EFFECT OF KINLDIN-1 DEFICIENCY ON INFLAMMATORY CYTOKINE
EXPRESSION IN GINGIVAL KERATINOCYTES IN THE PRESENCE OF ORAL
BIOFILM

by

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Abstract

Kindler syndrome (KS) is an autosomal recessive skin disorder of unknown etiology resulting in congenital skin blisters, photosensitivity, generalized progressive poikiloderma, and mucosal alterations. Early onset and severe periodontal disease has been noted as a common clinical finding. Kindlin-1 is an intracellular focal adhesion protein that functions in cellular adhesion in the epidermis and mucosal tissues through interaction and activation of integrins. Kindlin-1 deficiency, as occurs in KS, results in impaired adhesion of the junctional epithelium to the basement membrane and may contribute to severe periodontal disease. We hypothesize that kindlin-1 deficiency may affect other aspects of keratinocyte behavior, including expression of cytokines and mediators involved in inflammation and repair. Expression of kindlin-1 was down-regulated in human gingival keratinocytes (HGK) and confirmed via Western blot analysis and RT-PCR. HGKs were exposed to varying concentrations of a native and heat-inactivated oral biofilm extract as well as a 3-week live oral biofilm. mRNA was isolated from control and kindlin-1 deficient HGKs and RT-PCR was used to assess relative gene expression of the following gene products: β6 integrin, fibronectin ED-A and ED-B, IL-1α, IL-1β, IL-6, IL-8, TNFα, TGFβ-1, TGFβ-3, MMP-1, MMP-2, MMP-9, and tenasin C. Kindlin-1 deficiency in HGKs was found to significantly up-regulate the expression of genes encoding for β6 integrin, IL-1α, and IL-1β. β6 integrin expression was increased in K1-deficient cells in the absence of biofilm treatment and when exposed to native biofilm extract. The pro-inflammatory cytokines IL-1α and IL-1β were significantly up-regulated in K1-deficient cells as compared to control cells in keratinocytes exposed to native oral biofilm extract, and in control cells exposed to heat-inactivated biofilm.
MMP-2, MMP-9, IL-8, tenasin C, ED-B fibronectin, and TGFβ-3 were differentially regulated by the presence of biofilm extract or live biofilm in the gingival keratinocytes. TGFβ-3 and ED-B fibronectin expression were down-regulated in cell exposed to biofilm, while IL-1α, IL-1β, MMP-2, MMP-9, and tenasin C were up-regulated. Our findings suggest that the severe and early-onset periodontal disease found in patients with KS may be a result of enhanced expression of certain pro-inflammatory cytokines.
Preface

This is an *in vitro* laboratory experiment carried out using an immortalized human gingival keratinocyte cell line. This work has not been published previously. All parts of the study were completed by Angela Kehler.
Table of Contents

Abstract......................................................................................................................................................... ii
Preface........................................................................................................................................................ iv
Table of Contents ........................................................................................................................................ v
List of Tables ............................................................................................................................................... viii
List of Figures .......................................................................................................................................... ix
List of Abbreviations ............................................................................................................................... xvi
Acknowledgements ................................................................................................................................. xviii

Chapter 1: Introduction .............................................................................................................................. 1

Chapter 2: Review of the Literature ......................................................................................................... 3
  2.1 Kindler syndrome .............................................................................................................................. 3
  2.2 Kindlin family and structure .......................................................................................................... 4
  2.3 Implications of kindlin deficiency in animal models ................................................................... 6
    2.3.1 Kindlin-1 .............................................................................................................................. 6
    2.3.2 Kindlin-2 ............................................................................................................................ 7
    2.3.3 Kindlin-3 ............................................................................................................................ 8
  2.4 Kindlin proteins and their function in cellular adhesion .............................................................. 9
  2.5 Kindlin proteins and their role in integrin activation .................................................................. 10
  2.6 Kindlin deficiency and Kindler syndrome ............................................................................... 16
  2.7 Kindler syndrome and periodontal disease .............................................................................. 23
  2.8 Structure and function of the junctional epithelium ................................................................. 26
  2.9 Kindler syndrome and cancer .................................................................................................... 29
Chapter 3: Aim of the Study ........................................................................................................33

Chapter 4: Materials and Methods ..........................................................................................35

4.1 Cell lines and culture ........................................................................................................35

4.2 Downregulation of kindlin-1 by small interfering RNA (siRNA) transfection ............35

4.3 Preparation of oral biofilm ..............................................................................................37

4.4 Treatment of transfected gingival keratinocytes with oral biofilm extract ..................38

4.5 Treatment of transfected gingival keratinocytes with heat-inactivated oral biofilm extract ..........................................................................................................................39

4.6 Treatment of transfected gingival keratinocytes with 3-week live oral biofilm ..........40

4.7 RT-PCR to assess effect of kindlin-1 on inflammatory cytokine expression ..............42

4.8 Western blot analysis to confirm kindlin-1 downregulation ......................................43

4.9 Statistical analysis ...........................................................................................................44

Chapter 5: Results ..................................................................................................................45

5.1 Down regulation of kindlin-1 expression following transfection of gingival keratinocytes with siRNA ..............................................................................................................45

5.2 Effect of kindlin-1 deficiency on gene expression in human gingival keratinocytes .. 46

5.3 Gene expression in kindlin-1 deficient gingival keratinocytes treated with varying concentrations of oral biofilm extract as compared to normal gingival keratinocytes ..........46

   5.3.1 Effect of kindlin-1 deficiency on relative gene product expression in gingival keratinocytes .........................................................................................................................47

   5.3.2 Effect of biofilm extract on relative gene product expression in gingival keratinocytes .........................................................................................................................49
5.4 Gene expression in kindlin-1 deficient gingival keratinocytes treated with heat-inactivated oral biofilm extract

5.4.1 Effect of heat-inactivated and non-heat-inactivated biofilm extract on relative gene product expression in gingival keratinocytes

5.4.2 Effect of kindlin-1 deficiency on relative gene product expression in gingival keratinocytes when treated with oral biofilm extract and heat-inactivated oral biofilm extract

5.5 Gene expression in kindlin-1 deficient gingival keratinocytes treated with 3-week live oral biofilm

5.5.1 Effect of live biofilm on relative gene expression in gingival keratinocytes

5.5.2 Effect of kindlin-1 deficiency

Chapter 6: Discussion

Chapter 7: Conclusion

References

Appendices

Appendix A
List of Tables

Table 2.1 Summary of the effects of kindlin-1 deficiency on keratinocytes as determined through *in vitro* experiments ................................................................. 31

Table 2.2 Summary of the effects of kindlin-1 deficiency on keratinocytes as determined through *in vivo* experiments ................................................................. 32

Table 5.1 Statistically significant differences in relative gene expression between normal and K1-deficient keratinocytes treated with varying concentrations of oral biofilm extract ........... 85

Table 5.2 Statistically significant differences in relative gene expression between normal and K1-deficient keratinocytes treated with native oral biofilm extract and heat-inactivated oral biofilm extract ........................................................................................................... 85

Table 5.3 Statistically significant differences in relative gene expression between normal and K1-deficient keratinocytes treated with live oral biofilm ................................................................. 86
List of Figures

Figure 2.1 Kindlin domain structure and binding sites ILK, migfilin, β1-, β3, and β6-integrin .... 6

Figure 4.1 Transfection of gingival keratinocytes and treatment with biofilm extract .............. 39

Figure 4.2 Transfection of gingival keratinocytes and treatment with biofilm extract and heat-inactivated biofilm extract ................................................................. 40

Figure 4.3 Transfection of gingival keratinocytes and treatment with 3-week live biofilm ....... 41

Figure 5.1 Western blot analysis demonstrating 90% down-regulation of kindlin-1 protein in gingival keratinocytes transfected with siRNA targeted to kindlin-1 protein, as compared to reference protein β-actin ................................................................. 45

Figure 5.2 Relative mRNA expression in K1-deficient keratinocytes as compared to keratinocytes normal for K1 expression ......................................................... 60

Figure 5.3 Relative mRNA expression of β6 integrin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract .......................................................... 61
Figure 5.4 Relative mRNA expression of ED-A fibronectin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract................................................................. 61

Figure 5.5 Relative mRNA expression of ED-B fibronectin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract................................................................. 62

Figure 5.6 Relative mRNA expression of IL-1α in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract.... 62

Figure 5.7 Relative mRNA expression of IL-1β in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract.... 63

Figure 5.8 Relative mRNA expression of IL-6 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract.... 63

Figure 5.9 Relative mRNA expression of IL-8 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract.... 64

Figure 5.10 Relative mRNA expression of kindlin-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract................................................................. 64
Figure 5.11 Relative mRNA expression of MMP-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract

Figure 5.12 Relative mRNA expression of MMP-2 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract

Figure 5.13 Relative mRNA expression of MMP-9 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract

Figure 5.14 Relative mRNA expression of TGFβ-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract

Figure 5.15 Relative mRNA expression of TGFβ-3 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract
Figure 5.16 Relative mRNA expression of tenascin C in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract ................................................................. 67

Figure 5.17 Relative mRNA expression of TNFα in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract ........................................................................................................................................... 68

Figure 5.18 Relative mRNA expression of β6 integrin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. ........................................................................................................................................... 69

Figure 5.19 Relative mRNA expression of ED-A fibronectin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. ........................................................................................................................................... 69

Figure 5.20 Relative mRNA expression of ED-B fibronectin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. ........................................................................................................................................... 70

Figure 5.21 Relative mRNA expression of IL-1α in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. ........................................................................................................................................... 70
Figure 5.22 Relative mRNA expression of IL-1β in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. ........................................................................................................................................ 71

Figure 5.23 Relative mRNA expression of IL-6 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract............... 71

Figure 5.24 Relative mRNA expression of IL-8 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract ............... 72

Figure 5.25 Relative mRNA expression of kindlin-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. ........................................................................................................................................ 72

Figure 5.26 Relative mRNA expression of MMP-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract ........................................................................................................................................ 73

Figure 5.27 Relative mRNA expression of MMP-2 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. ........................................................................................................................................ 73
Figure 5.28 Relative mRNA expression of MMP-9 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. .......................................................... 74

Figure 5.29 Relative mRNA expression of TGFβ1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. .......................................................... 74

Figure 5.30 Relative mRNA expression of TGFβ3 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. .......................................................... 75

Figure 5.31 Relative mRNA expression of tenascin C in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. .......................................................... 75

Figure 5.32 Relative mRNA expression of TNFα in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. .......................................................... 76

Figure 5.33 Relative mRNA expression of β6 integrin in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm............. 77
Figure 5.34 Relative mRNA expression of ED-A fibronectin in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm............. 77

Figure 5.35 Relative mRNA expression of ED-B fibronectin in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm............. 78

Figure 5.36 Relative mRNA expression of IL-1α in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm........................................... 78

Figure 5.37 Relative mRNA expression of IL-1β in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm........................................... 79

Figure 5.38 Relative mRNA expression of IL-6 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm........................................... 79

Figure 5.39 Relative mRNA expression of IL-8 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm........................................... 80

Figure 5.40 Relative mRNA expression of kindlin-1 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm........................................... 80

Figure 5.41 Relative mRNA expression of MMP-1 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm........................................... 81
Figure 5.42 Relative mRNA expression of MMP-2 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm................................. 81

Figure 5.43 Relative mRNA expression of MMP-9 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm.............................................. 82

Figure 5.44 Relative mRNA expression of TGFβ1 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm.................................................. 82

Figure 5.45 Relative mRNA expression of TGFβ3 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm.................................................. 83

Figure 5.46 Relative mRNA expression of tenascin C in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm................................. 83

Figure 5.47 Relative mRNA expression of TNFα in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm................................. 84
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modification of Eagle’s medium</td>
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<tr>
<td>EBL</td>
<td>external basal lamina</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FA</td>
<td>focal adhesion</td>
</tr>
<tr>
<td>FERM</td>
<td>four point one protein, ezrin, radixin, moesin</td>
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<tr>
<td>HGK</td>
<td>human gingival keratinocytes</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<tr>
<td>IBL</td>
<td>internal basal lamina</td>
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<td>ILK</td>
<td>integrin linked kinase</td>
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<td>K1</td>
<td>kindlin-1</td>
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<tr>
<td>KS</td>
<td>Kindler syndrome</td>
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<tr>
<td>LAD</td>
<td>leukocyte adhesion deficiency</td>
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<tr>
<td>Mig-2</td>
<td>mitogen inducible gene-2</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDGFB</td>
<td>platelet derived growth factor B</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PH</td>
<td>plekstrin homology</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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I offer my enduring gratitude to the faculty, staff and my fellow students at UBC, who have continued to motivate and support me throughout my studies. I offer a special thanks to everyone in the Lab of Periodontal Biology for their contribution to this research by their ongoing support and answers to all my questions. I would also like to thank Dr. Larjava for all his knowledge, patience, and support throughout this project and his overall dedication to teaching and research.
Chapter 1: Introduction

Kindler syndrome (KS) is an autosomal recessive skin disorder of unknown etiology resulting in congenital skin blisters, photosensitivity, generalized progressive poikiloderma, and mucosal alterations (Lai-Cheong and McGrath, 2010; Wiebe et al., 2008). In addition to the cutaneous manifestations of the disease, severe periodontal disease has been noted as a common clinical finding (Wiebe et al., 1996). Severe periodontal disease that occurs early in life leads to early tooth loss and impairment of masticatory function. One report to date has documented the treatment of early and aggressive periodontal disease in a 16-year-old female with KS (Wiebe et al., 1996). Ultimately this patient lost most of her dentition prior to age 30; the remaining dentition was maintained with non-surgical periodontal treatment. No protocol for the prevention or management of severe periodontal disease in patients with Kindler syndrome has been established.

KS is a result of mutation of the gene encoding for kindlin-1 protein, ultimately leading to a deficiency in kindlin-1 protein (Jobard et al., 2003; Siegel et al., 2003). Kindlin-1 is a protein involved in the adhesion of the epidermis to the underlying basement membrane, although the exact mechanism for its role in the adhesion complex is not fully understood. Attachment of periodontal tissues to the tooth surface also involves an epithelial and basement membrane attachment apparatus. Immunohistological analysis of oral mucosa from a patient with KS has demonstrated discontinuities in the basement membrane zone (Wiebe et al., 2008). Structural deficiencies in the epithelial attachment to the tooth surface may account for the reduced defenses to oral bacterial challenge and subsequent periodontal breakdown. It is also possible
that altered gene expression in kindlin-1 deficient keratinocytes, including up-regulation of pro-inflammatory cytokines and down-regulation of anti-inflammatory mediators. Past research has found that kindlin-1 deficient keratinocytes may express increased levels of certain mediators of inflammation and fibrosis, including IL-20, IL-24, TGFβ-2, interleukin-1 family member 5 (IL1F5), PDGFB, and CTGF (Heinemann et al., 2011). Increased expression of pro-inflammatory mediators and altered expression of mediators involved in connective tissue maintenance may account for the exacerbated periodontal inflammation observed in patients with KS. The objective of this investigation is to determine if kindlin-1 deficiency has an affect on the expression of inflammatory mediators in human gingival keratinocytes.
Chapter 2: Review of the Literature

2.1 Kindler syndrome

Kindler syndrome (KS) is an autosomal recessive skin disorder of unknown etiology resulting in congenital skin blisters, photosensitivity, generalized progressive poikiloderma, and mucosal alterations (Lai-Cheong and McGrath, 2010; Wiebe et al., 2008). The condition was first noted in 1954 in a case report describing a 14-year-old female with a history of skin blisters since birth and sensitivity to sunlight (Kindler, 1954). The patient presented with generalized cutaneous atrophy with pigmentation irregularities on sun-exposed skin, syndactyly of the toes, and red, edematous gingiva since early childhood. Kindler noted that this condition included characteristics of both epidermolysis bullosa and poikiloderma congenitale and proposed that the disease was a combination of the two disorders. Early features of KS are similar to epidermolysis bullosa, however the similarities diminish with age. It has now been established that the Kindler syndrome is its own disease entity with clinical and histological findings unique from epidermolysis bullosa and poikiloderma congenitale (Shimizu et al., 1997).

Other common clinical manifestations of KS include hyperkeratosis of the palms of hands and soles of feet, and mucosal alterations involving the oral, ophthalmic, gastrointestinal, and urogenital mucosa (Wiebe and Larjava, 1999; Petricca et al., 2009; Wiebe et al., 2008). Skin atrophy of the dorsal hands is generally the first sign characteristic of KS in children, being recognized as early as 2 years of age (Has et al., 2011). Poikiloderma is usually identified around 10 years of age and progresses with time (Has et al., 2011). Early and rapidly progressive periodontal disease has also been described in KS patients (Ricketts et al., 1997;
Wiebe and Larjava, 1999; Wiebe et al., 2003). The skin blistering and photosensitivity present in early childhood improves into early adulthood with progressive skin atrophy and poikiloderma, however photosensitivity can be a variable finding amongst patients with KS (Siegel et al., 2003; Lai-Cheong et al., 2009). Skin fragility has been reported to be relatively mild in adult patients with KS with less susceptibility to blister formation (Has et al., 2011). Gastrointestinal symptoms may also be present and include severe colitis or bloody diarrhea (Sadler et al., 2006; Kern et al., 2007). Involvement of esophageal and genitourinary mucosa has been reported to increase with age. Further, an increased susceptibility for skin cancers has been found in patients with kindlin-1 deficiency (Arita et al., 2007; Emanuel et al., 2006).

Kindler syndrome is the result of mutations in the gene encoding for kindlin-1, KIND1, located at chromosome 20p12.3 (Jobard et al., 2003; Siegel et al., 2003). KIND1 is a human homologue of the Caenorhabditis elegans gene, UNC-112, which encodes for a protein that mediates attachment of the actin cytoskeleton to the ECM (Rogalski et al., 2000). Identification of the homology between these two genes initially provided insight into the potential functions of the kindlin proteins. More than 40 distinct FERMT1 mutations have been reported to cause KS, including large deletions, splice site, nonsense, and frameshift mutations, all leading to complete lack of kindlin-1 in epithelial cells (Heinemann et al., 2011).

### 2.2 Kindlin family and structure

Three kindlin proteins, kindlin-1, kindlin-2, and kindlin-3, comprise the kindlin protein family. All three kindlin proteins share significant homology, however each is encoded by a separate
gene: KIND1 (chromosome 20p12.3), KIND2 (chromosome 14q22.1), and KIND3 (chromosome 11q13.1) (Siegel et al., 2003; Rogalski et al., 2000).

Kindlin proteins are characterized by an evolutionarily conserved four-point-one protein, ezrin, radixin, moesin (FERM) domain, which is interrupted by a pleckstrin homology (PH) domain. Both the FERM and PH domains are similar to those found in talin, a key integrin-binding protein (Kloeker et al., 2004). FERM and PH domains are present in several other proteins involved in anchorage of the cytoskeleton to the ECM (Lai-Cheong et al., 2009). FERM domains are clover-shaped arrangement of three subdomains, F1, F2, F3 (Harburger et al., 2009). The F3 subdomain of the FERM domain of all kindlins contains a phosphotyrosine binding (PTB) fold, involved in localization of focal adhesion proteins to the plasma membrane, which is similar to that found in talin (Kloeker et al., 2004; Shi et al., 2007). Several mutations identified in KS are thought to affect the integrity of the kindlin-1 FERM domain, especially the F3 subdomain (Jobard et al., 2003; Kloeker et al., 2004).

Kindlin FERM domains are located at the carboxyl terminus, unlike talin, which has FERM domains in the N-terminal region (Jobard et al., 2003; Siegel et al., 2004; Larjava et al., 2008). Kindlin FERM domains are split in the F2 subdomain by a pleckstrin homology (PH) domain (Goult et al., 2009), a hydrophobic region of the protein that regulates the recruitment of kindlins to the cell membranes as it can bind phosphatidylinositol lipids of cell membranes (Lai-Cheong et al., 2009; Larjava et al., 2008).
Figure 2.1 Kindlin domain structure and binding sites ILK, migfilin, β1-, β3, and β6-integrin.

2.3 Implications of kindlin deficiency in animal models

2.3.1 Kindlin-1 deficiency

Kindlin-1 is highly expressed in epithelial cells of the epidermis and gastrointestinal tract (Siegel et al., 2003; Ussar et al., 2008; Lai-Cheong et al., 2008). Kindlin-1 protein has also been found in epithelial cells of other tissues, including the kidney, and placenta, although in lower amounts (Herz et al., 2006; Kern et al., 2007; Petricca et al., 2009; Ussar et al., 2008). Kindlin-1 is also expressed in fibroblasts, although in very low levels. In the skin, kindlin-1 is localized to the basal aspect of basal keratinocytes, observed in both phosphorylated and non-phosphorylated forms (Herz et al., 2006). Kindlin-1 knockdown in mice results primarily in a gastrointestinal phenotype characterized by perinatally lethal inflammatory bowel disease similar to that observed in ulcerative colitis (Ussar et al., 2006; Ussar et al., 2008). The cause for ulcerative colitis is secondary to impaired epithelial adhesion, which in turn is caused by defective integrin activation.

Zebrafish embryos present a unique model by which to study kindlin-1 deficiency as development of hemidesmosomes takes place five to six days following fertilization. It has been observed in humans and other animal models that hemidesmosomes can in part rescue the
adhesion defects caused by kindlin-1 deficiency. In zebrafish embryos, kindlin-1 deficiency results in epidermal fragility and progressive fin rupturing (Postel et al., 2013). Transmission electron microscopy (TEM) analysis showed that the basal epidermis of kindlin-1 deficient zebrafish embryos was malformed with microblisters between the basal epidermis and basement membrane and gaps between the cells forming the basal epidermis (Postel et al., 2013).

2.3.2 Kindlin-2 deficiency

Kindlin-2, also known as mitogen inducible gene-2 (Mig-2), is expressed widely in different tissues, including the heart, lung, skeletal muscle, kidney, bladder, stomach and skin. It is the closest homolog to kindlin-1 with 62% homology in amino acid sequence. In mammals, kindlin-1 and kindlin-2 both localize to integrin-containing focal adhesion complexes (Ussar et al., 2006).

Kindlin-2 plays an important role in formation of focal adhesions and cell-cell contacts, as well as in integrin activation. Kindlin-2 binds to integrins and to migfilin, which in turn interact with filamin, an actin-binding protein, recruiting migfilin into cell-extracellular matrix adhesions (Tu et al., 2003). This localization of migfilin and kindlin-2 to FAs is dependent on intergrin-linked kinase (ILK) (Mackinnon et al., 2002), a focal adhesion protein that was initially identified as a direct binding partner for β1 integrin cytoplasmic tails (Hannigan et al., 1996).

In this way kindlin-2 is involved in linking the actin cytoskeleton to extracellular matrix adhesions via the migfilin/filamin complex. When kindlin-2 is absent, recruitment of migfilin to these focal adhesions is blocked, while the localization of paxillin to FAs is not affected (Tu et
Whereas kindlin-1 is highly expressed in the epidermis and weakly in the dermis, kindlin-2 is more strongly expressed in the dermis as compared to the epidermis (Larjava et al., 2008).

Kindlin-2 knockdown in mice leads to death at or prior to 7.5 days due to severe detachment of the epiblast and endoderm, resulting in peri-implantation lethality (Dowling et al., 2008; Montanez et al., 2008).

Using a zebrafish model, kindlin-2 was shown to play an important role in cardiac development and function (Dowling et al., 2008). Kindlin-2 knockdown in zebrafish resulted in severe cardiac abnormalities. Ultrastructural analysis of the abnormal hearts showed disrupted intercalated disc formation and defective attachment of myofibrils to membrane complexes (Dowling et al., 2008). Overall this study demonstrates a critical role for kindlin-2 in cytoskeletal organization at sites of membrane attachment, and in vertebrate myocardial development and function.

2.3.3 Kindlin-3 deficiency

Kindlin-3 is exclusively expressed in hematopoietic cells and demonstrates 49% homology in amino acid sequence to kindlin-1. Kindlin-3 is present in spleen, thymus, lymph nodes, dendritic cells, macrophages, T-cells and B-cells but not in heart, brain, liver, skeletal muscle, kidney or testis (Ussar et al., 2006). Using a mouse model, kindlin-3 knockdown studies demonstrate an inability for platelets and leukocytes to activate integrins in kindlin-3 deficient mice (Moser et al., 2008; Moser et al., 2009). Other studies have demonstrated deficiencies in red blood cell
function in mice, with kindlin-3 knock-down resulting in severe GI, cutaneous, and bladder hemorrhages, leading to death shortly after birth (Kruger et al., 2008; Moser et al., 2008). The prolonged bleeding time in these kindlin-3 deficient mice is a result of platelet dysfunction, a consequence of an inability to activate αIIbβ3 integrin.

Defective function of kindlin-3 may also play a role in leukocyte adhesion deficiency (LAD-III), an autosomal recessive disorder characterized by impaired adhesion of leukocytes to inflamed endothelia, leading to severe bleeding and recurrent infections. Recent studies have shown that mutations in kindlin-3 are associated with LAD-III (Malinin et al., 2009; Svensson et al., 2009; Mory et al., 2008). The mechanism behind the disease is likely impaired binding of kindlin-3 to beta cytoplasmic tails of β2, resulting in defective integrin activation (Moser et al., 2009).

2.4 Kindlin proteins and their function in cellular adhesion

Hemidesmosomes are integrin-based complexes that mediate the adhesion of the epidermis to the basement membrane in skin and mucosa. Hemidesmosomes are composed of the α6β4 integrin, plectin, CD151, and the bullous pemphigoid antigens 180 and 230 (Margadent et al., 2008). β1-integrins are also expressed in keratinocytes and play a role in the connection of the actin cytoskeleton to the extracellular environment and are found in focal adhesions. Specifically, integrin α3β1 is involved in epidermal adhesion and basement membrane organization. Mice that lack the α3 or β1 subunit in the epidermis, as well as humans who carry mutations in the gene encoding α3, develop BM abnormalities and skin blisters; the blistering is less severe than when α4β6 integrin is deficient as adhesion of the BM can be partially rescued by
hemidesmosomes (DiPersio et al., 1997; Brakebusch et al., 2000; Margadant et al., 2009; Has et al., 2012).

In keratinocytes, kindlin-1 is localized to focal adhesions (Herz et al., 2006; Kloeker et al., 2004; Siegel et al., 2003; Ussar et al., 2006). Focal adhesions are multi-protein complexes that form around a transmembrane core of integrins, functioning in the connection of the actin cytoskeleton to the extracellular matrix. Focal adhesion complexes form through the binding of integrins to specific ligands, with the subsequent recruitment of various adapter, structural, and signaling proteins (Geiger and Yamada, 2011). Kindlin proteins have been localized to focal adhesions both in vitro and in vivo, where together with talin they bind to the cytoplasmic tails of integrin β subunits and take part in inside-out integrin activation (Kloeker et al., 2004; Larjava et al., 2008; Meves et al., 2009). Specifically, it has been identified that kindlin-1 co-localizes with filamentous actin and with vinculin, a marker of focal adhesions (Siegel et al., 2003). Kloeker et al. (2004) also found that kindlin-1 co-localizes with vinculin in HaCaT (immortalized human keratinocyte cell line) cells as well paxillin. Generally this evidence suggests a role for kindlin-1 in regulation of focal adhesion interactions.

2.5 Kindlin proteins and their role in integrin activation

Integrins are glycosylated transmembrane heterodimeric receptors made of an alpha and beta subunit (Hynes, 2002) involved in the regulation of cell adhesion, migration, and survival. Integrins function in the mechanical connection and communication of the cell to the external environment, mediating bidirectional signaling across the cell membrane, which is essential to maintenance of cell vitality (Lai-Cheong and McGrath, 2010). Cellular connection to the
extracellular matrix (ECM) is essential for maintenance of tissue integrity and function. Integrins are made up of an α and a β subunit, linked by non-covalent bonding. Humans express 18 α and eight β subunits that combine to form 24 different integrin heterodimers, each with varying ligand-binding specificities (Hynes, 2002; Calderwood, 2004). The larger extracellular domains of integrins interact with extracellular matrix proteins such as fibronectin and laminins. The shorter cytoplasmic domains of integrins are involved in binding ligand proteins, mediating communication between the intracellular and extracellular environments. For most integrins, the cytoplasmic tails are short sequences that form connections to the actin cytoskeleton. The β1 subunit cytoplasmic tail is around 50 amino acids however most alpha cytoplasmic domains are 25 amino acids. An exception is the β4 subunit, which has a much larger cytoplasmic domain, about 1000 amino acids (Hynes, 2002). Talin was one of the first proteins found to bind the cytoplasmic tails of integrins and play an essential role in integrin activation and regulation (Horwitz et al., 1986; Hynes, 2002). Talin has been identified to bind to β1, β2, β3, and β5 integrins, and weakly to β7 integrin (Critchley, 2000; Calderwood et al., 2000).

A key step in integrin activation is the binding of the FERM (four point one protein, ezrin, radixin, moesin) domain of proteins, including talin, to the cytoplasmic domain of the integrin (Calderwood et al., 1999; Calderwood, 2004; Nieswandt et al., 2007). This FERM domain was first identified in the talin protein, a well-recognized integrin-binding protein essential to integrin activation. The FERM domain of talin and other FERM domain-containing proteins binds the cytoplasmic tail of β integrin subunits. The binding of integrins to their ligands triggers a variety of signal transduction events which regulate many aspects of cellular behavior, including proliferation, apoptosis, cell shape, polarity, motility, gene expression, and differentiation. Most
integrins are not in a constant “active” state, but require binding of specific ligands to activate the signaling process (Hynes, 2002; Calderwood, 2004). Inactive integrins demonstrate a low affinity binding state. Upon extracellular ligand binding and cellular stimulation, a conformational rearrangement of the integrin extracellular domains ensues, shifting binding affinity from a low to high affinity state (Calderwood, 2004; Hynes, 2002; Lai-Cheong and McGrath, 2010).

Studies have shown that the cytoplasmic tails of α integrin and β integrin interact with one another forming a weak bond which maintains the integrin in a resting state, meaning the extracellular domains have weak affinity for ligands (Larjava et al., 2008; Hu and Luo, 2013). The disruption in this bond triggers integrin activation and increased affinity for ligands.

There is significant homology in the amino acid sequence between the talin and kindlin FERM domains, especially in the F3 subdomain region involved in integrin binding (Kloeker et al., 2004), implicating kindlins as integrin binding proteins that play a role in integrin activation. The localization of kindlin 1 protein to focal adhesion complexes may be mediated by FERM domain-integrin β tail interactions (Kloeker et al., 2004). It has been established that kindlins binds integrin beta subunits via the F3 subdomain of the FERM domain (Jobard et al., 2003; Kloeker et al., 2004; Shi et al., 2007), and that a direct interaction between kindlin-1 and β1 integrin is required for localizing kindlin-1 protein to these cell matrix adhesion complexes (Margadent et al., 2013). Kindlin-1 may also be involved in the internalization and redistribution of β1 integrins, a process also dependent on the direct interaction between the F3 domain of kindlin 1 and the integrin (Margadent et al., 2013).
To date, five kindlin-binding proteins have been identified and these include integrin linked kinase (ILK), migfilin, β1, β3, and β6 integrins (Larjava et al., 2008; Bandyopadhyay et al., 2012). Kindlin-1 binds all five of these proteins via the F3 subdomain of the FERM domain. Binding of the FERM domain of talin to the cytoplasmic tail of integrins is critical to integrin activation and cellular signaling. Other proteins may be involved in integrin activation that share this FERM domain. The FERM domain is conserved in the kindlin proteins (Jobard et al., 2003; Siegel et al., 2004; Meves et al., 2009) and subsequent studies have shown that kindlins function with talin as important components of cell-ECM adhesions by binding β integrin cytoplasmic tails.

Shi et al (2007) first demonstrated that kindlins are involved in integrin activation. Using Chinese hamster ovary (CHO) cells, kindlin-2 was found to directly interact the β3 and β1 integrin cytoplasmic tails (Shi et al., 2007). Specifically, the F3 subdomain of kindlin-2 is involved in binding β integrin cytoplasmic tails. The kindlin-2/integrin binding was found to weakly promote integrin activation and enhance cellular adhesion. This study also found that integrin binding is essential for the localization of kindlin-2 to focal adhesions; a mutated version of kindlin-2, with defects in integrin-binding, was unable to localize to focal adhesions as compared to a wild-type version. Subsequent research has found that kindlin-2 localizes to cell-ECM-adhesion sites and interacts directly with the focal-adhesion proteins ILK and migfilin (Larjava et al., 2008). Kindlin-2 and migfilin form an important connection between cell-ECM adhesions and the actin cytoskeleton. In the absence of kindlin-2, migfilin is unable to localize to focal adhesions (Meves et al., 2009).
Several studies have also shown that kindlin-1 binds directly to β1, β3, and β6 subunit integrin cytoplasmic tails using integrin pull-down assays (Has et al., 2008; Harburger et al., 2009; Bandyopadhyay et al., 2012) and affinity chromatography with CHO cell lysates (Kloeker et al., 2004). Specifically, the C-terminal domain of kindlin-1 is essential in the direct interaction with β subunit cytoplasmic tails (Harburger et al., 2009). The mechanism by which kindlins contribute to integrin activation appears to depend on levels of kindlin-1 expression; high levels of kindlin-1 have been found to inhibit integrin activation (Harburger et al., 2009). The F3 subdomain is important in kindlin-1 binding to integrin β tails and recruiting kindlin-1 to focal adhesions (Harburger et al., 2009; Margadant et al., 2013).

Kindlin-2 also has important functions in both the formation of FAs and cell adhesion. Kindlin-1 and kindlin-2 may partially compensate for each other in the activation of β1 integrin (Ussar et al., 2008). Although genes encoding for kindlin-1 and kindlin-2 share similarities in structure, Bandyopadhyay, et al. (2011) demonstrated functional differences between kindlin-1 and kindlin-2 using β1-knockdown keratinocytes. Using integrin pull-down assays, kindlin-1 was found to bind both β1 and β6 integrin tails while kindlin-2 specifically binds β1 integrin cytoplasmic tails; kindlin-2 and ILK recruitment to FAs is highly dependent on binding of kindlin-2 to β1 integrins (Bandyopadhyay et al., 2011). When β1 integrin was knocked down in keratinocytes, kindlin-2, ILK, and migfilin were not localized normally in the basal aspect of keratinocytes, while recruitment of kindlin-1 or talin were not affected. Normal keratinocyte function depends on binding of kindlin-2 to β1 integrins and recruitment to FAs. The
localization of kindlin-2 appears to be involved in cell spreading as an absence of kindlin-2 in keratinocytes resulted in reduced cell spreading (He et al., 2011).

Kindlin-1 and kindlin-2 may partially compensate for each other’s function in integrin activation in keratinocytes. He, et al. (2011) demonstrated that kindlin-1 and kindlin-2 have overlapping functions in adhesion, survival, and migration of keratinocytes. When both kindlin-1 and -2 were knocked down in keratinocytes, cells showed significant greater deficiencies in cell adhesion and spreading on laminin-332 and fibronectin than when kindlin-1 or kindlin-2 alone were down-regulated (He et al., 2011). Using an in vitro wound healing model, both kindlin-1 and kindlin-2 deficient cells showed delayed wound closure compared with control cells; kindlin-2 and kindlin-1 deficient cells showed more significant migration defects than when either protein alone was deficient. Using cultured keratinocytes cells from KS and healthy patients, Margadant, et al. (2013) found that kindlin-2 can partially rescue cell adhesion in the absence of kindlin-1 but defects in cell adhesion and spreading persist.

It has been established that environmental conditions, including UV exposure may aggravate the skin abnormalities associated with KS, especially early in life. Using cultured keratinocytes from normal and KS patients, UV-B irradiation resulted in down-regulation of kindlin-2 expression (He et al., 2011). These results suggest that UV exposure may modify kindlin-2 expression and therefore affect compensatory mechanisms that regulate the skin phenotype of these patients.
Kindlin-1 has also been shown to be involved in regulating cell shape and migration through control of lamellipodia formation (Has et al., 2009). Kindlin-1 regulates these processes through signaling via the Rho family of GTPases, specifically Rac1. Activation of Rac1 is induced by pathways involving both integrins and growth factors; activation of Rac1 was found to be significantly decreased in KS keratinocyte cells as compared to wild-type (WT) keratinocytes. In kindlin-1 deficient cells, authors found that lamellipodium were not extended as in normal keratinocytes due to lack of Rac1 activation (Has et al., 2008). This study is one of the first to implicate a role for kindlins in lamellipodia formation and function in keratinocytes and provides a mechanism for the migration and adhesion defects seen in kindlin-deficient keratinocytes.

2.6 Kindlin deficiency and Kindler syndrome

Loss of kindlin-1 causes Kindler syndrome in humans (Jobard et al., 2003; Siegel et al., 2003) and it therefore makes sense that kindlin-1 is involved in cell-ECM adhesion as disorders causing separation of overlying skin from basement membrane are usually a consequence of impairment of keratinocytes to adhere to the ECM. Kindlin-1 is localized to the basal cell layer of the epidermis. In skin, kindlin-1 is expressed in basal epidermal keratinocytes and participates in forming stable junctions between the epidermis and underlying basement membrane within the dermatoepidermal junction (DEJ) zone (Meves et al., 2009). Kindlin-2 distribution shows a lateral distribution in basal keratinocytes, while kindlin-1 distribution is predominantly basal (Margadant et al., 2013; Petricca et al., 2009).
In KS skin, keratinocytes become disorganized and lose their normal architecture, shape, polarity, and show reduced proliferation. Discontinuities are noted along the dermal-epidermal junction (Wiebe and Larjava, 1999).

Immunolocalization studies using human gingival tissues (Petricca et al., 2009) have localized kindlin-1 to the basal aspect of basal keratinocytes. Migfilin was also localized to the basal aspect of basal keratinocytes where it partially co-localized with kindlin-1, as well as in areas of cell-cell contact and suprabasal cell layers. The same study also found that paxillin, a focal adhesion protein that also interacts with the cytoplasmic tail of β integrin, was localized to the basal aspect of basal keratinocytes. In cultured keratinocytes (HaCaT cells), kindlin 1, kindlin 2, migfilin, and ILK-1 all localized to focal adhesion-like structures. When levels of kindlin-1 were increased by treatment with TGFβ-1, there was an increase in the number of focal adhesions containing ILK-1, kindlin-1, kindlin-2, and migfilin. ILK-1 staining was sparse as compared to staining for kindlin-1 and kindlin-2, suggesting that in keratinocytes, kindlin-1 and kindlin-2, but not ILK-1 are localized in the same focal adhesions with migfilin (Petricca et al., 2009). Authors also demonstrated that in vitro, kindlin-1 can physically interact with migfilin. In HaCat keratinocytes, kindlin-1 and migfilin were localized in focal adhesions at the termination points of actin filaments, suggesting that both kindlin-1 and migfilin are involved in the maintenance of the actin cytoskeleton in keratinocytes.

Kindlin-1 knockdown in HaCat cells does not appear to affect the numbers of focal adhesions per cell, however, kindlin-1-deficient keratinocytes show altered migfilin distribution in focal adhesions (Petricca et al., 2009). These findings suggest that kindlin-1 regulates migfilin.
behaviour in focal adhesions and that migfilin likely regulates integrin activity through indirect mechanisms.

Studies have shown, using both primary keratinocytes and HaCaT keratinocytes, that kindlin-1 deficiency results in altered cell morphology, decreased migration, adhesion, and cell spreading as compared to wild type cells (Herz et al., 2006; Has et al., 2011; Qu et al., 2012; Margadant et al., 2013). These defects are a result of altered β1-integrin signaling and impaired function of focal adhesions, both involved in anchoring the cytoskeleton to the ECM. Using kindlin-1-deficient keratinocytes isolated from patients with KS, Margadant et al. (2013) found β1 cell surface expression was reduced while the activation status of this integrin was slightly increased. Kindlin-2 may partially rescue the defects in cell adhesion in kindlin-1-deficient cells, however the defects in cell spreading are not affected. Integrins undergo continuous internalization and the recycling of internalized integrins is important for integrin-mediated processes such as cell spreading (Caswell et al., 2009; Margadant et al., 2011). Using an in vivo model, integrin internalization was found to be similar in KS and normal keratinocytes (Margadant et al., 2013). However, the redistribution of the internal integrin pool to the plasma membrane was observed only in a small percent of KS cells. This finding indicates that kindlin-1 regulates the redistribution of internalized integrins, which is dependent on the F3 domain (Margadant et al., 2013).

Cellular morphology of kindlin-1 deficient keratinocytes (both primary cells and HaCaT cells) appears to be altered as compared to wild type cells with a reduced ability to form intercellular adhesions. Increased membrane plasticity is observed in kindlin-1 deficient keratinocytes,
consistent with disruption of a regular cortical actin network (Qu, 2012). These findings suggest that kindlin-1 is required to maintain cellular shape and intercellular adhesions between keratinocytes. Transmembrane proteins, including integrin α6 and β4 subunits and collagen XVII (components of adhesion complexes), as well as E-cadherin and desmoglein-3 associated with intercellular junctions, are decreased in kindlin-1 deficient keratinocytes compared with controls (Qu et al., 2012). In this study, a strong correlation was observed between the reduced expression of α6β4 integrin, collagen XVII, and desmoglein-3 and the loss of K1.

Kloeker et al. (2004), using transfected HaCaT keratinocytes, found K1-deficient cells to have reduced surface area and cell spreading compared with normal keratinocytes. Decreased proliferation and reduced adhesion to fibronectin and laminin 332-coated substrates has also been demonstrated in kindlin-1 deficient keratinocytes (Herz et al., 2006; Has et al., 2008). Primary KS keratinocytes isolated from a patient with KS also appear to show undirected migration as compared to normal keratinocytes during wound closure (Has et al., 2008).

Kindlin-1 deficiency does not appear to have an effect on kindlin-2 expression and cellular localization in vivo or in vitro (Qu et al., 2012; Margadent et al., 2013). Gingival biopsy specimens from a patient with Kindler syndrome showed a reduction in the amount of migfilin and paxillin in the basal keratinocytes, suggesting that these proteins function together in the adherence of basal keratinocytes to the basement membrane (Petricca et al., 2009). Consistent with reduced cellular adherence, Margadent et al. (2013) also found reduced β1 integrin expression in cultured K1-deficient cells and epidermis biopsies. Interestingly, this study also
demonstrated enhanced motility in KS keratinocytes, consistent with reduced β1 integrin function and reduced integrin-mediated adhesion (Margadent et al., 2013).

As outlined in the previous section, kindlin proteins, along with talin, are essential for proper integrin function and activation. Integrins play an important role in cellular adhesion of overlying epithelial cells to the underlying basement membrane. Mucocutaneous disorders affecting this epithelial-connective tissue interface, including epidermolysis bullosa, result from mutations in genes that encode for basement membrane proteins or hemidesmosomes. The clinical manifestation of these disorders involves blistering of the skin and mucous membranes. The clinical presentation of Kindler Syndrome is similar to patients with junctional epidermolysis bullosa having mutations in α6 and β4 integrin genes. It is unclear how microblisters develop in the epidermis of patients with KS, and how kindlin-1 controls epidermal anchorage to the dermis.

The molecular defect associated with KS affects the basement membrane zone of the epidermis (Wiebe and Larjava, 1999). The defects seen in KS result from impaired function of β1 integrins, mediated by the absence of the kindlin protein. This defect leads to an abnormality of the actin cytoskeleton and its connection to the ECM (Jobard et al., 2003; Siegel et al., 2003). The result is a blistering skin disorder where the blisters localize to the lamina lucida aspect of the basement membrane zone; α6β4 integrin localizes in an area below the blister roof while laminin-1 and type IV collagen are seen at the blister floor (Wiebe and Larjava, 1999). Other histologic features include epidermal atrophy, focal vacuolization of the basal cell layer of the epithelium, and pigmentary incontinence in the upper dermis layer. Electron microscopy
demonstrates extensive reduplication and interruptions of the lamina densa beneath the basal cell layer (Wiebe et al., 1996). Abnormal distribution of type VII collagen in the connective tissue is another common finding in KS patients (Wiebe and Larjava, 1999). In healthy tissues, type VII collagen is found in a linear distribution along the basement membrane zone, while in patients with KS, type VII collagen is seen penetrating deep in the connective tissue (Shimizu et al., 1997; Wiebe and Larjava, 1999). Hemidesmosomes, desmosomes, and anchoring filaments are unaffected in KS (Shimizu et al., 1997).

Mechanisms responsible for the dermal aspect of the KS phenotype, including tissue fibrosis of the skin and the oral, ophthalmic, and urogenital mucosa remain unclear. It is known that fibroblasts express only very low levels of kindlin-1 and therefore the connective tissue fibrosis observed in patients with KS would have to be mediated by an interaction with the overlying epithelial cells. It is understood that epithelial-mesenchymal communication plays an important role in tissue formation and homeostasis, as the avascular epithelial layer receives both soluble mediators (cytokines, growth factors) and nutrients from the underlying vascular connective tissue layer. It has been hypothesized that an altered cytokine profile of kindlin-1-deficient keratinocytes may have an affect on the underlying connective tissue and mediate the fibrotic changes observed. In a study by Heinemann et al. (2011), authors hypothesized that kindlin-1 deficiency causes epithelial stress and consequently increased secretion of cytokines that mediate local inflammation and fibrosis.

IL-24, IL-20, TGFβ2, and IL1F5 were found to be up-regulated in keratinocytes isolated form a patient with KS as compared to normal keratinocytes (Heinemann et al, 2011). Fibroblasts are
known targets for IL-20 and IL-24 and therefore there is likely an epithelial-to-mesenchymal communication that may result in fibroblasts producing more collagen I and lead to the fibrosis observed in KS patients. TGFβ2 is a prominent mediator in the fibrotic process. Under varying conditions of cell stress, IL-20 and IL-24 were found to be up-regulated more strongly in KS keratinocytes as compared to normal keratinocytes. Fibroblasts isolated from patients with KS also showed greater type I collagen and tenascin C secretion as compared to normal fibroblasts, consistent with an activated fibroblast phenotype. Further, conditioned media from KS keratinocytes induced expression of Type I collagen and tenascin C in fibroblasts isolated from a normal patient; the slight increase in in Type I collagen synthesis and tenascin C indicates a slowly progressive fibrosis as is seen in KS patients. (Heinemann et al., 2011)

Kindlin-1 deficient keratinocytes also show increased levels of pro-MMP-2 and pro-MMP-9 in culture media (Qu et al., 2012). In the *in vitro* conditions of this experiment, MMP-9 activation was only slightly enhanced as was determined through zymography. It is important to consider that *in vivo* conditions may differ markedly and that in vivo these pro-enzymes may be activated by surrounding fibroblasts and exert significant effects, including a local inflammatory response.

The skin atrophy observed in KS patients may also be due to accelerated senescence of the kindlin-1 deficient keratinocytes (Piccinni et al., 2013). Using cultured primary keratinocytes, Piccinni et al. (2013) found that keratinocytes isolated from patients with KS had a much shorter lifespan in terms of cell generations observed. Kindlin-1 is likely involved in regulating molecular pathways of cell senescence and a deficiency in kindlin-1 plays a role in early senescence and premature aging of the skin.
A summary of the in vitro and in vivo literature detailing the effects of kindlin-1 deficiency on keratinocytes is provided in Tables 2.1 and 2.2.

2.7 Kindler syndrome and periodontal disease

In Kindler’s original case report on a 14-year-old girl presenting with what is today is known as Kindler syndrome, she described red, swollen gingiva that bled easily on provocation (Kindler, 1954). Since this report, few publications have included periodontal findings in their reports although several studies have suggested a possible link between Kindler syndrome and aggressive periodontal disease based on case reports (Ricketts et al., 1997; Wiebe et al., 1996; Wiebe et al., 2008), and one cross-sectional study (Wiebe and Larjava, 2003). In 1996, Wiebe et al. published a case report describing a 16-year-old female presenting with Kindler syndrome who was referred for periodontal assessment by her dermatologist. This patient had a history of blistering of the skin of her hands and feet and photosensitivity from birth to early childhood, with the situation improving over time. She presented with generalized skin atrophy with poikiloderma, hypopigmented lesions on the extremities, and syndactyly, all characteristic of Kindler syndrome. Dental findings included limited mouth opening, several missing mandibular teeth, generalized tooth mobility, generalized minimal plaque and calculus, and spontaneous gingival bleeding. A thin gingival biotype was described with areas of attached gingival that appeared transparent as well as areas where the gingival lifted way from the tooth with a gentle air blast. A radiographic examination showed severe bone loss at the maxillary central incisors and moderate-severe bone loss at posterior teeth. Non-surgical therapy was carried out along with the extraction of several non-vital, periodontal hopeless teeth. Improvements in gingival inflammation, probing depths, and tooth mobility were noted with mechanical therapy alone.
Although a complete dental history of the periodontal changes taking place in this individual were not available, it can be concluded that based on her good oral hygiene and the finding of severe periodontal disease at such a young age, her periodontal condition is likely related to her genetic condition.

A cross-sectional study conducted by Wiebe et al. in 2003 aimed to further clarify the relationship between Kindler syndrome and periodontal disease by examining a cohort of individuals with KS from a rural region of the Bocas del Toro province of Panama. This region showed a high prevalence of individuals with Kindler syndrome, with over 20 people identified with the disease. Eighteen of these individuals had periodontal assessments completed based on half-mouth periodontal examinations. Oral clinical findings in KS patients included spontaneous bleeding of the gingiva, gingival erythema, transparent appearance of attached gingiva, purulent exudate from the gingival margin, and severe periodontal attachment loss in early adulthood.

When periodontal disease was defined as clinical attachment loss ≥4mm, 13 of 18 Kindler syndrome patients and six of the 13 control subjects had periodontitis. KS patients showed earlier (usually around the time of permanent tooth eruption) and more rapid attachment loss over time when compared to individuals without KS. The significant difference in both the rate and clinical appearance of the periodontal disease in KS subjects as compared to the chronic periodontitis seen in control subjects suggests that the disease in these subjects is associated with systemic disease. Further, polymerase chain reaction (PCR) analysis of subgingival bacterial plaque samples demonstrated that KS periodontitis patients did not harbor typical periodontal pathogens usually associated with chronic periodontitis. An interesting finding was the inability to detect *Actinomyces actinomycetemcomitans* in subgingival plaque samples, a finding typically
associated with aggressive periodontal disease (Macheleidt et al., 1999), again suggesting the increased susceptibility to disease due to an underlying systemic condition.

In KS, skin blistering phenotype appears to improve with increasing age. While there is to date no molecular explanation for this observation, it is possible that other compensatory mechanisms develop over time. Along with FAs at the dermal-epidermal junction, other adhesive structures are present, including hemidesmosomes, which could act to compensate for the defects in FAs.

Oral biofilm (dental plaque) is the primary etiological factor for periodontal disease. The microbial species in plaque form communities, which establish an assortment of micro-niches, metabolic functions, and inter- and intra-species interactions, creating a biofilm (Hojo et al., 2009). Biofilms are dynamic communities where bacteria communicate and coordinate activities to optimize their adaptation to environmental demands. Gram negative and anaerobic bacterial species are found in greater numbers in the subgingival environment of patients with periodontal disease as compared to conditions of health (Listgarten, 1994). Key pathogens associated with periodontal disease include Porphyromonas gingivalis, Prevotella intermedia, Aggregatibacter actinomycetemcomitans (Slots and Listgarten, 1988; Listgarten, 1994), Treponema species, and Fusobacterium species (Listgarten, 1994). As discussed above, patients with KS and severe periodontal disease demonstrate minimal amounts of oral biofilm. Further, microbial analysis of the biofilm revealed low levels of known periodontal pathogens, including Porphyromonas gingivalis and Dialister pneumosintes, as compared to control individuals with periodontal disease (Wiebe et al., 2003). Based on these findings, it may be rejected that a particularly pathogenic subset of microbes is responsible for causing the severe periodontal disease observed
in these patients. The other possibility then is that cells of the periodontium in these patients exhibit an exacerbated inflammatory response to oral biofilm, resulting in rapid tissue destruction and disease.

2.8 Structure and function of the junctional epithelium

The gingiva is made up of both connective tissue and epithelium that form a collar around the tooth. There are three types of gingival epithelium – oral epithelium, sulcular epithelium, and junctional epithelium. The junctional epithelium (JE) is a highly permeable, nonkeratinized, stratified squamous epithelium, forming the floor of the gingival sulcus and seal between the epithelium and tooth surface (Shimono et al., 2003). Periodontal disease involves a chronic inflammation response triggered by pathogenic bacteria in subgingival areas, resulting in soft and hard tissue destruction. The JE plays a critical role in the host defense against pathogenic oral bacterial, protecting the space between the tooth and soft tissue from the external environment and invading pathogens. Consistent with this role, the JE is characterized by large intercellular spaces and high permeability, which increase under conditions of inflammation, allowing for the passage of tissue exudate, and inflammatory mediators and cells into the gingival sulcus (Bosshardt and Lang, 2005). Studies have demonstrated that these intercellular spaces may comprise up to 35% of the epithelial tissue, and likely are a result of a decreased number of desmosomes (Hashimoto et al., 1986). These intercellular spaces contain increased numbers of PMNs and fluid, as well as macrophages and lymphocytes (Schroeder and Listgarten, 1997). In inflammation, intercellular spaces increase and inflammatory cells, including PMNs, macrophages, and lymphocytes may occupy 30% or more of the space (Schroeder, 1970; Schroeder, 1973).
The junctional epithelium is formed from the reduced enamel epithelium during tooth eruption into the oral cavity (Shimono et al., 2003). As the tooth erupts through the oral mucosa, the reduced enamel epithelium fuses with the oral epithelium (Avery, 1987).

The JE demonstrates a high rate of cellular turnover and shows complete restoration within five days (Schroeder and Listgarten, 1997). The speed with which cells migrate up from the basal layer and exfoliate into the gingiva sulcus is controlled in part by the decreased number of desmosomes and gap junctions connecting the JE cells (Listgarten and Schroeder, 1997).

A unique feature of the JE is that it forms a basal lamina that establishes connections with both the tooth surface and the basement membrane of the gingival connective tissue. At the ultrastructural level, hemidesmosomes are involved in this attachment apparatus. The epithelial attachment at the tooth surface, be it cementum or enamel, is termed the internal basal lamina (IBL), and the attachment to the connective tissue side of the gingiva is termed the external basal lamina (EBL) (Shimono et al., 2003). The EBL demonstrates a structure typical of basement membranes found in other tissues while the IBL differs from the typical basement membrane with respect to the structural proteins present (Larjava et al., 2011). Okonsen et al. (2001) proposed that the internal basal lamina is not a true basement membrane, but is essentially a simple extracellular matrix without a network structure. Laminin-332 is a common cell adhesion protein in both the IBL and EBL, however it is the primary adhesion protein in the IBL (Hormia et al., 2001). While laminin-111 and Type IV collagen are components of the EBL, they are lacking in the IBL (Smola et al., 1998). It is hypothesized that lack of fibroblast
proximity to the IBL is responsible for the simpler protein make-up of the IBL as the soluble mediators from fibroblasts have less of an influence than on the EBL. Other structural proteins present in the IBL include Type VIII collagen and versican (Salonen et al., 1991), as well as tenascin-C (Ghannad et al., 2008).

Integrin expression in healthy junctional epithelium has been shown to differ from that of basal cells in oral epithelium (Larjava et al., 2011). Normal JE appears to express integrins that are similar to those expressed in keratinocytes in the oral mucosa during wound re-epithelialization, including αvβ6. This finding implies that JE cells remain in a perpetually activated state, similar cells found during wound healing (Larjava et al., 2011).

Integrin α6β4 is a critical component of hemidesmosomes and is involved in the basal keratinocyte attachment to the basement membrane. In the IBL, α6β4 integrin has been shown to co-localize with laminin 332, suggesting that this interaction may be involved in the attachment of the JE to mineralized tooth structure. Other integrins and proteins may also be involved in this attachment mechanism, including α3β1 and tenascin-C (Larjava et al., 2011). It has been demonstrated that basal keratinocytes of the JE interact with laminin-332 through integrins α3β1 and α6β4 (Aumailley et al., 2003). It has been established that kindlin-1 binds to β1 integrin in keratinocytes. When kindlin-1 is absent, as in Kindler Syndrome, hemidesmosome formation is not affected. It has also been suggested that in Kindler syndrome, the JE fails to form an attachment to mineralized tooth structure (Wiebe et al., 2008). These finding suggest that β1 integrin-mediated cell adhesion plays a critical role in adhesion of the JE to the tooth surface and
in Kindler Syndrome, where kindlin-1 is absent and β1 integrin function is impaired, the JE is unable to establish this firm attachment.

2.9 Kindler syndrome and cancer

Several studies have identified patients with KS to have an increased risk for development of mucocutaneous malignancies, especially squamous cell carcinoma (Lai-Cheong et al., 2010; Arita et al., 2007; Emanuel et al., 2006). In 2011, an analysis cohort of 59 patients with KS found that epithelial skin cancer affected 70% of subjects older than 45 years, while no patient was diagnosed with epithelial skin cancer before the age of 45 (Has et al., 2011). All patients were found to develop squamous cell carcinomas or precursor lesions. In the general population, the incidence rates for squamous cell carcinoma are 4.8/100 000 men and 0.6/100 000 in women for the age group of 45 years and over (Stang et al., 2003).

While data from cross-sectional studies of KS patients shows that a long-term kindlin-1 protein deficiency leads to an increased cancer risk, the mechanisms involved remain unknown. It is established that focal adhesions play important roles in preventing disease by controlling cell survival, growth, signaling, and cell invasion, making them central to the study and treatment of cancer (Kloeker et al., 2004). Impaired or altered function of focal adhesions may therefore result in unregulated cell growth, signaling, or invasion, resulting in malignancies. As kindlins play an essential role in the structure and function of FAs, kindlin proteins likely have a function in stem cell maintenance through control of cell cycle regulating process as well as the regulation of multiple growth factors that are associated with cancer and tumour cell survival (Lahn, 2005).
Weinstei, et al (2003) found an increased kindlin-1 mRNA expression in up to 60% of lung and 70% of colon cancers (Weinstein et al., 2003). Kindlin-2 has also been found to be highly expressed in a breast cancer cell line (TMX2-28) (Gozgit et al., 2006). When kindlin-2 was downregulated in this same cell line, a reduction in cancer cell invasiveness was found (Gozgit et al., 2006). KIND1 is a TGFβ1 inducible gene and increased kindlin-1 expression results from TGFβ1 treatment (Kloeker et al., 2004). This increased kindlin-1 expression results in increased cell spreading, indicative of an epithelial-mesenchymal transition, an integral step in carcinogenesis. Kindlin-2 overexpression has been found in leiomyomas (Kato et al., 2004), and increase kindlin-3 levels in some B cell lymphomas (Boyd et al., 2003). Research also indicates that kindlin-1 expression may play a role in the progression of pancreatic cancer by affecting cell migration and invasion (Mahawithitwong et al., 2012).

A recent study by Rognoni, et al (2014) looked at the effects of kindlin-1 deficiency on the regulation of cutaneous stem cells in mice. The study found that in the absence of kindlin-1, TGF-β secretion was significantly reduced and lead to hair follicle stem cell hyperproliferation (Rognoni et al., 2014). TGFβ is essential in stem cell quiescence. Further, kindlin-1 deficiency lead to increased Wnt–β-catenin–mediated signaling which results in loss of the resting phase of the hair cycle. Together, reduced TGFβ inhibitory effects and increased Wnt growth-promoting effects results in increased epithelial stem cell activity and increased tumour risk.
<table>
<thead>
<tr>
<th>Study design</th>
<th>Effect of kindlin-1 deficiency in keratinocytes</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Indirect immunofluorescence and cell spreading assays using K1-deficient immortalized human skin cell line | - Reduced surface area of cells  
- Reduced cell spreading                                                      | Kloeker et al., 2004 |
| Assays for cell proliferation, adhesion, migration, and apoptosis using human keratinocytes from normal or KS skin and an immortalized human keratinocyte cell line | - Loss of cell polarity  
- Reduced proliferation  
- Decreased adhesion  
- Undirected migration  
- Plasma membrane plasticity                                                 | Herz et al., 2006 |
| Keratinocytes isolated from patient with KS and *in vitro* wound healing assays performed | - Deficiencies in adhesion and proliferation  
- Abnormal cell morphology  
- Inability for cells to migrate in directed manner (undirected migration) | Has et al., 2008 |
| Analysis of primary human keratinocytes derived from normal control skin and skin from patients with KS; indirect immunofluorescence and cell motility assays performed. | - Abnormalities in lamellipodium extension due to increased activation of Rac1 | Has et al., 2009 |
| K1 knockdown in immortalized human keratinocyte cell line and cells assayed for proliferation, spreading, and migration. | - Alterations in migfilin distribution in focal adhesions  
- Deficiencies in cell spreading  
- Reduced cell proliferation  
- Decreased cell migration                                                  | Petricca et al., 2009 |
| Analysis of K1-deficient cells isolated from a patient with KS for gene expression using rPcR | - Up-regulation of IL-24, IL-20, TGFβ2, and IL1F5                                                          | Heinemann et al., 2011 |
| Analysis of cell adhesion, spreading, and wound closure using human keratinocyte cell lines both normal and deficient for K1 expression | - Deficiencies in cell adhesion and spreading on laminin-332 and fibronectin.  
- Deficiencies in wound closure in *in vitro* wound healing model            | He et al., 2011 |
| Analysis of immortalized human skin cell line an primary human keratinocytes derived from normal control skin and skin from patients with KS; immunofluorescence staining, cell adhesion, and protein analysis in cultured media performed | - Altered cellular morphology with increased cell membrane plasticity  
- Increased levels of pro-MMP-2 and pro-MMP-9 in culture media; slightly enhanced MMP-9 activation  
- Reduced expression of α6β4 integrin, collagen XVII, E-cadherin, and desmoglein-3  
- Increased expression of fibronectin and vimentin                          | Qu et al., 2012 |
| Serial culture of primary keratinocytes from patient with KS               | - Early cell senescence                                                                                       | Piccinni et al., 2013 |
| Analysis of K1-deficient cell line isolated from patient with KS for cell surface expression and activation status of β1 integrin | - Reduced β1 cell surface expression  | Margadant et al., 2013 |
| Analysis of primary keratinocytes derived K1-deficient mice                | - Reduced TGFβ secretion  
- Increased Wnt-β-catenin-mediated signaling resulting in increased epithelial stem cell activity | Rognoni et al., 2014 |

Table 2.1 Summary of the effects of kindlin-1 deficiency on keratinocytes as determined through *in vitro* experiments
<table>
<thead>
<tr>
<th>Study design</th>
<th>Effect of kindlin-1 deficiency in keratinocytes</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Examination of gingival biopsies from patient with KS through immunofluorescence microscopy | - Abnormal distribution of collagen type VII in basement membrane zone (BMZ)  
- Discontinuities along the dermal-epidermal junction | Wiebe and Larjava, 1999 |
| Analysis of epidermal tissue from patient with KS through immunostaining and immunoelectron microscopy | - Decreased proliferation  
- Loss of cell polarity  
- Weakened adhesion to dermis | Herz et al., 2006 |
| Histopathological analysis and immunohistochemistry of skin biopsies obtained from KS patient | - Abnormal cell morphology  
- Microblistering at dermal-epidermal junction | Has et al., 2008 |
| Immunolocalization investigation of oral mucosal tissue from patient with KS | - Absence of paxillin and reduced migfilin immunostaining | Petricca et al., 2009 |
| Immunostaining of skin biopsies obtained from KS patient                  | - Reduced β1 cell surface expression                                                                         | Margadant et al., 2013 |

Table 2.2 Summary of the effects of kindlin-1 deficiency on keratinocytes as determined through in vivo experiments
Chapter 3: Aim of the Study

The aim of this study is to further explore the mechanism through which kindlin-1 deficiency, as occurs in Kindler Syndrome, results in the early onset of severe periodontal disease in these patients. Kindlin-1 is highly expressed in dermal and oral mucosal keratinocytes and is involved attachment of keratinocytes to underlying basement membrane. It is established that in the absence of kindlin-1 protein this attachment apparatus is compromised, leading to the separation of the overlying epithelium from the underlying basement membrane and contributing to the predominant skin blistering phenotype (Larjava et al., 2008; Wiebe et al., 2008). Kindlin-1 deficiency has also been observed to compromise the adhesion of junctional epithelium of the periodontal attachment apparatus to the underlying basement membrane (Wiebe and Larjava, 1999). The impaired attachment of the junctional epithelium to the tooth surface observed in KS patients likely contributes to reduced periodontal defenses to bacterial challenge and the resulting severe periodontal attachment loss. It is hypothesized that as well as compromising the attachment apparatus, kindlin-1 deficiency in gingival keratinocytes may also have an effect on expression of cytokines and proteins involved in attachment and healing. Studies have found altered levels of certain cytokines and proteins in kindlin-1 deficient cells as compared to normal cells (Qu et al., 2012). The severe periodontal destruction observed in KS patients may be due to up-regulation of inflammatory cytokines and down-regulation of proteins involved in periodontal repair and defense.

Our general hypothesis is that the down-regulation of kindlin-1 protein in gingival keratinocytes will effect the expression of several pro-inflammatory cytokines and proteins involved in cell
adhesion and healing. Specifically, we hypothesize that (1) pro-inflammatory cytokine expression will be elevated in kindlin-1 deficient gingival keratinocytes, along with β6 integrin, (2) anti-inflammatory mediators, including TGFβ-1 and -3 will be down-regulated in kindlin-1 deficient gingival keratinocytes, and (3) proteins involved in cell adhesion and wound healing, including fibronectin and tenascin C may also show altered expression in kindlin-1 deficient keratinocytes.

To investigate these hypotheses, we will down-regulate expression of kindlin-1 in a human gingival keratinocyte cell line. In addressing our specific hypotheses as outlined above, our aims will be (1) to evaluate the expression level of several pro-inflammatory cytokines and proteins involved in wound healing in cultured human gingival keratinocytes normal for the expression of kindlin-1 and those deficient in kindlin-1 expression, and (2) to determine the effects of an oral biofilm challenge on cultured human gingival keratinocytes, both normal and deficient in kindlin-1 expression, with respect to expression of several pro-inflammatory cytokines and proteins involved in cell attachment. We plan to investigate whether there is a difference in pro-inflammatory cytokine expression and expression of several proteins involved in cell attachment between gingival keratinocytes deficient in kindlin-1 expression and those normal for kindlin-1 expression, in the presence or absence of oral bacterial challenge. Studies have shown that patients with KS experience severe early-onset periodontal disease even with minimal plaque levels (Wiebe et al., 2003). If keratinocytes in patients with KS demonstrate an exacerbated inflammatory response to bacterial challenge, this may be a mechanism through which severe periodontal disease occurs in these patients.
Chapter 4: Materials and Methods

4.1 Cell lines and culture

Human mucosal keratinocytes were obtained from surgical gingival biopsies and cultured as previously described (Salo et al., 1991). One mucosal keratinocyte cell line (HMK) was found to be spontaneously immortalized and these cells were used in the experiments between passages 28-33. Cultured keratinocytes were maintained in Dulbecco’s modification of Eagle’s medium (DMEM; Flow Laboratories, Irvine, UK) containing 23 mM sodium bicarbonate, 20 mM Hepes (Gibco, Biocult, Paisley, UK), antibiotics (50 μg/ml streptomycin sulfate, 100 U/ml penicillin), and 10% (v/v) heat-inactivated fetal calf serum (FCS). Cells were cultured on T75 tissue culture flasks (Sigma-Aldrich, St. Louise, MO, USA).

4.2 Down-regulation of kindlin-1 by small interfering RNA (siRNA) transfection

To analyze the effect of kindlin-1 on gingival keratinocyte function and behaviour, kindlin-1 expression was downregulated by transfection with siRNA. A 21-bp siRNA against human kindlin-1 was used (sense sequence: 5’ AAG ACA CAU CCA UAG CAU ACU 3’), synthesized by Invitrogen. A 21-bp non-specific siRNA (IR; sense sequence: 5’-ACU UCG ACA CAU CGA CUG CdTdT-3’; designed in the laboratory and synthesized by Invitrogen, Burlington, ON, Canada), which is not homologous to any human genes, was used as a negative control.

For siRNA transfections, approximately 2.5 million gingival keratinocytes were seeded in a T75 tissue culture flask and grown to almost reach confluence (3-4 days), renewing media every 2-3
days. Cells were then trypsinized and counted. In total, 125 pmol of siRNA oligonucleotide dimers (final concentration 50nM) and 2.25 μl of Lipofectamine RNAmax (Invitrogen, Burlington, ON, Canada) were diluted separately with Opti-MEM (Gibco) to a total volume of 250 μl each, mixed according to the manufacturers instructions, and added to cells (5 x 10^5) in suspension in 2ml of serum-free, antibiotic-free DMEM. The cells were gently shaken with the liposome–siRNA complexes at room temperature for 30 min and then plated onto 6-well tissue culture plates. When using other plate sizes, the transfection mixture volumes and cell numbers were adjusted accordingly. After 3 h of incubation and attachment at 37°C (5% CO₂), FCS was added to the cells (final concentration 8%).

Twenty-four hours following the transfection protocol (day 1), media in each well was replaced with 1.5ml fresh media. On day two of the transfection process, cell lysates from one well of IR siRNA transfected cells and one well of K1 siRNA transfected cells were collected to confirm down-regulation of kindlin-1 via RT-PCR and Western Blot analysis. On day two of the transfection process media from the remaining wells was collected and 1ml fresh DMEM (no FCS, with antibiotics) was added to the wells. At this point in the transfection (day two), cells were treated with various forms of oral biofilm through three different experimental settings (Figures 4.1 through 4.3). Each experiment evaluating either native biofilm extract, heat-inactivated biofilm extract, or the live biofilm, was repeated three times.
4.3 Preparation of oral biofilm

The culture for the oral biofilm followed the protocol previously described by Shen et al. (2009). Briefly, sterile hydroxyapatite (HA) discs (Clarkson Chromatography Products, Williamsport, PA, USA), 9.7 mm in diameter, 1.5 mm in thickness, were coated with bovine dermal type I collagen; 10 g/mL collagen in 0.012N hydrochloric acid in water (Cohesion, Palo Alto, CA, USA) by incubation overnight at 37°C in a 24-well tissue culture plate containing 2 mL of the collagen solution. Subgingival human dental plaque samples collected from the gingival sulcus were suspended in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA), to achieve a minimum bacterial cell concentration of $3.2 \times 10^7$ CFU/mL. The coated HA discs were rinsed and each immersed in the well of a 24-well tissue culture plate containing 2 mL of the BHI-plaque suspension and incubated anaerobically (AnaeroGen; Oxoid, Hampshire, UK) at 37°C. The media in each well was replaced each week.

The HA disks were removed from the 24-well plate after 3 weeks; rinsing briefly in PBS to wash off the bacterial culture medium. The bacteria were removed from the HA disks with a pipette. Pooled biofilm suspensions were placed into a mortar (pre-chilled with liquid nitrogen) and ground; resultant powder was collected into a 15ml tube. The biofilm suspension was then sonicated five times for 10 pulses (duty cycle 10%, power output 3) on ice, then transferred into Eppendorf tubes (1.5ml). Debris was removed from the biofilm suspension in the Eppendorf tubes by centrifugation (5000 rpm for 1 minute) at +4°C. Protein concentration was measured from 1 μl of biofilm in triplicates using Bio-Rad reagent. Biofilm suspensions were then stored at -20°C.
4.4 Treatment of transfected gingival keratinocytes with oral biofilm extract

Dental plaque biofilm extract was added to the cells in final concentrations of 30, 60, and 120μg/ml and incubated for 24 hours at 37°C. PBS (22 μl) was added to each one of the IR and K1 siRNA transfected cells to serve as a control (no biofilm extract).

Twenty-four hours following the addition of the biofilm extract to the transfected gingival keratinocytes, media was collected (stored at -80°C) and cell lysates collected for total RNA extraction. Total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA, USA). Total RNA (1 μg) was reverse transcribed with oligo (dT) primers using the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. The RT reaction products (cDNA samples) were diluted 20-fold with ultrapure H₂O and stored at -20°C.

In total, ten samples were collected from this experiment, two samples (one of each kindlin-1 and irrelevant siRNA transfections) on day two to confirm kindlin-1 down-regulation through RT-PCR, and eight on day three, following incubation with varying concentrations of dental plaque biofilm extract. The experimental groups and procedure are outlined in Figure 4.1.
4.5 Treatment of transfected gingival keratinocytes with heat-inactivated oral biofilm extract

Transfected gingival keratinocytes were treated with both heat-inactivated oral biofilm extract (120μg/ml) and non-treated oral biofilm extract (120μg/ml). Heat-inactivation was carried out by boiling the oral biofilm extract for 5 minutes to inactivate heat-sensitive proteins. Gingival keratinocytes were transfected with kindlin-1 siRNA and irrelevant siRNA as outlined above and plated in 6-well tissue culture plates. Two days following the transfection procedure the biofilm extract was added to the cells at a concentration of 120μg/ml. An equal volume of PBS was added to control samples. At this time cell lysate samples were collected for verification of the
kindlin-1 down-regulation. The cells were incubated at 37°C for 24 hours at which time cells were rinsed with PBS and collected for RNA isolation. An outline of the experiment and treatment groups is diagrammed in Figure 4.2.

![Diagram](image)

**Figure 4.2 Transfection of gingival keratinocytes and treatment with biofilm extract and heat-inactivated biofilm extract**

### 4.6 Treatment of transfected gingival keratinocytes with 3-week live oral biofilm

For the live biofilm experiments, 24-well tissue culture plates were used for the transfection process. Oral plaque samples were grown on hydroxyapatite discs to produce a 3 week-old oral biofilm (procedure outlined below). The biofilm-coated hydroxyapatite discs were added to the transfected cells in the wells 2 days following the transfection process, with the biofilm side of
the hydroxyapatite disc facing the cells. Sterilized hydroxyapatite discs without biofilm were added to cells to serve as control samples. The cells were incubated at 37°C for 5 hours. The discs were then removed, and cells rinsed with sterile PBS. Cells were then lysed with RNA lysis buffer and collected for RNA isolation using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA, USA) as detailed above. Total RNA (1 μg) was reverse transcribed with oligo (dT) primers using the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories) as above. The RT reaction products (cDNA samples) were diluted 20 fold with ultrapure H₂O and stored at -20°C.

![Figure 4.3 Transfection of gingival keratinocytes and treatment with live oral biofilm](image)

Figure 4.3 Transfection of gingival keratinocytes and treatment with live oral biofilm
4.7 RT-PCR to assess effect of kindlin-1 on inflammatory cytokine expression

Total RNA was extracted from cell cultures as detailed above. Cells were washed once with PBS and lysed with RA1 buffer containing 1% beta-mercaptoethanol at room temperature for 3–5 min. The lysate was then filtrated through a NucleoSpin Filter column at 11,000g for 1 min. The resultant supernatants were mixed with an equal volume of 70% ethanol and the mixture was centrifuged in the NucleoSpin RNA II Column at 11,000g for 1 min. Samples were desalted with MDB buffer, then washed with RA2 and RA3 buffer and total RNA was eluted from the column with 60μl of RNase/DNase-free water. Total RNA concentration and purity was measured by RNA/DNA Calculator (GeneQuant Pro, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). cDNA was synthesized using iScript Select cDNA Synthesis Kit (Bio-Rad) as outlined above. Briefly, 1.0 μg of total RNA was reverse transcribed by adding 4μl of 5x reaction buffer, 2 μl of random primers and 1.5 μl reverse transcriptase and nuclease-free water for a final volume of 20 μl. The cDNA was synthesized using Mastercycler gradient 5331 Reverse-Transcriptase PCR Instrument (Eppendorf AG, Hamburg, Germany) with the following program: 1 cycle at 25°C for 5 min, 1 cycle at 42°C for 30 min and 85°C for 5 min to heat-inactivate the reverse transcriptase.

RT-PCR was used to assess the expression of the following gene products in the transfected gingival keratinocytes: IL-1α, IL-1β, IL-6, IL-8, TNFα, MMP-1, -2, and -9, TGFβ1, TGFβ3, ED-A and ED-B fibronectin, β6 integrin, tenasin, and kindlin-1. The primers used for real-time PCR are listed in Appendix A. For the reaction, 5 μl of diluted reverse transcription products were mixed with 10 μl of 2x iQ SYBR Green I Supermix (Bio-Rad) and 5 μl of primers in a final volume of 20 μl. The reverse transcription products were diluted to a concentration such that
their cycle threshold values were well within the range of the standard curve. Quantitative PCR amplification was performed on the CFX Connect™ Real-Time System (Bio-Rad) using the program of 1 min at 95°C, followed by 35 cycles of 15 s at 94°C, 15 s at 60°C, and completion with reading plate and a melt-curve analysis from 65 to 95°C, 2 s for each 1°C increase. Amplification reactions were carried out for target genes with ubiquitin C (UBC), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and hypoxanthine phosphoribosyltransferase I (Hprt1) as reference genes. Non-transcribed RNA samples were used as a negative control. The amplification reactions were performed in triplicate for each sample. The data were analyzed and presented based on the comparative Ct method (CFX Manager Software Version 2.1, Bio-Rad).

4.8 Western blot analysis to confirm kindlin-1 downregulation

Western blot analysis was used to confirm the knockdown of the kindlin-1 protein. The siRNA-treated human gingival keratinocytes were cultured in a 6-well plate for 2 days. The cells were lysed in 100 μl 10% SDS solution. Equal volumes of cell lysates were separated by SDS/PAGE and transferred onto a Hybond ECL membrane (Amersham, Little Chalfont, Buckinghamshire, UK). Western blotting of the kindlin-1 protein was performed under non-reducing conditions using β-actin as a loading control. Membranes were immunoblotted with primary antibodies recognizing kindlin-1 and β-actin. Mouse anti-KIND1 antibody against human kindlin-1 protein (Norvus Biologicals, Littleton CO) and rabbit polyclonal antibody to β-actin were used as primary antibodies. IRDye 800CW conjugated goat polyclonal anti-mouse antibody and IRDye 680 conjugated goat (polyclonal) anti-rabbit antibody were used as secondary antibodies (LI-
COR Biosciences, Lincoln Nebraska USA). Membranes were viewed and analyzed with the Odyssey infrared imager (LI-COR) to determine relative protein concentrations.

4.9 Statistical analysis

All graphical data are expressed as the mean ± standard error of the mean (SEM) of parallel experiments. A two-tailed t-test was used to analyze for significant differences in gene expression between K1-deficient HGKs and normal HGKs (under control conditions). One-way analysis of variance (anova) with Tukey’s post-test was used to compare means of multiple treatment groups. The second log of the relative gene expression was used in the statistical analysis. Differences between means were considered statistically significant if the P-value was < 0.05.
Chapter 5: Results

5.1 Down-regulation of kindlin-1 expression following transfection of gingival keratinocytes with siRNA

Transfection of immortalized gingival keratinocytes with a custom siRNA targeted to the kindlin-1 mRNA, as described in the Materials and Methods section, resulted in a 80-85% down-regulation of kindlin-1 mRNA expression and a 90% down-regulation of kindlin-1 protein expression as compared to immortalized gingival keratinocytes transfected with irrelevant siRNA (Figure 5.1). Kindlin-1 mRNA expression was statistically significantly higher for all cells treated with the IR siRNA as compared to K1 siRNA (p<0.01). Kindlin-1 mRNA downregulation was 80% for the kindlin-1 siRNA transfected cells as compared to the irrelevant siRNA transfected cells.

![Western blot analysis demonstrating 90% down-regulation of kindlin-1 protein in gingival keratinocytes transfected with siRNA targeted to kindlin-1 protein, as compared to reference protein β-actin.]

Figure 5.1
5.2 Effect of kindlin-1 deficiency on gene expression in human gingival keratinocytes

Human gingival keratinocytes (HGK) were transfected with kindlin-1 siRNA to produce cells deficient in kindlin-1 protein and compared to cells treated with an irrelevant siRNA that resulted in cells normal for kindlin-1 expression. Data for non-treated K1-deficient and normal HGKs was compiled for control samples in the experiments looking at varying concentration of native biofilm extract and heat-inactivated biofilm extract. Kindlin-1 deficiency was found to have a statistically significant effect on the expression of ED-B ($p<0.01$), IL-1$\alpha$, IL-1$\beta$, and MMP-2 ($p<0.05$) (Figure 5.2). ED-B expression was found to be down-regulated by 2-fold and MMP-2 expression was down-regulated by 1.2-fold. Kindlin-1 deficiency resulted in up-regulation of IL-1$\alpha$ and IL-1$\beta$ by factors of 1.8 and 2, respectively. Expression levels of MMP-1 and MMP-9 were observed to be increased in K1-deficient cells by factors of 2.6 and 1.7, respectively. However these increases were not significant.

5.3 Gene expression in kindlin-1 deficient gingival keratinocytes treated with varying concentrations of native oral biofilm extract as compared to normal gingival keratinocytes

Human gingival keratinocytes (HGK) both deficient and normal for kindlin-1 were treated with varying concentrations of an oral biofilm extract. Cells were treated for 24 hours with three different concentrations of oral biofilm extract (30$\mu$g/ml, 60$\mu$g/ml, and 120$\mu$g/ml), cellular mRNA was isolated, reverse transcribed, and RT-PCR was used to analyze expression of the following gene products: $\beta 6$ integrin, ED-A and ED-B fibronectin, IL-1$\alpha$, IL-1$\beta$, IL-6, IL-8, MMP-1, MMP-2, MMP-9, TGF$\beta$-1, TGF$\beta$-3, TNF$\alpha$, and tenascin C. Relative expression of each gene product was determined in reference to the normal gingival keratinocytes, transfected with irrelevant siRNA, and not exposed to oral biofilm extract. The results from this experiment
are presented in Figures 5.3 through 5.17. Results are based on data from three independent experiments. One-way ANOVA testing showed statistically significant differences in expression levels between treatment groups for ED-A and ED-B fibronectin, IL-1α, IL-1β, kindlin-1, MMP-2, MMP-9, TGFβ1, TGFβ3, and tenascin C expression ($p<0.05$).

5.3.1 Effect of kindlin-1 deficiency on relative gene product expression in gingival keratinocytes treated with native biofilm extract

Expression levels for several of the gene products tested showed trends towards differential expression between kindlin-1 deficient keratinocytes and normal keratinocytes. However, no statistically significant differences were found in gene expression between normal and kindlin-1 deficient keratinocytes in the same group. There was a trend for increased expression of β6 integrin in kindlin-1 deficient keratinocytes as compared to normal keratinocytes, when exposed to native oral biofilm extract. However, the 1.5-fold increase in β6 integrin expression in kindlin-1 deficient keratinocytes treated with 60 µg/ml of biofilm extract did not reach statistical significance.

Expression of ED-A fibronectin was found to be significantly elevated, by about 1.5-fold, for keratinocytes normal for kindlin-1 expression and treated with 120 µg/ml biofilm extract as compared to kindlin-1 deficient keratinocytes not treated with biofilm extract ($p<0.01$) and those treated with 30 µg/ml biofilm extract ($p<0.05$).

There was a trend for elevated IL-1α and IL-1β expression in kindlin-1 deficient cells exposed to oral biofilm extract as compared to normal keratinocytes, however this relationship did not reach
statistical significance. When cells were treated with 60 and 120 µg/ml of biofilm extract, IL-1α expression was elevated 1.4-fold in K1-deficient cells as compared to normal keratinocytes treated with the same treatment. When cells were treated with 30, 60, and 120 µg/ml of biofilm extract, IL-1β expression was elevated 1.6-, 2.0-, and 1.7-fold, respectively, as compared to keratinocytes normal for kindlin-1 expression with the same treatment.

As shown in Figure 5.11, there was a trend for increased MMP-1 expression in kindlin-1 deficient cells treated with native biofilm extract, however this trend was statistically insignificant. When treated with 30, 60, and 120 µg/ml of native biofilm extract, expression of MMP-1 was elevated 2.0-, 2.8-, and 2.0-fold for kindlin-1 deficient keratinocytes as compared to normal keratinocytes. When looking at MMP-2 expression, there was a trend for increased expression levels in keratinocytes normal for kindlin-1 expression as compared to cells deficient in kindlin-1. Statistically significant increased expression levels were found for normal keratinocytes treated with 30, 60, and 120 µg/ml as compared to kindlin-1 deficient control cells ($p<0.05$), where MMP-2 expression was elevated approximately 2-fold. MMP-9 expression appeared to increase with increasing biofilm extract concentration. When treated with 30 and 60 µg/ml of biofilm extract, expression levels of MMP-9 were increased by 1.4- and 1.7-fold, respectively, for K1-deficient keratinocytes compared to K1-deficient cells not treated with biofilm extract, however statistical significance was not reached. Kindlin-1 deficient cells treated with 120 µg/ml of biofilm extract were observed to show statistically significant elevation of MMP-9, by 6-fold, as compared to keratinocytes normal for K1 expression and not treated with biofilm extract ($p<0.05$).
Figure 5.14 demonstrates a trend for elevated TGFβ-1 expression in K1-deficient keratinocytes treated with biofilm extract as compared to normal keratinocytes. TGFβ-1 expression was elevated 1.2-, 1.7-, and 1.4-fold for K1-deficient cells treated with 30, 60, and 120 µg/ml of biofilm extract, respectively, as compared to normal keratinocytes with the same treatment. Statistical analysis showed a significant 2-fold elevation in TGFβ-1 levels for K1-deficient cells treated with 60 and 120 µg/ml of biofilm extract as compared to normal keratinocytes not treated with biofilm extract (p<0.05). Differences in TGFβ-3, tenascin C, or TNFα expression were not observed between K1-deficient keratinocytes and normal keratinocytes. Figure 5.16 demonstrates a slight increase in tenascin C expression for K1-deficient cells treated with 60 and 120 µg/ml of biofilm extract, as compared to normal keratinocytes with the same treatment, however statistical significant was not reached.

### 5.3.2 Effect of biofilm extract on relative gene product expression in gingival keratinocytes

Expression levels of several gene products were significantly elevated when the gingival keratinocytes were treated with oral biofilm extract. Treatment of both the normal and kindlin-1 deficient keratinocytes with 30, 60, and 120 µg/ml of oral biofilm extract resulted in significantly elevated expression of IL-1α and IL-1β as compared to cells not treated with oral biofilm extract (p<0.001). In normal gingival keratinocytes, IL-1α expression was elevated 8-, 10-, and 12-fold, as compared to control cells, when treated with 30, 60, and 120 µg/ml of oral biofilm extract, respectively. In kindlin-1 deficient gingival keratinocytes, IL-1α expression was elevated 8-, 14-, and 17-fold, as compared to control cells, when treated with 30, 60, and 120 µg/ml of oral biofilm extract, respectively. The concentration of oral biofilm extract did not appear to have a
significant effect on IL-1α or IL-1β expression. Further, no significant differences in IL-1α or IL-1β expression were found between gingival keratinocytes normal and deficient for kindlin-1 expression.

Kindlin-1 deficient keratinocytes treated with 60µg/ml of biofilm extract demonstrated significantly higher expression of ED-A fibronectin (1.5-fold) as compared to kindlin-1 deficient keratinocytes without biofilm, and those treated with 30µg/ml of biofilm extract (p<0.05). Treatment with biofilm extract did not have an effect on ED-A fibronectin expression in gingival keratinocytes normal for kindlin-1 expression.

Treatment of gingival keratinocytes with native biofilm extract resulted in increased expression of MMP-9. Treatment of IR siRNA-transfected cells with 120 µg/ml of oral biofilm extract resulted in significantly elevated expression of MMP-9 (5.7-fold increase) as compared to control cells not treated with biofilm extract (p<0.05). Cells treated with 30 and 60 µg/ml of biofilm extract also resulted in elevated MMP-9 expression however this did not reach statistical significance. Kindlin-1 deficient keratinocytes treated with 60µg/ml and 120µg/ml of oral biofilm extract demonstrated significantly greater expression levels of MMP-9 as compared to the control (p<0.05). Expression of MMP-9 was elevated 4.9- and 6-fold when kindlin-1 deficient cells were treated with 60 and 120 µg/ml of biofilm extract, respectively. Kindlin-1 deficient cells exposed to 30 µg/ml biofilm extract also demonstrated elevated MMP-9 expression as compared to control cells, however this increase was not statistically significant.
Expression of TGFβ-3 was observed to decrease when cells were treated with native oral biofilm extract (Figure 5.15). Normal keratinocytes treated with oral biofilm showed significantly reduced expression of TGFβ-3 as compared to normal cells not exposed to oral biofilm extract ($p<0.05$). TGFβ-3 expression for biofilm extract treated cells was about half that of cells not exposed to oral biofilm. Kindlin-1 deficient keratinocytes that were treated with native oral biofilm extract showed significantly reduced expression of TGFβ-3 (approximately 0.5 times) as compared to kindlin-1 deficient cells not exposed to oral biofilm extract ($p<0.05$). No significant differences were observed for TGFβ-3 expression between the varying concentrations of biofilm extract.

For both normal and kindlin-1 deficient keratinocytes, treatment with oral biofilm extract resulted in significantly elevated expression of tenascin C as compared to control samples ($p<0.01$). Treatment of gingival keratinocytes normal for kindlin-1 expression with 30, 60, and 120 μg/ml of biofilm extract resulted in a 2.6-, 2.6-, and 2.8-fold increase in tenascin C expression as compared to control cells. Treatment of kindlin-1 deficient keratinocytes with 30, 60, and 120 μg/ml of biofilm extract resulted in a 2.6-, 3.8-, and 4.2-fold increase in tenascin C expression as compared to control cells. The effect of the biofilm extract on tenascin C expression was not observed to be dose-dependent with no statistically significant differences in tenascin C expression between oral biofilm extract concentrations.
5.4 Gene expression in kindlin-1 deficient gingival keratinocytes treated with heat-inactivated oral biofilm extract

Transfected gingival keratinocytes were treated with both heat-inactivated oral biofilm extract and non-heat-inactivated oral biofilm extract (120 µg/ml) to investigate the effects of heat-inactivation of the biofilm on the expression levels of the following gene products: β6 integrin, ED-A and ED-B fibronectin, IL-1α, IL-1β, IL-6, IL-8, MMP-1, MMP-2, MMP-9, TGFβ-1, TGFβ-3, TNFα, and tenascin C. Relative expression of each gene product was determined in reference to normal gingival keratinocytes, transfected with irrelevant siRNA, and not exposed to oral biofilm extract. The results from this experiment are presented in Figures 5.18 through 5.32. Results are based on data from three independent experiments.

One-way ANOVA testing showed statistically significant differences in expression levels between treatment groups for the following gene products: β6 integrin, ED-B fibronectin, IL-1α, IL-1β, IL-8, MMP-2, TGFβ-3, and kindlin-1.

5.4.1 Effect of heat-inactivated and non-heat-inactivated biofilm extract on relative gene product expression in gingival keratinocytes

Figure 5.18 demonstrates that heat-inactivated biofilm extract or biofilm extract failed to have a significant effect on β6 integrin expression in both normal and K1-deficient keratinocytes.

No significant differences in ED-A fibronectin expression were found between any of the treatment groups (Figure 5.18). K1-deficient cells without biofilm extract showed a 2-fold increase in ED-A fibronectin expression as compared to K1-deficient cells treated with heat-
inactivated biofilm extract or biofilm extract, however this difference did not reach statistical significance. Treatment with heat-inactivated biofilm extract or the native biofilm extract did not have any statistical significant effect on expression levels of ED-B fibronectin. Control K1-deficient keratinocytes showed a 2-fold increase in ED-B fibronectin expression as compared to K1-deficient cells treated with both biofilm extract and heat-inactivated biofilm extract, however statistical significance was not reached.

Normal keratinocytes treated with either native biofilm extract or heat-inactivated biofilm extract showed significantly greater expression of IL-1α and IL-1β as compared to control cells ($p<0.001$). Normal keratinocytes treated with biofilm extract and heat-inactivated biofilm extract resulted in a 3.8- and 3.7-fold increase, respectively, in IL-1α expression, and a 5.9- and 3.3-fold increase, respectively, in IL-1β expression. Heat inactivation of the biofilm extract did not have a significant effect on either IL-1α or IL-1β expression for either normal or K1-deficient keratinocytes. For kindlin-1 deficient keratinocytes treated with heat-inactivated biofilm extract, expression of IL-1α and IL-1β was significantly greater than control cells by factors of 2.2 and 2.0, respectively ($p<0.01$).

No significant effect of biofilm extract was found on IL-6 expression. IL-8 expression was significantly higher in kindlin-1 deficient keratinocytes treated with heat-inactivated biofilm extract as compared to kindlin-1 deficient keratinocytes treated with native biofilm extract by a factor of 1.4 ($p<0.05$). Kindlin-1 deficient control cells demonstrated significantly increased IL-8 expression as compared to kindlin-1 keratinocytes treated with native biofilm extract by a factor of 1.7 ($p<0.05$).
Treatment with either biofilm extract or heat-inactivated biofilm extract did not have an effect on kindlin-1 expression in either normal or K1-deficient keratinocytes (Figure 5.25).

Normal keratinocytes treated with both native biofilm extract or heat-inactivated oral biofilm extract showed significantly greater expression of MMP-2 as compared to control cells ($p<0.01$). MMP-2 expression was increased by 1.5-fold when normal keratinocytes were treated with biofilm extract and by 1.6-fold when treated with heat-inactivated biofilm extract. Kindlin-1 deficient keratinocytes treated with oral biofilm extract showed significantly greater expression of MMP-2 than kindlin-1 deficient control cells by a factor of 1.4 ($p<0.01$). K1-deficient keratinocytes treated with heat-inactivated biofilm showed a 1.3-fold increase in MMP-2 expression when compared to control cells however this relationship was not statistically significant. Treatment with biofilm extract did not have a significant effect on MMP-9 expression in either normal keratinocytes or K1-deficient keratinocytes (Figure 5.28). Treatment of K1-deficient cells with biofilm extract and heat-inactivated biofilm extract appeared to result in a decrease in MMP-9 expression as compared to control cells. However this decrease was not statistically significant.

Treatment with both native biofilm extract and heat-inactivated biofilm extract had no significant effect on TGFβ-1 expression in normal and K1-deficient keratinocytes. TGFβ-3 expression was significantly increased in kindlin-1 deficient control cells in comparison to kindlin-1 deficient keratinocytes treated with both native biofilm extract and heat-inactivated biofilm extract by a factor of 1.6 ($p<0.05$). A similar trend was observed for keratinocytes normal for K1 expression,
however this increase was not statistically significant. Treatment of normal and K1-deficient gingival keratinocytes with oral biofilm extract was not shown to have any significant effect on TNFα or tenascin C expression.

5.4.2 Effect of kindlin-1 deficiency on relative gene product expression in gingival keratinocytes when treated with native oral biofilm extract and heat-inactivated oral biofilm extract

Expression of β6 integrin was statistically significantly greater for kindlin-1 deficient keratinocytes as compared to normal keratinocytes both in the presence and absence of the biofilm extract ($p<0.01$), but not with treatment of the heat-inactivated biofilm extract. In the absence of biofilm extract, K1-deficient keratinocytes showed a 1.4-fold increase in β6 integrin as compared to normal keratinocytes ($p<0.01$); for cells treated with native biofilm extract there was a 1.8-fold increase in β6 integrin expression for K1-deficient cells ($p<0.01$).

Expression of ED-A fibronectin was not found to be statistically significantly different between normal and K1-deficient keratinocytes. Normal keratinocytes treated with heat-inactivated oral biofilm extract showed a 2.5-fold increase in the expression of ED-B fibronectin as compared to the kindlin-1 deficient keratinocytes with the same treatment ($p<0.05$). An increased expression of ED-B fibronectin (2.5-fold) was also found for normal keratinocytes treated with native biofilm extract as compared to K1-deficient cells, however this difference was not statistically significant.

Expression of IL-1α and IL-1β was found to be significantly increased in K1-deficient cells as compared to normal cells (Figure 5.2). However, when keratinocytes were treated with biofilm
extract, no significant difference in either IL-1α or IL-1β expression was found between normal keratinocytes and K1-deficient keratinocytes. No significant difference in IL-8 or IL-6 expression was noted between K1-deficient and normal keratinocytes.

Normal gingival keratinocytes that were treated with heat-inactivated oral biofilm extract demonstrated significantly increased expression of MMP-2 by a factor of 1.3 as compared to kindlin-1 deficient keratinocytes treated with heat-inactivated oral biofilm extract ($p<0.05$). No significant differences in MMP-2 expression were noted between normal and K1-deficient keratinocytes when cells were treated with native biofilm extract. No significant differences were found for MMP-9 expression between K1-deficient and normal keratinocytes. No significant differences in MMP-1, TGFβ-1, TGFβ-3, tenascin C, or TNFα were observed between keratinocytes normal and deficient in kindlin-1 expression (Figures 5.28 through 5.32).

5.5 Gene expression in kindlin-1 deficient gingival keratinocytes treated with 3-week live oral biofilm

Transfected keratinocytes, as described above, were treated with a 3-week live oral biofilm for 5 hours at 37°C. Cellular mRNA was isolated, reverse transcribed, and RT-PCR was used to analyze expression of the following gene products: β6 integrin, ED-A and ED-B fibronectin, IL-1α, IL-1β, IL-6, IL-8, MMP-1, MMP-2, MMP-9, TGFβ-1, TGFβ-3, TNFα, and tenascin C. Relative expression of each gene product as compared to a control (IR siRNA-transfected keratinocytes not treated with biofilm) was analyzed and data is displayed in Figures 5.33 through 5.47. One-way ANOVA testing showed statistically significant differences in
expression levels between groups for the following gene products: ED-A and ED-B fibronectin, IL-1β, kindlin-1, MMP-1, MMP-9, and TNFα.

5.5.1 Effect of live biofilm on relative gene expression in gingival keratinocytes

Statistical analysis failed to show a difference in β6 integrin expression between keratinocytes with the control or live biofilm treatment. However, Figure 5.33 demonstrates a trend towards increased β6 integrin for cells treated with live biofilm. For the normal keratinocytes, β6 integrin expression was increased by a factor of 1.9 in when treated with the live biofilm, and for K1-deficient cells by a factor of 1.8 as compared to control samples.

Statistically significant differences in ED-A fibronectin expression were not observed between keratinocytes treated with live biofilm compared to control samples. Figure 5.34 shows a slight increase in ED-A fibronectin expression for both normal and K1-deficient keratinocytes treated with live biofilm (1.3-fold increase), however this increase was not statistically significant. For ED-B fibronectin expression, Figure 5.35 demonstrates a similar trend of increased expression when both normal and K1-deficient cells were exposed to live biofilm, by factors of 1.7 and 2.0. Kindlin-1 deficient cells treated with live biofilm showed statistically significantly greater expression of ED-B fibronectin as compared to kindlin-1 deficient cells not treated with live biofilm (p<0.05); this increase was not found to be statistically significant for normal keratinocytes.

No significant difference in IL-1α was found when cells were treated with live biofilm as compared to control samples. However, Figure 5.36 demonstrates an increase in IL-1α
expression in both normal keratinocytes and K1-deficient keratinocytes treated with live biofilm as compared to control samples, by factors of 8 and 6, respectively. The lack of statistical significance may be attributed to the large standard deviation noted for live biofilm samples. A similar trend was noted for increased IL-1β expression in keratinocytes exposed to live biofilm. A significant 4.8-fold increase in IL-1β expression was found for normal keratinocytes exposed to live biofilm (p<0.05) and a 5.0-fold increase was found for K1-deficient cells treated with live biofilm (p<0.05). Again, for IL-6 and IL-8 expression, statistically significant differences were not observed between cells exposed to live oral biofilm and the control cells. However, both IL-6 and IL-8 expression were observed to be increased when both normal and K1-deficient keratinocytes were treated with live biofilm. When treated with live oral biofilm, IL-6 expression was increased by 2.7- and 2.4-fold for normal and K1-deficient keratinocytes, respectively. IL-8 expression was increased in both normal and K1-deficient keratinocytes when exposed to oral biofilm by factors of 10 and 8, respectively.

Treatment of keratinocytes with live oral biofilm had no statistically significant effect on MMP-1 or MMP-2 expression. When both normal and K1-deficient gingival keratinocytes were treated with live biofilm, expression of MMP-9 increased by factors of 1.9 (p<0.05) and 2.5 (p<0.01), respectively. Treatment of keratinocytes with live oral biofilm did not have a statistically significant effect on TGFβ-1 or TGFβ-3 expression as compared to control samples.

Tenascin C expression was found to be elevated by a factor of 1.4 in both normal and K1-deficient keratinocytes treated with live biofilm as compared to the control treatment, however this relationship did not reach statistical significance.
TNFα expression was increased in both normal and K1-deficient keratinocytes treated with live biofilm by factors of 5.9 and 7.4, respectively. The increase in TNFα expression was only found to be statistically significant for K1-deficient cells treated with live biofilm ($p<0.05$).

5.5.2 Effect of kindlin-1 deficiency in human gingival keratinocytes treated with live oral biofilm

Expression of β6 integrin was found to be unaffected by kindlin-1 deficiency in gingival keratinocytes. When looking at cells with the same treatment, either control or treatment with live biofilm, kindlin-1 deficiency had no significant effect on ED-A or ED-B fibronectin expression. However, normal keratinocytes treated with live biofilm demonstrated a statistically significant increase in ED-A and ED-B expression as compared to K1-deficient keratinocytes with the control treatment ($p<0.05$ and $p<0.01$, respectively). ED-A expression was increased by a factor of 1.8 in normal keratinocytes treated with live biofilm as compared to K1-deficient control cells, and ED-B by a factor of 2.2.

No significant difference in IL-1α or IL-1β expression was noted between normal and kindlin-1 deficient keratinocytes with the same treatment. A significant 3-fold increase in IL-1β expression was seen in K1-deficient keratinocytes treated with live biofilm as compared to normal keratinocytes not treated with live biofilm ($p<0.05$). Kindlin-1 deficiency did not have a significant effect on IL-6 or IL-8 expression in gingival keratinocytes.
MMP-1 or MMP-2 expression was not significantly different between normal and K1-deficient gingival keratinocytes. MMP-9 expression was observed to be significantly increased by a factor of 3.1 times in K1-deficient cells treated with live biofilm as compared to normal keratinocytes not exposed to live biofilm ($p<0.01$). Normal keratinocytes treated with live biofilm demonstrated significantly increased expression of MMP-9, by a factor of 3.5, as compared to K1-deficient control cells ($p<0.05$).

Kindlin-1 deficiency was not shown to have a statistically significant effect on TGFβ-1, TGFβ-3, tenascin C, or TNFα expression in gingival keratinocytes.

Figure 5.2 Relative mRNA expression in K1-deficient keratinocytes as compared to keratinocytes normal for K1 expression. *$p<0.05$, **$p<0.01$
Figure 5.3 Relative mRNA expression of β6 integrin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. $p = 0.290$ Cq 23.5-25.6

Figure 5.4 Relative mRNA expression of ED-A fibronectin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. $p = 0.0035$ Statistically significant differences in ED-A fibronectin expression found between the following groups: IR-120/K1-control ($p<0.01$); IR-120/K1-30, K1-60/K1-control, K1-60/K1-30 ($p<0.05$). Cq 18.8-24.0
Figure 5.5 Relative mRNA expression of ED-B fibronectin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract, \( p < 0.011 \) Statistically significant differences in ED-B fibronectin expression found between the following groups: IR-120/K1-control (\( p < 0.05 \)). Cq 22.2-27.2

Figure 5.6 Relative mRNA expression of IL-1\( \alpha \) in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract, \( p < 0.0001 \) Statistically significant differences in IL-1\( \alpha \) expression found between the following groups: IR-30/IR-control, IR-60/IR-control, IR-120/IR-control, K1-30/K1-control, K1-60/K1-control, K1-120/K1-control (\( p < 0.001 \)). Cq 23.1-25.7
Figure 5.7 Relative mRNA expression of IL-1β in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. $p < 0.0001$ Statistically significant differences in IL-1β expression found between the following groups: IR-30/IR-control, IR-60/IR-control, IR-120/IR-control, K1-30/K1-control, K1-60/K1-control, K1-120/K1-control ($p<0.001$). Cq 21.0-25.9

Figure 5.8 Relative mRNA expression of IL-6 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. $p = 0.1781$ Cq 23.8-27.4
Figure 5.9 Relative mRNA expression of IL-8 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. \( p \leq 0.0855 \) Cq 23.1-26.0

Figure 5.10 Relative mRNA expression of kindlin-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. \( p < 0.0001 \) Statistically significant differences in kindlin-1 expression found between the following groups: K1-control/IR-control, K1-30/IR-30, K1-60/IR-60, K1-120/IR-120 \( (p<0.001) \). Cq 19.9-25.2
Figure 5.11 Relative mRNA expression of MMP-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. \( p = 0.6996 \)  Cq 24.5-27.0

Figure 5.12 Relative mRNA expression of MMP-2 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. \( p = 0.0034 \) Statistically significant differences in MMP-2 expression found between the following groups: IR-30/K1-control \( (p<0.05) \); IR-60/K1-control, IR-120/K1-control \( (p<0.01) \).  Cq 27.5-28.8
Figure 5.13 Relative mRNA expression of MMP-9 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. $p \leq 0.0044$ Statistically significant differences in MMP-9 expression found between the following groups: IR-120/IR-control, K1-120/IR-control, IR-120/K1-control, K1-60/K1-control, K1-120/K1-control ($p<0.05$). Cq 27.9-29.1

Figure 5.14 Relative mRNA expression of TGFβ-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. $p \leq 0.0096$ Statistically significant differences in TGFβ-1 expression found between the following groups: K1-60/IR-control, K1-120/IR-control ($p<0.05$). Cq 20.8-23.6
Figure 5.15 Relative mRNA expression of TGFβ-3 in both Kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. $P < 0.0002$ Statistically significant differences in TGFβ-3 expression found between the following groups: IR-control/IR-30, K1-control/K1-30, K1-control/K1-120 ($p < 0.05$); IR-control/IR-60, IR-control/IR-120, K1-control/K1-60 ($p < 0.01$). Cq 29.9-31.5

Figure 5.16 Relative mRNA expression of tenascin C in both Kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. $p < 0.0001$ Statistically significant differences in tenascin C expression found between the following groups: IR-30/IR-control, IR-60/IR-control, IR-120/IR-control, K1-30/K1-control, K1-60/K1-control, K1-120/K1-control ($p < 0.01$). Cq 23.7-27.0
Figure 5.17 Relative mRNA expression of TNFα in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to varying concentrations of oral biofilm extract. $p = 0.3496$  Cq 19.9-25.2
Figure 5.18 Relative mRNA expression of β6 integrin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. $p = 0.0014$. Statistically significant differences in β6 integrin found between the following groups: K1-control/IR-control, K1-Bio/IR-Bio, K1-control/IR-HIBio ($p < 0.01$); K1-Bio/IR-HIBio ($p < 0.05$). Cq 21.2-27.4

Figure 5.19 Relative mRNA expression of ED-A fibronectin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. $p = 0.3908$. Cq 22.7-25.5
Figure 5.20 Relative mRNA expression of ED-B fibronectin in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. *p* 0.0059 Statistically significant differences in ED-B fibronectin expression were found for the following groups: IR-HIBio/K1-HIBio, IR-control/K1-Bio, IR-control/K1-HIBio, IR-HIBio/K1-Bio (*p*<0.05). Cq 24.7-29.0

Figure 5.21 Relative mRNA expression of IL-1α in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. *p* <0.0001 Statistically significant differences in IL-1α expression were found between the following groups: IR-Bio/IR-control, IR-HIBio/IR-control (*p*<0.001); K1-HIBio/K1-control, K1-control/IR-control (*p*<0.01). Cq 21.7-24.5
**IL-1β**

Figure 5.22 Relative mRNA expression of IL-1β in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. *p* < 0.0001 Statistically significant differences in IL-1β expression were found between the following groups: IR-Bio/IR-control, IR-HIBio/IR-control (*p*<0.001); K1-HIBio/K1-control (*p*<0.05); K1-control/IR-control (*p*<0.01). Cq 22.0-26.9

**IL-6**

Figure 5.23 Relative mRNA expression of IL-6 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. *p* 0.2038 Cq 24.2-28.2
Figure 5.24 Relative mRNA expression of IL-8 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. \( p = 0.0039 \) Statistically significant differences in IL-8 expression were found between the following groups: K1-control/K1-Bio, K1-HIBio/K1-Bio \((p<0.05)\). Cq 23.2-27.2

Figure 5.25 Relative mRNA expression of kindlin-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. \( p < 0.0001 \) Cq 21.6-25.8
Figure 5.26 Relative mRNA expression of MMP-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. $p = 0.1036$  Cq 23.6-27.4

Figure 5.27 Relative mRNA expression of MMP-2 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. $p <0.0001$ Statistically significant differences in MMP-2 expression were found between the following groups: IR-Bio/IR-control, IR-HIBio/IR-control, K1-Bio/K1-control ($p<0.01$); IR-HIBio/K1-HIBio ($p<0.05$).  Cq 26.9-30.4
Figure 5.28 Relative mRNA expression of MMP-9 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. \( p \ 0.8110 \)  Cq 28.2-30.9

Figure 5.29 Relative mRNA expression of TGFβ-1 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. \( p \ 0.6040 \)  Cq 22.1-24.4
Figure 5.30 Relative mRNA expression of TGFβ-3 in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. *p* 0.0015 Statistically significant differences in expression of TGFβ-3 were found between the following groups: K1-control/K1-Bio, K1-control/K1-HIBio (*p*<0.05). Cq 29.7-32.9

Figure 5.31 Relative mRNA expression of tenscin C in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. *p* 0.1256 Cq 25.9-28.4
Figure 5.32 Relative mRNA expression of TNFα in both kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to heat-inactivated oral biofilm extract. p 0.0668  Cq 27.6-30.2
Figure 5.33 Relative mRNA expression of β6 integrin in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm.  $p = 0.2269$  Cq 21.7-26.8

Figure 5.34 Relative mRNA expression of ED-A fibronectin in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm.  $p = 0.0195$  Statistically significant differences in ED-A fibronectin expression were found between the following groups: IR-Bio/K1-control ($p < 0.05$).  Cq 21.3-22.4
Figure 5.35 Relative mRNA expression of ED-B fibronectin in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. p 0.0067 Statistically significant differences in ED-B fibronectin expression were found between the following groups: IR-Bio/K1-control (p<0.01); K1-Bio/K1-control (p<0.05). Cq 21.9-25.4

Figure 5.36 Relative mRNA expression of IL-1α in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. p 0.1375 Cq 20.2-24.0
Figure 5.37 Relative mRNA expression of IL-1β in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. p = 0.0098 Statistically significant differences in expression of IL-1β were found between the following groups: IR-Bio/IR-control, K1-Bio/IR-control, K1-Bio/K1-control (p<0.05). Cq 22.3-25.7

Figure 5.38 Relative mRNA expression of IL-6 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. p = 0.0646 Cq 21.8-28.3
Figure 5.39 Relative mRNA expression of IL-8 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. \( p = 0.0995 \) Cq 19.5-23.5

Figure 5.40 Relative mRNA expression of kindlin-1 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. \( p = 0.0025 \) Cq 21.8-26.8
Figure 5.41 Relative mRNA expression of MMP-1 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. p 0.0393  Cq 24.3-28.6

Figure 5.42 Relative mRNA expression of MMP-2 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. p 0.6472  Cq 24.6-27.4
Figure 5.43 Relative mRNA expression of MMP-9 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. *p* 0.0024 Statistically significant differences in MMP-9 expression were found between the following groups: IR-Bio/IR-control, IR-Bio/K1-control (*p*<0.05); K1-Bio/IR-control, K1-Bio/K1-control (*p*<0.01). Cq 24.6-26.8

Figure 5.44 Relative mRNA expression of TGFβ-1 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. *p* 0.6617 Cq 22.2-23.9
Figure 5.45 Relative mRNA expression of TGFβ-3 in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. p 0.6204  29.2-30.8

Figure 5.46 Relative mRNA expression of tenasin C in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. p 0.1664  Cq 23.3-26.0
Figure 5.47 Relative mRNA expression of TNFα in kindlin-1 deficient gingival keratinocytes and normal gingival keratinocytes exposed to 3-week live oral biofilm. p 0.0207 Statistically significant differences in TNFα expression were found between the following groups: K1-Bio/K1-control (p<0.05). Cq 24.4-27.9
<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of kindlin-1 deficiency on the expression of selected genes as compared to control siRNA treated cells in absence of biofilm (control conditions)</td>
<td>Kindlin-1** 0.17</td>
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| Genes differentially regulated by native biofilm extract in control cells | IL-1α*** 12  
IL-1β*** 14  
MMP-9* 5.5  
TGFβ-3** 0.4  
Tenascin C** 2.8 |
| Genes differentially regulated by native biofilm extract in kindlin-1 deficient cells | ED-A* 1.5  
IL-1α*** 17  
IL-1β*** 24  
MMP-9* 6  
TGFβ-3** 0.5  
Tenascin C** 4 |
| Genes differentially regulated in kindlin-1 deficient cells by biofilm extract as compared to control cells with biofilm extract. | None |

Table 5.1 Summary of statistically significant differences in relative gene expression between normal and K1-deficient keratinocytes treated with varying concentrations of native oral biofilm extract. *p<0.05, **p<0.01, ***p<0.001  Relative increase represents maximum change in expression observed for each gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative fold change</th>
</tr>
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</table>
| Effect of kindlin-1 deficiency on the expression of selected genes as compared to control siRNA treated cells in absence of biofilm | B6 integrin** 1.4  
IL-1α** 2.2  
IL-1β** 2.4 |
| Genes differentially regulated by native biofilm extract in control cells | IL-1α*** 3.8  
IL-1β*** 5.9  
MMP-2** 1.5 |
| Genes differentially regulated by native biofilm extract in kindlin-1 deficient cells | IL-8* 0.7  
TGFβ-3* 0.6 |
| Genes differentially regulated in kindlin-1 deficient cells by native biofilm extract as compared to control cells with biofilm extract. | B6 integrin** 1.8 |
| Genes differentially regulated by heat-inactivated biofilm extract in control cells | IL-1α*** 3.6  
IL-1β*** 3.2  
MMP-2** 1.6 |
| Genes differentially regulated by heat-inactivated biofilm extract in kindlin-1 deficient cells | IL-1β* 2.1  
TGFβ-3* 0.6 |
| Genes differentially regulated in kindlin-1 deficient cells by heat-inactivated biofilm extract as compared to control cells with biofilm extract. | ED-B Fibronectin* 2.5  
MMP-2* 0.8 |

Table 5.2 Summary of statistically significant differences in relative gene expression between normal and K1-deficient keratinocytes treated with native oral biofilm extract and heat-inactivated oral biofilm extract. *p<0.05, **p<0.01, ***p<0.001  Relative increase represents maximum change in expression observed for each gene.
<table>
<thead>
<tr>
<th>Description</th>
<th>Gene</th>
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<td>Effect of kindlin-1 deficiency on the expression of selected genes as compared to control siRNA treated cells in absence of biofilm</td>
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<td>Genes differentially regulated by live biofilm extract in control cells</td>
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<td>MMP-9*</td>
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<td>MMP-9**</td>
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<td></td>
<td>TNFα*</td>
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<td>Genes differentially regulated in kindlin-1 deficient cells by live biofilm as compared to control cells with live biofilm.</td>
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Table 5.3 Summary of statistically significant differences in relative gene expression between normal and K1-deficient keratinocytes treated with live oral biofilm. *p<0.05, **p<0.01, ***p<0.001. Relative fold change represents maximum increase or decrease in expression observed for each gene.
Chapter 6: Discussion

This study investigated the behavior of kindlin-1 deficient human gingival keratinocytes in terms of expression of select genes involved in cellular defense against a microbial challenge. Structural irregularities have been identified in the basement membrane of oral mucosa in patients with Kindler syndrome (Wiebe and Larjava, 1999), which may contribute to the early and severe periodontal destruction seen in these patients. We hypothesized that along with structural deficiencies in the periodontal attachment to the tooth surface, kindlin-1 deficiency may contribute to an altered gene expression profile in gingival keratinocytes. Further, we exposed both control and kindlin-1 deficient HGKs to an oral biofilm challenge to determine if expression of certain genes and cytokines involved in the inflammatory response would be differentially expressed in kindlin-1 deficient conditions. We selected a subgingival biofilm cultured under anaerobic conditions as anaerobes are highly associated with periodontal disease. Wiebe et al. (2003) demonstrated that the biofilm isolated from patients with KS does not show a particularly pathogenic profile as compared to control individuals with periodontal disease. For this reason, it was not necessarily critical that the biofilm used in this experiment was isolated from conditions of periodontal disease. As patients with Kindler syndrome present early in life with aggressive periodontal disease, it was hypothesized that the absence of kindlin-1 protein may result in an exacerbated inflammatory response to oral bacterial biofilm, with elevated expression of pro-inflammatory cytokines that would induce destruction of the periodontal tissues.
Periodontitis is a destructive inflammatory disease that results from an interaction between gram-negative bacteria and the host immune and inflammatory response. Bacterial products, including lipopolysaccharide (LPS), are released and activate host cells such as macrophages, fibroblasts, keratinocytes, and endothelial cells to secrete pro-inflammatory cytokines (Page, 1991).

Increased expression of several pro-inflammatory cytokines in gingival keratinocytes has been shown to occur in the presence of bacterial biofilm and in patients with periodontal disease, initiating connective tissue and osseous destruction. These pro-inflammatory cytokines include IL-1α, IL-1β, IL-6, IL-8 (Eberhard et al., 2008, Eberhard et al., 2009), TNFα, and mediators of tissue destruction MMP-1, MMP-2, and MMP-9. Expression levels of IL-1β and TNFα are increased in periodontally diseased tissues as compared to healthy tissues (Roberts et al., 1997). Bacterial LPS activates macrophages to produce IL-1α and IL-1β, which then stimulate cells to amplify production of more inflammatory mediators (Page, 1991). The extent of tissue destruction is controlled by the expression levels of both pro- and anti-inflammatory cytokines at the site of microbial challenge.

Huang et al. (2004) looked at IL-8 mRNA expression in human gingival epithelial cells and found IL-8 to be up-regulated by *Fusobacterium nucleatum* and both up- and down-regulated by *Porphyromas gingivalis*, depending on the cellular pathway involved. The increase in IL-8 expression occurred within 4 hours after bacterial infection (Huang et al., 2004). Interestingly, authors found that heat treatment of *F. nucleatum* cells attenuated the IL-8 mRNA up-regulation while heat treatment of *P. gingivalis* enhanced IL-8 up-regulation. These findings point toward different gene regulation pathways for *F. nucleatum* and *P. gingivalis* in gingival epithelial cells. Using an immortalized human oral epithelial cell line, Yee, et al (2014) found that when cells
were co-cultured with both live and heat-inactivated *P. gingivalis*, IL-6 and IL-8 levels were increased in culture supernatants. Interestingly, heat-inactivation of *P. gingivalis* resulted in an even greater increase in IL-6 and IL-8 expression (Yee et al., 2014). Another study found that IL-8 and intercellular adhesion molecule 1 (ICAM-1) were up-regulated in gingival epithelial cells exposed to *A. actinomycetemcomitans* (Huang et al., 1998). However, when epithelial cells were challenged with *Porphyromonas gingivalis*, IL-8 and ICAM-1 were down-regulated (Huang et al., 1998, Darveau et al., 1998). It is possible that the up-regulation of IL-8 and ICAM-1 through *A. actinomycetemcomitans* challenge simulates the host immune response with subsequent neutrophil migration to the site as a form of defense against the bacteria. On the other hand, the inhibitory effects of *P. gingivalis* on IL-8 and ICAM-1 expression downplay the host immune response and defenses, allowing the bacteria to evade the host immune response and continue to invade and destroy host tissues.

Expression of IL-6 and IL-8 were not affected by treatment of HGK with native biofilm extract. Figures 5.7 and 5.8 illustrate a slight increase in IL-6 and IL-8 expression with native biofilm extract, however this was not statistically significant. K1-deficiency did not have an affect on IL-6 or IL-8 expression. Treatment of HGKs with heat-inactivated biofilm extract had no affect on IL-6 expression. In this experiment treatment of HGKs with native biofilm extraction resulted in increased IL-8 expression in K1-deficient cells. Interestingly heat-inactivation of the biofilm extract resulted in significantly increased IL-8 expression in K1-deficient cells as compared to K1-deficient cells treated with native biofilm extract (Figure 5.24). Treatment of HGKs cells with live oral biofilm resulted in elevated expression levels of IL-6 (3-fold) and IL-8 (8 to 10-fold), however these increases did not reach statistical significance. Overall, the biofilm
extract appeared to have no affect on IL-6 expression in HGKs. However, the live oral biofilm showed a greater increase in IL-6 expression for both K1-deficient and normal cells. It is likely that the live biofilm contains heat-sensitive bacterial elements that are involved in activating the host inflammatory response. As we cannot compare concentrations of bacterial products between the live biofilm and biofilm extract, it is also possible that the live biofilm treatment offered a higher concentration of bacterial products that were sufficient to regulate expression levels. There was a definitive tendency for increased IL-8 expression in HGKs exposed to native oral biofilm extract (Figure 5.9), however the high standard error likely contributed to a lack of significance. It appears that IL-8 up-regulation was variable across repeat experiments although all experiments showed up-regulation. When HGKs were treated with heat-inactivated biofilm extract, results conflicted with the previous experiment as IL-8 expression levels were shown to decrease with treatment. The difference in IL-8 expression observed for the native and heat-inactivated biofilm extract may be explained by results from Huang et al. (2004) and Yee et al. (2014). The study by Huang et al. demonstrated that different bacterial species can have different affects on IL-8 expression depending on the cellular pathways targeted. Depending on the relative proportions of various anaerobic microbes in the biofilm, IL-8 expression may have been up- or down-regulated.

Expression levels of both IL-1α and IL-1β were found to increase significantly when both K1-deficient and control HGKs were treated with native biofilm extract. IL-1α increased by 12- and 17-fold in control and K1-deficient cells treated with native biofilm extract, respectively, and IL-1β by 14- and 24-fold, respectively. No significant differences in IL-1α or IL-1β expression were noted between control and K1-deficient cells. When cells were treated with the native
biofilm extract, K1-deficient HGKs showed an increased expression of IL-1α and IL-1β as compared to control cells, however this was not significant. Conflicting result were found in the next experiment when HGKs were treated with heat-inactivated biofilm extract. K1-deficient cells not exposed to any biofilm extract showed significantly greater expression of IL-1α (2.1-fold) and IL-1β (2.2-fold) as compared to control cells (Figures 5.21 and 5.22). This difference in expression levels between K1-deficient and control cells was not found in the previous experiment looking at native biofilm extract only. Treatment of cells with heat-inactivated oral biofilm extract resulted in a significant increase in IL-1α in both K1-deficient and control HGKs, while treatment of cells with heat-inactivated biofilm extract resulted in a significant increase in IL-1β expression only in K1-deficient cells. When cells were treated with live biofilm, IL-1β was significantly up-regulated in both control and K1-deficient cells by 4.8- and 5-fold, respectively. However, while IL-1α expression was increased by a comparative factor in both control and K1-deficient cells, this increase was not found to be significant. Although the results are inconsistent between experiments, the findings from this study suggest that expression levels of IL-1α and IL-1β may be increased with kindlin-1 deficiency. As both IL-1α and IL-1β are potent pro-inflammatory cytokines involved in activating the host immune and inflammatory response and the release of tissue destructive factors, it is possible that their expression may be up-regulated in KS where severe periodontal disease occurs early in life. Figures 5.6 and 5.7 also show that there was a tendency for increased up-regulation of both IL-1α and IL-1β in kindlin-1 deficient keratinocytes when exposed to the native biofilm extract as compared to control cells. Again, this finding is consistent with an exacerbated inflammatory response to oral bacterial challenge with kindlin-1 deficiency.
TNFα expression in HGKs did not appear to be regulated by treatment with native oral biofilm extract. When HGKs were treated with heat-inactivated oral biofilm extract, expression levels of TNFα slightly decreased as compared to cells not treated with heat-inactivated biofilm extract, however this decrease was not statistically significant. Treatment of cells with live oral biofilm resulted in a 6-fold increase in TNFα expression for both control and K1-deficient cells, however this increase was not statistically significant. K1-deficiency did not affect TNFα expression. These findings are inconsistent with literature that demonstrates increased TNFα expression in response to microbial challenge (Cochran, 2008). There was a strong tendency for increased TNFα expression with the live oral biofilm. The difference in TNFα expression in response to the live biofilm and biofilm extract could be due to varying concentrations of bacterial products. It is possible that the live oral biofilm provided a greater concentration bacterial products and the concentration of these mediators in the extract was not enough to regulate the gene.

Expression of αvβ6 integrin is not normally expressed in epithelial cells but is found in these cells during wound healing (Hakkinen et al., 2004). Ghannad et al. (2008) observed that αvβ6 integrin was expressed in the junctional epithelium in healthy periodontium. This study found that αvβ6 integrin was down-regulated in tissues isolated from patients with chronic periodontal disease (Ghannad et al., 2008). Integrin β6 knockout in mice resulted in the development of chronic periodontal disease. Integrin αvβ6 is known to activate TGFβ-1, an anti-inflammatory mediator. It may be concluded that integrin αvβ6 is expressed in the junctional epithelium where it plays an important role in attachment of the gingival tissues to the tooth and resisting bacterial infection. Reduced expression levels of this integrin or impairment in its function can contribute
to the development of periodontal disease by compromising the periodontal attachment to the tooth, allowing bacteria to infiltrate the tissues or through decreased TGFβ-1 activation.

In this experiment, when keratinocytes were treated with native or heat-inactivated biofilm extract, β6 integrin expression was not affected. Interestingly, kindlin-1 deficient HGKs demonstrated significantly increased expression of β6 integrin both in the absence of biofilm extract and when treated with native biofilm extract (Figure 5.18). When cells were cultured with live oral biofilm, β6 integrin expression was not significantly regulated. Figure 5.33 shows increases in β6 integrin expression for both control and K1-deficient HGKs however this increase did not reach statistical significance.

Our *in vitro* findings conflict with the *in vivo* findings of Ghannad et al. (2008) where they showed down-regulation of β6 integrin in conditions of periodontal disease. Kindlin deficiency is associated with impaired integrin function, so expression levels of β6 integrin may be irrelevant to disease as it is assumed that the function is impaired in these cells. It is possible that in kindlin-1 deficient cells, β6 integrin expression was increased relative to controls in attempt to compensate for this impaired function.

Matrix metalloproteinases (MMPs) are produced by both infiltrating inflammatory cells (macrophages and neutrophils), as well as resident cells of the periodontium (epithelial cells and fibroblasts). MMP-1, -2, and -9 belong to a larger family of at least 16 matrix metalloproteinases, which are involved in the degradation and remodeling of extracellular matrix proteins (collagen, proteoglycans) in soft tissues and bone. ECM remodeling is an important
aspect in many developmental processes, including bone formation and angiogenesis, as well as in pathological situations including inflammatory periodontal disease (Birkedahl-Hansen et al., 1993). MMP-2 and MMP-9 (gelatinase B) are proteases involved in the degradation of type IV collagen, a key component of the basement membrane. Fibronectin type II domains are found in gelatinases and are critical sites which allow localization and binding to extracellular matrix components, especially gelatins (Allan et al., 1995).

MMPs, along with tissue inhibitors of metalloproteinases (TIMPs), control remodeling of connective tissues through a balance of degradation and remodeling. An imbalance between MMPs and TIMPs in favour of MMP activity leads to pathologic break down of extracellular matrix as occurs during periodontal disease. Studies have demonstrated that periodontal pathogens may regulate connective tissue destruction in periodontitis by stimulating cytokine production by host immune cells, including IL-1 and TNFα. These cytokines then stimulate MMP release from resident fibroblasts and keratinocytes and subsequent connective tissue degradation (Birkedal-Hansen et al., 1993). Both MMP-2 and MMP-9 have been shown to be elevated in tissues affected by periodontal disease (Mäkelä et al., 1994, Maeso et al., 2007).

MMP-1 expression was not found to be significant regulated in either control or K1-deficient human gingival keratinocytes when treated with native oral biofilm extract. There was a trend for MMP-1 expression to increase in K1-deficient cells treated with the biofilm extract, up to 1.8-fold, however this increase was not statistically significant. Further, there was a tendency for higher MMP-1 expression in kindlin-1 deficient cells when exposed to oral biofilm extract as compared to control cells, up to 2.7-fold (Figure 5.11). However, this difference between control
and K1-deficient cells in MMP-1 expression did not reach statistical significance. MMP-2 expression was found to be significantly increased in control cells exposed to both native and heat-inactivated oral biofilm extract as compared to control cells without biofilm extract (Figure 5.27). Although kindlin-1 deficient cells did demonstrate a slight increase in MMP-2 expression when exposed to native and heat-inactivated biofilm extract, this trend was non-significant. MMP-2 expression was not found to be significantly regulated by kindlin-1 deficiency either in the presence or absence of native biofilm extract. MMP-9 expression was increased significantly in both control and K1-deficient cells when treated with native biofilm extract (Figure 5.13). Heat-inactivation of the oral biofilm extract had no affect on MMP-9 expression in both control and K1-deficient cells as compared to cells not exposed to biofilm. Again, kindlin-1 deficiency had no affect on MMP-9 expression in the presence or absence of the native biofilm extract. Figure 5.28 illustrates a marked increase in MMP-9 expression in control cells as compared to K1-deficient cells with no biofilm extract. This increase of MMP-9 expression by 2.6-fold was not found to be significant, likely due to the high standard error observed for the K1-deficient cells not treated with biofilm. When control and K1-deficient HGKs were treated with live oral biofilm, only MMP-9 demonstrated significantly increased expression as compared to respective cells not treated with oral biofilm.

It was expected that levels of MMP-1, -2, and -9 would be elevated in HGKs exposed to both biofilm extract and live oral biofilm. Only MMP-9 expression was elevated in keratinocytes treated with native oral biofilm extract or live oral biofilm. MMP-2 expression was found to be significantly elevated in control cells exposed to native and heat-inactivated biofilm, but not in K1-deficient cells. Heat-inactivation of the biofilm extract denatures all heat-sensitive proteins,
leaving the heat stable lipopolysaccharide (LPS). Bacterial LPS is a potent stimulator of the host immune response to produce pro-inflammatory cytokines and factors involved in tissue destruction that ultimately lead to periodontal breakdown. Heat-inactivation of the oral biofilm extract may have resulted in other important factors required for activation of cell signaling pathways to produce MMPs. Further, it is possible that biofilm extract concentrations used in these experiments were not sufficient to result in increased expression levels of MMP-1 or MMP-2 in all cells. The live 3-week-old biofilm used in this study was not necessarily obtained from a patient with periodontal disease. Analysis of this biofilm for specific microbes was not carried out. This was a biofilm grown in anaerobic culture and it is known that anaerobic bacteria are associated with periodontal disease to a greater extent than aerobic bacteria. However, it is possible that key microbes, such as *P. gingivalis*, were not present in this biofilm in sufficient numbers to induce elevated expression of the MMPs.

Expression levels of TGFβ-1, and TGFβ-3, growth factors involved in inflammation and wound healing, were also investigated in human gingival keratinocytes both normal and deficient for kindlin-1. The TGFβ family is made up of three isoforms, TGFβ1-3. TGFβ growth factors play many important roles throughout life, including embryogenesis, growth and development of tissues, and wound repair. TGFβ-1 is expressed in many cells, however significant expression occurs in endothelial, hematopoietic and fibroblast cells (Halper, 2014). TGFβ-1 induces the proliferation of mesenchymal cells while inhibiting growth and activity of epithelial and endothelial cells (Halper, 2014). It stimulates production of collagen and development of the extracellular matrix through inhibiting expression of collagenases (MMPs) and up-regulating the production of tissue inhibitors of metalloproteinases (TIMPs) (Halper, 2014). Given the
prominent role of TGFβ in collagen synthesis and ECM production, it is a critical factor involved in tissue repair following injury. TGFβ-1 and TGFβ-3 are also anti-inflammatory mediators that are involved in down-regulating the host inflammatory response (Cardoso et al., 2008). We hypothesized that these factors may be down-regulated with kindlin-1 deficiency and that their low levels may contribute to an exacerbated inflammatory response and periodontal disease. Further, research by Rognoni et al. (2014) showed that kindlin-1 promotes TGFβ signaling and that in kindlin-1 deficient mice, TGFβ levels are lowered.

When both control and K1-deficient HGKs were exposed to a native oral biofilm extract, expression levels of TGFβ-1 were not significantly regulated. TGFβ-3 expression was found to be significant regulated by native biofilm extract; TGFβ-3 expression in both control and K1-deficient HGKs was down-regulated by 2-fold when cells were treated with oral biofilm extract \( (p<0.01) \); biofilm extract concentration had no effect on this down-regulation. Kindlin-1 deficiency in HGKs did not appear to affect either TGFβ-1 or TGFβ-3 expression both in the presence or absence of native oral biofilm extract. In the presence of heat-inactivated biofilm extract, TGFβ-3 expression was significantly elevated for kindlin-1 deficient HGKs as compared to K1-deficient cells not exposed to oral biofilm. This same trend was not observed for control cells. Treatment of both control and K1-deficient cells with live oral biofilm had no significant affect on TGFβ-1 or 3 expression.

These results are somewhat contradictory. The finding that TGFβ-3 was down-regulated by treatment with native oral biofilm extract is consistent with the role of TGFβ as an anti-inflammatory mediator; when HGKs were exposed to the biofilm extract, an up-regulation of
pro-inflammatory mediators likely occurred in defense to the microbial challenge while anti-inflammatory mediators were down-regulated. The finding that the heat-inactivated biofilm extract resulted in an increase in TGFβ-3 may be explained by a less potent biofilm extract due to denaturation of heat-sensitive proteins. This may have resulted in a more low-grade bacterial challenge to the HGKs with a response of repair.

Tenascin C (TN-C) is glycoprotein that is a component of the extracellular matrix and plays a role in the development of ECM. TN-C expression is increased in pathological situations including inflammation, wound healing, and cancer (Midwood and Orend, 2009; Midwood et al., 2011). The regulation of TN-C is controlled by various mediators that are expressed during tissue remodeling and repair including TGFβ-1, TNFα, IL-1, and keratinocyte growth factor (Rettig et al., 1989). Inflammatory mediators have been shown to induce TN-C expression, as well as mechanical stresses in bone, ligaments, and tendons (Sarasa-Renedo and Chiquet, 2005). Based on this information, we would expect to see TN-C levels elevated in periodontitis and as well in in vitro conditions simulating oral bacterial challenge. Further, TN-C plays a part in binding to the extracellular domains of integrins as opposed to kindlin-1, which binds integrin beta subunits inside the cell. We hypothesized that the lack of kindlin-1 and subsequent impairment of integrin function could also affect the binding of integrins to extracellular ligands, including tenascin C and fibronectin proteins. If integrins are unable to bind to extracellular ligands, expression levels of these ligands may be affected.

Expression of tenascin C was significantly elevated in control and K1-deficient cells when exposed to native biofilm extract. Relative expression was increased about 3-fold for control
cells and 4-fold for K1-deficient cells with biofilm extract as compared to with no biofilm extract. Treatment of cells with heat-inactivated biofilm extract had no effect on tenascin C expression. When control and K1-deficient HGKs were treated with live oral biofilm, expression of TN-C increased by about 1.4-fold, however this increase was not statistically significant. Kindlin-1 deficiency had no significant affect on TN-C expression. Keratinocytes exposed to native biofilm extract demonstrated increased expression of TN-C while no change in TN-C expression was observed to cells exposed to heat-inactivated biofilm. Heat-inactivation may have denatured proteins in the biofilm that contributed to the up-regulation of TN-C in the keratinocytes. It was expected that treatment of the HGKs with live oral biofilm would up-regulate expression of TN-C, similar to that observed for the native biofilm extract, however this was not observed. It is possible that the concentration of proteins in the live oral biofilm was not sufficient to up-regulate expression of TN-C in the in vitro environment.

Cellular fibronectin is another glycoprotein that makes up the extracellular matrix. Fibronectin is known to bind to integrins (Pankov and Yamada, 2002), along with other components of the ECM including collagen and fibrin. Fibronectin functions in cell adhesion, growth, migration, and differentiation, and also plays important roles in wound healing and embryonic development (Pankov and Yamada, 2002). Specifically, cellular fibronectin is highly expressed in the periodontium where it plays a critical role in maintaining periodontal attachment. Cellular fibronectin is secreted by a number of cells, however predominantly fibroblasts, as an insoluble protein and functions in attachment of cells to the ECM (Hynes, 1986; Wierzbicka-Patynowski and Schwarzbauer, 2003; Henderson et al., 2011).
Wu, et al (2013) found expression of fibronectin mRNA to be significantly decreased in patients with chronic periodontitis as compared to healthy controls. As kindlin-1 deficiency results in impaired integrin function, it is possible that extracellular integrin ligands, including fibronectin, would be affected.

Fibronectin ED-A expression was significantly up-regulated by native biofilm extract in K1-deficient HGKs, however not in control HGKs. Fibronectin ED-B was not significantly regulated by native biofilm extract. Heat-inactivated biofilm extract did not have a significant affect on ED-B or ED-A expression in K1-deficient or control HGKs. However, K1 deficiency was shown to have a significant affect on ED-B expression, decreasing expression by 2-fold, in the presence of heat-inactivated biofilm extract. A similar finding was also observed for cells exposed to native biofilm extract, but the difference was not significant. Also when looking at K1-deficient HGKs, treatment with either native or heat-inactivated biofilm extract was shown to decrease expression of fibronectin ED-B by about 2-fold when compared to K1 deficient HGKs not exposed to oral biofilm. Live biofilm treatment had no affect on ED-A expression in K1-deficient and control HGKs. However, for K1-deficient HGKs, treatment with live oral biofilm resulted in a 2-fold increase in ED-B fibronectin expression.

Interestingly, ED-A fibronectin expression was found to increase in response to native biofilm extract in K1-deficient HGKs by about 1.5-fold. ED-A fibronectin was increased by a factor of 1.3 in control keratinocytes when exposed to native biofilm extract, however this was not found to be significant. There is minimal difference between a 1.3- or 1.5-fold increase in gene expression so it cannot be confidently concluded that native biofilm extract significantly
regulated ED-A fibronectin expression in K1-deficient HGKs and not control HGKs. Figure 5.4 shows that control HGKs had about 2 times the ED-B fibronectin expression than K1-deficient HGKs, irrespective of native biofilm extract. This increased in expression was not found to be significant. However, the tendency for decreased expression in K1-deficient keratinocytes may be due to an increased inflammatory response in these cells. Kindlin-1, along with fibronectin, binds to integrins and is involved in cellular adhesion. Kindlin-1 deficiency may interfere with a pathway involved in fibronectin expression. ED-B fibronectin expression was also found to be decreased in K1-deficient cells when exposed to heat-inactivated biofilm extract by about 2.5-fold. However the corresponding control for this experiment did not show an effect of kindlin-1 deficiency on ED-B fibronectin expression (Figure 5.20). Contradictory to the previous results of this study with heat-inactivated biofilm extract, live oral biofilm was found to significantly up-regulate ED-B fibronectin expression only in K1-deficient cells. It is possible that the live oral biofilm presented a stronger microbial challenge to the keratinocytes which resulted in up-regulation of the fibronectin, much like in conditions of injury and wound repair than when the biofilm extract was used.

Overall, this study had several limitations. One of the limitations of this study was the in vitro design where many of the factors present in the gingival sulcus are absent. For example, many different cells work together in the periodontal tissues to defend against microbial challenge, including fibroblasts, macrophages, neutrophils, plasma cells, and endothelial cells. These cells each secrete their own unique array of mediators and enzymes that act on other cells to create a specific host response depending on the nature of the bacterial challenge. This complex
interplay between cells cannot be recreated in a laboratory setting and therefore our results may not reflect the true behavior of keratinocytes in their \textit{in vivo} environment.

The bacterial biofilm used in this study was not obtained from conditions of periodontal disease. Analysis of the biofilm was not carried out to identify specific bacterial species, so it is unknown if key periodontal pathogens were present. However, the biofilm used was cultured in anaerobic conditions and it is established that most microbes associated with periodontal disease are anaerobic. It is also possible that the bacterial concentrations used to treat the keratinocytes did not reach concentrations comparable to what would occur in \textit{in vivo} periodontal conditions.

As mentioned above, heat-inactivation of the biofilm extract denatures heat-sensitive proteins. Only heat-stable proteins, such as LPS would be present. It would then make sense that in some cases heat-inactivation of the biofilm extract may have a different affect on the keratinocytes than native biofilm extract.

HGKs were exposed to the biofilm extract and live biofilm for different amounts of time. Cells were treated with biofilm extract for a period of 24 hours while this was reduced to 5 hours for the live biofilm. This difference in incubation time may have accounted for differences in results seen between the two experiments. Regulation of cellular pathways controlling gene expression may have responded after at different times for various genes. Five hours of biofilm challenge may have been insufficient time for cellular pathways controlling expression of certain genes to be activated, or mRNA levels may not have reflected this change yet.
Chapter 7: Conclusion

In this experiment, kindlin-1 protein was successfully down-regulated in human gingival keratinocyte immortalized cell line. These kindlin-1 human gingival keratinocytes served as an in vitro surrogate for keratinocytes of the periodontal tissues in patients with Kindler Syndrome. Kindlin-1 deficiency in gingival keratinocytes was found to have a significant effect on the regulation of genes encoding for β6 integrin, fibronectin ED-B, MMP-2, IL-1α, and IL-1β. β6 integrin expression was increased in K1-deficient cells, while MMP-2 and fibronectin ED-B expression were decreased in K1-deficient cells. The pro-inflammatory cytokines IL-1α and IL-1β were significantly up-regulated in K1-deficient cells as compared to control cells.

IL-1α and IL-1β were up-regulated in both K1-deficient and normal keratinocytes exposed to native oral biofilm extract, and in normal keratinocytes exposed to heat-inactivated biofilm. Only IL-1β was up-regulated in K1-deficient cells exposed to heat-inactivated biofilm extract and in keratinocytes exposed to live biofilm. Expression levels of β6 integrin was also found to increase in K1-deficient cells exposed to native biofilm extract relative to control K1-deficient cells.

MMP-2, MMP-9, IL-8, tenascin C, ED-B fibronectin, TGFβ3 were differentially regulated by the presence of biofilm extract or live biofilm in the gingival keratinocytes. These results are consistent with past literature that demonstrates upregulation of MMP-2, and -9 in conditions of periodontal microbial challenge. Other studies point towards up- or down-regulation of IL-8 depending on the microbes involved, which is also consistent with the varying results of this
experiment. Expression of tenasin C was found to increase with biofilm treatment, although this was only significant for the live biofilm.

The overall aim of this study was to investigate the effects of kindlin-1 deficiency on regulation of the genes that mediate the periodontal inflammatory response as well as those involved with tissue repair and healing. Results indicate that pro-inflammatory mediators IL-1α and IL-1β may be up-regulated in kindlin-1 deficient keratinocytes and this up-regulation may be enhanced in conditions of biofilm challenge. Up-regulation of β6 integrin may also occur in kindlin-1 deficient cells both in the presence and absence of biofilm challenge and this may reflect a compensatory mechanism by the cell as kindlin-1 deficiency results in impaired β6 integrin function.

Our findings suggest that the severe and early-onset periodontal disease found in patients with Kindler Syndrome may be a result of enhanced expression of certain pro-inflammatory cytokines as compared to controls. Further investigation is required to confirm these results and explore the affects of kindlin-1 deficiency on other gene products that may be involved in the initiation and progression of periodontal disease.
References


## Appendices

### Appendix A  Primers used for RT-PCR

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