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## Quantifying the Effect of a Novel topical Hyaluronic-Acid Phosphatidylethanolamine Cream on the Epidermis


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A thesis submitted in partial fulfillment of the requirements for the Master of Clinical Science degree in Surgery

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QUANTIFYING THE EFFECT OF A NOVEL TOPICAL HYALURONIC-ACID  
PHOSPHATIDYLETHANOLAMINE CREAM ON THE EPIDERMIS

(Thesis format: Integrated Article)

by

Caitlin Jane Symonette

Graduate Program in Department of Surgery

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Surgery

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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## Abstract

With aging, keratinocytes have diminished proliferative capacity resulting in atrophic skin with reduced barrier function. This investigation evaluates the effect of daily topical applications of a novel high-molecular weight hyaluronan cream (HA-PE) on keratinocyte renewal and epidermal thickness. Unmodified hyaluronan and HA-PE were mixed separately into a vehicle cream. Each topical formulation was applied daily onto the shaved backs of aged female C57BL6 mice. Full-thickness biopsies of treated skin were obtained for analysis of keratinocyte proliferation, keratinocyte differentiation, and local inflammation at days 1, 5, and 10 of cream application. In addition, a cardiac puncture was performed for serum C-reactive protein analysis. The HA-PE group exhibited the highest level of keratinocyte proliferation and epidermal thickness at all time points. There was no significant difference in keratinocyte differentiation or local/systemic inflammation between groups. Thus, topical HA-PE cream is a novel skin care technology that increases epidermal thickness through enhanced keratinocyte proliferation/renewal.

**Keywords** topical hyaluronan, topical hyaluronic-acid, keratinocyte proliferation, epidermal thickness, skin renewal, skin aging; hyaluronan-phospholipid

## Co-Authorship Statement

Chapter 4: Manuscript “Hyaluronan-phosphatidylethanolamine polymers forms pericellular coats on keratinocytes and promotes basal keratinocyte proliferation”

All the experiments outlined in this manuscript were performed in Dr. Eva Turley’s laboratory. The experiments and analysis related to the formulation of HA polymers that form pericellular coats was performed by Dr. Eva Turley, Dr. Jenny Ma, and Dr. Cornelia Tolg. These preliminary studies were crucial to the discovery of the HA-PE molecule used as the foundation of this thesis.

In terms of the objectives of the current thesis outlined in Chapter 2: Carl Postenka paraffin-embedded and sectioned all of the skin specimens used in the analysis. Aman Kaur Mann and Cherie Tan assisted with the immunohistochemistry for the markers of keratinocyte proliferation, differentiation, and activated macrophages. In addition, Dr. Eva Turley, Dr. Cornelia Tolg, and Dr. Arjang Yazdani were involved in the data interpretation and manuscript preparation.



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First, I am indebted to my supervisor, Dr. Eva Turley. Your enthusiasm and commitment to innovation is inspiring. Thank you for teaching me the laboratory techniques, language of, and approach to basic science research. I plan to use these tools to form future collaborations between clinical and basic science teams.

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## List of Abbreviations

ACD	allergic contact dermatitis
BM	basement membrane
CD	cluster of differentiation
CO <sub>2</sub>	carbon dioxide
CRP	C-reactive protein
DAMP	Damage associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	enzyme-linked immunosorbant assay
EGF	epidermal growth factor
EPU	epidermal proliferative unit
GAG	glycosaminoglycan
GFP	green fluorescent probe
HA	hyaluronic acid/hyaluronan
HA-PE	hyaluronic acid-phosphatidylethanolamine
HAS	hyaluronan synthase
H&E	hematoxylin and eosin
HMWHA	high molecular weight HA
HYAL	hyaluronidase
LYVE	lymphatic endothelium specific hyaluronan receptor
ICD	irritant contact dermatitis



IGF-1	insulin growth factor-1
IL	interleukin
KGF	keratinocyte growth factor
KO	knockout
LMWHA	low molecular weight HA
MEF	mouse embryonic fibroblast
MES	2-( <i>N</i> -morpholine) ethanesulfonic acid
MMP	matrix metalloproteinase
MW	molecular weight
MWCO	molecular weight cut-off
NF- $\kappa\beta$	nuclear factor- $\kappa\beta$
NMF	natural moisturizing factor
PBS	phosphate buffered saline
PE	phosphatidylethanolamine
RHAMM	receptor of hyaluronan mediated motility
ROS	reactive oxygen species
SC	stratum corneum
SiRNA	silencing RNA
TA	transit-amplifying
TEWL	transepidermal water loss
TGF $\beta$	transforming growth factor
TLR	toll-like receptor
TNF $\alpha$	tumor necrosis factor

UV ultraviolet

## Chapter 1: Introduction

### **Epidermis**

#### Structure and Function

The skin is a specialized organ derived from both ectoderm and mesoderm precursors during embryogenesis. Skin has many important roles including providing a barrier to the external environment, sensation, immune surveillance, thermoregulation, endocrine functions and control of insensible fluid loss [1]. The integumentary system structure and physiology evolves with senescence. In contrast to young skin, aged skin is characterized as atrophic, dry, wrinkled, and fragile. However, it is not only the external appearance of skin that deteriorates with age- so does its function [2-4].

Anatomically, the epidermis (ectoderm) and dermis (mesoderm) are the primary layers of the skin. The epidermis is avascular and receives oxygen and nutrients by passive diffusion from the underlying dermis. In humans the epidermis forms a sinusoidal pattern with projecting fingerlike extensions, termed rete ridges, into the underlying dermis. The rete ridges provide an increased surface area of contact with, and thus adherence to, the underlying dermis. With aging, rete ridges become effaced and the epidermis is more vulnerable to external mechanical forces such as friction and shearing [3]. Simple skin tears, from innocent trauma such as removing an adhesive bandage for example, can result in significant morbidity for the elderly [5].

The epidermis is classified histologically as a stratified squamous epithelium. In humans, there are four identified layers of the epidermis. These layers are from deep (resting on the skin basement membrane (BM)) to superficial (exposed to the external environment):

stratum basale, stratum spinosum, stratum granulosum, and the stratum corneum (SC). The epidermis also lines dermal appendages including various sebaceous/ecrine sweat glands and hair follicles. This feature provides a reservoir for epidermal regeneration following wounding or extensive epidermal trauma such as a partial-thickness burn.

Young epidermis possesses a robust renewal capacity. The keratinocyte is the principal cell in the epidermis. Keratinocytes are generated from stem-cell progenitors lining the basal layer of the epidermis and the bulge of the hair follicle [6]. With respect to the epidermis, ongoing replication pushes terminally differentiated keratinocytes towards the SC. The SC is characterized by anucleate corneocytes embedded in a lamellar lipid matrix [7]. The thickness of the epidermis and concentration of natural moisturizing factors (NMF) improve the barrier role of skin [7, 8]. Flaggrin and hyaluronan (HA) are two examples of NMF important to SC hydration [7]. Desquamation, a process of shedding the outermost layer of the SC, allows the cornified envelope to balance the replicative potential of the stratum basale. The typical transit time of a keratinocyte through the epidermal layers is four weeks [1]. The self-renewal capacity of the epidermis is therefore tightly regulated to preserve homeostatic balance. However with aging, a new set-point is established as the thickness of the epidermis is diminished and this reflects a reduced proliferative capacity [4].

Between the cellular components of skin lies the extracellular matrix (ECM). The epidermal ECM is composed of proteoglycans and glycosaminoglycans. Glycosaminoglycans (GAG) are formed from disaccharide units of an amino and uronic

sugar. The simplest and most abundant GAG in the skin is HA. HA is found in the intercellular ECM and forms pericellular coats (discussed later in this chapter) around keratinocytes. Proteoglycans differ in that they contain a protein core with variable attachment of glycosaminoglycans. The BM, a specialized part of the ECM, separates the stratum basale from the dermis and is composed primarily of laminin, type IV collagen, and proteoglycans [9]. The differential structure of the BM helps identify the polarity of the epidermis. Overall, proteoglycans and GAG provides a structural framework to support cellular components of skin. As with keratinocytes aging, aging also affects the constituents of the ECM. Further details of GAG and aging are discussed later in this chapter.

Along with the keratinocyte, the epidermis contains other resident cell types including Langerhans cells, melanocytes, and Merkel cells. These cells are responsible for immune surveillance through antigen-presentation, protection from ultraviolet light exposure, and mechanosensation respectively.

#### Keratinocyte Proliferation and Differentiation

Keratinocyte proliferation and differentiation provide a foundation for epidermal repair and renewal. Keratinocyte renewal and maturation is identified by differential expression of cell surface markers as the keratinocyte proliferates, migrates to and ultimately integrates into the stratum corneum. Cutaneous keratins form intermediate filaments that provide structural support to keratinocytes. Different keratins are expressed during keratinocyte proliferation/differentiation. For example, keratin cell surface expression of

K5/K14 are hallmarks of epidermal proliferation and K1/K10 expression indicates suprabasal terminal differentiation [10, 11]. Additional proteins such as Ki67 are also commonly used to identify cellular proliferation [12-14]. A complex interaction of mechanical and cytokine ECM signaling regulates the keratinocyte proliferation/differentiation program [10, 15].

The basal layer of the epidermis contains epidermal proliferative units (EPU) [1, 16-18]. An EPU is formed from a single adult stem cell and its progeny. Epidermal adult stem cells can be found in niches in the basal layer of the epidermis and the bulge of the hair follicle. Through symmetric and asymmetric cell division the epidermal stem cell progenitor can replace itself and produce a transit-amplifying (TA) intermediate stem cell. The TA intermediate has a finite number of self-renewal cell divisions before being committed to a terminal differentiation pathway (represents keratinocytes with no further proliferative capacity). The adult stem cell forming the EPU can be identified using certain markers such as Oct4, which is expressed by the immortal stem cell but down regulated in the differentiated progeny (for example, TA intermediate stem cells)[19]. The self-renewal capacity of the epidermis is dependent on the organization and functional activity of the EPU. Various growth factors (epidermal growth factor (EGF) [20], keratinocyte growth factor (KGF) [12, 20], transforming growth factor beta (TGF $\beta$ )[21]), GAGs (HA), and mechanical stimuli [15] have been implicated in regulating keratinocyte growth.

## **Hyaluronan**

HA is an ubiquitous ECM non-sulphated GAG present in mammalian tissues. Although first discovered in the vitreous humor of the eye in the 1930's [22], the majority of total body HA is present in the skin [23, 24]. HA consists of linear chains of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid [25, 26]. Despite a simple chemical structure, endogenous HA can exist in a variety of conformations from simple extended chains to complex random entanglements [27, 28]. HA is also highly hydrophilic and can bind 10,000X its own weight in water [29, 30]. Both extracellular and intracellular HA exist, however the focus of this thesis will be limited to discussion of extracellular HA. Limited knowledge exists regarding the function of intracellular HA in epidermal rejuvenation and integrity. In comparison, extracellular HA is known to be important in preserving epidermal hydration as well as providing a scaffold for keratinocyte communication, proliferation, and differentiation.

Extracellular HA exists in a number of functional forms including cell associated pericellular coats, as extracellular structures bound to proteins, or as a small circulating pool of unoccupied HA [23, 31]. HA can modulate cellular activity through interaction with a variety of cell-surface receptors. CD44 is the major constitutively expressed HA receptor of the epidermis [23]. RHAMM is an additional HA receptor which is expressed during tissue inflammation and tissue repair [32]. HA receptor affinity, arrangement and downstream signaling cascades are influenced by the size of bound HA. CD44 binds HA through a single extracellular link domain [33]. High molecular weight HA (HMWHA) has multivalent sites for synchronous interactions with multiple CD44 receptors. HMWHA has thus been shown to induce clustering of CD44 receptors [34]. Yang *et al.*

(2012) through *in vitro* models showed that treatment with low molecular weight HA (LMWHA) disrupted the amount of CD44 clustering present on cells [35]. Thus, CD44 clusters create a configuration partial to HMWHA (anti-inflammatory) signaling, which appears inherently different to the single ligand-receptor interactions (pro-inflammatory) effects of LMWHA [35].

### Hyaluronan Homeostasis

HA is synthesized in the inner surface of the plasma membrane by HA synthases (HAS) [24, 31]. Three different isoforms of HAS, all of which are found in skin, have been identified (HAS1, HAS2 and HAS3) [36]. HAS2/3 are predominantly expressed by keratinocytes, whereas HAS1/2 are expressed by fibroblasts [37]. Each synthase isoform shares approximately 70% identical amino-acid sequences, but functionally each HAS produces HA of differing lengths [33]. HAS1/2 are known to produce HMWHA. Whereas, HAS3 produces shorter LMWHA [27, 33]. Each HAS is differentially expressed indicating distinct modes of regulation of expression [36, 38]. HAS2, for example, is crucial for both embryogenesis and life [27]. In terms of skin, cutaneous injury studies demonstrate increased HAS2/3 mRNA expression by mouse keratinocytes [39]. *In vivo* HAS1/3 null mice show impaired HA accumulation following cutaneous wounding providing further evidence for the importance of HAS3 in the acute inflammatory response of keratinocytes [37]. All HAS enzymes extrude nascent HA into the extracellular environment through an intra-protein pore present in their molecular configuration [28].

Dynamic HA metabolism balances ongoing HA synthesis. *In vivo* studies in rabbits demonstrated that the half-life of free HA is short, quoted at approximately 3-5 minutes in blood [23, 40], 24-72 hours in skin [29, 31, 41] and 1-3 weeks in cartilage [29]. In contrast, the stable configuration of HMWHA appears to resist rapid hyaluronidase degradation [23]. The majority of HA degradation occurs in the lymphatics, but can also take place in the host tissue and spleen/liver sinusoids [23, 31, 40, 41]. HA is fragmented into smaller units by either hyaluronidases (HYAL) [24, 25]. Six primary HYAL have been identified which act by hydrolyzing the  $\beta$  (1-4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid [29]. [33]. HYAL2 specifically hydrolyses HMWHA into smaller oligosaccharides which are further degraded in lysosomes by HYAL1 [33]. Fragmented HA units are subsequently cleared through lysosome endocytosis and glycosidase degradation into terminal sugars. Alternatively, HA can be metabolized by free-radical metabolism [24]. The process of HA degradation is partially mediated by HA-receptor interactions that differ depending on the tissue site. For example, HA binding to CD44 may signal HA endocytosis in the skin whereas LYVE-1 receptor activation provides the equivalent stimulus in lymph nodes [27].

#### Pericellular coats

Using erythrocyte exclusion assays, Clarris *et al.* (1968) identified a cleared zone surrounding human synovial cells which they described as analogous to a bacterial capsules [42]. Treatment with hyaluronidase obliterated this potential space, validating the association of HA to the integrity of pericellular coats [28, 42, 43]. HA associates with the pericellular membrane through into the plasma membrane by interactions with



HA receptors such as CD44, and nascent chain attachments to HAS2/3 [27]. Tammi and Tammi (2008) have made important contributions to our understanding of HA pericellular coats. Using cells transfected with a green fluorescent probe GFP-HAS3, their group demonstrated that the presence of HAS in the cell membrane promoted the formation of plasma membrane protrusions [43] (Figure 1). The plasma membrane protrusions add both surface area and complexity to the three dimensional structure of the HA pericellular coat (Figure 2).

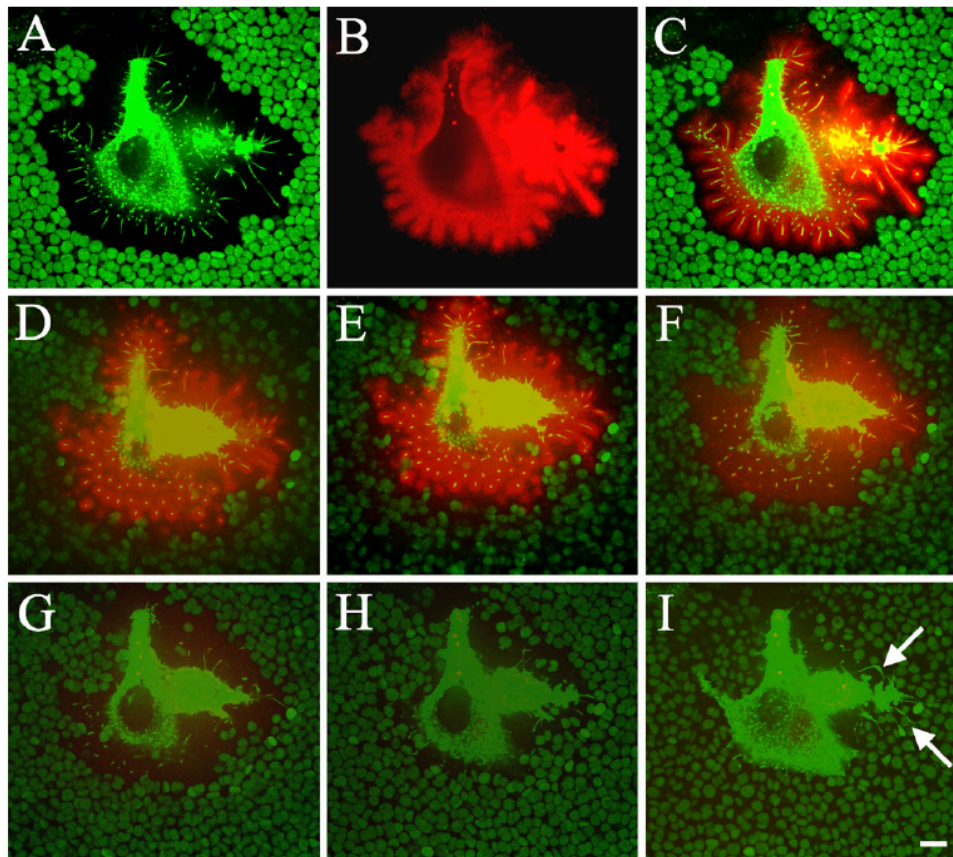


Figure 1. **Hyaluronan dependent pericellular coats.** Confocal images of hyaluronan-coated microvilli on live cells transfected with green fluorescent protein (GFP)-hyaluronan synthase (HAS)3. Erythrocyte exclusion space and GFP-HAS3 in cell protrusions around a live cell are shown (A), the use of a hyaluronan binding probe demonstrates HA associated with the same cell (B), a composite image (C). (D-I) Time-lapsed images of the same cell treated with *Streptomyces* hyaluronidase (10 TRU/ml) before treatment (D) and 1 (E), 2 (F), 3 (G), 4 (H) and 5 (I) min post-treatment. Arrows indicate microvilli projections resistant to degradation (I). Red, hyaluronan; green particles, erythrocyte autofluorescence; green in cell, GFP-HAS3 [43]. Image copyright was approved by SAGE publications (Appendix A).

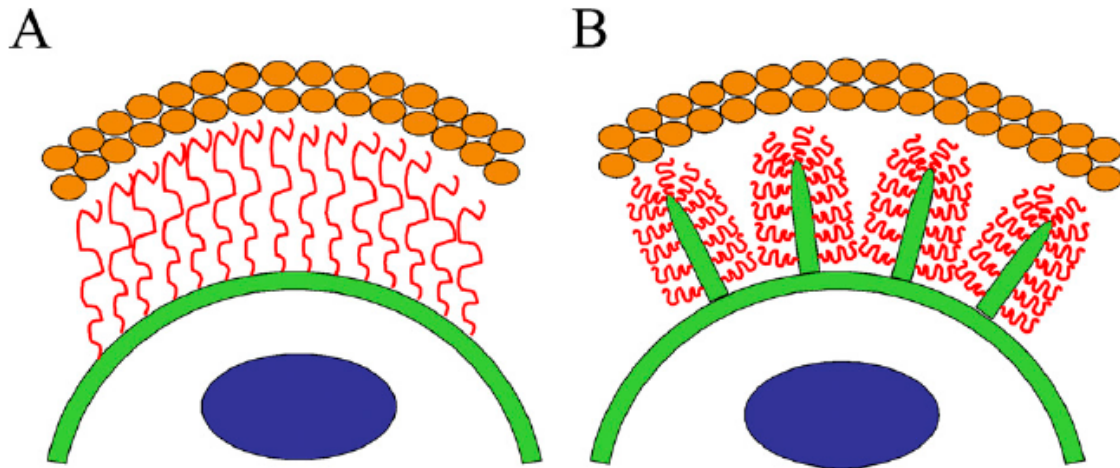


Figure 2. **Schematic diagram of proposed structure of pericellular hyaluronan coats.**

(A) Model whereby hyaluronan chains (red) attach directly into the plasma membrane (green) through hyaluronan receptors, hyaluronan synthases, or direct insertion creating an exclusion space for erythrocytes (orange). (B) Tammi and Tammi model whereby the presence of hyaluronan promotes plasma membrane protrusions creating a more complex scaffolding of the pericellular coat [43]. Image copyright is approved by SAGE publications (Appendix A).

Further study of the HA-dependent pericellular coat revealed its structure and function [28] [Figure 2]. The pericellular coats traps proteins, growth factors and other signaling factors in close proximity to the target cell that can stimulate downstream signaling cascades through target growth factor receptors and dynamic HA-receptor interactions. In addition to the interactions between CD44-HA, the HA pericellular coat can alter cell behavior through mechanotransduction signaling. For example, endothelial HA pericellular coats are known to be important in inducing intracellular nitric oxide production secondary to shear stress produced from blood flow [28]. The cell coat is fluid and can be rapidly formed or destroyed depending on the state of the tissue [28]. Thick HA pericellular coats can form from a linear orientation of HA caused by the negative valences from proteoglycan associations, such as aggrecan in chondrocytes and versican in fibroblasts [28]. The presence of proteoglycans can contribute to thick pericellular coats. However, epithelial cells also form 20  $\mu\text{m}$  thick coats with minimal aggregation of proteoglycans [43].

Plasma membrane CD44 is vital to pericellular coat formation. Blocking CD44 expression through antibodies, CD44-siRNA, and knockout (KO) CD44<sup>-/-</sup> models has been shown to result in abnormal histolocalization of HA [44]. Interestingly CD44 KO mice show normal epidermal development and thickness, perhaps identifying compensation from other HA signaling pathways contributing to epidermal development [44].

## Molecular weight and function

The multitude of identified functions of HA depend on its molecular size, concentration and configuration [23, 25]. The role of different molecular weight HA in skin hydration, aging, and acute and chronic wound repair been studied extensively [23, 30, 45-48].

### *HMWHA:*

The rheological properties of HMWHA are known to improve the overall quality and immune barrier function of skin. In terms of HA and wound healing, interestingly fetuses prior to 24 weeks gestation have markedly high expression of HA and are found to heal cutaneous wounds without scarring [30, 46, 47]. The majority of endogenous native HA is HMWHA ( $10^5 - 10^7$  Daltons) which forms random coils and entanglements depending on the size and molecular associations [23, 27]. Endogenous HMWHA is particularly present in pericellular coats [25, 28, 43] that create a fluid scaffold for enhanced cell motility and as noted above HA modulates cell-to-cell adhesion, migration, proliferation, and differentiation [23, 45, 49] through interactions with CD44 receptors [32]. Overall, HMWHA functions are anti-angiogenic, anti-inflammatory and immunosuppressive [35]. HMWHA is known to prevent immune cell recognition and inhibit phagocytosis by macrophages [50]. Studies using the naked mole rat model suggest that HMWHA also infers cancer resistance [51]. HMWHA can act as a free radical scavenger, the toxicity of free radicals is diminished because reactive oxygen species (ROS) complex with the double bond in the D-glucuronic acid unit of HA [52].

### *LMWHA:*

Under certain tissue states, smaller HA fragments (low molecular weight HA, LMWHA) are produced *de novo* by hyaluronidase degradation or oxidative metabolism of HMWHA. LMWHA activates the inflammatory cascade and promotes angiogenesis in response to tissue injury [23, 35]. The diverse spectrum of processes that involve HA, highlight its importance to preserving overall skin health.

### **Aging skin and hyaluronan**

Skin aging is a combination of the culmination of intrinsic and extrinsic influences [53, 54]. Intrinsic aging, or chronological aging, refers to aging related to cellular DNA damage and altered cellular biosynthetic function. Many theories have been developed to explain features of intrinsic aging. For example, the “oxidative stress theory” refers to impairment in the cell’s regulatory genetic program to reverse oxidative damage incurred in cellular DNA [55]. Alternatively, the “mitotic clock” theory also is used when discussing chronological aging, whereby telomere shortening of DNA with copious replication cycles is an inevitable cause of aging [53]. External factors such as sun exposure influence the degree of extrinsic aging. Photo-damage can also increase the amount of cellular oxidative damage that is poorly tolerated in aged skin given an intrinsic deficiency of anti-oxidant cellular machinery. The functional consequences of extrinsic and intrinsic aging are shared and include epidermal barrier dysfunction, atrophy, xerosis [3], delayed wound healing [2, 3], as well as an increased susceptibility to inflammatory or infectious pathologies and irritant contact dermatitis [2, 52, 53, 56, 57]. Overall with aging, skin integrity is diminished.

Histological changes in both the aged epidermis and dermis are evident. Both skin layers become atrophic [2, 3, 54, 55] with reduced cellularity [2, 55] as shown by decreased numbers of melanocytes, Langerhans cells and fibroblasts [53]. The thinner epidermis characterized in aged skin is due to a decrease in proliferation of basal keratinocytes [14, 22] and a longer SC transit time [4]. In young people, the SC transit time averages 20 days, whereas in elderly people it can be greater than 30 days [4]. Branchet *et al.* (1990) [4] used punch biopsies from a cross-sectional sample of healthy individuals age 20-80 to demonstrate the integumentary histological changes that occur with aging, including decreased epidermal thickness. Some studies have shown that despite decreased proliferation, keratinocyte differentiation appears unchanged with aging [58]. The regular honeycomb keratinocyte pattern of youthful skin becomes irregular with aging [54]. Flattening of the dermoepidermal junction is also a histological hallmark of aged skin [2, 53, 58]. In the dermis, aging results in decreased matrix synthesis by fibroblasts [14, 57, 58], decreased collagen type 1 expression [55], increased matrix metalloproteinase (MMP) expression and increased expression of collagenases [55]. Overall, dermal atrophy [3, 55, 58] and disorganization of the collagen/elastin framework contributes to skin laxity, fragility and poor wound healing [3, 58].

The relationship of HA and aging is well established in both normal skin and pathological conditions. Studies involving patients with conditions of premature aging, such as Werner's syndrome, have provided further evidence of the relationship of HA and aging [23, 59]. Skin biopsies from patients with progeria show abnormal GAG metabolism and reduced HA content [59]. In addition, patients with progeria have increased HA

metabolism reflected in higher urinary excretion of HA [59]. High circulating levels of fetal HA drop shortly after birth and are thought to remain in steady state until senescence [23, 60]. With aging, circulating levels of HA rise again [23] with notable changes in the histolocalization of HA [60]. In terms of the epidermis, the quantity of HA present in the epidermis decreases with age [23]. Dermal HA quantity remains constant throughout the lifespan, but becomes less extractable and fluid with aging [23]. Replacement of HA has been shown to improve some of the aesthetic and functional deficiencies of aged skin.

Controversy exists whether it is possible to stimulate senescent keratinocytes to undergo mitotic division to regenerate the skin barrier [55]. Growing evidence is emerging in favor of the mitogenic capability of HA in senescence [25]. Wohlrab *et al.* (2013) support the finding that HA has a stimulatory effect on keratinocyte and fibroblast proliferation *in vitro* [61]. Further animal studies show that topical application of HMWHA rejuvenates the skin through CD44-dependent expression of markers of keratinocyte proliferation and differentiation [56, 62, 63], as well as markers of SC integrity including the NMF marker Flagggrin [56]. HA is involved in dissociating basal keratinocytes from underlying substratum which promotes cellular replication [30]. Quan *et al.* (2013) [14] has shown that following injection of a cross-linked HA filler into individuals older than 70 years there was a 19% increase in epidermal thickness and increased Ki67 (keratinocyte proliferation marker) staining one-month post-injection. This study also provided evidence that HA injections also stimulated fibroblasts to undergo functional activation thus enhancing the structural support of the underlying



dermis [14]. Overall, it appears that replacement of HA which is lost in the aging process has beneficial effects on skin health.

### **HA and Cutaneous Immunobiology**

Skin provides a critical barrier role in protecting from external insults. Overall, cutaneous immunobiology is complex and tightly regulated. Innate and acquired immunity signaling cascades form the backbone to the host immune defense system [64]. Innate immunity provides a rudimentary mechanism whereby professional phagocytes such as neutrophils, mast cells, and macrophages recognize danger signals from infectious, physical, or chemical insults [65]. Keratinocytes are some of the first cells to secrete pro-inflammatory cytokines in response to a threatening stimulus. The ensuing temporary inflammatory reaction allows the individual to combat the potential damage to tissues. However, prolonged or uncontrolled inflammation can be detrimental. Cytokines are potent chemical messengers of the immuno-inflammatory cascade. The ultimate tissue effect of the immuno-inflammatory cascade depends on a multitude of factors including the inciting stimulus and the presence of other cellular mediators or receptors [64]. Acquired immunity refers to more sophisticated and targeted cell-mediated immunity involving T cell and B cell subsets. The discussion of this thesis will be limited to mediators of innate immunity and the local tissue inflammatory response.

## Macrophages

Macrophages are the most abundant immune cell population in skin [66]. Macrophages may either be recruited from a pool of circulating monocytes or alternatively exist as tissue-resident macrophages. Macrophages can be identified by expression of cell-surface markers including F4/80 [67], CD11b and CD18, CD68, and Fc receptors [68]. Macrophages are professional phagocytes which ingest and process foreign material and necrotic debris [68]. Through the production of various cytokines macrophages stimulate other immune cells such as dendritic cells, neutrophils, mast cells, T lymphocytes and lymphoid cells to assist with neutralizing threats to skin homeostasis [66].

Investigators have proposed that macrophages are heterogeneous and exhibit functional plasticity defined as polarity (e.g. M1 and M2) based upon different transcriptomes and markers [66, 68]. For example, M1 macrophages are generally pro-inflammatory, play a role in anti-tumor immunity, help defend against bacteria, protozoa and viruses and are present in early stages of response to injury processes [68, 69]. In contrast, M2 macrophages predominate in later stages of wound repair, and are involved in tissue healing and the resolution of inflammation, through secreting anti-inflammatory cytokines such as IL-10 [69]. Both infection and inflammation from sterile tissue damage provoke expression/accumulation of proinflammatory signals that attract macrophages. Macrophages express receptors that are part of the Damage-associated molecular pattern (DAMP) detection that include Toll-like receptors (TLRs, TLR2,4 also bind to HA), HA/motility receptors such as CD44 and RHAMM, and pattern recognition receptors [66, 68, 70]. In later stages of tissue repair the ratio of M1 macrophage pro-inflammatory

phenotype transitions to favoring the M2 macrophage anti-inflammatory and growth promoting phenotype. One result is, targeting of macrophage phagocytosis to apoptotic neutrophils, which contributes to resolution of inflammation [66].

### TNF $\alpha$

TNF $\alpha$  is a potent pro-inflammatory cytokine that is released by activated M1 macrophages [71] and Langerhan's cells [72]. TNF $\alpha$  is a cytokine involved in both the inflammatory phase of wound healing as well as acute and chronic skin inflammation. In addition, elderly patients have a two-fold to four-fold elevation in baseline levels of inflammatory markers [72] which could represent a state of low-grade inflammation secondary to systemic disease common in the elderly [73]. TNF $\alpha$  activates multiple downstream pro-inflammatory signaling pathways contributing to blood vessel dilatation, enhanced neutrophil lifespan and function, and induction of keratinocyte apoptosis [64, 71]. Researchers have searched for ways to temporize the detrimental tissue effects of prolonged inflammation, such as that seen in patients with cutaneous burns, by blocking TNF $\alpha$  activation. A rat model of partial-thickness burns, has shown promising results using topical HMWHA conjugated to an anti-TNF $\alpha$  antibody to reduce inflammation compared to an anti-TNF $\alpha$  antibody alone [71]. Cutaneous ulcers also demonstrate increased TNF $\alpha$  levels and provide an additional model of chronic wounding and prolonged inflammation. Acute wounding in patients with previous ulcers, have shown to have elevated TNF $\alpha$  up to day 7 post-wounding compared with age-matched controls [74]. The kinetics of TNF $\alpha$  expression can create challenges in using it as a marker of

inflammation in research. Initially thought to have a brief and transient acute response during inflammation, researchers have shown following wounding  $TNF\alpha$  can remain elevated for up to a week or longer [75]. Clinically, patient groups with burns and ulcers demonstrate that elevated  $TNF\alpha$  is both a marker of and target for clinical intervention in cases of impaired cutaneous healing and prolonged inflammation.

## HA and Inflammation

### *HA fragments:*

As previously mentioned, the functional effects of HA depend on its molecular size, localization, and availability of specific receptors [25]. HA fragmentation is known to be an important early regulator of inflammation that is part of the DAMP response to tissue stress. Elevated HA levels and likely fragmentation occur in rheumatoid joints, lung disease, as well as inflammatory bowel, liver, and vascular disease [50]. Fragmented or LMWHA produced from reactive oxygen species (ROS), catabolic degradation, or *de novo* synthesis through HAS3 contributes to the pro-inflammatory milieu in early stages of tissue injury [50]. Noble *et al.* (2010) and others (Savani *et al.* (2005)) have studied the pathogenic role of HA in sustaining inflammation and fibrosis in a model of non-infectious fibrotic lung injury [70, 76]. The profile of HA fragment accumulation mirrors the peak inflammatory response following damage to the ECM in a lung injury model. These fragments activate macrophages and signal expression of a subset of downstream pro-inflammatory cytokines through the transcriptional regulator  $NF-\kappa\beta$ : these function to recruit other mediators of the innate immuno-inflammatory cascade. When HA receptor-mediated endocytosis of these pro-inflammatory HA fragments are blocked

unremitting inflammation and consequent fibrosis ensues that eventually results in tissue destruction.

Thus, HA fragments regulate the innate immuno-inflammatory response through different receptors. For example, signaling through CD44 in murine macrophages augments TNF $\alpha$ , IL-1 $\beta$ , and IGF-1 production [50, 77]. TNF $\alpha$  activates fibroblasts further promoting collagen deposition, which if uncontrolled can lead to fibrosis [77]. In contrast, HA fragment interactions with TLR-2 and -4 are proinflammatory, whereas HMWHA interactions with TLR-2/4 are anti-inflammatory [78, 79]. CD44  $-/-$  macrophage models have a blunted inflammatory response, whereas double TLR-2 and -4 knockout models obliterated the inflammatory response from HA fragments but overall have increased susceptibility to non-infectious tissue injury [70, 80]. TLR-2 and -4 knockout models lose the anti-inflammatory protective effect of HMWHA cell-surface association [70]. HA fragments function to augment the immune-inflammatory cascade.

#### *Native HA:*

HMWHA is largely anti-inflammatory and evidence of its beneficial contribution to wound healing is evident in patients with burns, venous stasis or diabetic ulcers [81]. A rat model of inflammation also supports the anti-inflammatory effects of HMWHA. Topical HMWHA ( $4 \times 10^6$  kDa) applied directly to the laminectomy site prior to wound closure decreased the number of inflammatory cytokines and cells present during the acute phase of wound healing [82]. HMWHA dampens the magnitude of an inflammatory response by blocking the interaction of LMWHA with pro-inflammatory

receptors, inhibiting migration of white blood cells, and diluting the effect of inflammatory cell mediators [30, 82]. Although HMWHA is anti-inflammatory in experimental models, previous studies attempting to use topical HMWHA in the prevention of radiation dermatitis have shown contradictory results in terms of effects on inflammation [83-85]. Therefore further research is needed to clarify the relationship between exogenous HMWHA and supporting its potential anti-inflammatory effects.

### Proinflammatory Skin Disorders

Dysregulation of the immuno-inflammatory cascade, either from intrinsic or extrinsic influences, can contribute to pro-inflammatory skin disorders [66]. For example, unintentional increased levels of fragmented or LMWHA can support a proinflammatory tissue state. In addition, any topically applied molecule has a theoretical risk of causing either an allergic (ACD) or irritant contact dermatitis (ICD). Both ACD and ICD can result from contact with non-protein chemical molecules, and the severity of the cutaneous reaction depends on the characteristics of the contact itself and the robustness of individual inflammatory responses [65]. Contact dermatitis are common family of inflammatory skin diseases where damage to cutaneous integrity results in activation of the innate immune system [65]. A non-specific pro-inflammatory reaction ensues with hyper-production of cytokines such as IL-1 $\alpha$ , IL-6, IL-8 and TNF $\alpha$  leading to apoptosis of epidermal cells. Resolution usually occurs following elimination of the inciting stimulus. In the development of new skin care products, it is important to consider the likelihood of the product stimulating a local or systemic inflammatory reaction.

### **Commercial topical hyaluronan applications**

Biotechnology companies have capitalized using a plethora of HA applications to create a niche market valued at over a billion dollars annually [86]. The skin industry represents one of the primary stakeholders. Current commercial applications of HA aim to replace endogenous HA with aging, augment tissue volume by exploiting the known hydrophilic and anti-oxidant properties of HA, or as a component in wound dressings to promote healing. Injectable HA fillers used in cosmetic dermatology usually require cross-linking to improve stabilization and longevity. Overall, exogenous HA containing products have tried to emulate the physiological microenvironment of youthful skin to optimize tissue repair and rejuvenation.

HA can be produced from either animal or non-animal sources. The most common animal derived HA is from rooster coombs [87]. Whereas, non-animal derived HA is usually produced through a process of bacterial fermentation [52, 87]. HA has no species specificity and is non-allergenic [87]. It is also semi-permanent and naturally degraded in our skin. Thus, HA has a strong safety profile with few reported adverse outcomes.

It is well known that an intact SC is the primary barrier to topical administration of high molecular weight hydrophilic molecules. Current HA technology has circumvented this issue by using injectable formulations or topical penetration enhancers. Injectable cross-linked HA dermal fillers offer a painful and transient correction of volume, without addressing the remaining physiologic deficits of aged skin. Pavicic *et al.* (2011) looked at the effect of topical administration of HA creams of different molecular weights on

periorbital wrinkles in women aged 30-60 [52]. Their group only showed an effect with LMWHA (50-130 kDa), which they attributed to poor penetration of the HMWHA groups [52] yet HMWHA is potentially a safer, more effective skin replacement since homeostatic skin HA is largely in a native HMW form. Topical application of HMWHA requires the use of penetration enhancers. Established penetration enhancers to aid the delivery of HMWHA include chemical methods (e.g. DMSO), iontophoresis, microneedles, and lipid nanoparticles/microemulsions [88]. However, the use of penetration enhancers often leads to an unpredictable retention and activity of exogenously applied HA [89]. A major problem with liposomes, nanoparticles and other particulate enhancing formulations is that they are phagocytosed by cells and then HA is rapidly destroyed in lysosomes. To date topical HA delivery modalities have failed to reliably replenish native HMWHA in aged skin.

### **Identifying the problem**

Native epidermal HMWHA is linked with a youthful skin phenotype. Replenishing depleted HMWHA in the aged epidermis is optimal therapeutically but challenging as HMWHA is highly hydrophilic and does not easily penetrate the lipophilic stratum corneum (SC). Current penetration enhancers limit the retention or are rapidly destroyed by skin cells of HA in the skin. In addition, the stability of exogenous HA depends on interactions with HA receptors such as CD44, which show diminished expression with aging [57, 62]. A more ideal formulation for topical HMWHA delivery would have reliable penetration and retention in the skin and occur in a form that is not rapidly taken up and destroyed by skin cells.



**HA-PE: a novel skin care technology**

Hyaluronic acid phosphatidylethanolamine (HA-PE) is a patented technology (WO2011/140630A1) developed in Dr. Turley's lab. HA-PE is a modified hyaluronic acid with approximately 6% of the disaccharide units of the native hyaluronan covalently linked to phosphatidylethanolamine (PE). Phospholipids are the major components of the plasma membrane and are therefore biocompatible molecules and are non-allergic [89]. In addition, phospholipids have the advantage of being amphiphilic [89]. Soy lecithin is a heterogenous mixture of phospholipids, but most are neutral and do not react with HA. PE is the most common phospholipid in lecithin. PE has both an amino and a carboxyl group that can interact with the carboxyl group on the HA molecule. In the HA-PE reaction the amide-linking agent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), is also the limiting reagent and thus the ratio of EDC to HA ensure the consistency of the formulation. After mixing HA-PE in a 1:1 (v/v) ratio with a commercially available base cream, it is ready for topical application without the addition of further penetration enhancing agents. Unlike the cross-linked HA of injectable fillers, topical HA-PE transverses the skin barrier and forms pericellular coats mimicking endogenous HA configurations. In addition, HA-PE is retained in skin independent of HA receptors including CD44/RHAMM. HA-PE cream is a novel technology for topical penetration of HMWHA.

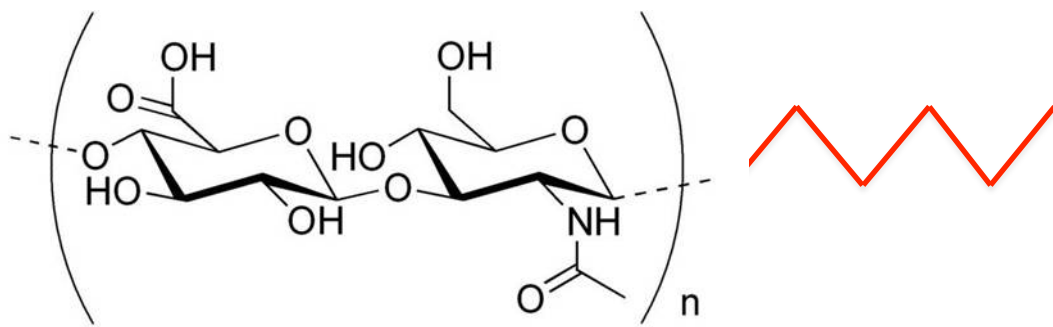


Figure 3. **Schematic diagram of HA-PE molecular structure.** Molecular structure of HA is shown with attached phospholipid tail (shown in red).

## Chapter 2: Thesis Objectives and Aims

An *in vivo* study design using a murine model coupled with molecular analysis techniques was used to assess the effects of a novel cream formulation, HA-PE. Once daily, 0.18g of cream from one of three treatment arms (HA-PE, HA, or Vehicle Cream alone) was applied to a shaved area on the back of retired breeder female mice. At three time points (24 hours, 5 days, and 10 days) following the first cream application, five mice from each treatment arm were sacrificed to harvest a full-thickness biopsy of the treated skin and a blood sample. Using an aged murine model, this thesis examined the effect of topical HA-PE cream on the epidermis using three primary objectives, which are outlined below.

### **Topical HA-PE and the skin barrier**

The addition of phosphatidylethanolamine groups to a high molecular weight (~ 500 kDa) hyaluronan will permit it to traverse the stratum corneum and be retained in the keratinocytes of the epidermis as a pericellular coat following a single topical application.

**Objective 1: To determine if HA-PE crosses the skin barrier following topical application.**

**Aim 1:** Following a single topical application of Alexa<sup>647</sup> tagged HA-PE and HA, harvest skin biopsies at 40 min, 2h, 4h, 8h, 24h, 48h, and 72h post application to quantify the amount of fluorescence in the epidermis.

### **Topical HA-PE and the epidermis**

High-molecular weight hyaluronan in the epidermis exists primarily in the extracellular matrix forming pericellular coats around keratinocytes. This configuration forms a fluid scaffold that plays an important role in cell proliferation, differentiation, and signaling.

**Objective 2: To characterize the effect of multiple applications of topical HA-PE on the rejuvenation capacity of the epidermis.**

**Aim 1:** Using hematoxylin and eosin stained skin specimens, quantify epidermal thickness.

**Aim 2:** Using paraffin processed skin specimens, quantify the amount of staining for immunohistochemistry markers of keratinocyte proliferation (Ki67) and keratinocyte differentiation (K10) using image analysis software (ImageJ).

### **Topical HA-PE and Inflammation**

High molecular weight hyaluronan can be degraded into proinflammatory low molecular hyaluronan fragments. This thesis aims to examine the possibility of a proinflammatory effect of topical HA-PE. Since localized inflammation manifests systemically, this was examined at the site of topical application and in blood.

**Objective 3: To determine if multiple topical HA-PE cream applications elicit either a local and/or systemic response.**

**Aim 1:** Using paraffin processed skin specimens, quantify the amount of staining of immunohistochemistry markers of activated Langerhans cells and macrophages (F4/80) as a marker of local inflammation.

**Aim 2:** Identify the presence of an additional downstream marker of local inflammation,  $TNF\alpha$ , in tissue samples using an ELISA.

**Aim 3:** Examine systemic inflammation by comparing serum C-reactive protein samples using an ELISA kit.

## Chapter 3: Methods

All experiments were approved by ethics and compliant with the standard operating protocols of the Animal Use Subcommittee at the University of Western Ontario, Canada.

### **Topical cream formulation**

HA-PE cream formulation:

For preparation of the HA-PE cream (CA Patent PCT/CIPO 2,703,532), 1.35 mL of unrefined liquid soy lecithin (Soy Lecithin GT non-GM IP, Imperial-Oel-Import, Germany) was mixed thoroughly at room temperature with 1.35 mL of 1% v/w Sodium Hyaluronate Solution (500 kDa, Medical Grade, Lifecore Biomedical, Chaska, MN, USA) and 252  $\mu$ L of isopropanolol. Subsequently, 3.78 mg of dry 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added and mixed thoroughly as a linking agent. After mixing for 10 – 15 minutes, 3 mL of vehicle cream was added and mixed for another 5 minutes. A water-based cream (Mango Face cream, Aquatech, Toronto) used throughout the study as the vehicle for mixing with unmodified HA or HA-PE. Creams were stored protected from light at 4° C.

HA cream formulation:

For preparation of the HA cream, 1.35 mL of 1% v/w Sodium Hyaluronate Solution was mixed thoroughly with 3 mL of vehicle cream. Creams were stored protected from light at 4° C.

### Alexa<sup>647</sup> mouse experiments

Preparation of Alexa<sup>647</sup>-HA:

In the preparation of Alexa<sup>647</sup>-HA, the solution was protected from ambient light. In a laminar flow hood, 0.0028g of EDC (Sigma Aldrich) was dissolved in a 1 mL solution of 20 mM MES and 30% ethyl alcohol (pH 4.5) in a 15 mL tube. Subsequently, 200 uL of pharmaceutical grade 1% v/w Sodium Hyaluronate Solution (500 kDa) (Lifecore Biomedical, Chaska, MN, USA) was added. After five minutes, 300 uL of Alexa Fluor® 647 Hydrazide, Tris (triethylammonium) salt (Life Technologies) was added. The Alexa<sup>647</sup>-HA solution was placed on a rocker at room temperature for 12 hours.

The Alexa<sup>647</sup>-HA solution was then injected into a Slide-A-Lyzer® dialysis cassette (10,000 MWCO, ThermoScientific) and placed in a 2 L dialysate of 1x PBS with a stirring bar in a 4°C room. The entire volume of 1X PBS buffer was exchanged at 1, 3, and 5 days. The Alexa<sup>647</sup>-HA solution was retrieved from the Slide-A-Lyzer® and stored in a 15 mL tube in the 4 °C fridge.

Animals:

Twelve retired breeder female C57BL/6 mice (The Jackson Laboratory) were used for the Alexa<sup>647</sup> mouse experiments. Older mouse skin is general defined as > 52 weeks of age [56]. Animals were individually caged in a temperature-controlled breeding room with a 12h light/dark cycle, and fed a standard diet. The breeding room had low ambient light exposure to prevent degradation of the fluorescent Alexa<sup>647</sup> probe while awaiting harvest of the tissue specimens.

Treatment of Alexa<sup>647</sup>-HA/HA-PE mice:

Twelve retired breeder female C57BL/6 mice were used for the Alexa<sup>647</sup> mouse experiments. For the initial cream application, each mouse was anesthetized using Isoflurane gas. While anesthetized, the upper dorsum of the back was shaven with an electric razor, leaving a strip of hair in midline to define the right and left sides. The left side was treated with Alexa<sup>647</sup>-HA cream and the right side was treated with Alexa<sup>647</sup>-HA-PE cream. A total of 0.18 g of treatment cream was applied to the respective treatment areas. Mice were individually caged and kept in the animal facility's breeding room to avoid high levels of ambient light exposure. Two mice were euthanized using a CO<sub>2</sub> chamber at each of six time points: 40 minutes, 2 hours, 4 hours, 8 hours, 24 hours, and 72 hours following cream application.

Preparation of Alexa<sup>647</sup> mouse tissue:

A full-thickness biopsy of the entire Alexa<sup>647</sup>-HA/HA-PE treated area was obtained and lightly fixed for 10 minutes in a solution of 1.5% w/v paraformaldehyde (Sigma Aldrich) and 0.5% w/v cetylpyridinium chloride monohydrate (Sigma Aldrich) in 1 x PBS. Each specimen was fixed in 4% paraformaldehyde (pH 7.4) and stored at -4 °C, follow which samples were paraffin embedded and sectioned at 10 µm thickness using a Microm HM 200 Ergostar Microtome (GMI; Ramsey, Minnesota, USA). A glass coverslip was mounted on each slide using Prolong® Gold anti-fade reagent with DAPI (LifeTechnologies).



Analysis of Alexa<sup>647</sup>-HA-PE mice:

Ten images of each sample were taken per a mouse at 20X magnification using a Nikon Eclipse motorized upright microscope (Nikon Canada Inc). Images were analyzed using ImageJ 1.47). The pixel density per an area of epidermis using the Alexa<sup>647</sup> channel was averaged per a mouse.

### **Multiple daily topical cream application experiments**

Animals:

Forty-five retired ten-month old female C57BL/6 mice (The Jackson Laboratory) were used for the multiple cream application studies. Animals were individually caged in a temperature-controlled environment with a 12 h light/dark cycle, and fed a standard diet.

Wounded mouse positive control for inflammation studies:

The inflammatory phase of wound repair in addition to acute and chronic models of cutaneous inflammation share similar profiles of immune cells and chemical messengers. For example, upregulated expression of TNF $\alpha$ , a pro-inflammatory cytokine, is observed during the inflammatory phase of wound healing [74] in addition to acute and chronic inflammatory skin disorders [72]. Activated macrophages known for their role in wound healing, are also recognized as an important mediator in chronic inflammatory skin disorders such as psoriasis [90]. Thus macrophages and pro-inflammatory cytokines, such as TNF $\alpha$ , appear to be ubiquitous players in cutaneous inflammation regardless of the inciting stimulus. A wounded mouse model was chosen as a positive control for the local inflammation studies in this experiment.

An additional wounded mouse was created as a positive control for the inflammatory marker evaluations. This was a single ten-month old C57BL/6 mouse (The Jackson Laboratory). After being anesthetized with Isoflurane gas, a 4 mm full-thickness skin wound was created on the upper back of the mouse. The wounded mouse was caged independently and stored in a temperature-controlled environment with a 12 h light/dark cycle, and fed a standard diet. The wounded mouse was euthanized via a CO<sub>2</sub> chamber 72 h post-wounding. The entire wounded area with a 2 mm perimeter of normal tissue was excised. This area was divided into two parts: one half was kept for permanent sectioning and the other half was stored at – 80 °C.

Treatment of multiple cream application mice:

At baseline, mice were divided into three sets of fifteen mice to define the three treatment arms: HA-PE cream, HA cream, and vehicle cream. For the initial cream application, each mouse was anesthetized using Isoflurane gas. While anesthetized, the upper dorsum of the back was shaven with an electric razor and 0.18 g of treatment cream was applied to the entire shaved area. Subsequent once daily cream applications were performed without anesthesia. Groups of five mice in each treatment arm (HA-PE, HA, and Vehicle cream) were sacrificed after one, five, and ten cream applications.

Animals were euthanized using a carbon dioxide chamber 24 h following the last cream application. A blood sample from each mouse was obtained via a cardiac puncture immediately following cessation of respiration. Subsequently, two four-millimeter punch

biopsies were obtained. The remainder of the shaven treatment area was excised and stored at  $-80^{\circ}\text{C}$ .

#### Tissue preparation:

Tissue biopsies were fixed in a 4 % paraformaldehyde solution at  $4^{\circ}\text{C}$  for 24 h. Samples were then embedded in paraffin blocks and  $4\ \mu\text{m}$  sections were cut using a Microm HM 200 Ergostar Microtome (GMI; Ramsey, Minnesota, USA).

#### **Effect of HA-PE on the epidermis**

##### Epidermal Thickness Analysis:

To evaluate epidermal thickness, sections stained with hematoxylin and eosin were uploaded to ImageJ 1.47 and analyzed at 4X magnification. For each mouse, fifty serial measurements at  $75\ \mu\text{m}$  intervals per section were made to determine epidermal thickness (stratum basale to the stratum granulosum).

##### Ki67 and K10 Immunohistochemistry:

Paraffin-embedded sections obtained from each mouse were processed for immunohistochemical staining. Sections were deparaffinized in xylene and rehydrated in a graded ethanol series. The sections were heated to a boil using a microwave oven in 0.01 M aqueous sodium citrate buffer (pH 6.0) for antigen retrieval. Following antigen retrieval, the sections were washed in 1X PBS three times for 5 min each and treated with 3.0 % hydrogen peroxide for 10 min. Further washes with 1 x PBS were performed

before sections were blocked with 3.0 % bovine serum albumin for 1 hour at 4 °C. Then, sections were incubated with diluted primary antibodies for 18-24h at 4 °C. The following primary antibodies were used: rabbit Ki67 monoclonal (1:100 dilution; Abcam ab16667) and rabbit K10 monoclonal (1:7000 dilution, Abcam ab76318). Following washes, sections were incubated for 2 h at room temperature with biotinylated polyclonal secondary antibodies (1:500 dilution, DAKO). Sections underwent further washes and were reacted with streptavidin-horseradish peroxidase (1 in 2000 dilution, Abcam) for 30 min. Staining was visualized with diaminobenzidine (DAB) (2-component DAB kit; DAKO). Sections were counterstained with hematoxylin and washed with distilled water. A final ascending ethanol series and xylene wash were performed before cover slip application using Richard-Allan Scientific Cytoseal 60 (ThermoScientific).

#### Keratinocyte Proliferation (Ki67) and Keratinocyte Differentiation (K10) Analysis:

Using Aperio© ImageScope (Aperio ePathology Solutions; <http://www.aperio.com/>) a ruler measuring 500 µm increments was placed parallel to and above the epidermis of each specimen. A total of two to three images per specimen were obtained at alternating 500 µm sections using 18.0X magnification. Specimens with significant ripping or damage were excluded from analysis. The Aperio image was saved and transferred to ImageJ 1.47. In the ImageJ 1.47 software, the epidermis area was traced along the 500 µm marked distance. The traced area was saved and isolated as a new image.

For the keratinocyte (Ki67) proliferation analysis, a total count of the basal keratinocyte nuclei in the selected area was recorded. Subsequently, the new image was deconvoluted

under the 'H DAB' setting. The deconvolution process allowed the software to separate the different colour channels of the image to allow for isolation of the positive brown staining from the Ki67 antibody. The deconvoluted image was set to a threshold pixel range of 10-31 arbitrary units, to set a conservative assessment for identifying positively stained cells. The number of positively stained nuclei was then recorded. The ratio of positively stained nuclei to the total nuclei count was used to determine the percentage of Ki67 staining per an area of epidermis.

For the keratinocyte (K10) differentiation analysis, an area measurement recorded in pixels of the new image of 500 um of traced epidermis was obtained. The image was deconvoluted under the 'H DAB' setting. The deconvoluted image was set to a threshold pixel range of 10-60 arbitrary units, to set a conservative estimate for identifying positively stained cells. Using the Histogram analysis tool, a pixel count of the amount of positive K10 staining was recorded. The ratio of the number of pixels of K10 staining to the total area pixels was used to determine the percentage of K10 staining per area of epidermis.

For both Ki67 and K10, five images analyses were performed and averaged per an individual mouse. Pooled average data was then calculated per treatment arm (HA-PE, HA, and Vehicle) and time point (1 day, 5 days, 10 days).

### **Effect of HA-PE on inflammatory markers**

#### **F4/80 Immunohistochemistry:**

The immunohistochemistry protocol was identical as previously described. The primary used in this series was a rat F4/80 monoclonal antibody (1:100 dilution, AbD Serotec). F4/80 antibody is used to detect a surface marker present on mature mouse macrophages.

#### **F4/80 Analysis:**

Aperio© ImageScope (Aperio ePathology Solutions; <http://www.aperio.com/>) was used to obtain images of F4/80 slides at 20X magnification. Specimens with significant ripping or damage were excluded from analysis. The Aperio images were saved and transferred to ImageJ 1.47. Using ImageJ, the area of the entire dermis and subcutaneous tissue was traced for each specimen. Subsequently, the number of F4/80 positively stained cells within this area were recorded. The wounded mouse specimen was used as a positive control for this experiment.

The number of F4/80 positive cells per area was expressed as a percentage of the number of F4/80 positively stained cells in the wounded mouse positive control. Seven to ten specimens were analyzed per a mouse.

#### **TNF- $\alpha$ ELISA Methods:**

##### *Protein Isolation*

Individual dissected tissue samples from each of the day 10 multiple application trial mice were mixed gently with 300  $\mu$ L of RIPA lysis buffer and a protease inhibitor tablet

(Roche) for 10 minutes. An additional tissue sample from the wounded C57BL/6 mouse was prepared as a positive control. Subsequently, each sample was lysed further using a sonicator for three 20-second pulses. Samples were put on a shaker in the 4 ° C cold room for an additional 45 minutes. Subsequently, samples were centrifuged at 4 ° C for 30 minutes at 13 000g. The resultant supernatant from each sample was transferred to a new eppendorf tube, and the pellet was discarded.

#### *TNF- $\alpha$ ELISA protocol*

A TNF- $\alpha$  mouse ELISA kit (Abcam (ab100747)) was used as per the manufacturers instructions to evaluate the presence of TNF- $\alpha$  in the experimental samples and wounded mouse positive control sample. Triplicates of 100 uL of each sample supernatant were used. The absorbance of each set of triplicates was read at 450 nm using a Wallace Victor<sup>2</sup> 1420 Multilabel Counter and Manager software (v 3.00.0.53, © 1997-2005 Perkin Elmer).

The average zero standard sample absorbance was subtracted from the average absorbance of each triplicate repeat. The final value for each mouse was then evaluated as a percentage of the average absorbance of the wounded mouse positive control.

C-Reactive Protein ELISA Methods:

#### *Sample Preparation*

Serum obtained via cardiac puncture at the time of euthanasia was collected in a sodium heparin coated vacutainer tube (BD, Franklin Lakes, NJ, USA). Within one hour of

collection, the serum samples were centrifuged at 3000 rpm at 4 °C for 10 minutes using an Eppendorf Centrifuge 5810R 15amp version. The supernatant was transferred to individual labeled containers and stored at – 80 °C until analysis.

#### *C-Reactive Protein (CRP) ELISA Protocol*

A C-reactive protein mouse ELISA kit (Abcam (ab157712)) was used as per the manufacturers instructions to quantify the presence of serum CRP in the experimental samples. Triplicates of 100 uL of each sample supernatant and standards were used. The absorbance of each set of triplicates was read at 450 nm using a Wallace Victor<sup>2</sup> 1420 Multilabel Counter and Manager software (v 3.00.0.53, © 1997-2005 Perkin Elmer).

The final serum CRP concentration (ng/mL) for each mouse was calculated using the slope of the curve (absorbance versus concentration) generated for the standard samples provided by the manufacturer.

#### **Statistical Analysis**

Parametric evaluations, including Student's T Test and OneWay ANOVA, were used to determine statistical significance between groups using a p value of 0.05. Tukey's post-hoc analysis was used to identify which groups exhibited a statistically significant difference. Data are expressed as mean  $\pm$  standard error of the mean (SEM) of at least five independent samples as described above. Statistical analysis was performed using GraphPad Prism 6 (GraphPad software).



## Chapter 4: Manuscript

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**Hyaluronan-Phosphatidylethanolamine polymers forms pericellular coats on keratinocytes and promotes basal keratinocyte proliferation.**

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**ABSTRACT**

Aged keratinocytes have diminished proliferative capacity and hyaluronan (HA) cell coats, which are losses that contribute to atrophic skin characterized by reduced barrier and repair functions. We formulated HA-phospholipid (phosphatidylethanolamine, HA- PE) polymers that form pericellular coats around cultured dermal fibroblasts independently of CD44 or RHAMM display. We investigated the ability of these HA-PE polymers to penetrate into aged mouse skin and restore epidermal function *in vivo*. Topically applied Alexa<sup>647</sup>-HA-PE penetrated into the epidermis and dermis, where it associated with both keratinocytes and fibroblasts. In contrast, Alexa<sup>647</sup>-HA was largely retained in the outer cornified layer of the epidermis and quantification of fluorescence confirmed that significantly more Alexa<sup>647</sup>-HA-PE penetrated into and was retained within the epidermis than Alexa<sup>647</sup>-HA. Multiple topical applications of HA-PE to shaved mouse skin significantly stimulated basal keratinocyte proliferation and epidermal thickness compared to HA or vehicle cream alone. HA-PE had no detectable effect on keratinocyte differentiation and did not promote local or systemic inflammation. These effects of HA-PE polymers are similar to those reported for endogenous epidermal HA in youthful skin and show that topical application of HA-PE polymers can restore some of the impaired functions of aged epidermis.

**KEYWORDS:** hyaluronan, hyaluronan-phosphatidylethanolamine, pericellular coats, keratinocyte, proliferation

**CONFLICT OF INTEREST:** The author(s) declare that there are no conflicts of interest regarding the publication of this article.

## INTRODUCTION

Hyaluronan (HA) is an ubiquitous extracellular matrix tissue polysaccharide belonging to the glycosaminoglycan family, which are characterized by repeating hexosamines and uronic acid [1-3]. Skin HA accounts for approximately 50% of total body HA and occurs in both the epidermal and dermal layers. Here it performs a variety of functions that are related to its rheological, viscoelastic and biological properties[4-6]. For example, its rheological properties contribute to the overall quality, hydration, permeability and immune barrier functions of skin while its unique viscoelastic properties protect skin cells from mechanical damage [7-11]. The biological properties of HA include a contribution to cell survival, proliferation and migration, and result from its ability to activate key signaling cascades through interactions with cellular HA receptors, which in keratinocytes is primarily CD44 [12-18]. HA is also an important regulator of skin immune surveillance [19] and response to injury processes [15, 20-25]. Although the primary structure of HA is simple and composed only of linear repeating N-acetyl-D- glucosamine and glucuronic acid disaccharides, its functions are complexly regulated and dependent upon its organization by extracellular and cellular proteins as well as by polymer size [14, 16, 21, 23].

The organization of HA in the extracellular matrix and around cells is a critical component of its skin functions [6, 26, 27]. On keratinocytes, HA is structured as compact pericellular coats that are maintained by CD44. Dermal HA is more

abundantly extracellular and linked to a variety of proteoglycans including versican [28-34]. The cellular and extracellular organization of skin HA is critical for its retention in the papillary dermal and keratinocyte layers. Quantitative loss of HA from these layers is associated with skin pathologies including poor wound healing, reduced skin elasticity/mobility, and loss of keratinocyte tight-junctions and permeability barrier functions [27, 29, 35-45]. Polymer size also contributes to the skin functions of HA. For example high molecular weight (HMW), native HA in skin protects against tumor initiation [46], provides intrinsic water binding properties of skin [26] and is required for dendritic cell functions [15, 19, 47]. It regulates the proliferation and differentiation of the basal keratinocyte layer during homeostasis and response to injury [4, 6, 48, 49], and contributes to the barrier/hydration function [26, 34, 50] and structure of the stratum corneum [51].

Most HA in homeostatic skin is high molecular weight but fragmentation occurs following injury or prolonged exposure to UV. In cooperation with the fragmentation of other extracellular matrix components, HA fragments activate signaling cascades in keratinocytes and dermal fibroblasts that control migration, survival and re-differentiation required for repair of injured skin [52-59]. HA fragments are also key regulators of innate immunity and are required for in-trafficking and pro-inflammatory cytokine expression of macrophages [15, 19]. The different functional effects of native versus fragmented HA likely result from selective interactions with specific receptors and differential effects of polymer size on the clustering/signal activation through these receptors [13, 15, 60]. The effects of native HA on homeostatic keratinocyte functions

are mediated through CD44 [30, 38] while repair functions of HA fragments involve coordination of signaling through CD44: RHAMM and TLR2, 4 complexes [56, 61, 62].

Chronological skin aging results in physiological alterations of keratinocytes and epidermal functions that contribute to epidermal thinning or atrophy, barrier dysfunction, delayed wound repair, as well as increased susceptibility to pathologies including ulceration, dermatitis and eczema [4, 61, 63]. Although age-associated epidermal dysfunction is not well understood, it is associated with changes in HA concentration and organization, and CD44 display [38, 64]. Experimental models have established that reduction or loss of keratinocyte CD44 results in epidermal changes that are similar to aging dysfunction such as thinning of the epidermal layer, barrier dysfunction, modified HA metabolism, reduced HA production, altered keratinocyte differentiation and decreased skin elasticity. Application of HA to aged mouse skin partially restores permeability barrier homeostasis and epidermal thickness [42, 43]. Topical or injected HMW HA products have had variable effects in restoring a sustained physiological and hydrated microenvironment of youthful skin required for optimizing tissue repair and rejuvenation [38, 63, 65, 66]. Trans-epidermal or dermal HA delivery modalities, although promising, have similarly failed to reliably replenish sustained high levels of native HMW HA in the epidermis or dermis of aged skin [67-69]. This failure is likely because of poor penetration of topically applied HA formulations of MW greater than 50 KDa [40, 70-72], reduced HA capture in the epidermis as a result of declining CD44 levels [38, 43, 73] and

aberrant organization as well as rapid clearance of the exogenous HA formulations [41, 61].

To address this problem, we developed a cell-based screening method for identifying HA-phospholipid (phosphatidylethanolamine, HA-PE) formulations that form pericellular HA coats on fibroblasts and keratinocytes in a CD44-independent manner [74]. We show that topical administration of HA-PE to the shaved skin of aged wild type or CD44<sup>-/-</sup> mice increased HA within the epidermal layer. This HA modification promoted basal keratinocyte and hair follicle proliferation as well as increased epidermal thickness but does not detectably alter the differentiation of keratinocytes. In addition, topical application of HA-PE *in vivo* did not result in either a local or systemic inflammatory response.

## METHODS

Animals:

Forty-five retired breeder ten-month old female C57BL/6 mice (Jackson Laboratory) were used for the multiple cream application studies. An additional twelve retired breeder twelve-month old female C57BL/6 mice were used for the Alexa<sup>647</sup> mouse experiments.

Animals were individually caged in a temperature-controlled environment with a 12h light/dark cycle, and fed a standard mouse chow diet. All experiments were

approved by and compliant with the standard operating protocols of the Animal Use Subcommittee at the University of Western Ontario, Canada (2009-051).

#### Preparation of HA-PE and HA:

For preparation of the HA-PE cream (Patent identification: WO2011140630 A1)[75], 1.35 mL of unrefined liquid soy lecithin (Soy Lecithin GT non-GM IP, Imperial-Oel-Import, Germany) was mixed thoroughly at room temperature with 1.35 mL of 1% v/w Sodium Hyaluronate Solution (500 kDa, Medical Grade, Lifecore Biomedical, Chaska, MN, USA) and 252  $\mu$ L of isopropanolol. Subsequently, 3.78 mg of dry 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added and mixed thoroughly as a linking agent. After mixing for 10 – 15 minutes, 3 mL of vehicle cream was added and mixed for another 5 minutes. For preparation of unmodified HA cream, 1.35 mL of 1% v/w Sodium Hyaluronate Solution was mixed thoroughly with 3 mL of vehicle cream.

A water-based cream (Mango Face cream, Aquatech, Toronto) was used throughout the study as the vehicle for mixing with unmodified HA or HA-PE. Formulated creams were stored protected from light at 4° C.

#### HA pericellular coat detection:

HA pericellular coats were detected using particle exclusion [76]. Dermal wild type, CD44<sup>-/-</sup>, RHAMM<sup>-/-</sup> and CD44:RHAMM<sup>-/-</sup> embryonic fibroblasts were plated onto 35 mm tissue culture dishes in DMEM + 10% FBS for 24 hrs. Cells were then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, 5 mM CaCl<sub>2</sub>, pH 7.2, for 30 min then

washed gently in Cacodylate buffer. 1 ml of either FITC-labeled 0.4  $\mu\text{m}$  microspheres (Invitrogen) or formalized sheep erythrocytes ( $1 \times 10^8$  erythrocytes/ml) were added to each 35 mm culture dish and swirled gently so that cells were evenly covered. Dishes were incubated for 15-30 min at 37 °C to let beads or erythrocytes settle around cells. As a control, cells were incubated with 200  $\mu\text{g/ml}$  bovine testicular hyaluronidase (Sigma) for 1 h at 37 °C prior to performing the particle exclusion assay. Cells were then photographed with a Nikon inverted microscope equipped with epifluorescence and Hoffmann optics.

#### Preparation of Alexa<sup>647</sup>-HA:

In the preparation of Alexa<sup>647</sup>-HA, the solution was protected from ambient light. In a laminar flow hood, 0.0028 g of EDC (Sigma Aldrich, <http://www.sigmaaldrich.com>) was dissolved in a 1 mL solution of 20 mM MES and 30% ethyl alcohol (pH 4.5) in a 15 mL tube. Subsequently, 200  $\mu\text{L}$  of pharmaceutical grade 1% v/w Sodium Hyaluronate Solution (500 kDa) (Lifecore Biomedical, Chaska, MN, USA) was added. After five minutes, 300  $\mu\text{L}$  of Alexa Fluor ® 647 Hydrazide, Tris (triethylammonium) salt (Life Technologies, <http://www.lifetechnologies.com>) was added. The Alexa<sup>647</sup>-HA solution was placed on a rocker at room temperature for 12 hours then dialyzed (10,000 dalton cut off, Thermoscientific) at 4 °C. The entire volume of 1X PBS buffer was exchanged at 1, 3, and 5 days. The retained Alexa<sup>647</sup>-HA solution was retrieved from the dialysis apparatus and stored in a 15 mL tube in the 4 °C fridge.



#### Application of Alexa<sup>647</sup>-HA/HA-PE to mice:

Mice were anesthetized using Isoflurane gas for application of cream. While anesthetized, the upper dorsum of the back was shaved with an electric razor, leaving a strip of hair in midline to define the right and left sides. 0.18 g of Alexa<sup>647</sup>-HA and Alexa<sup>647</sup>-HA-PE cream was applied to the left and right sides respectively. Two mice were euthanized using a CO<sub>2</sub> chamber at each of six time points: 40 min, 2 h, 4 h, 8 h, 24 h, and 72 h following cream application.

#### Preparation and analysis of Alexa<sup>647</sup>-HA-PE:

A full-thickness biopsy of the Alexa<sup>647</sup>-HA/HA-PE treated area was obtained and lightly fixed for 10 minutes in 1.5 % paraformaldehyde containing 0.5 % cetylpyridinium chloride monohydrate [76] in PBS. Each specimen was subsequently fixed in 4 % paraformaldehyde (pH 7.4) for an additional 30 min then stored at – 4 ° C. Tissue samples were paraffin processed, mounted then analyzed with a Nikon Eclipse motorized upright microscope. The penetration of Alexa<sup>647</sup>-HA/HA-PE into the epidermis was quantified using ImageJ, which converted fluorescent images to pixel density per a tissue area. A total of twelve C57BL/6 retired breeder female mice were used for this experiment.

#### Treatment of mice with HA-PE:

Three treatment arms were used for these experiments: HA-PE cream, HA cream, and vehicle cream and 15 mice were used/group. For the initial cream application mice

were anesthetized, shaved and treated as described above. Subsequent once daily cream applications were performed without anesthesia. Five mice in each treatment arm were sacrificed after one, five, and ten applications. At each of these times, mice were euthanized, a blood sample via a cardiac puncture and two four-millimeter punch skin biopsies were obtained. The remainder of the shaven treatment area was excised and stored at  $-80^{\circ}\text{C}$ . To provide a positive control for inflammation markers, an additional mouse was wounded with a 4 mm punch biopsy as described previously [77], then tissue harvested using an 8 mm punch biopsy 3 days after wounding. All tissue biopsies were fixed in 4 % paraformaldehyde/PBS at  $4^{\circ}\text{C}$  for 24 h. Samples were then processed for paraffin histology sections. For immunohistochemistry, tissue sections were deparaffinized and antigen retrieval performed by heating tissue sections in a microwave oven in 0.01 M aqueous sodium citrate buffer (pH 6.0). Tissue sections were washed then incubated with one of the following primary antibodies: rabbit Ki67 monoclonal (1:100 Abcam), rabbit K10 monoclonal (1:7000 dilution, Abcam), and rat F4/80 monoclonal (1:100 dilution, AbD Serotec) followed by the appropriate biotinylated secondary antibody. Sections were counterstained with hematoxylin then mounted in Richard-Allan Scientific Cytoseal 60 (ThermoScientific). Digital images of stained tissue sections were obtained using an Aperio Scanscope. Five representative areas were taken per mouse and analyzed using ImageJ.

#### Measurement of Epidermal Thickness:

To evaluate epidermal thickness, the above tissue sections stained with hematoxylin and eosin (4X magnification images) were analyzed using Image J. For each mouse,

fifty serial measurements at 75  $\mu\text{m}$  intervals per section were made to determine epidermal thickness (*stratum basale* to the *stratum granulosum*) [78].

#### TNF- $\alpha$ ELISA:

Activated macrophages and proinflammatory cytokines, such as TNF $\alpha$ , appear to be ubiquitous players in cutaneous inflammation regardless of the inciting stimulus whether wounding or sterile inflammation [79-81]. Dissected tissue samples from each of treatment and group and wounded tissue were mixed gently RIPA lysis buffer and a protease inhibitor tablet for 10 min then sonicated for three 20-second pulses. Samples were incubated on a rotary shaker at 4 ° C in lysis buffer for an additional 45 min then centrifuged at 13,000 g (4 °C) for 30 min. The pellet was discarded and supernatant used for the TNF $\alpha$ -ELISA assays (Abcam), which were performed per the manufacturers instructions. Triplicate samples of 100  $\mu\text{L}$  of each were used for these assays.

#### C-Reactive Protein ELISA:

Serum was obtained from blood harvested via cardiac puncture. The serum samples were centrifuged at 3000 rpm at 4 °C for 10 mins using an Eppendorf Centrifuge and stored at – 80 °C until analysis. Samples were analyzed for the presence of C-Reactive protein using an ELISA kit (Abcam) and assays were performed using the manufacturers instructions. Triplicates samples (100  $\mu\text{L}$ ) of each sample were used for these assays.

### Statistical Analysis:

OneWay ANOVA and Tukey test, as a post-hoc analysis, were used to determine statistical significance between groups using a p value of 0.05. Data are expressed as mean  $\pm$  standard error of the mean (SEM) of at least five independent samples as described above. Statistical analysis was performed using GraphPad Prism 6 (GraphPad software).

## RESULTS

### A. Formulating and characterizing HA-PE polymers that forms pericellular coats

Using a cell-based screen, HA-PE polymer formulations were prepared. The polymer formulations that formed nanoparticles were discarded while those that were non-particulate and formed pericellular coats on fibroblasts were identified using particle exclusion assays (Figure 1A). Addition of HA-PE polymer resulted in a significant increase in the number of cells that formed pericellular coats compared to those formed when PBS alone was added. A light but significant increase in the number of cells forming coats was also stimulated by exogenous unmodified HA of the same size used for HA-PE formulations. Nevertheless, the addition of HA-PE stimulated coat formation to a significantly greater extent than HA alone and a trend to formation of larger coats was also observed (data not shown).

CD44 has been demonstrated to be critical for keratinocyte, fibroblast and smooth muscle cell HA coat formation [32-34, 41, 43] and both CD44 and RHAMM [12, 14, 62] have recently been implicated in the binding of HA to tumor cells and fibroblasts. The role of these HA receptors in the formation of endogenous HA and HA-PE coats was examined by comparing wild type primary mouse embryonic fibroblasts (MEF) with those lacking RHAMM, CD44 or both receptors due to genetic deletion of these genes (Figure 2A). Loss of RHAMM resulted in highly variable numbers of MEF forming endogenous pericellular coats but these were not significantly different from wild type fibroblasts. Loss of CD44 however resulted in a 6-fold reduction in the number of MEF exhibiting HA coats. Dual loss of CD44 and RHAMM did not further reduce coat loss from that observed in CD44<sup>-/-</sup> MEF. These results show that CD44 is the major HA receptor that facilitates endogenous HA coat formation in MEF consistent with previous studies of other cell types including keratinocytes [33, 40, 43]. The effect of HA-PE on RHAMM<sup>-/-</sup>, CD44<sup>-/-</sup>, RHAMM: CD44<sup>-/-</sup> and wild type MEF coat formation was compared next to begin to identify the mechanisms for HA-PE-promoted pericellular HA coats. As shown in Figure 2B, the addition of HA-PE to these different fibroblast genotypes resulted in a similar number of cells with HA coats indicating that HA-PE effects were not dependent upon HA receptor display including CD44, which has previously been shown to mediate endogenous coat formation (Figure 2 B, C). However, HA-PE stimulated coat formation in CD44<sup>-/-</sup> MEF was sensitive to hyaluronidase as were endogenous coats (Figure 2C). These results predict that addition of a PE group to HA promote its direct association with cells in a CD44-independent manner.

To determine if HA-PE promotes coat formation in other cell types particularly *in vivo*, its effect on skin keratinocyte HA pericellular coats, which have previously been shown to require CD44 expression [30, 43] was next evaluated. Adult mouse back skin was chosen for these studies since it has been reported to produce very little endogenous HA [29], permitting more sensitive detection of exogenous applications of HA-PE using staining methods to detect accumulated HA. Consistent with previous studies [29], very little HA staining was observed in control (PBS) keratinocytes. Topical application of HA-PE to wild type mice significantly increased HA staining in the epidermis (Figure 3) but did not detectably increase HA staining in the dermis when compared to PBS-treated controls likely because the dermis produces large amounts of HA and the staining method was not sensitive enough to detect elevation above this high background. These results show that HA-PE not only enhances pericellular HA coat formation in cultured MEF but also in epidermal keratinocytes *in vivo*. These results also show that HA-PE crosses the outer cornified epidermal layer more efficiently than unmodified HA.

The association of HA with keratinocytes in mouse skin *in vivo* depends upon CD44 expression [30]. We therefore next assessed if the loss of CD44 altered HA-PE mediated increases in keratinocyte associated HA *in vivo*. As shown in Figure 3B, topical application of HA-PE increased hyaluronan staining of CD44<sup>-/-</sup> epidermis compared to PBS controls suggesting that epidermal accumulation of HA-PE is CD44 independent *in vivo* similar to cultured MEF. To more directly follow the association of small amounts of HA-PE with the epidermal and dermal skin layers, we labeled HA-PE

with Alexa-dye and analyzed its distribution after a single topical application to mouse skin.

**B. Alexa<sup>647</sup>-HA-PE accumulates in the epidermis and penetrates into the dermal and sub-dermal skin layers.**

Alexa<sup>647</sup>-HA was prepared, linked to PE (A<sup>647</sup>-HA-PE) or not (A<sup>647</sup>-HA) and applied as equal amounts of HA to the shaved back skin of mice as described in methods. Skin biopsy samples were collected from 40 min to 72 h after the single application, processed for histology and examined with a confocal microscope. Confocal images showed accumulation of A<sup>647</sup>-HA-PE and particularly A<sup>647</sup>-HA on the stratum corneum but A<sup>647</sup>-HA-PE also penetrated into the stratum granulosum as well as the basal keratinocyte layers (Figure 4). Fluorescence was also observed in dermal fibroblasts and even within the subcutaneous muscle layer. Further analysis of the epidermis revealed that A<sup>647</sup>-HA-PE was most strongly associated with subpopulations of basal keratinocytes, and formed coats around these cells (Figure 4, arrows). A<sup>647</sup>-HA-PE also was detected in fibroblasts of the upper dermis. In contrast, A<sup>647</sup>-HA primarily accumulated in the outer stratum corneum with much smaller amounts penetrating into the epidermis and dermis. In particular, A<sup>647</sup>-HA accumulation was not concentrated in the basal layer (Figure 4).

Since the boundary of the epidermal layer is clearly identified, the amount of A<sup>647</sup>-HA-PE in this layer was quantified using image analysis as described in methods. For these analyses, the fluorescent intensity of Alexa<sup>647</sup> HA, Alexa<sup>647</sup> HA-PE and a negative

control vehicle cream were compared from the stratum basale to stratum granulosum layers of the epidermis. A<sup>647</sup>-HA-PE penetrated into the epidermis as early as 40 min after application and could be detected up to 72 h after application (Figure 4). Accumulation of A<sup>647</sup>-HA-PE in skin was significantly greater than A<sup>647</sup>-HA at all time points but reached a maximum difference of 5 fold at 2 h. This significantly elevated accumulation of A<sup>647</sup>-HA-PE versus A<sup>647</sup>-HA was sustained for 24 h suggesting that HA-PE was able to establish a stable organization within the epidermis. Although A<sup>647</sup>-HA-PE levels were still greater than A<sup>647</sup>-HA at 72 h after application, the difference did not reach statistical significance (Figure 4).

Confocal analysis (24 h shown, Figure 4) showed that A<sup>647</sup>-HA primarily accumulated in the outer stratum corneum with little penetration into the dermis. A<sup>647</sup>-HA-PE was also present in the stratum corneum but unlike unmodified A<sup>647</sup>-HA, accumulated around dermal fibroblasts. These results show that HA-PE readily penetrates into and is retained in the epidermal and deeper skin layers

The formation of pericellular HA coats have been linked to cellular detachment during mitotic rounding of proliferating fibroblasts and smooth muscle cells[12, 16, 82]. HA coats have also been linked to migration and differentiation of keratinocytes in organotypic cultures[13, 14, 30, 38, 83, 84]. We therefore next assessed if increasing keratinocyte HA coat formation by application of HA-PE affects the proliferation or differentiation of this skin cell type.



### **C. HA-PE increases epidermis thickness and basal keratinocyte proliferation but does not affect keratinocyte differentiation or dermal cell proliferation.**

The consequence of repeated HA-PE topical application on epidermal thickness was first quantified. As shown in Figure 5, daily topical application of HA-PE significantly enhanced epidermal thickness by 24 h, 5 days and 10 days after treatment initiation when compared to application of unmodified HA or vehicle control. Maximal epidermal thickness occurred between 24 h and 5 days and was sustained throughout the treatment period (Figure 5). The tissue samples were dehydrated prior to analysis of epidermal thickness, therefore did not account for any *in vivo* contribution of increased tissue hydration from hyaluronan.

Consistent with this thickening effect on the epidermal layer, topical application of HA-PE stimulated basal keratinocyte proliferation compared to unmodified HA or vehicle controls as detected by Ki67 staining, which is a cell proliferation marker. In addition, increased Ki67 staining of the suprabasal keratinocytes was observed in the HA-PE group. The majority of epidermal proliferation and regeneration occurs by activation of stem cells in the basal layer of the epidermis. The suprabasal levels contain transit-amplifying (TA) intermediate stem cells which are defined by a finite number of cell divisions before entering a terminal differentiation pathway (85). The HA-PE cream appears to stimulate both the basal layer stem cells in addition to the TA stem cells. Increased keratinocyte proliferation was observed 1 day after treatment initiation and was sustained throughout the treatment period, corresponding well with the time

frame observed for the increase in epidermal thickness (Figure 6). Analysis of DAPI-stained skin sections 24 and 72 h after application of HA-PE or HA showed that dermal cell number was similar in both treatments suggesting that HA-PE does not stimulate fibroblast or other dermal cell proliferation. This predicts that HA-PE effects on proliferation are limited to the epidermal layer.

Growth factors such as keratinocyte growth factor (KGF) that promote keratinocyte proliferation coincidentally inhibit keratinocyte differentiation and hyaluronan production [86, 87]. We therefore next assessed if elevating HA around keratinocytes affects their differentiation cycle. The consequence of HA-PE application on the expression of keratin-10 (K-10) was quantified using immunohistochemistry. No detectable effect of HA-PE application on K-10 staining was observed suggesting that HA-PE does not modify keratinocyte differentiation (Figure 7).

#### **D. Topical application of HA-PE does not affect local skin or systemic inflammation.**

Since HA and its fragments are potent regulators of the immune system in particular innate immunity [15, 19, 47, 88], which could indirectly affect keratinocyte proliferation, we next determined if topical application of HA-PE affected local or systemic inflammation. F4/80 staining of skin tissue sections and TNF $\alpha$  was used to identify localized activation of macrophages while serum CRP levels were monitored to determine effects on systemic inflammation. As shown in Figure 9, the application of HA-PE did not increase F4/80 staining levels compared to unmodified HA or

vehicle controls. Furthermore, the levels of macrophage activation detected by this method were very low compared to those detected in skin wounds, which are known to contain high levels of activated macrophages [89] and were used as a positive control. Similarly local TNF $\alpha$  and systemic CRP inflammation levels were not increased by topical application of HA-PE compared to unmodified HA or vehicle controls (Figure 8 A, B).

## DISCUSSION

Our results identify a method for preparing HA-phospholipid (PE) polymers that do not require CD44 display for forming pericellular coats around fibroblasts in culture and aged keratinocytes in wild type and CD44 $^{-/-}$  mice. These results are consistent with a mechanism whereby HA-PE inserts directly into the cell membrane via the phospholipid entity. The topical applications of this HA-PE polymer increased epidermal thickness of aged female mouse skin as a result of stimulating the proliferation of the basal keratinocyte layer in the absence of detectable dermal or systemic inflammation or changes in keratinocyte differentiation rates.

Topical application of unmodified HA to intact skin in situ penetrated into the epidermis and dermis as previously reported [43, 70-72] but this was limited compared to HA-PE polymers. In addition, most of the unmodified HA remained associated with the stratum corneum and was not retained in skin as long as HA-PE. The ability of unmodified HA to penetrate the skin in small amounts, which can occur in both rodent and human skin, is dependent upon molecular weight. Thus, HA polymers smaller

than 50,000 Da readily penetrate human skin ex vivo while larger HA chains do not [70]. The average MW of HA used in the present study was 500,000 Da and therefore the limited association of unmodified HA with the epidermis is likely due to the polymer size restriction. The penetration of this HA size through the outer stratum corneum is facilitated by the additional of a phospholipid moiety and this addition likely increases solubility of HA in lipids (e.g. ceramides, cholesterol and fatty acids) [90] that are present in the stratum corneum. Retention within the epidermis and dermis is likely promoted by the ability of keratinocytes and fibroblasts to capture and retain HA-PE as demonstrated in culture. Since HA-PE is able to organize as a pericellular coat in the absence of CD44 display, HA-PE polymers are able to spontaneously organize within the epidermis and we predict that keratinocyte CD44 acts at least in part as an anchor for attaching HA to outside of cells.

Dermal and epidermal homeostasis is compromised in aging and a variety of disease processes. Aged skin is characterized as dry, atrophic, inelastic, and wrinkled. The key molecule required for water retention within skin is HA. Both the amounts and organization of HA change in skin with intrinsic (chronological) and extrinsic (photodamage) aging [4, 91, 92]. With chronological aging while HA is retained within the aged dermis, it is precipitously lost from the epidermis. HA retained within the aged dermis is however modified in its organization and this change together with epidermal loss are thought to contribute to some of the above malfunctions of aged skin [38]. Additionally, expression of skin HA receptors including CD44 are increasingly reduced with age. Even though intrinsic and extrinsic skin aging are

distinctive processes, they share similarities in molecular mechanisms. For example, extrinsic causes of premature aging such as chronic exposure to UV also results in loss of skin moisture, HA and HA receptor expression. Importantly, photoaging is also associated with a decrease in the size of HA that will likely affect its ability to organize into structures such as pericellular coats [73]. HA performs a variety of functions in skin and this age dependent loss is considered to impact upon skin moisture, barrier functions, epidermal thinning and sluggish response to injury [34]. The ability of HA-PE polymers to increase rodent epidermal thickness and epidermal proliferation predict that this formulation has the potential for reversing some of the intrinsic and extrinsic age-related epidermal defects such as epidermal thinning and reduced repair response [93-95].

Our results showing that HA-PE promotes epidermal thickening and keratinocyte proliferation are similar to the ability of exogenous high molecular weight HA to affect functions of aged keratinocytes in culture and *in vivo* [33, 35, 40, 43] although in the present study topical HA-PE had a significantly greater effect than unmodified HA. Our data suggest that this results from increased basal keratinocyte proliferation rather than enhanced differentiation. Previous reports have similarly noted an increase in keratinocyte proliferation in response to topical HA but noted this effect was maximal for intermediate (50,000-400,000 Daltons) and no effect was noted for large HA (>400,000 Daltons) [40]. In another study, topical application of large HA promoted epidermal barrier function and keratinocyte differentiation [43]. The different effects of high versus intermediate HA fragments in these studies may have

been due to the documented poor penetration of high molecular weight HA [70] that is confirmed here. The ability of 500,000 Da HA-PE to promote keratinocyte proliferation is likely because it efficiently penetrated to and was retained in the basal keratinocyte layer to a much greater extent than unmodified HA.

A number of topical HA formulations have been developed in the past decade most of which are composed of intermediate-small HA fragments (e.g. < 50,000 Da) or nanoparticulate formulations [4, 96]. Although these have shown efficacy (e.g. [97]) in terms of treating skin disorders, we pose that HA-PE formulations will be more effective since they are designed to replace the naturally occurring HA pericellular coat that is depleted during aging and other conditions that cause epidermal atrophy [35, 40], and will also therefore be retained within the epidermis for longer times. Particulate formulations by their nature tend to stimulate endocytosis and such formulations are rapidly depleted from tissues [98-100]. Topical application of intermediate unmodified HA has the advantage of being non-particulate and able to penetrate skin readily but is dependent upon the expression of keratinocyte CD44 for effects on keratinocyte function, which is depleted with age-related or other causes of epidermal atrophy.

The consequences of exogenous HA or inhibition of endogenous HA show that CD44 mediates the consequences of HA on keratinocyte function [6, 38, 61, 85]. For example, the effects of topical HA on keratinocyte differentiation and proliferation are ablated when CD44 function is blocked or expression is lost [35, 40, 43].

CD44 appears to regulate multiple downstream signaling pathways to control these keratinocyte processes and these include coordination of signaling through EGFR, activation of RHO GTPase and translocation of activated ERK1,2 to the cell nucleus [17, 35, 38, 85, 101]. The mechanisms by which HA-PE control basal keratinocyte proliferation and the dependence of this effect on CD44 expression are currently being investigated.

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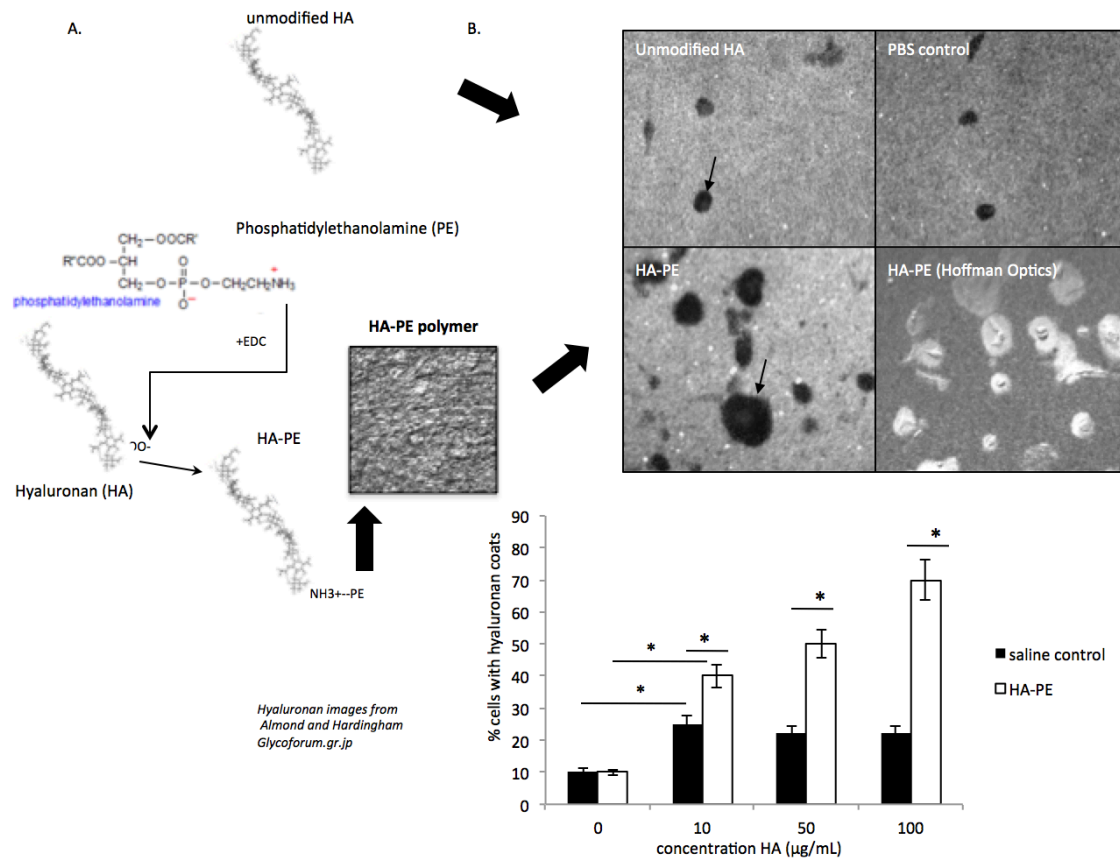


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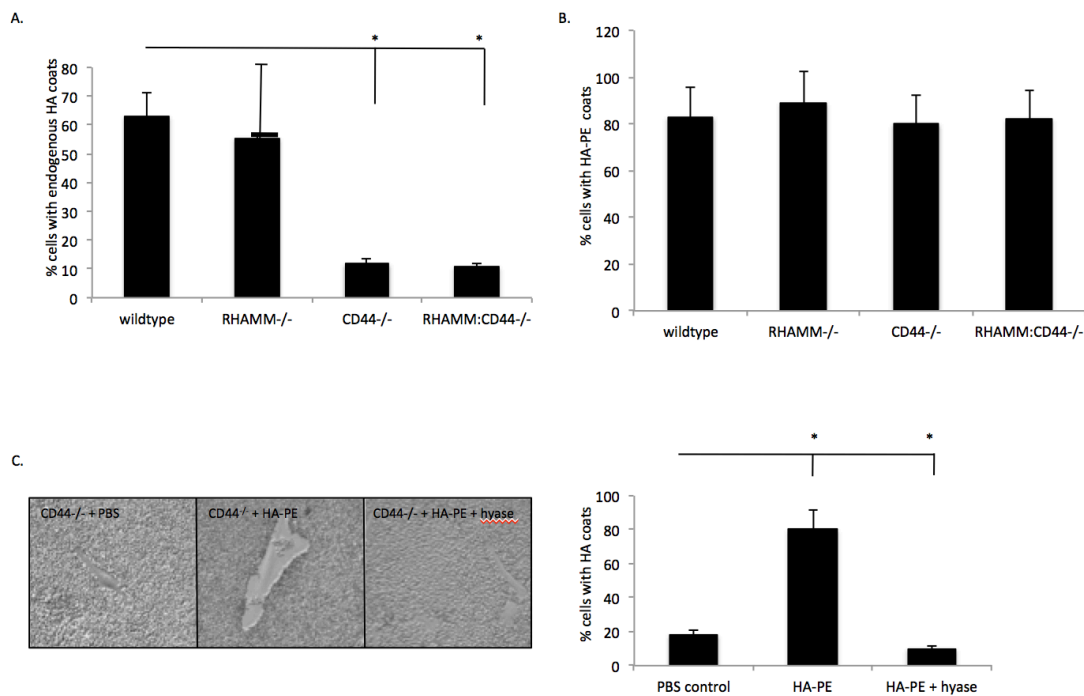


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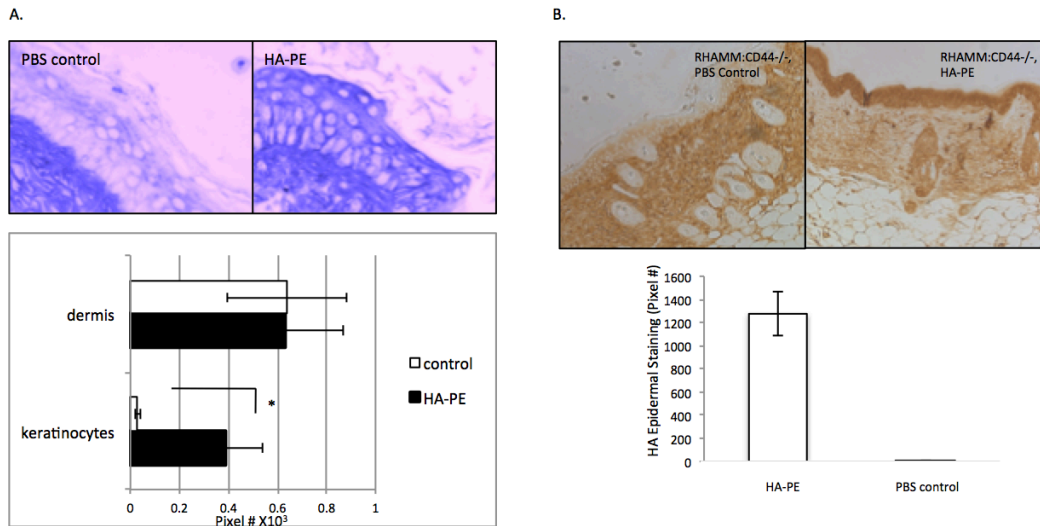
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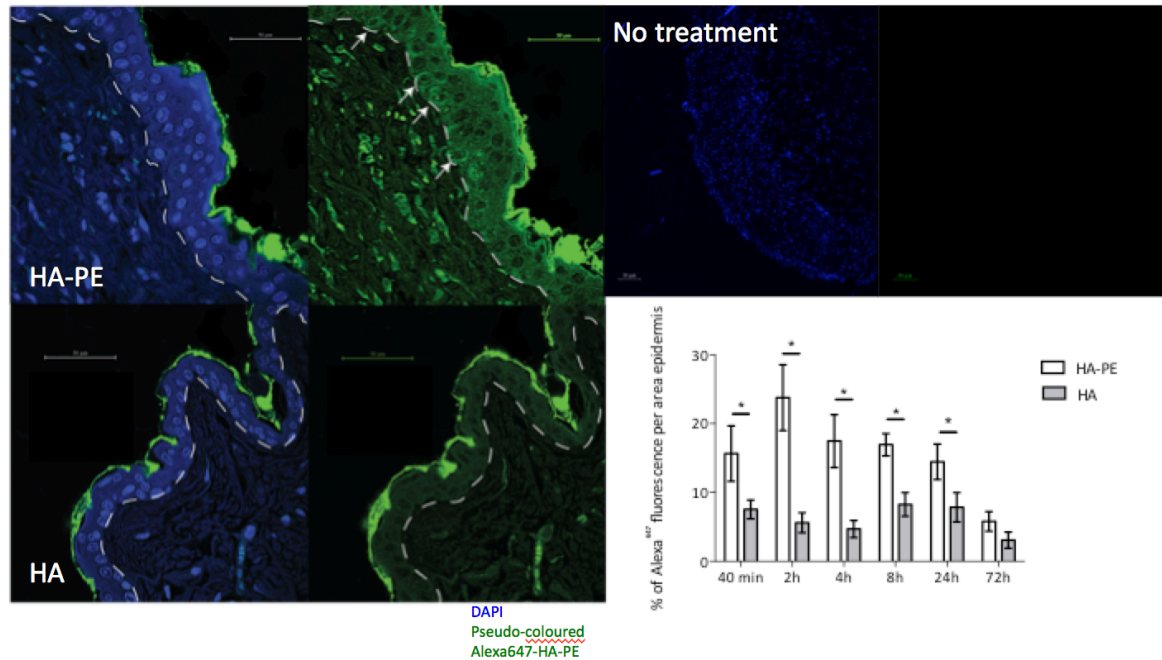
Manuscript: Figure 1. **HA-PE simulates pericellular coat formation in cultured fibroblasts.** 500 kDa HA was linked to PE using EDC to achieve a stoichiometry of 1:10 (HA:PE) (A). This ratio was selected because it resulted in the largest pericellular HA coats, which were detected using fluorescent particle exclusion assays (B). The black patches on the epi-fluorescent images are areas of pericellular coats. A Hofmann image is included which shows cells at the center of the pericellular coats. Images were taken with a 10X objective. The percentage cells/10x field that were surrounded by HA coats were calculated using Hoffman optics (C). Values are Mean and S.E.M n = 3 fields. Asterisks indicate statistical significance (p < 0.01).



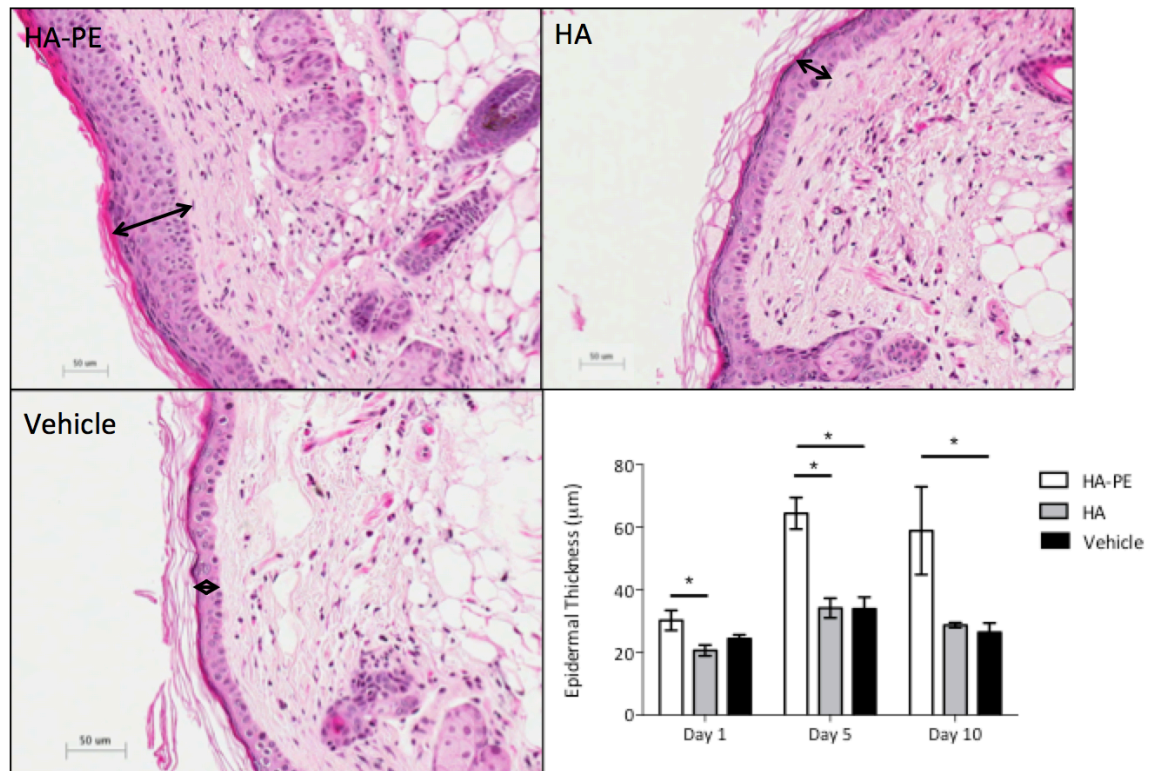
Manuscript: Figure 2. **Endogenous HA coats require expression of CD44 while HA-PE generated coats do not.** The numbers of wild type and RHAMM<sup>-/-</sup> fibroblasts that form endogenous HA coats are not significantly different. In contrast, loss of CD44 significantly reduces the numbers of fibroblasts forming coats. This is not further reduced by loss of both CD44 and RHAMM (A). However, loss of CD44 has no effect on the number of cells forming coats as a result of HA-PE addition (B). The pericellular coats formed by CD44<sup>-/-</sup> fibroblasts in the presence of HA-PE are destroyed by hyaluronidase (C). Images were taken with a 20X Hoffmann objective. Values are the Mean and S.E.M. n = 75 cells.



Manuscript: Figure 3. **Topical application of HA-PE stimulates HA accumulation in wild type and CD44<sup>-/-</sup> keratinocytes in mouse skin *in vivo*.** Topical application of HA-PE to shaved B57/BL6 wild type mice increases HA in the keratinocyte layer as detected by biotinylated HABP (blue staining indicates hyaluronan detected by HABP probe) (A). Staining 3 days after application is not detectably different in the dermis and although patchy and variable in the epidermis is significantly different in HA-PE versus vehicle controls (brown staining indicates hyaluronan detected by HABP probe) ( $p < 0.05$ ). Images were taken with a 20X bright field objective. Similar results were obtained when the epidermal layer was analyzed for hyaluronan after HA-PE was applied to B57/BL6 RHAMM:CD44<sup>-/-</sup> mice. Significantly greater HA staining was observed in HA-PE treated vs. vehicle controls ( $p < 0.0001$ ) (B) Images were taken with a 10X brightfield objective. Values in A and B are the Mean and S.E.M.  $n = 3$  mice, 5 tissue section/mouse.

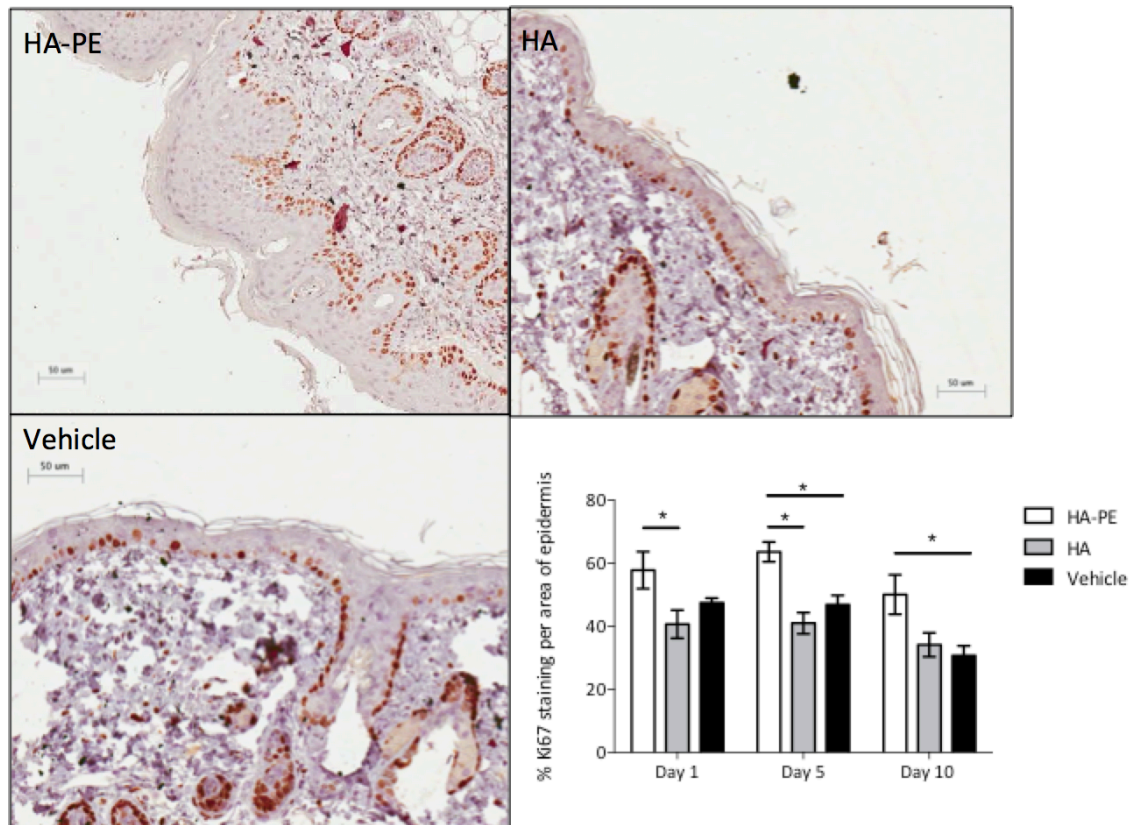


Manuscript: Figure 4. **Topical Alexa<sup>647</sup>-HA-PE enters the epidermis.** Significantly increased fluorescent staining is observed in the epidermis (stratum basale to stratum granulosum) of the Alexa<sup>647</sup>-HA-PE group at all time points up to 24 h (mean ± S.E.M, 20 skin images) (\*  $p < 0.05$ ). Representative confocal images of Alexa<sup>647</sup>-HA-PE and Alexa<sup>647</sup>-HA treated mice sectioned at 24 h. Dashed line indicates the junction of the epidermis and dermis. Arrowheads indicate areas of enhanced pericellular fluorescence. Alexa<sup>647</sup>-HA-PE and Alexa<sup>647</sup>-HA staining was pseudo-colored green. Blue staining is DAPI.



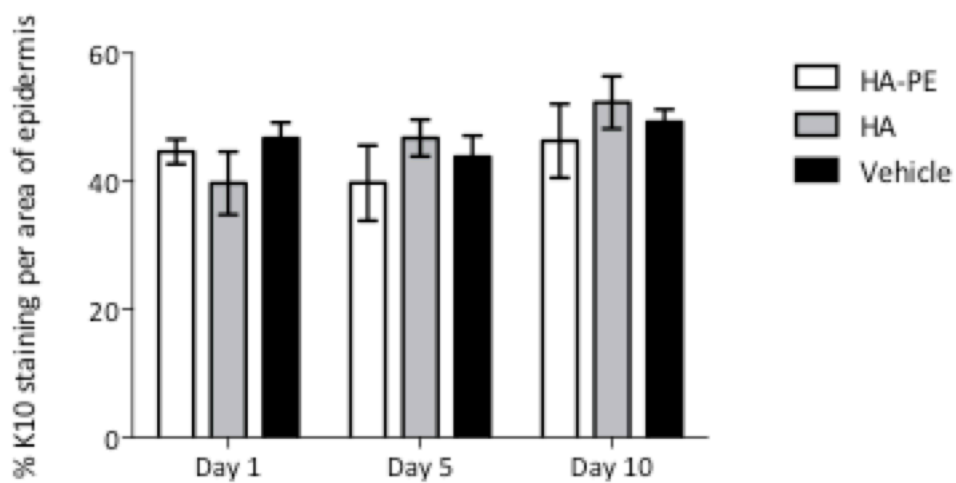
Manuscript: Figure 5. **HA-PE cream results in a thicker epidermis.** There is a significant increase in epidermal thickness ( $\mu\text{m}$ ) of mice treated daily with HA-PE for 1, 5, and 10 days compared with control groups (HA and Vehicle cream) (mean  $\pm$  SEM, 5 mice per group) ( $*p < 0.05$ ). A single representative H&E section from each of a day 10 HA-PE, HA, and Vehicle Cream mouse is shown, arrows indicate epidermal thickness.



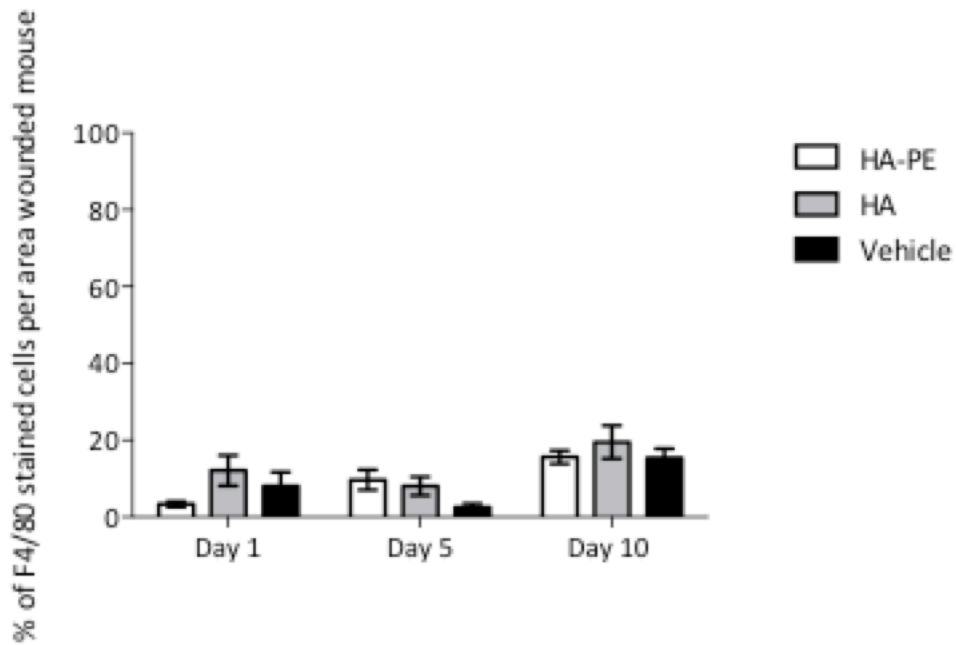


Manuscript: Figure 6. **HA-PE cream results in increased keratinocyte proliferation.** There is a significant increase in the percentage of positive Ki67 staining per an area of epidermis of mice treated daily with HA-PE for 1, 5, and 10 days compared with HA or Vehicle Cream (mean  $\pm$  SEM, 5 mice per group) (\* $p < 0.05$ ). A single representative Ki67 section from each of a day 10 HA-PE, HA, and Vehicle Cream mouse is shown.



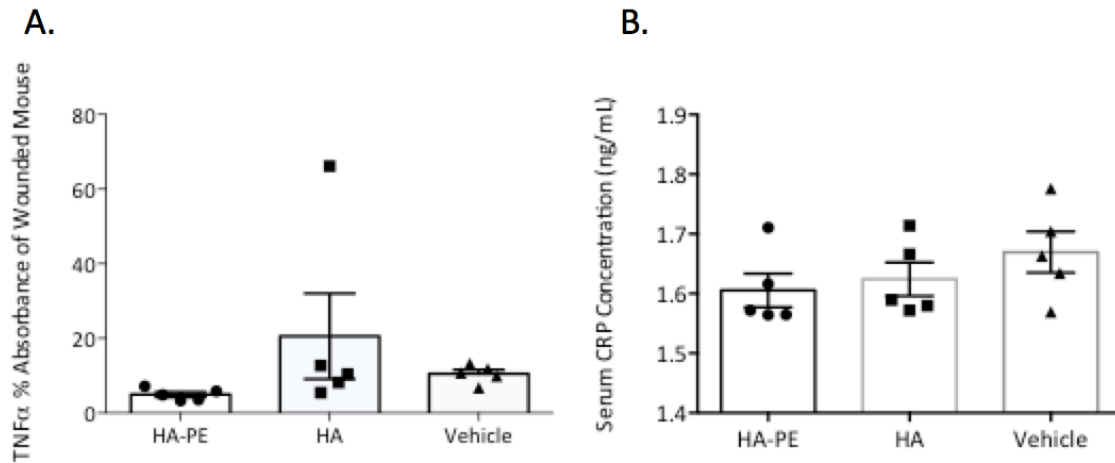


Manuscript: Figure 7. **HA-PE cream does not affect suprabasal keratinocyte differentiation.** The percentage of K10 staining per an area of epidermis for mice treated daily for 1, 5, and 10 days with either HA-PE, HA, or Vehicle Cream (mean  $\pm$  SEM, 5 mice per group).



Manuscript: Figure 8. **HA-PE cream does not elicit a local inflammatory response.**

The percentage of positive F4/80 staining per an area of a wounded mouse for mice treated daily for 1, 5, and 10 days with either HA-PE, HA, or Vehicle Cream (mean  $\pm$  SEM, 5 mice per group).



Manuscript: Figure 9. **HA-PE cream does not elicit a local or systemic inflammatory response.** The TNF $\alpha$  expression as a percentage of a wounded mouse control for day 10 treated mice with HA-PE, HA, or Vehicle Cream using an ELISA (mean  $\pm$  SEM, 5 mice per group) (A). The serum CRP concentration (ng/mL) of day 10 treated mice with HA-PE, HA, and Vehicle Cream using an ELISA (mean  $\pm$  SEM, 5 mice per group) (B).

## Chapter 5: Discussion

Hyaluronan, particularly HMWHA, has proven benefits in wound repair [45, 46, 48], as a skin surface depot for transdermal drug delivery [92], and when injected as a cross linked form, cosmetic tissue rejuvenation [87, 93]. With aging, the quantity of endogenous HA in the epidermis is decreased and the HA in the dermis becomes more cross-linked. Topical penetration of exogenous hydrophilic HMWHA has been a challenge to date because of limited penetration of the negatively charged polymer through the outer hydrophobic skin layers. This study evaluated the role of a novel exogenous HA formulation in addressing the functional and aesthetic consequences of aging. In summary, our study found that modified HA-PE cream penetrated the skin barrier resulting in increased keratinocyte proliferation and epidermal thickness, without resulting in a local/systemic inflammatory response compared with control groups (unmodified HA, vehicle cream).

Our study assessed the consequences of a novel hyaluronic acid phosphatidylethanolamine (HA-PE) cream (CA Patent PCT/CIPO 2,703,532) designed to facilitate topical penetration and retention of HMWHA in the skin on keratinocyte renewal and skin inflammation. HA-PE is a modified hyaluronic acid with approximately 6% of the disaccharide units of native hyaluronan (~500kDa) covalently linked to phosphatidylethanolamine. Phospholipids, such as PE, act as amphipathic vehicles in skin delivery with a strong safety profile including low immunogenicity [89]. A stable HA-PE formulation was accomplished by using the water soluble crosslinking agent, EDC as the

limiting reagent in the reaction. HA-PE was formulated in a 1:1 (v/v) ratio with a standard skin base cream and then topically applied to shaved mouse skin.

Evidence that HA-PE cream traversed the outer skin barrier and incorporated into the epidermal layers was assessed using confocal analysis following a single topical application Alexa<sup>647</sup> fluorescently labeled HA (Alexa<sup>647</sup>HA and Alexa<sup>647</sup>HAPE). Our results show that HA-PE penetrates into the epidermis and is retained for up to 72h following a single application, suggesting that the amphipathic qualities of the PE linkage modifications facilitate the permissibility of the 500 kDa HA into the epidermis. These findings are preserved in CD44<sup>-/-</sup> mice, demonstrating that the retention of HA-PE appears independent of CD44 interactions, which is the HA receptor that primarily mediates endogenous keratinocyte pericellular coats.

Some authors argue that molecules greater than 500 Daltons are impermeable to the skin barrier [94]. In contrast using a murine model, Brown *et al.* (1999) showed that [<sup>3</sup>H]hyaluronan, weighing approximately 400 kDa, penetrated the skin barrier and small quantities of 360-400 kDa polymers were recovered in both the skin/blood after application [95]. The permeability of HA-PE (~500 kDa) likely depended on its structural composition as opposed to its molecular weight. Modifying the structural composition of a molecule has been successfully used by other researchers to increase penetration of hydrophilic compounds. For example, Billich *et al.* (2005) found that the addition of phosphocholine to topical Cyclosporin A (1203 Da), an immunosuppressant drug, increased its penetration into skin compared to an unmodified version [96]. Overall, HA-

PE appears to penetrate the skin barrier and be retained in the extracellular space independent of CD44 receptor interactions.

The addition of phosphatidylethanolamine to HA-PE results in retention of exogenous HA-PE in the epidermis. The retention of HA-PE is likely a result of interactions with receptors, inserting into the plasma membrane via the PE tails and native HA in the extracellular environment. The rate of HA-PE rate of decline appears reduced compared to unmodified HA, however this was not measured directly and should be the focus of future investigations. Native epidermal HA turnover ( $t_{1/2}$ ) is approximately 24h [41, 97]. Our findings are consistent with these previous results with declining levels of both exogenous HA-PE and HA occurring 24h after a single application. Overall, HA-PE cream delivers HMWHA to the epidermis, which behaves in a similar fashion to native HA.

Qualitatively in addition to accumulating in the basal keratinocyte layer of the epidermis, a strong signal for HA-PE remained in the SC up to the final 72h time point. In addition to a role in providing a permeability barrier, the SC is important in preventing xerosis [7]. Skin hydration in the SC is dependent on the configuration of lipids and the presence of hygroscopic molecules such as NMF [98]. Saki *et al.* (2000) deposed earlier theories that hyaluronan was absent from SC [99]. Their group provided evidence that along with NMF, HA was a key player in the moisturization of the outermost layer of the epidermis [99]. In addition to providing a reliable carrier of HMWHA to the epidermis, retention of

HA-PE in the SC has the potential added benefit of improving skin hydration through reinforcing the outermost protective barrier.

Further evidence supporting the penetration of HA-PE into the epidermis rests in its effect on epidermal physiology. Multiple applications of HA-PE result in a two-fold increase in epidermal thickness compared with unmodified HA. This increase in epidermal thickness correlates to increased keratinocyte proliferation without detectable differences in suprabasal terminal keratinocyte differentiation per area. The thickened epidermis is likely to reflect both indirect (hydrophilic properties of HA, not measured in the current study) and direct (stem cell mobilization/cell proliferation) effects of HA-PE cream. Thus, the increased keratinocyte proliferation observed in the current study is likely secondary to stimulation of EPU in the basal layer of the epidermis by HA-PE. However, the exact mechanism and confirmation of the effect of HA-PE on EPU is a potential topic for future study.

Stimulation of epidermal proliferation may be negatively linked to either tumorigenesis or hyperproliferative skin disorders such as psoriasis. Skin tumor biology is complex. Repetitive ultraviolet radiation damage is widely known to be an important risk factor for the development of basal and squamous cell cancers. It is unlikely that HMWHA promotes skin tumorigenesis. First, HMWHA acts as a free radical scavenger and is protective against DNA damage which is a common etiology linked to skin cancer [100]. In a rodent model, HMWHA production in the naked mole rat confers resistant to cancer throughout its extended lifespan (30+ years) [51]. When production of HMWHA is

blocked, the cancer resistance is lost. However, future research is needed to refute any unlikely potential link with HA-PE cream application and tumorigenic potential. In terms of promoting a negative hyperproliferative skin phenotype, fortunately, the structure of the skin layer histology appears qualitatively to remain intact following HA-PE cream application. In comparison, marked hyperplasia of the epidermis and incomplete keratinocyte differentiation can be negatively associated characteristics of psoriasis-like skin inflammation. In psoriasis these changes are also accompanied by a loss of the granular cell layer of the epidermis in addition to the presence of neutrophils in the epidermis[90], which are not changes evident in our experimental model.

One potential mechanism by which topical HA-PE recovers an attenuated epidermis is by attracting water to the ECM. HA can bind up to 10,000x its weight in water [100]. Exogenous HA-PE traverses the skin barrier and contributes to the keratinocyte pericellular coat mimicking an endogenous HA configuration. Röck *et al.* (2010) demonstrated that intradermal injection of non-crosslinked HA incorporated into the pericellular matrix and promoted proliferation of fibroblasts *in vitro* [101]. Similarly, our non-crosslinked topical HA-PE transverses the skin barrier and incorporates into the pericellular matrix. The immediate increase in epidermal thickness evident at 24h post-application may therefore reflect the increased water content of the epidermis treated with HA-PE.

Thinned epidermis characteristic of senescent skin, in both human and murine models, is a result of decreased keratinocyte proliferation [2, 3, 56]. Topical HA-PE has a direct



effect on restoring the proliferative capacity of aged keratinocytes contributing to a thickened epidermis. Pericellular hyaluronan coats are known to create a fluid scaffold, which enhances cell migration and proliferation [28, 43]. Exogenous HA-PE may facilitate diffusion and interactions of keratinocyte growth and differentiation factors in the restored pericellular matrix. Alternatively, a mechanical stimulus from increased ECM volume may exist which stimulates keratinocyte proliferation. The added ECM structural support from crosslinked dermal HA fillers have been associated with a modest increase in epidermal thickness one to three months post-injection [14]. The added ECM volume from HA-PE could incite a similar stimulus for epidermal proliferation. Our findings of HMWHA resulting in increased keratinocyte proliferation conflict with the results from studies which show only LMWHA and not HMWHA enhance keratinocyte proliferation in aged skin [56, 62]. For example, Kaya *et al.* (2006) used *in vivo* and *in vitro* models to demonstrate that only topical HA cream < 400 kDa resulted in keratinocyte proliferation and skin hyperplasia, whereas HA > 400 kDa had no effect. This finding could be due to poor penetration of HA > 400 kDa in their experimental models which included both mouse and human skin. The addition of phosphatidylethanolamine in the HA-PE cream overcomes penetration barriers allowing HMWHA to enter the skin and impact downstream mediators of skin rejuvenation and growth.

Topical HA-PE: HA receptor interactions, may also promote up-regulation of endogenous HA production to further amplify its effects and establish a positive autocrine feedback loop. In support of this injected HA has been shown to have a

stimulatory effect on endogenous synovial cell HA synthesis [102] . Rilla *et al.* (2013) demonstrated that activate HA synthesis is associated with cells shedding microvesicles containing HA synthetic machinery which can propagate HA production at a distance from the original cell source [103]. Therefore, HA-PE could promote HA production in the dermis and other points distance from the epidermis by this type of mechanism. Further studies are required to elucidate potential mechanism whereby exogenous HA-PE restores a critical quantity of HA in the epidermis, which is permissive to functional activation of endogenous HA synthesis.

Consistent with my results, previous studies have indicated increased keratinocyte proliferation and thickened epidermis can result from factors that promote HA synthesis, such as EGF, whereas factors that inhibit HA synthesis result in decreased proliferation and enhanced differentiation [21]. Our results show despite increased proliferation there was no detectable difference per area of suprabasal keratinocyte differentiation compared with control groups. Thus, the downstream signaling effects of HA-PE stimulate keratinocyte proliferation without negatively affecting the suprabasal differentiation expression. This is important because preservation of keratinocyte differentiation is crucial to maintain epidermal integrity [104].

If exogenously applied HMWHA is rapidly metabolized into pro-inflammatory LMWHA fragments it has the potential for stimulating inflammation. Our study has provided evidence that topical HA-PE cream does not elicit either a local or systemic inflammatory response. In mimicking the endogenous configuration of HA, HA-PE preserves the anti-

inflammatory properties of HMWHA. The wounding literature best supports the anti-inflammatory nature of HMWHA. Topical HMWHA added to a rat laminectomy model before final skin closure resulted in a reduction of macrophages and inflammatory cytokines at the wound site during the acute phase of healing [82]. Schimizzi *et al.* (2006) theorized that HMWHA blocked the interaction of LMWHA pro-inflammatory signaling cascades [82]. In addition, the hydrophilic properties of HMWHA were thought to provide a dilutional effect on inflammatory cell mediators [82]. The anti-inflammatory properties of HA have been used clinically as a component in wound dressings for burns and skin ulcers since the 1960s [71, 102]. Injectable HA has also been used as a therapy to temporize joint inflammation in arthritis [102]. In addition, HMWHA has well documented anti-oxidant properties as a free radical scavenger which protects cellular DNA from damage [105]. Our study has confirmed the absence of an inflammatory reaction caused by HA-PE cream when applied to normal aged skin. However, future studies are needed to determine if anti-inflammatory and anti-oxidant properties of HA-PE cream have benefit in clinical models of cutaneous inflammation or wounding.

## Limitations and Future Directions

One of the limitations of the current study is technical. A wild-type murine model of aged skin was chosen because unlike nude murine models, the wild-type mice have the full complement of their immune system. Compared with human skin, the mouse epidermis and dermis are thinner and thus penetration of the experimental creams may be facilitated. However, due to economic and practical administrative reasons of a pilot study, more homologous models to human skin were not selected. In addition, as this is a pilot experiment, the variability of the expected findings was not known at the time of model selection. Extrapolating the study results broadly to other animal models or humans should be done with caution. We recommend that future studies repeat and add to our results in a model whose skin more closely resembles that of human tissue. In addition, given the proven beneficial effects of HMWHA on certain pathologic conditions such as radiation dermatitis, burns, cutaneous wounds, and ulcers, it would be interesting to study whether topical HA-PE had a beneficial effect in these settings.

Another limitation is confirming if HA promotes skin renewal by mobilizing stem cell and/or TA populations in the basal layer of the epidermis. Future studies could incorporate markers of stem cell populations such as integrins[106] or Oct4[19] to further evaluate this theory.

Skin integrity and health is a multidimensional construct. For our studies, we selected a handful of relevant markers as a starting point to understand the potential effects of HA-PE cream on the epidermis. However, further research is needed to explore additional

markers of skin integrity and dermal markers. Our results suggest that HA-PE penetrates through the dermis but we did not test for its effects in this layer. Future studies for example could use markers of collagen production or Flaggrin expression in the SC could provide further support of the trophicity of HA-PE on aged skin. In addition, it will be necessary for future studies to examine the potential mechanisms of action of HA-PE. Examining known pathways of native HA activity could help elucidate whether HA-PE is acting through an analogous mechanism.

Our analysis methodology included primarily immunohistochemistry and ELISA tissue specimens. Given that these methodologies rely on preserved and dehydrated specimens- they can only at best provide a snapshot of the dynamic state of live tissue. Furthermore, the immunohistochemistry analysis software relies on calculating pigment density as an indicator of the presence of the markers of interest. There is always some variability in the uptake and penetration of the stain given a particular staining cycle and the particular section of tissue. To mitigate this variability, all specimens from a single time point were stained and analyzed as a cohort.

Given that HA is known to improve cutaneous hydration and barrier function- we recommend that future studies consider adding a functional measure of cutaneous hydration and transepidermal water loss (TEWL) in a live animal model. In the current study, the necessary equipment for these measurements was cost prohibitive to obtain for the pilot data.

The current investigation provides a foundation for future studies to investigate the positive effect of HA-PE on replacing the native cutaneous HA lost with age.

## Conclusion

Aged skin, characterized by diminished hyaluronan, has both functional and aesthetic deficits. Topical HA-PE is a novel skin care technology that reliably carries HMWHA (500 kDa) into the epidermis to restore a youthful phenotype to aged skin. The biological effect of HA-PE results in a thicker epidermis from enhanced keratinocyte proliferation. The suprabasal differentiation markers appear unchanged compared to unmodified HA. In addition, HA-PE does not result in either a local or systemic inflammatory response. Findings from our investigation provide a foundation for further research to elucidate additional clinically relevant benefits of HA-PE cream.

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## Appendix A



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**Title:** Pericellular Hyaluronan Coat Visualized in Live Cells With a Fluorescent Probe Is Scaffolded by Plasma Membrane Protrusions:

**Author:** Kirsi Rilla, Riikka Tiihonen, Anne Kultti, Markku Tammi, Raija Tammi

**Publication:** Journal of Histochemistry & Cytochemistry

**Publisher:** SAGE Publications

**Date:** 10/01/2008

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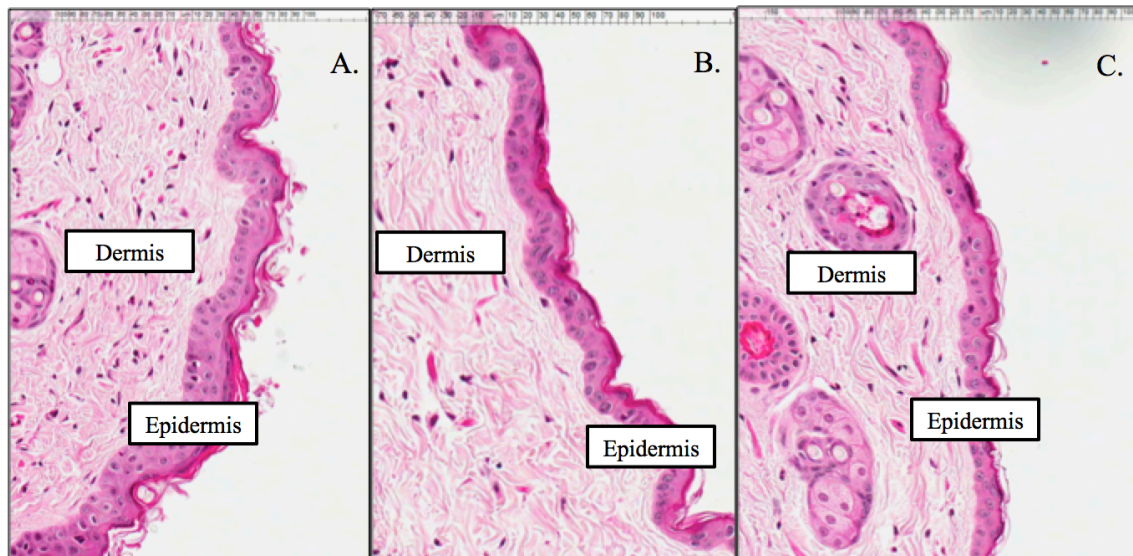
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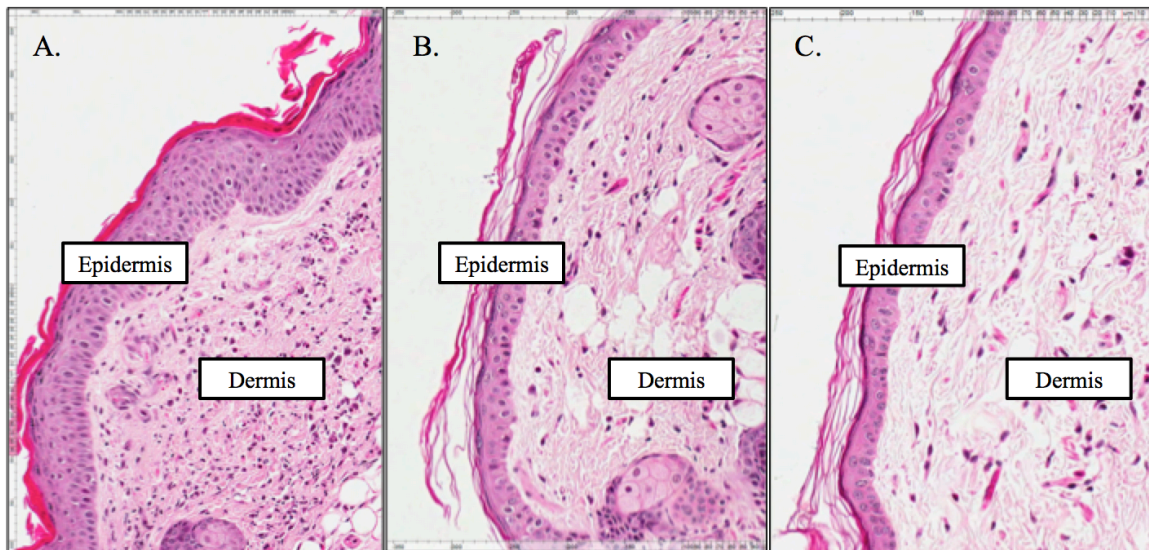
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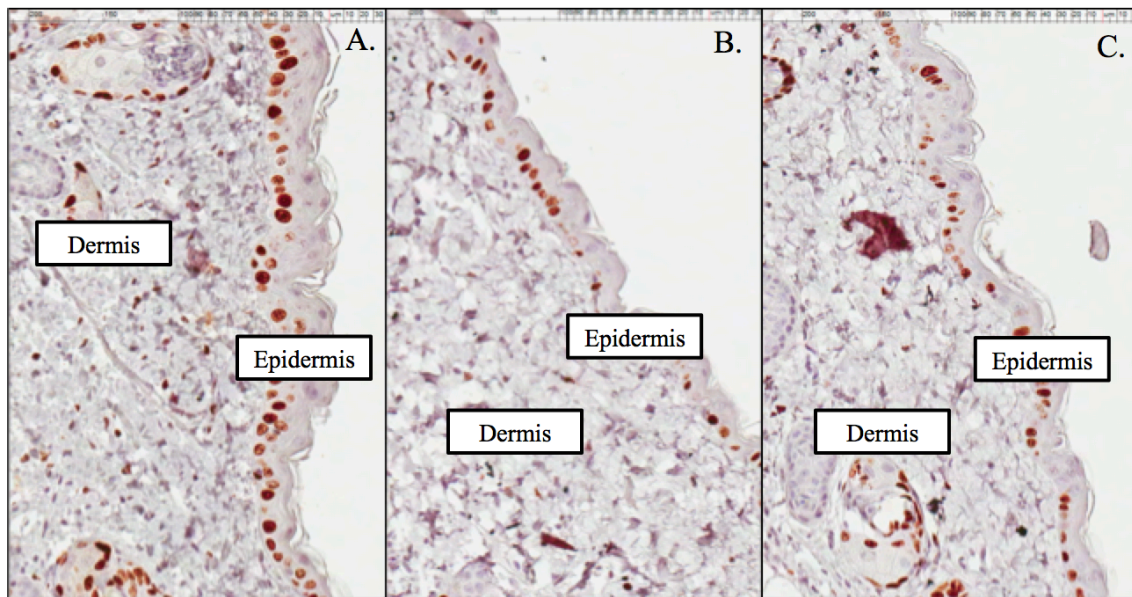
## Appendix B



Supplemental: Figure 1. **Epidermal thickness representative H&E sections from each of day 1 HA-PE (A), HA (B), and Vehicle Cream (C) groups.**

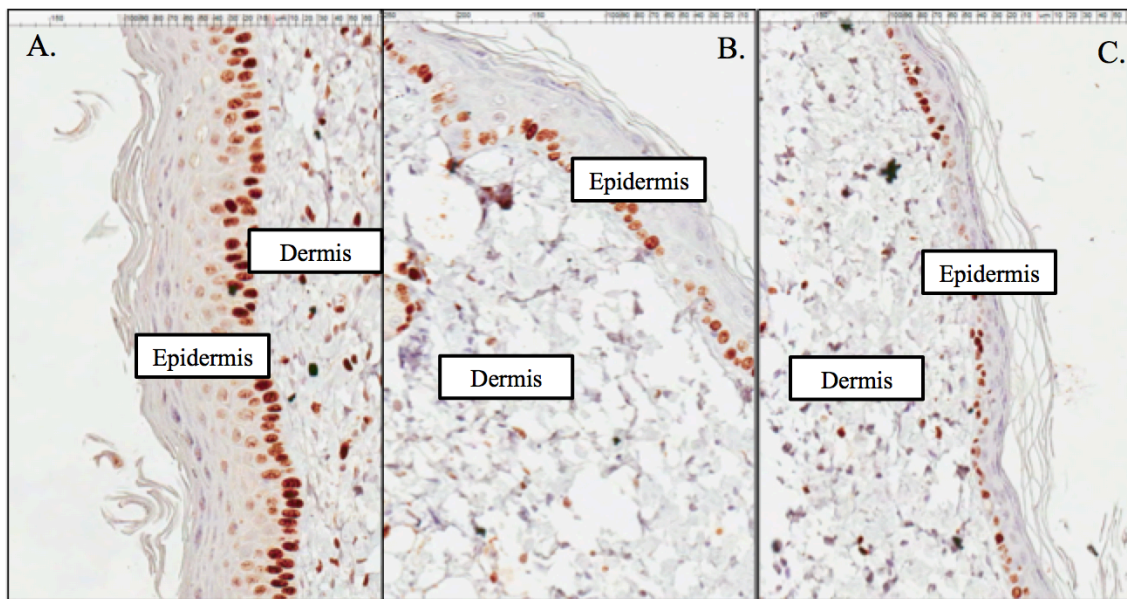


Supplemental: Figure 2. **Epidermal thickness representative H&E sections from each of day 5 HA-PE (A), HA (B), and Vehicle Cream (C) groups.**

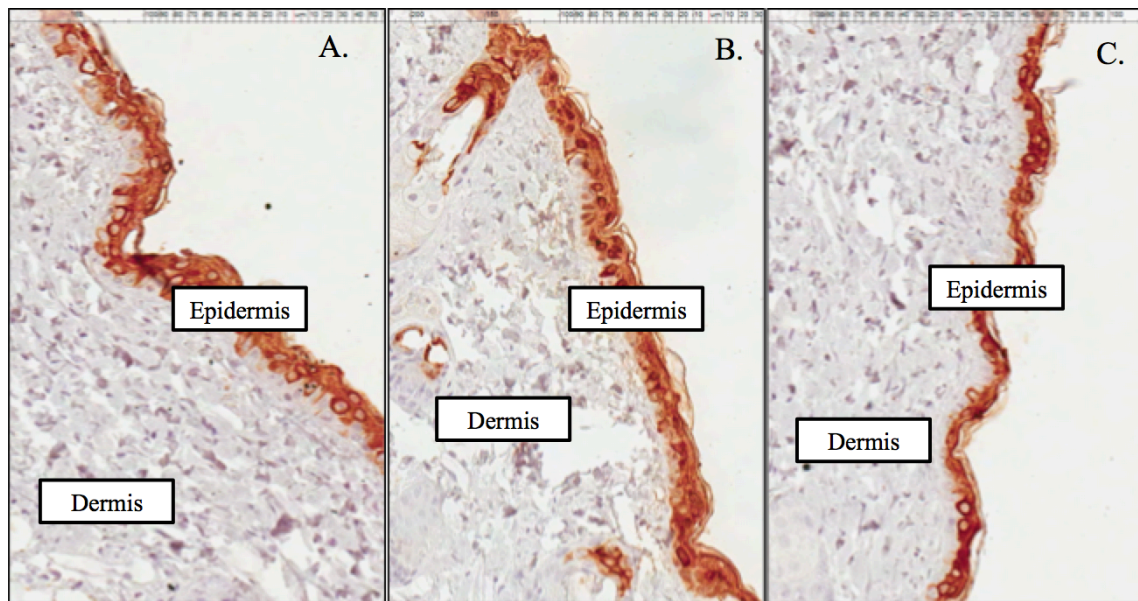


Supplemental: Figure 3. **Ki67 representative sections from each of day 1 HA-PE (A), HA (B), and Vehicle Cream (C) groups. Ki67 – brown staining.**

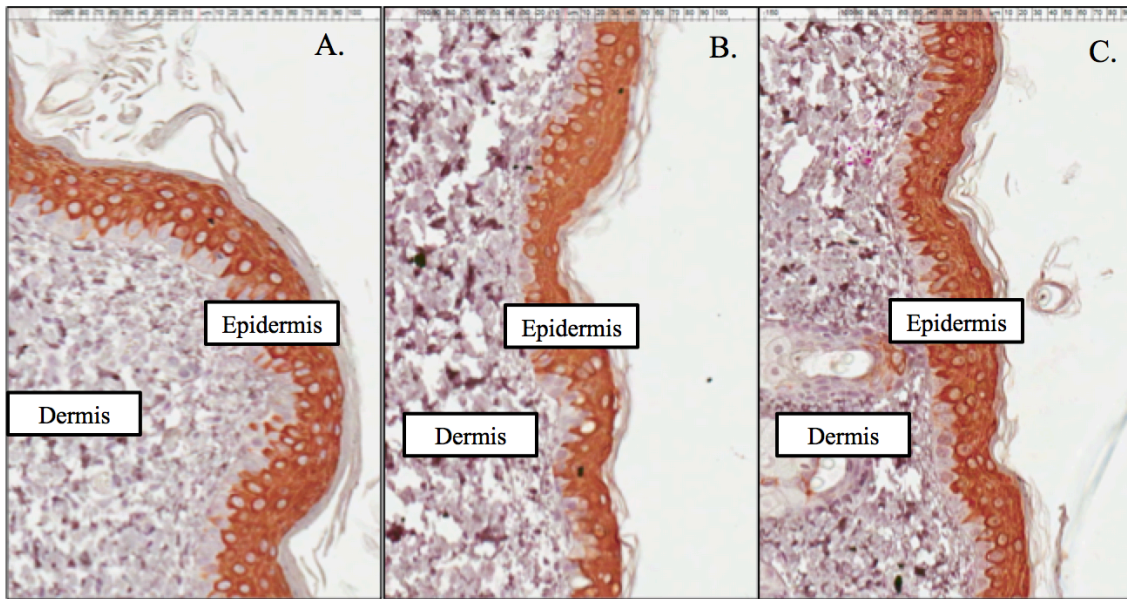




Supplemental: Figure 4. **Ki67** representative sections from each of day 5 HA-PE (A), HA (B), and Vehicle Cream (C) groups. Ki67 – brown staining.

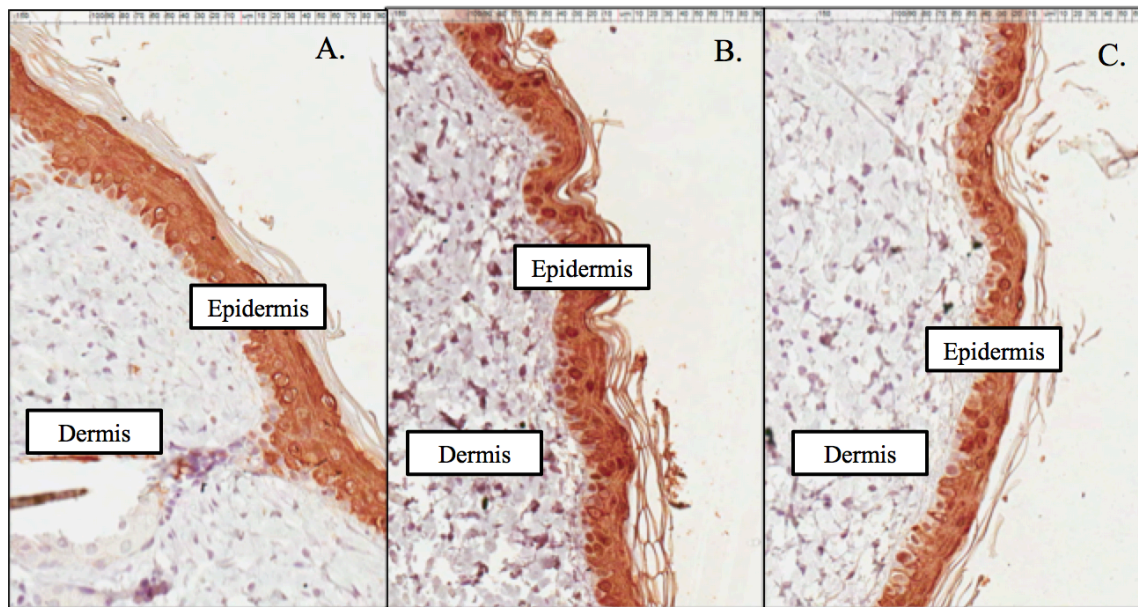


Supplemental: Figure 5. **K10** representative sections from each of day 1 HA-PE (A), HA (B), and Vehicle Cream (C) groups. K10 – brown staining.

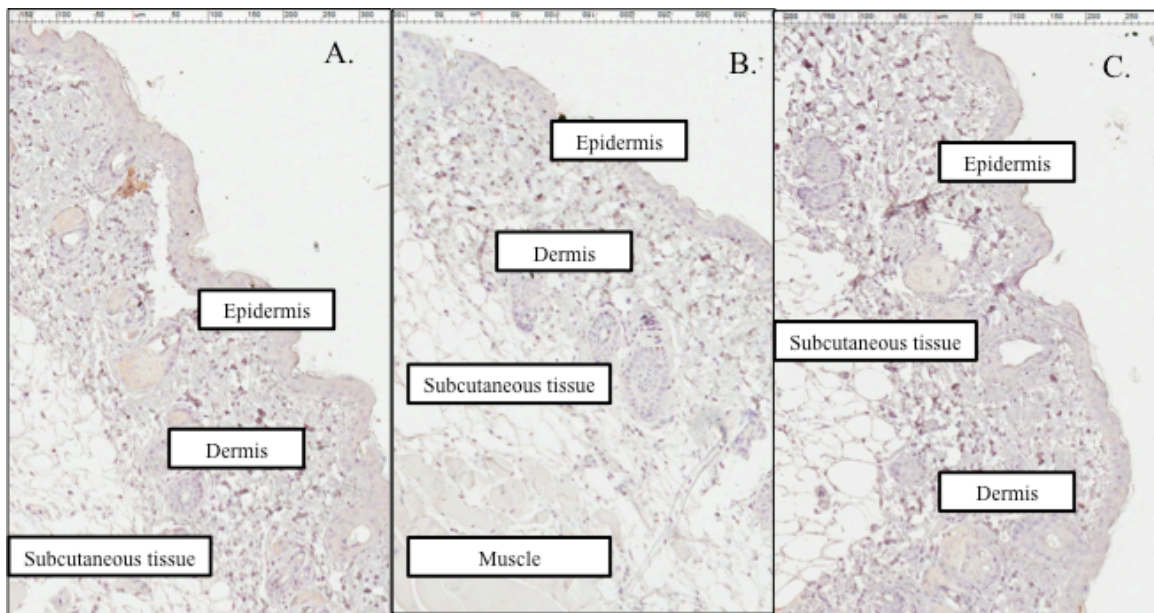


Supplemental: Figure 6. **K10** representative sections from each of day 5 HA-PE (A), HA (B), and Vehicle Cream (C) groups. K10 – brown staining.

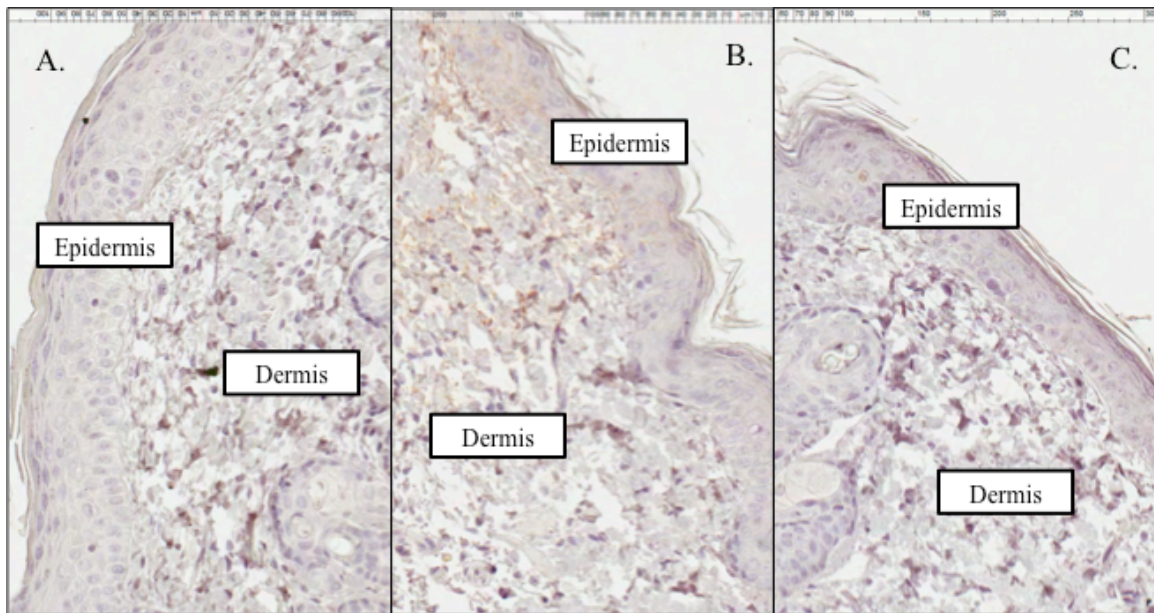




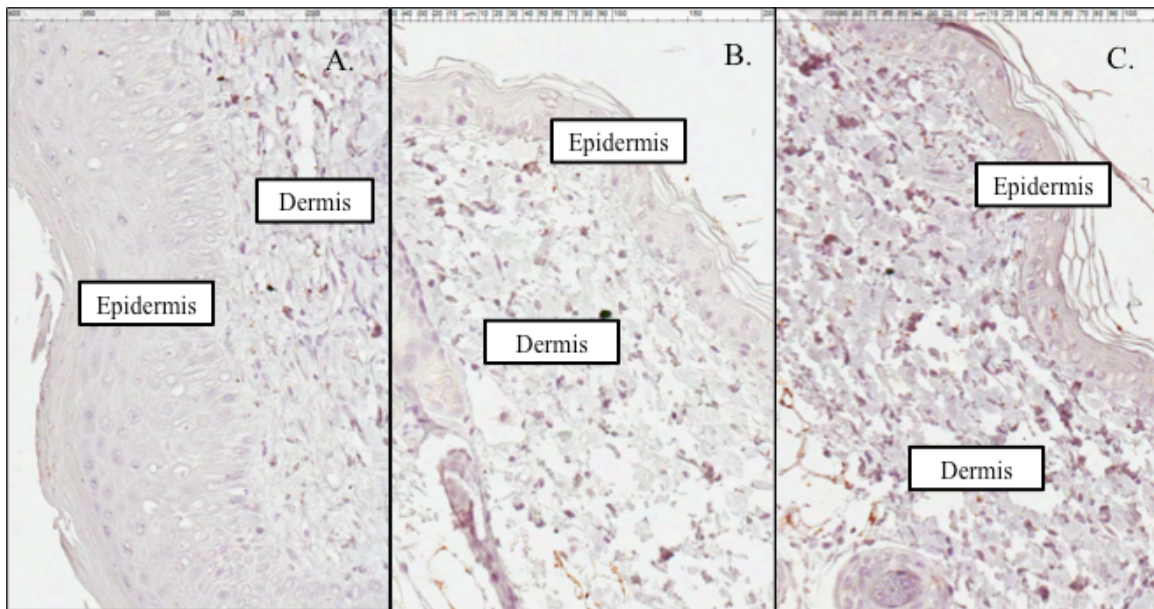
Supplemental: Figure 7. **K10** representative sections from each of day 10 HA-PE (A), HA (B), and Vehicle Cream (C) groups. K10 – brown staining.



Supplemental: Figure 8. **F4/80 representative sections from each of day 1 HA-PE (A), HA (B), and Vehicle Cream (C) groups.** F4/80 – brown staining.

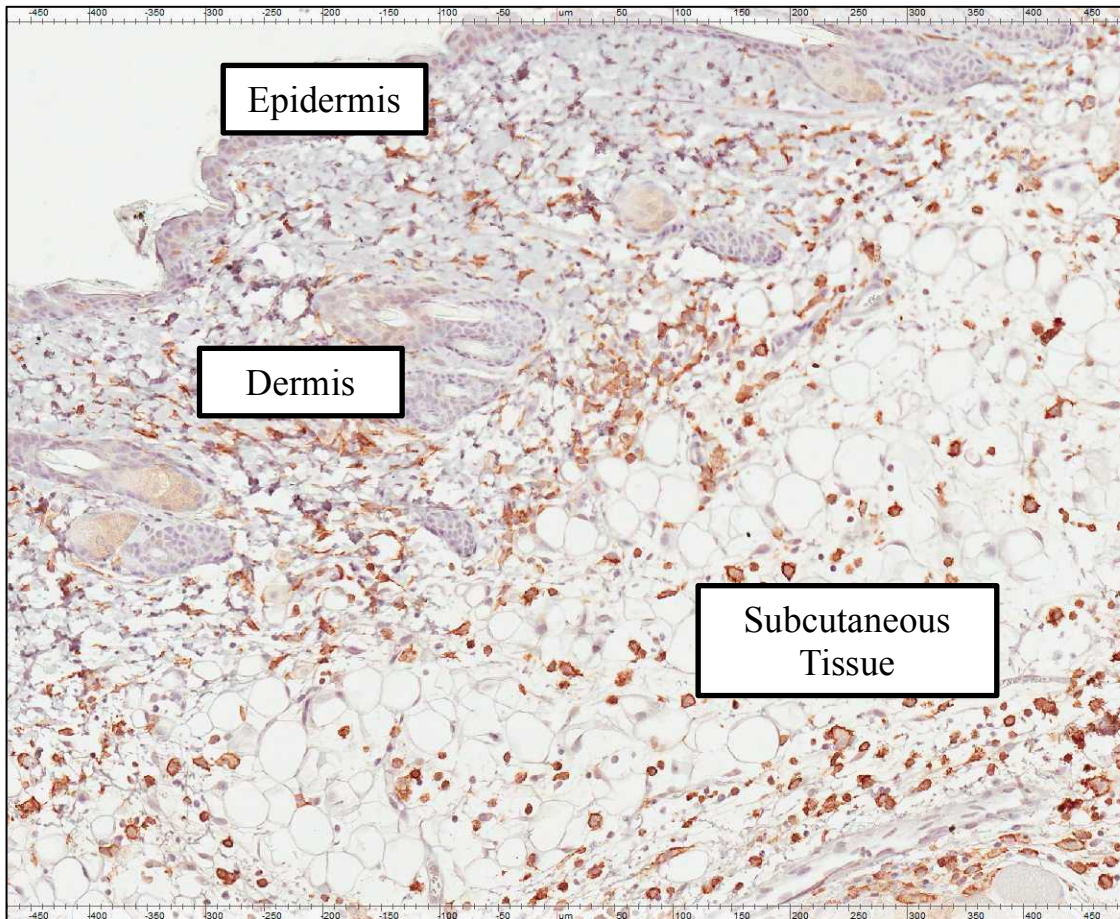


Supplemental: Figure 9. **F4/80 representative sections from each of day 5 HA-PE (A), HA (B), and Vehicle Cream (C) groups. F4/80 – brown staining.**



Supplemental: Figure 10. **F4/80** representative sections from each of day 10 HA-PE (A), HA (B), and Vehicle Cream (C) groups. F4/80 – brown staining.





Supplemental: Figure 11. **F4/80** representative sections from wounded mouse positive control. F4/80 – brown staining.

## Curriculum Vitae Caitlin J. Symonette

### EDUCATION

Plastic and Reconstructive Surgery Resident, Western University (2012 – present)  
 Master of Surgery, Western University (2013 – 2014 (anticipated))  
 Doctor of Medicine, Schulich School of Medicine and Dentistry (2008 – 2012)  
 Master of Science, School of Kinesiology, Western University (2006 – 2008)  
 Bachelor of Medical Sciences (Honors), Western University (2001- 2006)

### AWARDS & SCHOLARSHIPS

Dr. Robert McFarlane Resident Research Award	June 2014
Best Basic Science Paper, Plastic Surgery Research Day	June 2014
Resident Travel Reimbursement Fund (\$1000)	October 2013
The Roche Scholarship	May 2012
PAIRO Trust Fund Citizenship Award	May 2012
Honour Society Award, Hippocratic Council	March 2012

### STUDENT RESEARCH GRANTS

Cancer Research & Technology Transfer, CIHR Strategic Training Program 2013-2014  
 Department of Surgery Resident Research Grant (\$5000) December 2012  
 Schulich Research Opportunities Program, Undergraduate Medicine (\$5000) 2009 - 2011

### PEER-REVIEWED PUBLICATIONS

1. Lutz K, Symonette C, Yeoh K, MacDermid JC, Grewal R. **Complications associated with operative versus non-operative treatment of distal radius fractures in patients aged 65 years and older.** *Journal of Hand Surgery* 2014; 39(7): 1280-1286.
2. Symonette CJ, MacDermid J, Grewal R. **Emotional support contributes to outcomes following distal radius fractures.** *Rehabilitation Research and Practice* Accepted September 2013, *In Press*.
3. Symonette CJ, Gan BS. **Computed tomography-based vascular imaging in autologous breast reconstruction: a Canadian perspective.** *Canadian Journal of Plastic Surgery*. Spring 2013. 21(1): 11.
4. Symonette CJ, Adams PC. **Do all hemochromatosis patients have the same origin? A pilot study of mitochondrial DNA and Y-DNA.** *Can J Gastroenterol* 2011;25(6): 324-326.
5. Symonette CJ, Watson BV, Koopman WJ, Nicolle MW, Doherty TJ. **Muscle strength and fatigue in patients with generalized myasthenia gravis.** *Muscle and Nerve* 2010; 41(3): 362-369.