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The Effect of Topical Estriol on Human Inner Foreskin

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The Effect of Topical Estriol on Human Inner Foreskin

Cheryl LaKeysha Bell, Ph.D.

University of Connecticut, 2014

Human immunodeficiency virus (HIV) is a global health concern that prompts an urgent need to develop simple, effective, economical preventive measures. In spite of success with current prevention strategies, HIV continues to disproportionately affect men in certain sub-populations. The research described in this thesis evaluated the potential for topical estriol to induce keratinization of the inner foreskin, the primary site of HIV entry in males. My thesis examined the results of a phase 1a clinical trial to determine the effects of estriol on the biology of the inner foreskin mucosa.

Increased keratinization of the vaginal epithelium in monkeys was shown to effectively protect against simian immunodeficiency virus (SIV) infection through the female reproductive tract. We hypothesized that topical estrogen would have a similar effect on the inner foreskin. Using histology, we found that topical estriol can induce keratinization of the human inner foreskin by up-regulating the production of keratins per cell without increasing skin proliferation. Furthermore, the quantity and position of HIV target cells in the foreskin were not adversely affected by the treatment.

Estrogen receptors (ERs) are transcription factors that are capable of regulating a substantial number of target genes when bound by estrogen. Transcriptomic analysis of genes differentially affected by our treatment revealed that estrogen primarily acts on the keratinocyte epidermal differentiation process. We saw no evidence for an increase in

skin proliferation as a result of estrogen exposure. Estriol also induced early differentiation of keratinocytes in an immortalized keratinocyte cell line, HaCaT. Taken together, our results suggest that treatment of the inner foreskin with estrogen increases keratin production in keratinocytes without affecting cellular proliferation. While some adverse effects of the treatment were observed, the results of our phase 1a clinical trial were vastly positive. We recommend the continuation of this line of study with a larger scale clinical trial to determine the long-term effects of treatment. Similarly positive results could have a global impact as they imply that treatment provides a safe, cheap, non-invasive method for reducing the transmission of HIV while simultaneously aiding in controlling the spread of human papillomavirus (HPV).

The Effect of Topical Estriol on Human Inner Foreskin

Cheryl LaKeysha Bell

B.S., Jackson State University, **2004**

M.S., University of Connecticut, **2012**

A Dissertation

Submitted in Partial Fulfillment of the

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Doctor of Philosophy

at the

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2014

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Cheryl LaKeysha Bell

2014

APPROVAL PAGE

Doctor of Philosophy Dissertation

The Effect of Topical Estriol on Human Inner Foreskin

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2014

To my children:

Jamaria, Sean, and Chad

“You are my strength”

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Throughout my graduate career, I was constantly asked the question, “How do you do it?” “It” referred to balancing the seemingly insurmountable task of working on a PhD, teaching classes, raising three amazing children as a single mom, while still finding time to attend church, present at conferences, coach baseball games, and be an active leader in the parent teacher organization (PTO). My response: “I can do all things through Christ who strengthens me.” To the Trinity (God the Father, the Son, and the Holy Spirit), thank You for healing me when I was broken and strengthening me when I was weak. All of the glory belongs to You.

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To my wonderful mother and father, Lillian Howell-Bell and Curtis Bell, thank you for your unconditional love and support through the good times and the bad. Your daily phone calls, words of encouragement, and willingness to go through hell and high water for me are unmatched. I thank God every day for you! I love you more than you’ll ever know! To my sisters, Katrina and Christy, and my brother, Curtis, thank you for being proud of your little sister, even though it felt like I would be in school forever. To my grandmother, grandfather, aunts, and uncles who have all wondered when I’m moving back to Michigan, if you build it, I will come.

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TABLE OF CONTENTS

Abstract	
Title page	i
Copyright page	ii
Approval Page	iii
Dedication	iv
Acknowledgements	v
Table of Contents	viii
List of Figures	x
List of Tables	xvi
 Chapter 1. Introduction	
1.1 Overview	1
1.2 Skin	3
1.3 HIV	12
1.4 Estrogen	20
1.5 Scope of Thesis	24

Chapter 2: Elucidating the effect of topical estriol on foreskin epithelium

2.1 Background	28
2.2 Methods	31
2.3 Results	38
2.4 Discussion	45

Chapter 3: Transcriptional response of the human inner foreskin to topical estrogen

3.1 Background	48
3.2 Methods	53
3.3 Results	58
3.4 Discussion	82

Chapter 4: Modeling the effect of estriol on keratin production in keratinocytes in vitro using the HaCaT cell line

4.1 Background	90
4.2 Methods	93
4.3 Results	100
4.4 Discussion	118

Chapter 5: Summary and Future Directions

References

LIST OF FIGURES

Figure 1.1	Layers and structures of the skin. There are three layers of the skin: epidermis, dermis, and subcutaneous tissue (hypodermis). The skin is comprised of many structures that carry out important functions in each layer.	4
Figure 1.2	Human mucosal (thin) epithelium and full thickness skin. The epidermis of thin skin is narrower than thick skin, therefore the stratum corneum is thinner and less organized.	5
Figure 1.3	Creating a protective barrier through keratinocyte differentiation. Daughter cells of proliferating keratinocytes (epidermal stem cells) move out of the stratum basal and migrate up toward the stratum spinosum. As the cells undergo terminal differentiation, they lose vital organelles and begin to flatten. These flattened keratinocytes comprise a network of dead cells in the stratum corneum that are linked together with junction proteins and lipids.	7
Figure 1.4	Genes critical to the differentiation process. Keratin expression is specific to the stage of cell differentiation. As cells begin to differentiate, expression of the proliferating keratin pair (K5/14) is down-regulated, as expression of the differentiating keratins (K1/10) is up-regulated. The differentiation process is further characterized by the expression of keratin binding, crosslinking, and cornified cell envelope precursor proteins.	8
Figure 1.5	Retraction of the outer and inner foreskin. The foreskin is attached to the penis at the frenulum. In its relaxed state, the penis is fully or partially covered by the outer foreskin (thick skin). During an erection, the outer foreskin is retracted exposing the inner foreskin (thin skin). When the male has sexual intercourse with an HIV infected partner, the inner foreskin is presumably the site where the virus enters the body.	11
Figure 1.6	Langerhans cell near the apical surface of the epithelium. Langerhans cells survey the inner surface of the skin with their dendritic arms to detect the presence of pathogens.	13

These cells are capable of engulfing and degrading foreign material through specialized receptors and compartments with subsequent antigen presentation to T-cells circulating in the lymph nodes.

- Figure 1.7** HIV Co-receptors. The HIV protein, env, is made up of glycoproteins gp120 and gp41 that are required for viral entry into the cell. Binding of gp120 to CD4 induces a conformational change, allowing the virus to bind to the co-receptor CCR5 on macrophages or CXCR4 on T-cells. 15
- Figure 1.8** Genomic and non-genomic actions of estrogen. Estrogen diffuses into the cell and acts on its receptors to activate or repress transcription of target genes. Estrogen can also bind to receptors on the cell membrane to exert non-genomic effects through signal transduction. The latter mechanism induces rapid changes in gene expression. 22
- Figure 2.1** Dorsal Slit Method for Circumcision. 34
- a.) A circumferential cut is made on the inner and outer preputial skin slightly above the corona. A dorsal slit is made along the foreskin from the tip to the corona.
 - b.) The foreskin is removed and the glans penis is permanently exposed.
 - c.) Biopsies were collected from dorsal, frenular, and midventral areas of the excised foreskin.
- Figure 2.2** Histological staining of the inner foreskin. 37
- a.) Ayoub Shklar (AS) staining of inner foreskin tissue (top panels). AS differentially stains keratin and pre-keratin. The stratum corneum (keratin layer) is stained red; the stratified squamous epithelium is gray; the stratum spinosum (pre-keratin) is orange; the connective tissue is blue; erythrocytes are red. Keratin thickness was randomly measured at six sites across the intact epithelial keratin layer
 - b.) Immunofluorescence staining for Langerhans cells in inner foreskin tissue using CD1a antibody (bottom panels). CD1a (green) was used to identify Langerhans' cells present throughout the epithelium. DAPI (blue) was used to counterstain nuclei. Langerhans' cell projections can be seen as green staining not associated with a

nucleus (White arrow head in inset: B'). Langerhans' cell in the inner foreskin at 100X magnification. Yellow lines indicate measurement of Langerhans' depth within the cell.

- Figure 2.3** Intra- and intervariation in stratum corneum (keratin) thickness of the inner foreskin. Boxplot of keratin thickness differences before (striped) and after treatment (black) for each patient and among treatment groups. The black line in the middle of the boxes represents the median. The bottom and top of the boxes are the lower and upper quartiles, respectively. The whiskers represent the lower and upper quartile ranges, circles represent the outliers, and stars are extreme outliers. 40
- Figure 2.4** Stratum corneum (keratin) thickness pre and post treatment. 41
- a.) Each colored line represents the change in stratum corneum thickness for an individual patient.
- b.) Percent change in stratum corneum thickness across groups. Error bars represent standard deviation of the mean. Kruskal-Wallis test was used to determine significance. $p < 0.05$
- Figure 2.5** Langerhans cell distribution pre and post treatment. 43
- a.) Comparison of average Langerhans' cell density pre- and post- treatment per group. Error bars represent standard error.
- b.) Comparison of Langerhans' cell depth pre- and post-treatment. Error bears represent standard error. Mann-Whitney test was used to determine significance.
- Figure 3.1** Co-localization of filaggrin and involucrin in human inner foreskin. Immunohistochemistry was employed to determine the expression pattern of epidermal differentiation proteins, filaggrin and involucrin, before and after treatment with estrogen. Despite a thicker epithelium post-treatment, the expression of the proteins do not appear to be altered. 47
- Figure 3.2** Principal Component Analysis (PCA) plot of differentially expressed genes in foreskin tissues with or without 60

estrogen treatment. Trends exhibited by the expression profiles of treated (GB and RK) and non-treated (JS and PK) tissues. Each label represents a sample and the type of run.

- Figure 3.3** Differential expression analysis of estrogen treated and control inner foreskin tissues. 63
- a.) Scatterplot of genes expressed in foreskin tissue with or without estrogen treatment. Each dot represents a gene that is expressed in the foreskin. Dots along the center line represent genes that are expressed in both treated and non-treated tissues. Red dots are genes that are differentially expressed, genes in gray are not differentially expressed, and genes in black dots are non-differentiated genes whose expression values overlap.
- b.) MA plot of average gene expression level vs fold change in foreskin tissues. M, on the y-axis, represents the fold change in treated vs non-treated tissues. A, on the x-axis, represents the mean of the treated and non-treated values of a particular gene. Dot colors follow the description written above. This plot shows that genes with both low and high expression levels are differentially expressed.
- Figure 3.4** qRT-PCR validation of three differentially expressed genes from estrogen-treated inner foreskin RNA-Seq data. Fold changes determined from the relative Ct values for C-C chemokine receptor type 5 (CCR5), Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), and Late cornified envelope 3A (LCE3A) were compared to those detected by RNA-seq. Replicates (n = 3) of each sample were run and the Ct values averaged. All Ct values were normalized to beta-actin. 65
- Figure 3.5** Top ten Gene Ontology terms for genes significantly up-regulated in estrogen treated human foreskin tissues. Genes were grouped according to their biological processes. The top ten groups were ranked based on p-value. 70
- Figure 3.6** Top ten Gene Ontology terms for genes significantly down-regulated in estrogen treated human foreskin tissues. Genes were grouped according to their biological 71

processes. The top ten groups were ranked based on p-value.

Figure 3.7	Significantly differentially expressed genes turned on only in estrogen treated tissues from the Gage family of genes.	74
Figure 3.8	Expression patterns of estrogen responsive genes. We compared the expression data of ESR1, ESR2, and EEIG1 generated by a.) transcriptome sequencing and b.) qRT-PCR.	76
Figure 3.9	Expression patterns of HIV-co receptors. We compared the expression data of CD4, CCR5 and CXCR4 generated by a.) transcriptome sequencing and b.) qRT-PCR.	77
Figure 3.10	Expression patterns of Langerhans cell markers by a.) transcriptome sequencing and b.) qRT-PCR.	79
Figure 3.11	Expression patterns of keratin genes by a.) transcriptome sequencing and b.) qRT-PCR.	80
Figure 3.12	Expression patterns genes involved in keratinocyte proliferation and differentiation by a.) transcriptome sequencing and b.) qRT-PCR.	81
Figure 4.1	ESR 2 expression in untreated HaCaT cells.	102
Figure 4.2	Northern blot demonstrates ESR 1 expression in untreated HaCaT cells.	103
Figure 4.3	Untreated HaCaT cells express ESR1 and ESR2. A.) Cytoplasmic staining of ESR1 in untreated HaCat cells cultured for 24 hours. B.) Punctate nuclear staining of ESR2 in untreated HaCat cells cultured for 24 hours.	104
Figure 4.4	ESR1 expression in HaCaT cells treated with estriol at 24 and 48 hours. In the absence of estrogen, ESR1 localizes in the cytoplasm. High levels of estrogen can induce translocation of ESR1 to the nucleus in as little as 24 hours. All forms of estrogen have this effect by day 2.	105

Figure 4.5	ESR2 expression in HaCaT cells treated with estriol at 24 and 48 hours. ESR1 responds to both low and high levels of estrogen treatment in 24 hours. This expression is maintained at 48 hours.	106
Figure 4.6	ESR1 mRNA expression in treated HaCaT cells.	107
Figure 4.7	ESR2 mRNA expression in treated HaCaT cells.	109
Figure 4.8	Cell Proliferation Assay using treated HaCaT cells.	110
Figure 4.9	Western Blot analysis of keratin proteins.	112
Figure 4.10	Keratin 5 mRNA expression in treated HaCaT cells.	113
Figure 4.11	Keratin 14 mRNA expression in treated HaCaT cells.	114
Figure 4.12	Keratin 6 mRNA expression in treated HaCaT cells.	115
Figure 4.13	Keratin 16 mRNA expression in treated HaCaT cells.	116
Figure 4.14	Ki67 mRNA expression in treated HaCaT cells.	117
Figure 4.15	Keratin 1 mRNA expression in treated HaCaT cells.	119
Figure 4.16	Keratin 10 mRNA expression in treated HaCaT cells.	120
Figure 4.17	Filaggrin mRNA expression in treated HaCaT cells.	121
Figure 4.18	Involucrin mRNA expression in treated HaCaT cells.	122

LIST OF TABLES

Table 2.1	Randomized Treatment Group Assignments.	32
Table 2.2	Keratin thickness of each patient pre- (baseline) and post-treatment. Group assignments are shown in Table 2.1.	41
Table 2.3	Langerhans cell (LC) density and depth in human inner foreskin.	44
Table 3.1	Sequences of the qRT-PCR primers used in this study.	58
Table 3.2	Table 3.2 Top 25 significantly up-regulated genes in estrogen treated human foreskin tissues. The differentially expressed genes in estrogen treated human foreskin tissues compared to those in control foreskin tissues were determined by G-fold. For each gene, the fold change was calculated by dividing the RPKM of estrogen treated tissues to the RPKM of untreated tissues. The fold change was then log ₂ transformed. Genes with positive log ₂ values represent those that are over-expressed in the data set. The differentially expressed genes were ranked on their fold change and the 25 with the highest fold changes are shown here.	67
Table 3.3	Table 3.3. Top 25 significantly down-regulated genes in estrogen treated human foreskin tissues. The differentially expressed genes in estrogen treated human foreskin tissues compared to those in control foreskin tissues were determined by G-fold. For each gene, the fold change was calculated by dividing the RPKM of estrogen treated tissues to the RPKM of untreated tissues. The fold change was then log ₂ transformed. Genes with negative log ₂ values represent those that are under-expressed in the data set. The differentially expressed genes were ranked on their fold change and the 25 with the lowest fold changes are shown here.	68
Table 3.4	KEGG pathways overexpressed in treated tissue.	71
Table 3.5	KEGG pathway underexpressed in treated tissue.	72

Table 4.1 Sequences of the qRT-PCR primers used in this study.

98

Chapter 1

Introduction

1.1 Overview

Human immunodeficiency virus (HIV) is a significant global health concern that continues to devastate communities over 30 years after its discovery, in spite of extensive research in prevention and treatment. Over 2 million people became newly infected with the virus in 2013 [1]. In 2011, men accounted for about 75% of all new diagnoses in the United States [2]. Research suggests that in males the primary entry point of HIV virions is through the inner foreskin of the penis which contains a dense population of Langerhans cells (LCs), a subset of specialized immune cells that act as our body's first line of defense against invading viruses [3, 4]. LCs are highly efficient in degrading HIV in the absence of a direct infection with high concentrations of HIV [5].

In males, the foreskin protects the internal environment from outside forces. The outer foreskin has a thick keratin layer that provides a barrier against viral pathogens, thereby acting as a safeguard to the LCs. The inner foreskin is characterized by a thin keratin layer that yields little protection for the underlying cells [6]. Surgical removal of the foreskin (circumcision) provides at least 50% reduced risk of infection [7-9] presumably due to the removal of the LCs and the mucosal tissue. Unfortunately, implementing circumcision in developing countries that have high infection rates is challenging, particularly in populations where it may be culturally or religiously unacceptable [10]. Therefore, there remains a critical need to identify countermeasures for preventing HIV transmission that are simple to implement, acceptable, economical, readily available, and applicable for long-term usage.

One potential HIV intervention, and the topic of this thesis, is the use of topical estrogen to enhance keratin production of the inner foreskin epithelium. Estriol, the weakest naturally occurring estrogen, is highly effective in preventing simian immunodeficiency virus (SIV) transmission across the vaginal epithelium (an analogous mucosal epithelium to the inner foreskin) in monkeys [11, 12]. However, excess estrogen in women can increase the risk of breast, uterine, and cervical cancers [13-16]. The foreskin epithelium is characterized by the presence of stratified squamous cells as is the vaginal epithelium [6]. To date, very few studies have evaluated how foreskin biology is affected by estrogens. The work in this thesis examined the structural, cellular, and molecular effect of estriol treatment on the physiology of on a subset of epithelial cells, including HIV target cells, of the inner foreskin using *in vivo* and *in vitro* models. We have demonstrated that the human inner foreskin can respond to topical estriol by increasing epithelial and keratin thickness. This effect, in turn, may decrease susceptibility to HIV infection by reducing HIV virion penetration of the skin and presentation to the underlying target cells. Our proposed intervention could help control HIV spread in developing countries using a non-invasive procedure and may also aid in reducing the incidence of human papillomavirus (HPV). Our research supports the potential benefit of using topical estrogen in a phase 2 clinical trial to assess the efficacy of estriol applied to the inner foreskin in reducing the risk of HIV infection.

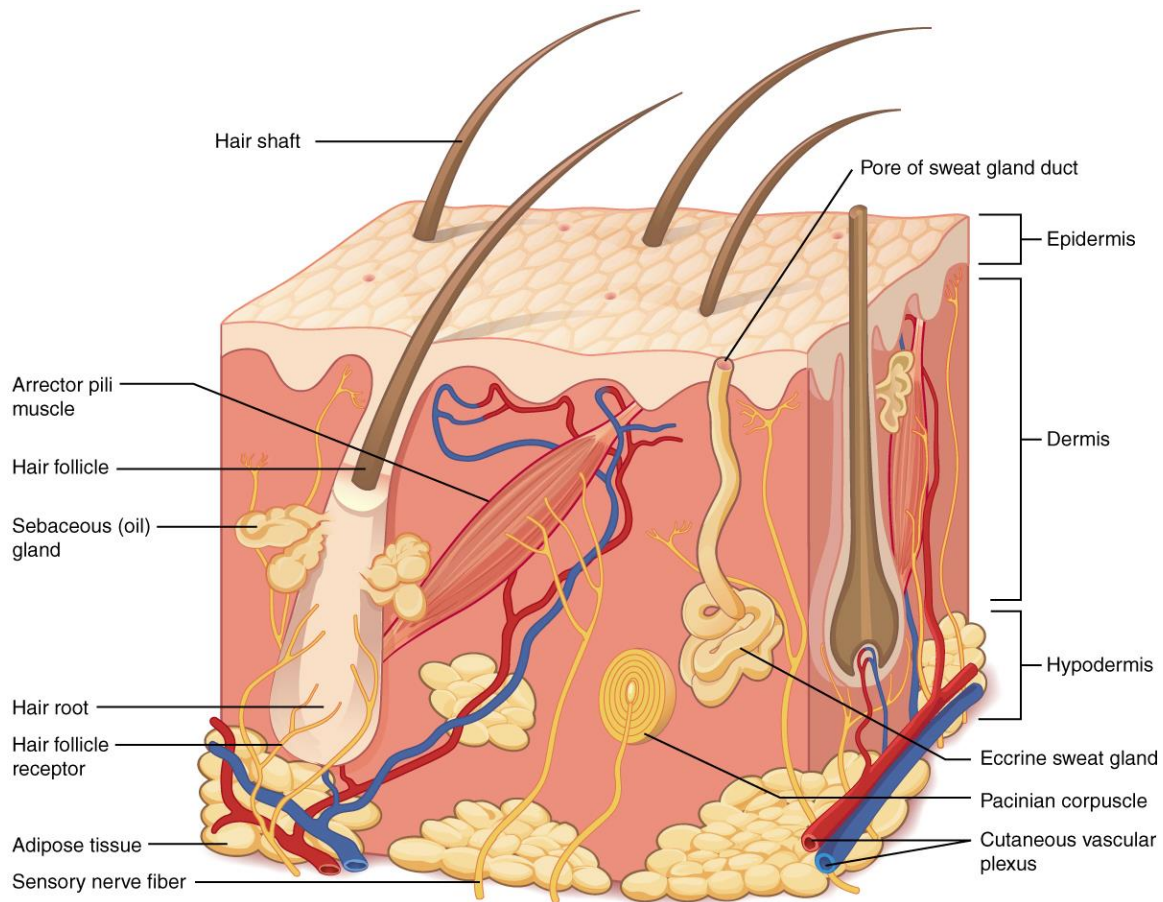
1.2 Skin

Anatomy and Function

Skin, the largest organ of the body, is responsible for protecting internal structures from the external environment [17]. Among its many important functions, the skin regulates the passage of water and other molecules into and out of the body, while simultaneously protecting against pathogens, toxins, and injury [18, 19]. The skin is typically divided into three layers (Figure 1.1). The subcutaneous tissue, also known as hypodermis, is the base layer of the skin and is composed of fat and connective tissue. It provides an energy reserve, insulation, and cushion. Above it lies the dermis, which also contains connective tissue, giving strength and elasticity to the skin. The epidermis is the outermost layer and serves as a waterproof barrier for protection. It is this layer of skin that will be the focus of this project.

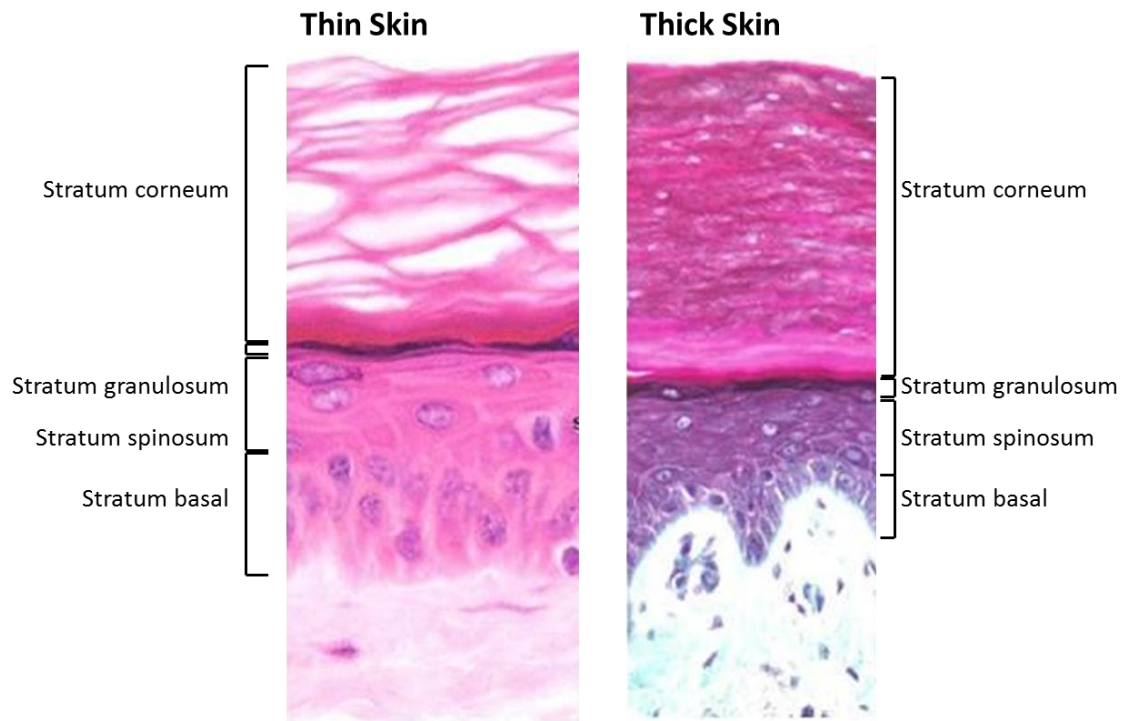
The protective nature of the epidermis can be attributed to its ability to continuously shed and replace its structural cells as they age. These structural cells, keratinocytes, are the most abundant cells in the epidermis [20]. As keratinocytes divide, they migrate upward toward the surface of the skin forming four layers: the stratum basal, the stratum spinosum, the stratum granulosum, and the stratum corneum (Figure 1.2). Epidermal keratinocytes maintain the integrity of the skin by producing keratins, a family of structural, water-insoluble proteins [21]. Keratins assemble into bundles of intermediate filaments consisting of a specific combination of type I (acidic) and one type II (neutral/basic) keratin proteins to provide mechanical strength [22]. Each layer of the skin can be characterized by the keratin pairs being expressed.

Figure 1.1 Layers and structures of the skin. There are three layers of the skin: epidermis, dermis, and subcutaneous tissue (hypodermis). The skin is comprised of many structures that carry out important functions in each layer.



http://philschatz.com/anatomy-book/resources/501_Structure_of_the_skin.jpg

Figure 1.2 Human mucosal (thin) epithelium and full thickness skin. The epidermis of thin skin is narrower than thick skin, therefore the stratum corneum is thinner and less organized.



Modified from <http://www.studyblue.com/notes/note/n/histology/deck/3704969>

Keratinocytes in the stratum basal are stem cells that divide mitotically to produce two identical daughter cells. One daughter cell stays in the stratum basal to constantly renew keratinocytes, while the other migrates up toward the stratum spinosum and begins to terminally differentiate (Figure 1.3). Skin-specific keratin proteins 5 and 14 are expressed in the mitotically active basal cell layer where they play a role in cell proliferation [23]. During the process of epithelial differentiation, expression of keratins 5 and 14 decreases and cells begin to express a different set of skin keratins, 1 and 10 [20]. As the cells age, they begin to flatten, the nucleus and cytoplasmic organelles disappear, and transcription ceases. The dying cells become cornified as keratin protein is incorporated into longer keratin intermediate filaments. These keratin filaments make up the fully differentiated, outermost layer of the skin, the stratum corneum. The dead cells containing the keratin are called corneocytes and are constantly replaced as they continuously slough off the surface of the skin in a process known as desquamation. Throughout this dissertation, this region of the skin may be referred to as the keratin layer or the stratum corneum.

During skin development, keratin protein production may be up-regulated in response to wound healing and disease. For example, keratins 6 and 16 are normally expressed in hyperproliferative stratified epithelia or epithelia with abnormal differentiation. In normal stratified epithelia, only basal levels can be detected [24]. Following injury, expression of keratins 6 and 16 is up-regulated and maintained during the repair process [25-27]. In addition to the keratin proteins, there are other specialized proteins in the skin that are responsible for adhering keratinocytes within the epidermis and supporting the structure of the stratum corneum (Figure 1.4).

Figure 1.3 Creating a protective barrier through keratinocyte differentiation. Daughter cells of proliferating keratinocytes (epidermal stem cells) move out of the stratum basal and migrate up toward the stratum spinosum. As the cells undergo terminal differentiation, they lose their nucleus and begin to flatten. These flattened keratinocytes comprise a network of dead cells in the stratum corneum that are linked together with junction proteins and lipids.

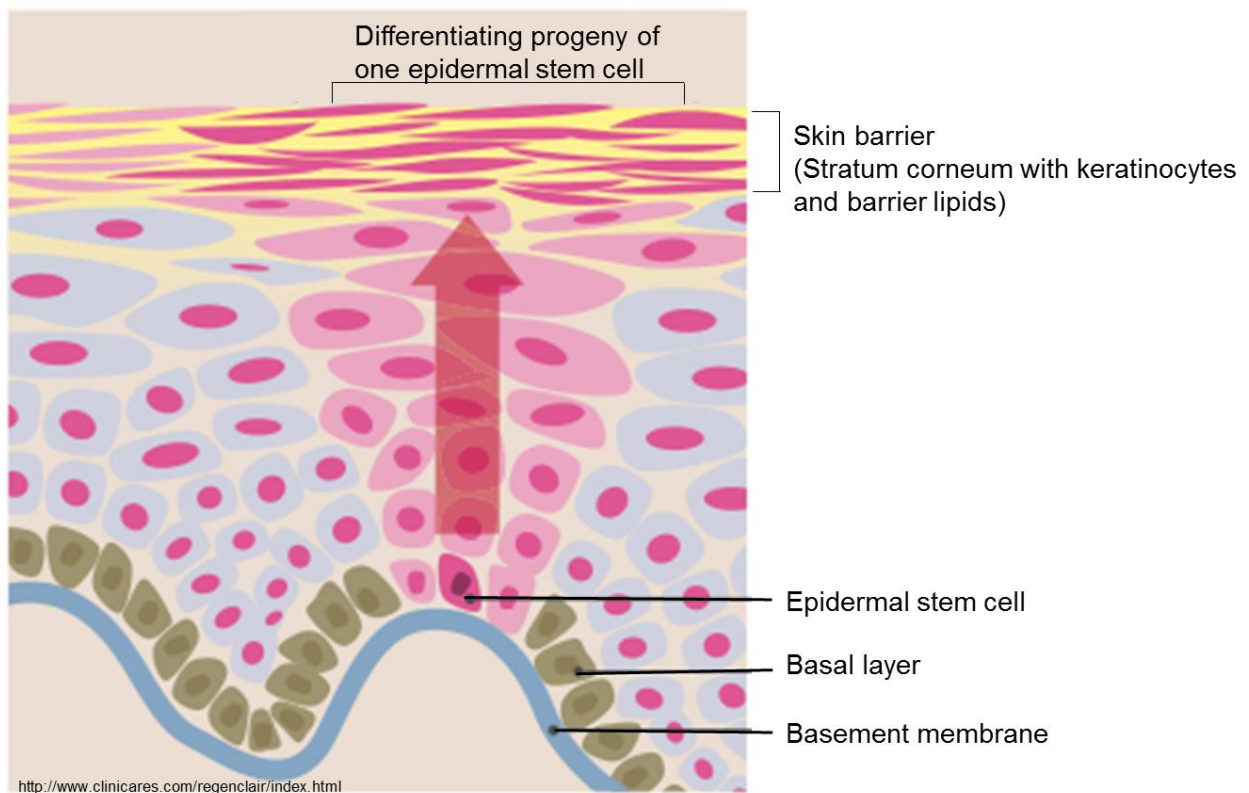
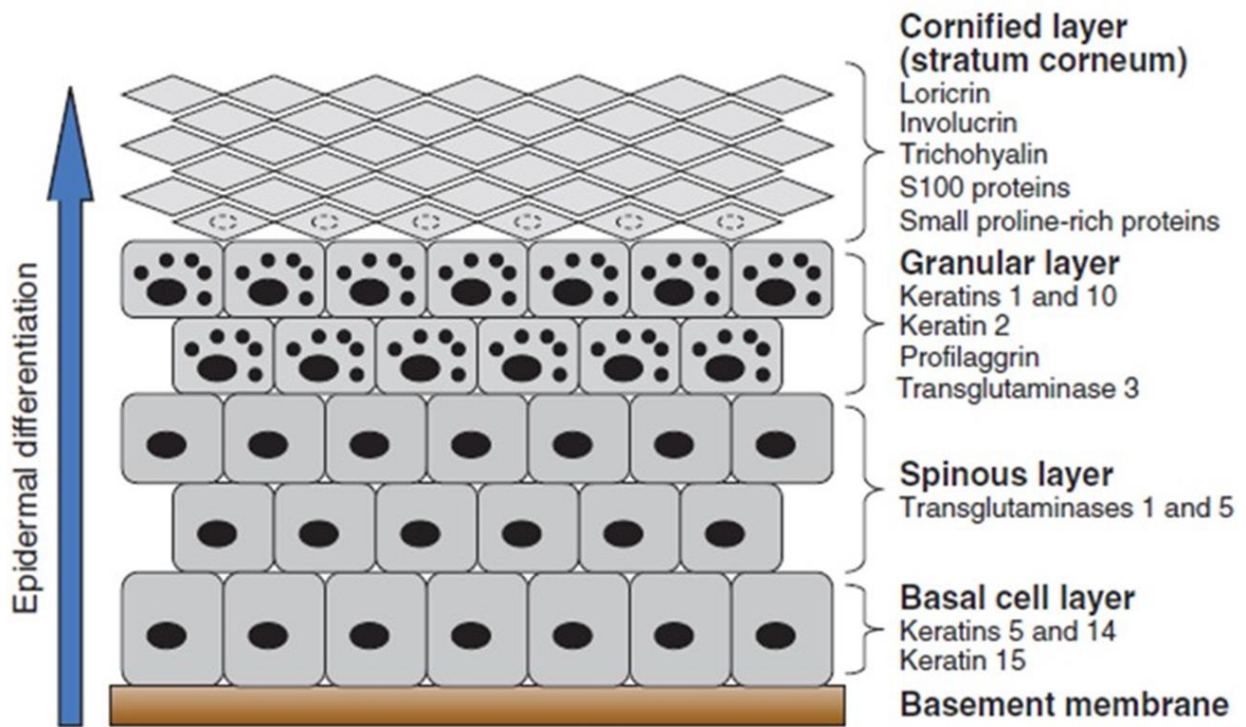


Figure 1.4 Genes critical to the differentiation process. Keratin expression is specific to the stage of cell differentiation. As cells begin to differentiate, expression of the proliferating keratin pair (K5/14) is down-regulated, as expression of the differentiating keratins (K1/10) is up-regulated. The differentiation process is further characterized by the expression of keratin binding, crosslinking, and cornified cell envelope precursor proteins.



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Hemidesmosomes are cell adhesion structures that bind keratinocytes to the basement membrane of the epidermis, while desmosomes link keratinocytes together [28, 29]. Filaggrin binds to keratin to aid in the aggregation of the keratin filaments, thereby providing additional structural support to the stratum corneum [30]. Involucrin, a protein synthesized during terminal differentiation, aids in formation of an insoluble envelope, by crosslinking to membrane proteins [31]. As the outer surface of the epithelium comes in contact with harsh conditions from the external environment, the cornified cell envelope serves as a major contributor to the epithelial barrier protecting the inner surface of the skin [20].

The expression of the aforementioned genes that are involved in the formation of the cornified envelope, such as the keratins, filaggrin, and involucrin, have been extensively studied in cell culture. HaCaT (human adult skin keratinocytes propagated under low Ca^{2+} conditions and elevated temperature) cells, immortal, nontumorigenic keratinocytes, are a common keratinocyte cell line used to study proliferation and differentiation [32]. Their growth pattern in culture is similar to normal human keratinocytes *in vivo*, so they are commonly used to study skin response to various stimuli. To determine what effect the stimulus has on the skin, the normal condition of the skin must be taken into consideration.

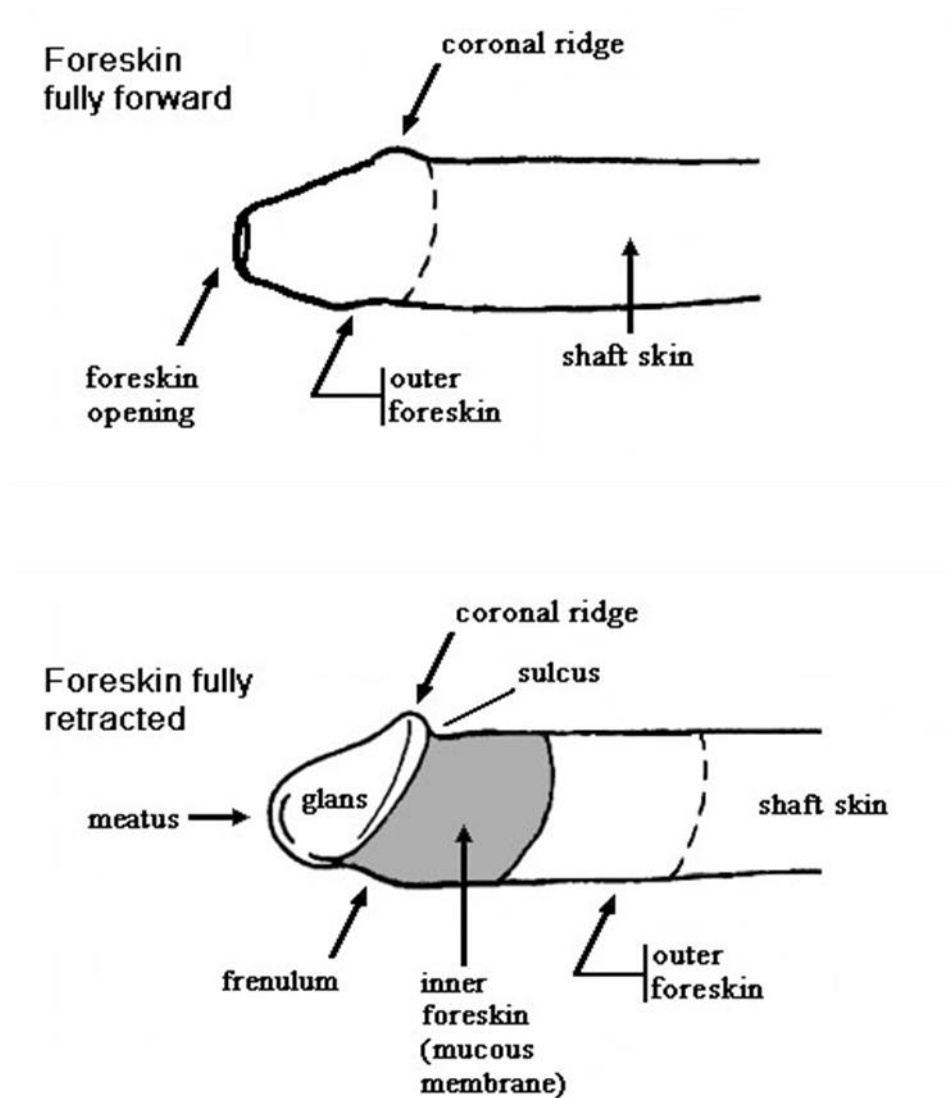
1.2.1 Foreskin anatomy, physiology, and immunology

Skin covers the entire body and is contiguous with the mucous membrane of the genitals [6]. Although males and females have distinct reproductive physiology, the prepuce is an

external genitalia tissue that is inherent to both male and female anatomy. The prepuce forms the clitoral hood of the female genitalia, while its counterpart in males is the foreskin. The foreskin is comprised of two distinct layers: the outer and the inner [6] (Figure 1.5). The outer foreskin, an extension of the skin covering the penile shaft, serves as a protector of the glans penis (the head of the penis) and the inner foreskin [6]. The inner foreskin is a mucosal epithelium that forms an interface with the outer foreskin and the glans penis. It functions as a barrier against moisture loss as well as infection with pathogens and harmful agents. This region of the foreskin is characterized by a thin layer of keratin covering the underlying tissue [33]. Keratin aids in protection from exposure to external environmental conditions. When the outer foreskin is folded over the inner foreskin (as in the flaccid state) a moist environment is formed which aids in protection of the glans penis from the external environment [6].

In addition to this physical barrier, the foreskin plays an important role in the innate immune system. The foreskin epithelium is thought to be the main site of HIV entry into the penis, presumably because it is densely packed with Langerhans cells (LCs) [34]. LCs, derived from bone marrow, are a subset of dendritic cells that survey the epithelium for foreign antigens. They are capable of recognizing and engulfing pathogens like viruses present on the cell's surface through endocytosis [35]. Upon internalization, the pathogen is degraded and a fragment of the antigen is displayed on the outer surface of the cell [36]. LCs present the antigen to the T-cells to elicit an immune response [37, 38]. In addition LCs express cytokine and chemokine receptors that aid in innate and adaptive

Figure 1.5 Retraction of the outer and inner foreskin. The foreskin is attached to the penis at the frenulum. In its relaxed state, the penis is fully or partially covered by the outer foreskin (thick skin). During an erection, the outer foreskin is retracted exposing the inner foreskin (thin skin). When the male has sexual intercourse with an HIV infected partner, the inner foreskin is presumably the site where the virus enters the body.



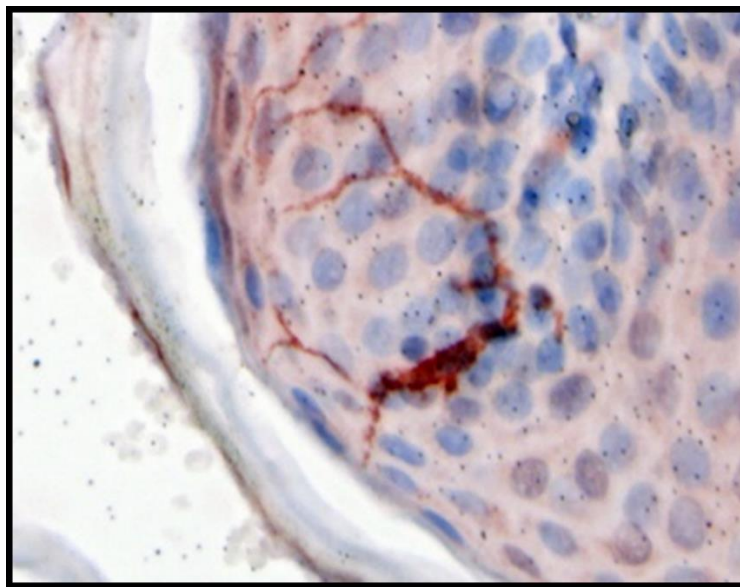
Modified from <http://coloradonocirc.org/foreskin>

immune responses. LCs are present in all epithelial tissues throughout the body, but are more concentrated in the mucosal epithelia (foreskin, vagina, mouth, anus etc.) most likely due to the increased level of exposure to microbes than other skin [39]. Being in close proximity to the thin, mucosal layer of the inner foreskin renders LCs susceptible to infection by viruses and other infectious agents, which can result in disease [40] (Figure 1.6). Viruses cannot survive for long periods of time outside of a host cell. The foreskin may create a permissible environment for infection, particularly when the penis returns to the relaxed state after a sexual encounter with an infected partner. The warm, moist environment favors survival of viruses, therefore, many infectious agents are commonly transmitted during unprotected sexual intercourse. Human immunodeficiency virus (HIV) is a well-studied sexually transmitted viruses that cannot be cured. The prevention of this viral infection is of utmost importance in many communities.

1.3 HIV

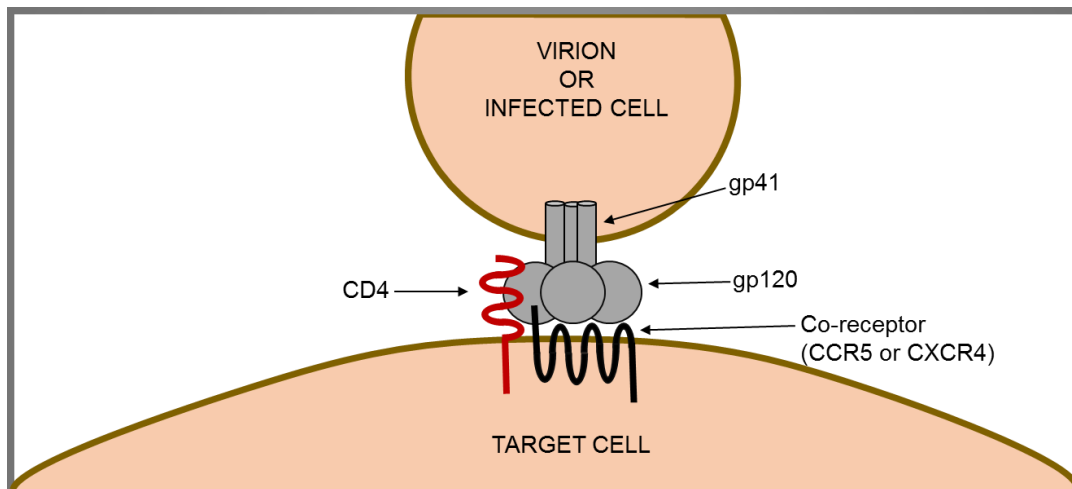
Human immunodeficiency virus (HIV) has infected over 75 million people and caused over 35 million deaths worldwide [41], making it one of the greatest health crises in human history. There is a desperate need to develop simple and effective strategies to prevent HIV infection. Attempts to combat the disease are taking place on many fronts, from the development of HIV vaccines to the therapeutic use of microbicides, prophylactics, and extensive sexual education programs. However, there have been very few breakthroughs in these areas and we are far from being able to control the epidemic by any of these means.

Figure 1.6 Langerhans cell near the apical surface of the epidermis. Langerhans cells survey the inner surface of the skin with their dendritic arms to detect the presence of pathogens. These cells are capable of engulfing and degrading foreign material through specialized receptors and compartments with subsequent antigen presentation to T-cells circulating in the lymph nodes.



In the early 1980s, there was a sudden surge in male patients presenting with severe pneumonia, sudden weight loss, and in some cases, a rare cancer called Kaposi sarcoma [42-44]. Over time, these men experienced a decline in the function of their immune system. These symptoms along with opportunistic infections that were seen in this patient population were categorized as acquired immunodeficiency syndrome (AIDS). It was not long before scientist discovered that HIV was the virus that caused this illness. HIV is an enveloped, retrovirus with an RNA genome [42]. The envelope contains glycoprotein spikes that are essential for the attachment of the virus to the host cell [45]. Untreated HIV develops into AIDS when the virus destroys essential immune cells responsible for initiating the body's response to infection called CD4⁺ T-cells [46]. The virus can only infect cells displaying a specific combination of receptors, a main receptor, CD4, and a co-receptor [47] (Figure 1.7). The co-receptor varies depending upon the target cell, but the two major types are CCR5 and CXCR4 [47]. Both receptors are expressed on cells that play an essential role in the immune response to viruses. CCR5 is the predominant co-receptor targeted in HIV transmission and is mainly expressed on dendritic cells and macrophages, while CXCR4 tends to be associated with T-cells [48-51]. Once the virus successfully enters the host cell, it hijacks the cell's transcriptional machinery to make copies of itself [52]. These new virus particles are released from the cell, spreading and infecting nearby cells. As cells become damaged, the decline in the number of functioning CD4⁺ cells results in a weakened immune system that is more susceptible to secondary infections [53].

Figure 1.7 HIV Co-receptors. The HIV protein, env, is made up of glycoproteins gp120 and gp41 that are required for viral entry into the cell. Binding of gp120 to CD4 induces a conformational change, allowing the virus to bind to the co-receptor CCR5 on macrophages or CXCR4 on T-cells.



Modified from *Frontiers in Bioscience* 3, d44-58, January 1, 1998

As HIV multiplies and spreads throughout the body, it can be transmitted to other people through direct contact with body fluids such as blood, semen, or vaginal secretions [54]. However, it cannot survive very long outside of the host [55]. In order to establish infection, the virus must complete a six-step process that is described in brief here [56]. To enter a host cell, the virus must bind to CD4 and a co-receptor. Once the virus enters the cell, reverse transcriptase will convert the RNA genome to DNA. The viral DNA may integrate into the host cell's DNA. During new synthesis, the viral DNA is transcribed into mRNA, which is translated into one long polypeptide. Protease cleaves the polypeptide into individual viral proteins that make up the virion: spikes, capsid and reverse transcriptase. New viruses are assembled and released from the cell to infect more cells. Each step is a potential target for drug therapy to prevent the replication and spread of HIV.

1.3.1 Current Prevention Strategies

Although, HIV is a devastating disease, survival rates have continuously improved in recent years due to the advancement in the development of effective treatments. There have been a handful of cases in which HIV patients have been “cured” of the disease by eliminating the viral reservoirs in the body.

In one study, a baby born to a HIV infected mother was given immediate and aggressive treatment with an anti-HIV drug regimen called highly active anti-retroviral therapy (HAART) [57]. HAARTs success is primarily due to its employment of a combination of

drugs to block several steps of the viral replication cycle, reducing the likelihood of drug resistance. However, there are many adverse effects of these medications which can lead to non-compliance and misuse of the drugs. Soon after the treatment began, examination of the baby's blood confirmed that the virus had been transmitted to the baby during birth. One month after the baby was born, HAART treatment had been successful at lowering the levels of HIV below the detection limit. After adhering to the HIV regimen for at least 15 months, the mother began to miss medical appointments and reported discontinuing the use of the ARTs when the baby was 18 months old. When the mother resumed medical care for the baby at 23 months, the doctors were unable to detect HIV RNA or antibodies in the baby's blood, despite being off of medication for about 5 months [57]. Sadly, recent news reports indicate that after being off of medication for 27 months, the child has again tested positive for the disease [58].

In another case, an HIV infected patient taking ARTs who also had acute myeloid leukemia was given a bone marrow transplant from a donor who was CCR5 negative [59]. The patient continued with his ART regimen up to the day before his transplant. Twenty months after the transplant and no ART treatment, the patient had no detectable levels of the virus [59]. Both of these cases were unique as they were instrumental in demonstrating that HIV can be reduced to undetectable levels after establishing infection.

HIV is still considered an incurable infection, and even the best treatment will only delay the progression of HIV to AIDS. In addition treatments like those described above are

very expensive and largely inaccessible to areas where HIV/AIDS has become endemic. Controlling the increasing rates of infection in susceptible populations rely on preventing exposure to the virus through abstinence, monogamy, and most importantly, using prophylactics [60]. I choose to live by the mantra, “prevention is better than a cure.” As such, the research project detailed in this paper focuses on a novel method to potentially prevent transmission of HIV.

1.3.3 Circumcision

Efficient means for reducing new HIV infections should prevent or reduce transmission of the virus. To date, the most significant advance in HIV/AIDS prevention has been the surprising correlation of circumcision and a reduced risk for HIV infection. Circumcision began in ancient Egyptian times as a ritualistic practice but is currently performed as a cosmetic and/or hygienic practice in Western cultures [61, 62]. It is a common medical procedure in which the foreskin is surgically removed from the glans penis [63]. It is well known that there are many benefits to circumcision, including lower risks of urinary tract infections [64], penile cancer [65], and some sexually transmitted infections [66-68]. In September 2012, the American Academy of Pediatrics published its circumcision policy stating that the potential health benefits of circumcising newborn males outweigh the risks associated with the surgical procedure [69].

The results of three large randomized trials of male circumcision, carried out in Kenya [7], Uganda [8], and South Africa [9] leave no doubt that circumcision more than halves a

man's risk of HIV infection [70, 71]. The protective effect is thought to be due to the physical removal of most of the foreskin epithelium containing HIV target cells [34, 72]. Removal of the foreskin, the primary site of HIV infection in men, through circumcision results in keratinization of the remaining epithelium covering the glans penis. This may confer a level of protection against infections with external pathogens [6].

While male circumcision is undoubtedly effective in reducing the risk of male HIV infection, implementing such a surgical preventative strategy is a daunting task, particularly in countries like India, China and most of South-East Asia where circumcision may be culturally unacceptable [73]. In addition, a circumcision program requires extensive medical training, personnel, and equipment. Studies suggests that the populations who would most benefit from the protective effect of circumcision may be high-risk populations (e.g. sex workers, men who have sex with men, and drug users) and female to male transmissions due to lower rates in the general population [74]. Other interventions will likely be required in these populations and those with high rates of HIV infection to control its spread. Male to female transmission tends to be the primary mode of transmission for new infections, so preventative methods need to be explored in this populations as well [75]. Rather than removing the LCs by circumcision, another method to prevent infection would be to create a barrier to reduce the exposure of the LCs to the virus.

Many researchers are studying the potential of microbicides as an effective means of preventing the spread of sexually transmitted diseases. Microbicides reduce infection in a number of ways by creating a physical barrier that keeps HIV and other microbes from reaching target cells and thereby preventing replication within the cell [76-78]. Although microbicides can be produced in many forms, most have been developed for the vagina and the rectum just before intercourse. Unfortunately, this limits protection to women and men who have sex with men [79]. Additionally, these regions of the human anatomy are subject to micro tears during sexual intercourse, providing direct access of the virus to the circulatory system [80]. An ideal microbicide would be one that can be applied at any time prior to the sexual encounter. There is a need to explore additional effective means of preventing disease.

1.4 Estrogen

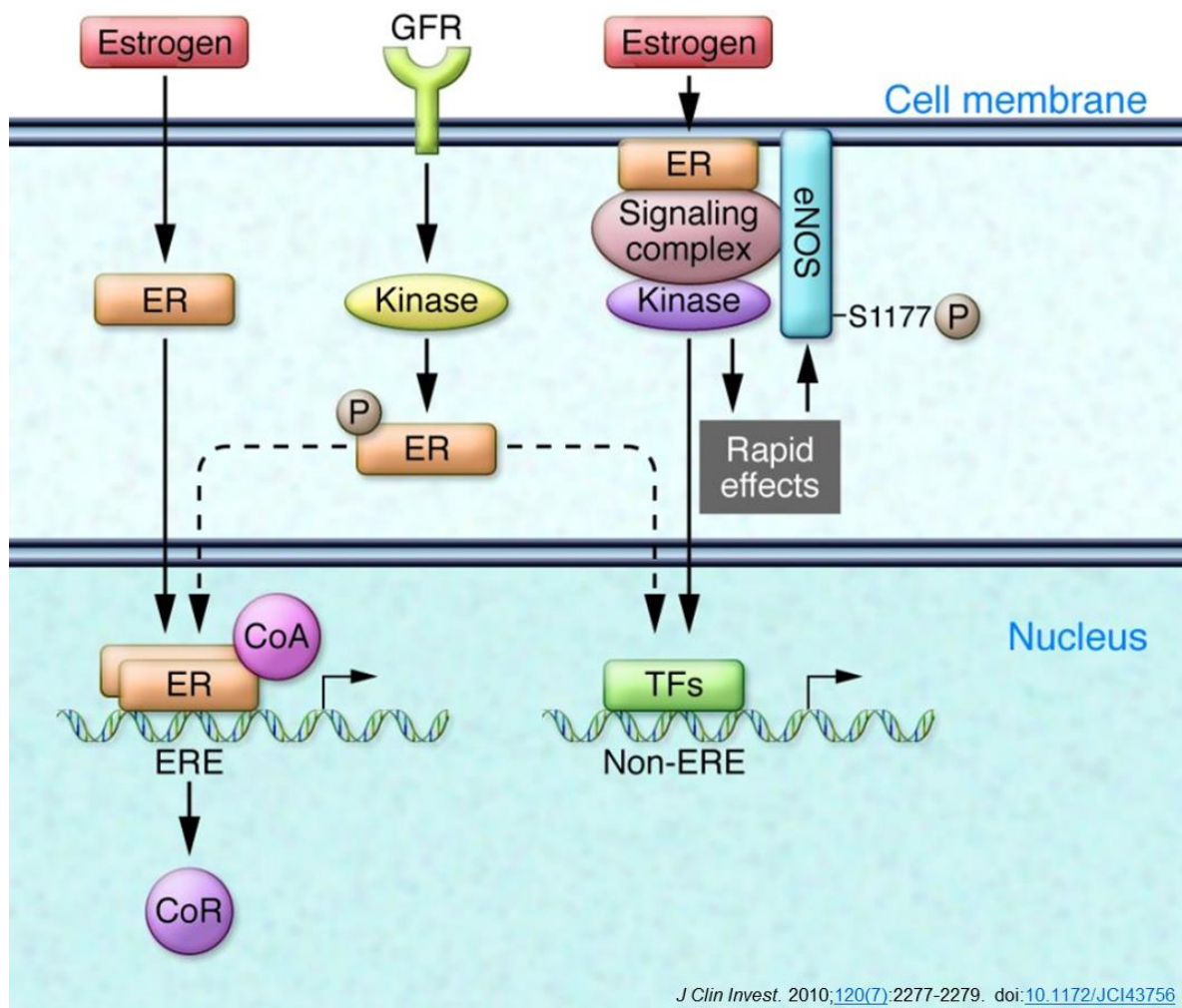
Estrogens are essential in the development and maintenance of reproductive tissues. In addition, they have multiple targets so their biological effects are widespread throughout the body. Because estrogens are capable of regulating the activity of distal organs and tissues, they can also affect the activity of the cardiovascular, musculoskeletal, immune, and central nervous systems [81]. There are three major forms of natural estrogen produced by the body [82]. The most potent form, 17 β -estradiol (E2), is the predominant estrogen found in the circulation of reproductive aged women. When a woman becomes pregnant, she begins to secrete higher levels of estriol (E3), the weakest of the three forms [82]. When a woman reaches menopause, estrone (E1) becomes the primary form

of circulating estrogen [82]. Men also produce estrogen, although at much lower levels than women [83].

1.4.1 Mechanism of Action

Estrogens can diffuse across the outer cell membrane and carry out their action in the cytoplasm and nucleus [84]. The biological effects of estrogen are mediated through two cytoplasmic estrogen receptors (ER), estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2). When the ERs are bound by their endogenous hormones, they become activated and the newly formed complex translocates to the nucleus where it regulates transcriptional activity of many genes, often resulting in increased proliferation of cells [85]. All estrogens have an affinity for the ER, however, E2 exhibits the strongest relative binding affinity followed by estriol, and lastly, estrone [86]. Estrogen signaling can occur through genomic or non-genomic actions (Figure 1.8). The classical genomic signaling pathway takes place inside of the cell when the estrogen-estrogen receptor complex binds to specific DNA sequences called estrogen response elements (EREs) in the promoter of target genes to regulate gene expression [87]. Alternatively, ERs can also regulate gene expression without directly binding to DNA, through protein-protein interactions with DNA-binding transcription factors in the nucleus [82]. Genomic signaling may take hours or days to produce an effect, while, rapid responses are usually due to non-genomic signaling initiated at the cell membrane. Here, the ER interacts with signaling molecules, activate signal transduction pathways, which could affect the transcription of genes that lack EREs in their promoters [88]. The ultimate action of estrogen depends on its target tissue. The main targets are the female reproductive tract,

Figure 1.8 Genomic and non-genomic actions of estrogen. Estrogen diffuses into the cell and acts on its receptors to activate or repress transcription of target genes. Estrogen can also bind to receptors on the cell membrane to exert non-genomic effects through signal transduction. The latter mechanism induces rapid changes in gene expression.



however, estrogen also acts on many organs including the brain, bone, liver and heart [89]. Estrogen has significant effects, positive and negative, on skin biology, including the modulation of epidermal keratinocytes [90].

Estrogen decreases the amount of time needed for wounds to heal [91, 92]. Topical administration of estradiol increases epidermal thickness, presumably by inducing keratinization [93, 94]. It is well known that estrogens can stimulate uterine epithelial proliferation [95-97]. In addition, the hormone is used to improve inflammatory conditions that affect both women and men, such as vaginal atrophy and phimosis [98, 99]. Vaginal estrogen cream or tablets are widely used by postmenopausal women to treat vaginal atrophy by thickening and keratinizing the vaginal epithelium [100, 101]. There is a well demonstrated link between the vaginal epithelial structure and its susceptibility to HIV infection. The vaginal epithelium contains a high density of LCs and, as in the foreskin, the LCs are thought to be the primary site of HIV entry into the female reproductive tract [102]. Furthermore, an European study evaluating HIV-1 discordant couples found that women over the age of 45 had a 4-fold higher risk of acquiring HIV than their younger counterparts [75]. Thus, the thinning of the vaginal epithelium as a result of a decline in estrogen levels after menopause may contribute to this increased risk [103].

To study how estrogen affects the susceptibility of the vaginal epithelium to HIV infection, ovariectomized Rhesus monkeys were treated with either intravaginal estriol or a placebo base cream and then challenged intravaginally with simian immunodeficiency virus (SIV)

[11]. Estriol treated animals showed a dramatically increased resistance to SIV infection; only one of the twelve estriol treated animals became infected when challenged with SIV, compared to six of the eight ovariectomized controls treated only with placebo [11]. The estriol treated animals all became infected if the SIV was injected beneath the vaginal epithelium, showing that the resistance to infection was occurring at the epithelial surface. The increased keratinization of the vaginal epithelium induced by the topical estriol treatment is hypothesized to have reduced the number of SIV virions coming into contact with the LCs, thus preventing infection. Despite the strong protective effect of estrogen in these ovariectomized monkeys, the same response was not observed in animals with intact ovaries and normal cycling hormone levels. Moreover, the vagina is frequently subjected to trauma during intercourse causing tears in the epithelial lining [104], negating any protective effect of an increased keratin thickness through the use of topical estriol treatment. Finally, and perhaps most importantly, estrogen is a known co-factor in many vaginal and cervical cancers and its long-term administration to the vagina would be inadvisable making this intervention unlikely to be of use in humans for reducing the susceptibility of women to HIV [16].

1.5 Scope of Thesis

Our studies propose to validate topical estrogen as a medical alternative to circumcision to aid in the reduction of HIV transmission in uncircumcised men. While the role of estrogen in regulating epithelial keratinization in women is well established, comparable studies in men have not been well documented. The preputial mucosa of the male inner foreskin is an analogous mucosal epithelium to that of the vagina, with a very thin layer

of overlying keratin [33]. However, unlike the vagina, the foreskin has a lower risk of trauma during intercourse, and it is not exposed to significant levels of systemic hormone cycling. A number of prior studies have utilized the epithelium modifying properties of estrogen to treat phimosis (a constriction of the foreskin preventing its complete retraction) in children, adolescents and adults [99, 105]. Estrogen treatment was effective in reversing foreskin constrictions in the vast majority of cases. Estrogen is very effective in increasing moisture and collagen content of the skin, leading to its increased elasticity as well as increasing keratin thickness [106]. The increased elasticity of the foreskin caused by such treatments (in addition to the keratin increase) could also be beneficial to controlling HIV as it would reduce the likelihood of foreskin sustaining micro tears or epithelial breakage during intercourse. Several studies of transgender males as well as children given topical estrogen to treat foreskin infections reported negative side effects of high estrogen treatment. However, these side effects are usually reversible upon cessation of the treatment [99, 107]. These findings support the need to determine minimal doses of weak estrogenic compounds to avoid any adverse systemic estrogenic side effects of topically applied estrogen in males.

As most studies were performed over a short time period, there is still a need to examine the effect of chronic treatment in men. We propose the use of estriol since it is a weak, naturally occurring estrogen metabolite that is normally present in human male urine, and is thus unlikely to cause hypothalamic inhibition or gynecomastia at low levels. To support this statement, studies have been performed to assess the systemic effect of topical estrogen treatment. Luteinizing hormone is responsible for stimulating the production of

estrogen [108]. Similar treatment regimens to those proposed in our study have been assessed in women and were shown to have no effect on circulating luteinizing hormone (LH) concentrations indicating no systemic effects from the treatment and only a local response [109]. Pask *et al.* has previously examined the ability of topical estrogen to increase keratin production of the inner foreskin epithelium [110]. Their results showed that topical administration of estriol (Ovestin cream, 1mg/ml, Organon) for 14 days, caused a rapid and sustained increase in inner foreskin keratinization (similar to the estrogen response observed in the vagina). However, contact smears only provide an indirect examination of keratinization of the foreskin, therefore a more direct means of examining this phenomenon is needed. The studies mentioned above assessing the response of the vaginal epithelium of Rhesus monkeys to estrogen reported a similar keratinization response to that induced by estriol in men after 74 days of estradiol treatment [11]. Although that study used a more potent form of estrogen, we hypothesized that small amounts of topically applied estriol would be sufficient to induce an increased keratin thickness on the foreskin without yielding any measurable systemic effects.

While the primary effect of estrogen on the thickness of the epithelium in humans has been documented, how it affects foreskin biology and particularly susceptibility to virus infection, is less well-understood. This will be thoroughly investigated in Chapters 2 and 3 of this thesis. The foreskin, like the vagina and most other epithelial surfaces, contains a high density of estrogen receptors, necessary for transducing the estrogen signal and affecting gene transcription. Estrogen acts as a mitogen in the skin, stimulating the proliferation and decreasing terminal differentiation of keratinocytes under stressful

conditions such as wounding or infection [111-113]. This in turn results in increased thickness of the stratum corneum. Estrogens have also been shown to affect Langerhans cell maturation and antigen presenting capacity [114]. Macrophages treated with estrogen display decreased migratory capacity and increased apoptosis [115]. Estradiol has also been shown to affect HIV target cells through their estrogen receptors and to regulate the expression of HIV-1 receptor molecules, and the release of chemokines and cytokines [114]. The estrogen receptors themselves are widely distributed in multiple layers of both the epidermis and dermis as well as on LCs and macrophages. Treatment of ovariectomized mice with estradiol increases CD4+ T lymphocyte expression of CCR5 [114]. The stratified epithelia of genital tissues are populated by T cells, LCs and macrophages, all of which express the CD4, CCR5 and CXCR receptors. In addition, the sub-epithelium is densely populated with dendritic cells, T cells and macrophages expressing CD4, CCR5 and CXCR4 receptors. Due to the expression of these known HIV-1 receptors, immune cells of the epithelium and sub-epithelium are highly susceptible to HIV-1 infection. How each of these cell types and their HIV-1 receptor expression respond to topical estriol treatment is unknown and must be evaluated before assessing the efficacy our proposed intervention. We will characterize how topical estriol mediates reduced epithelial susceptibility to viral infection through keratinization and epithelial thickening. The aim of this thesis is to determine the role of estrogen in modifying the inner foreskin epithelium. I will provide definitive evidence to support the need of a phase 2 clinical trial to further define estrogen's role as an adjunct treatment to reduce the risk of HIV infection.

CHAPTER 2

Elucidating the effect of topical estriol on foreskin epithelium

2.1 Background

The results of three large randomized trials of male circumcision and its protective effect on female-to-male HIV infection, carried out in South Africa [9], Kenya [7] and Uganda [8], leave no doubt that circumcision at least halves a man's relative risk of HIV infection. The protective effect of circumcision is thought to be due to the physical removal of the majority of the inner foreskin epithelium, the main site of HIV entry into the penis [33, 116]. The inner foreskin is a mucosal membrane [6] with little overlying keratin. It is richly supplied with Langerhans cells, whose dendritic processes lie close beneath the epithelial surface. Langerhans cells are a vital part of the body's natural epithelial defense against HIV infection since they contain the c-type lectin, Langerin, which can degrade viral particles entering the cell [117]. The risk of HIV transmission through non-mucosal skin is significantly reduced due to a thicker stratum corneum that forms a physical barrier against virion entry [33, 110]. If keratin production in the inner foreskin mucosa could be increased to that of non-mucosal skin it could significantly reduce the risk of HIV infection in uncircumcised men. In this study, we investigated the use of a topical treatment to increase keratin production in the inner foreskin mucosal epithelium.

It is well established that the human vaginal epithelium (a mucosal epithelium containing many Langerhans cells [118] that is analogous to the foreskin epithelium) responds to topical estrogen administration by thickening presumably as a result of keratinization. A decline in estrogen levels in post-menopausal women is associated with vaginal atrophy and a four to eightfold increased risk of HIV infection compared to premenopausal women [75, 119]. The protective role of the estrogen exposed epithelium in preventing HIV

infection was verified in a series of experiments in Rhesus monkeys. Monkeys were ovariectomized to remove endogenous estrogen production and then treated intravaginally with topical estriol or a control base cream. Following the treatment regimen, both groups were given an intravaginal challenge with an inoculum of simian immunodeficiency virus (SIV) [11]. Only one of the twelve treated animals became infected, compared to six of the eight untreated controls ($p=0.0044$). However, estrogen treated animals did become infected if the SIV was injected sub-epithelially, showing that the resistance to infection was occurring at the epithelial surface [12]. Thus, the increased thickness of the vaginal epithelium's stratum corneum induced by the topical estrogen produced an effective barrier to prevent infection.

The majority of estrogen action is mediated via the estrogen receptors (ERs) alpha and beta. We have previously reported that both ERs are abundant in the human inner foreskin and respond to topical estrogen [110]. Furthermore, treatment accelerated the rate of keratinization of the inner foreskin. However, this study was limited to two participants and keratinization was only assessed using the indirect measure of determining the amount desquamated keratinocytes from the inner foreskin. Despite its limitations, this preliminary study suggested that the foreskin could elicit an increase in keratin production and thicken the stratum corneum similar to what has been observed in the vaginal epithelium, following topical application of estriol [11, 120]. As such, the foreskin's natural barrier to viral entry could be enhanced.

We have determined the effects of topical estrogen on inner foreskin biology using a low dose of the naturally occurring and weak estrogen metabolite, estriol. Estriol was chosen to reduce the risk of any adverse systemic side effects such as gynecomastia or infertility observed with high dose or long-term exposures. Lutenizing hormone (LH) is known to stimulate estrogen production in women. Similar estriol treatment regimens to those used in this study had no effect on circulating LH concentrations, suggesting that the treatments are unlikely to have systemic side effects [121]. In addition, a significant keratinization response was detected in the vaginal epithelium of Rhesus monkeys using as little as 25 µg of estriol applied twice weekly [11].

Here, we report the results of a phase 1 clinical trial (clinical trial number H2007/02979) examining the response of the foreskin to topical estrogen by performing a direct assessment of stratum corneum thickness *in vivo* both pre- and post-treatment. We sought to determine the minimal effective dose needed to induce keratinization of the stratum corneum. Since estrogen has also been reported to affect Langerhans cell maturation and migration [122], we also examined how estrogen affected Langerhans cell density and depth in the inner foreskin epithelium. Our findings show that topical estriol is able to induce a significant increase in the thickness of the stratum corneum without altering the Langerhans cell number or distribution.

2.2 Methods

Study Participants

Twenty-five adult men aged 24-65 scheduled for elective circumcisions at the Urology Clinic at the Austin Hospital in Melbourne, Australia were recruited to participate in the study. Eligibility criteria for the study was: (1) male; (2) aged 18 and above; (3) already scheduled for elective circumcision; (4) acceptable condition of the foreskin tissue based on the surgeon's assessment of medical history and the presenting condition. Study exclusion criteria included severe balanoposthitis/ phimosis and unacceptable foreskin condition, based on the surgeon's assessment at the time of recruitment and the patient's medical history. All participants signed an institutional review board approved consent form declaring their willingness to participate in the trial.

Study Design

The participants provided a 3mm standard punch-hole biopsy taken from the inner foreskin before treatment began (hereafter referred to as the pre-treatment biopsy) to establish a baseline of their individual keratin thickness before treatment. The men were then randomly assigned to one of 5 groups to determine minimal dose; placebo (base cream without estriol) or an identical base compound, commonly used as an ointment base, containing estriol as an active ingredient in varying concentrations (500 µg, 50 µg, 25 µg, or 12.5 µg; each diluted in 0.5ml of base cream) for fourteen days prior to circumcision (Table 2.1). Specific dose concentrations were blinded from the participants,

Table 2.1 Randomized Treatment Group Assignments.

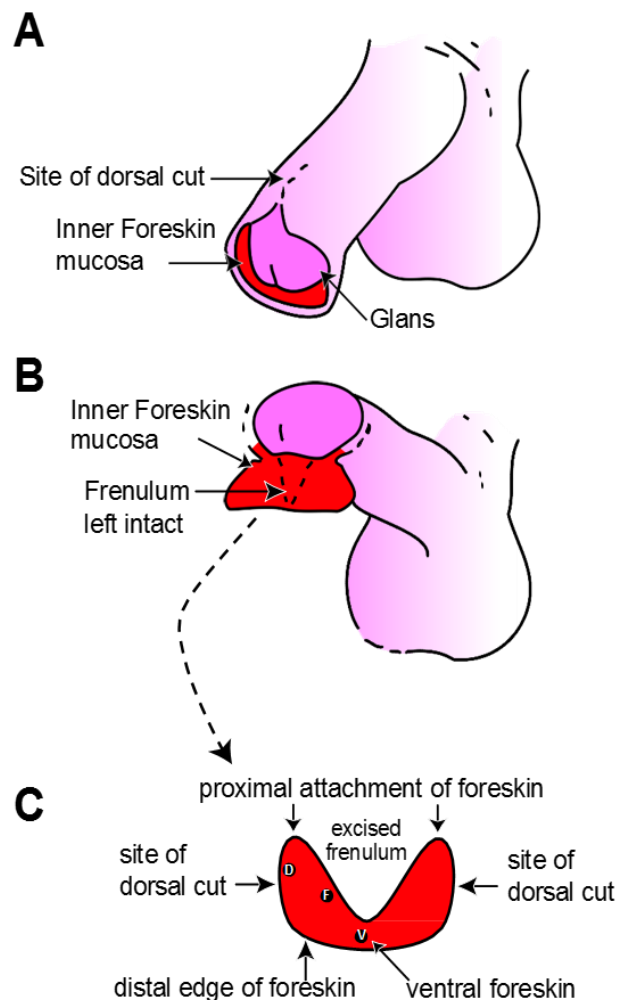
Group	Estriol Conc. (mg/ml)	Daily Dose (μ g estriol)	N
A	1.000	500	5
B	.100	50	2
C	.050	25	5
D	.025	12.5	4
E	control	0.00	4

the consulting surgeons, and the research team until all data had been collected. To produce the desired concentrations for each treatment group, estriol (Sigma-Aldrich, Sydney, Australia) was diluted in 0.5ml of the base cream Cetomacrogol (Skin Basics, Laverton North, Australia), which was mixed with a small amount of liquid paraffin (David Craig, Laverton North, Australia). The cream was supplied to participants in preloaded 1ml plastic disposable syringes, marked for each day of administration so that an accurate dose would be applied. Participants were asked to return all syringes (used and unused) at the conclusion of the trial to track compliance. The procedure for application of the cream was as follows: 1.) Uncap a pre-filled single-dose applicator. 2.) Holding the applicator in one hand, pull back the foreskin with the other. 3.) Apply the cream to the exposed inner foreskin by depressing the applicator plunger. 4.) Distribute the cream evenly over the entire surface of the exposed inner foreskin using your finger. 5.) Allow the foreskin to retract/relax back over the glans. 6.) Place the used applicator in the container provided.

At the conclusion of the treatment period (14 days), circumcision was performed using the dorsal slit method [123]. Inner foreskin tissues from the dorsal, ventral, and frenular regions (Figure 2.1) of the inner aspect of the foreskin were collected using a 5 mm punch hole biopsy tool (here after referred to as post-treatment biopsy) and processed to determine the effect of treatment on stratum corneum thickness and the number and localization of Langerhans cells. All data was compared to patient-matched control pre-treatment biopsies.

Figure 2.1 Dorsal Slit Method for Circumcision.

- a.) A circumferential cut is made on the inner and outer preputial skin slightly above the corona. A dorsal slit is made along the foreskin from the tip to the corona.
- b.) The foreskin is removed and the glans penis is permanently exposed.
- c.) Biopsies were collected from dorsal, frenular, and midventral areas of the excised foreskin.



Tissue Processing

Tissues were fixed in 4% paraformaldehyde overnight at 4°C, then washed several times in cold phosphate buffered saline before paraffin embedding. Eight micron sections of each sample were cut and placed onto superfrost-plus glass slides (Fisher Scientific, Agawam, MA) for downstream analyses. Twenty slides containing post-treatment tissue from each region mentioned above were randomly chosen from a larger batch for analysis using a random number generator. Due to the small size of the pre-biopsy sample (3mm), twelve sections were selected from each patient for comparison.

Assessment of Stratum Corneum Thickness using the Ayoub-Shklar Staining

Ayoub Shklar staining [124] was performed to assess the thickness of the stratum corneum, the outermost region of the skin made up of corneocytes (dead cells packed with keratins). All solutions were freshly prepared and filtered using filter paper prior to use in staining protocol. Sections were deparaffinized in Citrisolv (Fisher Scientific, Agawam, MA) for a total of 30 minutes and rehydrated with 100% ethanol, followed by 95% and 70% dilution. Slides were washed in 1xPBS and stained with 5% acid fuchsin for 3 minutes, then in aniline blue-orange G (Acros Organics, Fair Lawn, NJ) for 45 minutes. Stained slides were dehydrated with 95% and 100% ethanol, respectively, and coverslips were mounted using DPX (CTL Scientific Supply Corp., Deer Park, NY). Images were collected using an Olympus BX40 microscope equipped with a Moticam 5 mega pixel camera and the accompanying Motic software (British Columbia, Canada). Each image was examined for keratin accumulation in the stratum corneum by collecting

measurements randomly along twelve sites across the intact apical surface of the epithelium (Figure 2.2a). The closest distance between the apical and basal edges of the stratum corneum were manually measured in μm using Image J software (NIH, Bethesda, MD). Over 900 data points were collected per patient. The mean keratin thickness was compared between pre- and post-treatment biopsy samples for individual patients as well as within and across treatment and control groups.

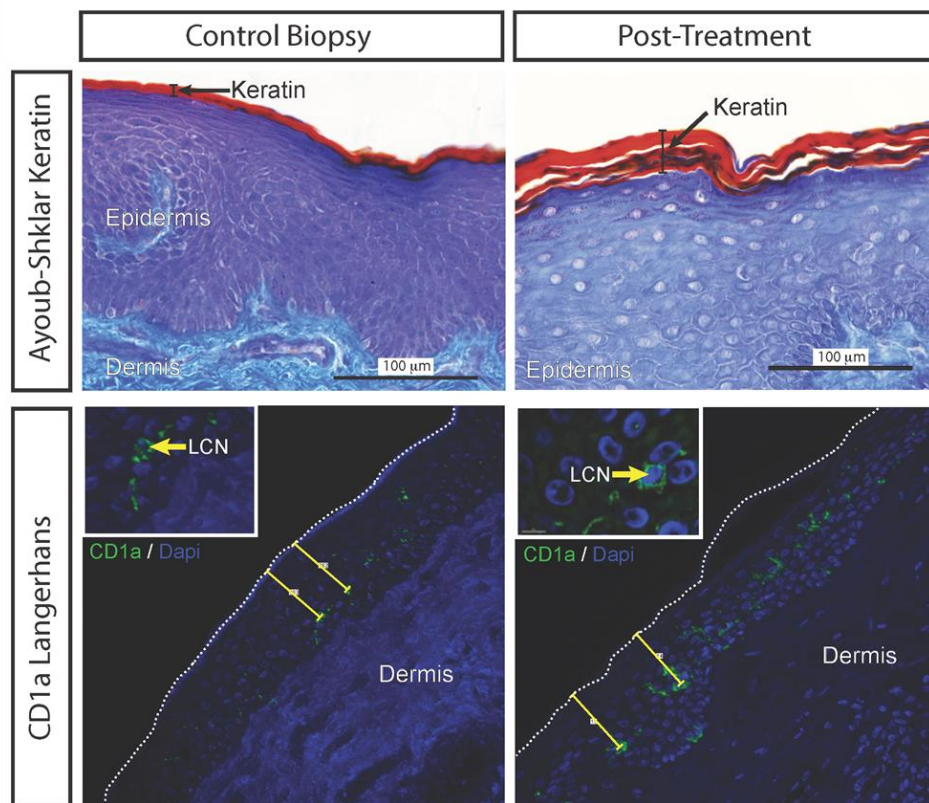
Assessment of Langerhans Cell Number and Location

Immunofluorescence was performed to assess Langerhans cell density and distribution within the inner foreskin epithelium. Sections were dewaxed, rehydrated, and blocked with 10% BSA (Sigma Aldrich, St. Louis, MO, USA). Langerhans cells present in the skin and epithelia were identified using a mouse monoclonal anti-human Cd1a antibody (Leica Biosystems, United Kingdom). To enhance staining, two secondary antibodies, FITC-conjugated chicken anti-mouse and FITC-conjugated goat anti-chicken (Abcam, Cambridge, MA, USA), were applied consecutively using a 1:100 dilution prepared in 1X PBS. Slides were washed with 1X PBS in between antibody applications. To visualize the nuclei, 4',6-Diamidino-2-Phenylindole (Dapi, Invitrogen, Carlsbad, CA, USA) was applied to each section. Each slide was cover slipped using Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA, USA) to preserve the fluorescent signal during long-term storage. Images were captured using DeltaVision RT Systems with SoftWorx software (Applied Precision, Issaquah, WA). Seventy-two sections of foreskin per patient were evaluated to determine the number of Langerhans cell within the epithelium in a field of

Figure 2.2 Histological staining of the inner foreskin.

a.) Ayoub Shklar (AS) staining of inner foreskin tissue (top panels). AS differentially stains keratin and pre-keratin. The stratum corneum (keratin layer) is stained red; the stratified squamous epithelium is gray; the stratum spinosum (pre-keratin) is orange; the connective tissue is blue; erythrocytes are red. Keratin thickness was randomly measured at six sites across the intact epithelial keratin layer

b.) Immunofluorescence staining for Langerhans cells in inner foreskin tissue using CD1a antibody (bottom panels). CD1a (green) was used to identify Langerhans' cells present throughout the epithelium. DAPI (blue) was used to counterstain nuclei. Yellow lines indicate measurement of Langerhans' depth within the cell. Langerhans' cell projections can be seen as green staining not associated with a nucleus (yellow arrow head in inset). Inset shows Langerhans cell in the inner foreskin at 100X magnification.



view and the distance of the cell from the apical surface (Figure 2.2b). Cells were manually counted and the density per mm² determined using Image J (NIH, Bethesda, MD) was tabulated in Microsoft Excel. To determine the depth of the cells, the distance of the Langerhans cell nucleus to the apical surface of the epithelium was manually measured in μm using the straight line tool in Image J (NIH, Bethesda, MD).

Statistical Analyses

A Welch's t-test was performed to compare the mean Langerhans cell density and depth between pre and post treatment samples for each individual. GraphPad InStat version 3.10 for Windows (GraphPad Software, San Diego, CA) was used to analyze the mean keratin thickness of the stratum corneum. Since initial assessment determined that the data was non-parametric, the Kruskal-Wallis test (a non-parametric equivalent of ANOVA), was performed to determine the percentage change between baseline and post-treatment within each treatment group. Bonferroni correction was used to control the family-wise error rate. Dunn's Multiple Comparisons test was used post analyses to determine which specific group means differed. A p-value of less than 0.05 was considered statistically significant.

2.3 Results

Twenty-five adult men participated in the study, however, due to concerns with proper consent, the data reported here is for only 20 of the patients. We analyzed inner foreskin samples collected from twenty adult men scheduled for elective circumcision. Sixteen

patients received active hormone therapy; 5 in the 500 µg estriol/day cohort (Group A), 2 in the 100 µg/day cohort (Group B), 5 in the 50 µg/day cohort (Group C), 4 in the 25 µg/day cohort (Group D) and 4 patients were assigned to the control/placebo cohort (Group E) (Table 2.1).

As expected, the thickness of the stratum corneum exhibited considerable inter- and intra-variation across the tissue (Figure 2.3; Table 2.2). To control for individual variation, the change in thickness was calculated for each patient (Δ -keratin thickness), by determining the individual baseline (pre-treatment thickness) and comparing it to post treatment thickness (Figure 2.4a). At the conclusion of the study, patients in group A (500 µg estriol/day) showed the strongest response to treatment, with a 65% average increase above baseline ($p < 0.5$) (Figure 2.4b).

For each patient, the number of Langerhans cells were evaluated in 72 microscopic fields at 10X magnification, and the final counts expressed as density of cells per mm² of inner foreskin epithelium (Figure 2.5a; Table 2.3). Six of the twenty patients (one from group A, B, C, E and two from group D) were excluded from the density analyses due to the presence of an insufficient number of Langerhans cells. The results of the Welch's t-test indicated that (of the remaining samples) there was no statistically significant difference in the number of Langerhans cells among individuals either pre- or post-treatment. Furthermore, there was no statistically significant change in Langerhans cell depth among individual pre- and post-treatments (Figure 2.5b, Table 2.3).

Figure 2.3 Intra- and intervariation in stratum corneum (keratin) thickness of the inner foreskin. Boxplot of keratin thickness differences before (striped) and after treatment (black) for each patient and among treatment groups. The black line in the middle of the boxes represents the median. The bottom and top of the boxes are the lower and upper quartiles, respectively. The whiskers represent the lower and upper quartile ranges, circles represent the outliers, and stars are extreme outliers.

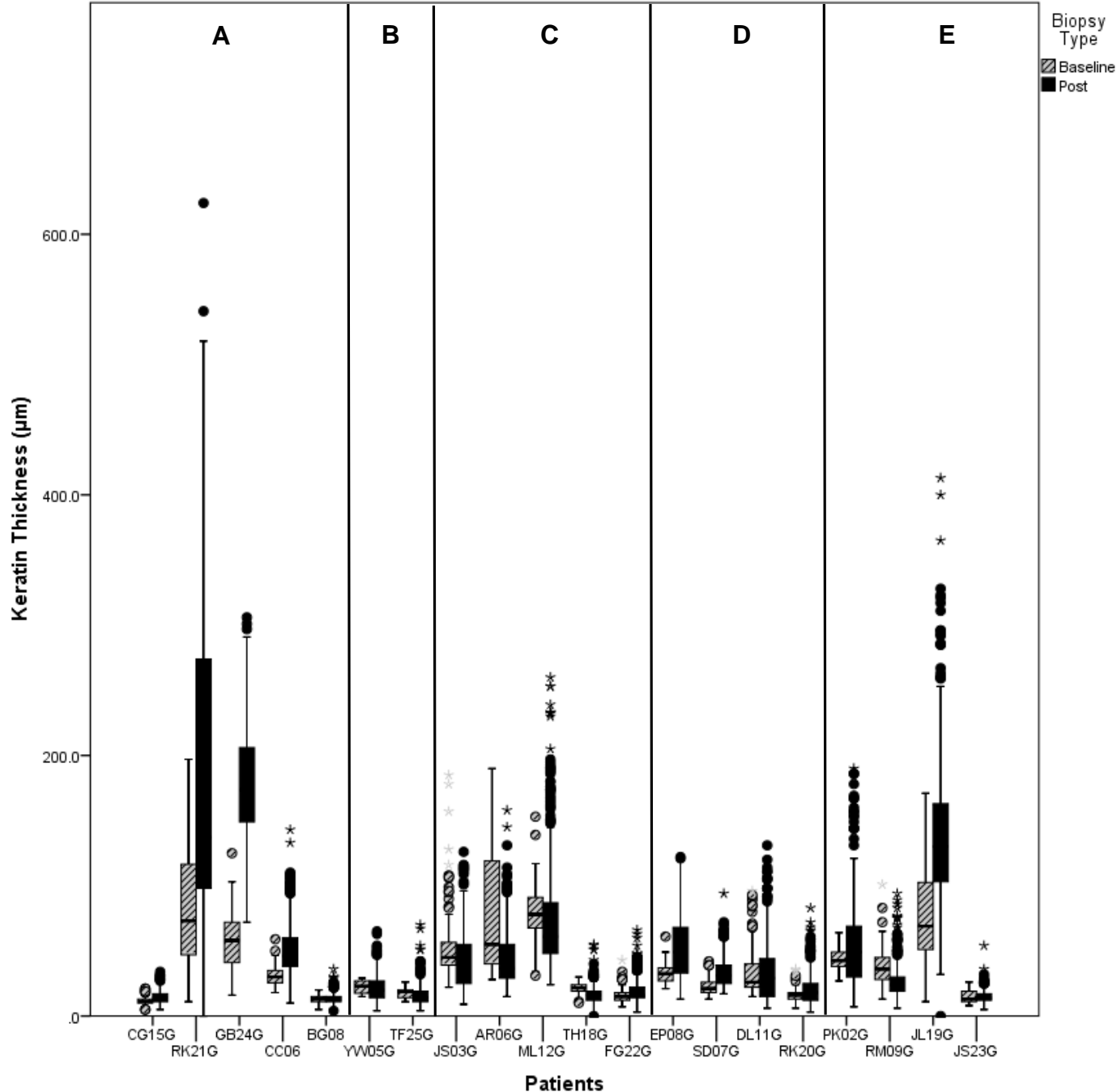


Table 2.2 Stratum corneum thickness of each patient pre- (baseline) and post-treatment.

Treatment groups are shown in Table 2.1.

Group	Patient	Baseline Mean \pm SD	Post Mean \pm SD
A	CG15G	11.58 \pm 3.23	14.15 \pm 4.44
A	RK21G	82.37 \pm 44.40	187.64 \pm 115.46
A	GB24G	59.04 \pm 20.64	176.34 \pm 44.32
A	CC06	30.89 \pm 7.17	50.60 \pm 18.31
A	BG08	13.04 \pm 2.83	13.48 \pm 3.95
B	YW05G	22.33 \pm 5.23	21.41 \pm 9.46
B	TF25G	17.58 \pm 4.09	16.08 \pm 7.90
C	JS03G	51.43 \pm 23.03	42.14 \pm 21.13
C	AR06G	79.45 \pm 46.83	44.88 \pm 22.79
C	ML12G	79.79 \pm 19.51	74.46 \pm 39.61
C	TH18G	21.19 \pm 4.21	15.96 \pm 7.15
C	FG22G	16.14 \pm 6.02	18.74 \pm 8.98
D	EP08G	32.48 \pm 7.13	52.72 \pm 24.62
D	SD07G	22.54 \pm 5.89	33.16 \pm 10.45
D	DL11G	34.79 \pm 20.52	32.49 \pm 20.65
D	RK20G	16.22 \pm 4.79	19.94 \pm 10.94
E	PK02G	43.44 \pm 8.02	53.44 \pm 30.00
E	RM09G	37.42 \pm 14.51	26.11 \pm 11.20
E	JL19G	76.31 \pm 34.11	138.94 \pm 52.38
E	JS23G	15.00 \pm 5.43	14.52 \pm 4.49

Figure 2.4 Stratum corneum (keratin) thickness pre- and post-treatment.

A.) Each colored line represents the change in stratum corneum thickness for an individual patient.

B.) Percent change in stratum corneum thickness across groups. Error bars represent standard deviation of the mean. Kruskal-Wallis test was used to determine significance.

$p < 0.05$

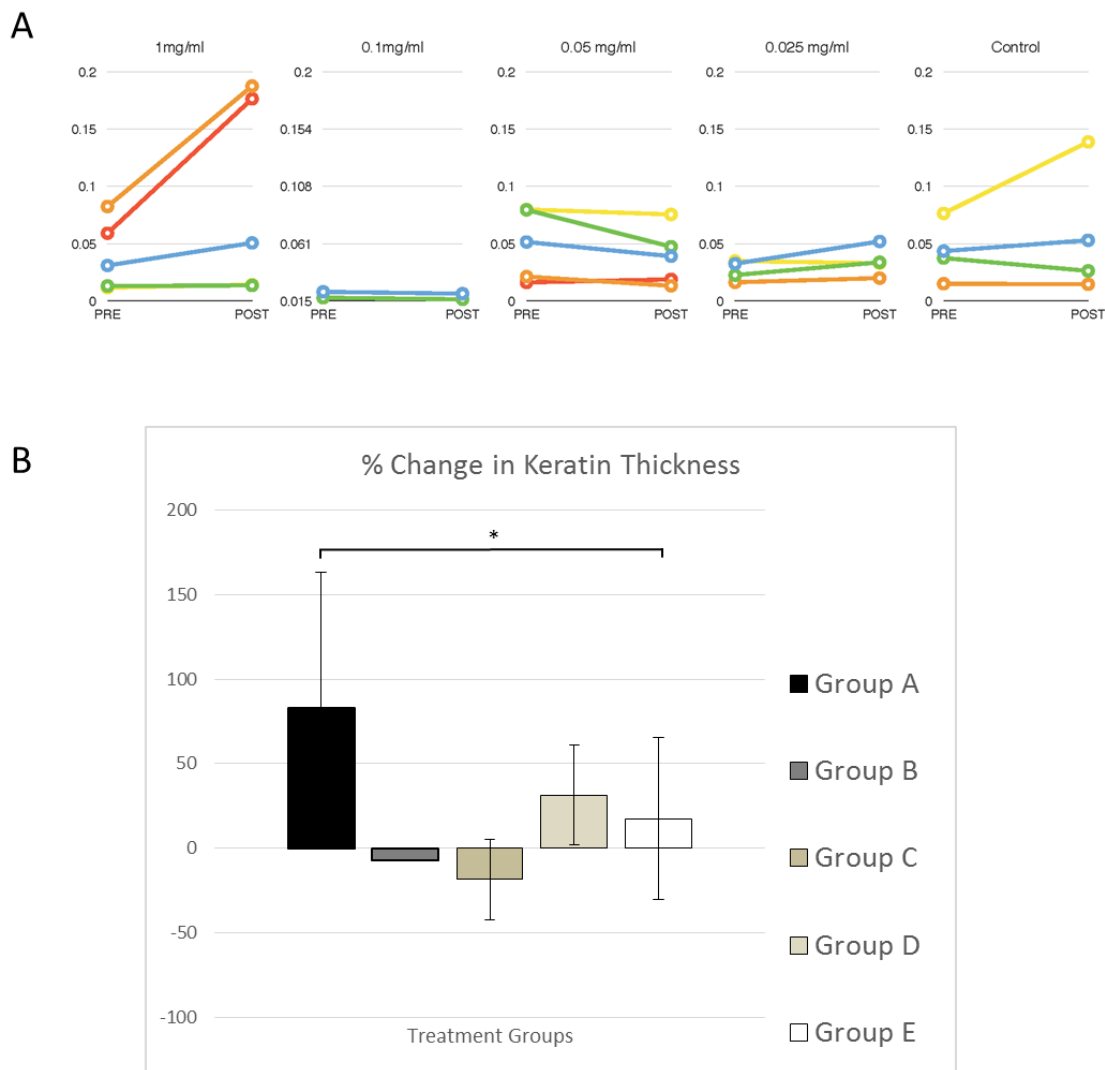
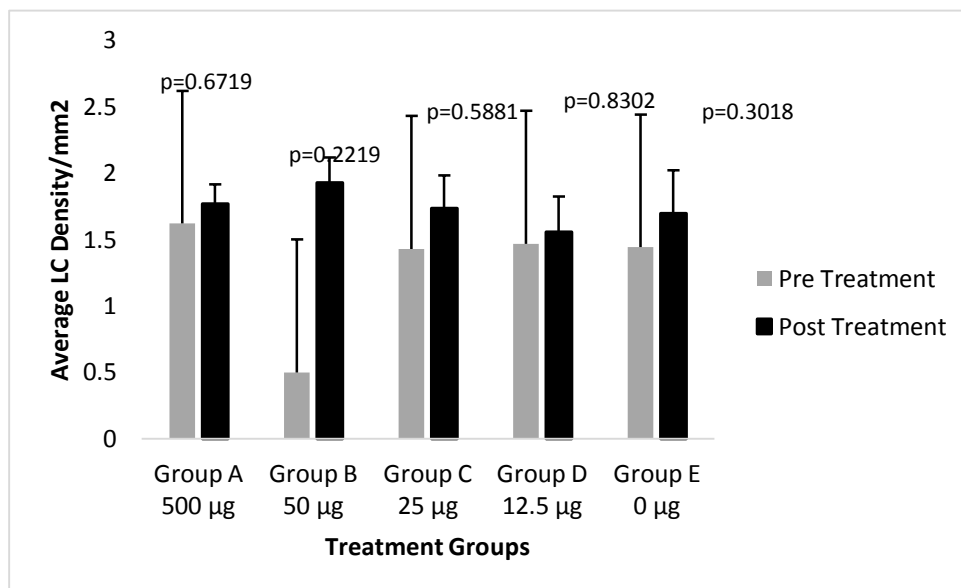


Figure 2.5 Langerhans cell distribution pre- and post-treatment.

A.) Comparison of average Langerhans cell density pre- and post-treatment per group. Error bars represent standard error. Mann-Whitney test was used to determine significance.

B.) Comparison of Langerhans cell depth pre- and post-treatment. Error bears represent standard error. Mann-Whitney test was used to determine significance.

A.



B.

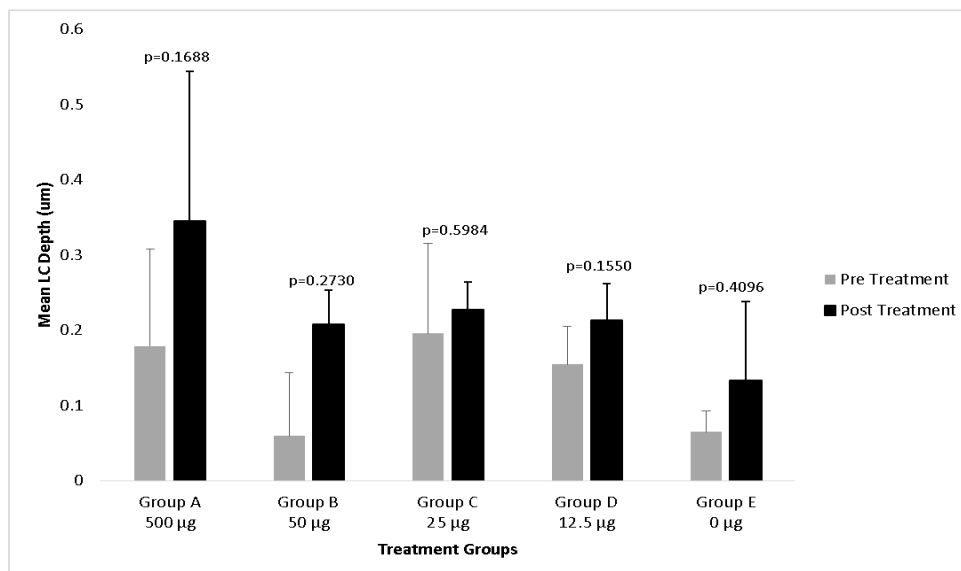


Table 2.3 Langerhans cell (LC) density and depth in human inner foreskin.

Group	Patient	LC NUMBER		LC DISTANCE		
		Baseline ^a Mean (cells/mm ²)	Post ^b Mean (cells/mm ²)	Baseline ^a Mean ± SD	Post ^b Mean ± SD	Baseline:Po p-value
A	CG15G	1.0	1.6	0.19 ± 0.02	0.29 ± 0.13	0.2593
A	RK21G	2.0	1.4	0.12 ± 0.03	0.14 ± 0.06	0.2755
A	GB24G	0.0	1.8	†N/A	†0.62	‡N/A
A	CC06	2.1	2.1	0.34 ± 0.14	0.48 ± 0.17	***< 0.000
A	BG08	3.0	2.0	0.25 ± 0.08	0.19 ± 0.09	***0.00070
B	YW05G	1.0	1.8	0.12 ± 0.04	0.18 ± 0.11	0.1764
B	TF25G	0.0	2.1	†N/A	†0.24	‡N/A
C	JS03G	1.0	1.9	0.26 ± 0.02	0.29 ± 0.14	0.9528
C	AR06G	1.1	2.0	0.24 ± 0.13	0.24 ± 0.14	0.8812
C	ML12G	0.0	1.3	†N/A	†0.2	‡N/A
C	TH18G	3.0	1.7	0.30 ± 0.01	0.21 ± 0.11	*0.0472
C	FG22G	2.0	1.8	0.17 ± 0.09	0.20 ± 0.09	0.5037
D	EP08G	1.0	1.6	†0.08	†0.17	‡N/A
D	SD07G	1.0	1.8	†0.21	†0.26	‡N/A
D	DL11G	1.4	1.5	0.16 ± 0.06	0.18 ± 0.08	0.8007
D	RK20G	2.5	1.2	0.17 ± 0.09	0.25 ± 0.14	0.2386
E	PK02G	1.7	2.1	0.22 ± 0.07	0.25 ± 0.12	0.711
E	RM09G	1.0	1.4	†0.19	†0.22	‡N/A
E	JL19G	1.6	1.9	0.24 ± 0.10	0.33 ± 0.15	0.1374
E	JS23G	1.5	1.5	0.26 ± 0.04	0.25 ± 0.02	0.7621

N/A: No data available; †Insufficient data, ‡p value cannot be calculated

SD, Standard Deviation

*p<0.5, ***p<0.0001

^aPre-treatment keratin thickness; ^bPost-treatment keratin thickness

2.4 Discussion

A highly keratinized stratum corneum of the penis shaft, in addition to the removal of the foreskin pocket, appear to be the major contributing factors aiding in the protection of circumcised men against epithelial transmission of HIV. We present here the first study directly assessing the *in vivo* response of the inner foreskin of uncircumcised men to topical estriol, and its subsequent effect on increased thickness of the stratum corneum. Since estrogens are known to affect immune cell migration and maturation, we also examined how treatment affected Langerhans cell number and distribution in the inner foreskin epithelium. Estriol was chosen for the treatment since it is the weakest occurring, natural form of estrogen identified in men, typically circulating in the range of 10-40pg/ml in blood. Estrogen has been shown to affect keratin production in the rat vaginal epithelium [125]. Furthermore, studies in the vaginal mucosa (an analogous mucosal epithelium to the foreskin) in rhesus macaques showed that estrogen can induce a significant keratinization response that was sufficient to prevent SIV transmission [11, 12, 126]. If the human inner foreskin can respond in a similar way, topical estrogen treatment might be able to reduce the risk of HIV infection through the intact mucosa in uncircumcised men.

We have previously reported that the human male inner foreskin contains estrogen receptors and responds rapidly to topical estriol application by increased desquamated cells [110]. These findings suggested that human inner foreskin is indeed able to respond to estrogen by thickening the stratum corneum. No adverse reactions to the estriol treatment were reported by the men. This is likely due to the low dose of estriol applied,

its easy metabolism, and the short overall length of the study (two weeks). Our data shows that patients given the highest dose of estriol exhibited an average of 80% increase in stratum corneum thickness over the placebo group. The mean thickness after treatment also appeared to decrease in some patients in the lower treatment groups, although none of the values were significantly different from placebo. This is most likely due to the effect of small sample size coupled with large amounts of individual variation [127]. In addition, the lower treatment groups received significantly less estriol than group A. This was intentional, as we were attempting to define a minimum dose and effective epithelial responses to estrogen have been reported at low doses in female rhesus macaques [11]. However, it would appear that the human male inner foreskin mucosa is much less responsive to estriol compared to the vaginal epithelium of female macaques and requires a higher dose to elicit a keratin response. In subsequent studies, we would recommend the use of a reduced dose, but higher than 100 µg/day of estriol.

Having confirmed that our highest dose of estrogen can increase the keratin barrier of the inner foreskin, we next investigated if it had any negative impact on Langerhans cells. Langerhans cells are the primary target of HIV entering the intact mucosa. Previous studies have shown that estrogen may affect the differentiation of Langerhans cells *in vivo* which in turn could increase the number of HIV target cells in the treated epithelium [128]. Such changes could affect the safety of estrogen treatment by increasing the ability of HIV to infect the intact foreskin, especially if there was break in the stratum corneum. Previous studies suggest that estrogen, produced locally in the skin, can promote Langerhans cell differentiation *ex vivo* [128]. If this same mechanism is operating in the

mucosa, then the number of LCs present in the epithelium could be altered following treatment with estrogen. However, we found that there was no difference in the total number or distribution of Langerhans cells between treatment and placebo groups. The difference in our results could be due to the potency of the estrogen used (estriol versus estradiol benzoate) or the route of administration (topical versus systemic). We chose to use estriol instead of estradiol because it is a weaker estrogen that exhibits a rapid metabolic breakdown, resulting in very low serum levels which may not be enough to affect LC maturation. A topical route of administration allows us to focus our study on the local effects without the risk of triggering broader physiological responses that would be observed following systemic administration of estrogen.

Together, these data indicate that topical estriol administration is an effective method for specifically eliciting increased keratinization of the inner foreskin mucosa without affecting the biology of the underlying immune cells. Despite our small sample size, we were able to see an increase in stratum corneum thickness in the inner foreskin using the highest dose. This result is in agreement with the effect of estrogen on other skin types. Although statistical significance was not achieved in the lower treatment groups compared to the placebo in this limited data set, if the sample size is expanded in future studies, it may possibly allow for a more thorough investigation of estriol's effect on keratinization at low concentrations. A phase II clinical trial is warranted to further evaluate the efficacy of topical estriol in inducing keratinization of the inner foreskin. In addition, studies will need to be performed to evaluate the potential for topical estriol to protect the inner foreskin

against HIV transmission. If proven effective, estriol could provide a useful adjunct to circumcision to decrease the uncircumcised male's risk of acquiring HIV infection.

Chapter 3

Transcriptional response of the human inner foreskin to topical estrogen

3.1 Background

Several studies have been conducted demonstrating the effects of estrogen on the physiology of the skin. Estrogen has the ability to affect the skin at both the cellular and molecular levels; it can increase cell proliferation, keratin production, and the migration and maturation of immune cells, all of which have implications for the susceptibility of the skin layer to infection. Although some of these estrogen-driven effects have been modeled in other areas of skin, to our knowledge, they have never been described in the foreskin mucosa.

Estrogen's genomic effects are largely mediated through intracellular receptors present in various cells in the skin, including the most abundant cells in the epidermis, the keratinocytes [113]. Upon ligand binding, the estrogen receptor complex translocates into the nucleus where it binds to specific consensus sequences (known as estrogen response elements; EREs), within regulatory regions of target genes [129] (Figure 1.8). Transcription of estrogen responsive genes can be either activated or repressed in a locus- and cell-specific manner in a number of ways, including activation or repression of the promoter, co-factor binding and occasionally modification of chromatin structure [130]. Although estrogen can also induce non-genomic effects on gene expression resulting in regulation of signal transduction pathways and ion channels, this pathway is less likely to affect transcriptional processes involved in keratinocyte development and function [130].

Two estrogen receptors have been isolated and characterized in humans, ER-alpha (ESR1) and ER-beta (ESR2). These receptors share a high degree of sequence

homology and can function as heterodimers. However, estrogenic ligands have preferential binding affinities. 17β -estradiol (E2), the most potent form of estrogen, binds to both receptors with similarly high affinities [131]. In contrast, estriol (E3), the weakest form, preferentially binds to ESR2 while estrone (E1) prefers ESR1 [131]. Preferential binding among the estrogens may contribute to receptor-specific expression in different cell types, resulting in varied biological effects [131]. ESR1 promotes cellular proliferation and thus is necessary for development of the reproductive tract and mammary glands [132]. In stark contrast, ESR2 suppresses cell proliferation [132]. In addition, it has been shown that ESR2 is more widely expressed in the skin than ESR1 [130, 132].

There has been a vast amount of research published describing the effects of E2 on the skin, particularly in postmenopausal women and in cell culture. The effects of estrogen on the vaginal epithelium have been widely studied in post-menopausal women as estrogen is critical for the maintenance of urogenital skin [133-136]. Cessation of menstruation in women is associated with a decrease in estrogen production accompanied by decreased water and collagen content as well as loss of elasticity, which can all lead to vaginal atrophy [136]. Women with this condition experience thinning of the vaginal mucosa accompanied by dryness and itching. Topical hormone replacement therapy can effectively alleviate most symptoms associated with declining estrogen levels [133, 134]. Thinning of the mucosal skin is thought to contribute to the increased risk of HIV infection seen in postmenopausal women [75] as it contains densely packed LCs, HIV target cells in the epidermis.

Protection of LCs from infectious agents primarily comes from the outer protective barrier, the stratum corneum, formed by keratin and other structural proteins in the epithelium. Keratins, the predominant cytoskeletal proteins in the epithelium, are differentially expressed by keratinocytes [137] (Figure 1.4). Basal keratinocytes in the proliferative state express keratins 5 and 14 [137], as well as the proliferation marker, Ki-67 [138]. As cells lose their ability to proliferate, they begin to undergo terminal differentiation and keratins 5/14 are replaced by the expression of keratins 1 and 10 [137]. Under normal conditions, all suprabasal keratinocytes express these keratins, however, during wound repair and hyperproliferative skin diseases, the cells may begin to aberrantly express keratins 6 and 16 [139, 140]. As cells begin to senesce, they transition from the granular layer to a cornified state through crosslinking of several structural proteins responsible for forming the stratum corneum. Filaggrin (FLG) aggregates keratin filaments, causing the cells to collapse and flatten [141]. S100 calcium-binding proteins, such as S100A12, helps to control the differentiation of epithelial cells as the epidermal structural proteins begin to be expressed [142]. Loricrin (LOR), the most abundant cornified envelope protein, along with involucrin (IVL), the small proline-rich proteins (SPRRs), and late cornified envelope (LCE) proteins, function exclusively in the cornified envelope and are responsible for the maintenance of the stratum corneum [143].

Enhancing the strength of the stratum corneum may improve its ability to resist infection. It has been noted in the literature that the thickness of the epithelium may correlate affect susceptibility to infection [144]. Female rhesus macaques challenged with SIV after treatment with estrogen have a decreased rate of infection compared to their untreated

counterparts and exhibit vaginal epithelial thickening [12]. In Chapter 2, we demonstrated that estriol augments the thickness of the stratum corneum in the inner foreskin. We hypothesized that there are two ways to increase keratin production: 1.) accelerated cell division or 2.) an accumulation of more keratin than normal in each cell. Understanding estrogen's effect on keratinocyte function and foreskin biology as a whole may shed light on its ability to reduce HIV transmission in uncircumcised males.

Estrogen responsive genes, such as CCND1 and IGFBP4, are involved in cell cycle regulation, which can influence cell proliferation, differentiation, and apoptosis [145, 146]. Estradiol has been reported to act as a keratinocyte mitogen, stimulating proliferation and decreasing apoptosis *in vitro* [111-113]. This role suggests that exogenous estrogen could be advantageous in the re-epithelialization process during wound healing [92]. In fact, estrogen has been shown to affect wound healing by regulating genes associated with a variety of biological processes including epidermal function [147]. TGF- β 1 is another key gene involved in wound healing that is essential for development and maintenance of steady-state Langerhans cells (LCs), the primary immune cells in the epidermis [148]. Estrogen has been shown to affect Langerhans cell maturation and antigen presenting capacity through the release of chemokines and cytokines [149]. However, the LC migration marker CCR7, co-stimulatory marker CD86, and maturation marker CD83 are not affected by its presence [150]. LC maturation and antigen presentation are critical for initiating a proper adaptive immune response to pathogens, such as HIV.

Productive HIV infection requires the fusion of the virus membrane to the target cell membrane mediated by viral envelope proteins (e.g. gp120) and target cell surface receptors such as CD4 and its co-chemokine receptors, CCR5 and CXCR4 (Figure 1.7). The CCR5 receptor facilitates the entry of macrophage-tropic HIV-1 strains (R5 viruses) while CXCR4 mediates entry of T-cell tropic strains (R4 viruses). Treatment of ovariectomized mice with estradiol increases CD4⁺ T-lymphocyte expression of CCR5 [149]. The stratified epithelia of genital tissues are populated by T cells, LCs and macrophages, all of which express the CD4, CCR5 and CXCR receptors. In addition, the sub-epithelium is densely populated with dendritic cells, T cells and macrophages expressing CD4, CCR5 and CXCR4 receptors. Due to the expression of these known HIV-1 receptors, immune cells of the epithelium and sub-epithelium are highly susceptible to HIV-1 infection. Estrogen has been shown to elevate expression of CCR5 on CD4⁺ T-cells [114] which could potentially increase T-cell susceptibility to HIV infection. How each of the cell types described and their HIV-1 receptor expression is affected by topical estradiol treatment needs further investigation.

Almost nothing is known of the molecular pathways that control foreskin proliferation and keratin deposition to protect against pathogens. There is a need to take a global look at estrogen's effect on gene regulation in the foreskin to help determine efficacy of this treatment in reducing HIV infection in uncircumcised males. Whole transcriptome sequencing allows researchers to perform a comprehensive, systematic comparison of similarities and differences in expression of a substantial number of genes across treated and untreated samples. The data generated could reveal many pathways that could be

modified to increase resistance of the epithelium to infection. We have assessed how topical estriol treatment regulates keratinization and epithelial thickening by defining changes in the transcriptional profile of normal foreskin tissue and that of treated tissue after exposure to estriol, the weakest naturally occurring estrogen. We surveyed the transcriptome for epithelial pathways that were affected by estrogen treatment, most notably the terminal differentiation pathway. In our data analysis, we explored the expression of markers corresponding to both proliferation and differentiation processes. In addition, we analyzed genes involved in HIV susceptibility in the epidermis. These data can be correlated with the morphological and physiological changes discussed in Chapter 2 to add a greater depth to our understanding of foreskin biology and its modulation with estrogen treatment. This approach may also enable us to identify other molecules that can potentially be used to target keratin production or regulate epithelial immunity.

3.2 Methods

Localization of Filaggrin and Involucrin in the Inner Foreskin

Double immunofluorescence was performed to assess filaggrin and involucrin expression within the inner foreskin epithelium. Sections were dewaxed, rehydrated, and blocked with 10% BSA (Sigma Aldrich, St. Louis, MO, USA). Two primary antibodies, a rabbit polyclonal anti-filaggrin antibody (Abcam, Cambridge, MA, USA) and a mouse monoclonal anti-involucrin antibody (Abcam, Cambridge, MA, USA), were mixed in 1X PBS in a 1:250 and 1:100 dilution, respectively and applied to the tissue for one hour at room temperature. After three washes in 1X PBS, a mixture of two secondary antibodies, FITC-conjugated goat anti-rabbit (Abcam, Cambridge, MA, USA) and FITC-conjugated

chicken anti-mouse, were applied using a 1:100 dilution prepared in 1X PBS. Slides were washed three times with 1X PBS. To visualize the nuclei, 4',6-Diamidino-2-Phenylindole (Dapi, Invitrogen, Carlsbad, CA, USA) was applied to each section. Each slide was cover slipped using Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA, USA) to preserve the fluorescent signal during long-term storage. Images were captured using DeltaVision RT Systems with SoftWorx software (Applied Precision, Issaquah, WA).

Tissue Samples for RNA Seq

Foreskin from two patients (GB and RK) treated with the highest estrogen dose (500 µg estriol/day) and two (PK and JS) from the placebo group were selected for whole transcriptome sequencing. The patients selected from the highest treatment group were most affected by the treatment (measured by the highest response to treatment), while those selected from the treatment group were the least affected by treatment. All samples were handled in accordance with the guidelines of the Austin Health Institutional Review Board (Melbourne, Australia).

RNA Extraction

Total RNA was extracted using the Qiagen RNeasy Midi Kit's protocol for isolation of total RNA from heart, muscle, and skin tissue. RNA concentration and integrity was determined via Nanodrop 1000 spectrophotometry and gel electrophoresis, respectively. A DNase digestion was performed using the Ambion Turbo DNA-free Kit to ensure that any residual DNA was removed. Again, the RNA concentration was determined via

Nanodrop 1000 spectrophotometry. About 10 µg (Invitrogen RiboMinus Transcriptome Isolation Kit Human/Mouse) or 5 µg (Epicentre Ribo-Zero rRNA Removal Kit Human/Mouse/Rat) of DNased total RNA was subjected to ribosomal RNA depletion according to the manufacturer's protocol. A set of external RNA standards (External RNA Control Consortium [ERCC] RNA Spike-In Control Mixes, Life Technologies) were mixed with the total RNA during the initial step of the enrichment process. A 1:10 dilution of Mix 1 was added to the treated samples and a 1:10 dilution of Mix 2 was added to the control samples in the Ribominus preparation. In the Ribozero preparations, a 1:10 dilution of Mix 2 only was added to both the treated and control tissues. The quality and quantity of the purified RNA was determined using the RiboGreen RNA Quantification Reagent and Kit (Molecular Probes) and the Experion RNA HighSens Analysis Kit (BioRad) according to the manufacturer's protocol.

Library Preparation

Two whole transcriptome libraries were constructed using the SOLiD Total RNA-Seq Kit according to manufacturer's instructions: one for the treated sample and the other for the control sample. Briefly, rRNA-depleted total RNA for all samples was fragmented using RNase III, ligated, and reverse transcribed into cDNA. The cDNA library was purified and size-selected using the Agencourt AMPure XP Reagent, then amplified by PCR using barcoded SOLiD 3' primers from a SOLiD RNA Barcoding Kit which adds unique sequences to the PCR product to distinguish between multiple libraries being sequenced simultaneously. The amplified DNA was purified using the PureLink PCR Micro Kit (Invitrogen) and the concentration measured on the NanoDrop Spectrophotometer. The

Agilent DNA 1000 Kit and the 2100 Bioanalyzer were used to determine the size and quantity of the purified library. RNA-Seq mapping is more efficient when library inserts are longer than 200 bp. The protocol recommends the use of libraries with less than 50% of the DNA falling into the 25-200 bp range. Our libraries were within range, so we proceeded with the SOLiD template bead preparation.

SOLiD Sequencing

Whole transcriptome next-generation sequencing was performed at the Center for Applied Genetics and Technology, University of Connecticut. Briefly, the barcoded cDNA libraries were pooled and libraries were amplified on P1 beads by emulsion PCR, generating bead clones. The enriched beads were deposited on a glass slide and single-end sequencing (up to 50 bases) was performed on the Applied Biosystems SOLiD 4 System (Life Technologies).

Mapping Reads

Cutadapt was used to locate and remove adaptor sequences and low quality bases (a quality score of less than 20). After trimming any remaining reads that were less than 20bp long were removed. The reads were then aligned to the human genome (hg19) using TopHat2.

Expression quantification and identification of differentially expressed genes

GFOLD was used to quantify the mapped reads and normalize read counts by calculating the reads per kilobase per million reads (RPKM). RPKM, an expression of the relative abundance of transcripts, is calculated by dividing the number of reads mapped per kilobase of exon model by the total number of mapped reads in the entire dataset [151]. Differential gene analysis was performed using GFOLD which ranks differentially expressed genes and is particularly useful when there is no biological replicate samples available. GFOLD estimates the relative change in expression levels of genes based on their fold change to determine which genes are differentially expressed. All genes that were identified as statistically significantly different by GFOLD were entered into DAVID to identify the enriched (overrepresented) ontological categories (GO terms) in the dataset and to explore any Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were affected by the treatment.

Validation of Select Differentially Expressed Genes

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) was performed to validate genes of interest using β -actin as the housekeeping gene. In addition, we also sought to validate the SOLiD results of genes involved in HIV binding, Langerhans cell maturation and migration, as well as those related to the keratinization pathway. Briefly, total RNA from the treated and control samples was DNased as described above and reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Primers were designed using NCBI's primer construction software

Primer-BLAST (Table 3.1). mRNA abundance was determined using iQ Sybr Green Master Mix (BioRad). Reactions were run in triplicates on the Bio Rad CFX96 Real Time PCR machine. The fold change in expression of each gene from the housekeeping gene was calculated using the $2^{-\Delta\Delta CT}$ method. Significance was determined using t-test. $p < 0.05$ was considered statistically significant. Error bars represent standard deviation (SD).

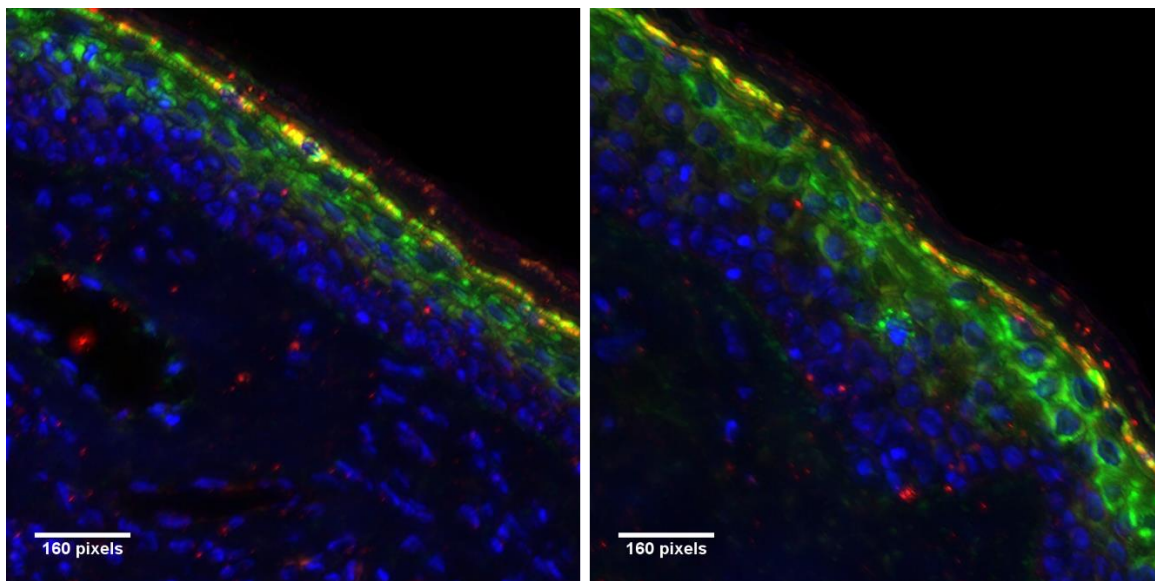
3.3 Results

Immunostaining was performed on control and treated inner foreskin tissues to determine if estriol treatment affects the expression of epidermal terminal differentiation markers, filaggrin and involucrin (Figure 3.1). In both the treated and control tissues, cytoplasmic staining of filaggrin was observed in the upper stratum spinosum and throughout the stratum granulosum as expected. Treated and untreated tissues also had similar and expected staining patterns using the involucrin antibody, where staining was observed in the upper stratum granulosum and the stratum corneum of all tissues analyzed. However, the epithelium as a whole appeared to be thicker after treatment due to an increase in keratinocyte cell volume. Immunostaining allowed us to identify the presence of these proteins, however, it cannot be used to accurately quantify the specific expression of the genes. To quantify gene expression we used a combination of next-generation whole transcriptome sequencing and quantitative RT-PCR. To quantify the change in expression level of genes from estrogen and placebo treated inner foreskin, we examined the transcriptome from two patients assigned to the highest estrogen treatment group and two patients from the placebo group. Total RNA was subjected to ribosomal RNA depletion and the resulting RNA pool was deep-sequenced using the Applied Biosystems

Table 3.1 Sequences of the qRT-PCR primers used in this study.

Gene Symbol	Gene Name	GenBank number	Forward Primer	Reverse Primer
Estrogen Responsive Genes				
EEIG1	Early estrogen-induced gene 1	NM_001035254.2	CAGACTCAGGGAACTCGCTC	GCCGGTTAGCACTCATCTT
ESR1	Estrogen receptor 1	NM_001122742.1	AAATGCAGGCTCCATGCTCA	CAGTCCGGAGAATGTGAAGA
ESR2	Estrogen receptor 2	NM_001291723.1	GGCTTCCAGGCAGTAATGGG	ATTCTAAAGCGCGGTGCTG
HIV Co-Receptors				
CCR5	Chemokine (C-C motif) receptor 5	NM_000579.3	TGATTTCACACAGCTCATCTGG	GGCTGCGATTTCGCTTCACAT
CD4	CD4 molecule	NM_000616.4	GAGCTCCAAAGTCTCTACACA	TAAAAGGGACTCCCCGGTTC
CXCR4	Chemokine (C-X-C motif) receptor 4	NM_003467.2	CAGCACGTAGCAAAAGTGACG	GTAGATGGTGGGCAGGAAGA
Langerhans Cell Markers				
CCR7	Chemokine (C-C motif) receptor 7	NM_001838.3	GTGGTGGCTCTCC TTGTCAT	GTAGGCCCCACGAAACAAATG
CD40	CD40 molecule, TNF receptor superfamily member 5	NM_001250.4	GGCTTTGGGGTCAAGCAGAT	GGACCCACAGACAACATCAGT
CD80	CD80 molecule	NM_005191.3	GCTGGCTGGTCTTCTCACT	GTCCGGTCTTGTAATCTCGGG
CD83	CD83 molecule	NM_001040280.1	TGCAACTCGGGGACATACAG	ACTCTGTAGCCGTGCAAACT
CD86	CD86 molecule	NM_176892.1	TGGGAATGCTGCTGTGCTTA	GGCAGGCTGTCAGTCTCAT
CD207	CD207 molecule, langerin	NM_015717.4	CCTGGTATAGTGCCGAGCAG	TGGAATCCAGAAAGCTCGCAC
TGFβ	Transforming growth factor, beta 1	NM_000660.5	TACCTGAACCCCGTTGCTC	GCGAAAGCCCTCAATTTC
Keratin Genes				
KRT1	Keratin 1	NM_006121.3	CCTGTCTGCCCTCCTGGT	CTGCAGCAGCTCCCCATTTTG
KRT5	Keratin 5	NM_000424.3	GAGATCGCCACTTACCGAA	GTAGCTTCCACTGCTACCTCC
KRT6A	Keratin 6	NM_005554.3	ATCGACCACGTCAAGAAGCA	ATTCAGCCTGCACCTCCTCAC
KRT10	Keratin 10	NM_000421.3	ACAAAGTTCTGGGCTCTGGAAG	CGCAGAGCTACCTCATCTCTCA
KRT14	Keratin 14	NM_000526.4	AACGCCGACCTTGAAGTGAA	AATCTGCAGAAGGACATTGGC
KRT16	Keratin 16	NM_005557.3	AGCATCTGGCCCAATCCTATTCT	GCAGAGGAGCAGGGGAGATA
Other Proliferation/Differentiation Markers				
CCND1	Cyclin D1	NM_053056.2	GATGCCAACCTCCTCAACGA	GGAAGCGGTCCAGGTAGTTC
IGFBP4	Insulin-like growth factor binding protein 4	NM_001552.2	ACCCACGAGGACCTCTACAT	CTCTCGAAAGCTGTCAGCCA
IVL	Involucrin	NM_005547.2	CTCTGCTCAGCCTTACTGTGA	ACTGGCAGTGTGTGTTGCTG
MKI67	Marker of proliferation Ki-67	NM_002417.4	GCCTGTACGGCTAAACATGG	AGGCCTTGGAAATCTTGAGCTTT
qRT-PCR Validation				
CCR5	Chemokine (C-C motif) receptor 5	NM_000579.3	TGATTTCACACAGCTCATCTGG	GGCTGCGATTTCGCTTCACAT
CYP11A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	NM_000499.3	CTTGGAAACCTTCCCTGATCCT	GACAGAAGATGACAGAGGCCA
LCE3A	Late cornified envelope 3A	NM_178431	GCTGCCTGAGTCAACACAGAT	GTTGGAGCTCTTGCCAACGG

Figure 3.1 Co-localization of filaggrin and involucrin in human inner foreskin. Immunohistochemistry was employed to determine the expression pattern of epidermal differentiation proteins, filaggrin and involucrin, before and after treatment with estrogen. Despite a thicker epithelium post-treatment, the expression of the proteins do not appear to be altered.



SOLiD 4 platform. On average, 50 million or more reads were generated for each sample across the two runs. Reads were quality filtered by removing low quality base pairs, adaptors, and any reads shorter than 20bp. Reads were mapped to human rRNA using Bowtie2 and any aligned reads were removed (about 5%). The total remaining reads (202 million) were mapped to the University of California Santa Cruz (UCSC) hg19 human reference assembly using TopHat2. A combined 129 million reads (65%) were generated from every library that passed the filter (PF) and were aligned to the reference genome. Furthermore, 33% of mapped bases corresponded to exons, 31% to introns, and 35% to intergenic regions.

A principal components analysis (PCA) was performed using RPKM values for all four samples across the two runs to reduce the dimensionality of the data into a three dimensional space (Figure 3.2). In doing so, we were able to observe similarities in gene expression patterns across the groups of samples. PCA showed that the two runs for each sample along with two technical replicates, returned very similar expression patterns. The PCA analysis also showed tight clustering of the placebo-treated samples compared to the estrogen-treated samples, supporting differences between the transcriptional profiles as indicated by GFOLD. A scatterplot (Figure 3.3a) and MA plot (derived from the Bland–Altman plot; Figure 3.3b) confirm that the values for both groups are highly correlated. Our quality control measures indicate that our RNA-seq method provides high-quality data for further exploration of the human foreskin transcriptome and determination of the effects caused by estrogen exposure.

Figure 3.2 Principal Component Analysis (PCA) of differentially expressed genes in foreskin tissues with or without estrogen treatment. Trends exhibited by the expression profiles of treated (GB and RK) and non-treated (JS and PK) tissues. Each label represents a sample and the type of run.

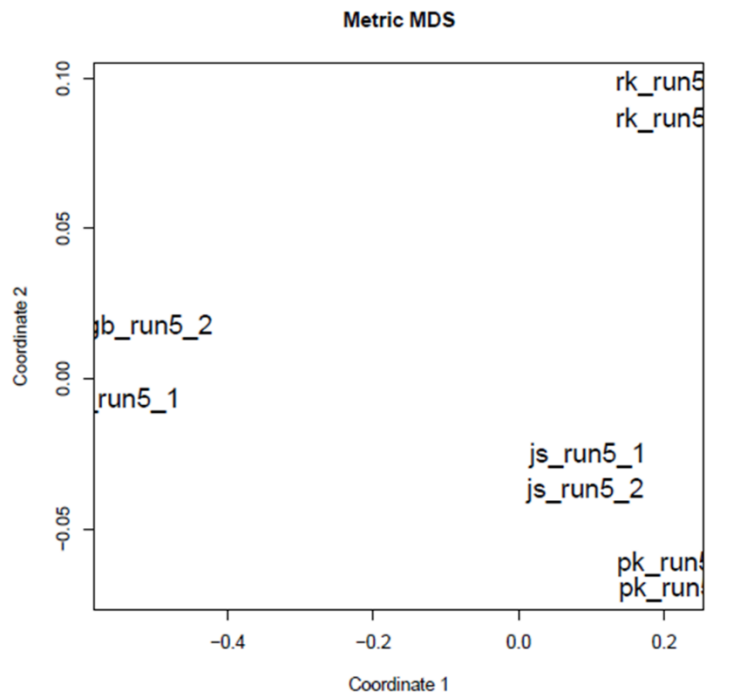
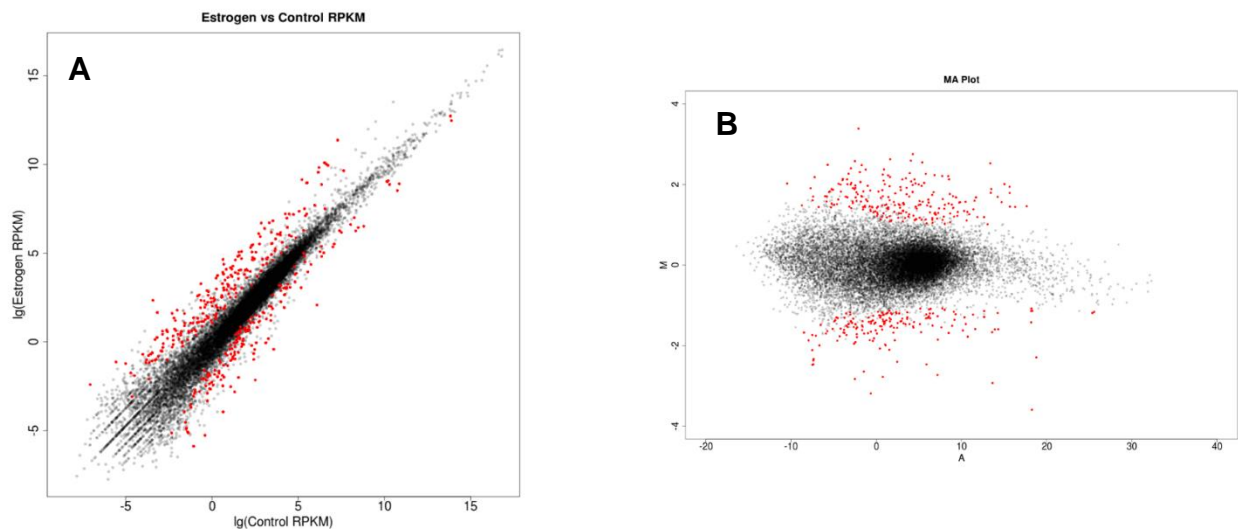


Figure 3.3 Differential expression analysis of estrogen treated and control inner foreskin tissues.

a.) Scatterplot of genes expressed in foreskin tissue with or without estrogen treatment. Each dot represents a gene that is expressed in the foreskin. Dots along the center line are genes that are expressed in both treated and non-treated tissues. Red dots are genes that are differentially expressed, genes in gray are not differentially expressed, and genes in black dots are non-differentiated genes whose expression values overlap.

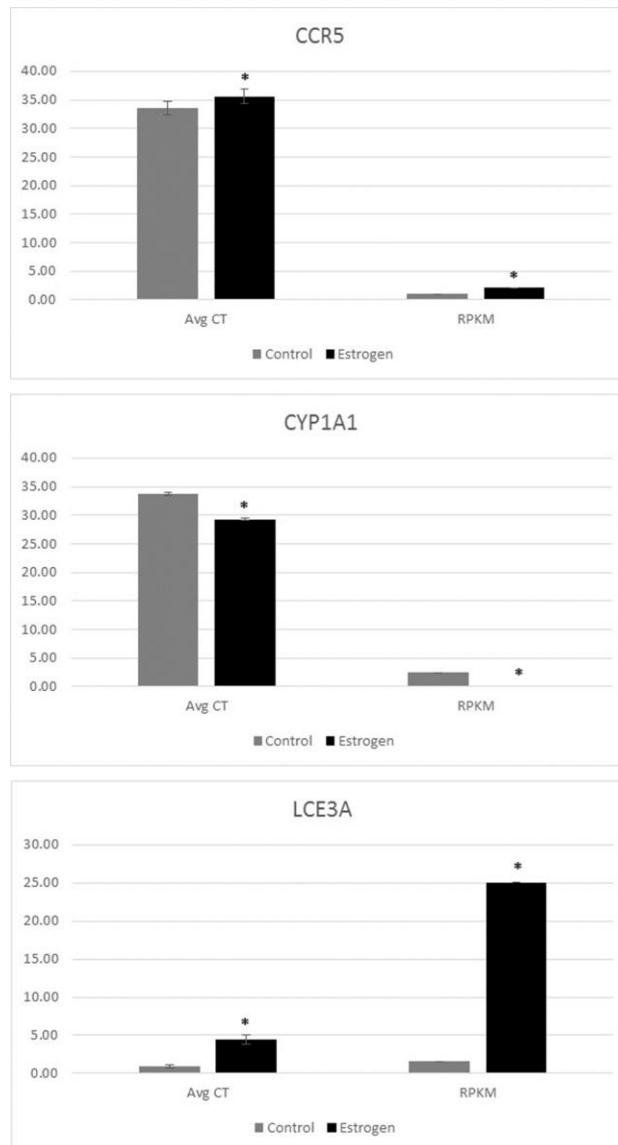
b.) MA plot of average gene expression level vs fold change in foreskin tissues. M, on the y-axis, represents the fold change in treated vs non-treated tissues. A, on the x-axis, represents the mean of the treated and non-treated values of a particular gene. Dot colors follow the description written above. This plot shows that genes with both low and high expression levels are differentially expressed.



To measure gene expression and identify the differentially expressed genes (DEGs) between estrogen-treated and control inner foreskin tissues, we used GFOLD [152]. The normalized expression level of each gene was measured as reads per kilobase of exon per million reads mapped (RPKM). GFOLD, a bioinformatic software package, was used to calculate fold change (FC) defined as the ratio of the RPKM values obtained for individual genes in treated versus untreated tissues followed by a log 2 transformation of the data. The software also determines the statistical significance of the expression values.

To experimentally confirm the differentially expressed genes identified by RNA-seq, the expression levels of select genes were validated by quantitative real-time PCR (qRT-PCR) using the same RNA samples that were prepared for the SOLiD library construction. We chose three candidate genes that were differentially expressed by GFOLD. Beta-actin was used as an endogenous control in these reactions. In RNA-Seq data, CCR5 was up-regulated 0.5 fold; CYP1A1 was down-regulated 3 fold; and LCE3A was also up-regulated 3 fold. In qRT-PCR data, the results were nearly 5 fold, 5 fold, and 3.4 fold, respectively. Although there were differences in the magnitude of expression differences detected, the qRT-PCR results validated a consistent pattern in differential gene expression compared to those observed using RNA-seq (Figure 3.4).

Figure 3.4 qRT-PCR validation of three differentially expressed genes from estrogen-treated inner foreskin RNA-Seq data. Fold changes determined from the relative Ct values for c-c chemokine receptor type 5 (CCR5), cytochrome p450, family 1, subfamily A, polypeptide 1 (CYP1A1), and late cornified envelope 3A (LCE3A) were compared to those detected by RNA-seq. Replicates (n = 3) of each sample were run and the Ct values averaged. All Ct values were normalized to beta-actin.



We identified a total of 23,802 transcripts in the sequenced pools, of which 1.7% (398 transcripts) were significantly differentially expressed. Of the differentially expressed transcripts, 66 showed a 2 fold or higher change in expression. The statistically significant differentially expressed genes in the estrogen treated tissues compared to the placebo treated controls ranged in fold expression differences from -2.45 to 3.39. Further analysis of these genes revealed that 230 were up-regulated while 168 were down-regulated. Interleukin 20 (IL20) and late cornified envelope 3a (LCE3A) were the top two most up-regulated genes in estrogen treated inner foreskin tissue, respectively. IL20 was up-regulated by 3 fold, while LCE3A was up-regulated by 2.5 fold. Among the top up-regulated genes were the cytokine secretion gene S100A12 (2 fold), and the cytokines IL19, IL36A, IL22, all of whose expression increased 2.5 fold. There was also a 2.5 fold up-regulation of small proline rich protein 2c (SPRR2C) which is associated with keratinocyte differentiation and keratinization. Surprisingly, filaggrin (FLG) and uroplakin 1A (UPK1A), both involved in epithelial cell differentiation, were the top two most down-regulated genes. They were down-regulated about 3.4 and 3 fold, respectively. Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), involved in cell proliferation, was down-regulated about 3 fold. A few of the significantly differentially expressed genes have not yet been annotated including one in the top 25 most up-regulated genes (LOC648691; 2.5 fold) as well as one in the top 25 most down-regulated genes (C8orf56; 2 fold). The top 25 most up- and down-regulated genes are shown in Tables 3.2 and 3.3, respectively.

Table 3.2 Top 25 significantly up-regulated genes in estrogen treated human foreskin tissues. The differentially expressed genes in estrogen treated human foreskin tissues compared to those in control foreskin tissues were determined by G-fold. For each gene, the fold change was calculated by dividing the RPKM of estrogen treated tissues to the RPKM of untreated tissues. The fold change was then log2 transformed. Genes with positive log 2 values represent those that are over-expressed in the data set. The differentially expressed genes were ranked on their fold change and the 25 with the highest fold changes are shown here.

*MiRBase Accession Number

**PubMed Gene ID

NOTE: Biological Processes presented in the table may not be an exhaustive list. Please visit the Go ID for more information.

Gene Symbol	Gene Name	GO ID	GO: Biological Process	SOLID Log2 (FC)
IL20	Interleukin 20	Q9NYY1	Positive Regulation of Keratinocyte Differentiation; Regulation of Inflammatory Response	3.39
LCE3A	Late Cornified Envelope Protein 3A	Q57A76	Keratinization	2.76
IL19	Interleukin 19	Q9UHD0	Immune Response; Apoptotic Pathway	2.63
IL36A	Interleukin-36 Alpha	Q9UHA7	Immune Response; Inflammatory Response	2.59
GAGE12E	G Antigen 12E	**729431	Anti-apoptotic Properties	2.59
LOC648691	Uncharacterized LOC648691	**648691	Noncoding RNA	2.58
GAGE12G	G Antigen 12G	**645073	Anti-apoptotic Properties	2.58
PRG4	Proteoglycan 4	Q92954	Cell Proliferation	2.55
MIR650	MicroRNA 650	*MIMAT0003320	*microRNA associated with Keratin Genes	2.53
GAGE12D	G Antigen 12D	**100132399	Anti-apoptotic Properties	2.52
GABRB2	Gamma-aminobutyric Acid (GABA) A Receptor, Beta 2	P47870	Ion Transport	2.49
GAGE12C	G Antigen 12C	**729422	Anti-apoptotic Properties	2.49
IL22	Interleukin 22	Q9GZX6	Cell-Cell Signaling; Inflammatory Response	2.48
CENPA	Histone H3-like Centromeric Protein A	P49450	Host-Virus Interaction; Mitotic Cell Cycle	2.40
CXCL6	Chemokine (C-X-C Motif) Ligand 6	P80162	Chemotaxis; Immune Response; Inflammatory Response	2.37
CARD17	Caspase Recruitment Domain Family, Member 17	Q5XLA6	Regulation of Apoptotic Pathway	2.37
WT1-AS	WT1 Antisense RNA	Q06250	Not Determined	2.31
CLDN17	Claudin-17	P56750	Cell Junction Assembly	2.28
GPR50	G protein-coupled receptor 50	Q13585	Cell-Cell Signaling	2.28
PGA3	Pepsinogen 3, Group I (Pepsinogen A)	P0DJ08	Digestion	2.27
S100A12	Protein S100-A12	P80511	Innate Immune Response; Inflammatory Response; Cytokine Secretion	2.27
NOS2	Nitric Oxide Synthase, Inducible	P35228	Innate Immune Response; Inflammatory Response	2.25
DEFB103A	Beta-defensin 103A	P81534	Innate Immune Response	2.23
TMC5	Transmembrane Channel-Like 5	Q6UXY8	Ion Transport	2.23
DEFB103B	Beta-defensin 103B	P81534	Innate Immune Response	2.21

Table 3.3. Top 25 significantly down-regulated genes in estrogen treated human foreskin tissues. The differentially expressed genes in estrogen treated human foreskin tissues compared to those in control foreskin tissues were determined by G-fold. For each gene, the fold change was calculated by dividing the RPKM of estrogen treated tissues to the RPKM of untreated tissues. The fold change was then log2 transformed. Genes with negative log 2 values represent those that are under-expressed in the data set. The differentially expressed genes were ranked on their fold change and the 25 with the lowest fold changes are shown here.

**PubMed Gene ID

† NOTE: Biological Processes presented in the table may not be an exhaustive list. Please visit the Go ID for more information.

Gene Symbol	Gene Name	GO ID	GO: Biological Process	SOLID Log2 (FC)
FLG	Flaggrin	P20930	Keratinocyte Differentiation; Establishment of Skin Barrier	-3.59
UPK1A	Uroplakin-1a	O00322	Epithelial Cell Differentiation	-3.18
FLG2	Flaggrin-2	Q5D862	Establishment of Skin Barrier	-2.93
CYP11A1	Cytochrome P450, Family 1, Subfamily A, Polypeptide 1	P04798	Cell Proliferation	-2.82
AADAC	Amycetamide Deacetylase	P22760	Positive Regulation of Triglyceride Catabolic Process	-2.77
HBD	Beta-defensin 1	P60022	Immune Response; Inflammatory Response	-2.73
CYP2C19	Cytochrome P450 2C19	P33261	Steroid Metabolic Process	-2.64
DUX2	Double Homeobox 4 like 8	O75505	Sequence Specific DNA Binding	-2.47
SERPINA12	Serpin A12	Q8IW75	Regulation of Proteolysis	-2.47
DUX4L3	Double Homeobox 4 like 3	P0CJ86	Transcription Regulation	-2.46
IGFL1	Insulin growth factor-like Family Member 1	Q6UW32	Signaling Molecule involved in Protein Binding	-2.40
DUX4L6	Double Homeobox 4 like 6	P0CJ89	Transcription Regulation	-2.37
DUX4L2	Double Homeobox 4 like 2	P0CJ85	Transcription Regulation	-2.33
SNORD116-18	Small Nucleolar RNA, C/D Box 116-18	**100033430	Small Nucleolar RNA	-2.29
DUX4L5	Double Homeobox 4 like 5	P0CJ88	Transcription Regulation	-2.11
CYTL1	Cytokine-like Protein 1	Q9NRR1	Signal Transduction; Inner Ear Development	-2.10
SPINK7	Serine Peptidase Inhibitor, Kazal Type 7 (Putative)	P58062	Protein Binding	-2.07
CDX1	Caudal Type Homeobox 1	P47902	Positive Regulation of Transcription from RNA Polymerase II Promoter	-1.99
SOSTDC1	Sclerostin Domain-containing Protein 1	Q6X4U4	Wnt Signaling Pathway	-1.99
C8orf56	BAALC Opposite Strand	P0C853	Noncoding RNA	-1.97
PADI1	Peptidyl Arginine Deiminase, Type I	Q9JLC6	Protein Citrullination	-1.95
UPK1A-AS1	UPK1A antisense RNA 1	**100862728	Noncoding RNA	-1.91
SFRP5	Secreted Frizzled-related Protein 5	Q5T4F7	Cell Differentiation; Negative Regulation of Cell Proliferation	-1.88
LCNL1	Lipocalin-like 1	Q6ZST4	Not Determined	-1.88
KRT33A	Keratin, Type I Cuticular Ha3-I	O76009	Structural Molecule Activity	-1.87

An enrichment analysis was conducted to determine which Gene Ontology (GO) terms were related to over-represented or under-represented genes in our annotated transcriptome from treatment and placebo samples. *Keratinocyte Differentiation* and *Immune Response* were the most over-represented biological processes up-regulated by estrogen treatment (Figure 3.5), while down-regulated genes were mostly involved in the more generic category of *Developmental Processes* (Figure 3.6). DAVID was also used to determine which KEGG pathways were enriched in up- and down-regulated gene sets. The pathway most enriched with up-regulated DEGs was cytokine-cytokine receptor interactions. The fourteen genes contributing to this pathway are listed in Table 3.4. The pathway most affected by down-regulated DEGs is metabolism of xenobiotics by cytochrome P450. The five genes contributing to this pathway are listed in Table 3.5.

In addition, we examined the data for potential negative effects of the treatment such as increased expression of proto-oncogenes. Among the significantly differentially expressed genes, there were none that were expressed only in the control tissues. However, there were 9 genes that were turned on only in the estrogen treated tissues (Figure 3.7). All 9 genes are members of the g antigen 1 (GAGE) gene family, which are not expressed in normal tissue, except in germ cells and certain cancer cells [153].

We used our transcriptome data set to examine the expression of known estrogen targets as well as genes involved in HIV binding, Langerhans cell maturation, and keratinization, all of which are critical to our assessment of treatment efficacy.

Figure 3.5 Top ten Gene Ontology terms for genes significantly up-regulated in estrogen treated human foreskin tissues. Genes were grouped according to their biological processes. The top ten groups were ranked based on p-value.

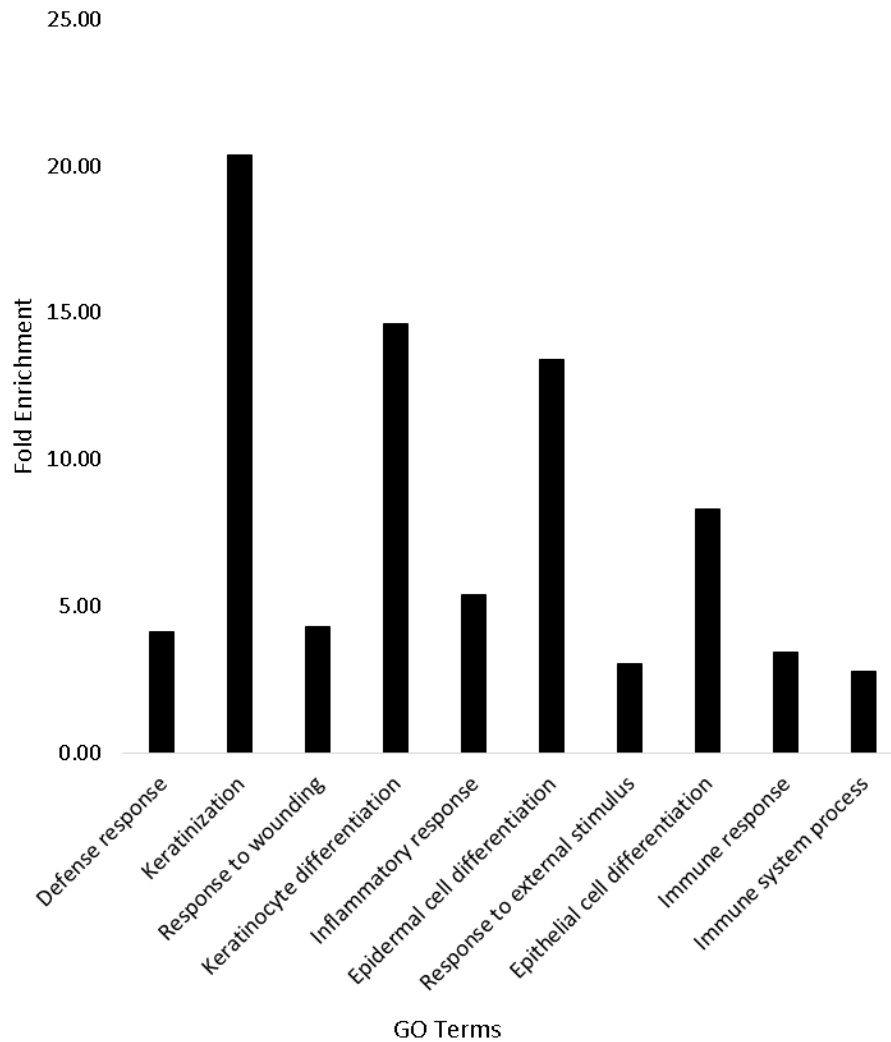


Figure 3.6 Top ten Gene Ontology terms for genes significantly down-regulated in estrogen treated human foreskin tissues. Genes were grouped according to their biological processes. The top ten groups were ranked based on p-value.

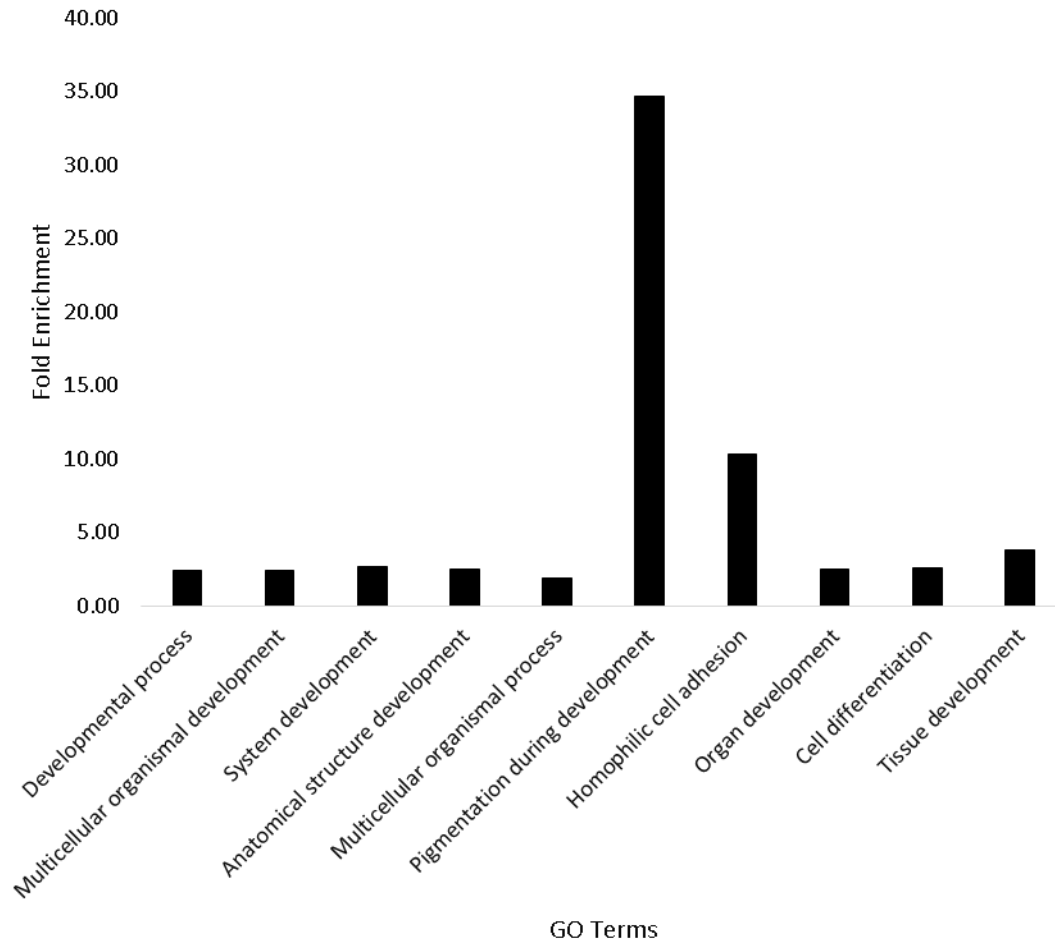


Table 3.4 KEGG pathways overexpressed in treated tissue.

DAVID IDs	Category	Term	Count	%	PValue	Fold Enrichment
201	KEGG_PATHWAY	hsa04060:Cytokine-cytokine receptor interaction	12	5.970149254	6.07E-05	4.312977099
201	KEGG_PATHWAY	hsa00591:Linoleic acid metabolism	5	2.487562189	1.79E-04	16.81547619
201	KEGG_PATHWAY	hsa00592:alpha-Linolenic acid metabolism	4	1.990049751	7.81E-04	20.92592593
201	KEGG_PATHWAY	hsa00590:Arachidonic acid metabolism	5	2.487562189	0.002588489	8.407738095
201	KEGG_PATHWAY	hsa00565:Ether lipid metabolism	4	1.990049751	0.005530623	10.76190476
201	KEGG_PATHWAY	hsa04621:NOD-like receptor signaling pathway	4	1.990049751	0.026252574	6.075268817
201	KEGG_PATHWAY	hsa00564:Glycerophospholipid metabolism	4	1.990049751	0.033309906	5.539215686
201	KEGG_PATHWAY	hsa04730:Long-term depression	4	1.990049751	0.034573313	5.458937198
201	KEGG_PATHWAY	hsa04370:VEGF signaling pathway	4	1.990049751	0.04267043	5.022222222
201	KEGG_PATHWAY	hsa04062:Chemokine signaling pathway	6	2.985074627	0.043994504	3.021390374
201	KEGG_PATHWAY	hsa04664:Fc epsilon RI signaling pathway	4	1.990049751	0.047046789	4.829059829
201	KEGG_PATHWAY	hsa05340:Primary immunodeficiency	3	1.492537313	0.050958008	8.071428571
201	KEGG_PATHWAY	hsa04640:Hematopoietic cell lineage	4	1.990049751	0.059755113	4.379844961
201	KEGG_PATHWAY	hsa04912:GnRH signaling pathway	4	1.990049751	0.081505779	3.843537415

Table 3.5 KEGG pathway underexpressed in treated tissue.

DAVID IDs	Category	Term	Count	%	pValue	List Total	Fold Enrichment
150	KEGG_PATHWAY	hsa00980:Metabolism of xenobiotics by cytochrome P450	5	3.333	1.56E-04	25	16.95
150	KEGG_PATHWAY	hsa00350:Tyrosine metabolism	4	2.667	0.001077838	25	18.49090909
150	KEGG_PATHWAY	hsa00830:Retinol metabolism	4	2.667	0.001957086	25	15.06666667
150	KEGG_PATHWAY	hsa00982:Drug metabolism	4	2.667	0.002911112	25	13.12258065
150	KEGG_PATHWAY	hsa00590:Arachidonic acid metabolism	3	2	0.028157568	25	10.89642857
150	KEGG_PATHWAY	hsa04916:Melanogenesis	3	2	0.078501327	25	6.163636364

Figure 3.7 Significantly differentially expressed genes turned on only in estrogen treated tissues from the Gage family of genes.

GeneName	GFOLD(0.01)	E-FDR	log2fdc	Control RPKM	Estrogen RPKM
GAGE12E	0.681015	1	2.59096	0	0.408532
GAGE12G	0.559425	1	2.57809	0	0.408532
GAGE12D	0.760844	1	2.52292	0	0.393867
GAGE12C	0.819573	1	2.48699	0	0.408532
GPR50	0.417766	1	2.27935	0	0.410636
GAGE2A	0.132708	1	2.06222	0	0.425115
GAGE2C	0.123426	1	1.90108	0	0.428276
GAGE12H	0.0316476	1	1.80213	0	0.408532
GAGE12B	0.111357	1	1.75551	0	0.49551

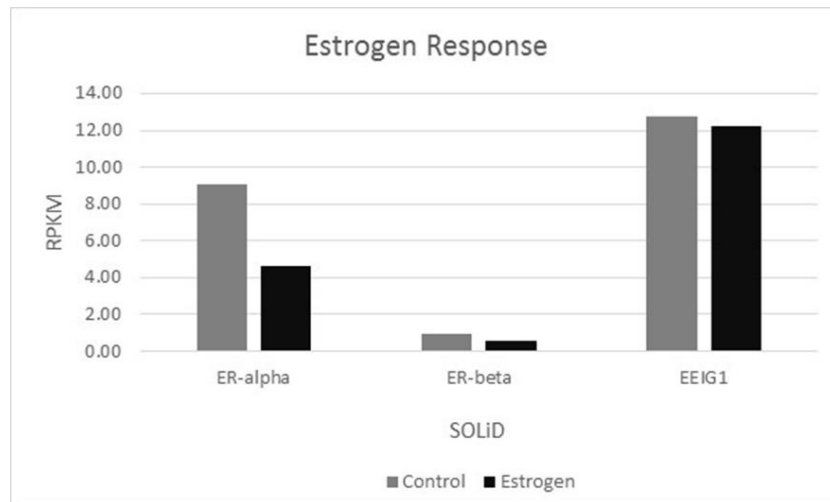
Estriol must bind to an estrogen receptor to affect gene expression. In order to determine estriol's ability to affect the genes regulating the process of keratinization, we sought to confirm the expression of estrogen receptors alpha and beta in the inner foreskin (as previously reported [110, 154]). In addition, we examined if transcription levels were affected by the treatment. No significant difference was observed in the expression of either receptor in the treated or placebo foreskin (Figure 3.8).

Our ultimate goal is to use this treatment as an adjunct to circumcision, to aid in reducing HIV transmission. The major factor contributing to HIV entry into the body is its ability to bind to the HIV co-receptors located on the outside of target cells. We sought to examine any fluctuations in the expression of these genes to determine if the treatment could alter HIV binding and infectivity (Figure 3.9). CD4 is the main receptor needed for viral attachment, while CCR5 and CXCR4 are co-receptors that can be utilized by HIV to complete the attachment process. The sequencing data indicated no significant change in the expression of CD4 and CXCR4. However, CCR5 showed a small but significant increase after estrogen treatment (G-fold value 0.01) in the SOLiD data but not qPCR.

Langerhans cells are HIV target cells present in the epithelium of the foreskin. When LCs uptake viral components, the cells mature and rapidly degrade the foreign material. The cells then migrate to the lymph nodes to present the antigen to the T-cells. To ensure that estrogen treatment does not affect this process, we examined the following genes involved in LC maturation and migration: CD207, CCR7, CD40, CD80, CD83, CD86, and

Figure 3.8 Expression patterns of estrogen responsive genes. We compared the expression data of ESR1, ESR2, and EEIG1 generated by a.) transcriptome sequencing and b.) qRT-PCR.

A



B

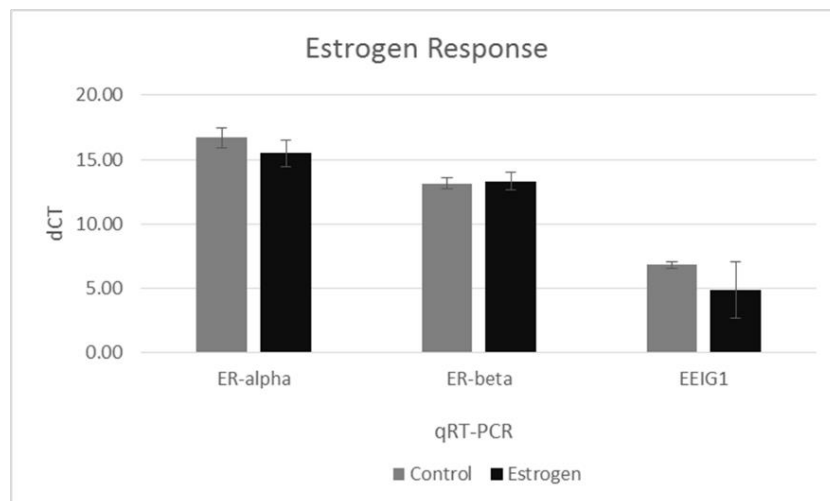
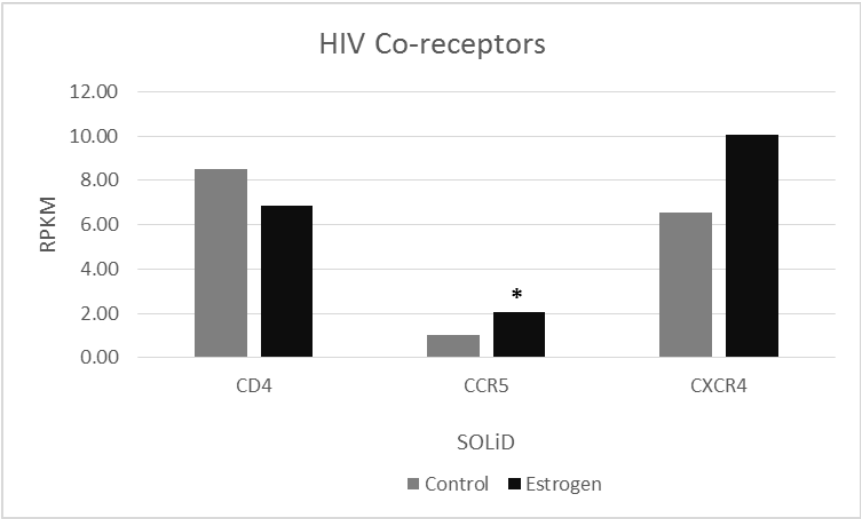
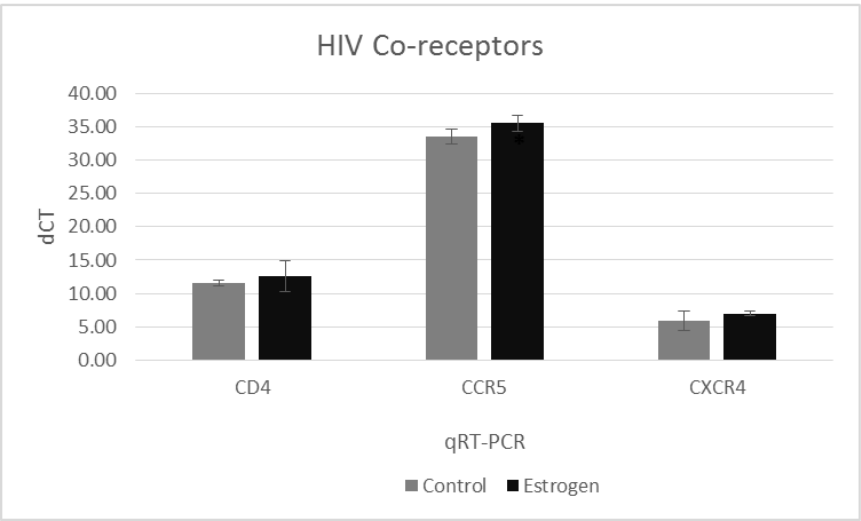


Figure 3.9 Expression patterns of HIV-co receptors. We compared the expression data of CD4, CCR5 and CXCR4 generated by a.) transcriptome sequencing and b.) qRT-PCR.

A



B

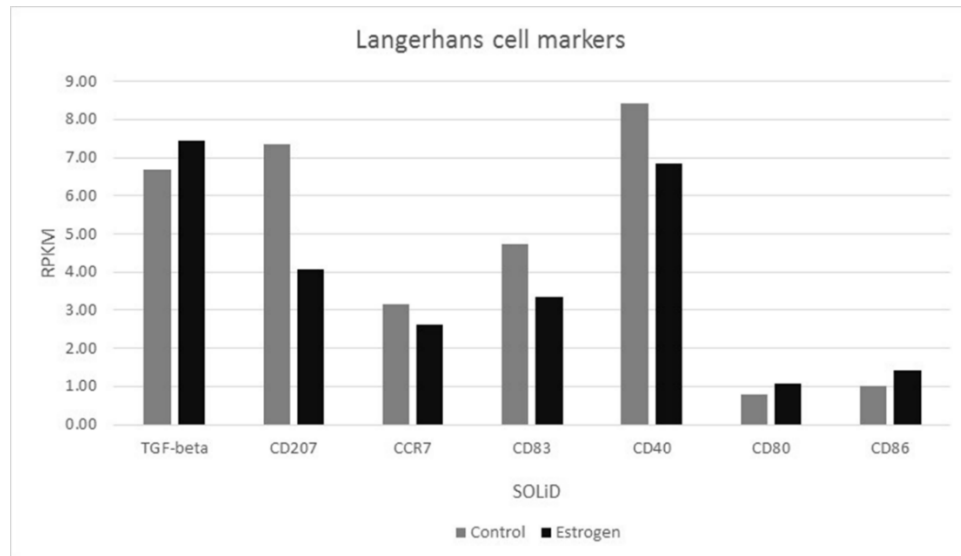


TGF- β . LC development and ability to uptake antigens is largely mediated by TGF- β and CD207, respectively (Figure 3.10). There was no statistical difference in the expression of these LC markers. CCR7, a marker of LC migration, was found to be slightly down-regulated, although not significantly. Neither of the maturation markers, CD40 and CD83, showed differential gene expression. Finally, expression of the co-stimulatory markers, CD80 and CD86 increased slightly (0.5 fold), however this change was not significant. In contrast, expression profiles generated from qPCR demonstrate significant differential expression of CD207, CCR7, CD83, CD40, and CD86.

Next, we examined markers of keratinocyte proliferation and differentiation (Figure 3.11). Analysis of keratins typically expressed in skin revealed contrasting results between the SOLiD data and the qPCR. The basal cell keratins 5 and 14, expressed during proliferation of basal keratinocytes, increased slightly after estrogen exposure in our SOLiD samples. In contrast, they decreased slightly in the qPCR analysis. Neither of these differences were significant. Cellular differentiation markers, keratins 1 and 10, expressed in suprabasal keratinocytes were slightly down-regulated in the SOLiD data, but not significantly. However, in the qPCR data, their expression increased significantly. Keratins 6 and 16, markers of hyperproliferation, are up-regulated in suprabasal cells during wound healing. These genes were up-regulated after estriol treatment, however, significance was only observed for keratin 6a in the qPCR data. Other genes involved in the proliferation and differentiation processes, Ki67, CCND1, IGFBP4 and IVL, were not significantly affected by the treatment (Figure 3.12) in the SOLiD data. Quantitative PCR data revealed a significant increase in the latter two genes.

Figure 3.10 Expression patterns of Langerhans cell markers by a.) transcriptome sequencing and b.) qRT-PCR.

A



B

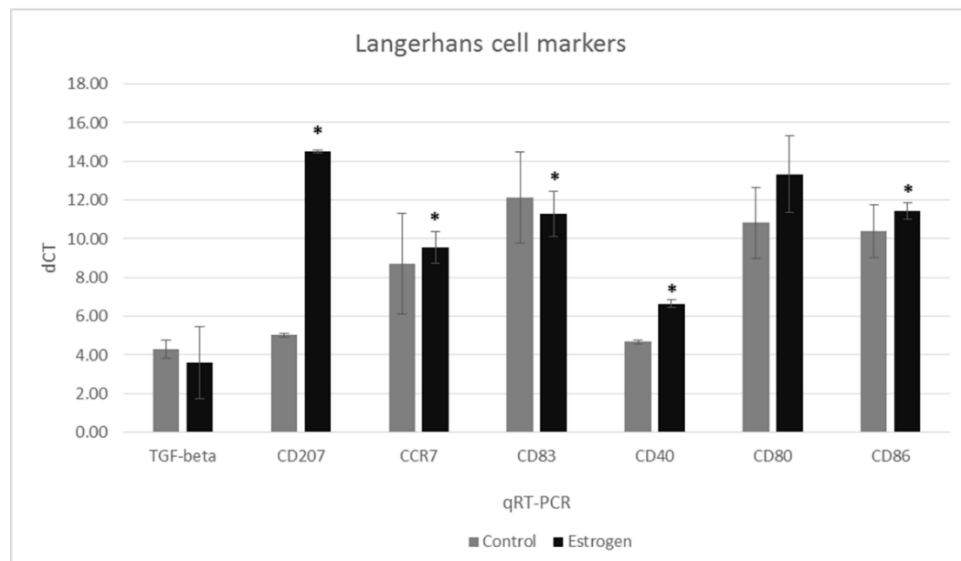
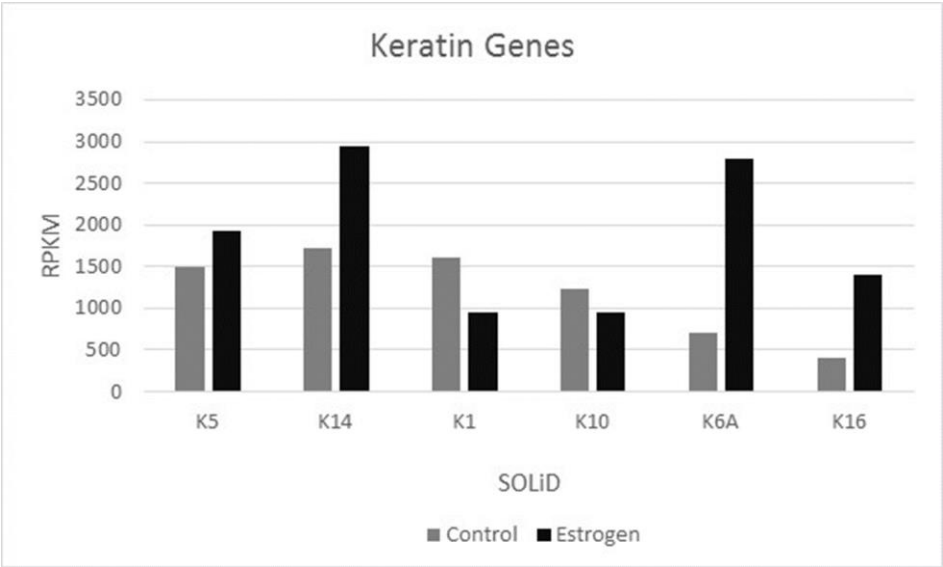


Figure 3.11 Expression patterns of keratin genes by a.) transcriptome sequencing and b.) qRT-PCR.

A



B

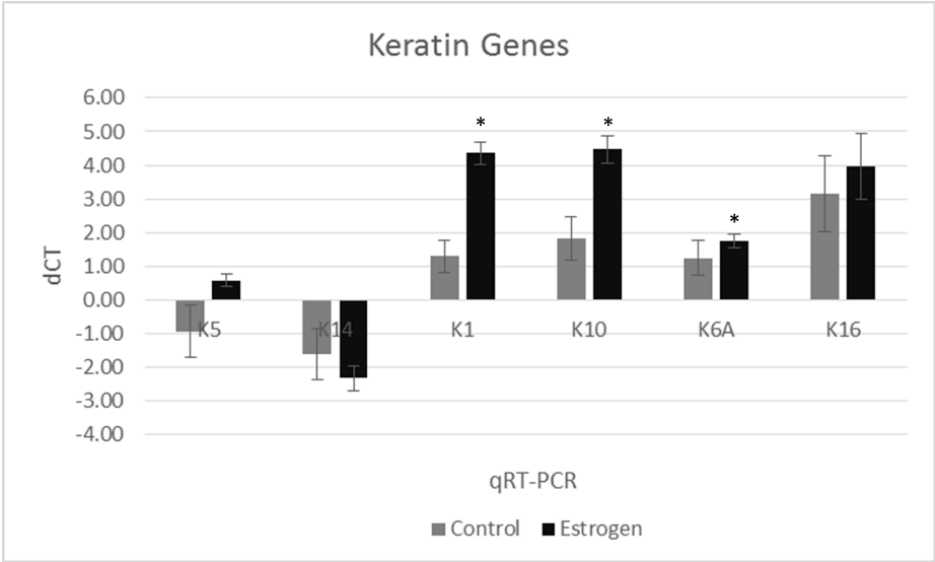
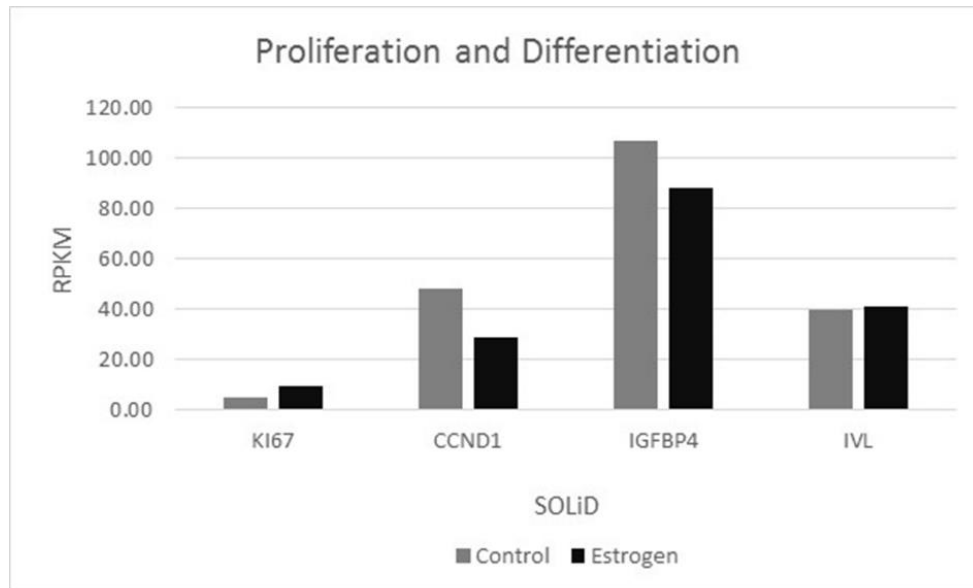
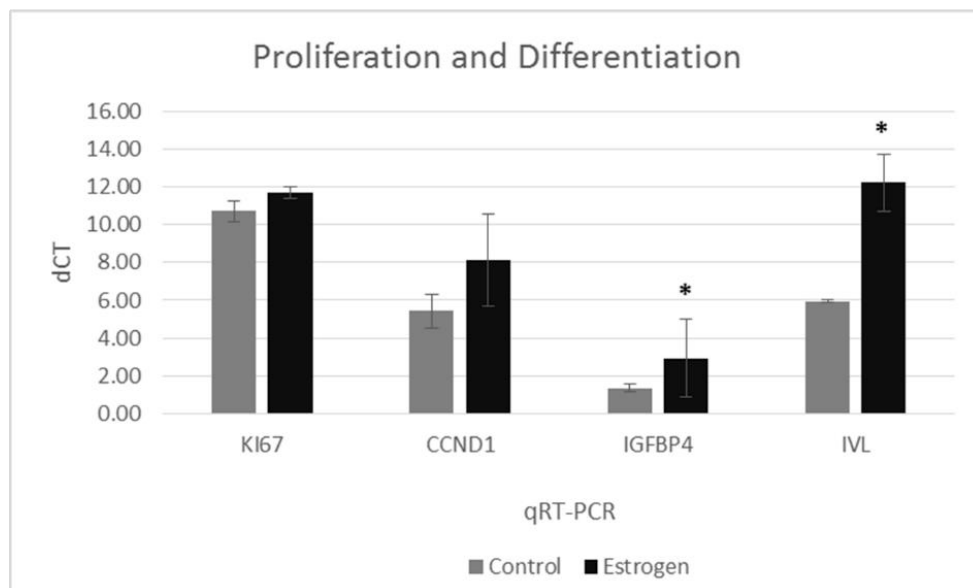


Figure 3.12 Expression patterns genes involved in keratinocyte proliferation and differentiation by a.) transcriptome sequencing and b.) qRT-PCR.

A



B



3.4 Discussion

The incidence of many sexually transmitted diseases in uncircumcised men has been linked to the presence of the foreskin. Transmission rates of these STIs decreases dramatically after removing the foreskin through circumcision. The foreskin not only creates a warm and moist environment, permissive to bacterial growth and virus survival, but also has a thin mucosal stratum corneum permitting microbes to more readily enter the body. This realization led to the hypothesis that increasing the protective barrier of the foreskin may aid in the reduction of sexually transmitted diseases in uncircumcised men [72].

It is well known that the outermost layer of the epithelium serves as a barrier, protecting the inside of the body from exposure to harmful agents such as pathogens. Estrogen has been shown to prevent intravaginal SIV infections in macaques by increasing the thickness of the vaginal epithelium [11]. In chapter 2, we determined that topical estriol has a similar effect on the analogous inner foreskin epithelium. However, the mechanism by which estriol induces this effect at a molecular level was not clear. Based on our histological analyses, the thicker epithelium appeared to be the result of a thicker stratum corneum (keratin layer) and not due to a change in the number of keratinocytes populating the epithelium, suggesting that estriol induces increased keratin deposition during differentiation rather than increasing skin proliferation. To further clarify this result, here we performed IHC to compare protein distribution of the epidermal terminal differentiation markers, filaggrin and involucrin, before and after treatment with topical estriol. Surprisingly, we found no difference in the localization of these markers after treatment.

It has been reported that estrogen increases the expression of filaggrin in fetal rat skin [155, 156]. These opposing results may be attributed to differences in the thickness of the skin type being analyzed, as the mucosal skin in humans may not mirror the expression patterns in the non-mucosal skin of rats. In addition, another study has reported that involucrin is expressed in all suprabasal layers of the epidermis after estrogen treatment [157], compared to its normal limitation to the stratum granulosum. This effect was observed in tissues sampled from human female tongues. The tongue is a very different epithelial structure to the foreskin mucosa, and in addition, all our samples are from men, making comparisons between the two studies difficult. But together, our data suggests that both filaggrin and involucrin show varied responses to estrogen stimulation depending on the tissue context.

To further characterize how estriol affects the molecular response of the foreskin epithelium tissue as a whole, we performed transcriptome sequencing of treated and control inner foreskin tissues. Here, we provide the first insight into the transcriptome of human inner foreskin tissue treated with estrogen using RNA-seq, a powerful high-throughput DNA deep-sequencing technology. A search through the PubMed database with the combination of key words of estrogen, foreskin, and RNA-seq retrieved zero results as of Sept. 1, 2014. Understanding how estrogen affects the foreskin is essential for assessing the efficacy of the treatment and for defining how estriol activates the keratinization pathways. In addition, such data may help to identify potential non-hormonal targets that could be used to increase keratinization.

For RNA-seq, we used the Applied Biosystems SOLiD 4 System to perform a single-end run of ribo-depleted total RNA extracted from our samples. We used GFOLD to determine the differentially expressed genes between estrogen treated tissues and controls based on the mean RPKM values across the two individual biological replicate samples (from separate patients) in each group. We obtained at least 50 million reads per sample, which is generally sufficient coverage for accurate transcriptome profiling of genes of interest [158]. PCA analysis showed that the two estrogen treated samples had some differences in their gene expression profiles, suggesting the presence of skewed results in our sequencing data. For example, one reason why we saw up-regulation in genes associated with inflammatory and immune response could be due to a pre-existing, but unreported foreskin-associated infection in one of the patients. We could examine this assumption by performing qRT-PCR for genes associated with inflammation or infection across the two samples to see if one shows higher expression. In addition, our foreskins were collected from men of various ages with different previous pathologies related to the foreskin and ethnic backgrounds. The differences observed could indicate that the two control samples came from men with similar backgrounds, while estrogen may have induced the differential expression pattern observed in the treated patients, where 'normal' state may be for them to be similar.

To support the reliability of our transcriptome based DEG analyses, we performed qRT-PCR for three differentially expressed genes (CCR5, CYP1A1, and LCE3A) and confirmed comparable trends to those identified through the RNA-seq analyses. Furthermore, estrogen has been shown to increase expression of CCR5 [114] and

decrease expression of CYP1A1 [159] in female mice and human endometrial cells, respectively. We see the same trends in our data from the foreskin transcriptome. We also observed an increase in the expression of the cornified envelope protein LCE3A in line with previous studies demonstrating its up-regulation after estrogen treatment [160, 161]. Together these data confirm the accuracy of our transcriptome analyses and that estrogenic effects can be readily measured in our samples. We next proceeded with untargeted analyses of the transcriptome.

The two most highly up-regulated differentially expressed genes identified across our data set were interleukin 20 (IL20) and late cornified envelope protein 3A (LCE3A), both known to be involved in the keratinocyte proliferation and differentiation processes. IL20 plays a role in hyperproliferation of keratinocytes associated with inflammation in the skin [162, 163]. Several other interleukins involved in hyperproliferation and inflammation were also up-regulated in our study, including IL8, IL17F, IL19, and IL36A. LCE3A is a part of a family of genes that encode structural proteins incorporated into the maturing envelope late into terminal differentiation [164]. Proteins that cross-link structural proteins to the cornified envelope, such as those from the small proline-rich protein (SPRR) family were also up-regulated in our study, suggesting that we have induced epidermal keratinocyte differentiation. When all DEGs are examined as a whole, the Gene Ontology (GO) categories *Keratinocyte Differentiation*, *Inflammatory Response*, and *Immune Response* were overrepresented in the estrogen treated tissues. This is in alignment with estriol's demonstrated ability to induce differentiation and accelerate wound healing. In addition, the cytokine-cytokine receptor interaction pathway was most up-regulated in the presence

of estriol. Cytokines are released in response to a stimulus to regulate cells involved in innate and adaptive inflammatory host defenses, cell growth, differentiation, as well as development and repair process. Keratinocytes are the major source of cytokine receptors although they are present on all cell types in the epidermis.

The two most down-regulated differentially expressed genes across the data set were filaggrin (FLG) and uroplakin 1A (UPK1A), both key players in keratinocyte terminal differentiation. FLG is responsible for bundling the keratin filaments in keratinocytes to form the flattened cells comprising the stratum corneum [30, 165]. Decreased filaggrin expression, with normal expression of other epidermal differentiation markers has been reported in the literature, although this was observed in association with human skin diseases such as ichthyosis vulgaris and atopic dermatitis [166, 167]. In these conditions, a decrease in mRNA expression was observed, leading to decreased protein synthesis. Upon further investigation, local inflammatory responses marked by an increase in cytokines was determined to be responsible for the abnormal expression [166, 167]. As mentioned above, we also observed a marked increase cytokine expression and the subsequent decrease in FLG expression. However, our immunohistochemistry data combined with normal or increased expression of other structural genes, indicates that decreased expression of FLG does not cause disruption of the stratum corneum in the foreskin as it does in non-mucosal skin. This is supported by other studies using topical estrogen applications on the inner foreskin and vaginal epithelium, which did not report abnormalities in the integrity of the epithelium following treatment [99, 105, 109, 161]. UPK1A is a member of a family of urothelial membrane proteins present in the urinary

tract that are involved in the regulation of cell development, differentiation, and proliferation [168, 169]. Decreased UPK1A expression is associated with an increase in cell growth and differentiation. The expression of UPK1A in the foreskin has not been described to date, thus the novel role of uroplakin in this tissue needs to be explored. Another key keratinocyte differentiation gene that is found in the stratum corneum, loricrin (LOR), was also significantly down-regulated [170]. Although, LOR is a major constituent of the stratum corneum, aberrant expression does not affect formation of the stratum corneum [171]. Furthermore, in its absence other key proteins important in constructing the stratum corneum, such as the SPRRs, are up-regulated to compensate for the loss [172]. Our data supports these findings as we also observed an up-regulation of SPRRs.

The GO category *Organism Development Processes* was most underrepresented in the estrogen treated tissues, suggesting that estriol down-regulates the transcription of genes involved in metabolic processes and development of the skin. Terminal differentiation of epidermal keratinocytes requires many posttranscriptional regulatory mechanisms [173]. Interestingly, many microRNAs (miRNAs), which can modulate the availability of RNA, were expressed in our data set. Two miRNAs, mir4709 and mir650, were found to be significantly up-regulated by estrogen treatment. MiRBase reports a number of predicted targets for both miRNAs, and while there are no validated targets for mir4709, mir650 has been shown to target two genes involved in cell proliferation: inhibitor of growth protein 4 (ING4) and N-Myc downstream-regulated gene 2 (NDRG2) [174, 175]. Mir936 and mirlet7c were significantly down-regulated after treatment. Mir936 has only predicted targets, while mirlet7c has over 195 validated targets including genes involved in cell

cycle regulation like cyclin-dependent kinase 6 (CDK6) and high-mobility group AT-hook 2 (HMGA2) [176]. Although the role of these miRNAs have not been studied in the foreskin, they may have played a role in the differential expression observed in this study. MicroRNA sequencing would provide a more thorough analysis to infer their role and the role of other miRNAs in this tissue. Interestingly, xenobiotic metabolism was the most down-regulated pathway in estrogen treated tissue. Drug metabolism can be affected by a number of physiological factors including the age of the patient and individual inherited variations in drug metabolic pathways. Interestingly, CYP1A1, an enzyme responsible for metabolizing estrogen was most affected by the treatment. This may indicate that the estrogen degradation is delayed with this treatment. Excess estrogen can have a negative impact on some tissues in the body, therefore this discovery warrants further exploration.

Modification of transcriptional regulators may be responsible for the aberrant expression of the GAGE genes in the estrogen treated tissue. Their function is not understood, but at least one gene in the family has been reported to have anti-apoptotic properties [153]. In addition, they may control cellular proliferation, differentiation and survival of human germ line cells [177]. These tumor antigens are normally only expressed by male germ cells and by some types of cancers [178]. In other tissue types, their closely related counterparts, MAGE genes, are silenced by DNA hypermethylation [179]. It has been widely reported in the literature that estrogen plays a role in chromatin modifications [180, 181]. It is important to point out that GAGE genes do not cause cancer, rather the demethylation of proto-oncogenes. The overexpression of GAGE genes in our tissue samples following treatment suggests that estriol or miRNAs could be involved in

demethylation of these genes. Of note, we did not observe altered expression in any known proto-oncogenes in our data set. Hence, the nature of GAGE gene expression in the foreskin will need to be explored further.

The predominant way that the effects of estrogens are mediated in the cell is by direct binding to the estrogen receptors α and β , which homo- or heterodimerize and translocate to the nucleus to interact with estrogen response elements. This stimulates the transcription of target genes, including those important for the development and maintenance of the some target tissues as well as cellular proliferation and differentiation. Although ER expression has been observed in the foreskin [154], there are no studies to my knowledge that have shown whether their expression is regulated by estriol. In this study, we report that there was no statistically significant difference in ER mRNA levels between the estriol treated foreskin tissues and the control tissues. Some of the differential expression seen in our study may be caused by non-genomic effects of estrogen independent of its receptor. We can perform ChIP seq to study gene expression that could be caused by other transcription factors interacting with the DNA.

The increased thickness of foreskin after estrogen treatment may serve to enhance protection of the HIV target cells present in the epithelium. Langerhans cells are the primary HIV target cells present in the skin, as they express CD4, CCR5, and CXCR4. The maintenance of CD4 and CXCR4 expression suggests that these receptors are not affected by estriol. However, we did observe a small but significant increase in CCR5

expression, warranting a closer look at why this is occurring. Our findings from Chapter 2 suggests that the increased expression is not due the presence of more Langerhans cells (LCs), the primary HIV target cells in skin. Mature LCs emigrate from the epidermis to the draining lymph nodes where they present antigens to T-cells which also express CCR5. It has been shown that CCR5 expression on T-cells increases after exposure to estrogen [182]. This up-regulation could mean an increased risk of HIV transmission should the virus be allowed contact with the cells, however the likelihood of this happening is diminished because of our enhanced barrier. Furthermore, an absence of statistically significant differential expression in genes involved in migration and maturation suggests that estriol treatment does not affect LC function nor distribution. A more likely explanation, consistent with the data reported thus far, is that CCR5 is acting as a canonical chemokine receptor to control the inflammatory response either initiated by the treatment or by an existing infection [183].

We showed in Chapter 2 that estrogen increases the thickness of the stratum corneum of the inner foreskin. Thickening of the stratum corneum could result from increased proliferation of the keratinocytes (more cells increases amount of keratin fibers packed into the stratum corneum) or increased keratinization of the cells during differentiation (more keratin per cell). Keratin genes are markers of proliferation and differentiation in the epithelium as they are specifically expressed in different skin layers. We did not find any significant difference in the expression of any of the keratin genes or other genes involved in the development of keratinocytes in our SOLiD data. However, the qPCR data revealed a significant up-regulation in the expression of keratins 1, 10, and 6a as well as

IGFBP4 and IVL. This discrepancy may be due to variability in the number of transcripts generated by each preparation procedure. Quantitative PCR may be more adept at capturing higher transcript numbers, as it amplifies a specific gene. As reported above, we also found differential expression of stratum corneum development genes, such as LCE3a and SPRR2C.

Taken together, our findings demonstrate that topical estrogen not only enhances the protective barrier of the foreskin but also elevates important defense mechanisms. Here we show that differential expression of genes in response to estriol elicit an initial inflammatory/immune response that is being regulated by expression of anti-inflammatory and anti-defense genes like DEFBs and LTF (data not shown). Furthermore, the key cells present in the skin, keratinocytes and Langerhans cells, are not significantly affected by this process. The results obtained thus far, which strongly support our hypothesis that estriol can be used as a potent stimulator of keratinization, are promising. However, further studies are needed to clarify the role of GAGE and CCR5 in our data set. In addition, we need to repeat transcriptomics on additional samples to confirm consistent results.

Chapter 4

**Modeling the effect of estriol on keratin
production in keratinocytes *in vitro* using the
HaCaT cell line**

4.1 Background

Keratinocytes, the most abundant cells in the epidermis, play two important roles in the skin: to replenish the epithelium and to form the outer protective barrier of skin [184]. These cells are packed with intermediate filaments called keratins that serve as a scaffold to the cytoskeleton [185]. All keratinocytes begin their existence in the basal cell layer of the epidermis where they are characterized by the expression of proliferative markers, Ki-67, keratin 5, and keratin 14 [138]. During proliferation, the keratinocytes go through mitosis to produce an identical daughter cell that eventually undergoes terminal differentiation. When the cells exit the cell cycle and migrate into the suprabasal layer of the epidermis, expression of the proliferative markers decline and markers of epidermal differentiation increase [184]. Early epidermal differentiation is marked by expression of keratins 1 and 10, while filaggrin and involucrin are late markers of differentiation [138, 186]. In late differentiation, keratinocytes begin to flatten and eventually only long fibers of keratin proteins remain along with junctional and structural proteins. These components comprise the primary constituents of the stratum corneum [138].

The stratum corneum marks the outermost layer of the epidermis where water retention is regulated and the body comes in contact with pathogens in the external environment [187]. Estrogen has been identified as a compound that can induce thickening of the stratum corneum [11, 12, 110, 188]. Studies in humans and monkeys have shown that a thicker protective barrier prevents transmission of viral particles into the epithelium and may also protect the underlying immune cells from contact with viruses [11, 12, 33, 110]. Our results reported in Chapters 2 and 3 of this thesis corroborates these studies by

demonstrating that estrogen can induce keratinization of the inner foreskin mucosa through stimulation of epidermal differentiation. Uncircumcised males could greatly benefit from this treatment, as they are more likely than their circumcised counterparts to experience bacterial and viral infections [189].

Studying keratinocyte proliferation and differentiation can be complicated by the complex structure of the dermis and epidermis *in vivo*. Cell culture experiments can be employed to reduce the complexity of cell interactions and the factors involved in keratinocyte proliferation and differentiation. Studies in cultured keratinocytes report that many factors promote differentiation, including vitamin D [190, 191] and calcium [186, 192] among others. The effect of estrogens are largely mediated through activation of the two key estrogen receptors (ERs), estrogen receptor alpha (ER α) and estrogen receptor beta (ER β). Once activated, the receptors translocate to the nucleus where they bind to estrogen response elements and regulate gene transcription.

Estrogen may increase keratin thickness in the skin by activating keratin production in the keratinocytes. Alternatively, estrogen may affect the keratinocytes in the basal layer of the epidermis, causing increased proliferation of the mitotic cells. In order for the keratinocytes to respond to topical estriol, they must express ERs. Assessing keratinocyte proliferation and differentiation in culture before and after treatment with estriol can reveal relevant information about its effect on keratinization at the cellular level.

The spontaneously immortalized human keratinocyte cell line, HaCaT, has been used extensively to study keratinocyte function [186, 193-195]. Unlike primary cells that have a finite life span and require supplementary growth factors to survive *in vitro*, HaCaT cells can be maintained in culture for longer periods of time and can be grown in conventional media. HaCaT cells were derived from histologically normal, male skin located in the distant periphery of a melanoma [32]. The name describes the specific culture conditions used to create the cell line; originating from human adult skin keratinocytes propagated under low Ca²⁺ conditions and elevated temperature [32]. These conditions encouraged prolonged cell proliferation, while suppressing differentiation. After several passages, the cells overcame their dependency on the calcium and temperature requirements. Despite, their immortalized nature, the cells never became tumorigenic. In subsequent experiments, they were shown to be capable of differentiating [32] and forming a stratified epidermal monolayer in organotypic culture [195]. Although HaCaT cells are aneuploidy with duplicated chromosomes and a missing Y chromosome, they exhibit the same functionality and keratinocyte surface markers of proliferation and differentiation found in the upper layers of normal human skin [194].

HaCaT cells have been regarded as the ideal model system to study cell proliferation and differentiation in response to exogenous factors, such as calcium and vitamin D [186, 193, 196]. Estrogen also has the ability to influence keratinocyte function *in vivo*, however, few studies have examined the ability of estradiol to alter proliferation and differentiation of HaCaTs [197-199]. Additionally, no studies have been performed using its weaker counterpart, estriol. We demonstrated in Chapters 2 and 3 that estriol can be used as a

potent stimulator of keratinization *in vivo*. Keratinocytes are the main cells in the epidermis, so we presume that they serve as the target cells upon which estriol exerts the majority of its regulatory effects. Langerhans cells, Merkel cells, and melanocytes are also very important cells present in the skin as they are responsible for viral detection and degradation, somatosensory, and pigment production, respectively [200] and they express estrogen receptors. Therefore, to accurately assess the effect of a drug treatment on specific biological processes, it is necessary to isolate the cells of interest. The aim of this study was to test the suitability of HaCaT cells as a model system to examine keratinocyte proliferation and differentiation in response to estriol.

4.2 Methods

Cell Culture Maintenance

HaCaT cell stock cultures were maintained at 37°C, under 5% CO₂ in 1X Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen) and supplemented with 100 U/ml penicillin-100 mg/ml streptomycin, as well as 10% Fetal Bovine Serum (FBS) (Invitrogen). Throughout the methods, this mixture will be referred to as growth media (GM).

Hormone Preparation

Estriol (E3) (Cayman Chemical) and 17- β estradiol (E2) (Sigma-Aldrich) were solubilized in absolute ethanol to make a 1000X stock. E3 was diluted further in absolute alcohol to

0.1 μ M and 10 μ M working stock solutions and E2 was diluted to a 10 μ M working stock solution.

Cell Treatments

HaCaT cells were trypsinized using 0.02% trypsin/EDTA and seeded in duplicate to a density of 0.4×10^5 cells per chamber in 8-well culture slides, 6.8×10^5 cells per well in 6-well culture plates, or 0.7×10^5 cells per well in 24-well culture plates for immunohistochemistry, Western Blot analysis, and RT-qPCR, respectively. All cells were grown in phenol red-free DMEM (Invitrogen) and supplemented with 100 U/ml penicillin-100 mg/ml streptomycin, as well as 10% charcoal stripped FBS (Invitrogen). All cells were grown at 37°C in a 5% CO₂ humidified incubator for 24 hours before treatment. Cells grown on the culture slides were treated with equivalent amounts of vehicle (100% ethanol), 0.1 nM E3, 10 nM E3, or 10 nM E2 for either 24 or 48 hours. Cells were stored at room temperature for use in immunostaining. For Western Blot and RT-qPCR analyses, cells were treated with vehicle (100% ethanol), 0.1 nM E3, 10 nM E3, or 10 nM E2 for 24, 48, or 72 hours. Following treatment, the cells for immunoblot analysis were lysed on ice with 8.5 M urea for 5 minutes. The lysate was collected and centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant containing the protein was collected for downstream analysis. For RT-qPCR, cells were harvested and total RNA was isolated using the attached cells protocol of the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Briefly, the cells were incubated in the lysis solution/2-mercaptoethanol (2-ME) mixture for 2 minutes and the lysate was filtered to release the DNA and remove cellular debris. RNA was isolated and DNased using the Turbo DNA-free Kit (Ambion) to

remove any remaining DNA. The SuperScript III First-Strand Synthesis System for RT-PCR was utilized to synthesize cDNA to be used in the qRT-PCR.

Subcloning

Untreated HaCaT cells were subcultured from a confluent plate containing about 8×10^6 cells. Cells were collected in an RNase free tube and the RNA was extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Briefly, the cells were incubated in the lysis solution/2-mercaptoethanol (2-ME) mixture for 2 minutes and the lysate was collected for downstream analyses. Total RNA was isolated and DNased using the Turbo DNA-free Kit (Ambion) to remove any remaining DNA and then converted to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR. The resulting cDNA was used to confirm expression of the estrogen receptors α and β in the HaCaT cells using β -actin as a housekeeping gene (Table 4.1). The PCR products were analyzed by standard gel electrophoresis, and bands corresponding to the estrogen receptor excised and purified using the Qiaquick Gel Extraction Kit (Qiagen). Purified PCR products were ligated into the pGEM-T Easy Vector System (Promega). The plasmid was used to transform JM109 *Escherichia coli* (*E. coli*) (Promega) competent cells and plated onto Luria broth (LB) agar, containing 100 $\mu\text{g/ml}$ ampicillin and supplemented with 80 $\mu\text{g/ml}$ X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and 0.5mM IPTG (Isopropyl β -D-1-thiogalactopyranoside). The plates were incubated overnight at 37° C and white colonies containing the genes of interest were selected and grown overnight in LB containing 100 $\mu\text{g/ml}$ ampicillin in a 37° C shaking incubator. Following incubation, 1.5 ml of the culture was used to isolate plasmid DNA using the QIAprep Spin Miniprep

Table 4.1 Sequences of the qRT-PCR primers used in this study.

<i>Gene Symbol</i>	<i>Primer</i>	<i>Sequence</i>
<i>ESR1</i>	Forward	AAATGCAGGCTCCATGCTCA
	Reverse	CAGTCCCGGAGAATGTGAAGA
<i>ESR2</i>	Forward	GGCTTCCAGGCAGTAATGGG
	Reverse	ATTCTAAAAGCGCGGTGCTG
<i>FLG</i>	Forward	TGAGGGCACTGAAAGGCAA
	Reverse	TGGCCACATAAACCTGGGTC
<i>IVL</i>	Forward	CTCTGCCTCAGCCTTACTGTGA
	Reverse	ACTGGCAGTGTGTGTTGCTG
<i>KRT1</i>	Forward	CCTGTCTGCCCTCCTGGT
	Reverse	CTGCAGCAGCTCCCATTTG
<i>KRT5</i>	Forward	GAGATCGCCACTTACCGCAA
	Reverse	GTAGCTTCCACTGCTACCTCC
<i>KRT6A</i>	Forward	ATCGACCACGTCAAGAAGCA
	Reverse	ATTCAGCCTGCACTCCTCAC
<i>KRT10</i>	Forward	ACAAAGTTCGGGCTCTGGAAG
	Reverse	CGCAGAGCTACCTCATTCTCAT
<i>KRT14</i>	Forward	AACGCCGACCTGGAAGTGAA
	Reverse	AATCTGCAGAAGGACATTGGCAT
<i>KRT16</i>	Forward	AGCATCTGGCCAATCCTATTCT
	Reverse	GCAGAGGAGCAGGGGAGATA
<i>MKI67</i>	Forward	GCCTGTACGGCTAAACATGG
	Reverse	AGGCCTTGGAATCTTGAGCTTT
<i>ACTB</i>	Forward	AACTCCATCATGAAGTGTGACG
	Reverse	GATCCACATCTGCTGGAAGG

Kit (Qiagen). Clones containing the appropriate inserts were sent for DNA sequencing at the DNA Biotechnology Facility at the University of Connecticut.

Northern Blot

Total RNA extracted from a HaCaT cell pellet was fractionated on a denaturing 1% agarose gel containing 37% formaldehyde at 40V for one hour. The RNA was transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore) during an overnight incubation. After briefly washing in 2X saline-sodium citrate (SSC) buffer, the RNA was cross-linked to the membrane using Stratalinker (Stratagene). A ³²P-labeled probe complimentary to the ER-alpha sequence was generated via random priming. The membrane containing the immobilized RNA was hybridized with the labeled probe overnight at 62°C in Church's hybridization buffer. After removing the blot from the hybridization solution and washing in 2X SSC/0.1% SDS, the bands were visualized using autoradiography. Briefly, the blot was placed in a film cassette, covered with a sheet of X-ray film and exposed at -80C for 7 days. Following exposure, the film was brought to room temperature before developing.

Immunohistochemistry

Immunofluorescence was performed on treated and untreated HaCaT cells to determine the localization of estrogen receptors α and β . The cells were fixed on their slides in 4% paraformaldehyde/1XPBS for 20 minutes, permeabilized in 0.2% Triton-X100/1X PBS for 5 minutes, and blocked with 1% bovine serum albumin (BSA) for 1 hour. After an

overnight incubation at 4°C with a 1:250 dilution of ER- α (Santa Cruz Biotechnology) or 1:250 dilution of ER- β (Pierce Biotechnology) rabbit polyclonal antibodies in 1 X PBS, slides were washed with 1X PBS and incubated with a secondary FITC-conjugated goat anti-rabbit antibody (AbCam) in 1X PBS containing 1% BSA/2% FBS for 1 hour. A second wash with 1X PBS was followed by an incubation with a FITC-conjugated donkey anti-goat antibody (AbCam) in 1X PBS containing 1% BSA/2% FBS for 1 hour to enhance the signal. Slides were washed with 1X PBS, then incubated with 4',6-Diamidino-2-Phenylindole (Dapi) (Invitrogen) to counterstain the cell nuclei. The slides were mounted using Prolong Gold antifade reagent (Invitrogen) to preserve the fluorescent signal during long-term storage. Control sections were processed using the same protocol with the exclusion of the primary antibody. Images were captured using DeltaVision RT Systems with SoftWorx software (Applied Precision).

Quantitative Real Time-Polymerase Chain Reaction

Real-time reverse-transcription polymerase chain reaction (RT-qPCR) was performed to evaluate the expression of estrogen receptors (ESR1 and ESR2), epidermal keratins (5, 14, 1, 10, 6, and 16), terminal differentiation genes (involucrin and filaggrin) and proliferation gene Ki-67 in treated HaCaT cells. Beta-actin was employed as the housekeeping gene. Primers were designed using NCBI's primer construction software Primer-BLAST (Table 4.1). The mRNA was quantified using iQ Sybr Green Master Mix (BioRad) which contains Taq DNA polymerase, dNTPs, and MgCl₂. Reactions were run in triplicates on the Bio Rad CFX96 Real Time PCR machine. Relative expression to a

housekeeping gene was calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = \Delta C_T$ treated - ΔC_T untreated, where $\Delta C_T = \text{Avg } C_T$ of gene of interest - $\text{Avg } C_T$ of β -actin.

Cell Proliferation Assay

To evaluate the effect of estriol on proliferation of HaCaT cells, the MTS colorimetric assay was performed as described using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega). This test is based on the reduction of the tetrazolium compound, MTS (tetrazolium, inner salt), into an aqueous, soluble formazan, which can only be performed by living cells. Briefly, cells were incubated at an initial density of 2.5×10^4 cells per well in 96-well plates. One day later, the medium was replaced by fresh GM and cells were treated with vehicle, 0.1 nM estriol or 10 nM estriol for 24, 48, or 72 hours. Following treatment, cells were incubated for 3 hours at 37° C in a humidified 5% CO₂ chamber in the presence of MTS (20 μ l/100 μ l of medium). The quantity of formazan product formed (directly proportional to number of living cells) was measured at 490nm absorbance.

Western Blots

Protein concentrations of treated cells were determined using the Quant-iT Protein Assay Kit (Invitrogen) and equal amounts of protein (10 μ g lysate) mixed with 2X sodium dodecyl sulfate (SDS) buffer was loaded onto a polyacrylamide gel for each sample. The proteins were blotted onto polyvinylidene difluoride membranes (PVDF) (EMD Millipore) and blocked with 5% non-fat dry milk for 1 hour. The membranes were incubated overnight

at 4°C with monoclonal anti-epithelial keratin-AE1/AE3 mixture primary antibody (1:1000) (MP Biomedicals) in 1X PBS. After washing with 1X PBS, the blots were incubated with stabilized, peroxidase conjugated goat anti-mouse IgG antibody (1:10,000) (BioRad) in 5% non-fat dry milk for 1 hour at room temperature. Bands were detected using the Western Lightning-Plus Enhanced Chemiluminescence (ECL) substrate system (Perkin Elmer Life and Analytical Sciences) under UV.

Statistical Analysis

The data was analyzed using InStat Version 3 (GraphPad) and One-way Analysis of Variance (ANOVA) was used to compare results across treatment groups, followed by Bonferroni Multiple Comparisons Test post analyses to determine which group means differed from one another. A p-value of less than 0.05 was considered statistically significant. Error bars represent 1 standard deviation (SD) of the mean.

4.3 Results

HaCaT cells express estrogen receptors alpha and beta

In order to determine if estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2) are present in HaCaT cells, untreated cells were subcultured after reaching confluence. Total RNA was extracted from cell pellets and converted to cDNA, followed by PCR. Clones were generated, sequenced, and data obtained from the inserts were entered into BLAST (Basic Local Alignment Search Tool) to compare the sequence

generated to the known sequences for ESR1 and ESR2 genes in the human genome. The sequence generated for ESR2 matched the known sequence but not for ESR1 (Figure 4.1). Northern blot analysis showed that ESR1 is also expressed in HaCaT cells (Figure 4.2). Immunohistochemistry analysis of untreated cells confirmed protein localization of both ESR1 and ESR2 (Figure 4.3). Moreover, ESR1 exhibited cytoplasmic staining (Panel A) while ESR2 showed punctate staining in the nucleus (Panel B).

Estrogen activates and up-regulates the estrogen receptors alpha and beta in keratinocyte culture

The expression of the estrogen receptor alpha and estrogen receptor beta genes in response to estrogen treatment were studied in HaCaT cells using immunostaining and RT-qPCR. Cytoplasmic staining of ESR1 and ESR2 was observed in the absence of estrogen at 24 and 48 hours (Figures 4.4 and 4.5; Panels A and E). Low dose estriol (0.1 nM E3) activates both ESR1 and ESR2 within 48 hours as shown by the predominant nuclear localization in Panel F. Higher doses of estrogen, 10nM E3 and 10 nM 17 β -estradiol (E2) causes complete translocation of ESR1 and ESR2 into the nucleus with 24 hours (Panels D).

After treatment, ESR1 mRNA levels were significantly up-regulated across all treatments and time points, with a marked increase in cells treated with a high concentration of E3 or the main endogenous estrogen, E2 (Figure 4.6). After 24 hours of treatment with 0.1nM E3, 10nM E3, or 10 nM E2, ESR1 expression increased 1.7 fold ($p < 0.05$), 4.7 fold

Figure 4.1 ESR 2 expression in untreated HaCaT cells.

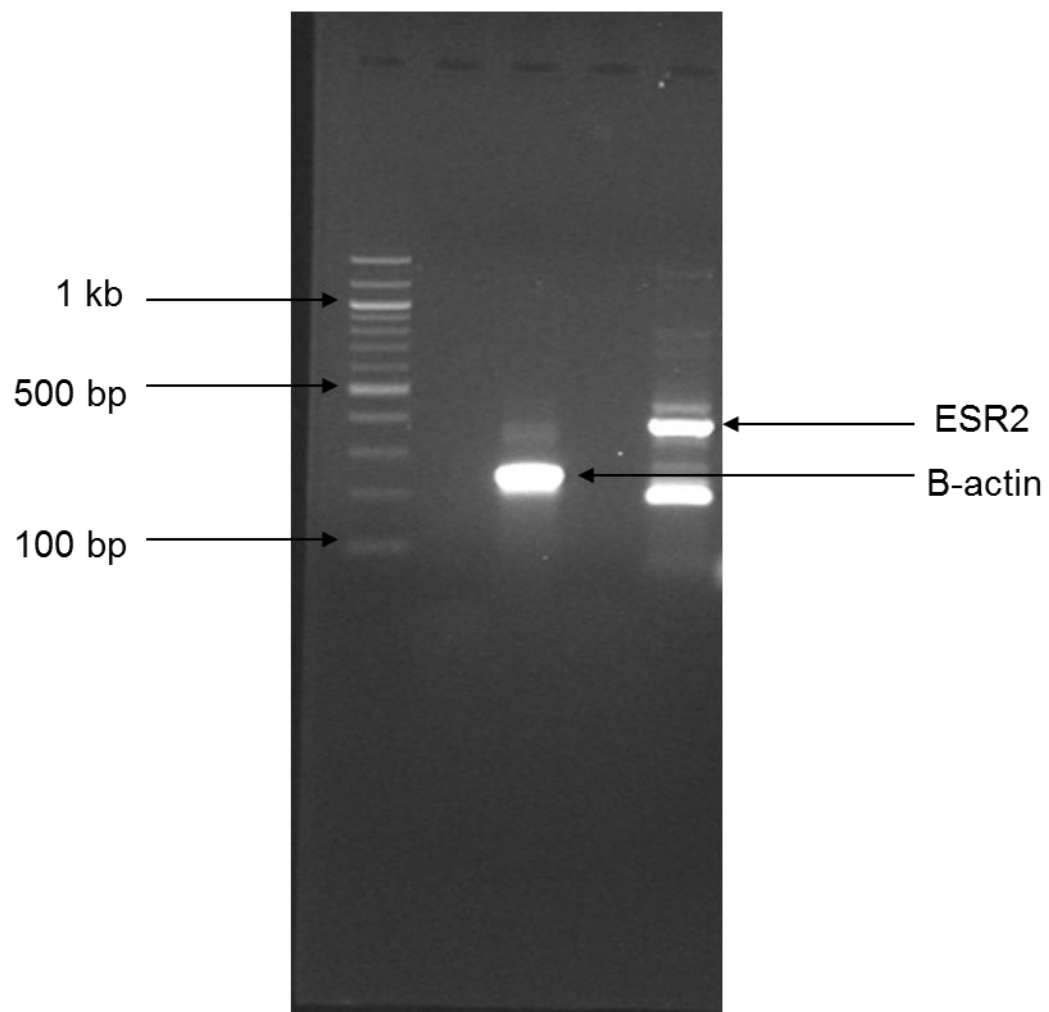


Figure 4.2 Northern blot demonstrates ESR1 expression in untreated HaCaT cells.

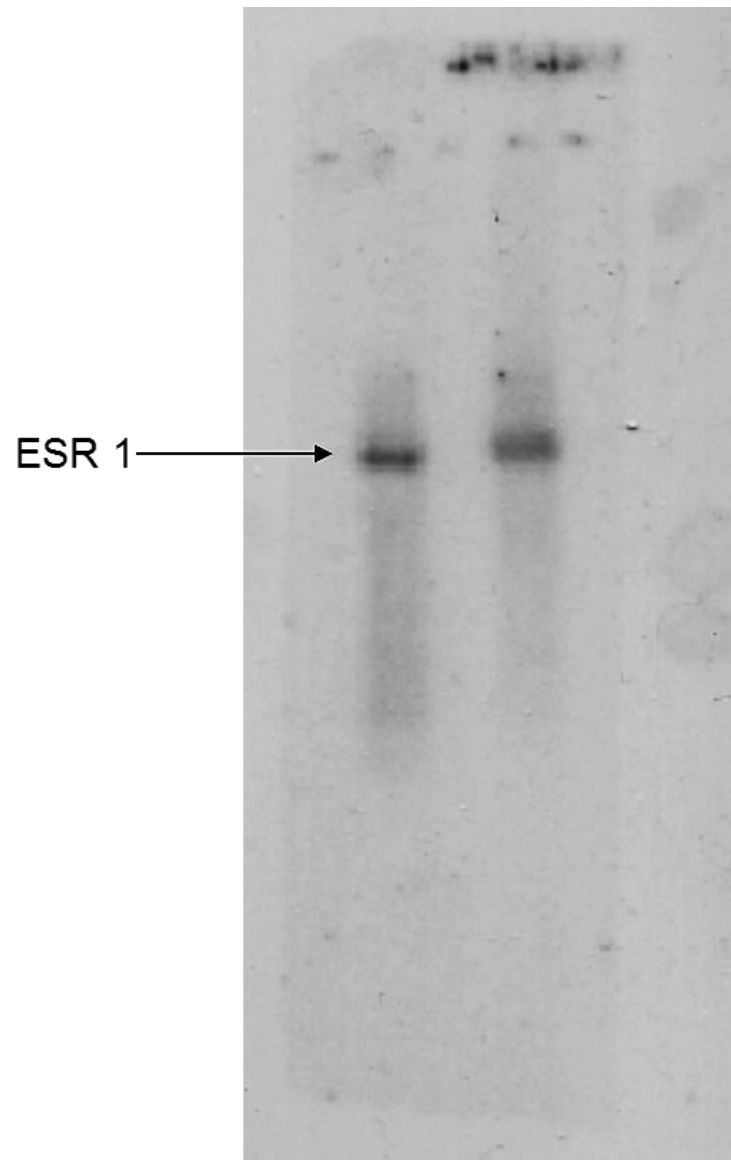


Figure 4.3 Untreated HaCaT cells express ESR1 and ESR2.

A.) Cytoplasmic staining of ESR1 in untreated HaCat cells cultured for 24 hours.

B.) Punctate nuclear staining of ESR2 in untreated HaCat cells cultured for 24 hours.

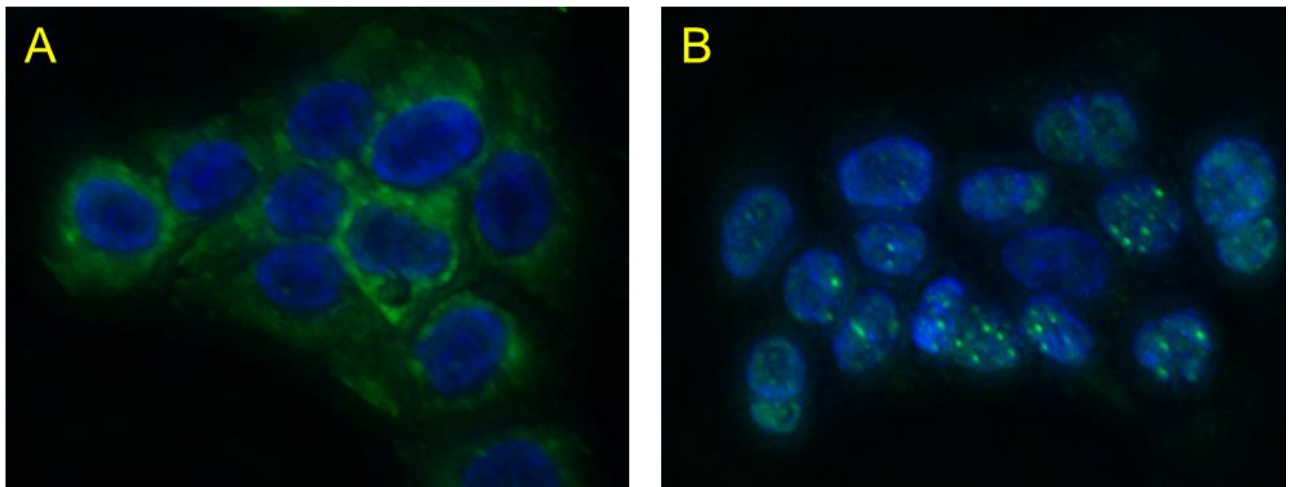


Figure 4.4 ESR1 expression in HaCaT cells treated with estriol at 24 and 48 hours. In the absence of estrogen, ESR1 localizes in the cytoplasm. High levels of estrogen can induce translocation of ESR1 to the nucleus in as little as 24 hours. All forms of estrogen have this effect by day 2.

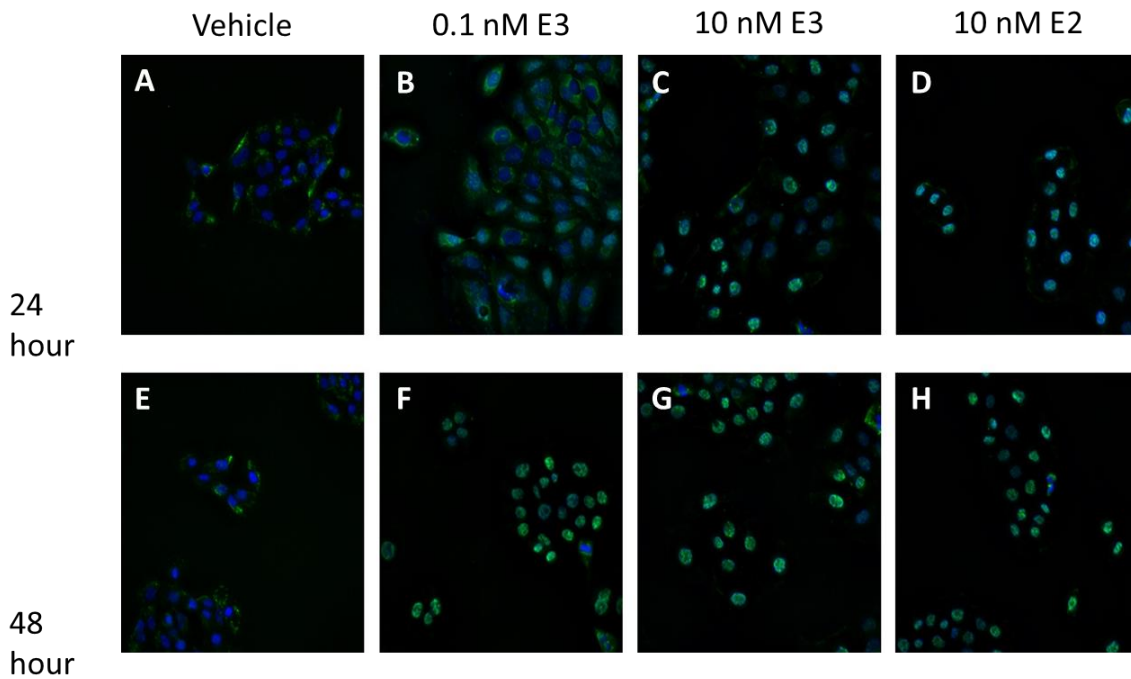


Figure 4.5 ESR2 expression in HaCaT cells treated with estriol at 24 and 48 hours. ESR1 responds to both low and high levels of estrogen treatment in 24 hours. This expression is maintained at 48 hours.

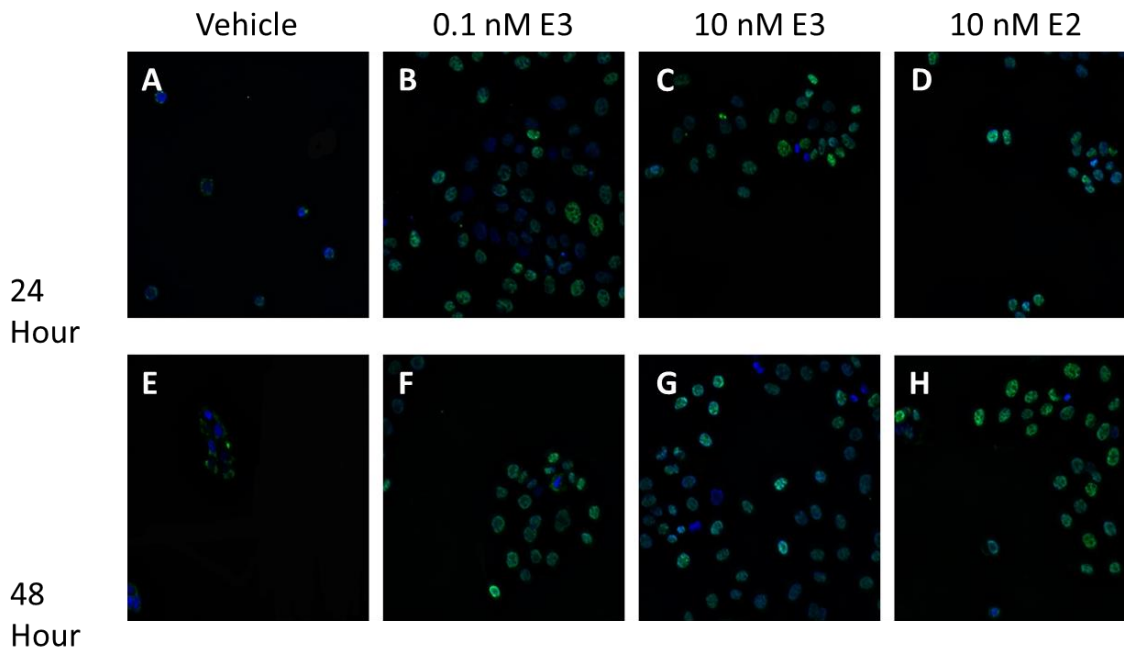
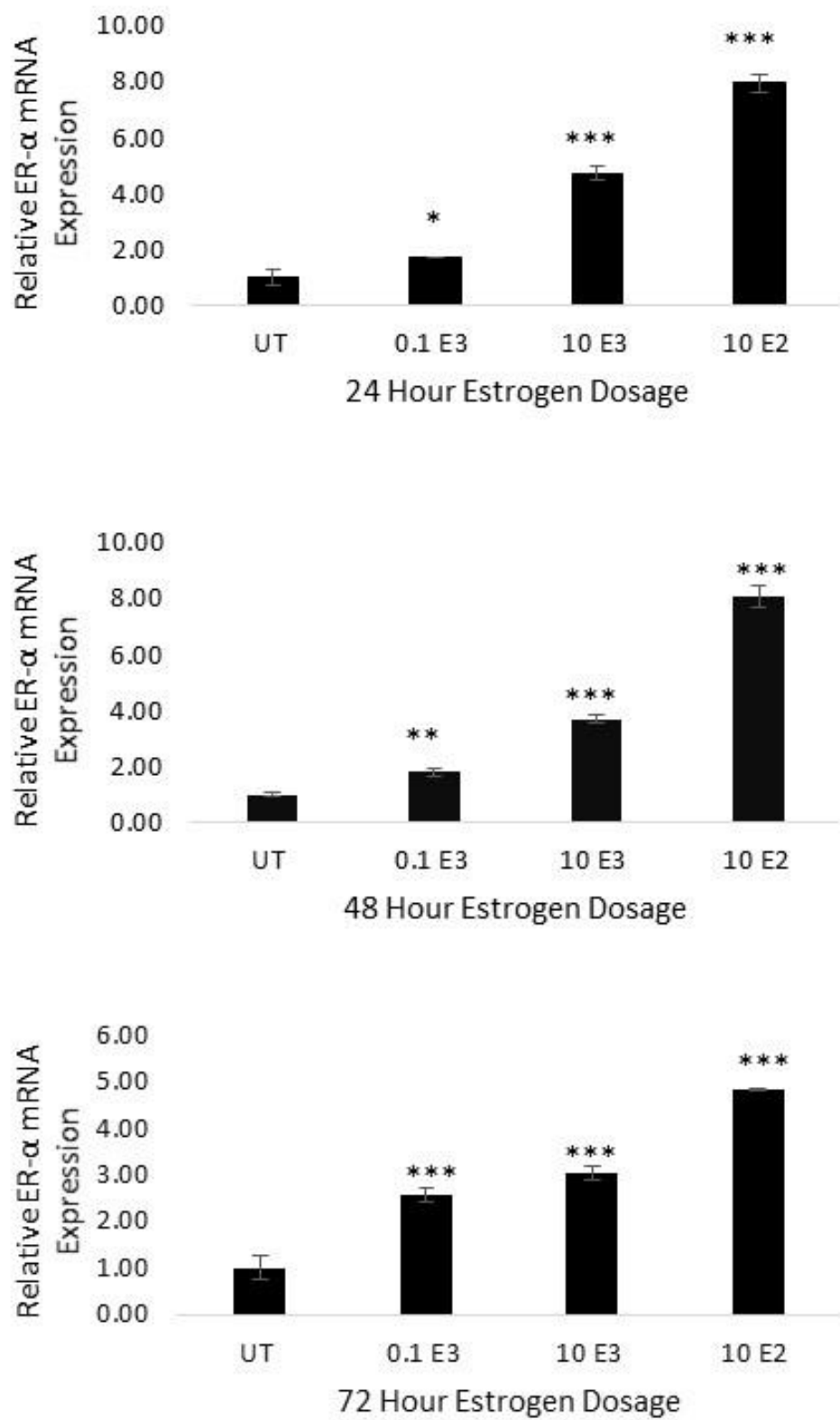


Figure 4.6 ESR1 mRNA expression in treated HaCaT cells.



($p < 0.001$), and 8 fold ($p < 0.001$), respectively. The same treatments after 48 hours, induced a 1.8 fold ($p < 0.01$), 3.7 fold ($p < 0.001$), and 8.1 fold ($p < 0.001$) increase in expression. After 72 hours, ESR1 expression continued to increase above the control at 2.6 fold ($p < 0.001$), 3 fold ($p < 0.001$), and 4.8 fold ($p < 0.001$).

A similar increase in ESR2 expression was observed, however, only the higher concentrations of E3 and E2 were able to stimulate a significant up-regulation (Figure 4.7). In 10 nM E3 treated cells, ESR2 expression increased 2.1 fold ($p < 0.05$), 3.3 fold ($p < 0.01$), and 2.8 fold ($p < 0.001$) after 24, 48, and 72 hours, respectively. E2 treated cells showed a 2.7 fold, 3.4 fold, and 3.1 fold increase across the same time points.

Estrogen promotes terminal differentiation but not proliferation of keratinocytes

MTS is a tetrazolium used to monitor cell viability in culture. Measurement of MTS reduction by cells revealed that estriol does not stimulate proliferation of HaCaT cells, regardless of dosage (Figure 4.8). Although, there was a trending decline in the number of cells present, no statistical significance was reached when compared across treatments. Furthermore, the more potent E2 treatment also failed to induce significant proliferation. To further assess the effect of estrogen on keratinocyte proliferation, we performed a Western Blot to analyze the production of basal keratins 5 and 15, markers of proliferating keratinocytes in skin as well as keratins 6 and 16, markers of hyperproliferation in culture. A pan keratin antibody was used for immunoblotting that detects every known keratin. Specific keratins can normally be identified by their

Figure 4.7 ESR2 mRNA expression in treated HaCaT cells.

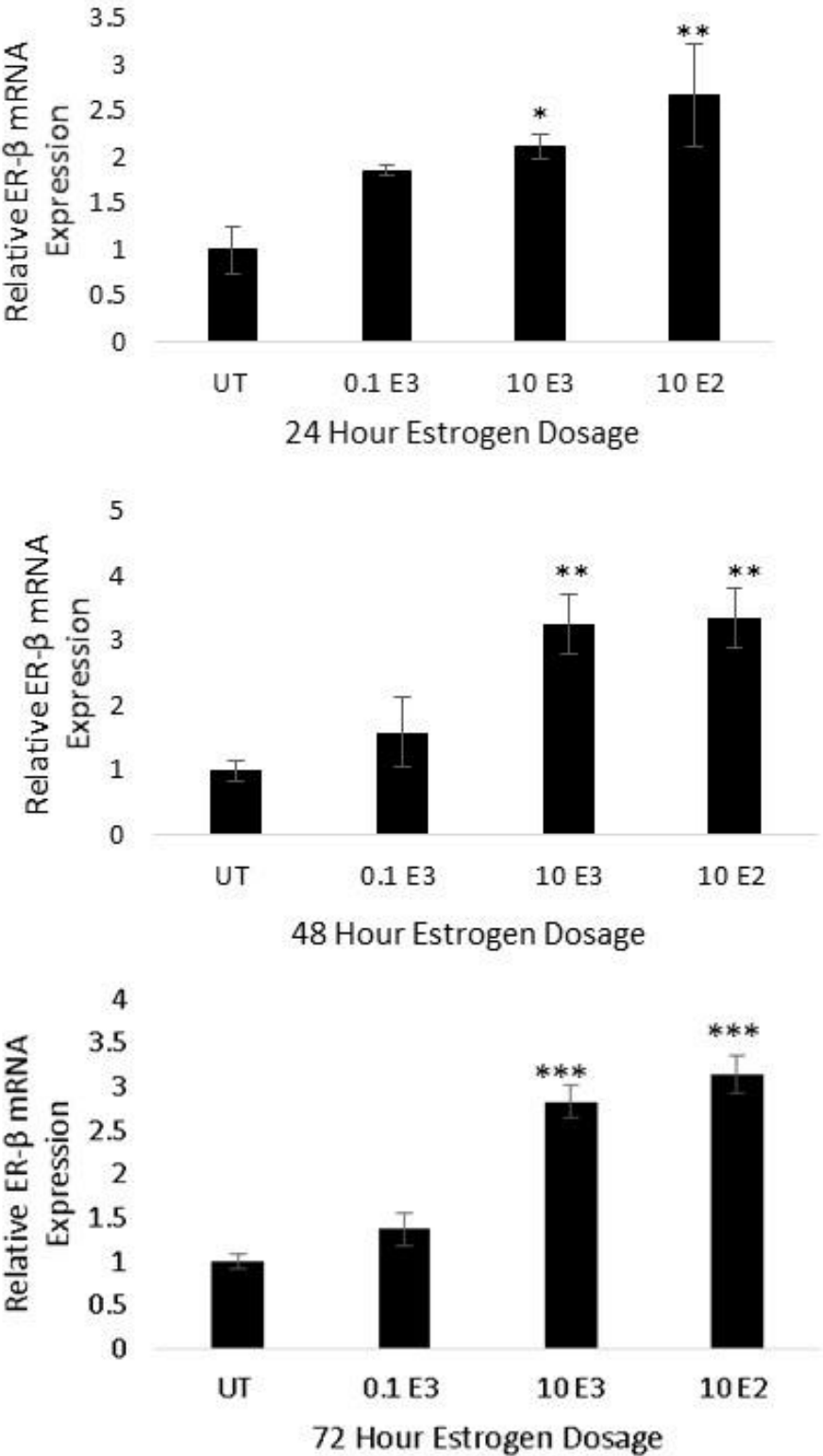
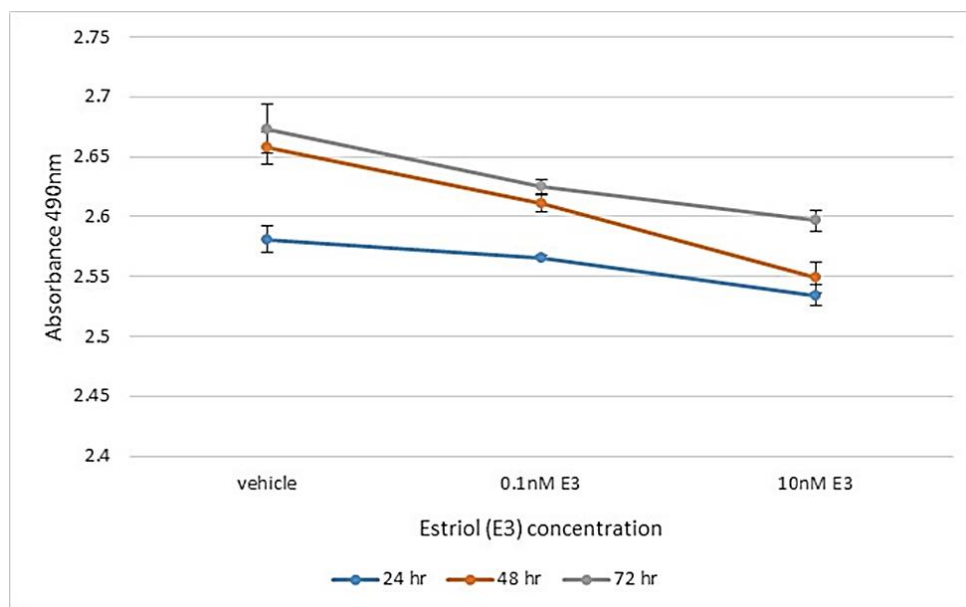


Figure 4.8 Cell Proliferation Assay using treated HaCaT cells.



molecular weight, however, we were unable to resolve the bands to identify keratins of interest (Figure 4.9). We did observe keratins being produced at earlier time points than normal when treated with estrogen. This expression continued to increase over longer treatment times. Quantitative mRNA analysis of the keratins revealed a significant decrease (nearly 1 fold; $p < 0.01$) in keratin 5 production after treating with high dose estrogens for 48 hours, however, this decrease was not evident at 24 or 72 hours (Figure 4.10). Keratin 14 showed a three quarter fold decrease (10 nME3: $p < 0.05$; 10 nM E2: $p < 0.01$) in the presence of high levels of estrogen 24 hours after treatment (Figure 4.11). The decline observed at 48 and 72 hours failed to reach significance. Both keratins 6 and 16 also decreased significantly after 24 hours of treatment in all treatment groups (UT v 0.1 E3: $p < 0.05$; UT v 10nM E3: $p < 0.01$; UT v 10 nM E2: $p < 0.001$) (Figures 4.12 and 4.13). Keratin 6 expression did not change significantly at later time points. Keratin 16 expression at later time points only changed significantly after E2 treatment at 48 hours., where there was a nearly one fold ($p < 0.05$) decline in mRNA abundance. Quantitative mRNA analysis of the standard marker of proliferation, Ki-67, showed that cell proliferation did not change after exposure to estrogen (Figure 4.14).

To determine estrogen's effect on terminal differentiation of keratinocytes in culture, production of suprabasal keratins 1 and 10 were examined in HaCaTs using Western Blot analysis, followed by RT-qPCR. Because keratins in aging cells are critical for proper stratum corneum formation, we also investigated whether mRNA expression of filaggrin (FLG) and involucrin (IVL), two epidermal differentiation markers, is altered in estrogen-treated HaCaT cells. As stated above, we were unable to resolve bands of the

Figure 4.9 Western Blot analysis of keratin proteins.

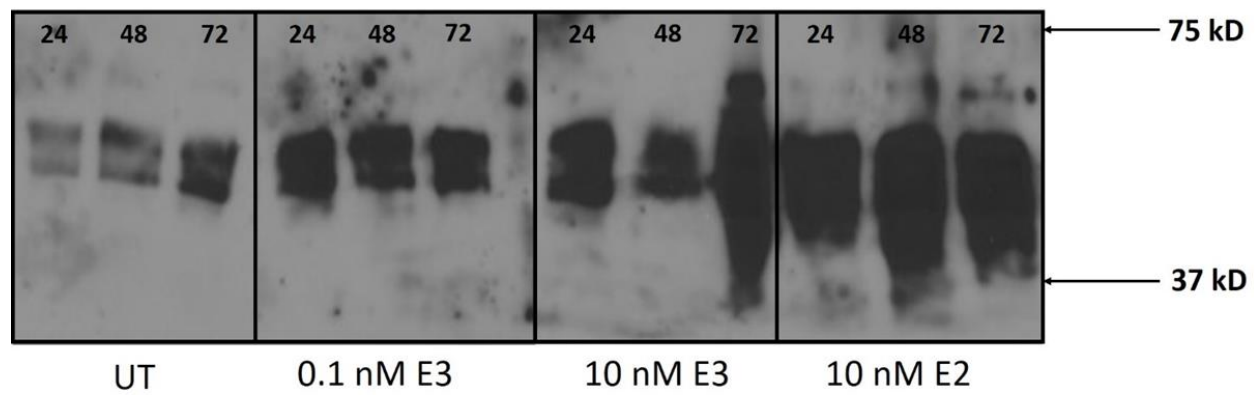


Figure 4.10 Keratin 5 mRNA expression in treated HaCaT cells.

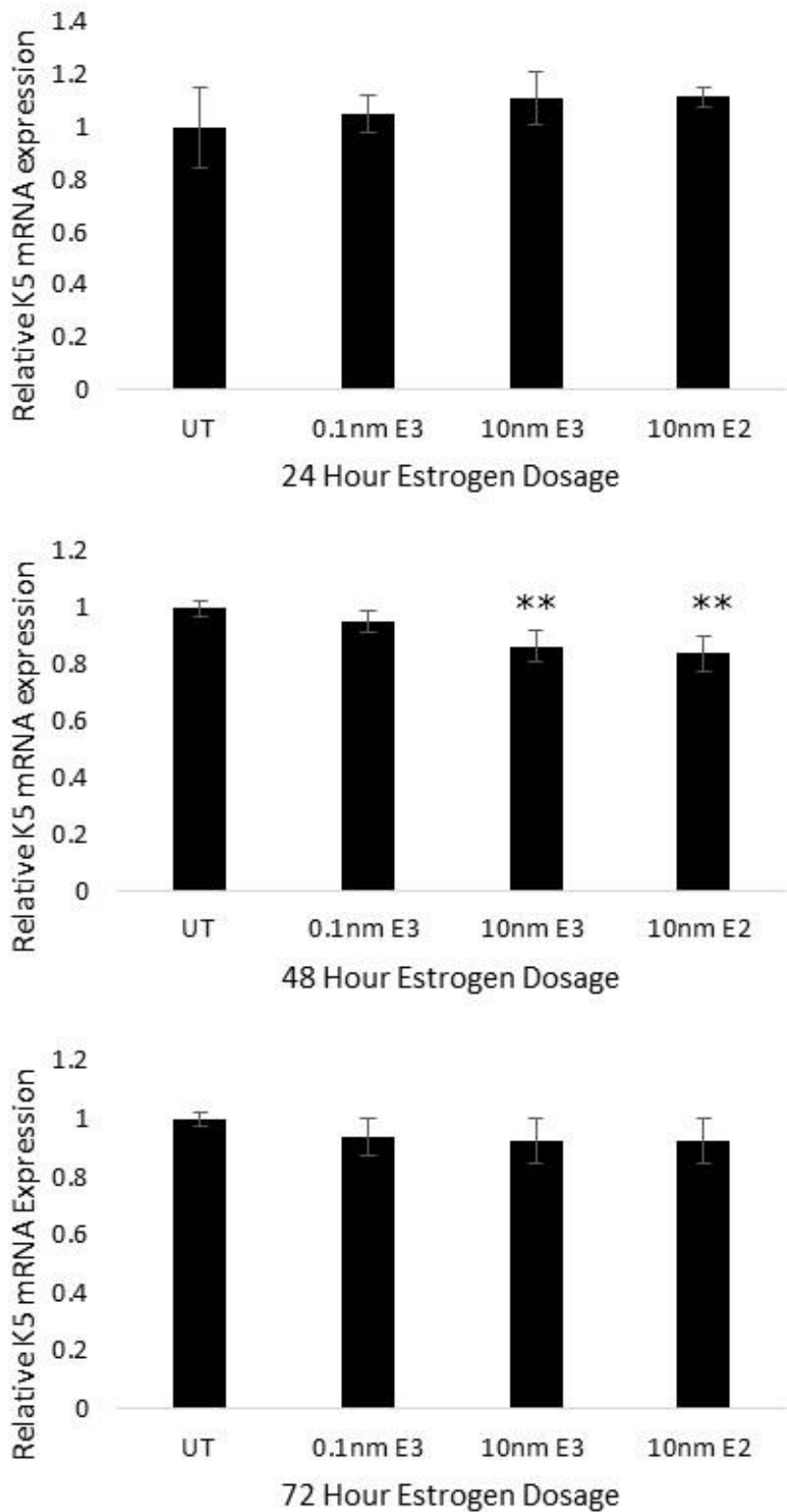


Figure 4.11 Keratin 14 mRNA expression in treated HaCaT cells.

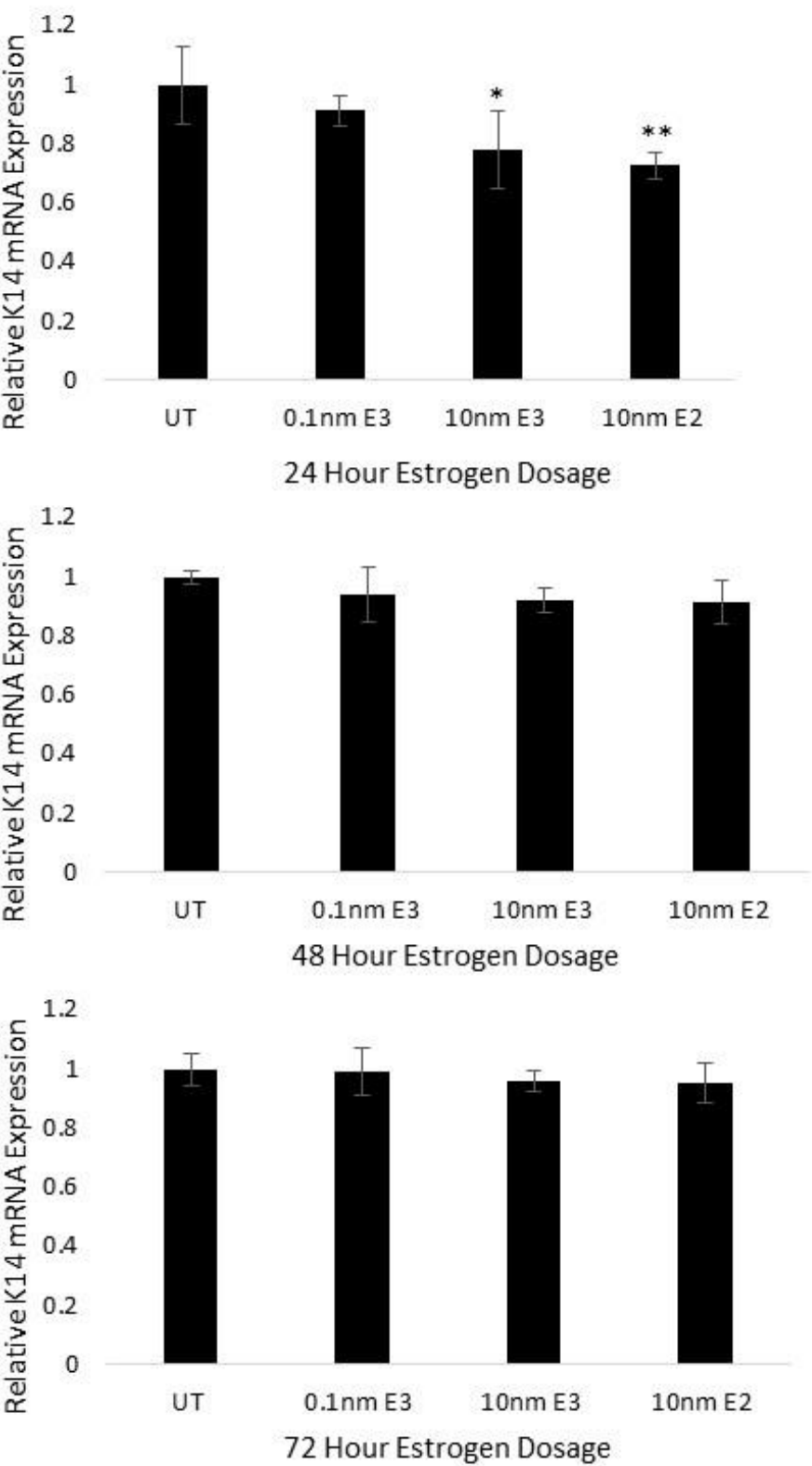


Figure 4.12 Keratin 6 mRNA expression in treated HaCaT cells.

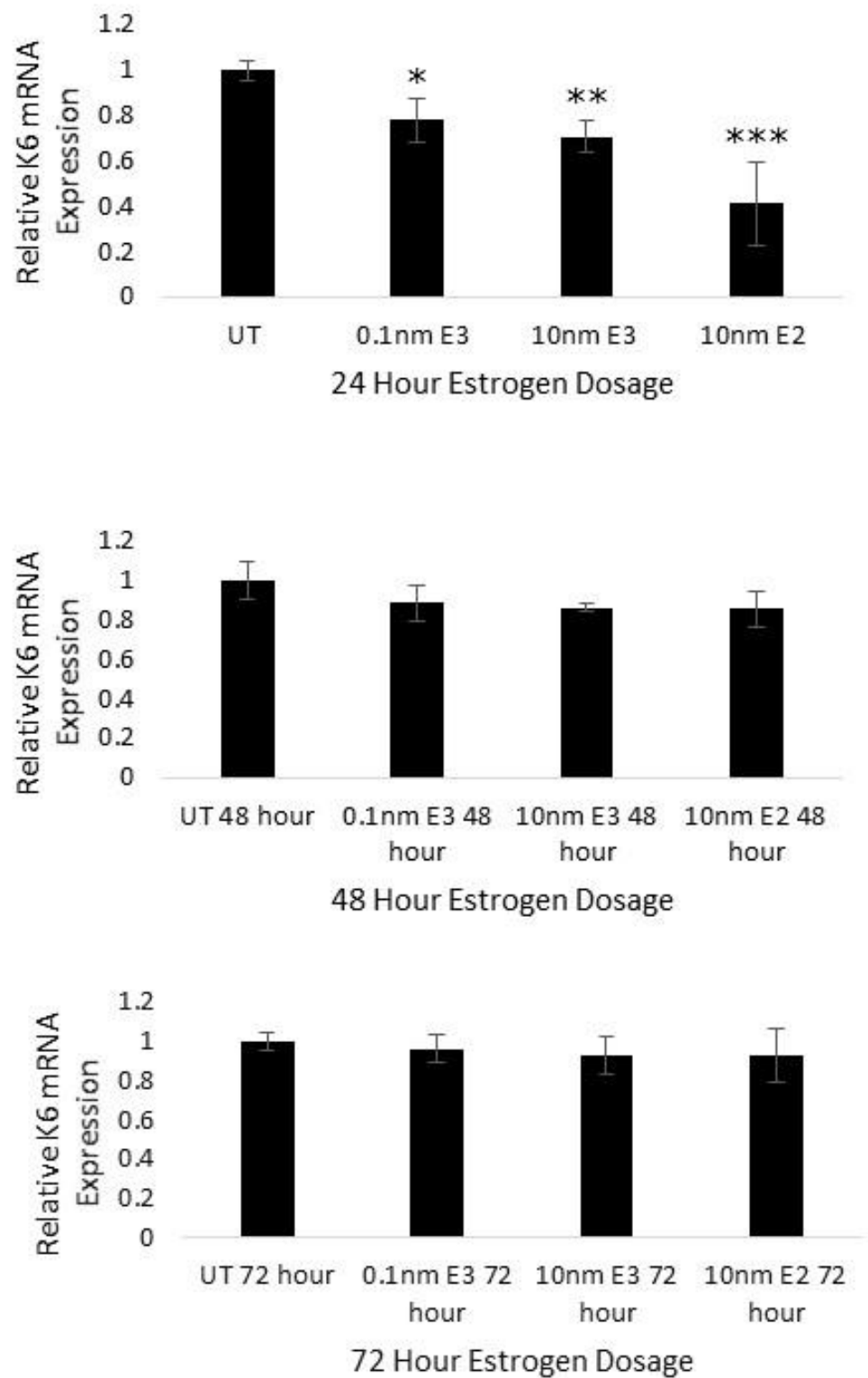


Figure 4.13 Keratin 16 mRNA expression in treated HaCaT cells.

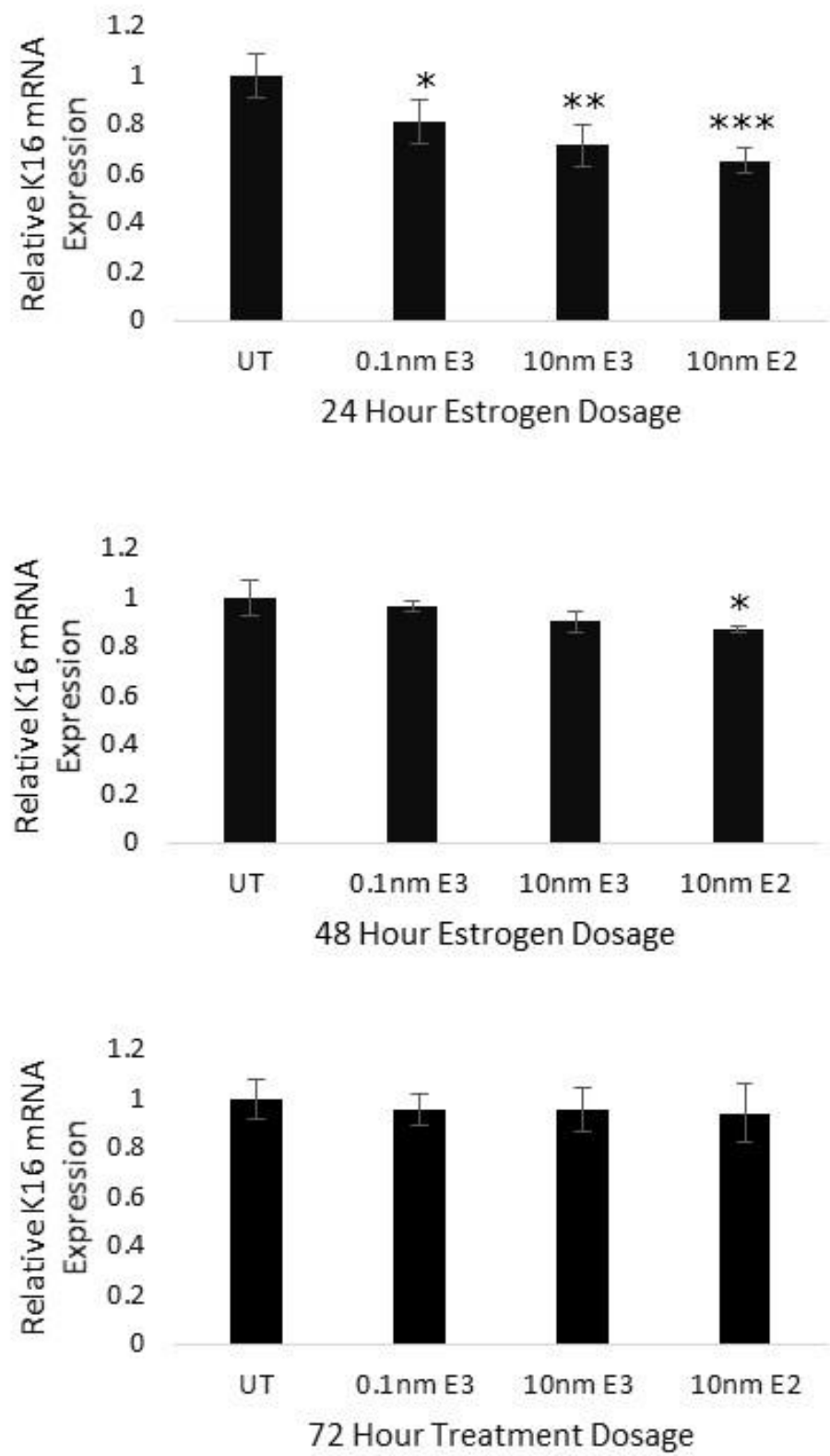
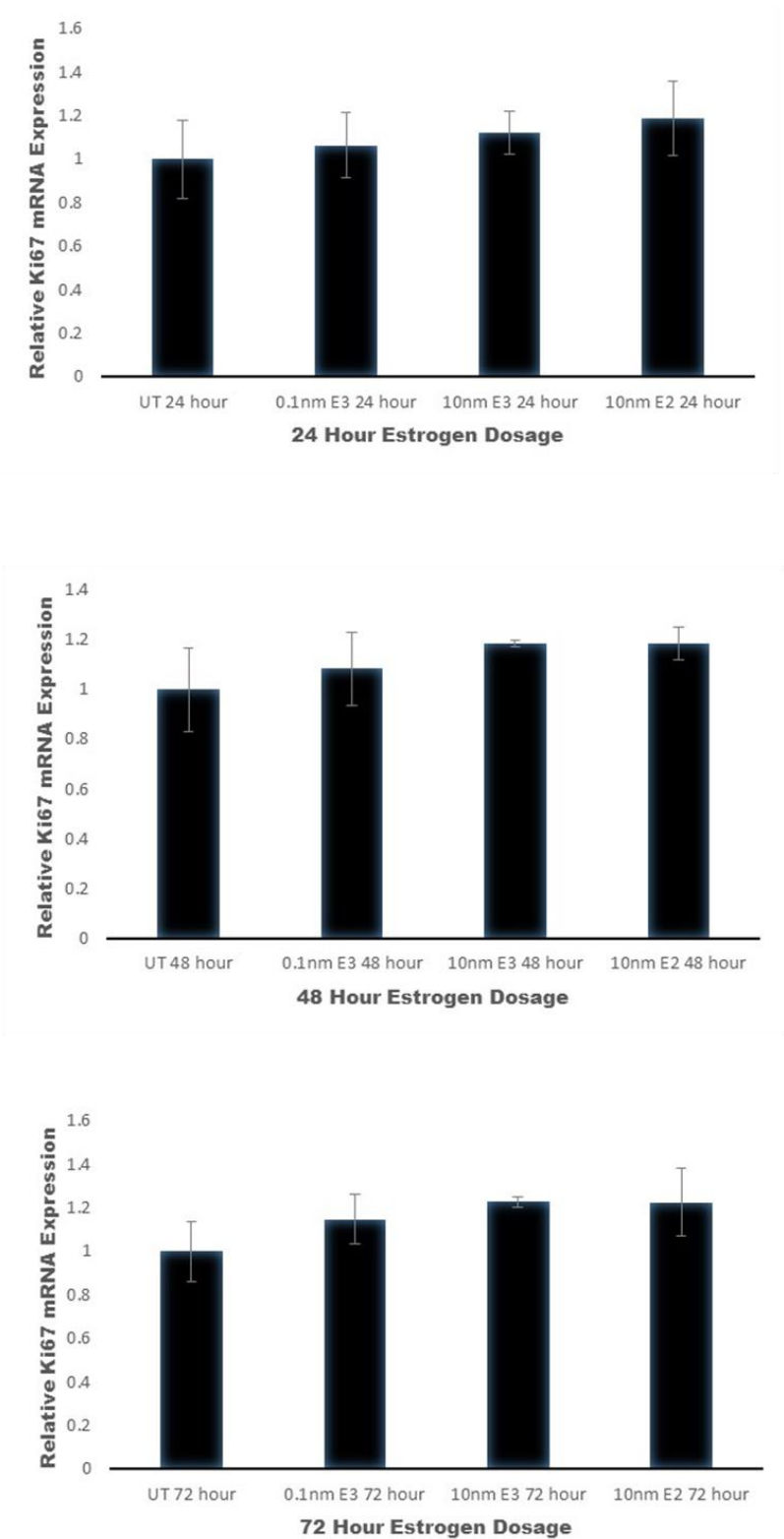


Figure 4.14 Ki67 mRNA expression in treated HaCaT cells.



immunoblot, but we did notice a trend for increased keratin production in the presence of estrogen. There was a near 2 fold increase in keratin 1 mRNA production after the 24 hour treatment with E2 ($p<0.05$) (Figure 4.15) and a 1.5 fold increase in keratin 10 treated with high doses of estrogen E3 and E2 ($p<0.05$) (Figure 4.16). RT-PCR results demonstrated a rapid up-regulation of both filaggrin and involucrin. Filaggrin exhibited a small increase in all treatment groups following 24 and 48 hours of treatment with all 3 estrogen doses (Figure 4.17). Involucrin showed a significant increase only with 10nm E2 treatment after 24 hours ($p<0.05$), but maintained a significant increase in all treatments at the longer time points (Figure 4.18).

4.4 Discussion

The aim of these studies was to assess the viability of using an *in vitro* model system to further define the effects of estriol on the production of keratin. The skin is a complex tissue comprised of multiple cell types that express ERs, making them potentially responsive to estrogen. Keratinocytes, the primary producers of the stratum corneum, are known to be estrogen responsive and greatly outnumber all other cells in the skin. We reasoned that estrogen exerts the majority of the actions we have observed thus far on this cell type. Chapter 3 of this thesis suggests that estriol stimulates differentiation of keratinocytes, leading to a thicker stratum corneum, which could protect against viral and bacterial infections. However, the tissues used in Chapter 3 contained a diverse array of cell types within the skin from both the dermis and epidermis, making it difficult to determine the precise effect on our primary target cell, the keratinocytes. Therefore, to directly assess estriol's effect on keratinocyte development, we used the spontaneously

Figure 4.15 Keratin 1 mRNA expression in treated HaCaT cells.

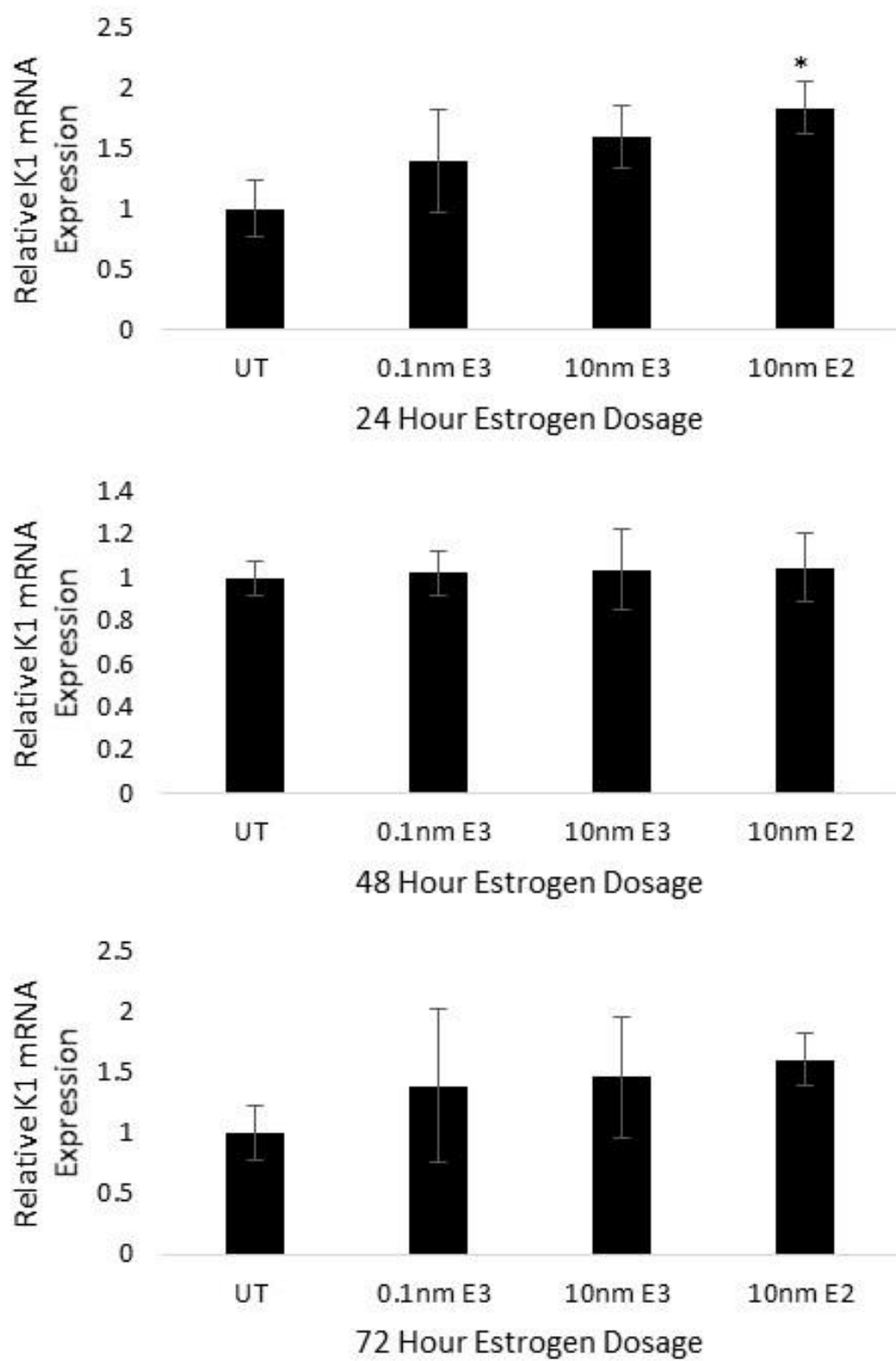


Figure 4.16 Keratin 10 mRNA expression in treated HaCaT cells.

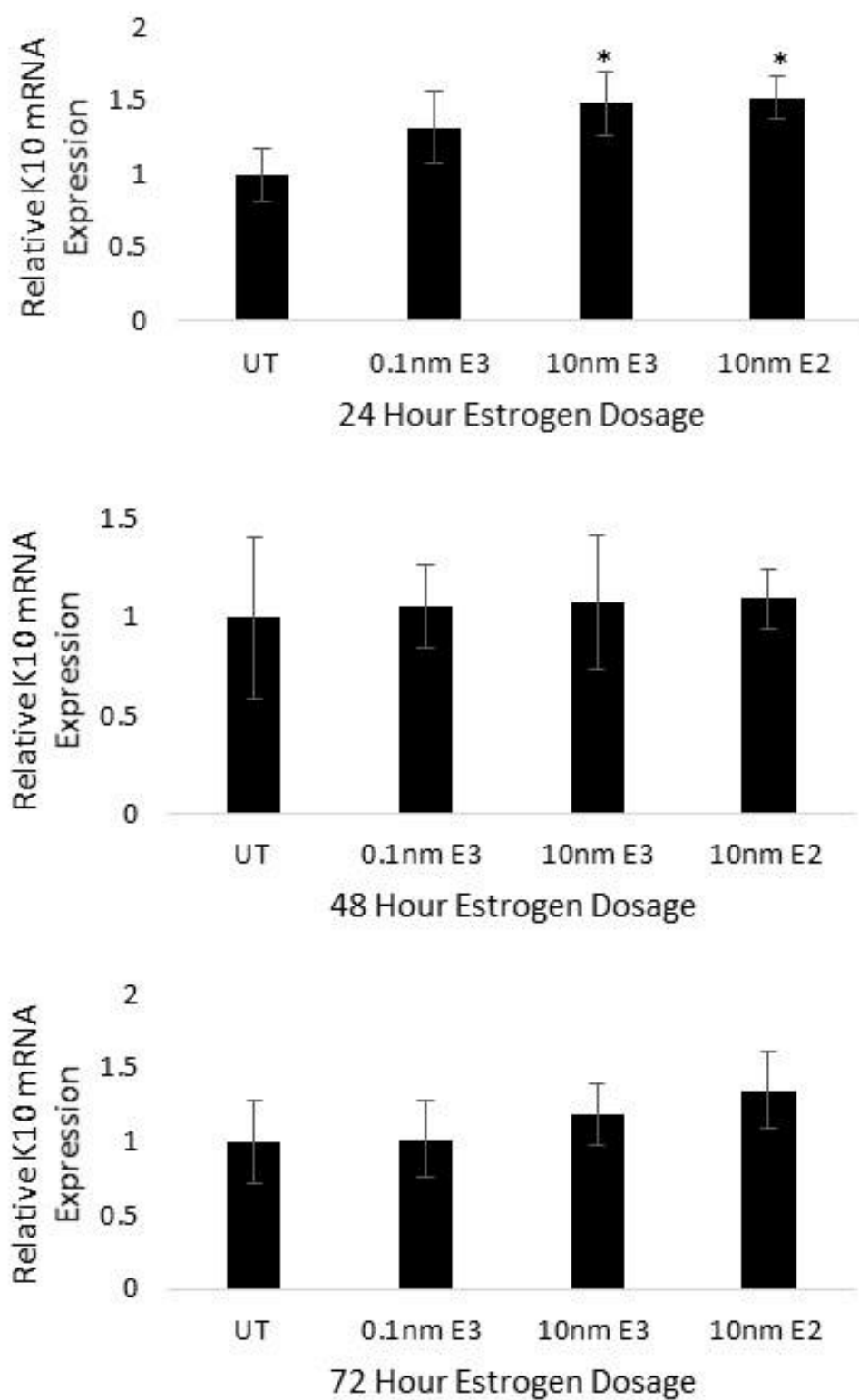


Figure 4.17 Filaggrin mRNA expression in treated HaCaT cells.

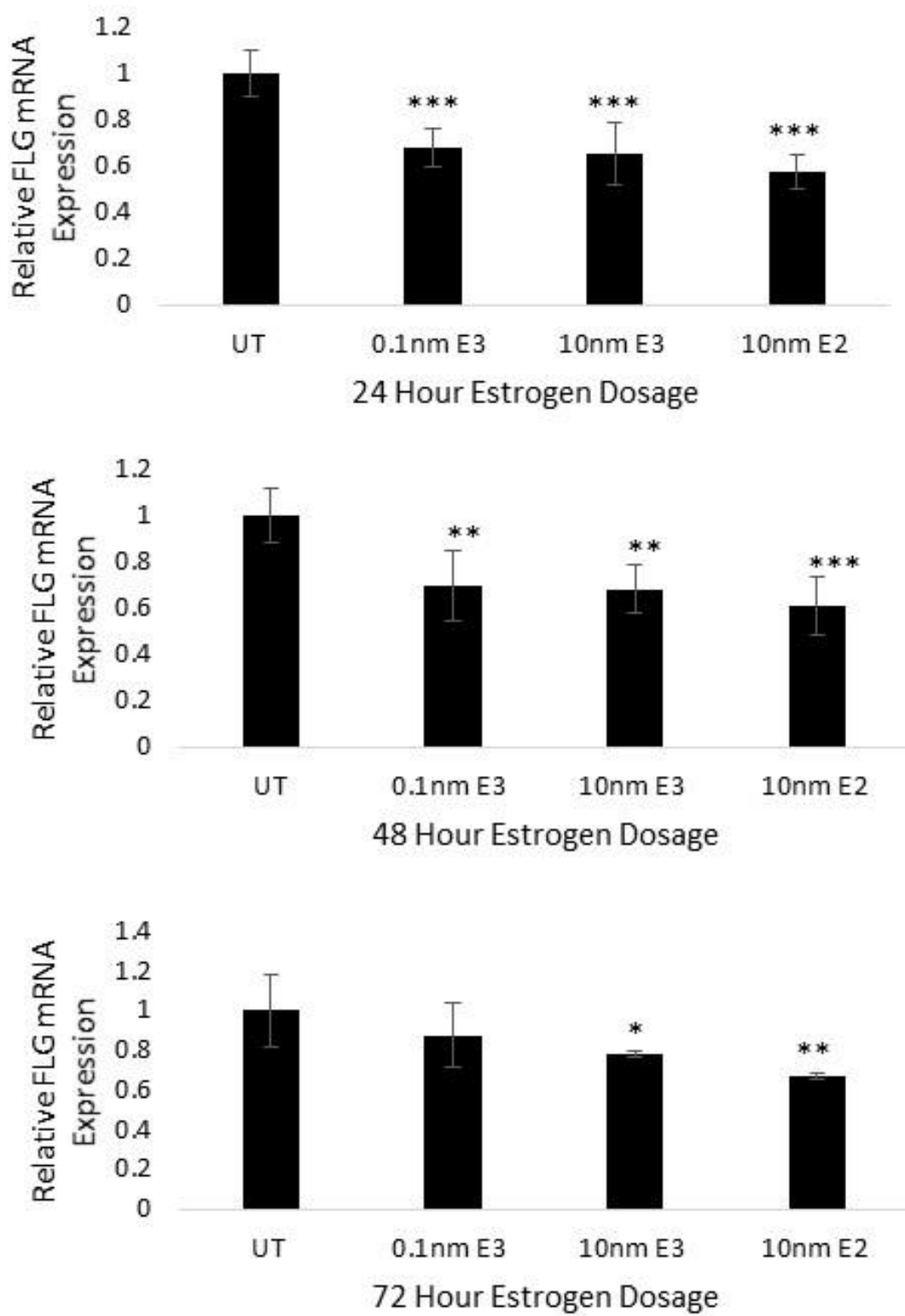
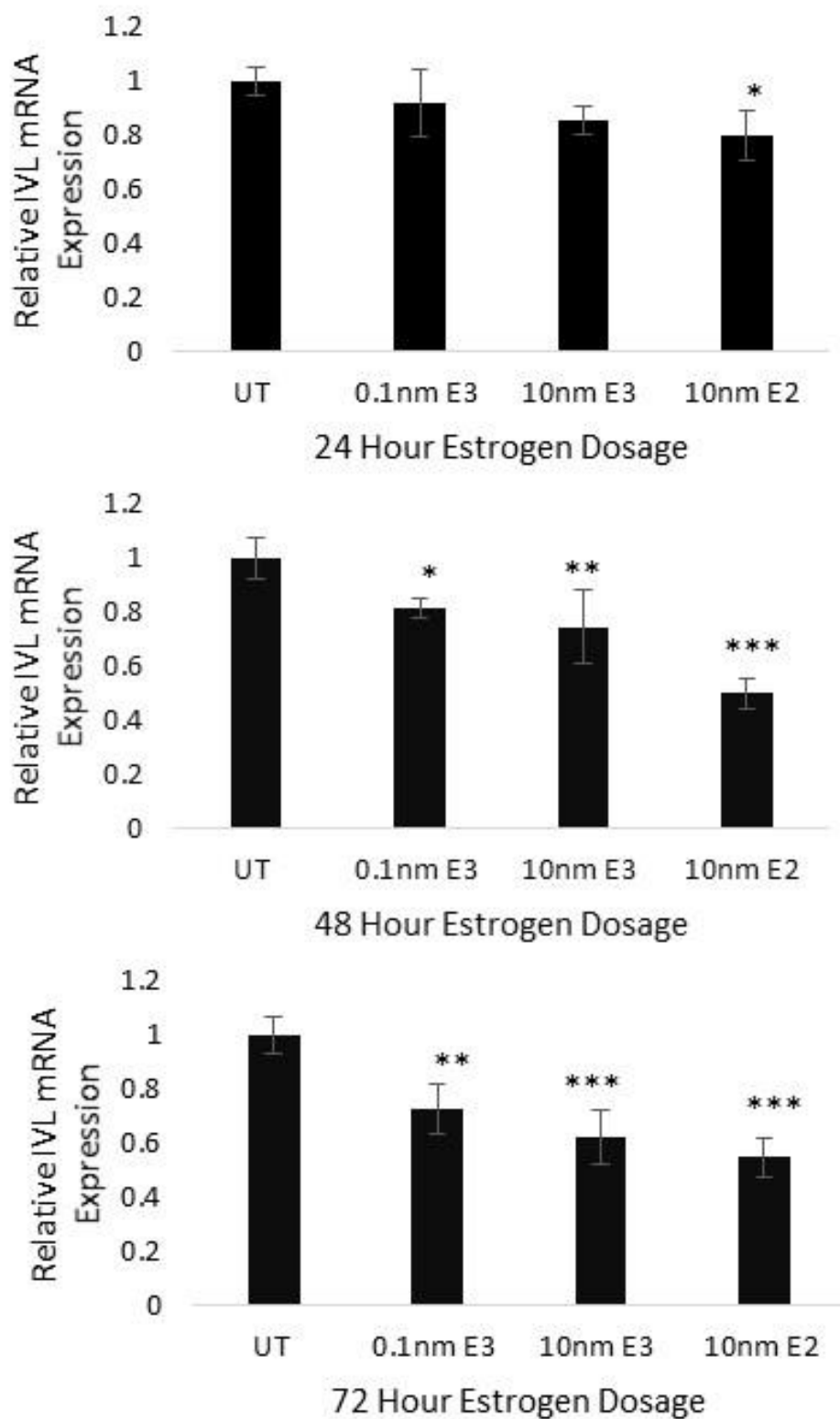


Figure 4.18 Involucrin mRNA expression in treated HaCaT cells.



immortalized and very well characterized HaCaT cell line. We examined the expression of cell proliferation and differentiation genes in the presence of the estriol. Here I describe the effect of low dose estriol (0.1 nM E3), high dose estriol (10 nM E3), and high dose 17 β -estradiol (10 nM E2) on cell growth and differentiation in this cell line and discuss how it relates to the treatment of inner foreskin *in vivo* and the overall suitability of HaCaT to assess estrogen effects on the mucosa. The results of these studies could be expanded to *in vitro* foreskin organotypic culture to test estriol's ability to reduce HIV transmission in a controlled manner. Our ultimate goal is to translate this new knowledge into a novel clinical use of estriol for uncircumcised men.

HaCaTs are similar to normal keratinocytes, however, as with most immortalized cell lines, it exhibits chromosomal abnormalities resulting in aneuploidy and duplications of certain chromosomal regions [32]. Despite being immortalized, the HaCaT cell line is regarded as a suitable and stable model for studying keratinocyte physiology *in vitro* as it retains the capacity to undergo full epidermal differentiation while remaining non-tumorigenic. In addition, the presence of proliferation marker Ki-67, proliferation specific keratins (K5 and K14), differentiation specific keratins (K1 and K10) and epidermal markers of differentiation (filaggrin and involucrin) have been confirmed in this cell line [23, 201, 202].

We verified through immunohistochemistry that HaCaT cells express the estrogen receptors alpha (ESR1) and beta (ESR2) supporting previous work from Alexaki *et al*

[203] using immunoblotting. In the absence of exogenous estrogen, ESR1 appeared to localize to the cytoplasm, while ESR2 showed punctate staining in the nucleus. Nuclear staining of ESR2 suggests activation of the receptor by an endogenous factor in the media. Indeed, it is known that phenol red, a pH indicator in culture media, is a weak estrogen receptor agonist and normal serum typically contains estrogens [204]. Therefore all cell work performed beyond this point was carried out in phenol red-free, charcoal-stripped serum media to abolish the effect of any endogenous hormones present in the samples.

We have demonstrated that both receptors respond to weak (E3) and potent (E2) estrogens. ESR1 and ESR2 mRNA was detected in the keratinocyte cultures with a higher mean level of ESR1 expression than ESR2. In addition, ESR1 expression was up-regulated as early as 24 hours after exposure to the lowest dose estrogen treatment (0.1nM E3), while ESR2 showed a significant response only in the presence of higher treatments (10nM E3 and 10nM E2) during the same time frame. This suggests that ESR1 co-expressed with ESR2 may play a dominant role in early gene regulation in HaCaT cells. These results are in alignment with, Verdier-Sevrain *et al.* who also observed increased ESR1 and ESR2 mRNA levels in human neonatal keratinocytes treated with E2 [113]. Taken together, these findings suggest that treatment of keratinocytes with estriol causes transient up-regulation in the expression of the estrogen receptors.

We were primarily interested in determining the effect of estriol downstream of ER activation, regulating genes driving proliferation and differentiation in keratinocytes. Western blot analysis revealed an acceleration in overall keratin production in the HaCaT cells in response to estrogen treatment, supporting the results of chapter 2 and 3 and previous papers on wound healing and alterations of the vaginal epithelium. Our cell proliferation data did not show a significant change in the number of keratinocytes under different estrogen exposures during the time frame examined. This supports our *in vivo* data from Chapter 2 and 3 which also showed no increase in skin proliferation rates. However, these results are in conflict to what has been previously reported in the skin. One study using epidermal keratinocytes from newborn foreskin reported an increase in proliferation following 0.1 nM E2 treatment [113, 205]. A second study using normal human epidermal keratinocytes from adult female breast skin showed increased proliferation at lower concentrations of E2 (0.01 nM and 0.1 nM) but this switched to a decrease in proliferation in the presence of higher concentrations (1 nM, 10 nM, and 100 nM) [205]. Further investigation is needed to uncover the cause of the differences observed between the studies, which could be due to a number of factors including *in vivo* versus *in vitro*, donor age (newborn versus adult), gender disparities, or anatomical site (back skin vs. breast tissue). It is also important to note that the previous studies were carried out in primary cell culture in which keratinocytes rapidly die following differentiation [193]. A decline or maintenance in proliferating keratinocytes could indicate that most of the HaCaT cells have converted into a differentiated state following estrogen treatment. Our cell cultures did not reach confluency at the time points we sampled. To confirm that HaCaT cells were differentiating, we showed by RT-PCR that the late terminal

differentiation markers, filaggrin and involucrin, are up-regulated at 24 hours and 48 hours post-treatment regardless of dosage. Up-regulation of these markers is consistent with previous reports in the literature during differentiation of this cell line [193, 194, 206]. However, the HaCaT data is in contrast to our *in vivo* sequencing data from Chapter 3 that showed filaggrin expression was reduced in the presence of estriol, while involucrin expression was unchanged. The complexity of the skin *in vivo* versus the simplicity of the culture system *in vitro* could play a large role in this difference. Estriol may exhibit different effects on cellular pathways depending upon the environmental context of the keratinocyte. The mucosa of the inner foreskin is a highly specialized epithelium that is likely maintained by multiple intercellular signaling pathways which are lost in the HaCaT monoculture. Expression of other terminal differentiation markers could provide better insight into this phenomenon and the suitability of HaCaT to model for mucosal tissues.

Taken together, our results indicate that estriol accelerates keratin production in HaCaT cells and therefore in keratinocytes. This supports the results of chapter 2 and 3 and confirms that keratinocytes are likely to be the primary target for estrogen action in the skin in terms of increasing keratin production. Furthermore, we see no evidence for increased rates of proliferation of HaCaTs exposed to estrogen, indicating that the observed increase in keratin produced on the western blot is due to a net increase in keratin per cell. This is again corroborates our data from chapters 2 and 3 and suggests that keratinocytes respond to estriol by increasing keratin production per cell and not by increasing proliferation. In alignment with our human studies reported in Chapter 3, it appears to confer this action on keratinocytes by a receptor-mediated mechanism to

modulate the expression of genes involved in keratinocyte differentiation. We observed marked differences in the expression of terminal differentiation markers in HaCaT cells exposed to estrogen that were at odds with what we observed to estrogen exposed foreskins *in vivo*. Obviously, HaCaT cell monoculture can never hope to fully recapitulate the complexity of signaling events and intercellular signaling seen in skin *in vivo* and suggests that extrapolation of findings back to *in vitro* models must be done with caution. Nevertheless, this is the first study to document the ability of estriol to accelerate keratin production and differentiation in HaCaT cells. Furthermore, acceleration of keratin production, without altering cellular proliferation rates provides an ideal way to increase keratinization without an increased risk of the cells becoming tumorigenic *in vivo* and/or *in vitro*.

HaCaT cells have previously been reported to be a great model for studying keratinocyte differentiation of normal skin, however, our studies suggests that estrogen responses in mucosal skin may be quite different. More fundamental studies are needed to fully understand the production of keratins in the foreskin mucosa and to identify the potential targets of activated estrogen receptors in these pathways. If time was permitted, I would have liked to perform ER chromatin immunoprecipitation (ChIP) alongside transcriptome sequencing of HaCaT cells in treated and untreated cohorts to examine more global changes in expression as a result of estrogen exposure, as well as the likely direct targets of activated ERs.

Chapter 5

Summary and Future Directions

Over 2 million people became infected with HIV in 2013 alone, and nearly as many died from complications of AIDS [207]. These devastating diseases plague just about every country in the world. Although the latest reports reveal that rates of new infection are declining, about 35 million people are currently living with HIV [207, 208]. Each one of these people, has the potential to transmit the disease. In 2013, men accounted for about 53% of new infections globally [208]. Reducing HIV transmission in populations where it remains a major health threat hinges on developing or improving safe and effective treatments that are reliable, cost-effective, and available to all who seek them. In spite of the success and availability of current HIV intervention strategies to prevent, manage, and/or treat infections, HIV continues to disproportionately affect men in certain sub-populations. Two of the best lines of defense against HIV in sexually active males, condoms and circumcision, are effective because they enhance the barrier between the penis and its external environment.

Estriol, a naturally occurring compound, is currently being used to treat a number of conditions, including inflammatory conditions that compromise the protective barrier of the male and female reproductive tracts [105, 209]. This weak hormone has also been shown to accelerate wound healing and induce keratinization [12, 210]. The long term research goal of this project is to test and implement topical estriol as a preventive therapy that can be used to significantly reduce the incidence of new HIV infections in uncircumcised men. Using data generated from a phase I clinical trial, our objective was to determine how topical estriol affects the epithelium of the human foreskin, thereby

defining the effect of treatment on epithelial and target cell physiology. Topical estriol offers the advantage of being a ready-made, FDA approved treatment that may be a useful medical adjunct to condoms and circumcision for reducing HIV transmission.

Through extensive histology in **Chapter 2**, we provided the first report of direct evidence demonstrating that estriol is capable of inducing keratinization of the inner foreskin resulting in increased thickness of the stratum corneum. Furthermore, this effect was exerted without affecting the quantity or position of the underlying primary immune cells. Our findings are significant because we observed this effect using our highest dose of 500 μ g estriol. This dose is half of what is currently prescribed clinically to treat menopausal symptoms and phimosis in males. It is possible that an even lower dose may also be beneficial, however, this was not the case for our small number of participants. Overall, the positive benefits of estriol treatment on the inner foreskin provides a foundation to propose a phase 2 clinical trial to further investigate this treatment regimen for reducing HIV infections in uncircumcised males.

In **Chapter 3**, expression profiling of genes and proteins involved in formation of the stratum corneum shed light on the mechanism by which exogenous estriol could mediate increased viral resistance of the mucosal epithelium. To our knowledge, this is the first transcriptomic analysis to assess the effect of estriol on the foreskin. We have shown that estriol induces terminal differentiation of epidermal keratinocytes, producing enhanced keratinization of the stratum corneum. The pathway by which estriol exerts this effect

was not limited to keratin protein production, but rather a more complex epidermal terminal differentiation system, including the presence of inflammatory and immune response markers. Our small sample size makes it difficult to ascertain the biological relevance of what we have observed. More work is needed to examine estrogen's effect on each marker. In addition to proving estriol's ability to regulate stratum corneum development, we also sought alternate means to target keratinization without the use of hormones. Our analysis suggest that an immune/inflammatory response characterized by up-regulation of cytokines may not negatively affect the integrity of the stratum corneum. However, cytokines can interfere with differentiation, therefore, its beneficial role needs to be explored further. We also observed an up-regulation in late epidermal crosslinking genes, thus identifying other compounds that can increase the production of crosslinking proteins that may also be beneficial. ChIP-seq can be used to study all estrogen targets identified.

Establishing HaCaT cells as a sufficient model for characterizing, in the absence of compounding factors, estriol's ability to enhance or inhibit cellular proliferation and differentiation processes in keratinocytes was of paramount importance. Our findings in **Chapter 4** suggests that HaCaTs are a readily available platform that can enable us to improve our understanding of the dynamics involved in the formation of the stratum corneum, however, the findings in our data set suggest that they may not be suitable as a stand-alone model for mucosal tissues. Treatment of HaCaT cells with estriol promotes keratinocyte differentiation corroborating our results in Chapter 3. However, differential expression of filaggrin and involucrin as compared to our *in vivo* results may suggest that

the presence of other estrogen responsive cells in the epidermis may alter signaling of these proteins. Furthermore, HaCaT cells exhibit aneuploidy with duplicated chromosomes which could contribute to the differential gene expression observed. Although estriol's stimulation of mucosal skin *in vivo* and normal skin *in vitro* result in contrasting differentiation patterns, the end result could be the same: increased thickness of the stratum corneum. To establish biological relevance, all results generated from the HaCaT data will need to be re-evaluated *in vivo*.

The limitations of our study strongly demonstrate the need for more insight into basic foreskin biology before our data can be fully interpreted for biological relevance. Overall, the results reported in this thesis strongly support our hypothesis that estriol can be used as a potent stimulator of keratinization in the foreskin. A phase 2 clinical trial is warranted to assess the hormone's effect on a larger sample size. Although, we observed an up-regulation in the expression of potentially negative genes, CCR5 and GAGE, there was a lack of evidence correlating their expression with increased risk of HIV infectivity or cancer susceptibility. Nevertheless, it is absolutely necessary to explore their role in our data set before proceeding with large scale treatments.

Data obtained from these studies will allow us to define how the effects of exogenous estrogen mediate increased viral resistance of the mucosal epithelium at the histological and protein level. Previous studies have reported that human foreskin grown in tissue culture is susceptible to HIV-1 [72, 211]. Furthermore, migratory cells originating from the

HIV-1 treated explants are able to disseminate the virus [211]. Both studies also suggest that the inner foreskin is highly susceptible to infection. Our studies have shown that estrogen increases the thickness of the stratum corneum on the inner foreskin, potentially limiting exposure of the Langerhans cells to viruses, and therefore may reduce HIV infectivity rates. However, no studies have been performed to evaluate the implications of protection through estrogen treatment for males. Future aims of this study could assess the effects of topical estrogen on the inner foreskin in the presence of HIV-1 using foreskin explants as an *in vivo* model system.

Comparative studies in a primate model system would yield the best results for determining biological relevance. We would like to determine the suitability of the Rhesus Macaque as a model in which to define efficacy of estriol treatment *in vivo* in the presence of simian immunodeficiency virus (SIV). While the protective effect of estrogen treatment on the epithelium has been clearly demonstrated in the vagina of the Rhesus Macaque, it is necessary to investigate if it is equally effective in the foreskin. The phallus or penis is one of the most variable structures throughout mammals. However, the rhesus monkey penis is similar in morphology to that of humans, but has a comparatively elongated foreskin covering the glans penis in the flaccid state. As in humans, this is retracted exposing a large inner foreskin surface during intercourse. Therefore, preliminary efficacy trials, to directly assess the effectiveness of increased keratin in increasing resistance to SIV infection, could be performed in the Rhesus monkey model system. We will determine if the Rhesus monkey foreskin is sufficiently similar to humans in histology and immunology to be a suitable model to test efficacy of the estriol treatment in clinical trials.

If the histology and immunology of the monkey and human penises are suitably similar, it would support the use of the Rhesus monkey to investigate the efficacy of the treatment and suitability of the intervention for human clinical trials. In the event that the rhesus macaque tissues do not compare favorably to the human foreskin samples, *in vitro* studies using human explant penile tissues can be grown in culture and treated with estriol to determine its effect.

The skin is vital for life as it is key in keeping moisture in and preventing entrance of pathogens. The penile skin of uncircumcised men has a thin stratum corneum and viral target cells are present in the epithelium. As a result, this population of men are at a higher risk of acquiring sexually transmitted diseases, such as HIV and HPV, compared to their circumcised counterparts. Estriol is capable of enhancing the stratum corneum specifically through up-regulation of differentiation, thus it is a promising medical adjunct for reducing HIV transmission in uncircumcised men. The results reported in this thesis can have a global impact by providing a safe, non-invasive method for controlling the spread of HIV in developing countries and may aid in controlling the spread of HPV in developed countries. As I stated in the introduction to this thesis, “prevention is always better than a cure.”

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