PATHOPHYSIOLOGY OF THE CYCLICAL EPIDERMOLOYTIC PALMOPLANTAR KERATODERMA (EPPK) IN THE KERATIN 9 MOUSE MODEL

By

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ABSTRACT

Keratin 9 (KRT9/Krt9) is a type I intermediate filament protein that is constitutively expressed in the suprabasal layer of the thicker and specialized epidermis of the palmoplantar skin. Mutations at the KRT9/Krt9 locus cause epidermolytic palmoplantar keratoderma (EPPK), a rare autosomal dominant disorder characterized by diffuse palmoplantar keratoderma. Krt9−/− mice exhibit hyperpigmented calluses on the major stress-bearing footpads that form, progress, and slough off in a precise cyclical fashion (Fu et al., 2014), and thus offer a useful model to study the pathophysiology of EPPK related to KRT9/Krt9 mutations. Previously, our laboratory has shown that oxidative stress associated with hypoactive NRF2, a transcription factor that regulates the cellular stress response, precedes the development of lesions in Krt16−/− mice, a model of non-epidermolytic palmoplantar keratoderma (NEPPK) (Kerns et al., 2016). The finding of increased oxidative stress associated with impairment of NRF2 activity in a model of NEPPK raised the possibility of similar alterations in EPPK. Here, we conducted an in-depth analysis of lesion progression in the Krt9−/− mouse model that expanded the work of Fu et al. and enabled us to pinpoint critical time points to assess the status of NRF2 signaling and NRF2-mediated stress-related proteins. Biochemical and histological analysis of pre-lesional paw skin revealed normal NRF2 activity and redox status. Moreover, treatment with an NRF2 inducer failed to impact lesion formation in Krt9−/− mice. These findings suggest that unlike NEPPK, oxidative stress and hypoactivity of NRF2 may not be a major driving force in EPPK. Thus, although EPPK and NEPPK share the common
feature of palmoplantar keratoderma, distinct molecular mechanisms underlie each disorder.

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INTRODUCTION

Keratins belong to the superfamily of intermediate filament (IF) proteins

Intermediate filaments are cytoskeletal elements composed of related proteins that provide mechanical strength for cells and protect tissues against mechanical force and other forms of stress (Chung, Rotty, & Coulombe, 2013; Fuchs & Weber, 1994; Fuchs & Cleveland, 1998; Herrmann, Hesse, Reichenzeller, Aebi, & Magin, 2003; Herrmann, Strelkov, Burkhard, & Aebi, 2009; Koster, Weitz, Goldman, Aebi, & Herrmann, 2015; Omary, Coulombe, & McLean, 2004). Seventy genes encode IF proteins in the human genome, and their DNA sequences and expression patterns are evolutionarily conserved. Although IF genes and proteins share similar traits in their sequence feature and basic properties, their tightly regulated expression and posttranslational modifications make them cell-type-specific (Fuchs & Weber, 1994; Fuchs, 1994; Herrmann & Aebi, 2016; Homberg & Magin, 2014; Omary et al., 2004; Szeverenyi et al., 2008).

Keratins belong to the superfamily of IF proteins (Schweizer et al., 2006). There are two types of keratins: type I and type II (Figure 1). Type I keratins (40-60 kDa) number 28 and generally are acidic proteins. Type II keratins (50-70 kDa) number 26 and tend to be neutral to basic proteins. Keratins form obligatory heteropolymers, meaning that type I keratin interacts with type II keratin to form a heterodimer heterodimer (Chung et al., 2013; Fuchs & Weber,
1994; Hatzfeld & Franke, 1985; Hatzfeld, Maier, & Franke, 1987; Herrmann & Aebi, 2016; R. Moll, Franke, Schiller, Geiger, & Krepler, 1982). Since dimers generated from different combinations of keratins have distinct physical properties, it is widely believed that they can adapt to tissue-specific structural requirements regarding tensile strength, flexibility, and dynamics.

Figure 1. Two types of keratins in the intermediate filament family.
From (Wang, Zieman, & Coulombe, 2016).

The tripartite structure of keratins

Similar to all intermediate filament proteins, keratin proteins exhibit a tripartite domain organization (Figure 2). The central $\alpha$-helical rod domain, a highly-conserved region with ~310 amino acids, is flanked by non $\alpha$-helical amino (N-) head and carboxy (C-) tail domains (Bader, Magin, Hatzfeld, & Franke, 1986; Fuchs, 1995; Geisler & Weber, 1982; Hanukoglu & Fuchs, 1983; Herrmann & Aebi, 2016; Homberg & Magin, 2014). The $\alpha$-helical rod domain
consists of four coiled coil-forming segments (1A, 1B, 2A, and 2B) that are linked by three non α-helical short sequences (L1, L1-2, and L2). Interestingly, there are sequence motifs (15~20 amino acids) at the start of the 1A segment and at the end of the 2B segment that have been highly conserved across IF proteins throughout evolution (Wu et al., 2000). Mutations in both of these regions of the central rod domain can cause devastating results, including impairment of keratin assembly, organization, and dynamics, conveying their critical role for keratin intermediate filaments (KIFs) (Fuchs, 1995; Herrmann et al., 2009; Herrmann & Aebi, 2016; Homberg & Magin, 2014; Kim & Coulombe, 2007; Omary et al., 2004; Parry, Strelkov, Burkhard, Aebi, & Herrmann, 2007; Wagner et al., 2007).

The non α-helical domains of head and tail, in contrast to the rod domain, are variable. In general, these regions are comprised of three subdomains: high homology (H), special variability (V), and highly charged termini (E). Interestingly, compared to type II keratins (50 ~ 70 kDa), type I keratins (40 ~ 60 kDa) have shorter H sequences, due to the loss of the H2 region near the end of the 2B region and the shorter H1 sequence. However, only type I keratins contain two caspase (Casp) cleavage sites, and Casp has shown to cleave keratin proteins (i.e., K18) during apoptosis (Chung et al., 2013; Fuchs & Weber, 1994; Fuchs, 1995; Homberg & Magin, 2014; Leers et al., 1999; Omary et al., 2004; Schutte et al., 2009).

Although the tripartite domain structure largely defines the physical and chemical properties of keratin proteins, their post-translation modification, e.g., phosphorylation, O-linked N-acetylglucosamine glycosylation (O-GlcNAC),
sumoylation, ubiquitination, transglutamination and others, allows them to play roles in cell signaling and redox balance under stress (Fuchs, 1995; Homberg & Magin, 2014; Omary et al., 2004; Snider & Omary, 2014). For example, keratins 8 and 18 (K8 and K18) account for the keratin IF network in hepatocytes and can be site-specifically and rapidly phosphorylated during metabolic stress and detoxification responses. Phosphorylated forms of K8 and K18 become capable of interacting with stress proteins, including Mrj, Hsp 40, Hsp70, PKCε, and c-Jun (Akita et al., 2007; Coulombe & Wong, 2004; Guldiken et al., 2015; Homberg & Magin, 2014; Izawa et al., 2000; Liao, Lowthert, Ghori, & Omary, 1995; Omary et al., 1992; Song, Cho, Yoo, Park, & Lee, 1998).

Figure 2. The tripartite domain structure of type I and type II keratins.

The α-helical rod domains in both types of keratin consist of four coiled coil-forming segments (1A, 1B, 2A, and 2B) that are linked by three non α-helical short sequences (L1, L1-2, and L2). The non α-helical head (N-) and tail (C-) domains comprise of high homology (H), special variability (V), and highly charged termini sequences. Interestingly, there is no H2 in the type I keratin. From (Homberg & Magin, 2014).
Differentiation-specific expression of keratins in epidermis

In the human genome, the genes coding for type I and type II keratins are located in two separate clusters on chromosomes 12q13 and 17q21.2 (Arin, 2009; Corden & McLean, 1996; Fuchs et al., 1987; Fuchs, 1995; Herrmann & Aebi, 2016; McLean & Moore, 2011; R. Moll, Divo, & Langbein, 2008; Schweizer et al., 2006). Moreover, of these 54 functional keratin genes, 17 code for cysteine-rich "hard" (trichocyctic) keratins, and the remaining 37 genes are expressed in "soft" epithelia (liver, kidney, skin, etc.). Hard keratins, in particular, are restricted to hair, nail, lingual papilla and the thymic reticulum (Coulombe & Omary, 2002; Heid, Moll, & Franke, 1988a; Heid, Moll, & Franke, 1988b; Herrmann & Aebi, 2016; I. Moll, Heid, Franke, & Moll, 1988).

Keratin gene expression in the epidermis is tightly regulated in a differentiation-specific and thus spatially-defined manner (Figure 3). There are four layers within the epidermis: basal layer, spinous layer, granular layer, and stratum corneum. The stratum corneum is composed of corneocytes, which are terminally differentiated and enucleated keratinocytes that are embedded in a hydrophobic matrix. The stratum corneum layer provides a barrier that protects the epidermis from water loss, chemical irritation, and infection by various pathogens (Eckhart, Lippens, Tschachler, & Declercq, 2013; Fuchs, 1995; Homberg & Magin, 2014; Lippens, Denecker, Ovaere, Vandenabeele, & Declercq, 2005; Lopez-Pajares, Yan, Zarnegar, Jameson, & Khavari, 2013; R. Moll et al., 2008; Sandilands, Sutherland, Irvine, & McLean, 2009; Wang et al., 2016). Basal keratinocytes are mitotically-active and relatively undifferentiated
cells, and they express K5 (Type II) and K14 (Type I). As basal keratinocytes differentiate, they migrate towards the skin’s surface and undergo a progressive transformation associated with the expression of distinct keratin pairs and, of course, several other types of proteins. Spinous layer keratinocytes express K1 (Type II) and K10 (Type I), whereas granular layer keratinocytes express K2e (Type II).

This basic blueprint for keratin gene expression in epidermis varies according to determinants such as body site and homeostatic status. For instance, the type I K9 occurs exclusively in the differentiating layers of the epidermis of glabrous skin, e.g., the spinous layer.

When the epidermis is damaged, wound-proximal epithelial cells express the wound-inducible keratins K6 type II, and K16, and K17 (type I) (Cai et al., 2006; Coulombe & Wong, 2004; Kim, Wong, & Coulombe, 2006; Kim & Coulombe, 2007; Lippens et al., 2005; Lopez-Pajares et al., 2013; Mazzalupo, Wong, Martin, & Coulombe, 2003; Pan, Hobbs, & Coulombe, 2013; Sandilands et al., 2009; Wang et al., 2016; H. H. Zhang et al., 2009).
Mutations in \textit{KRT9} cause epidermolytic palmoplantar keratoderma (EPPK)

Epidermolytic palmoplantar keratoderma (EPPK, OMIM: 144200) is an autosomal dominant skin disorder caused by mutations in the \textit{KRT9} gene (Fu et al., 2014; Leslie Pedrioli et al., 2012; Leslie Pedrioli et al., 2012; McLean & Moore, 2011; Reis, Kuster, Eckardt, & Sperling, 1992). Keratin 9 (K9) is a type I IF protein that is specifically expressed in the suprabasal layers of the palmoplantar epidermis in humans (Knapp et al., 1986; R. Moll et al., 2008; Swensson et al., 1998). EPPK is characterized by acanthosis (diffuse skin thickening) of the palms and soles that has erythematous borders and develops during the first weeks or months after birth (\textbf{Figure 4 A and B}; Braun-Falco, 2009; Hamm, Happle, Butterfass, & Traupe, 1988; Kuster, Reis, & Hennies, 2002a; Lopez-Valdez, Rivera-Vega, Gonzalez-Huerta, Cazarin, & Cuevas-
Covarrubias, 2013). It was first described by Vörner in 1901, and its current incidence is estimated to be 2.2 to 4.4 cases per 100,000 newborns in different populations (Braun-Falco, 2009). EPPK cases have been reported in Britain, China, Germany, Japan, Korea, Mexico, Pakistan, Spain, and Taiwan (Ke et al., 2014; Requena, Schoendorff, & Sanchez Yus, 1991; Rugg et al., 2002; Sakabe, Nakamura, & Tokura, 2009; Shimomura, Wajid, Weiser, Kraemer, & Christiano, 2010; Torchard et al., 1994; Wevers, Kuhn, & Mahrle, 1991; Yang, Lee, Lin, & Chao, 2003). The histological features of EPPK include perinuclear vacuolization of keratinocytes and suprabasal epidermolytic hyperkeratosis in the palmoplantar skin (Figure 4D; Carvajal-Huerta, 1998; Mochida, Rivkin, Gil, & Kierszenbaum, 2000; Shimazu et al., 2006; Terrinoni et al., 2004; Tsunemi et al., 2002). There is no cure for EPPK, but current treatment strategies include topical calcipotriol, oral retinoids, and surgical reconstruction with skin graft (Happle, van de Kerkhof, & Traupe, 1987; Lucker, van de Kerkhof, & Steijlen, 1994; Tropet, Zultak, Blanc, Laurent, & Vichard, 1989). Interestingly, Lucker et al. reported that EPPK patients treated with topical calcipotriol (50 µg/g) and urea (40 µg/g) helped improve their tactile sensitivity due to an unknown mechanism. In contrast to the debilitating pain in pachynonychia congenital (PC), non-epidermolytic palmoplantar keratoderma, EPPK patients have mild pain at affected sites (Goldberg, Sprecher, Schwartz, & Gaitini, 2013; Kuster, Reis, & Hennies, 2002b). However, physicians notice that their EPPK patients with frequent mechanical insults on their palms and soles can develop increasing sensitivity to mechanical stress (Rothnagel et al., 1995).
In mice, *Krt9* expression is concentrated in footpads, which are equivalent to the thick skin of the human foot (Fu et al., 2014; Schweizer, Baust, & Winter, 1989). The restriction of *KRT9/Krt9* expression to palms and soles in both humans and mice suggests that K9 protein plays a crucial role in not only locally supporting palmoplantar epidermis against the highest degree of mechanical stress, but also mechanically reinforcing the cellular and structural integrity in this specific anatomical site via the promotion of keratin filament bundling (Fu et al., 2014; Schweizer et al., 1989; Swensson et al., 1998). Fu et al. reported that ablation of the *Krt9* gene in mice caused paw lesions characterized by hyperpigmentation, acanthosis, and hyperkeratosis occurring in the third week after birth. Hyperpigmented callouses intensified for three weeks, and then they sloughed off. Under scanning electron microscopy, Fu et al. observed cytolysis-associated splits within the spinous layer in the *Krt9*−/− paw tissue. Fu et al. (2013) also indicated that the loss of *Krt9* triggered abnormally high proliferation in basal keratinocytes and rapid transition toward terminal differentiation. The absence of *Krt9* resulted in the upregulation of the wound-inducible keratins, *Krt6* and *Krt16*, which are markedly upregulated in mouse EPPK (Fu et al., 2014). The *Krt9*−/− mouse model offers a unique opportunity to explore the function of *Krt9* as well as the contributing factors underlying the formation of EPPK.
Lessons from studies on non-epidermolytic palmoplantar keratoderma (NEPPK)

Recent progress in the study of a non-epidermolytic form of palmoplantar keratoderma (NEPPK) associated with a distinct keratin-based skin disorder, pachyonychia congenita, may shed some light on the pathogenesis of EPPK. Pachyonychia congenita (PC) is caused by mutations in the genes encoding for the so-called wound-inducible keratins, KRT16, KRT17, and KRT6. Like EPPK, NEPPK is also typified by palmoplantar keratoderma (PPK); however, NEPPK-
associated PPK can be diffuse, striate, or focal and lacks an erythematous border. As its name suggests, NEPPK lesions do not exhibit lysis of the palmoplantar epidermis. Also, compared to EPPK, the PPK associated with PC is accompanied by excruciating pain ((Das, Kumar, & Das, 2013; Kelsell et al., 1995; Kelsell et al., 1999; Kroigard et al., 2016; Plassais et al., 2015; Shamsher et al., 1995). The development of a Krt16−/− mouse model that phenotypically and molecularly mimics the PPK lesions in PC patients has fueled substantial progress towards understanding the role of K16 in epidermal homeostasis and the mechanisms underlying NEPPK formation. Lessard et al. (2013) showed that K16 plays a role in regulating the innate immunity and inflammatory response in the human and murine skin. In particular, ablation of Krt16 in murine paw skin resulted in the upregulation of damage-associated molecular patterns (DAMPs), which subsequently contributed to PPK lesion development (326 Lessard, J.C. 2013; 328 Hobbs, R.P. 2012;).)

Kerns et al. (2016) subsequently found that PPK lesion formation in the Krt16−/− mouse model was preceded by increased levels of oxidative stress associated with hypoactivity of the NRF2 signaling pathway, a key regulator of the cellular stress response. NRF2 (nuclear factor erythroid 2-related factor 2) is a transcription factor that falls into the "cap-n-collar" (CNC) subgroup of the basic region-leucine zipper-family (Pitoniak & Bohmann, 2015). Under normal conditions, NRF2 is bound to the cytoplasmic inhibitor "KEAP1", which promotes its ubiquitination and proteosomal degradation (Katsuoka & Yamamoto, 2016; A. Kobayashi et al., 2004; Suzuki & Yamamoto, 2015). Under stress conditions,
KEAP1 is modified, leading to release, stabilization, and phosphorylation of NRF2. The latter allows for translocation of NRF2 into the nucleus where it can activate the transcription of cellular stress genes containing the antioxidant response element (ARE) (Figure 5A). Remarkably, topical application of the NRF2 inducer sulforaphane prevents the formation of PPK lesions in \( K16^{--} \) male mice. Furthermore, analysis of PPK biopsies obtained from individuals with PC confirmed the hypoactive status of NRF2 in lesional skin (Kerns et al., 2016).

The genes targeted by the NRF2 transcription factor encode proteins that are vital to the cellular stress response, including enzymes regulating the synthesis and regeneration of glutathione (GSH), the most abundant endogenous antioxidant in the cell (Figure 5B). GSH protects the cell from reactive oxygen species (ROS) through a reaction in which reduced GSH is converted to its oxidized form (GSSG), with concurrent reduction of cellular substrates. In \( Krt16^{--} \) murine paw skin, hypoactive NRF2 is associated with a specific impairment of GSH synthesis. In addition to the regulation of GSH homeostasis, NRF2 also modulates a myriad of other related cellular stress pathways, including the pentose phosphate pathway (PPP)/NADPH production, quinone detoxification, and direct ROS clearance (Itoh, Mimura, & Yamamoto, 2010; Kerns et al., 2016; M. Kobayashi & Yamamoto, 2006; Maher & Yamamoto, 2010; Mitsuishi, Motohashi, & Yamamoto, 2012; Schafer & Werner, 2015; Suzuki, Motohashi, & Yamamoto, 2013; Suzuki & Yamamoto, 2015; Taguchi, Motohashi, & Yamamoto, 2011; Vriend & Reiter, 2015). These findings of increased oxidative stress associated with impairments of NRF2 activity in the
Krt16 null mouse model of NEPPK raised the possibility of similar alterations in EPPK. This is the hypothesis to be tested in my thesis.

Figure 5. Schematic of NRF2 signaling pathway and NRF2-mediated protection.

(A) NRF2 is regulated by KEAP1. Under normal condition, NRF2 binds to KEAP1 and subsequently undergoes NRF2 degradation. However, under conditions associated with oxidative stress, NRF2 releases from KEAP1 and becomes phosphorylated. Phosphorylated forms of NRF2 translocate into the nucleus and activate the genes containing antioxidant response elements (AREs) or electrophile responsive element (EpRE). (B) NRF2 can activate several mechanisms to protect the body, including direct ROS clearance, quinone detoxification, cytoprotection, NADPH production, and GSH homeostasis. From (Kobayashi, Suzuki, & Yamamoto, 2013) and (Haslam et al., 2017).
METHODS

Animals and antibodies

*Krt9<sup>-/-</sup>* mouse line in the C57BL/6 strain background were obtained from Dr. Irvin McLean's laboratory at the University of Dundee. They were maintained under specific pathogen-free conditions with ad libitum access to food chow and water supply. Genotyping was performed using *Krt9*-specific oligonucleotide primers and the polymerase chain reaction (PCR) as described (Fu et al., 2014).

The protocol to generate K16 has been described (Bernot, Coulombe, & McGowan, 2002). Commercial antibodies used are listed below:

<table>
<thead>
<tr>
<th>Company</th>
<th>Antibody</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Santa Cruz Biotechnology</td>
<td>NRF2</td>
<td>sc-13-32</td>
</tr>
<tr>
<td></td>
<td>γ-GCSc</td>
<td>sc-28965</td>
</tr>
<tr>
<td>Bioss</td>
<td>NRF2-P</td>
<td>bs-2013R</td>
</tr>
</tbody>
</table>

The secondary antibodies were Alexa Fluor-conjugated goat anti-rabbits and goat anti-mouse, purchased from Life Technologies.

Mouse studies

Macroscopic analysis of PPK-like lesions spontaneously arising in footpads of *Krt9<sup>-/-</sup>* mice entailed photographing male *Krt9<sup>-/-</sup>* mouse paws once a week for ten weeks. *WT* and *Krt9<sup>-/-</sup>* mice were harvested at three-time points: 1.5 weeks of age (clinically pre-lesional), 3.5 weeks of age (clinically lesional), and six weeks of age (clinically post-lesional) (see “Results” section for an explanation).
**Sulforaphane (SF) treatments**

Two-day-old *Krt9^-/-* or WT mice were treated with either 100 µl of 1 µmole SF or jojoba oil vehicle control on their front paws twice weekly for four weeks (Kerns et al., 2016).

**Biochemical and morphological analyses**

A glutathione fluorometric assay (BioVision) was used to measure the levels of GSH and GSSG. Using TRIzol reagent and protocol (Life Technologies) together with the NucleoSpin Kit (Macherey-Nagel), total RNA was extracted from male footpad skin tissue and treated with DNase (RNAse free DNase kit: Macherey-Nagel). RNA concentration was measured by spectrophotometry (IMPLEN P330 spectrophotometer) (Hobbs et al., 2015; Kartha, Trotier, Kreuz, & Huber, Kerns et al., 2016). Using reverse transcriptase from the iScript cDNA synthesis kit (Bio-Rad) and following the iScript protocol, 1µg of the purified RNA sample was converted to cDNA. Quantitative RT-PCR was performed as described (Hobbs et al., 2015; Kerns et al., 2016). The target-specific oligonucleotide primers used in experiments are listed below:

<table>
<thead>
<tr>
<th>Control targets:</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actin</em></td>
<td>5’-TGG-AAT-CCT-GTG-GCA-TCC-ATG-AAA-C-3’</td>
<td>5’-TAA-AAC-GCA-GCT-CAG-TAA-CAG-TCC-G-3’</td>
</tr>
<tr>
<td><em>18S</em></td>
<td>5’-CCT-GTG-CCT-TCC-TTG-GA-3’</td>
<td>5’-CAT-TCG-AAC-GTC-TGC-CCT-ATC-3’</td>
</tr>
</tbody>
</table>
Experimental targets:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krt16</td>
<td>5'-GGT-GGC-CTC-TAA-CAG-TGA-TCT-3'</td>
<td>5'-TGC-ATA-CAG-TAT-CTG-CCT-TTG-G-3'</td>
</tr>
<tr>
<td>NRF2</td>
<td>5'-TCT-CCT-CCGC-TGG-AAA-AAG-AA-3'</td>
<td>5'-AAT-GAG-CTG-GCT-GTG-CTT-TA-3'</td>
</tr>
<tr>
<td>GS</td>
<td>5'-CAA-AGC-AGC-TAG-ACA-GGG-3'</td>
<td>5'-AAA-AGC-GTG-AAT-GGG-GCA-TAC-3'</td>
</tr>
<tr>
<td>GCLC</td>
<td>5'-ACA-TCT-ACC-ACG-CAG-TCA-AGG-ACC-3'</td>
<td>5'-CTC-AAG-AAC-ATC-GCC-TCC-ATT-CAG-3'</td>
</tr>
<tr>
<td>G6PD</td>
<td>5'-ACC-ATC-TGG-TGG-CTG-TTC-C-3'</td>
<td>5'-CAT-TCA-TGT-GGC-TGT-TGA-AGG-3'</td>
</tr>
<tr>
<td>GSR</td>
<td>5'-AGA-TGT-CGA-TTG-CCT-GCT-CTG-3'</td>
<td>5'-CCT-ATT-GTC-GAA-GTC-GGC-CTT-3'</td>
</tr>
</tbody>
</table>

For histological analysis, surgically-harvested mouse paw tissues were embedded in optimal cutting temperature compound (OCT, Sakura Finetek), flash-frozen in liquid nitrogen, and stored at -20° upon sectioning. Sections of 6μm were cut longitudinally in a precise and consistent orientation relative to paw anatomy and stained with either Hematoxylin & Eosin (H&E) or Fontana & Masson (F&M) for routine histopathology or subjected to indirect immunofluorescence. For indirect immunofluorescence staining, sections were rehydrated using PBS (pH 7.4) and washed three times for 5 minutes each at room temperature. After these washing steps, samples were incubated with 5%
normal goat serum (a non-specific blocking agent) for 30 minutes at room temperature and then incubated with primary antibodies overnight at 4°C. The primary antibodies were diluted in 2.5% NGS to reach antibody-specific desired dilutions. The primary antibody to NRF2 (H-300) was diluted 1:200 in PBS. The primary antibody to NRF2 (Ser40) was diluted 1:100, the primary antibody to GCSc (H-300) was diluted 1:250, and the antibody to mouse K16 was diluted 1:500 in PBS. The following day, the specimens were washed with PBS three times for five minutes each. The samples were then incubated with Alex-Fluor-conjugated goal antibody for one hour at room temperature. Then, specimens were incubated with DAPI stain (nuclear marker) for five minutes. DAPI was diluted with PBS in 1:1000. The specimens were washed with 0.5% TBST twice for ten minutes. The final stage was to use Calbiochem Fluro-Save Reagent solution to mount the samples and stored them at 4°C. A Zeiss fluorescent microscope with an Apotome attachment was used to visualize the samples.

Statistics

Unpaired 2-tailed Student’s t test was performed when appropriate. The statistical analyses involved calculations of $P$ values and SEM. The results of mRNA measurement and GSH assays were presented in mean ± SEM. Asterisks above graphic bars represent significant differences between two groups. F&M staining samples were quantified by ImageJ (NIH).
Study approval

All mouse studies were granted approval by the Johns Hopkins University Institutional Animal Care and Use Committee, and the approval number is MO16H264.
RESULTS

Lesion onset and progression in the *Krt9*<sup>−/−</sup> mouse model

Previously, Fu et al. reported that adult *Krt9*<sup>−/−</sup> mice begin to develop hyper-pigmented calluses on the stress-bearing forepaw footpads between the ages of three and four weeks old (Fu et al., 2014). Aiming to expand their effort and, in particular, determine when the lesions first appear in footpad skin, we conducted an in-depth clinical assessment and histological analysis of the timeline of lesion development in *Krt9*<sup>−/−</sup> mice (N=6).
Clinical assessment

At 1.5 weeks of age, the paws of the Krt9\(^{-/-}\) mice appear normal with no lesions detected by visual inspection (Figure 6). Hyperpigmented calluses are first seen shortly thereafter, at two weeks of age in the Krt9\(^{-/-}\) mice. This trait is fully penetrant and is never seen in littermate Krt9\(^{+/-}\) or Krt9\(^{++/+}\) mice. Calluses on the stress-bearing footpads continue to expand and thicken in the Krt9\(^{-/-}\) mice, with an intensification of pigmentation, until four weeks of age. From 5 to 6 weeks of age, the lesions slough off resulting in normal appearing footpads. At seven weeks of age, there is a reoccurrence of hyperpigmentation which marks the beginning of another cycle of lesion onset, progression, and resolution. Interestingly, in our experiments, the PPK lesion development is evident one week earlier than the observation from Fu’s report, and is associated with histologically disrupted palmoplantar epidermis that is evident before the PPK lesion becomes visible. The sloughing-off process in our findings has more visible outcomes during the stage of resolution of hyperpigmentation (between the fifth and sixth weeks), compared to Fu’s macroscopic observation on the Krt9\(^{-/-}\) mouse model (Fu et al., 2014).
Figure 6. The timeline of the cyclical epidermolytic palmoplantar keratoderma (EPPK) in the *Krt9*<sup>−/−</sup> mouse model, as seen by the author.

The hyperpigmentation begins to develop on the stress-bearing footpads at the age of two weeks (Week 2). The pigmentation intensifies in the following three weeks (between Week 2 and Week 4). At the age of Week 5, the sloughing-off process starts and continue to normalize the palmoplantar skin until Week 6, which we can barely detect the PPK lesion. However, the hyperpigmentation on the *Krt9*<sup>−/−</sup> mouse paw tissue is observed again at Week 7.
**Histological analysis**

Despite the lack of a clinically apparent lesion in *Krt9−/−* mice at 1.5 weeks of age, histological analysis revealed thickening and disorganization of the footpad epidermis in comparison to *WT* controls (**Figure 7**). Additionally, fissures could clearly be seen in the suprabasal layers of epidermis. What the fissures later reflect tissue fragility is unclear at this time. Thus, even though we cannot detect macroscopic abnormalities in the footpad of the *Krt9−/−* mouse at 1.5 weeks of age, significant subclinical histological changes are occurring and likely contribute to lesion formation.
Figure 7. The histology of palmoplantar epidermis at the age of 1.5 weeks.

(A) H&E staining of footpad skin for male WT mouse. The suprabasal layers are in an integral shape without disruption. (B) H&E staining of footpad skin for male Krt9⁻/⁻ mouse at the age of 1.5 weeks. There is hyperkeratosis on the top of the epidermis with a disrupted epidermis and several fissures. E, epidermis; H, hyperkeratosis; SG, sweat glands.
At 3.5 weeks of age, hyperpigmented calluses are present on the stress-bearing footpads of Krt9⁻/⁻ mice. In comparison to WT control skin, there is substantial thickening of the epidermis, particularly the stratum corneum. Fissures in the epidermis are once again noted. Moreover, there are pockets of dark blue staining that are suggestive of aggregated keratin material, but confirming so would require further investigation (e.g., scanning electron microscopy) (Figure 8).
Figure 8. The histology of palmoplantar epidermis at the age of 3.5 weeks.

(A) H&E staining of footpad skin for male WT mouse at the age of 3.5 weeks. The WT mouse does not exhibit any disruption of the suprabasal layers, and there is not visible aggregation in the epidermis. (B) H&E staining of footpad skin for male Krt9−/− mouse at the age of 3.5 weeks. There is a thicker epidermis with more substantial hyperkeratosis on the top of the palmoplantar tissue. E, epidermis; H, hyperkeratosis. SG, sweat glands.
Remarkably, and based on visual inspection, the hyperpigmented calluses we have seen on stress-bearing footpads disappear by 6 weeks of age in Krt9⁻/⁻ mice. This phenomenon was not noted in the study by Fu et al. (2013). Histologically, there is less hyperkeratosis in the palmoplantar epidermis of Krt9⁻/⁻ paw skin when it is compared with that at earlier ages; however, there are still substantial differences when the paw skin is compared with that of WT controls. Perinuclear vacuolization of keratinocytes is present, and the suprabasal keratinocytes, the sites of normal Krt9 expression, are abnormally shaped (Figure 9).
Figure 9. The histology of palmoplantar epidermis at the age of 6.0 weeks.

(A) H&E staining of footpad skin for male WT mouse at the age of 6.0 weeks. The WT mouse does not exhibit any disruption of the suprabasal layers, and there is not visible aggregation in the epidermis. (B) H&E staining of footpad skin for male Krt9⁻/⁻ mouse at the age of 6.0 weeks. Compared to earlier ages of 3.5 weeks, here you can see a thinner hyperkeratosis in the Krt9⁻/⁻ paw tissue. However, the disruption of suprabasal layers does not normalize after the sloughing-off process. E, epidermis; H, Hyperkeratosis; P, perinuclear vacuolization of keratinocytes.
In summary (Figure 10), our clinical and histological analysis adds to the understanding of the lesion progression in the Krt9⁻/⁻ mouse model. Although 1.5-week-old Krt9⁻/⁻ mice do not exhibit clinical manifestations of hyperpigmentation or hyperkeratosis, there is already substantial thickening and disorganization of the epidermis at the early postnatal time point. At 3.5 weeks of age, Krt9⁻/⁻ mice have visible footpad lesions and exhibit a worsening of histological changes. Once again, at 6 weeks of age, the mild clinical presentation of Krt9⁻/⁻ mice belies the significant histological alterations that are present.
Figure 10. Summary of Macroscopic & Histological Findings in the Krt9⁻/⁻ mouse model.

(A) At the pre-lesional stage (1.5 weeks), there is no apparent clinical phenotype of PPK lesion on the left paw of the male Krt9⁻/⁻ mouse; however, the histology shows hyperkeratosis and a disrupted suprabasal layers of palmoplantar skin. In addition, there are fissures in the epidermis due to unknown reasons. (B) At the lesional stage (3.5 weeks), PPK lesions with hyperpigmentation on the left stress-bearing footpads of the male Krt9⁻/⁻ mouse become visible. Under the histological analysis, there is a thicker hyperkeratosis and some cautious keratin aggregations in the epidermis which require further investigation. (C) At the post-lesional stage (6.0 weeks), the left paw of the male Krt9⁻/⁻ mouse shows that hyperkeratosis becomes thinner than the earlier age of 3.5 weeks, and more visible perinuclear vacuolization of keratinocytes locate at the bottom of the palmoplantar epidermis. Scale Bar = 50 μm.
Measurement of melanin density in the palmoplantar epidermis

The fluctuating nature of the presence of focal hyperpigmentation in the \( Krt9^{-/-} \) mouse footpad has two potential explanations. First, the clinical resolution of hyper-pigmentation could reflect a loss or removal of melanin in the palmoplantar epidermis. Second, the hyperpigmentation may not be resolved but rather visibly obscured by the increased thickness of palmoplantar epidermis. To test these two possibilities, Fontana-Masson (F&M) staining, which detects melanin in tissues, was performed and revealed that there is increased melanin in \( Krt9^{-/-} \) paw skin relative to \( WT \) controls at all ages assessed (Figure 11). Thus, the more likely scenario is that there is no removal of melanin via the normal turnover and sloughing off of keratinocytes. Rather the clinical resolution of hyperpigmentation is more likely due to the thickness of the palmoplantar epidermis obscuring the increased melanin. This finding suggests a further disruption of epidermal homeostasis and keratinocyte differentiation.
Figure 11. Fontana-Masson (F&M) Staining at the three stages.

On the left (A), Fontana-Masson staining of WT and Krt9-/- footpad skin. On the right (B), quantitation of fold change of melanin in Krt9-/- paw tissue relative to WT controls and normalized for epidermal thickness. Error bars = standard deviation.
RNA levels of Krt16, NRF2, and genes encoding redox balance related enzymes in Krt9−/− paw skin

Previously, Fu et al. reported an upregulation of the wound-inducible Krt16 in Krt9−/− paw epidermis (Fu et al., 2014). Given that mutations in KRT16 underlie the non-epidermolytic form of PPK (NEPPK), the potential effect of increased K16 levels on the Krt9−/− phenotype as well as how this induction may contribute to molecular differences between the two forms of PPK, we assessed Krt16 mRNA levels at multiple ages in Krt9−/− paw epidermis (Figure 12). Consistent with the work of Fu et al., Krt16 expression is increased in the paw skin of Krt9−/− mice relative to WT controls throughout the stages of lesion development. To evaluate whether alterations in NRF2 and redox balance contribute to the pathogenesis of EPPK lesions in Krt9−/− mice, as is the case for NEPPK lesions in Krt16−/− mice, we assessed the RNA levels of NRF2 and two NRF targets glutathione synthetase (GS) and glutamate-cysteine ligase catalytic subunit (GCLC), which regulate GSH synthesis (Kerns et al., 2016). Kerns et al. reported that although NRF2 is upregulated in pre-lesional Krt16−/− paw skin, it is hypoactive and associated with impaired glutathione synthesis. Likewise, we find that NRF2 mRNA levels are elevated 1.5 ± 0.04 fold in pre-lesional Krt9−/− paw skin at the age of 1.5 weeks. NRF2 mRNA levels are also elevated 4 ± 0.03 fold in post-lesional paw skin at the age of 6 weeks. However, the mRNA levels for GS and GCLC in Krt9−/− paw skin were not significantly different from WT controls throughout lesion formation. Compared to WT, the mRNA levels of glucose-6-phosphate dehydrogenase (G6-PD), an NRF2 target that plays an important role in the pentose phosphate pathway (DeBerardinis & Chandel, 2016; Patra & Hay,
were slightly reduced in pre-lesional, lesional, and post-lesional $Krt9^{-/-}$ paw skin. mRNA levels for glutathione disulfide reductase (GSR), an NRF2 target involved in GSH regeneration (Haslam et al., 2017; Telorack et al., 2016), were not significantly different from WT in pre-lesional or lesional $Krt9^{-/-}$ paw skin.

![Figure 12. The impact of Krt9 expression on Krt16, NRF2, and NRF2-mediated gene expressions.](image)

(A) Relative fold change of mRNA for $Krt16$, $NRF2$, GS, GCLC, G6-PD, and GSR in pre-lesional 1.5-week-old male $Krt9^{-/-}$ paw tissue relative to WT control. Data represent mean ± SEM of 3 mice. Student’s test. (B) Relative fold change of mRNA for $Krt16$, $NRF2$, GS, GCLC, G6-PD, and GSR in lesional 3.5-week-old male $Krt9^{-/-}$ paw tissue relative to WT control. Data represent mean ± SEM of 7 mice. Student’s test. (C) Relative fold change of mRNA for $Krt16$, $NRF2$, GS, GCLC, G6-PD, and GSR in post-lesional 6.0-week-old male $Krt9^{-/-}$ paw tissue relative to WT control. Data represent mean ± SEM of 6 mice. Student’s test. *$P < 0.01$; **$P < 0.04$. 

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Assessing levels of glutathione (GSH) in Krt9−/− paw skin

Kerns et al. (2016) reported decreased levels of reduced glutathione (GSH) in pre-lesional Krt16−/− paw skin (4-week old mice) compared with WT controls, which was indicative of increased cellular stress (Kerns et al., 2016). We assessed GSH levels in Krt9−/− paw skin and determined that there is no significant difference (P > 0.05 at all three stages) from WT controls throughout the time course of lesion development (Figure 13).

Figure 13. The GSH synthesis or regeneration in male Krt9−/− mouse paws are relatively normal to WT at the three stages.

Fold change of GSH levels in Krt9−/− paw skin relative to WT controls. Error bars = SEM. Although there are fluctuations of GSH levels at the three stages, the P values at the three stages are larger than 0.05 (at the 1.5-week pre-lesional stage, the P value is 0.188; at the 3.5-week lesional stage, the P value is 0.17; at the 6-week post-lesional stage, the P value is 0.22); thus, we conclude there may be no significant defect in the GSH synthesis and regeneration mechanism in the Krt9−/− mouse model.
Assessing the levels of K16 and NRF2 proteins in Krt9\textsuperscript{-/-} paw skin

To confirm the finding of elevated RNA levels of Krt16 in Krt9\textsuperscript{-/-} paw skin, we evaluated protein levels using indirect immunofluorescence. The signal for K16 was elevated in pre-lesional (1.5 weeks old) and lesional (3.5 weeks old) Krt9\textsuperscript{-/-} mouse paw tissue at the lesional stage, but was undetectable in post-lesional (6.0 weeks old) Krt9\textsuperscript{-/-} paw skin (Figure 14). This latter finding is surprising and warrants more investigation to rule out a technical error. The protein expression and phosphorylation status of NRF2, the latter of which is indicative of its active state, were also assessed in Krt9\textsuperscript{-/-} mouse paw tissue. NRF2 was present in the suprabasal layer of palmoplantar epidermis of WT controls at 1.5, 3.5, and 6 weeks of age. Positive staining for NRF2 was detected in pre-lesional (1.5 weeks of age) Krt9\textsuperscript{-/-} paw skin, but not in lesional or post-lesional Krt9\textsuperscript{-/-} paw skin (Figure 15). An immuno-fluorescence signal for phosphorylated NRF2 (pNRF2) was present in the epidermis of WT paw skin at all three ages and the pre-lesional Krt9\textsuperscript{-/-} paw skin, and absent in lesional and post-lesional Krt9\textsuperscript{-/-} paw skin (Figure 16).
Figure 14. The impact of Krt9 expression on K16.

Indirect immunofluorescence for Krt16 in WT and Krt9Δ paw skin. DAPI = nuclear staining. Dashed line marks dermo-epidermal junction. Epi, epidermis; derm, dermis. Scale bar: 50 μm.
Figure 15. The impact of Krt9 expression on NRF2.

Indirect immunofluorescence for NRF2 in WT and Krt9<sup>-/-</sup> paw skin. DAPI = nuclear staining. Dashed line marks dermo-epidermal junction. Epi, epidermis; derm, dermis. Scale bar: 50 μm.
Figure 16. Impact of Krt9 expression on pNRF2.

Indirect immunofluorescence for pNRF2 in WT and Krt9<sup>−/−</sup> paw skin. DAPI = nuclear staining. Dashed line marks dermo-epidermal junction. Epi, epidermis; derm, dermis. Scale bar: 50 μm.
Topical application of NRF2 inducer sulforaphane in Krt9−/− mouse model

In contrast to the Krt16−/− mouse model of PPK, our characterization of the Krt9−/− murine model of EPPK suggests that NRF2 is present and active in the pre-lesional (at the age of 1W) paw skin and that the redox balance is relatively normal. Kerns et al. (2016) reported that topical treatment with the NRF2 inducer sulforaphane (SF) (Houghton, Fassett, & Coombes, 2013; Houghton, Fassett, & Coombes, 2016; Y. Zhang, Talalay, Cho, & Posner, 1992) is capable of activating NRF2 in the Krt16−/− mouse model and preventing the non-epidermolytic form of PPK lesion development (Kerns et al., 2016). To examine whether the epidermolytic form of PPK has a similar NRF2- or redox balance-pathophysiological pathway to NEPPK, we used the SF regimen in the Krt9−/− mouse model. To do so, we treated Krt9−/− mice with topical SF twice a week beginning at birth and continuing until 3.5 weeks of age. The regimen had no discernable effect on the onset or progression of paw lesions in the Krt9−/− mice (Figure 17). These findings further strengthen the previous hypothesis of Kerns et al. that the success of the SF treatment in the Krt16−/− mouse model is likely due to its ability to activate NRF2 and normalize redox balance, which can limit any other NRF2 off-target effects.
Figure 17. The timeline of the SF treatment in the Krt9−/− mouse model, as seen by the author.

Images of representative paws from 1-week-old, 1.5-week-old, 2.5-week-old, and 3.5-week-old male Krt9−/− mice. At the age of 2.5 weeks, the hyperpigmented calluses become visible, indicating that the SF regimen has no effect on the onset or progression of paw lesions in Krt9−/− mice.
DISCUSSION

The site-specific expression of keratins has critical implications for human health and disease. K9 is exclusively expressed in the suprabasal keratinocytes of the palmoplantar epidermis. Mutations in KRT9 cause EPPK, which is typified by diffuse hyperkeratosis of the palms and soles, indicating the important of K9 in maintaining the structural integrity and providing additional mechanical reinforcement for the palmoplantar epidermis (Fu et al., 2014; Schweizer et al., 1989; Swensson et al., 1998), which covers body sites that are exposed to large amounts of daily stress. Our lab previously reported that oxidative stress associated with impairments of NRF2 signaling precede and contribute to the development of non-epidermolytic PPK (NEPPK) lesions in the Krt16−/− mouse model (Kerns et al., 2016). Utilizing the Krt9−/− mouse model, we assessed whether similar alterations of NRF2 activity and redox balance underlie the development of EPPK lesions.

First, we conducted an in-depth macroscopic characterization and histological analysis of lesion formation in Krt9−/− mice. We observed that EPPK lesions in Krt9−/− mice were visible as early as the second week after birth, which is approximately one week earlier than noted by Fu et al. In addition, Fu et al. did not assess histology of samples taken prior to lesion onset. Our histological analysis of pre-lesional (1.5 weeks old) Krt9−/− mouse paws revealed substantial changes, including increased thickness and disorganization of palmoplantar epidermis and fissures that precede lesion development. Compared to Fu’s report, we observed a more substantial regression of hyperpigmentation on the
stress-bearing footpads during the post-lesional stage (6 weeks of age) (Fu et al., 2014).

This work allowed us to pinpoint time points of interest to evaluate NRF2 activity and redox status of the palmoplantar skin of the Krt9−/− mice, specifically pre-lesional (1.5 weeks of age), lesional (3.5 weeks of age), and post-lesional (6 weeks of age). One of the key findings of our study is that, unlike in the Krt16−/− model of PPK, NRF2 appears to be present and active in pre-lesional Krt9−/− paw skin. In further contrast to the Krt16−/− model, GSH levels and the RNA levels of redox related enzymes in Krt9−/− paw skin are similar to WT controls. Furthermore, topical treatment with SF, an NRF2 inducer, had no impact on lesion formation in the Krt9−/− mice (Table 1). This further strengthens the notion that the rescue of PPK-like lesions in Krt16−/− mice is due to the NRF2 component of SF’s action. Moreover, the elevated levels of K16 in the Krt9−/− paw skin may explain the normal activity of NRF2 and reinforces the specificity of the connection between K16 and NRF2 activity.

Although EPPK and PPK share a common feature of palmoplantar keratoderma, they each have distinct clinical and histological features. For instance, excruciating pain is a key characteristic of PPK, whereas the pain associated with EPPK is considered mild. Oxidative stress has been implicated as a key pathological trigger of nerve damage and pain in diabetic neuropathy (Naziroglu, Dikici, & Dursun, 2012). The lack of indicators for oxidative stress in the Krt9−/− mouse paws suggests a possible explanation for the differences in pain associated with EPPK and PPK that is worth further investigation.
Future studies are needed to explore other NRF2-independent pathways correlating with the development of EPPK lesions. The massive upregulation of K16 in the Krt9\textsuperscript{-/-} paw skin has also raised the possibility that K9 may directly or indirectly regulate the expression of K16. Investigating the levels of K9 in Krt16\textsuperscript{-/-} paw skin may be particularly revealing. Lastly, a major limitation of this study is its focus on male mice. A similar in-depth analysis of female Krt9\textsuperscript{-/-} mice is needed to rule out any sex based differences.

<table>
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<th>Nature of Lesions</th>
<th>NEPPK (Krt16 Model)</th>
<th>EPPK (Krt9 Model)</th>
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<td></td>
<td>Non-Epidermolytic</td>
<td>Epidermolytic</td>
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<td>Palmoplantar Keratoderma; Lesions are “Persistent”</td>
<td>Palmoplantar Keratoderma; Lesions are “Cyclical”</td>
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<tr>
<td>GSH Regeneration</td>
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<td>Relatively similar to WT</td>
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<tr>
<td>Pentose Phosphate Pathway</td>
<td>Relatively similar to WT</td>
<td>Relatively similar to WT</td>
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<td>The efficacy of SF Regimen</td>
<td>Prevent PPK lesion development</td>
<td>Does not prevent PPK lesion development</td>
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Table 1. Summary of Krt16\textsuperscript{-/-} and Krt9\textsuperscript{-/-} mouse model studies.
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Curriculum Vitae

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EDUCATION

The Johns Hopkins University Bloomberg School of Public Health
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Master of Science (ScM) in Biochemistry & Molecular Biology

ScM Thesis:
“Pathophysiology of the cyclical epidermolytic palmoplantar keratoderma (EPPK) in the Keratin 9 mouse model.”

Advisor: Pierre A. Coulombe, PhD
Secondary Reader: Michael Caterina, MD, Ph.D.

MHS Thesis:
“A New Gingivitis Treatment with IL-1β siRNA.”

Advisor: Paul Miller, PhD
Secondary Reader: Anthony K. L. Leung, Ph.D.

University of Washington Post-baccalaureate studies – Seattle, WA.
Courses: Year-long human physiology and anatomy with lab

University of Washington, Seattle – Seattle, WA.
Bachelor of Science (B.S.): Biochemistry and Microbiology
Minors: Chemistry, Bioethics and Humanities

HONORS & AWARDS

The Johns Hopkins School of Public Health Master’s Tuition Scholarship 2016
- It awards students in good academic standing to pursue their second year of study in a two-year master’s program.

The Johns Hopkins SOURCE Service Scholar 2016-2017
RESEARCH EXPERIENCE

The Johns Hopkins University Bloomberg School of Public Health, 2015-2017
Department of Biochemistry and Molecular Biology, Baltimore, MD.
- **The Krt9⁻⁻ Mouse: A Model for Cyclical Epidermolytic Palmoplantar Keratoderma (EPPK)**
  - Elucidating the molecular mechanism behind the cyclical EPPK.
  - Advisor: Dr. Pierre A. Coulombe – The Johns Hopkins University.

University of Washington School of Medicine, 2013-2015
Department of Otolaryngology, Seattle, WA.
- **Laryngeal Trauma: A Ten Year Experience from a Level I Trauma Center.**
  - Collected laryngeal trauma data from Washington, Wyoming, Alaska, Montana, and Idaho to identify increasing trends to improve patients' surgical outcomes over the past ten years.
  - Advisors: Dr. Maya Sardesai – University of Washington.

University of Washington School of Medicine, 2008-2012
Department of Bioengineering, Seattle, WA.
- **Designing siRNA Delivery Systems and Molecular Diagnostic Tools with Polymers.**
  - Project 1: investigated the efficacy of controlling macrophage activity in the acute lung injury model through gene silencing with siRNA.
  - Project 2: modifying polymers to seek an affordable and sensitive HIV diagnostic device that can be used in developing countries.
  - Advisor: Dr. Patrick Stayton – University of Washington.

University of Washington, 2009-2010
Department of Biology, Seattle, WA.
- **Senior Thesis: The Arabidopsis thaliana Clock.**
  - Studied how the photoperiodic flowering response was accomplished based on genetic and molecular mechanisms.
  - Advisor: Dr. Takato Imaizumi – University of Washington.

University of Washington School of Medicine, 2007-2008
Department of Comparative Medicine, Seattle, WA.
- **The metabolism of Vitamin A: Interactions between RBP4 and Type 2 Diabetes and Obesity.**
  - Investigated the interaction of retinol-binding protein 4 (RBP4) and Type 2 diabetes.
  - Advisor: Dr. Jisun Paik – University of Washington.
PRESENTATIONS

The Triological Society Combined Sections Meeting Program
– San Diego, CA.

January 2015

• Poster 1: "Laryngeal Trauma: A Ten Year Experience from a Level I Trauma Center."
  ▪ Presented a retrospective study of the presentation, management, and treatment outcomes associated with laryngeal trauma treated at a level I trauma center over a 10-year period.
  ▪ The 1st Authorship.

• Poster 2: "The Evolution of Craniofacial Actinomyces Osteomyelitis: A Case that Implies its Natural History."
  ▪ This craniofacial actinomyces osteomyelitis case elaborated how the disease can progress and highlighted the need for a multidisciplinary approach to patient care.
  ▪ The 1st Authorship.

• "Factors Affecting Patient Decision Making Involving Elective Surgery: Implications for Educating Referring Providers."
  ▪ To determine the relevance of factors that influences a patient’s decision to pursue elective surgery involving the head and neck.
  ▪ The 3rd Authorship.

SERVICE & LEADERSHIP

The Johns Hopkins SOURCE Service Scholar
Baltimore, MD.

• Recruiting and managing volunteers to provide official documents for returning citizens in Baltimore.

HIV and Family Planning Counselor
– The Johns Hopkins’ Harriet Lane Clinic
Baltimore, MD.

• Counseling sexually active adolescents on how to protect their health through correct contraceptive methods and routine STD testing.

The Thread – Academic/GED Tutor
Baltimore, MD.

• Helped students who have dropped out of high school prepare the GED tests and locate their career goals.

The Otolaryngology-Head and Neck Surgery Clinic – Volunteer
Harborview Medical Center, Seattle, WA

• Helped patients comprehend laryngopharyngeal reflux and gastroesophageal reflux disease (GERD) by explaining pamphlets on silent reflux in three languages—English, Mandarin, and Formosan.
The Oral and Maxillofacial Surgery Clinic – Volunteer 2013-2015
Harborview Medical Center, Seattle, WA
• Taught patients how to maintain oral hygiene when they dealt with oral trauma and surgery.
• Helped patients to exercise their jaw movement with a stack of tongue blades to improve trismus, a tonic contraction of the jaw-closing muscles.

The Emergency Department – Volunteer 2008-2009
Harborview Medical Center, Seattle, WA.
• Escorted patients and their family members to different departments.

Dr. Paul Brand Centre for Leprosy Reconstructive Surgery - Volunteer 2009
Christian Medical College, Vellore, India.
• Took care of leprosy patients.

English Camp Organizer & English Teacher Summer, 2007
Phnom Penh, Cambodia.
• Helped local children develop their English literacy.

CLINICAL CERTIFICATE

HIV Counseling Skills Level I Prevention & Test-Decision Counseling. 2015
• Issued by the Maryland Department of Health and Mental Hygiene Prevention and Health Promotion Administration.

PERSONAL INTERESTS
• Jogging, Traveling, and Community Services.