressed in gingival tissues comparing samples from experimental gingivitis and periodontitis subjects to those from healthy individuals.

Methods: Total RNA was extracted from gingival biopsies from twenty-six participants including: 8 periodontally healthy, 9 experimental gingivitis, and 9 periodontitis subjects. Gene expression patterns were evaluated using a PCR array panel to examine 84 candidate genes involved with glucose metabolism, insulin resistance, and obesity. Array data were evaluated using t-test adjusted by FDR (P<0.05) and Ingenuity Pathway Analysis (IPA) was performed for statistical testing of pathways. Although tissue samples were not sufficient to enable protein
quantification, we confirmed the up-regulation of the key gene using lipopolysaccharides (LPS) stimulated primary gingival epithelial cells by western-blot.

**Results:** The mRNA expression patterns of genes that are associated with insulin response and glucose metabolism are markedly different in experimental gingivitis subjects compared to healthy controls. 32 genes are up-regulated significantly by at least 2-fold adjusted for FDR ($P<0.05$). Periodontitis subjects show similar but attenuated changes in gene expression patterns and no genes meet the significance criteria. IPA demonstrates significant activation of carbohydrate metabolism network in experimental gingivitis, but not in periodontitis. Glucose-6-phosphate dehydrogenase (G6PD) protein increases in response to LPS stimulation in primary gingival epithelial cells, which is in the same direction as up-regulated mRNA in tissues.

**Conclusion:** Acute gingival inflammation may be associated with tissue metabolism changes, but these changes are not evident in chronic inflammation. This study suggests that acute gingival inflammation may induce localized changes, which modify tissue insulin/glucose metabolism.
Introduction

Periodontal disease is a biofilm-initiated inflammatory condition that affects the tooth supporting apparatus. Two major periodontal diseases, gingivitis and periodontitis respectively affect 75% and 47% of the adult population in the United States (Albandar et al., 1999; Eke et al., 2012). The pathogenesis of periodontal disease has been described in many aspects, but little is known regarding periodontal tissue glucose metabolism and insulin responsiveness.

Glucose is a major energy source for human cells besides fat and protein and inflammatory signals are likely to affect glucose metabolism. Insulin is critical for cellular uptake of glucose through the glucose transportation system and for activating enzymatic pathways of glucose metabolism to produce energy for cellular activation and survival. Within gingival tissues, the existence of major glucose transporters GLUT1 and GLUT4 is an indication that glucose metabolism may have a profound effect on periodontal tissue metabolism (Kuroki et al., 2009; Rao et al., 2012). Many studies have shown that certain pro-inflammatory cytokines that are elevated in gingivitis and periodontitis can modulate glucose utilization and metabolism (Page, 1991; Shoelson et al., 2006). Tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1beta (IL-1β) and increased level of oxidative stress are all known to cause insulin resistance by disrupting molecular pathways including c-Jun N-terminal Kinase (JNK) and insulin signaling pathways (Evans et al., 2005; Hotamisligil et al., 1993; Lee et al., 2003). The modulatory roles of inflammatory cytokines on glucose metabolism appear to be cell and tissue specific. TNF-α inhibits glucose uptake by decreasing GLUT4 expression in adipose tissue (Ruan et al., 2002). However, in noninsulin-sensitive tissues TNF-α or IL-1β has been proved to increase glucose utilization (Shikhman et al., 2001; Yu et al., 1995). TNF-α is also known to
block lipoprotein lipase production, which may as a result increase glucose utilization (Kim et al., 2001).

Gingivitis and periodontitis are both known to be associated with dramatic elevations in inflammatory cytokines and chemokines (Page, 1991). However, whether gingival inflammation, comparing acute and chronic gingival inflammation, affects glucose metabolism and insulin response gene expression locally is still unknown. Therefore, we conducted this study to begin to explore whether genes associated with glucose metabolism and insulin response were differentially expressed in gingival biopsies comparing experimental gingivitis and chronic periodontitis to healthy.
Material and Methods

Study Participant Selection and Gingival Tissue Biopsies

Twenty-six participants, aged 18 to 64, provided informed consent and were recruited in the dental clinics from School of Dentistry at University of North Carolina at Chapel Hill. All procedures were approved by Institutional Review Board (IRB) of the University of North Carolina at Chapel Hill. Participants were sequentially enrolled with either periodontally healthy, experimental gingivitis or chronic periodontitis with the following exclusion criteria: 1. No use of antibiotics within one month before the screening examinations; 2. No signs of systemic diseases with any oral manifestation at the time of the enrollment; 3. No self-reporting treatment for systemic diseases three months prior to the collections of gingival biopsies. Besides obtaining demographic information and periodontal clinical parameters, examiners also recorded all participants’ weight and height to compute Body Mass Index (BMI).

8 periodontally healthy participants were either healthy volunteers or patients who were undergoing crown-lengthening procedures and they exhibited ≤4mm probing depth (PD), no signs of bleeding on probing (BOP). 9 chronic periodontitis patients representing chronic inflammation were enrolled with exhibitions of ≥5mm of PD, presence of BOP and radiographic demonstration of bone loss. 9 experimental gingivitis patients representing acute inflammation were acquired from a 3-week stent-induced biofilm overgrowth procedure (Offenbacher et al., 2009). A gingival biopsy was obtained from each subject at the site of marginal gingiva. Periodontitis gingival biopsies were acquired at the severely inflamed sites during a periodontal flap osseous surgery. For experimental gingivitis subjects, gingival biopsies were obtained at the end of 3 weeks of induction. Upon harvesting, all biopsies were immediately preserved in RNA-later (Ambion) overnight at 4°C, and then transferred to −80°C for longer storage.
RNA Isolation and Assays for Insulin Response Genes Expression Profile

Total RNA was isolated from gingival biopsies using RNeasy Mini kit (Qiagen). 600ng of the RNA were reverse transcribed to cDNA using RT² First Strand Kits (Qiagen). To examine genes of interests, we used a diabetes-pathway focused RT² Profiler PCR array (PAHS-023) in a 7500 Sequence Detection System (ABI Prism, Applied Biosystems). The mRNA expression levels were normalized using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. We performed the delta-delta Ct method to calculate fold-changes of regulated genes. The cut-off Ct value for qPCR was set at 35 and all genes within this threshold were included in the analysis. The identified genes were then mapped to known molecular networks in IPA to obtain statistical significance estimates for pathways and networks ranked by Fisher Exact scores.

Cell culture, LPS stimulation, and Western blot

Primary human gingival cells (HGEPs) were purchased from CellInTec and maintained according to the manufacture’s instructions. 1ug/ml Escherichia coli (E. coli, O55:B5) LPS (Sigma-Aldrich) was added to stimulated HGEP for 0, 4, 8, and 24 hours. Whole cell extracts were prepared by lysing the cells with ice-cold RIPA buffer (Cell signaling 10x buffer and Roche protease inhibitor mixture). The protein concentrations of the lysates were determined by DC protein assay (Bio-rad). The same amounts of protein were separated by 4-12% Bis-Tris gels and transferred to a nitrocellulose membrane (BD). The membrane was blocked 1h in 5% milk powder/TBST solution and then overnight blotted with anti-G6PD (H160, Santa Cruz) or anti-β actin antibodies (13E5, Cell signaling). Membranes were washed three times with TBST, and incubated with anti-rabbit HRP-labeled secondary antibody in TBST and developed using ECL system (SuperSignal West Pico; Pierce).
Statistical Analysis

To compare gene expression in different groups, we used web-based RT² profiler PCR array data analysis version 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). This web-based analysis by default employs the student t-test to examine the differences between groups, which increase type I error. To correct for this error, we applied false discover rate (FDR) test to calculate a stringent statistical significance and the significance level was set at 0.05. To explore potential confounding effects of age on expression patterns, we used multiple linear regression models. For network analysis using IPA, the significance of the networks was calculated using the right-tailed Fisher Exact Test.
Results

Participants

The demographic information and periodontal parameters of the participants are shown in Table 2.1. No statistically significant differences in age, gender, race, BMI, and smoking history were found among participants with either healthy or diseased gingival tissues. As expected, clinical parameters such as probing depth and attachment loss exhibited higher readings in chronic periodontitis subjects compared to healthy and gingivitis participants.

Gene Expression Patterns in Gingivitis and Periodontitis Subjects.

We compared gene expression patterns between healthy, experimental gingivitis, and periodontitis gingival biopsies. The volcano plot showing the log2 (fold change) plotted versus the –log10 (p value) for the 76 genes that were within the Ct value threshold can be seen in Figure 2.1. Genes that were up-regulated by at least 2-fold are illustrated in Figure 2.1 as red dots with the clustering of genes within the upper right quadrant for statistically significant genes. Only a few genes were down-regulated as indicated as green dots. In Figure 2.1A which compared gingivitis to healthy subjects, 45 genes were up-regulated >2-fold and 32 of them were at p<0.05 after FDR adjusted t-test. In this comparison, 5 genes were down-regulated >2-fold but none of them reached statistical significance (p>0.05). In Figure 2.1B, periodontitis group showed similar directional but much attenuated changes in gene expression with 15 genes being up-regulated >2-fold. None of them reached statistical significance by FDR. Only 1 gene was found down-regulated >2-fold but did not reach statistical significance as well (p>0.05). In Table 2.2, we reported the complete expression data for each individual gene in gingivitis and periodontitis groups. To explore potential confounding effects of age on gene expression, we
chose the top 4 regulated genes in overall comparisons (\textit{G6PD, IFNG, CEBPA, DUSP4}) and analyzed their fold regulation differences adjusted by age. In Table 2.3, age-adjusted fold change for these genes did not differ much from the unadjusted fold change. When comparing gingivitis to healthy subjects, the age-adjusted fold change differences were even slightly increased. For instance, adjusted G6PD fold regulation was increased by 11%. This gene expression profile suggests that genes that are associated with glucose metabolism and insulin responsiveness are more highly expressed in acute gingival inflammation, but not significantly up-regulated in chronic inflammation.

\textbf{Molecular Networks Analysis in Gingivitis and Periodontitis Subjects}

To further understand the molecular pathways associated with glucose metabolism and insulin responsiveness, we used IPA to analyze molecular networks. To include regulated genes that are more relevant in the network analysis, we used fold regulation at 1.5 as the cutoff threshold. Figure 2.2A shows the top 5 activated networks in gingivitis subjects comparing to their healthy counterparts. Each network is endowed with a score and in IPA score of 2 or higher suggests that networks have at least a 99% confidence of not being generated by random chance. Since we used the targeted array, the highest ranked network was endocrine system disorder, metabolic disease and carbohydrate metabolism ($p=1\times10^{-25}$). As delineated in Figure 2.2A, the majority of key molecules in this network were up-regulated (red nodes) by acute inflammation with only \textit{AKT2} and \textit{NFKB1} genes being down-regulated (green nodes). Figure 2.2B shows the top 4 activated networks in periodontitis subjects comparing to healthy controls. There are some shared functions within these networks, but more potent activations were observed in gingivitis subjects. It further proves that activation of glucose metabolism and insulin responsiveness was more potently induced by acute gingival inflammation.
G6PD protein expression increases in response to LPS stimulation

To confirm that the elevated mRNA expression data seen in experimental gingivitis likely is mirrored by increased protein expression we created an in vitro acute inflammatory by stimulating HGEPs with *E. coli* LPS and detected G6PD protein expression by Western blot. G6PD was selected as a marker because it was the top up-regulate mRNA species identified (Table 2.2). As demonstrated in Figure 2.3, G6PD protein expression increased in response to LPS stimulation starting from 4 hours of stimulation, suggesting that inflammatory stimuli likely induce G6PD on both mRNA and protein levels.

The Modulating Roles of Obesity

To address the potential role of obesity in modulating insulin responses, we divided all three groups of subjects to obese and non-obese subgroups based on BMI. After normalizing against non-obese healthy subjects, fold change ratio of obese/non-obese diseases subjects are presented in Figure 2.4. It appears that majority of the fold change differences between obese and non-obese subjects are within 2-fold differences. In gingivitis group, more genes are regulated greater than 2 fold (Figure 2.4A). Due to limited sample sizes, we focused more on trend instead of performing statistical analysis.
Discussion

This study suggests that during the induction of experimental gingivitis, the acute gingival inflammatory response is locally activating glucose metabolism and insulin response genes, but in chronic inflammation there has been a re-establishment of a relative balance in insulin/glucose metabolism within the local gingival tissues.

The experimental gingivitis group has an overall markedly up-regulated gene profile for these glucose/insulin response genes. As can be seen in Table 2, the top 4 up-regulated genes in gingivitis biopsies are \textit{G6PD}, \textit{IFNG}, \textit{CEBPA}, and \textit{DUSP4}. Both tissue mRNA data and gingival epithelial cells protein data suggest that gingival inflammation affects G6PD expression (Table 2.2 and Figure 2.3). The augmentation in G6PD is likely a response to attenuate oxidative stress by generating NADPH, rather than participate in the pentose or carbohydrate driven pathway for 5-carbon sugars. The increased \textit{INF} \text{G} mRNA is a clear sign for inductions of gingival inflammation since interferon-\(\gamma\) is an activator for both innate and adaptive immunity. Wong N et al demonstrated that restricting interferon-\(\gamma\) in mice improved their glucose metabolism (Wong et al., 2011). There was also a significant increase in CEBPA, which encodes for CCAAT enhancer binding protein-\(\alpha\) that controls transcriptions of GCSF and IL6R genes, therefore affects granulocytes differentiation (Zhang et al., 1998). LPS from \textit{P. gingivalis}, IL-1\(\beta\), IL-1\(\alpha\) and TNF-\(\alpha\) all enhance DNA binding activity of CEBPA in vitro, suggesting that CEBPA may be linked to periodontal inflammation (Kido et al., 2005). DUSP4 is a downstream transcription factor of MAPK pathway and it forms a negative feedback loop on limiting MAPK activity (Arthur and Ley, 2013). In periodontal cells, MAPK participates in the regulation of the expression of IL-6 and enzymes like MMP-1 and MMP-13, and elevated DUSP4 could be a protective mechanism to control MAPK activity (Craig et al., 2000; Johansson et al., 2000).
However, a previous study found that DUSP4 protein expression in gingival epithelial cells was decreased by periodontal pathogens, which was in contrast with our data showing a significant up-regulation of DUSPS mRNA in gingivitis (Hasegawa et al., 2007). One limitation of our study is that we did not have adequate biopsy samples to confirm protein expression levels, as our emphasis was on transcriptomic analysis.

In addition, the expression of key insulin signaling molecules, namely IRS1, IRS2, SLC2A4 (GLUT4), STX4, STXBP2 and PIK3R1 are all enhanced in experimental gingivitis. This suggests that locally acute inflammation up-regulates glucose metabolism by increasing local cellular uptake and glucose utilization. The IPA analysis as shown in Figure 2.2 confirms that pathway associated with carbohydrate metabolism is activated by acute inflammation. However, chronic inflammation associated with periodontitis was not as capable as acute inflammation in regulating glucose/insulin response genes as shown in Table 2.2. This suggests that in chronic inflammation there is some adaptation in the localized tissue response to create a more homeostatic glycolic local environment. Importantly, we had no naturally occurring gingivitis samples to compare in this study. However, we might expect them to have similar insulin/glucose profiles as periodontitis, if the naturally occurring gingivitis represents a chronic inflammatory process. The finding of altered metabolism during acute induction might suggest that acute episodes of increased inflammation superimposed upon an existing chronic gingivitis or periodontitis may also be associated with enhanced insulin resistance and glucose utilization of the local periodontal tissues, but that will require experimental confirmation.

In gingival tissues, glucose metabolism may be important not only for tissue metabolism but also affect microenvironment that supports the growth of periodontal pathogens. We appreciate that most subgingival species and most of the pathogens are assacharolytic. However,
the extension of the supragingival adherent plaque that contains saccharolytic bacteria into the subgingival pocket provides an important “feeder bed” to provide nutrients of intermediate metabolism including lactate and formate to support the metabolism of the assacharolytic organisms.

We conducted a secondary analysis to consider the potential effects of obesity, because obesity affects both insulin responsiveness and periodontal disease (Franchini et al., 2011; Pischon et al., 2007). In Figure 2.4, certain genes are differentially regulated by obesity and this trend is more apparent in gingivitis group. More subjects are needed to study the modulatory roles of obesity. Studies have shown that obesity interacts with local gingival inflammation. Mizutani K et al showed that obese mice had selective insulin resistance in gingival tissues (Mizutani et al., 2014). In gingiva, specific miRNAs are differentially expressed in obese periodontitis compared to non-obese periodontitis subjects, suggesting that obesity may regulate genes post-transcriptionally (Perri et al., 2012).

There were no statistical differences in race, gender, age, smoking history, or BMI in the study subjects. BMI were matched in subjects from each group to eliminate potential effects of obesity on insulin/glucose metabolism gene expression locally. It should be noted that the mean age of gingivitis group varies from those of the other groups, which raises questions about confounding effects of age on gene expression. Our analysis shows that adjusted fold regulation did not differ much from unadjusted fold regulation. This suggests that the markedly differential expression pattern we observed in gingivitis subjects was not affected by age. To further prove the interactions between age and insulin response gene expression, larger study subjects are needed. The application of PCR array allows us to have a more quantitative data compared to microarray. However, limitations of this study was that relatively few subjects were involved
limiting the generalizability of the findings, and we did not design the study to enable protein analysis. Although this is a pilot study with relatively few subjects (26 subjects), it demonstrates the utility of pathway analysis, which allows each molecular observation to contribute to the overall significance of networks.

In conclusion, this study suggests that acute gingival inflammation may induce localized changes, which modify tissue insulin/glucose metabolism. But these changes are not evident in chronic periodontitis. Additionally, larger studies will be needed to confirm these findings to provide insights into understanding the association between gingival inflammation and periodontal tissue metabolism.
Table 2.1 Subjects demographics and clinical parameters

<table>
<thead>
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<th></th>
<th>Healthy (N=8)</th>
<th>Gingivitis (N=9)</th>
<th>Periodontitis (N=9)</th>
<th>P</th>
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<td>Body mass index</td>
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<td>30.4±2.4</td>
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<td>&lt;30</td>
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<td>25.2±1.4</td>
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<td>Probing depth</td>
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<td>3.9±0.6</td>
<td>&lt;0.001</td>
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Values are presented in mean ± standard error or n.
## Table 2.2 Gene regulation in gingivitis and periodontitis subjects

<table>
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<th>Gene</th>
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<th>Fold change</th>
<th>P value</th>
<th>FDR</th>
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Fold changes are calculated through the delta-delta threshold cycle method; P values are generated by Student’s t tests, followed by a false discover rate adjustment. *Statistical significance after the false discover rate adjustment, P < 0.05.
## Table 2.3 Fold Regulation Adjusted by Age for 4 Genes

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* $P$<0.05, computed from multiple linear regression models; FR: Fold Regulation
A

Experimental Gingivitis vs. Healthy

B

Chronic Periodontitis vs. Healthy
Figure 2.1 Volcano plot presenting regulation of insulin response genes comparing diseased to healthy subjects. The vertical axis refers to the -log10 (p-value) for t-test of differences between groups; the horizontal axis refers to log2 (fold change) as expression levels. Red and green dots represent up-regulated (>2-fold) and down-regulated (<2-fold) genes respectively. Black dots are genes that are within 2-fold regulation. The clustering of red dots within the upper right quadrant are statistically significant genes (p<0.05). A) 45 genes are up-regulated >2-fold in experimental gingivitis group and 32 of them are statistically significant; 5 genes are down-regulated >2-fold in the same group but none of them reach significant. B) 15 genes are up-regulated >2-fold and 1 gene is down-regulated >2-fold in chronic periodontitis group, but none of them is significant.
Figure 2.2 Ingenuity pathway analysis comparing diseased to healthy subjects. Red nodes are up-regulated genes; green shapes are down-regulated genes. Genes that are associated with these key network molecules but not included in the polymerase chain reaction array are not colored. Straight lines represent direct interaction, and dotted lines represent indirect interaction. A) Functions and scores of 5 activated networks in gingivitis subjects are listed. The top-ranked network comprises endocrine disorders, metabolic disease, and carbohydrate metabolism functions (score=25). The majority of molecules in this network are up-regulated. Only 2 molecules are down-regulated. B) Functions and scores of 4 activated networks in periodontitis subjects are listed. The 2nd ranked network comprises carbohydrate metabolism, small molecule biochemistry, cell death and survival (score=14). The majority of molecules in this network are up-regulated.
Figure 2.3 G6PD protein increases by *E. coli* LPS stimulation. HGEPs were treated with *E. coli* LPS (1μg/ml) for 0, 4, 8 and 24h and blotting for G6PD showed protein up-regulation.
Figure 2.4 Fold change ratio of obesity/non-obese diseased subjects. Gene fold changes are normalized against non-obese healthy subjects. A) The ratio of obese/non-obese in gingivitis subjects is plotted. If the ratio is below zero, gene expression in obese subjects is lower than non-obese subjects; if the ratio is above zero, gene expression in obese subjects is higher than non-obese subjects.
CHAPTER 3: EFFECTS OF ADVANCED GLYCATION END PRODUCTS ON GINGIVAL FIBROBLAST MIGRATION AND GENE REGULATION

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Curriculum of Oral Biology\textsuperscript{1}, Department of Periodontology\textsuperscript{2}, Center for Oral and Systemic Diseases\textsuperscript{3}, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Abstract

Background: The presence and accumulation of advanced glycation end products (AGEs) in gingiva has been evident in chronic periodontitis patients, which is even more drastic among those who have diabetes. We conducted this study to examine whether transcription of key regulatory genes in gingival wound healing and migration were affected by AGEs in gingival fibroblast.

Methods: AGEs were prepared in vitro using bovine serum albumin (BSA) as a carrier. Human gingival fibroblast (HGF-1) was pretreated with AGEs-BSA (1ug/ml) for 24h, then stimulated with AGEs-BSA (1ug/ml) with \textit{E.coli} LPS (0, 100, 1000ng/ml) for 24h. Total RNA was extracted and reverse transcribed to cDNA. qRT-PCR was performed to detect the transcription of prostaglandin-endoperoxide synthase 2 (PTGS2), matrix metalloproteinase 1 (MMP1), and...
fibroblast growth factor 2 (FGF2) genes. Scratch assay and transwell assay were conducted to examine the migration activity of gingival fibroblast affected by AGEs. HGF-1 that was pretreated and stimulated with BSA served as controls.

**Results:** Compared to BSA treated cells, HGF-1 that was pretreated and stimulated by AGEs-BSA and LPS showed significantly increased transcription of *PTGS2*, *MMP1* genes, but not *FGF2* gene. AGEs-BSA treated cells displayed a delayed migration as proved by the scratch assay and transwell assay.

**Conclusion:** AGEs inhibit gingival fibroblast migration. *PTGS2*, *MMP1* mRNA is increased by AGEs and LPS stimuli, which may explain the accelerated periodontium destruction during chronic periodontitis, especially under the diabetic background.
**Introduction**

Periodontal disease and diabetes mellitus especially type 2 diabetes (T2DM) share a bidirectional relationship (Mealey et al., 2006). Periodontal disease adds a burden of systemic inflammation by inducing pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β (Page, 1991). These cytokines are known to activate insulin resistance, which occurs at the beginning state for T2DM and a later state for T1DM. Periodontal disease may lead to severe consequences in the diabetic population. In a study conducted among Pima Indians with T2DM, severe periodontitis significantly increased the mortality rate from diabetic nephropathy and ischemic heart disease (Saremi et al., 2005). Treating periodontal disease, on the other hand, improves the glycemic controls for diabetic patients (Teeuw et al., 2010). From the perspective of how diabetes affects periodontal disease, many mechanisms have been proposed and one possible mechanism is the influence of advanced glycation end products (AGEs).

AGEs are a heterogeneous group of non-enzymatically formed proteins from having a sustained contact with sugars. For diabetic subjects, their tissues and organs have higher AGEs accumulations than those of non-diabetic subjects. Receptor for AGEs (RAGE) has been found to interact with AGEs and triggers the downstream NF-κB pathway (Schmidt et al., 1994). As a result, inflammation and reactive oxygen species are elevated, which subsequently change the physiology of the tissues. Accumulations of AGEs are evident in gingival tissues. AGEs aggregate more potently in the gingival epithelium and the underlying connective tissues among diabetic subjects compared to their non-diabetic counterparts (Abbass et al., 2012; Zizzi et al., 2013).

Emerging evidence has shown that AGEs might be associated with impaired wound healing in diabetes. Systematically blocking RAGE improves skin repairs in diabetic mice (Goova et al., 2014).
Chang PC et al found that anti-AGEs agents facilitated the palatal gingival wound healing by reducing inflammation (Chang et al., 2014). During the gingival wound healing process, connective tissue has a unique role in tissue remodeling. Gingival fibroblast, as the most abundant resident cells in the connective tissue, is a reservoir for inflammatory cytokines (eg. IL-1β, TNFα, PGE2), effector molecules (eg. MMPs, TIMPs), and growth factors (eg. FGF, CTNF). All these molecules actively participate in the tissue remodeling process. Fu Y et al found that AGEs induced MMP1 productions in human gingival fibroblast through activating NF-κB pathway, which further indicated the importance of AGEs on periodontal disease. However, whether AGEs affect anabolic growth factors and whether AGEs inhibit gingival fibroblast migration in vitro remain unknown.

The objective of this study is to investigate effects of AGEs on gingival fibroblast migration and gene regulation. We hypothesized that AGEs inhibited gingival fibroblast migration. Additionally, AGEs increased transcription of genes that were associated with inflammatory cytokines and effector molecules, but not anabolic growth factor genes.
Material and Methods

Preparation of AGEs-BSA compounds

AGEs-BSA compounds were prepared by incubating BSA at a 1mM concentration with 1M glucose, 1.5mM phenylmethanesulfonyl fluoride, and 1% penicillin-streptomycin in phosphate-buffered saline (PBS) solutions (ph=7.4), at 37°C for 10 weeks. Same compositions without glucose were incubated simultaneously as BSA controls. Both AGEs-BSA and BSA solutions were dialyzed against PBS using 20K MWCO dialysis cassettes, at 4°C overnight. We then used Bradford protein assay (Sigma) to quantify for the concentration of BSA in both groups. BSA and AGEs-BSA were diluted to a concentration of 1mg/ml and stored at -80°C until use.

Cell culture and in vitro stimulations

HGF-1 was purchased from ATCC (CRL-2014) and maintained in DMEM low glucose medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Invitrogen). We seeded HGF-1 in 6 well plates at a density of $2 \times 10^5$ and changed the medium every other day until AGEs challenges began. AGEs-BSA or BSA compounds were added to HGF-1 culture for 24h. The medium was then switched to *E. coli* LPS (0, 100ng/ml, 1000ng/ml, strain 55 O:H) containing medium in the continuing presence of AGEs-BSA or BSA for 24h. At the end, cells were harvested for RNA isolation.

RNA isolation and qRT-PCR

Total RNA was isolated from HGF-1 cells using RNeasy Mini kit. RNA quality and quantity were measured by nanodrop. 500ng total RNA was reverse transcribed to cDNA. Gene expression was examined using qRT-PCR in a 7500 Sequence Detection System (ABI Prism, Applied Biosystems). Taqman primers for *PTGS2*, *MMP1* and *FGF2* genes were purchases from
ABI expression (Hs00153133_m1, Hs00899658_m1, Hs00266645_m1). The mRNA expression levels were normalized using 18s ribosome RNA as the housekeeping gene.

**Migration Assay**

In the transwell migration assay, HGF-1 cells were undergoing BSA or AGEs-BSA treatment in the absence of serum overnight. 2x10^4 cells were added to the upper well of the transwell inserts (Millipore, 6.5mm²). Inserts were then immersed into medium containing BSA or AGEs-BSA (10, 100, 1000ng/ml) in the presence of 30% FBS (chemo-attractant) in a 24 well plate. Following 12h of migration, inserts were removed from the culture plate and fixed with 4% paraformaldehyde at room temperature for 10mins, followed by 1XPBS washing 3 times. We applied a cotton swap to clean the remaining cells apically. The last step was to stain the nuclei using DAPI solution (D1306, Invitrogen) for 5mins, avoiding lights. Nuclei staining were read under a fluorescent microscopy (EVOS). For each insert, 5 fields (four corners and a central spot from a cross) were chosen to count for the average cell numbers. In the scratch assay, 2x10^5 HGF-1 cells were seeded into 6-well plates until they formed a monolayer. After overnight serum starvation, cell monolayers were scraped with a sterile 200ul pipette tip in the midline. We immediately aspirated the culture medium, rinsed the cells with 1XPBS 3 times, and added medium containing either BSA or AGEs-BSA. Both medium were supplemented with 30% FBS. After 12h of migration, cell images were captured from an inverted phase contrast microscopy. Two scratch wounds were made for each treatment and two independent experiments were performed. Image J software was used to quantify for the wound closure percentage.

**Statistical Analysis**

Fold changes are calculated through the delta-delta threshold cycle method. P values are generated by Student’s t-tests. Significance level was set at 0.05.
Results

Predisposing to AGEs enhances gingival fibroblast PTGS2 and MMP1, but not FGF2 mRNA expression in response to LPS

In order to investigate effects of AGEs on gingiva fibroblast gene regulation, we stimulated HGF-1 with AGEs-BSA or BSA for 24 hours and examined mRNA expression of representative genes. To optimize the dosage of AGEs-BSA, we treated HGF-1 with AGEs-BSA at 10, 100 and 1000ng/ml for 24h and measured PTGS2, MMP1 and FGF2 mRNA expression. HGF-1 tolerated all three dosages of AGEs-BSA without visible cellular changes (data not shown). To this end, we continued using AGEs-BSA at a concentration of 1000ng/ml. To our surprise, PTGS2, MMP1 and FGF2 mRNA levels did not differ between AGEs-BSA and BSA treatments (Figure 3.1A). We then adjusted the in vitro model by pretreating HGF-1 with AGEs-BSA or BSA for 24h and stimulated cells with E. coli LPS (0.1ug/ml, 1ug/ml) in continuing presence of AGEs-BSA or BSA for another 24h. We found that LPS significantly increased MMP1 and PTGS2 mRNA expression in a dosage dependent manner while pretreatment of AGEs-BSA significantly enhanced their mRNA expression (Figure 3.1B and 3.1C). However, FGF2 mRNA was not significantly altered by either AGEs-BSA or LPS treatment (Figure 3.1D).

AGEs inhibit gingival fibroblast migration

In addition to gene regulation, we hypothesized that AGEs may inhibit gingival fibroblast migration in vitro. In order to test our hypothesis, we utilized two types of migration assays. In the transwell assay, HGF-1 cell migration was clearly inhibited by AGEs-BSA with fewer numbers of cells counts (Figure 3.2A, $P<0.05$). In the scratch assay, the wound closure percentage was significantly smaller in AGEs-BSA treatment compared to BSA treatment, which confirmed with the transwell assay results (Figure 3.2B).
Discussion

In this study, we have proved that predisposing gingival fibroblast to AGEs enhances PTGS2 and MMP1, but not FGF2 gene transcription in response to LPS stimulations. AGEs appear to inhibit gingival fibroblast migration. Our data suggests that accumulations of AGEs in gingival tissue during diabetes may accelerate periodontium destruction and impair gingival wound healing.

The transcription of PTGS2 gene is tightly coupled with PGE2 productions. PGE2 is a vasodilator that engages in increasing vascular permeability and promoting inflammation that leads to periodontium destruction (Offenbacher et al., 1993). Therefore, PGE2 is a well-established inflammatory biomarker for periodontal disease. Empirically, monocytes and macrophages are thought be the major source for PGE2 production. However, gingival fibroblast potently produces PGE2 in gingival tissues as well (Bage et al., 2011). MMP1 is a collagenase that regulates ECM, therefore influences both gingival wound healing and periodontium destruction. Yu S et al has shown that AGEs induce MMP1 mRNA through RAGE/ NF-κB pathway in gingival fibroblast (Yu et al., 2012). Since MMP1 degrades collagen, it is not surprising to observe that AGEs inhibit type I and III collagen synthesis in gingival fibroblast (Ren et al., 2009). FGF2 is an anabolic growth factor that promotes fibroblast migration during periodontal regeneration (Kitamura et al., 2008). FGF2 is absent from gingival epithelium, but stained potently in connective tissue fibroblast (Takayama et al., 2002).

Our data shows that AGEs modifications alone do not affect gene transcription. Since AGEs compounds are not commercially available, every laboratory has different formulas for making AGEs. This is likely to be a reason for the different responses we have observed in the study. However, this does not suggest AGEs are not functioning. It is clear that LPS increase both
*PTGS2* and *MMP1* transcription and predisposing fibroblast to AGEs almost doubles the expression (Figure 3.1B and 3.1C). Elevations of *PTGS2* and *MMP1* cause periodontal destruction. When AGEs accumulate in the gingival tissues during diabetic periodontitis, gingival fibroblast becomes more sensitive to a 2nd stimulus from bacterial challenges. According to this study, FGF2 is unlikely to be regulated by LPS or AGEs (Figure 3.1.D). Failure of induction of FGF2 supports that in periodontitis especially when combined with diabetes, repair of gingival fibroblast might be delayed.

Hyperglycemic condition in diabetes impairs wound healing and AGEs are potential regulators. The interaction between AGEs and RAGE activates the downstream signaling pathways that lead to pro-inflammatory cytokine productions. These chains of events may alter the cytoskeleton architecture and eventually regulate migratory abilities of cells. This study confirms with the existing concepts that AGEs are causally associated with severe periodontium destruction. The innovative discoveries of AGEs inhibiting gingival fibroblast migration further shed lights on potential drug development for improving gingival wound healing.
A

BSA AGEs-BSA

Fold Change

PTGS2
MMP1
FGF2

B

MMP1 transcription

LPS 0 100ng/ml 1000ng/ml

Fold Change

BSA
AGEs-BSA

C

PTGS2 transcription

LPS 0 100ng/ml 1000ng/ml

Fold Change

BSA
AGEs-BSA
Figure 3.2 Prior exposures of AGEs enhance gingival fibroblast response to LPS. A) Gene regulation fold changes do not differ between AGEs-BSA and BSA treatments. B) and C) *PTGS2* and *MMP1* transcriptions increase by LPS stimulations in a dosage dependent manner and AGEs significantly enhance the responses (* indicates $p<0.05$). D) LPS or AGEs do not regulate *FGF2* transcription in HGF-1. Error bar represents standard error.
Figure 3.1 AGEs inhibit gingival fibroblast migration. A) Representative staining images show that AGEs inhibit fibroblast from migrating out of the transwell inserts. There are significantly more migratory cells in the BSA group than those of AGEs-BSA group (* indicates $p<0.05$). Scale bar represents 400um. B) The percentage of scratch closure is relatively smaller in BSA group compared to those from AGEs-BSA group.
CHAPTER 4: IMPAIRED FUNCTION OF EPITHELIAL PLAKOPHILIN-2 IS ASSOCIATED WITH PERIODONTAL DISEASE

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Curriculum of Oral Biology¹, Department of Periodontology², Center for Oral and Systemic Diseases³,
School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Abstract

Objectives: Plakophilin-2 (PKP2) is a structural protein of desmosomes that assists in maintaining the integrity of the epithelium barrier. A previous genomic wide association study in our lab has discovered a statistically significant association between multiple SNP variants of PKP2 gene and periodontal disease. Key desmosome molecules Desmoglein 1 and Desmocolin 1 were compromised in periodontitis. For these reasons, we initiated studies to elucidate the role of PKP2 in periodontal disease.

Methods: Gingival biopsies were harvested from 11 healthy, 10 experimental gingivitis and 10 chronic periodontitis subjects. We examined PKP2 mRNA and DNA methylation patterns in
these biopsies. Primary human gingival epithelial cells (HGEPs) were challenged by *Porphyromonas gingivalis* (P.g), *Campylobacter rectus* (C.r), or multiple TLR agonists. Medium was switched to high calcium condition to examine the effect of calcium on desmosome molecules. The alteration of protein was detected by western blot or immunofluorescence staining. We further knocked down PKP2 in HGEPs using short hairpin RNA (shRNA) to study cell proliferation, spreading, and barrier functions in the lack of PKP2.

**Results:** PKP2 mRNA was significantly decreased in gingival biopsies from chronic periodontitis comparing to healthy subjects (*p*<0.05). Using the same biological samples, we found an increase of DNA methylation in the promoter region of PKP2 that might be associated with PKP2 down-regulation. Epithelial PKP2 mRNA was enhanced by P.g stimulation, but protein expression differed. Both PKP2 and DSG1 proteins were decreased by viable P.g, but not by C.r or heat killed organisms, suggesting their selective degradations by P.g. The addition of the proteasome inhibitor MG132 and lysosome inhibitor NH4Cl did not prevent degradation, but cysteine proteases inhibitors greatly attenuated PKP2 degradation by viable P.g. In vitro stimulation of P.g and C.r both increased the overall PKP2 DNA methylation level. Epithelial cells with deficient PKP2 had inhibited cell proliferation and spreading. Decreased PKP2 was associated with impaired cell barriers.

**Conclusions:** PKP2 protein expression is suppressed in gingival epithelial cells associated with periodontal disease. Cysteine proteases made by *P. gingivalis* selectively degrade PKP2. PKP2 deficiency potentiates epithelial homeostasis. These data suggest that PKP2 may play an important role in maintaining epithelial integrity thus have an important protective role in the etiopathogenesis of periodontal disease.
Introduction

An imbalanced biofilm-gingival interface is the key to the onset of periodontal disease. Gingival epithelium, the outmost layer of gingiva, creates a physical barrier to minimize bacterial invasions thus is regarded as an early line of defense against periodontal pathogens (Schroeder and Listgarten, 1997). Disruptions of gingival epithelial cells (GEC) barrier allow bacteria and bacterial products to enter into the underlying tissues, trigger immune responses and cause bone resorptions. Intact cell-to-cell adhesions are crucial for GEC barrier and are consist of many intercellular junctions, including tight junctions, adherens junctions, gap junctions, and desmosome junctions.

Desmosome junctions reside in gingival epithelium and have complex structural components (Green and Gaudry, 2000; Hatakeyama et al., 2006). In desmosomes, Desmoglein (DSG) and Desmocollin (DSC) are extracellular cadherin molecules, while plakophilin (PKP), plakoglobin, and desmoplakin are intracellular components that bridge intermediate filaments and desmosomes. DSG1 and DSC1 mRNA were found to be decreased in gingival biopsies from periodontitis patients compared to healthy subjects (Abe et al., 2011). However, whether PKP is altered in periodontal disease remains unknown and whether the change leads to the pathogenesis of periodontal disease is yet to be discovered.

PKP has four members (PKP1, PKP2, PKP3, and p0071) and PKP2 is the best-characterized member. Mutations of PKP2 cause a severe inherited heart disease-arrhythmogenic right ventricular cardiomyopathy, and PKP2 null mice exhibit embryonic lethality due to heart morphogenesis defects, suggesting that lack of PKP2 leads to severe consequences (Grossmann et al., 2004; van Tintelen et al., 2006). Functions of PKP2 are not limited to structural molecule of desmosome junctions. PKP2 interacts with epidermal growth factor receptor, a growth factor
that is tightly coupled with cell proliferation (Arimoto et al., 2014; Kazlauskas, 2014). PKP2 deficient keratinocytes display a delayed migration through altering the focal adhesion (Koetsier et al., 2014). Additional study has shown that PKP2 directly interacts with β-catenin to potentiate TCF/LEF1 mediated transcription of target genes as part of Wnt signaling pathways (Chen et al., 2002).

DNA methylation is an epigenetic modification that controls gene expression. Many groups including us have found that aberrant DNA methylation patterns of several inflammatory genes are associated with periodontitis (Andia et al., 2010; Zhang et al., 2013). Periodontal pathogens can alter DNA methylation machinery to regulate genes encoding antimicrobial peptides as well (Yin and Chung, 2011). In a recent study, membrane bound protein TLR2 can be modified by DNA methylation mechanism in periodontitis patients (Benakanakere et al., 2015). This finding suggests that structural protein could be modified by DNA methylation. Therefore, the goal study is to investigate the association between PKP2 function and periodontal disease and what mechanisms are involved in PKP2 regulation.
**Material and Methods**

**Participants Selection**

Thirty-one participants, aged 19 to 64, provided informed consent and were recruited in the dental clinics from School of Dentistry at University of North Carolina at Chapel Hill. All procedures were approved by Institutional Review Board (IRB) of the University of North Carolina at Chapel Hill. Participants were sequentially enrolled with either periodontally healthy, experimental gingivitis or chronic periodontitis with the following exclusion criteria: 1. No use of antibiotics within one month before the screening examinations; 2. No signs of systemic diseases with any oral manifestation at the time of the enrollment; 3. No self-reporting treatment for systemic diseases three months prior to the collections of gingival biopsies. Basic demographic information and routine periodontal clinical parameters were recorded for each subject.

Eleven periodontally healthy participants were either healthy volunteers or patients who were undergoing crown lengthening procedures and they exhibited ≤ 4mm probing depth (PD), no signs of bleeding on probing (BOP) at the biopsy site, and no evidence of bone loss radiographically. Ten chronic periodontitis patients representing chronic inflammation were enrolled with exhibitions of > 5mm of PD, presence of BOP and radiographic demonstration of bone loss. Ten experimental gingivitis patients representing acute inflammation were acquired from a 3-week stent-induced biofilm overgrowth procedure (Offenbacher et al., 2009). A gingival biopsy was obtained from each subject at the site of interproximal papillae and composed of epithelium and connective tissues. Gingival biopsies were acquired at the severely inflamed interproximal site during a periodontal flap surgery for periodontitis subjects and for experimental gingivitis patients, gingival biopsies were obtained at the end of 3 weeks of
inductions. Upon harvesting, all biopsies were divided into three portions for genomic DNA isolation, RNA isolation, and immunofluorescence staining according to different preservation methods.

**In vitro stimulation of HGEPs with bacteria**

P.g strain A7436 were grown in defined Wilkins-Chagren (Thermo Scientific) broth medium anaerobically under 37°C for up to 5 days. When the optical density reached 0.5, the concentration of P.g equaled 10^9 CFU/ml. C.r strain 314 were grown on an ETSA plate (Thermo Scientific) anaerobically for up to 3 days. When the optical density reached 1.0, the concentration of C.r equaled 10^9 CFU/ml. HGEPs were purchased from CellInTec and maintained according to the manufacture’s instructions. Bacterial cells were pelleted at a speed of 6000g for 15mins, washed with 1XPBS twice, and reconstituted in the cell culture medium. HGEPs were seeded at a density of 5X10^5 in 6 well plates and treated with P.g at the multiplicity of infection (MOI) at 10, 50, or 100 for up to 24h. HGEP were treated with C.r at MOI 50 or 100 for up to 24h. Both P.g and C.r were placed in a 70°C heat block to be heat-killed to serve as control bacteria. MG132 and NH_4Cl were directly added into the cell culture; pefabloc SC, cathepsin B inhibitor, and leupeptin (Roch) were co-cultured with P.g in the anaerobic chamber for 1h before being added into the cell culture.

**RNA isolation and quantitative real-time PCR**

Total RNA was isolated from gingival biopsies or HGEP cells using RNeasy Mini kit (Qiagen). RNA quality and quantity were measured by nanodrop. 500ng total RNA were reverse transcribed to cDNA using Invitrogen vilo kit (Invitrogen). Gene expression was examined using quantitative real-time PCR in a 7500 Sequence Detection System (ABI Prism, Applied
Biosystems). The mRNA expression levels were normalized using 18s ribosome RNA as the housekeeping gene.

**Cell lysates preparation and western blot**

Whole cell lysates were collected using RIPA buffer (cell signaling) that were supplemented with protease inhibitor cocktail and phospho stop (Roch). We then used DC protein assay (Biorad) to quantify the total protein concentrations. The same amount of protein from each sample was aliquoted, mixed with 4x sample buffer (Invitrogen), and boiled on a heater (80°C) for 10mins to reduce the protein contents. Proteins were loaded into the NuPAGE Novex 4-12% Bis-Tris Gels to separate protein bands and transferred to a nitrocellulose membrane paper. Membranes were blocked with 5% skim milk in TBST for 45mins. Anti-PKP2 antibody (1:200 dilution, C-1, santa cruz), anti-DSG1 antibody (1:200 dilution, H-290, santa cruz), anti-DSC2/3 antibody (1:200 dilution, 7G6, santa cruz), and anti-β actin antibody (1:1000 dilution, 13E5, cell signaling) were incubated with the membranes overnight at 4°C. Specific HRP conjugated secondary antibodies (anti-mouse Sigma; anti-rabbit Jackson Lab) were incubated for 1hour at room temperature and ECL system (SuperSignal West Pico; Pierce) was used to detect the intensity of the bands.

**Genomic DNA isolation, bisulfite conversion, bisulfite specific PCR, and Pyrosequencing**

Genomic DNA was isolated from the collected gingival biopsies samples using a DNeasy Mini Kit (Qiagen). A total of 500ng genomic DNA was quantified and undergoing bisulfite conversion using Epitect Bisulfite kit (Qiagen). After the conversion, all unmethylated cytosines in the non-CpG sites of the genomic DNA were converted to thymines. We chose a promoter region about 600bp upstream of the transcription starting site of PKP2 gene and this was illustrated in Figure 4.1. For methylation analysis, we amplified a designated region (referred as
amplicon), which contained a total of 5 CpG sites using a HotStar Taq kit (Qiagen). Primers needed for this bisulfite-specific PCR were listed in Table 4.1. PCR condition for this amplicon can be found in Table 4.2. Direct quantification of the ratio of methylated to unmethylated cytosine nucleotide for each analyzed CpG site present was determined by pyrosequencing with the PSQ HS 96 Pyrosequencing System (Biotage) and Pyro Gold CDT Reagents (Qiagen) as previously described (Zhang et al., 2013).

**Methylation PCR assay**

To study whether bacteria could alter DNA methylation machinery for *PKP2* gene in a short period of time, we treated HGEPs with P.g or C.r for 24h and detected the overall methylation levels of *PKP2* gene. 500 ng of genomic DNA were isolated and digested using EpiTect Methyl II DNA Restriction Kit (Qiagen). Digested DNA was then quantified by qPCR using EpiTect Methyl II PCR Primer Assay for Human PKP2 (CpG Island 103034, EPHS103034-1A). Data analysis was done using integrated Excel-based templates provided by the manufacturer, which provided gene methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. M represents the fraction of input genomic DNA containing two or more methylated CpG sites in the targeted region of a gene. A relative methylation fold change to controls was reported.

**Calcium switch and immunofluorescence staining**

5X10^4 HGEPs cells were seeded in 24-wells glass bottom plates that were coated with 10µg/ml Fibronectin (Sigma). When cells reached 80% confluence, the initial low calcium (0.07um) containing cell culture medium were switched to high calcium (1.8mM) containing medium over night to get a monolayer sheet. Cells were then stimulated with P.g at MOI: 50 for another 24h. At the end of the stimulation, cells were fixed with 4% paraformaldehyde for
10mins, permeabilized with 0.1% Triton X-100 in PBS for 30m, and blocked with 1%
BSA/PBS/0.1% triton x-100 at RT for 1h. Anti PKP2 and anti DSG1 antibodies (1:200 dilutions)
were incubated overnight at 4°C. Secondary antibodies (Alexa Fluoro 488 for Anti-PKP2, Alexa
Fluoro 590 for Anti-DSG1) were incubated at the1:200 dilution at RT for 1h, avoiding the light.
We then observed cell staining under a confocal micros copy (Zeiss LSM 710).

**PKP2 knocked down in HGEPs**

In order to test our hypothesis that the reduced epithelial PKP2 is functionally associated
with periodontal disease, we knocked down PKP2 using short-hairpin RNA (shRNA) and
examined the alterations of cell physiology. One scramble shRNA (Addgene) and five PKP2
targeted shRNA bacterial glycerol stocks (Sigma Aldrich) were grown in lysogeny broth for 16h
at 37°C and plasmids DNA were isolated using NucleoBond Xtra Midi kit (Clonetech). Target
sequences of shRNA and scramble RNA were listed in Table 4.4. We sent these plasmids DNA
to the lenti-shRNA core facility at UNC-Chapel Hill to assemble lentiviral particles. Upon
receiving the lentiviral containing medium, we preserved them in -80°C until use. HGEPs were
treated with the designated lentiviral medium for 48h and switched to medium containing
puromycin (2ug/ml, MP Biomedicals) for up to 7 days for selections. Knocked down efficiency
was determined by western blot. We compared cell proliferation using MTS assay (CellTiter 96
® Aqueous) and cell spreading using direct phase contrast microscopic images between the
scramble control and PKP2 knocked down cells.

**Epithelial barrier test**

We conducted a barrier test using Fluorescein isothiocyanate-dextran (FITC-Dextran, sigma)
to test for whether lack of PKP2 impaired the epithelial barrier. 4x10⁵ HGEPs that were either
treated by PKP2 shRNA and scramble shRNA were seeded into fibronectin-coated transwell
inserts. FITC-Dextran (1mg/ml) was added to the top compartment of each inserts. In a parallel experiment, we seeded 4x10^5 normal HEGPs were added into the transwell inserts. FITC-Dextran, together with different P.g (live 10^{10} CFU/ml, live 10^{9} CFU/ml, heat-killed 10^{9} CFU/ml) was added to the top compartment of the inserts. Inserts were all immersed in culture medium in 24-well plates. 5 hours later, cell culture medium were removed from the bottom well and measured for fluorescence on a microplate reader using 485nm/530nm excitation-emission filter.

**Statistical Analysis**

Statistical significance was determined by student t-test or one-way ANOVA for most assays. Chi square test was used for categorical variables. In gene expression data, the differences within groups were evaluated by LSD test. Statistical significance was established at p<0.05. Three replicates were used in each treatment and every experiment was repeated at least twice.
Results

Epithelial PKP2 transcription is decreased in gingival biopsies from periodontitis

For three groups of biopsies, subjects’ demographics and clinical parameters were listed in Table 4.3. Compared to gingival biopsies from healthy and experimental gingivitis subjects, those of chronic periodontitis subjects had significantly decreased PKP2 mRNA transcripts (Figure 4.2A). To quantify for the epithelium portion of the gingival biopsies, we used Keratin 5 (KRT5) as a marker. No significant differences were detected in KRT5 mRNA transcripts between three comparison groups (Figure 4.2B). We later divided PKP2 fold induction by KRT5 fold induction for each sample and plotted the adjusted fold inductions and still found that periodontitis biopsies displayed significantly fewer PKP2 mRNA transcripts (Figure 4.2C). These results suggest that decreased epithelial PKP2 mRNA is associated with periodontitis.

PKP2 protein is degraded by P. gingivalis through cysteine proteases

PKP2 and DSG1 proteins were decreased when P.g challenges were at MOI of 50 or 100 (Figure 4.3A). Contrary to the protein data, viable P.g increased PKP2 mRNA expression (Figure 4.3B). This discrepancy suggests PKP2 protein degradation instead of decreased protein synthesis. Heat-killed P.g did not alter PKP2 on mRNA or protein levels, indicating effects of viable P.g may be due to active proteinases. We then conducted a time and dosage course experiment and confirmed that the degradation of PKP2, DSG1 was in a time and dosage dependent manner (Figure 4.3C). Since sustained bacterial challenges enhance inflammation, our next step is to see whether inflammation changes PKP2 expression. LPS is an ideal stimulus to mimic the in vitro inflammation. LPS from E.coli, P.g W83, and P.g A7436 LPS did not affect PKP2 and DSG1 protein expression, but TNF-α seemed to induce protein expression (Figure 4.3D). Both viable and heat-killed C.r challenges did not alter PKP2 and DSG1 proteins (Figure
4.3E). Since viable P.g specifically degraded PKP2 and DSG1 proteins, we next looked into the potential extracellular and intracellular degradation pathways. Extracellular degradation may be caused by cysteine or serine proteases, which are possessed in the Gingipain-a major virulent factor of P.g. Two major intracellular degradation mechanisms are proteasomal degradation and lysosomal degradation pathways. Both MG132 (proteasomal degradation inhibitor) and NH$_4$Cl (lysosomal degradation inhibitor) did not restore the protein of PKP2 and DSG1 degradation by P.g (Figures 4.4A and 4.4B). This suggests P.g may not trigger the intracellular degradation pathway to recycle desmosome molecules, but rather possess with a direct proteolytic activity.

To confirm this, we co-cultured Pefabloc SC (serine protease inhibitor), Cathepsin B inhibitor (cysteine protease inhibitor), and Leupeptin (inhibitors for both) with P.g in the anaerobic chamber for 1h prior to bacterial challenges to HGEPs. In Figure 4.5C, both Leupeptin and Cathepsin B inhibitors restored PKP2 to an extent that mimicked the endogenous expression, but only partially restored DSG1 degradations. This may due to the fact that DSG1 is the extracellular component of the desmosome, which is more subjected to proteolytic activities. At the same time, Pefabloc SC did not restore any of these molecules degradation by P.g, suggesting that the degradation was due to cysteine proteases not serine proteases.

**Overall PKP2 DNA methylation is increased by bacterial challenges**

Emerging evidence has shown that DNA methylation is one mechanism regulating genes that are important to periodontal disease. The in vitro model can help revealing this mechanism. In this session, we studied whether P.g or C.r challenges altered the overall PKP2 DNA methylation in short period of time. P.g at MOI of 10 and 50 and C.r at MOI of 100 slightly increased PKP2 DNA methylation (Figure 4.5A and 4.5B).

**Specific PKP2 CpG DNA methylation is not altered in periodontitis gingival biopsies**
We have observed a hypermethylation pattern for PKP2 in response to P.g and C.r. Additionally, down-regulation of PKP2 mRNA in periodontitis gingival biopsies suggested a potential association with DNA hypermethylation of PKP2. Therefore, we tested for methylation patterns of specific CpG sites. For these five positions, periodontitis biopsies exhibited a trend of DNA hypermethylation pattern, but no differences reached the statistical significance (Figure 4.5C). We further plotted the mRNA fold induction to the percentage of DNA methylation for each sample at position 2 and did not see a significant association (Figure 4.5D).

**Calcium switch results in co-localizations of PKP2 and DSG1**

Calcium levels affect cadherin proteins, but whether PKP2 is affected by calcium is unknown. The culture medium used to maintain HGEPs in this study contains low calcium levels (0.7uM). In this session, we aimed to study whether high calcium conditions affected PKP2 expression or interfered with bacterial challenges. After the overnight calcium switch, PKP2 presented a punctated staining pattern and co-localized with DSG1 at the cell adjunctions (Figure 4.6A). High Ca\(^{2+}\) increased DSG1 and DSC2, but not PKP2 protein. After P.g challenges, DSG1 and DSC2 proteins decreased in both low and high Ca\(^{2+}\) conditions. At MOI of 50, P.g degraded PKP2 in low Ca\(^{2+}\), but not in high Ca\(^{2+}\) (Figure 4.6B). When increasing MOI to 100, P.g degraded PKP2 in high Ca\(^{2+}\) as well (data not shown). These results suggest that calcium may strengthen desmosome junctions.

**PKP2 deficiency potentiates cell proliferation, spreading, and permeability**

Among the five shRNA constructs we screened, three constructs (sh1, sh2, sh4) were able to knock down PKP2 expression in HEGPs without interfering with β-actin expression. This was confirmed in HGEPs at 3 individual experiments. In one representative experiment (Figure 4.7A), sh1, sh2, and sh4 cells had reduced PKP2 proteins than scramble RNA control. We then seeded
2x10^4 HGEPs with normal or deficient PKP2 to 96 well plates and let them stay for 2 days before the start of the MTS assay. At 0h, 1d and 3d, MTS assay was performed. Compared to the scramble control, all three knocked down cells displayed significantly inhibited cell proliferation (Figure 4.7B). Cells with intact PKP2 tend to attach and spread better than those with deficient PKP2 (Figure 4.7C). We initially seeded shRNA and scramble RNA HGEPs into fibronectin-treated transwell inserts and planned to apply FITC-dextran to test for cell barrier activity. However, we noticed that knocked down cells attached poorly to transwell inserts (data not shown), which provided another piece of evidence showing the impaired barrier in lack of PKP2. To compensate for this, we utilized P.g to decrease PKP2 in HGEPs in the transwell system. P.g treated HGEPs were more permeable to FITC-dextran in a dosage dependent manner, while heat-killed P.g did not alter barrier activity (Figure 4.7D).
Discussion

This study unravels an innovative role of PKP2 in periodontal disease. Our data strongly suggest the association between impaired functions of epithelial PKP2 and periodontal disease. Gingival biopsies from periodontitis subjects have decreased epithelial PKP2 mRNA expression (Figure 4.2). Abe D et al had similar findings showing that desmosome molecules DSG1 and DSC1 mRNA were down-regulated in gingival biopsies from periodontitis compared to those of healthy subjects (Abe et al., 2011). For decades, studies have found that major intercellular junctions are affected in periodontitis. For instance, gingival biopsies from periodontal disease subjects have aberrant staining patterns for tight junctions proteins, including occludin, claudin-4 and 15, ZO-1 and 2, and JAM-A (Choi et al., 2014; Ye et al., 2014). Several groups have reported that E-cadherin is reduced in periodontitis gingival biopsies than healthy controls (Arun et al., 2010; Loo et al., 2010; Nagarakanti et al., 2007). Connexin 26 and 43 are reduced in the pathological lining epithelium of periodontal pockets, indicating the alterations of gap junctions (Ye et al., 2000).

In order to delineate how PKP2 expression is regulated, we co-cultured gingival epithelial cells with P.g and other bacteria. PKP2 protein is reduced while mRNA is induced (Figure 4.3A and B). This discrepancy can be attributed to several reasons, including mRNA instability, inhibition of protein translation, or protein degradation. P.g are known to produce proteases to facilitate periodontium destructions (Grenier and La, 2011). Heat-killed P.g have no effect on PKP2, suggesting that protein products of P.g may affect PKP2 protein. Proteases are proved to be one of the products (Figure 4.3A). On the other hand, C.r do not regulate PKP2 or DSG1 expression, suggesting the specificity of P.g on desmosome degradation (Figure 4.3E). P.g invade and spread among cells, therefore intracellular degradation mechanism may also lead to
desmosome degradation. Two major intracellular degradation pathways have been described in the literature. One is autophagy or lysosomal degradation, which delivers structural proteins to lysosome to degrade. The other is proteasome degradation, in which target proteins get ubiquitinated and transported to proteasome to be degraded. Inhibitors targeting these two pathways fail to prevent PKP2 or DSG1 degradation driven by P.g, eliminating the possible regulatory mechanisms of proteasome or autophagy (Figure 4.4A and 4.4B). These findings allow us to infer that P.g may degrade PKP2 and DSG1 by proteases directly. Indeed, cysteine proteases inhibitors prevent PKP2 degradation while serine proteases inhibitors fail to exert similar functions (Figure 4.4C). DSG1 degradation is partially prevented by cysteine proteases inhibitors. It is likely due to the fact that DSG1 is an extracellular component, which is subjected to proteases cleavages. Cysteine proteinases of P.g exist in the form of gingipains and the strain A7436 used in this study contains functional gingipains (Genco et al., 1998).

Periodontal disease is a multifactorial disease that is controlled by many mechanisms. Several groups including us have reported that DNA methylation is involved in regulating key genes in periodontal disease (Andia et al., 2010; Zhang et al., 2013). Structural molecule like TLR2 is also a target for DNA methylation (Benakanakere et al., 2015). In accordance with decreased PKP2 mRNA, five CpG sites of PKP2 DNA methylation levels are slightly increased, but the differences are not statistically significant (Figure 4.5C). Additionally, short-term exposures (24h) to P.g and C.r result in increased overall DNA methylation of PKP2 gene, suggesting that bacteria could alter DNA methylation machinery. However, whether long-term exposures of bacteria can continue altering DNA methylation or rather brings it to a homeostatic stage is still unknown. More in-depth studies are needed to discover the DNA methylation mechanism in PKP2 regulation.
Cadherin proteins like DSG and DSC are calcium-dependent. As expected, high Ca\(^{2+}\) increases DSG1 and DSC2 in gingival epithelial cells. PKP2 becomes punctated and co-localizes with DSG1 at cell junctions under high Ca\(^{2+}\), even though the total amount stays the same (Figure 4.6). These data suggest that calcium enhances desmosome junctions in vitro. P.g degrade PKP2 in low Ca\(^{2+}\), but not in high Ca\(^{2+}\) when MOI is at 50 (Figure 4.6A). P.g degrade DSG1 and DSC2 more potently in low Ca\(^{2+}\) (Figure 4.6B). Low calcium allows us to observe apparent PKP2 degradation by P.g, thus is primarily used in experiments.

The final part of this study focuses on studying the altered cell physiology after PKP2 is knocked down by shRNA. Three PKP2 knock down cells all demonstrate inhibited proliferation and cell attaching abilities, even the knocked down efficiency is only moderate (Figure 4.7A-C). PKP2 knocked down HGEPs fail to form a monolayer on transwell inserts, which indicates that lack of PKP2 is associated with impaired barrier functions (data not shown). Viable P.g impair cell barrier in a dosage dependent manner (Figure 4.7D). This evidence indirectly proves that lack of PKP2 is associated with impaired barrier functions.

In summary, PKP2 expression is suppressed in gingival epithelial cells, which is associated with periodontal disease. P.g cysteine proteases selectively degrade PKP2 in the cell culture system. These data suggest that PKP2 may play an important role in maintaining epithelial integrity and play an important protective role in the etiopathogenesis of periodontal disease.
Table 4.1 Oligonucleotides used for bisulfite specific PCR and pyrosequencing

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<th>CpG sites (*)</th>
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*CpG sites indicate nucleotides position in relation to transcription start. “-” indicates upstream of transcription start site.

Table 4.2 Bisulfite specific PCR conditions for CpG containing amplicon

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Table 4.3 Subjects demographic and clinical parameters

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Values are presented in mean ± standard error or n.
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<td>scramble</td>
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Figure 4.1 Genomic sequence of *PKP2* promoter fragment. Sequence ranging from -572bp to +13bp relative to transcription start site (TSS) before bisulfite modification is presented. This promoter fragment upstream of TSS contains five investigated CpG dinucleotides that are marked with “*” and in bold. Their position in relation to TSS is also indicated above. Sites for potential transcriptional factor binding are underlined.
Figure 4.2 Epithelial *PKP2* mRNA transcription is decreased in gingival biopsies from chronic periodontitis. A) Gingival biopsies from periodontitis subjects have significantly reduced *PKP2* mRNA compared to healthy and gingivitis biopsies. * indicates $P<0.05$; ** indicates $P<0.01$; NS is not significant. B) *KRT5* mRNA is not significantly regulated between three conditions (NS). C. The fold change ratio of *PKP2/KRT5* is significantly lower in chronic periodontitis biopsies ($P<0.05$).
Figure 4.3 PKP2 protein is degraded by viable *P. gingivalis*. A) After 24h of bacterial challenge, PKP2 protein is decreased by live P.g (MOI:50) but not heat-killed P.g. DSG1 protein decreases in response to P.g (all 3 MOIs). B) *PKP2* mRNA increases in response to live P.g (MOI:50) not heat-killed P.g (* indicates *P*<0.05). C) PKP2 and DSG1 proteins are degraded in a time-dependent manner. D) LPS from *E. coli*, P.g W83, and P.g A7436 (100ng/ml) do not affect PKP2 and DSG1 protein expression but TNF-α (10ng/ml) seems to increase protein expression. E) PKP2 and DSG1 proteins are not affected by *C. recuts*. Densitometric measurements were shown to the right of each immunoblot. Data are representatives of at least three independent experiments.
A

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PKP2

DSG1

Actin
Figure 4.4 Degradation of PKP2 protein depends on cysteine proteases. A) The addition of the proteasome inhibitor MG132 does not prevent PKP2 and DSG1 degradation. B) The addition of lysosome inhibitor NH₄Cl does not prevent PKP2 and DSG1 degradation. C) P.g were co-cultured with different protease inhibitors for 1h anaerobically at 37°C before treating HGEPs. Cysteine proteases inhibitors (Leupeptin and Cathepsin B inhibitor) greatly attenuate PKP2 degradation by viable P.g. Both inhibitors partially restore DSG1 degradation by viable P.g.
A. PKP2 DNA methylation level

B. PKP2 DNA methylation level

C. PKP2 Methylation Level

D. Position 2

Fold Induction vs. Methylation%
Figure 4.5 *PKP2* DNA methylation is associated with periodontal disease. A) and B) HGEPs display a global DNA hypermethylation pattern for *PKP2* gene in response to *P.g* and *C.r* stimulation at 24h. C) The methylation percentage in periodontitis biopsies has an increased trend compared to healthy biopsies without meeting statistical significance. D) The *PKP2* mRNA of each biopsy sample is plotted against its methylation level at position 2. Regression analysis indicates that the transcriptional level of *PKP2* is not significantly related to the methylation level at position 2.
Figure 4.6 PKP2 and DSG1 co-localize under high calcium condition. A) Fluorescent staining for PKP2 shows a punctated pattern under high calcium and forms co-localization with DSG1 at the cell adjunctions. B) High calcium increases cadherin molecules DSG1 and DSC2 proteins, but not affects the total amount of PKP2. \textit{P.g} decrease PKP2 protein in low calcium but not in high calcium. DSG1 and DSC2 proteins are degraded by \textit{P.g} in both high and low calcium conditions, but more potent degradations occur in low calcium.
Figure 4.7 Lack of PKP2 is associated with disrupted HGEPs homeostasis and impaired barrier functions. A) PKP2 is knocked down in sh1, 2, and 4. B) PKP2 deficiency inhibits HGEPs cell proliferation. C) PKP2 deficiency impairs cell attaching after 1h of seeding on fibronectin-coated dishes. D) \textit{P.g} treated HGEPs have impaired cell barriers in a dosage dependent manner.
CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

Periodontal disease is a multifactorial disease that is regulated by complex etiopathogenesis mechanisms. In this study, we covered many aspects of periodontal disease from both cellular and tissue changes. Gingival epithelium provides an early line of defense against bacteria in the gingival sulcus, while gingival connective tissue fibroblast plays an integral role in tissue remodeling and host responses. On the other hand, gingival tissues need to maintain glucose homeostasis to survive and act.

Genetic risk burdens are also of great significance in periodontal disease. Over the years, many candidate genes have been identified that either predispose or protect subjects from developing periodontal diseases. These genes include but not limited to IL-1, IL-6, Fc gamma receptor, TNF-α, human vitamin D receptor, MMP-1, TLRs, COX-2, and C-reactive protein genes (Laine et al., 2012; Zhang et al., 2011). Mixed results are reported and extents of risks conferred by genetic differences are still under debate. It has been widely accepted that genetic risks of periodontitis may depend on a mixed pool of genes. To search for more candidate genes for periodontal disease, we conducted a genome-wide association study and have generated many interesting findings. Many new candidate genes have been found to be associated with severities of periodontitis (Divaris et al., 2013). We also have unpublished data suggesting that polymorphisms of human genes are associated with bacterial colonization. PKP2 gene is one of those genes. 5 SNPs variants (rs6488099, rs4931044, rs2389107, rs7962334, rs7299220) of PKP2 gene are significantly associated with the highest colonization of P. gingivalis among 909
subjects. In chapter 4, we have found that PKP2 expression is decreased in periodontitis gingival biopsies and *P. gingivalis* degradation is a potential driven force. Therefore, we further generate a hypothesis that hosts with differential PKP2 SNPs variants respond differently to *P. gingivalis*, which could predispose them to periodontal disease.

For the future directions, we can acquire primary gingival epithelial cells from gingival explants of periodontitis and healthy subjects from designated genetic backgrounds. After genotyping each individual’s PKP2 SNPs, we will evaluate gingival epithelial cells for cell proliferation, attaching and spreading, and barrier activity. As demonstrated in Figure 4.7, we have already established a system that allows us to use different experimental approaches to test for potential roles of PKP2 SNPs variants. Similarly, hosts with differential PKP2 DNA methylation patterns may respond differently to *P. gingivalis* or other periodontal pathogens. In human gingival epithelial cells, TLR2 DNA hypermethylation is associated with dampened immune responses to *P. gingivalis* (Benakanakere et al., 2015). Although we did not capture a significant hypermethylation pattern of PKP2 gene in gingival biopsies, we did observe an elevation of overall methylation after a short term of bacterial challenges. For the future study, we can group gingival epithelial cells based on their methylation pattern and examine potential high responders or low responders. Additionally, a long-term bacterial culture system should be established to investigate the alteration of DNA methylation machinery affected by periodontal pathogens.

PKP2 exerts multiple functions beyond being a desmosomal structural protein. PKP2 induces the phosphorylation of epidermal growth factor receptor (EGFR) to promote cell proliferation (Arimoto et al., 2014; Kazlauskas, 2014). Deficient PKP2 in keratinocytes leads to delayed migration through altered focal adhesion (Koetsier et al., 2014). An additional study has shown
that PKP2 directly interacts with β-catenin to potentiate TCF/LEF1-mediated transcription of target genes (Chen et al., 2002). PKP2 also associates with RNA polymerase III in the nucleus (Mertens et al., 2001). Since pol III regulates tRNA, rRNA 5S, and some small RNAs, PKP2 may present post-translational functions as well.

We have data showing that PKP2 is also potently expressed in gingival fibroblast, where DSG1 is lacking. Therefore, PKP2 may present multiple roles in periodontal disease, which is another direction to pursue.
REFERENCES


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