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Characterization of Changes in Hyaluronan Following Epidermal Barrier Injury in an Organotypic Model

Gati Ajani
Cleveland State University

CHARACTERIZATION OF CHANGES IN HYALURONAN FOLLOWING EPIDERMAL BARRIER INJURY IN AN ORGANOTYPIC MODEL

GATI AJANI

Bachelor of Medicine and Bachelor of Surgery (M.B.B.S)

University of Mumbai-Bombay

Dec, 2000

Master of Technology in Biomedical Engineering (M.Tech)

Indian Institute of Technology (IIT)-Bombay

Aug, 2002

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This dissertation has been approved
for the Department of Chemical and Biomedical Engineering
and the College of Graduate Studies by

_____

Dissertation Committee Chairperson, **Edward Maytin, M.D. Ph.D.**
Department of Chemical and Biomedical Engineering

_____
Date

_____

Joanne Belovich, Ph.D.
Department of Chemical and Biomedical Engineering

_____
Date

_____

Roy Silverstein, M.D.
Department of Biological and Geological Sciences

_____
Date

_____

Marc Penn, M.D. Ph.D.
Department of Chemical and Biomedical Engineering

_____
Date

_____

Amit Vasanji, Ph.D.
Department of Chemical and Biomedical Engineering

_____
Date

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CHARACTERIZATION OF CHANGES IN HYALURONAN FOLLOWING EPIDERMAL BARRIER INJURY IN AN ORGANOTYPIC MODEL

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ABSTRACT

The stratum corneum, the outermost layer of the epidermis of skin, provides a functional permeability barrier that is essential for our survival and must be rapidly repaired after injury. Of interest to us, a ubiquitous matrix molecule called hyaluronan (HA) has come to the forefront in wound healing research because it is induced after epidermal injury and may be crucial for barrier repair. This thesis examines the role of HA during recovery from the most fundamental form of skin injury, i.e. selective damage to the stratum corneum. The overall question is whether this form of stratum corneum injury can lead to changes in HA similar to that of a full thickness skin wound. To investigate this question, a bioengineered model of barrier injury in organotypic epidermal cultures was first characterized. Changes in HA, in terms of an increase in overall amount and molecular weight, and increased levels of the HA-metabolizing enzymes (HA Synthases 2 and 3) were then measured and defined. Finally, the signalling mechanisms responsible for induction of HA synthesis were explored, revealing that activation of the Epidermal Growth Factor Receptor is essential for the up-regulation of HA after barrier injury.

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Introduction

1.1.   Overview

The epidermis and its outermost layer, the stratum corneum, is the outer barrier of the skin that protects the inner layers against environmental factors. This barrier behaves like a shield that protects higher organisms from external insults such as infectious pathogens and UV radiation, and also prevents water loss and aids in thermoregulation. In short the epidermal barrier is vitally important for our survival. This thesis examines the most basic and fundamental form of injury to this barrier, i.e., injury that produces selective damage to the stratum corneum.

To address some important biological questions that exist regarding barrier injury of the skin, we have in this thesis created a bioengineered model. This model is described in detail in section 2.4 and 3.2. In this overview, we will describe the general features of the model, its advantages and disadvantages, and then introduce the specific questions

(unknown aspects) about the barrier injury responses in the skin that will be experimentally addressed in this thesis. At the end of this overview, we list the three specific hypothesis (specific aims) of the thesis.

The REK (Rat Epidermal Keratinocyte) organotypic model of barrier injury:

Monolayer cell cultures that are generally used to study keratinocyte biology and wound healing lack many of the normal physiological features (including the ability to build a cornified layer) that are present in the *in vivo* epidermis (see section 2.1.4. for details). Instead, tissue engineered models, i.e., three-dimensional (3-D) constructs that mimic the mammalian epidermis in both structure and function, are more suitable to study wound healing and epidermal biology. Although a number of 3-D models exist, such as Apligraf^{TM} from Organogenesis, Epiderm^{TM} and EpidermFT^{TM} from Mat-Tek, and a skin substitute genetically engineered to synthesize and deliver keratinocyte growth factor (Andreadis, Hamoen et al. 2001; Erdag and Morgan 2004); the main disadvantage of all 3-D systems with human keratinocytes is that co-culture with fibroblasts is required. For mouse keratinocytes, 3-D cultures are not possible at all unless performed using dead de-epidermized dermal substrates.

Instead we have developed a new 3-D rat keratinocyte model which has several distinct advantages over other systems. In this system, an immortalized line of oral keratinocytes (REK) is grown at the air-liquid interface for a period of five days on a well-formed basement membrane, and these cells fully stratify and express all the markers that are seen in the normal epidermis *in vivo* (see Fig.2-14 and 2-15 for details). Permeability barrier characteristics of REK organotypic cultures correspond to those in a human skin equivalent model and are similar to normal cadaver skin. The REK model

has three distinct advantages, (1) First it has the ability of growing autonomously, without the need for any fibroblasts in coculture (unlike for human keratinocyte 3-D cultures). Benefits of this include a faster experimental turnaround (7 days for REKs instead of 3 weeks for human keratinocytes), and the advantage of knowing that all observed effects (including changes in HA) are due to the activity of the keratinocytes alone.

(2) The second advantage is that the model contains an intact, functional basement membrane (BM). The advantage here is that one obtains a physiologically accurate epidermal compartment. The REK system has both a BM (to seal in large molecules such as hyaluronan (HA) into the epidermal compartment from below) and an intact stratum corneum (to seal in the HA from above). (3) The third advantage is that REK cells are immortalized, so that for future studies one can contemplate the cloning of REK cells that are stably transfected with HA synthases (or any other gene products that one might wish to study), and know that they will be expressed in every cell in the system.

Disadvantages of the REK 3-D model lead to certain limitations. Since the model does not have dermal components which are normally present in the intact skin (such as collagen, fibroblasts and blood vessels), the response after barrier injury in this model might not be physiologically accurate. Hence, it would be necessary to validate any responses seen in this system after barrier injury to responses seen *in vivo*. Also our model is made of a transformed cell line so that changes observed following injury might not be the true reflection of an *in vivo* response.

A third way in which the model may not accurately reflect real life is that the *in vivo* epidermal compartment is composed of many more cells within each layer as compared to the REK model. This feature along with minor differences in the extra cellular epidermal matrix as well as the basement membrane might influence the response observed after barrier injury in the REK model. A final point that needs to be mentioned here is that the model contains no hair follicles nor bulge stem cells, which are thought to be important for wound healing. Interestingly though, a recent study shows that hair follicles may not be essential for full thickness wound healing (Langton et.al., 2008), suggesting that wound and injury responses in the REK model may be very relevant *in vivo*.

Unanswered questions about barrier injury to be addressed by the REK 3-D model:

Acute disruption of the permeability barrier can be achieved using acetone, detergent or successive application of cellophane tape. Within several hours to days, the epidermal tissue responds by becoming thicker, due to increased cell proliferation and upward migration. In addition to the hypertrophic response there is a significant increase in certain extracellular matrix molecules such as hyaluronan and proinflammatory cytokines within the epidermal compartment after barrier injury. A number of receptor pathways like the peroxisome-proliferator activated receptors, G-protein-coupled receptors, and cytokine receptors are also indirectly implicated in this healing response (discussed in detail in section 2.1.). Even though a lot is known about epidermal barrier injury and it is implicated in many cutaneous disorders like atopic dermatitis and hand eczemas, the mechanisms that trigger this injury response as well as the role of keratinocytes, the principal effector cells of the epidermis, are poorly understood.

Along with the newly recognized role of the epidermal barrier, a ubiquitous polysaccharide molecule called HA has come to the forefront in wounding and injury research. The fact that significant amounts of HA exist in the epidermis was only discovered in the late 1980's (Tammi, Ripellino et al. 1988). Further investigations by that group (Pasonen-Seppanen, Karvinen et al. 2003; Rilla, Pasonen-Seppanen et al. 2004) and by us (Maytin, Chung et al. 2004; Passi, Sadeghi et al. 2004) are exploring possible functions for HA in the epidermis. Our group has observed that HA rapidly accumulates in the epidermis after full thickness (scalpel incision) as well as barrier (acetone treatment) injury. Mouse skin sampled at various times after scalpel incision and examined histologically displays a dramatic increase in levels of HA within the epidermis at the wound edge. It is believed that one or more soluble factors in the EGF family are involved in this mechanism and up-regulate HA after this form of full thickness epidermal injury (Monslow J. and Maytin E., communication). As discussed in chapter II (Background), it is known that acetone, when applied to the skin of mice, disrupts the barrier (stratum corneum) and causes an epidermal hyperplastic response to injury. This leads to an increase in HA levels within 24 h after skin wounding, with the largest apparent increases occurring in the epidermis at the wound edge (Passi, Sadeghi et al. 2004).

Interactions between keratinocytes and the extracellular matrix molecule hyaluronan (HA), and activation of the internal/external signaling systems that influence HA synthesis and keratinocyte activity, are critical components of the epidermal healing responses after injury. As one step towards understanding these interactions, this work investigates the effect of acetone-induced barrier disruption in an *in vitro* REK epidermal

model system. This work uses tissue-engineering principles to create a 3-D model of epidermal barrier injury in an *in vitro* setting which provides a simple but elegant tool for studying the role of extracellular matrix, e.g., hyaluronan (HA) in injury response phenomena. It is established for the first time that stratum corneum barrier injury causes profound changes in the quantity and properties of epidermal HA, and propose that the REK organotypic model system represents an ideal model to study the biology of HA following epidermal barrier injury.

1.2.   Specific Aims

The Specific Aims are listed as follows:

Specific Aim 1: To create and characterize an *in vitro* model of epidermal barrier injury.

Hypothesis: Barrier injury leads to epidermal hyperplasia with an increase in cellular proliferation in REK lift cultures.

To assess the effects of acetone mediated barrier injury on the REK system, and relate these to the physiological changes that are expected in the intact epidermis, the following hypotheses will be investigated: (I) Barrier injury leads to epidermal hyperplasia, and this phenomena occurs in the absence of any dermal input, (II) repeated barrier injury is required to develop hyperplasia of the epidermis, (III) and phenotypic changes post-injury can be understood in terms of changes in cellular proliferation and differentiation.

Specific Aim 2: To examine the effect of epidermal barrier injury upon hyaluronan, including its quantity, size and metabolizing enzymes.

Hypothesis: Epidermal barrier injury results in an increase in the total HA quantity and a change in its molecular weight distribution in REK lift cultures.

To assess the effect of barrier injury upon the HA molecule in terms of quantity, molecular weight and metabolizing enzymes, the following hypotheses will be investigated: (I) barrier injury increases matrix hyaluronan, (II) the molecular weight distribution of hyaluronan changes with a shift to higher molecular weight structure, (III) an increase in expression of one or more HA-synthetic enzymes contributes to the increase in HA after barrier injury.

Specific Aim 3: To investigate whether barrier injury releases a soluble factor, this by signaling through the Epidermal Growth Factor Receptor leads to an increase in HA.

Hypothesis: Barrier injury leads to the release of a soluble factor that signals via the Epidermal Growth Factor Receptor pathway leading to the increase in HA in REK lift cultures.

To begin to understand the mechanisms by which barrier injury leads to increased HA accumulation, the following hypothesis will be investigated: Barrier injury to keratinocytes in a 3-D tissue model causes the release of soluble protein factors and activates a mechanism that involves epidermal growth factor receptor (EGFR) activity, ultimately leading to enhanced HA synthesis.

Chapter II
Background

The purpose of this chapter is to provide the reader with sufficient background information to understand the biology of epidermal (stratum corneum) disruption, the biochemistry/biology of HA, and the potential involvement of growth factors in HA responses. Also described is the creation of a tissue-engineered model to be used for these studies.

2.1. The skin and epidermal responses to barrier injury

2.1.1. Epidermis and its supportive matrix (dermis)

The skin is the largest organ of the body, accounting for about 15% of the total body weight in adult humans. It is a complex organ consisting of an outer epithelial layer, the epidermis and an inner supportive matrix called the dermis (Fig. 2-1). The epidermis is a keratinized stratified squamous epithelium that renews itself continuously over 3-4 weeks. It is made up of different cell types, predominantly around 90-95% are the
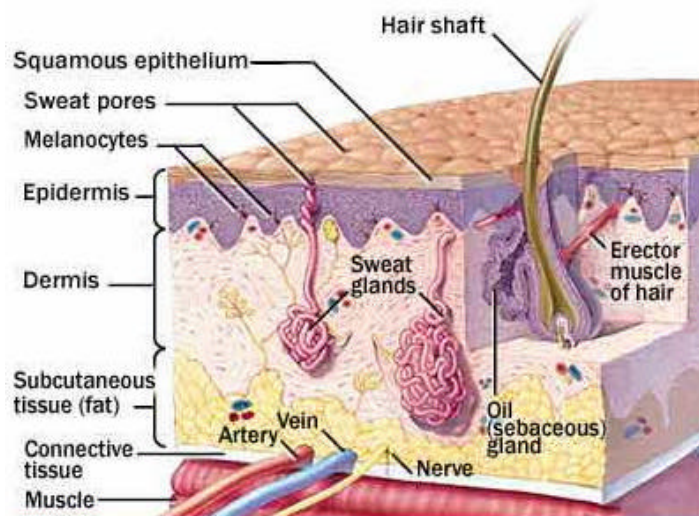
Figure 2-1: Histology of the skin
The cross-section of the skin shows the epidermis (purple), dermis (pink), fat layer (yellow) and the connective tissue fascia. Depicted in this figure are the sweat and sebaceous glands with their ducts opening on the surface, along with the hair bulge and the shaft. Adapted from www.revolutionhealth.com, content provided by Mayo clinic.

keratinocytes while the remaining 5-10 % are mainly Langerhans cells, melanocytes and Merkel cells.

Keratinocytes are epithelial cells derived from the ectoderm, which undergo a specific differentiation process, resulting in the production of flattened, anucleate corneocytes. These well-arranged continuous layers of keratinocytes constitute the epidermis where each layer of cells exhibits a specific program of differentiation. The layers are arranged from bottom to top as follows: the basal layer, the stratum spinosum, stratum granulosum and the cornified layer (Fig. 2-2). The stratum corneum is further subdivided into a deep, compact layer stratum compactum and a superficial, loose stratum disjunctum. In some body areas such as on the palms and soles an additional layer, the stratum lucidum, can be seen between the granular and the cornified layers (although this is now thought to be a fixation artifact of electron microscopy).
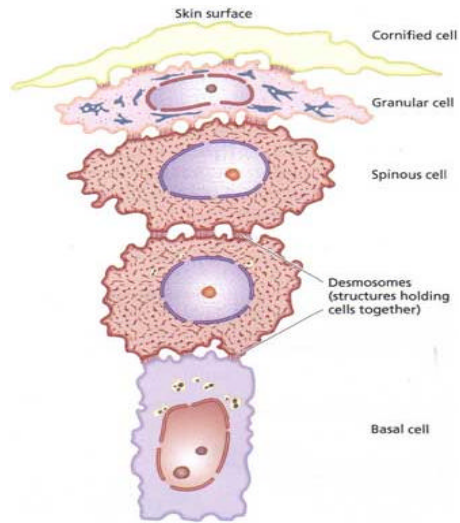
Figure 2-2: The epidermis
This subcomponent of the skin comprises of different cell types stacked together on top of one another to form specific layers. Adapted from www.pg.com/science/skincare/Skin_tws_13.htm

The basal cells represent one of the stem cell compartments of the epidermis. Small numbers of stem cells within the basal layer give rise to transiently amplifying cells (TACs), keratinocytes that constitute the majority of the basal layer. Similar to other somatic stem cells, epidermal stem cells are assumed to have the ability to undergo asymmetric cell division, a process in which one daughter cell is regenerated, while the other daughter cell is termed a transit-amplifying cell (TAC) (Fig.2-3). The TACs are derived from stem cells, and after three to six cycles of division are committed to terminal differentiation. They express a specific marker, CD98. TACs detach themselves from the basement membrane and start migrating towards the skin surface.

Another location of the epidermal cells, different from the basal layer but continuous with it, is the epithelial portion of hair follicles known as the bulge. The bulge region contains morphologically undifferentiated cells and marks the lowermost permanent portion of the hair follicle during cycling. Stem cells of the bulge are known to be multi-potent, migrating upwards to generate the sebaceous glands and epidermis,
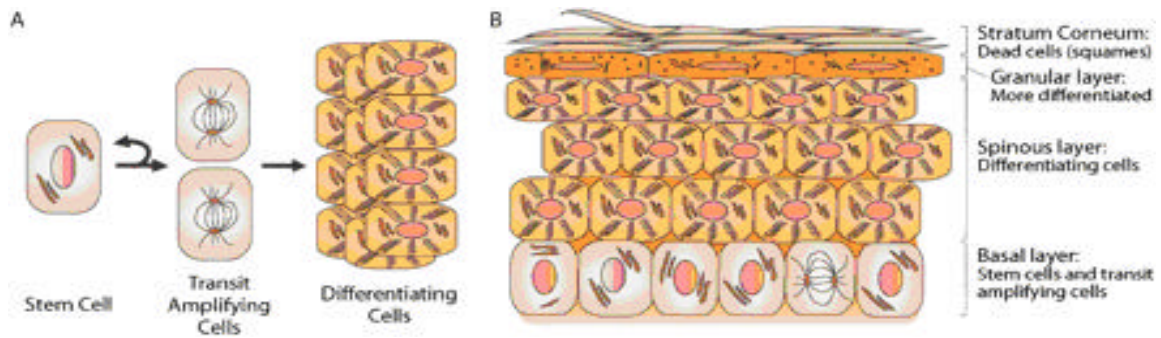
Figure 2-3: Germinative potential of keratinocytes in specific epidermal layer
The basal layer represents the location of the stem cell compartment and consists of the TACs, which start their journey of differentiation as they climb successive layers and terminally form the corneocytes. Cited from (Alonso and Fuchs 2003).

and downward to produce the hair follicle. They can also be induced to differentiate *in vitro* into sebaceous glands (Wang, Chen et al. 2006).

The proliferating compartment of epithelial tissues is characterized by the specific expression of cell cycle regulators and integrin family members responsible for the attachment of the epithelium to the basement membrane. In the skin, a2ß1 integrin, a collagen and laminin receptor, is expressed in high amounts in the basal cell layer of the epidermis and is required for re-epithelialization of human skin wounds (Parks 2007). Another important molecule is extracellular ATP, which functions as an important messenger via purinergic receptors and can regulate proliferation and differentiation in keratinocytes (Inoue, Hosoi et al. 2007).

ATP2C1 is a calcium/manganese-ATPase localized in the Golgi apparatus. It plays an essential role in the undifferentiated state of basal keratinocytes and its reduction evokes differentiation, with an upward migration of basal cells to suprabasal layers most likely triggered via manganese starvation in the Golgi apparatus of keratinocytes (Yoshida, Yamasaki et al. 2006). AP-2 is a transcription factor whose binding sites have

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been found in a myriad of genes differentially expressed in both basal and suprabasal compartments of mammalian epidermis (Mack, J et al. 2005 review, Maytin, EV et al. 1999). AP-2a is in the EGFR signal transduction cascade and plays a role in the switch between epidermal cell proliferation and differentiation (Wang, Bolotin et al. 2006).

As keratinocytes undergo differentiation, there is a change in the expression of certain proteins within and on the surface of the cell. Terminal differentiation is accompanied by synthesis of an array of keratinization-specific lipids in the granular layer. These are synthesized and accumulate in the trans-Golgi apparatus from which they bud off as cytoplasmic lamellar bodies. Upper layer keratinocytes express epithelial-specific antigens such as profilaggrin, the main component of keratohyalin granules, along with other soluble cytoplasmic protein precursors of the cornified envelope and desmosomal proteins (e.g., desmoplakins, desmogleins, desmocollins), while the corneocytes express corneodesmosin, a protein stored in lamellar bodies. Desmosomes play a key role in the adhesion of corneocytes, and to each other; the digestion of desmosomes by two different types of serine proteases furthers desquamation. This differentiation program and its perturbation via injury is further discussed in subsequent paragraphs.

2.1.2. Epidermal-Dermal junction

At the junction between epidermis and dermis is a complex basement membrane (BM) synthesized by basal keratinocytes and dermal fibroblasts. It plays a primary role as a mechanical support for the adhesion of the epidermis to the dermis and regulates the exchanges of metabolic products between these two compartments. It supports

keratinocyte migration during wound healing, and is traversed by different cell types such as Langerhans cells and lymphocytes during inflammatory processes.

The antigenic composition of the BM is complex; presently over twenty macromolecules have been well characterized at the biochemical and genomic level. The BM is hardly visible by light microscopy due to the presence of neutral mucopolysaccharides, but can be stained by a PAS stain. The BM is made of four distinct layers. These are i) the outer most layer, composed of the cell membrane of basal cells and their hemidesmosomes, to which are attached cytoskeletal filaments, e.g., keratin and plectin; ii) the lamina lucida, an electron-lucent space crossed by anchoring filaments, and 2-4 nm thick; iii) the lamina densa, ~50-70 nm thick; and iv) the sub-basal lamina filamentous zone, mainly composed of anchoring fibres.

2.1.3. Dermis

The dermis is an elastic connective tissue with fibroblasts as the principal cell. It supports the epidermis and its appendages as well as the vascular and nerve plexuses that run through it. The dermis also contains three distinct structural units that are epithelial in nature: the eccrine sweat glands, sebaceous glands and hair follicles. These complex structures have specific biological properties and functions, but are in some ways analogous to keratinocytes of the epidermis in terms of their renewal, differentiation, and response to external stimuli.

2.1.4. Epidermal barrier: Its structure and function

One of the most critical components of the skin is the epidermis. The epidermis is important because it produces the stratum corneum which supplies the functional permeability barrier (also called the epidermal barrier) of the skin. The stratum corneum,

(outermost layer of dead, crosslinked epidermal cells), is primarily responsible for providing this barrier function and is absolutely essential to protect the body from UV radiation, noxious chemicals, water loss and thermal dysregulation. Most of the biodefensive functions of the epidermis also localize to this barrier, which limits pathogen colonization through its low water content, acidic pH, resident micro flora, and surface-deposited antimicrobial lipids.

The stratum corneum arises via programmed cellular differentiation, and is composed of terminally differentiated keratinocytes interspersed with intercellular lipids (mainly ceramides and sphingolipids) (Lampe, Williams et al. 1983). The stratum corneum was once regarded as degenerate and inconsequential, but is now respected as a structurally heterogeneous and metabolically active tissue. The structural basis of the permeability barrier in mammalian epidermis was examined by tracer and freeze-fracture techniques as early as the 1970's (Elias and Friend 1975). We now know that the stratum corneum is composed of corneocytes which are devoid of all organelles and are encapsulated within a highly specialized, tough, ~10 nm thick peripheral protein structure called the cornified envelope (CE). The interior surface of this cornified envelope is linked to bundles of keratin intermediate filaments that fill the interior of keratinocytes. On the external surface of the CE is a covalently bound layer of long-chain omega-hydroxyl-ceramides; this layer is known as the lipid envelope. The cornified cells, rich in proteins, lie embedded within an extracellular matrix rich in highly nonpolar lipids (Williams and Elias 1987); together they form the complete CE complex (protein plus lipid envelope plus intercellular lipids) that provides the essential aqueous barrier function for the skin and the organism as a whole.

2.1.5. Lipids of the stratum corneum

Using thin-layer chromatography and glass capillary gas-liquid chromatography, the lipids in the germinative, differentiating, and fully cornified layers in human epidermis have been studied. The sphingolipids account for about 20% of the lipid in the stratum corneum, and are the major repository for long-chain fatty acids that predominate in the outer epidermis. Cholesterol sulfate, previously considered only a trace metabolite in epidermis, is also found in significant quantities, with peak levels immediately beneath the stratum corneum in the stratum granulosum (Lampe, Williams et al. 1983). In effect, a decrease in phospholipids and triglycerides is accompanied by enrichment in ceramides, cholesterol, and free fatty acids in the stratum corneum (Elias and Feingold 1992). The latter, nonpolar lipids are sequestered in the intercellular spaces of the stratum corneum, transforming a relatively polar lipid mixture into a more hydrophobic environment. Results of X-ray diffraction analysis done on the membrane couplets isolated from newborn mouse stratum corneum, support the findings that stratum corneum lipids are intercellular in location rather than associated with the intracellular filamentous protein (Elias, Bonar et al. 1983).

The increased lipid synthesis by the uppermost epidermal layer, the stratum granulosum, provides the key lipids- cholesterol, phospholipids, and glucosyl ceramides required for the formation of lamellar bodies, one of the distinctive features of the granulocyte. The term "secretory granulocyte" was coined to encompass the specialized features of these cells (Elias, Cullander et al. 1998). When studied under confocal microscopy, granulocytes display a widely disbursed trans-Golgi-like network, associated with arrays of contiguous lamellar bodies. The lamellar bodies are delivered selectively

to the intercellular spaces at the stratum granulosum-stratum corneum interface (Elias, Cullander et al. 1998). These lamellar bodies are secreted in an accelerated fashion, generating the precursors of the stratum corneum lamellar membranes that are essential for permeability barrier function.

The lamellar bodies were first isolated by utilizing staphylococcal epidermolytic toxin to produce intact sheets of outer epidermis, followed by controlled homogenization
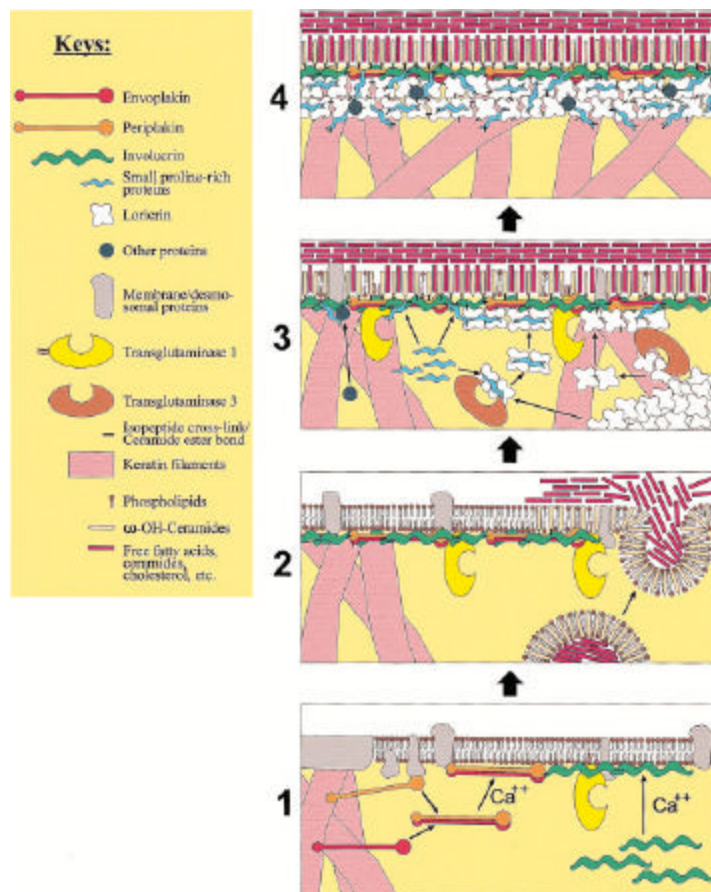
Figure 2-4:   Model for the cornified envelope assembly.
One of the first documented changes upon commitment to the terminal differentiation is expression of envoplakin (red) and periplakin (orange), which associate with keratin intermediate filaments (large pink rods) and desmosomal junctions (gray),respectively (panel 1). Coincident with initiation of scaffold assembly, in the case of the epidermis (panel 2), extrusion of LB takes place. CE reinforcement begins (panel 3). The mature epidermal CE (panel 4) has a mass density of 7 kDa/nm^2 and is uniformly 15 nm thick, of which 10 nm is the protein envelope and 5 nm is the ceramide lipid envelope. Cited from (Kalinin, Kajava et al. 2002).

in a cell disrupter, and passage of the homogenates through a graded series of nuclepore filters. This isolation protocol was published in 'Science' and allowed investigators to characterize the lipid profile of these organelles. This work demonstrated that lamellar bodies deliver precursors of barrier lipids to intercellular domains (Grayson, Johnson-Winegar et al. 1985). Also, lamellar bodies possess phospholipase activity, which may be crucial for inducing both the initial fusion and elongation of lamellar body disks and the subsequent formation of the hydrophobic membrane bilayers found in the mid-to-outer stratum corneum (Elias, Menon et al. 1988). It was observed that Ca++ is associated with the lamellar body disc membranes and its contents, suggesting that this divalent cation may contribute to both lamellar body secretion and to the formation of inter-corneocyte membrane bilayers (Fig.2-4) (Menon, Grayson et al. 1985).

It is important to note that a distinct calcium gradient is present in the epidermis, beginning with a low calcium content in the basal and spinous layers, followed by a progressive increase in calcium content to a maximal density in the outer stratum granulosum (Denda, Hosoi et al. 2000). In normal skin, both calcium and magnesium are localized with high concentration in the upper epidermis. The striking intercellular Ca^{++} accumulation in the mid granular layer, coupled with Ca^{++} influx in the upper granular layer, supports the view that changes in intracellular Ca^{++} may regulate epidermal differentiation (Menon, Grayson et al. 1985). Now there is direct evidence that acute and sustained fluctuations in epidermal calcium regulate expression of differentiation-specific proteins *in vivo* and coordinately regulate events late in epidermal differentiation, thereby forming the barrier (Elias, Ahn et al. 2002).

In the extracellular compartment, lipids secreted from lamellar bodies, such as glucosyl-ceramides, phospholipids, and cholesterol sulfate, are metabolized to ceramides by ß-glucocerebrosidase. These are then converted to free fatty acids by one or more secretory phospholipase A2 enzymes and to cholesterol by steroid sulfatase. This critical step, termed "lipid processing," is required for the formation of mature functional lamellar membranes and for normal permeability function.

2.1.6. Proteins of the stratum corneum

In epidermis from most body sites, 80% of CE protein mass is comprised of the protein, loricrin. Another protein component that becomes cross-linked to the CE is filaggrin, usually in the form of keratin intermediate filament-filaggrin complexes. There is good evidence that Ichthyosis vulgaris, a condition with poor barrier repair, is due to a profound deficiency in filaggrin (Madison 2003). A recent report in 'Nature Genetics' talks about the two common filaggrin null mutations that cause ichthyosis vulgaris and predispose to eczema and secondary allergic diseases (Sandilands, Terron-Kwiatkowski et al. 2007).

Together these protein systems (loricrin, filaggrin, and keratins) form the bulk of the terminally differentiated keratinocytes, which are then extensively cross linked by enzymes known as Transglutaminases (TGases). TGases 1,2,3,5,6, and 7 are expressed in the epithelia, but to date only 1, 3 and 5 are proven participants in the assembly of the cornified envelope. The characteristic insolubility of the cornified envelope is due to the formation of gamma-glutamyl lysine isopeptide bonds (cross-links) catalyzed by the calcium dependent TGases (Kalinin, Kajava et al. 2002). It is not clear if TGases perform most of the reactions *in vivo*, or whether other schemes, such as simple condensation in

an anhydrous lipid environment, also occur. One possibility is that TGase enzyme might be crucial for initiating the process, which is completed by other yet unknown mechanisms.

The mouse knockout model of the TGase 1 gene results in neonatal death due to loss of water barrier function. Similarly mutations that destroy TGase 1 activity cause the life threatening human disease lamellar ichthyosis. Based on the importance of TGase 1 in epidermal morphogenesis, its role has been assessed in wound healing. It is reported that in neonatal mouse skin, TGase 1 mRNA as well as keratin 6α is induced in the epidermis at the wound edges as early as two hours after injury and that expression continues in the migrating epidermis until completion of re-epithelialization. It is suggested that activation of the TGase 1 gene is essential to facilitate repair of skin injury (Inada, Matsuki et al. 2000).

2.1.7. Stratum corneum and innate immunity

In addition to the physical barrier provided by the stratum corneum, the skin also contains a biological barrier consisting of antimicrobial peptides that control microbial growth on the surface (Namjoshi, Caccetta et al. 2007). In normal skin, human beta defensin (HBD)-1 is localized to the perinuclear region of keratinocytes. HBD-2 is seen primarily in the basal layer of the epidermis, while HBD-3 is found in dendritic cells of the stratum spinosum (Poindexter, Bhat et al. 2006). Another study shows that a neuroendocrine peptide, catestatin, has antimicrobial function against a wide assortment of skin pathogens. It is up-regulated upon injury, thus indicating a direct link between the neuroendocrine and cutaneous immune systems (Radek, Lopez-Garcia et al. 2008).

Among inflammatory skin diseases the ones most studied and with altered ceramides and lipids are atopic dermatitis and psoriasis (Ghadially, Reed et al. 1996). For example, non-eczematous atopic dry skin shows an enhanced transepidermal water loss denoting an impaired water permeability barrier function (Fartasch and Diepgen 1992). Also, there are several genetic skin conditions with known defects in lipid metabolism that have scaly or ichthyotic skin as part of the clinical picture. A few examples are Gaucher's disease (Type 2), Niemann-Pick disease, Refsum's disease, Sjogren-larrson syndrome, CHILD syndrome and Netherton syndrome.

2.1.8. Cutaneous permeability barrier homeostasis

The stratum corneum is such an efficient barrier that only 2-5 g per h per cm^2 of transepidermal water loss (TEWL) occurs in normal skin. Essential for the maintenance of this tissue is its ability to continually self-renew and to regenerate after injury. This ability is dependent upon the principal epidermal cell type, the keratinocyte, which must proliferate and respond to differentiation cues. Through its ability to signal and communicate with the underlying keratinocytes, the stratum corneum is not simply an inert end product of keratinocyte differentiation, but a sophisticated biosensor that responds to external stimuli and environmental perturbations.

2.1.9. Physical injury: Acute disruption of the stratum corneum

Barrier function of the stratum corneum (SC) can be perturbed in a number of ways, through direct physical injury and indirect physiological responses. In daily life, chemicals such as detergents and organic solvents potentially perturb the SC. Acute disruption of the SC can be achieved by treating the skin with chemical or mechanical

irritants. Friction, sheer, blistering, dry environments and other mechanisms leading to mechanical damage also disrupt the permeability barrier.

A dry environment or exposure to low humidity (10%) induces profound changes in epidermal proliferation and function (Denda, Sato et al. 1998). Epidermal DNA synthesis is known to increase within the first 12 h such that it reaches twice the original level, and this increase is maintained for up to 5 days (Sato, Denda et al. 1998). Also, there is an increase in the number of lamellar bodies and their exocytosis from stratum granulosum cells. Furthermore, the dry weight of the SC and the thickness of the epidermis also increase in a dry environment (Denda 2000). Conversely, immersion of the stratum corneum in water for prolonged periods of time leads to its disintegration, observable as maceration.

Injuries that initially occur in living cell layers of the epidermis can later manifest as a disrupted, poorly formed stratum corneum, observed when the damaged and or hyperproliferative layers come to the surface. Examples include radiation injury and cold damage (frostbite). A prospective study (Schmuth, Sztankay et al. 2001) looked at the epidermal permeability barrier function of the skin via exposure to ionizing radiation. The subjects were women who received local radiation therapy (5000-6000 rad [50-60 Gy]) following breast-conserving surgery. The onset of increased TEWL preceded the onset of radiation dermatitis, and the maximal TEWL measurements preceded the peak of skin changes. Even when the appearance of the skin later normalized, transepidermal water loss remained abnormal. Persistence of abnormalities in the intercellular lipid complexes and corneocyte maturation were attributed to the sustained effects of the injury.

Physical breakage of the SC is another form of barrier disruption. Particle bombardment with gold beads can cause microwounds that span the stratum corneum of the skin. Damage to the SC persists even after 24h in organ culture. The implications of particle-mediated delivery to permeability barrier functions of the SC are discussed in this work (Menon, Brandsma et al. 2007). Tape stripping leads to physical removal of the corneocytes and is used quite frequently to injure the barrier in an experimental setting.

Environmental humidity influences the sensitivity of skin to topical application of sodium dodecyl sulfate (SDS) although the increased sensitivity is not always associated with altered water permeability of the stratum corneum (Denda 2001). Benzalkonium chloride (BKC), a widely used antiseptic and preservative, is a rare sensitizer; It is reported that BKC causes a high frequency of pustular or bullous reactions with consequent scarring (Wahlberg and Maibach 1981).

Any lipid-extracting solvent, such as acetone or detergents that remove the lipids and proteins from the stratum corneum, also leads to barrier disruption. For example, sodium dodecyl sulfate (SDS) and other detergents are often associated with barrier perturbation, e.g., as a cause of irritant dermatitits and thickening of the skin. Acetone is another agent frequently used to disruption of the permeability barrier, resulting in a homeostatic response in the viable epidermis that ultimately repairs the barrier. One feature of the response to acetone mediated barrier injury in mice is marked epidermal hyperplasia (Maytin, Chung et al. 2004). In animals treated topically with acetone, DNA synthesis is increased, the extent of the burst in DNA synthesis is proportional to the degree of barrier abrogation, and an increase in epidermal DNA synthesis occurs without altering bulk protein synthetic rates (Proksch, Feingold et al. 1991). Subacute epidermal

injury induced by acetone also results in a significant increase in protein prenylation (Denda, Brown et al. 1997). Interestingly, a single application of 5% tranexamic acid, a well known anti-plasmin reagent, accelerates barrier recovery in both hairless mice and human skin after acetone induced barrier injury (Denda, Kitamura et al. 1997).

2.1.10. Barrier injury: chronic alterations of the stratum corneum

Barrier function can be impaired by stress-induced changes in hormonal profiles, for example by adrenal glucocorticoids that reduce the production of lipids and intercellular lamellae in the stratum corneum. Psychological stress induced by a novel environment, can delay barrier recovery rates (Denda, Tsuchiya et al. 2000). Aging (Denda, Koyama et al. 1993) and circadian rhythmicity (Denda and Tsuchiya 2000) can also influence cutaneous barrier function, supporting the popular notion that stress and some other systemic physiological conditions can trigger or aggravate skin disease. On the other hand, pre-treatment with a phenothiazine sedative, chlorpromazine, prior to exposure to the novel environment, restores the kinetics of barrier recovery towards normal, as does the application of the sedative drugs diazepam and chlorpromazine (Denda, Tsuchiya et al. 1998). Rather strangely, odorant inhalation affects cutaneous barrier homeostasis in mice and humans; odorants with a sedative effect prevented the delay of skin barrier recovery induced by stress after acute barrier disruption (Denda, Tsuchiya et al. 2000).

Previous studies have demonstrated that sex hormones modulate homeostasis of the epidermal permeability barrier. Skin sensitivity and barrier function are altered at menopause or during the menstrual cycle. One study examined the effects of topical application of sex hormones on permeability barrier recovery after tape stripping in the

hairless mouse. It was found that application of testosterone or androsterone delayed barrier recovery, and the delay was overcome by co-application of beta-estradiol. Progesterone also delayed barrier recovery, but in this case the delay was enhanced by beta-estradiol (Tsutsumi and Denda 2007).

2.1.11. Role of receptors in recovery from barrier injury

Recent studies have demonstrated a role for Peroxisome proliferator-activated receptors (PPAR, best known as transcriptional regulators of lipid and glucose metabolism) in skin homeostasis. Activation of PPAR improves barrier homeostasis via mechanisms that involve increased activity of enzymes required for epidermal lipid synthesis and lamellar body formation and secretion (Schmuth, Jiang et al. 2008) .

Several ionotropic receptors play an important role in homeostasis of the skin permeability barrier. For example, topical application of NMDA receptor agonists after barrier disruption delayed the barrier repair whereas NMDA receptor antagonists accelerated it (Fuziwara, Inoue et al. 2003). GABA (A) receptor and purinergic receptor also play roles in skin permeability barrier homeostasis (Denda, Inoue et al. 2002; Denda, Inoue et al. 2002). Also, an increase in the intracellular levels of cAMP in the epidermis was shown to delay barrier repair, whereas a decrease in cAMP accelerated barrier recovery (Denda, Fuziwara et al. 2004). Thus, neurotransmitters that activate Gs or Go/Gi proteins might affect skin barrier homeostasis. Histamine H2 receptor antagonists famotidine and cimetidine accelerate the recovery of skin barrier function, while histamine and histamine H2 receptor agonist dimaprit delayed it (Ashida, Denda et al. 2001).

2.1.12. Role of ions in recovery from barrier injury

In human epidermis, a characteristic distribution of ions for calcium, sodium, potassium, magnesium, and other ions is established under normal conditions (Denda, Hosoi et al. 2000). After barrier disruption, these gradients are perturbed. For example, calcium concentrations are normally highest in the granular layer of epidermis (Menon, Grayson et al. 1985). Barrier disruption causes passive loss of calcium through the damaged stratum corneum, which then signals the outer stratum granulosum cells to secrete their pool of lamellar bodies (Lee, Elias et al. 1992). Conversely, restoration of calcium levels in the outer epidermis during after barrier disruption prevents the release of lamellar bodies.

Changes in the intracellular levels of K^+ can alter barrier repair. Applications of K^+ channel blockers (i.e. glibenclamide) delayed barrier recovery in one study. Development of epidermal hyperplasia, a downstream consequence of barrier disruption, was also inhibited by agents that reduced intracellular K^+ levels. These results demonstrate that changes in K^+ levels that occur after barrier disruption can signal metabolic responses, i.e. Lamellor body secretion, which might accelerate normalization of barrier function (Denda, Tsutsumi et al. 2007). Some magnesium salts and a mixture of magnesium and calcium salts are also known to accelerate barrier recovery (Denda, Katagiri et al. 1999).

2.1.13. Role of cytokines, growth factors, and ECM molecules in barrier injury

A number of proinflammatory cytokines are increased after barrier injury. TNF expression is known to increase in response to barrier disruption and wound healing. The TNF-Receptor 55 signaling pathway contributes to cutaneous permeability barrier repair

through sphingomyelinase‑mediated generation of ceramides (Jensen, Schutze et al. 1999). Another example of this is an increase in epidermal IL‑1 alpha mRNA and protein levels and release of IL‑1 alpha from skin after tape stripping, in hairless mice kept under low or high humidity. Additionally, the release of IL‑1 alpha from skin immediately after tape stripping was significantly higher in animals kept in a low‑humidity than in a high‑humidity environment (Ashida, Ogo et al. 2001). Interestingly, the SC of facial skin constantly exposed to the environment shows an abnormal ratio of IL‑1/IL‑1 receptor, suggesting the presence of mild inflammation even in normal individuals (Tagami, Kobayashi et al. 2001). IL‑6 and IL‑6R immunostaining and STAT3 tyrosine phosphorylation are induced in epidermal keratinocytes by tape‑stripping (Wang, Schunck et al. 2004). Interestingly, in the IL‑6‑deficient mice, there is a decrease in epidermal barrier repair even at 24 h after treatment (Wang, Schunck et al. 2004). These results indicate that the IL‑6 cytokine system, particularly trans‑signalling via the soluble IL‑6R, is critically involved in barrier repair after skin injury

An increasing body of evidence suggests that many cytokines, including IL‑6 and others, exert their effects via interactions with the epidermal growth factor (EGF) receptor pathway. The EGFR, in turn, has strong effects upon hyaluronan synthesis and metabolism. The important topic of EGFR and HA is reviewed at length in section 2.3.

2.2. Role of hyaluronan (HA) in epidermal responses to injury

In this section, the current state of knowledge regarding HA in cutaneous biology, including the role of HA in normal skin development, skin diseases, wounding, and repair is reviewed. The final two subsections highlight changes in HA following severe skin wounding (2.2.8) or after superficial epidermal disruption of the stratum corneum (2.2.9).

2.2.1. Hyaluronan: Structure and physical properties

Hyaluronan is a high molecular mass extracellular glycosoaminoglycan (GAG) expressed in most tissues of the body. This high molecular weight, polyanionic polymer consists of a disaccharide unit (-1,4-glucuronic acid-1,3-N-acetylglucosamine-) repeated several thousand times (Fig. 2-5) (Hascall and Laurent 1997).

The number of repeat disaccharides in a completed HA chain can approach 30,000 units in some tissues. It can therefore have a molecular mass of up to 10×10^6 Da and an extended length of 25 µm (Fig. 2-6), much larger than any other member of the GAG family (Toole, 2000). In free solution under physiological conditions of pH and strength, HA forms a stiffened and expanded random coil due to hydrogen bonding of adjacent sugar units and mutual repulsion between carboxyl groups.

In dilute solution it occupies a very large solvent domain but as the concentration increases, individual molecules entangle and form a continuous network. Its conformation can also be modified by interaction with numerous specific binding proteins (Toole, 2001).

HA solutions of concentrations similar to those found in soft connective tissue exhibit interesting physical and chemical properties. HA has therefore been assigned various roles in the homeostasis of the extracellular space (Comper and Laurent 1978), e.g., the non-ideal osmotic behaviour of HA makes it efficient as an osmotic buffer. The osmotic contribution of polysaccharide networks gives them a role in the homeostasis of tissues (Laurent and Fraser 1992). At physiological concentrations, HA molecules entangle and form a random network of chains. These networks interact sterically with other macromolecular components (Comper and Laurent 1978). HA excludes other
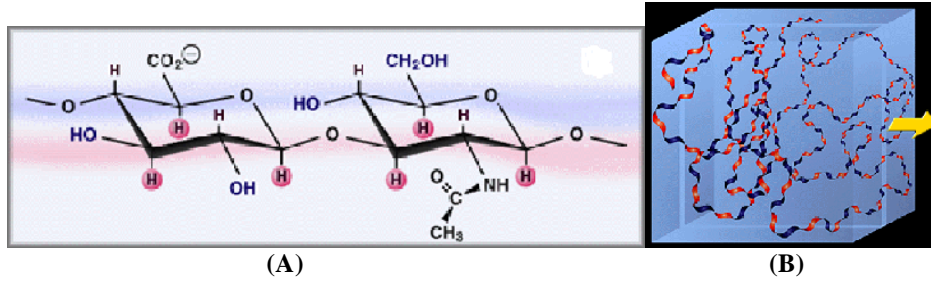
Figure 2-5: Hyaluronan fundamentals.
(A) The repeating unit of HA (see text). (B) Model of HA in solution. The alternating blue and red strand represents the ribbon structure with blue (hydrophilic) and red (hydrophobic) faces. Adapted from http://www.glycoforum.gr.jp/science/hyaluronan/HA01/HA01E.html.

macro-molecules that cannot find space in the network, and also retards the diffusion of other substances trying to penetrate it. By these properties, HA regulates the distribution of plasma proteins in tissues (Laurent and Fraser 1992). The rheological properties that HA solutions exhibit (e.g., in synovial fluid) have also led to speculations about its role in the lubrication of joints and tissues. HA solutions demonstrate similar visco-elastic properties shown by joint fluid. Reports also show that HA separates many tissue surfaces that slide along one another, e.g., fibrils in skeletal muscle (Laurent, Laurent et al. 1996).

The interaction of HA with specific extracellular molecules is important in the
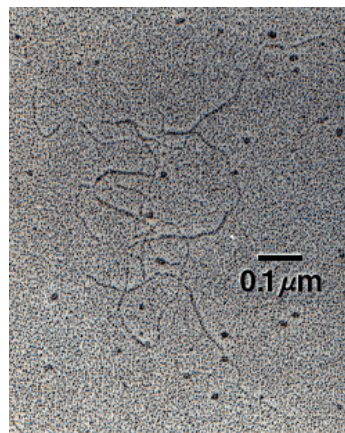
Figure 2-6: Electron micrograph of intertwined HA cables.
They have been deposited on a flat surface and rotary shadowed with heavy metal for contrast (Hascall and Laurent, 1997).
Adapted from http://www.glycoforum.gr.jp/science/hyaluronan/HA01/HA01E.html.

assembly of a functional extracellular or pericellular matrix. These HA binding proteins are known collectively as hyaladherins. Among the best-characterised hyaladherins are the proteoglycan link protein family, in particular cartilage link protein, and the aggregating proteoglycan family, such as aggrecan, versican, brevican and neurocan. The receptors that are known to bind to HA are: CD44, RHAMM, LYVE and PH20. In particular, CD44 is expressed by a number of different cell types and is the principal hyaluronan binding receptor.

2.2.2. HA: Synthesis and degradation

Unlike other GAGs, HA is not assembled in the rough endoplasmic reticulum and Golgi apparatus, and is not covalently linked to a protein backbone during synthesis (Toole, 2000). Instead, HA synthesis occurs by hyaluronan synthases (HAS1, HAS2, and HAS3), which are glycosyl transferase enzymes located within the plasma membrane. HA synthesis begins on the cytoplasmic side of the plasma membrane, and the growing polymer chain is extruded out of the cell. As an important molecular detail, one should note that HA is elongated at the reducing, rather than the non-reducing terminus [Weigel et al, 1997]. Epidermal keratinocytes abundantly express both HAS2 and HAS3 from the basal to the granular layers, signifying that HAS3 functions in the epidermis. Therefore, HA is primarily synthesized in the basal and spinous layers, although some of it ends up in the stratum corneum (Sakai, Yasuda et al. 2000).

The HA synthesizing enzymes, especially HAS2, can be induced by cytokines and growth factors; examples include all-trans-RA (tRA) and TNF-alpha (Saavalainen, Tammi et al. 2007). Several reports have shown that tumor necrosis-alpha (TNF-a), interferon-gamma (IFN-?), and interleukin-beta (IL-1ß) are capable to induce hyaluronan

synthases (HASs) mRNA expression in different cell culture types. The consequence of this stimulation is a marked increment in hyaluronan production.

HA-degrading enzymes include the mammalian and bacterial hyaluronidases (HYALs). HYAL 1, 2 and 3 have been cloned in mammals, with HYAL 1 and 2 being the most abundant and best characterized. The degradation of HA occurs in a stepwise fashion with quantum decreases in polymer size. From the cluster on chromosome 3, HYAL1 and HYAL2 constitute the major hyaluronidases of somatic tissues. HYAL2 cleaves high molecular weight HA to a limit product of approximately 20 kDa (~50 mer size) and is bound to the plasma membrane by a GPI-anchor (Stern 2000; Lepperdinger et al. 2001). These fragments have proinflammatory and angiogenic activity. HYAL1 is thought to be lysosomal, cleaving the 20 kDa chains into small HA disaccharides (Stern 2000). Very little is known about HYAL3. Transcripts have been found in the brain and liver, but its protein product remains uncharacterised [Triggs-Raine et al, 1999]. PH20 is expressed in testis and has a role in fertilization (Cherr et al. 2001). While originally thought to be sperm-specific, PH20 has now been shown in the prostate (Vines et al. 2001), female genital tract (Zhang and Martin-DeLeon 2003), breast (Beech et al. 2002) and fetal tissues (Csoka et al. 1999).

HA has an extraordinarily high rate of turnover that varies from tissue to tissue. Metabolic studies have shown that the half-life of a HA molecule in cartilage, for example, is normally 2-3 weeks, whereas in keratinocytes of the epidermis the half-life is less than 24 hours. Catabolism of hyaluronan is very well described by a pathway that includes the cell surface hyaluronan receptor (CD44), two hyaluronidases (Hyal-1 and Hyal-2), and two enzymes within lysosomes (beta-glucuronidase and beta-N-

acetylglucosaminidase). The catabolism begins after palmitoylation of CD44, which is important for the invagination of lipid rafts at the surface of the cell membrane. High-molecular-weight extracellular HA is degraded in a series of steps, breaking down the molecule into successive size categories. The oligomers in each size range have different biological functions. Thus, undegraded HA has space-filling, hydrating, anti-angiogenic, and immunosuppressive properties. Intermediate sizes (down to 20-kDa) are highly angiogenic, immuno-stimulatory, and inflammatory. The small oligomers (8-20 mers) can induce heat shock proteins and are anti-apoptotic. Single sugar products, glucuronic acid and a glucosamine derivative, are released from lysosomes to the cytoplasm, where they become available for other metabolic cycles (Stern 2004).

The half-life of HA in the blood is extremely short, only a few minutes. In addition, certain cells may predominantly synthesise or catabolise HA, rather than keep a constant concentration in the tissue. For example, cells in the dermis actively synthesise more HA than they degrade. A larger proportion of HA escapes from this tissue only to be rapidly captured by receptors on reticuloendothelial cells in lymph nodes and in the liver, which internalise them for subsequent catabolism in lysosomes. It has been estimated that approximately one-third (~5 g) of total HA in the human body is metabolically removed and replaced during an average day (Fraser et al. 1997; Hascall 2000).

Systemic hyaluronan is removed from the lymph and serum, and its local turnover is dependent upon the same cells that are responsible for its synthesis. HA and chondroitin sulfate (CS) clearance from lymph and blood in mammals is mediated by the HA receptor for endocytosis (HARE), which is present as two isoforms in rat and human

in the sinusoidal endothelial cells of liver, spleen, and lymph nodes (Zhou, B., et.al. Glycobiology 13, 339-349). The small rat and human HARE proteins are not encoded directly by mRNA but are derived from larger precursors.

Local turnover is dependent upon receptor-mediated uptake of HA and its delivery to the lysosomes. Of the many hyaluronan binding proteins/receptors known, the participation of CD44 in the internalization of HA has been best described. As discussed before, palmitoylation of CD44 is a critical step in its turnover from the cell surface and is required for its association with the lipid rafts. This process in turn determines the rate of hyaluronan endocytosis, wherein some fraction of the hyaluronan bound to CD44 becomes internalized and is delivered to lysosomes by a mechanism that is not dependent on clathrin, caveolae or pinocytosis (Thankamony and Knudson 2006). Different cells have differing mechanisms of control over HA internalization. Regulation may involve the participation of alternatively spliced isoforms of CD44, changes in CD44 phosphorylation, changes in cytoskeletal binding proteins, or extracellular proteolytic activity (Knudson, Chow et al. 2002).

2.2.3. Extracellular proteins that bind HA

2.2.3.1. Cell surface receptor proteins that bind HA

Despite its structural simplicity, hyaluronan exhibits a broad spectrum of biological activity that is achieved by interacting with a large number of hyaluronan-binding proteins and proteoglycans. SHAP, a protein that corresponds to the heavy chains of inter-alpha-trypsin inhibitor (ITI) molecules circulating in blood, can covalently modify and form a complex with HA. TSG-6 (the product of tumor necrosis factor-
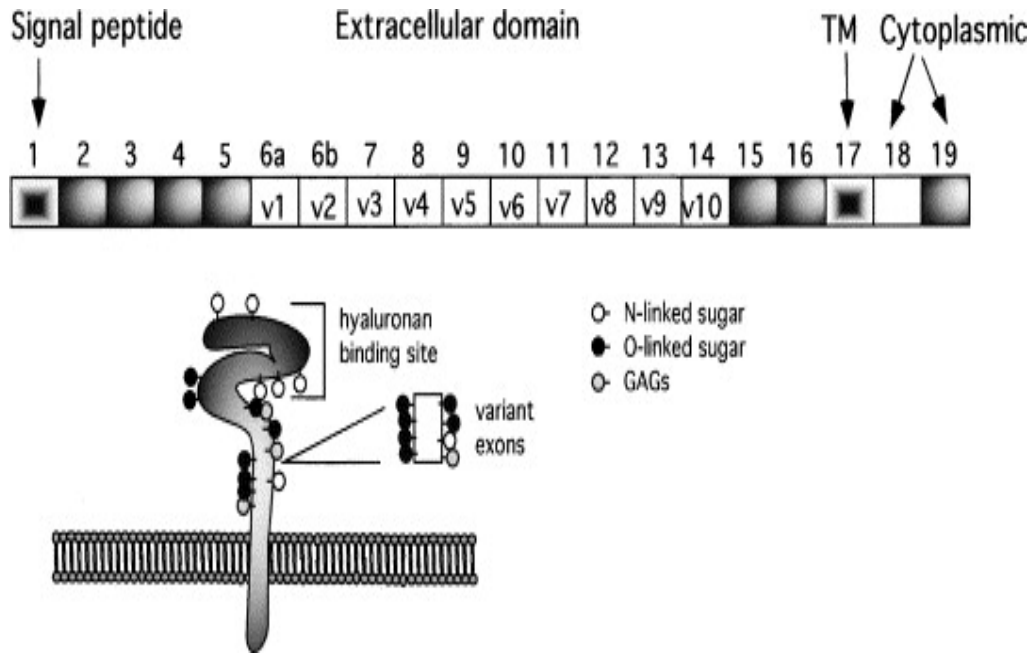
Figure 2-7: Protein structure of the hyaluronan receptor, CD44.
The extracellular domain of CD44 is extensively glycosylated and subject to alternative splicing due to variant exon insertion. The amino terminal ~180 amino acids contain the hyaluronan-binding "link module" or cartilage link protein domain so called because of its homology (~35%) to the ligand binding domain of other hyaluronan binding proteins such as cartilage link protein and TSG-6. This is followed by a mucin-like membrane proximal region which contains the site of insertion for additional amino acid sequences by alternative splicing among the "variable" exons. (Isacke and Yarwood 2002).

stimulated gene-6) is not present in normal tissue but is expressed in a setting of an inflammatory environment. It can be found in complexes with various forms of ITI.

CD44 is very widely distributed in the body and is recognized to be the major cell surface receptor for hyaluronan. CD44 is a cell-surface glycoprotein that contains an extracellular domain, a trans-membrane domain and a cytoplasmic domain (Stamenkovic et al. 1989; Aruffo et al. 1990; Ponta et al, 2003). Many variant isoforms are possbile via alternative RNA splicing (Fig. 2-7).

CD44 has been implicated in many adhesion-dependent cellular processes including tumor growth, metastasis and tissue injury. Soluble forms of CD44 have been identified in the serum of normal individuals which may have physiological significance,

because these may influence hyaluronan binding to the cell surface (Cichy and Pure 2004).

Receptor for Hyaluronan Mediated Motility (RHAMM), also called CD168, is alternatively spliced and is expressed in most normal avian and mammalian cell types. RHAMM is found on cell surfaces, inside the cell, and in the nucleus (Turley et al. 2002). Interestingly, deletion of either CD44 or RHAMM does not result in embryonic lethality. Therefore, these two proteins either share some functions and/or other cellular hyaladherins are able to compensate for the loss of CD44 or RHAMM (Turley et al. 2002).

2.2.4. Functions of HA in wound healing and responses to injury

The molecular functions of HA fall into three partially overlapping categories. Firstly, HA can bind to several specific cell surface receptors that activate intracellular signalling pathways and therefore influence cell behaviour. Secondly, HA can interact with a variety of extracellular molecules, such as link proteins and proteoglycans, to form a composite extracellular or pericellular matrix. Thirdly, HA can promote cell migration, proliferation or differentiation depending upon its molecular size. All three of these functions contribute to cell adhesion, proliferation, migration, differentiation, tissue remodelling, inflammation and diseases such as cancer and atherosclerosis (Toole 2001; Itano et al. 2002; Lee and Spicer 2000) Subsequent sections will focus upon the specific effects of HA as a function of HA size class, and the different roles that each HA class plays in varied cellular systems.

2.2.5. Pericellular/ High molecular weight HA

Hyaluronan can form a voluminous pericellular matrix or "coat", which has also been termed the "glycocalyx". The HA-dependent coat has multiple important roles, from serving structural and mechanochemical functions, to the regulation of cell division and motility, as well as cancer progression and metastasis. Time-lapse studies show that pericellular matrix formation facilitates cell detachment and cell rounding during mitosis.

Epithelial cells can form relatively thick pericellular coats when hyaluronan synthesis is induced by growth factors like EGF (Tammi, Rilla et al. 2001). Furthermore, microvillous or filopodial membrane protrusions are created by active hyaluronan synthesis, and in turn form the scaffold of hyaluronan coats in cells (Evanko, Tammi et al. 2007). The length of the microvilli can exceed 20 µm and they are extremely thin, almost undetectable in ordinary phase contrast microscopy, and easily destroyed during fixation.

An unusual form of high molecular size HA, is the so-called "cable structure". HA cables occur at sites of inflammation and appear to originate from within the cells. Hyaluronan cables are synthesized by latent HAS enzymes which exists perhaps in association with the ER or the nuclear membrane. Under conditions that induce cellular stress, such as ER stress, the latent HAS enzyme is modified, becomes activated and begins synthesizing HA cables that extend out into the extracellular matrix (Hascall, Majors et al. 2004). These "stress cables" arise from the peri-Golgi region, with interweaving strands extruded from multiple cells. They become cross-linked and complexed into cables and fibrils at sites of inflammation. Inflammatory cells bind to the
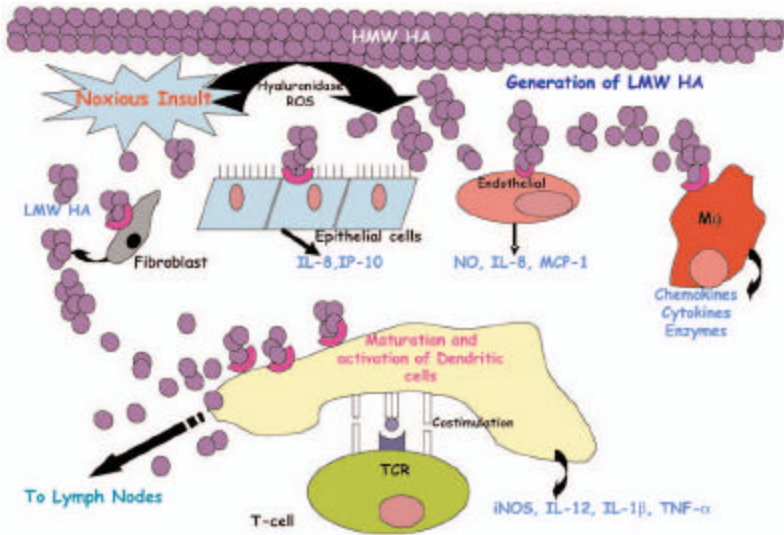
Figure 2-8 HA as a danger signal.
In health, high-molecular-weight (HMW) HA supports tissue integrity and water and solute homeostasis. Upon a noxious insult such as an infection, ischemia, or environmental toxin, the generation of reactive oxygen species and hyaluronidases act to break down HA into lower-molecularweight (LMW) forms. The LMW HA activates epithelial cells, endothelial cells macrophages, and fibroblasts to secrete chemokines, cytokines, and degradative enzymes, all which serve to enhance inflammation. (Powell and Horton 2005)

cables. These phenomena are now well-documented in inflammatory bowel disorders, Crohn's disease and ulcerative colitis (de la Motte et al. 2003).

2.2.6. Low molecular weight HA

Although HA usually exists as a high molecular mass polymer, HA of a much lower molecular mass that shows a variety of biological activities can be detected under certain pathological conditions (Fig. 2-8). It has been reported that low molecular weight HAs (LMW-HAs) of a defined size range can induce the proteolytic cleavage of CD44 from the surface of tumor cells and promote tumor cell migration. It is well documented that tumor cells generate HA oligosaccharides via their own hyaluronidases, which then bind to the tumor cell CD44. This further enhances CD44 cleavage, cell motility, and tumor progression (Sugahara, Hirata et al. 2006).

Fragment size is important in signalling. In particular, HA fragments larger than 500 kDa or smaller than six sugars do not signal. Some breakdown products of HMW HA have the ability to induce a diverse array of inflammatory mediators. For example, low MW HA fragments lead to up-regulation of a wide array of inflammatory genes in mouse macrophages (McKee, Penno et al. 1996; Noble, McKee et al. 1996).

2.2.7. HA in normal skin development, differentiation, and disease

A large proportion of HA is found in the skin and constitutes ~56% of the total extracellular matrix in the skin (Fraser, Laurent et al. 1997). Hyaluronan, known for 50 years to be a major constituent of the skin, was thought to be solely of dermal origin until recently, when highly specific and sensitive techniques to localize HA in histological tissue sections revealed unexpectedly high signals around keratinocytes in the epidermis (Tammi, Ripellino et al. 1988).

HA is synthesized in the basal and spinous cellular layers through enzymatic activity of HA enzymes (see section 2.2.2) and migrates eventually to the stratum corneum of the epidermis, where it may plays a role in moisturizing the stratum corneum and/or regulating its mechanical properties (Sakai, Yasuda et al. 2000).

However, the close involvement of HA in regulating all aspects of epidermal homeostasis, including keratinocyte proliferation and differentiation (see Chapter 4) and HA metabolism itself (see Chapter 5) speak to an important role during abnormal states in disease and injury.

The major HA receptor, CD44, is important for epidermal homeostasis and is therefore expected to play a major role in disease and injury. CD44 is very widely distributed in the body, and the two major functions of CD44 in skin are the regulation of
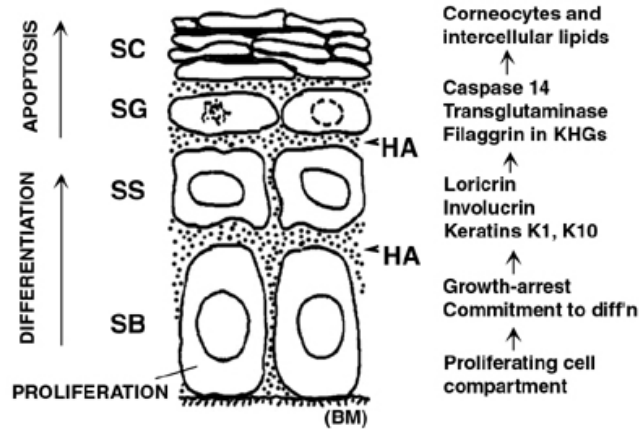
Figure 2-9: Hyaluronan in the epidermis.
HA is distributed between the keratinocytes in the basal, spinous and granular layer in the extracellular matrix. It supports cellular proliferation in the basal layer.
Adapted from http://www.glycoforum.gr.jp/science/hyaluronan/HA04/HA04E.html

keratinocyte proliferation in response to extracellular stimuli and the maintenance of the local HA homeostasis (Kaya, Rodriguez et al. 1997). In adult epithelium, CD44 and hyaluronan are seen abundantly in stratified, but not simple, epithelia. This suggests that CD44 is a marker for epidermal growth and normal differentiation. RHAMM on cell surface has been associated with cell locomotion and is cited in literature for its involvement in tumorigenesis. It has been observed that loss of keratinocyte differentiation in advanced squamous cell carcinomas is associated with down-regulation of CD44 and hyaluronan loss from cell surfaces (Karvinen, Kosma et al. 2003).

Hydrocortisone (cortisol), a widely used anti-proliferative and anti-inflammatory agent for skin diseases, inhibits HA catabolism (at all doses) and HA synthesis (at pharmacological doses). Pharmacological doses of hydrocortisone reduce the content of HA in the epidermis and enhance normal differentiation (Agren, Tammi et al. 1995). Current data are consistent with the possibility that hyaluronan interacts with its cell surface receptors and serves as a survival signal for keratinocytes, delaying terminal differentiation under normal circumstances.

2.2.8. Role of HA in full-thickness wound healing

Much evidence points to an important role for HA in wound healing. For example, higher than normal amounts of HA are found in healing skin, especially at early stages of tissue regeneration. A study has recently reported a 6-fold increase in levels of hyaluronan on day three of epidermal injury by tape stripping in adult mice (Tammi, Pasonen-Seppanen et al. 2005). They report a strong induction of Has2 and Has3 mRNA, a slightly higher CD44 expression following epidermal injury and considerable epidermal hyperplasia that is associated with an increase in levels of hyaluronan. Transgenic mice with lower than normal expression of RHAMM in epidermal keratinocytes show delayed wound healing, suggesting that cell migration in the reepithelialization phase is not functioning properly. Mice in which the epidermal CD44 receptor is inhibited also show a defective response to wounding. These results suggest that RHAMM and CD44 are involved in the migratory signals stimulated by hyaluronan in keratinocytes.

A study on HA expression in human skin wounds showed that hyaluronidase activity in pressure ulcers was significantly elevated as compared with the acute wounds. These results suggest a role for increased enzymatic degradation of hyaluronan during wound repair (Dechert, Ducale et al. 2006). The importance of HA in keratinocyte migration was examined in scratch-wounded cultures of keratinocytes that had been engineered to constitutively overexpress Has2; the data indicated a correlation between HA expression and the rate of cell migration into the 'wounded' spaces (Rilla, Lammi et al. 2002).

Another aspect of HA in wound healing may be an important role in scarless healing. It was observed early on that HA levels are higher in fetal vs. adult skin. Over a

decade ago, Estes et al. used Hunt-Schilling wound chambers to measure the HA content of fetal lamb wound fluid at 75 and 120 days of gestation (in sheep, term =145 days). The transition between scarless healing and healing with a scar occurs somewhere around 100 days. In wound fluids collected in a time course from 75-day ("fetal") and from 120-day ("adult-like") animals, peak HA levels were significantly higher in the younger animals and persisted longer than in the older animals. From these data, both the high levels and prolonged presence of HA in the younger (fetal) wounds appear to promote scarless healing.

The hypothesis that high levels of HA support scarless healing is supported by studies conducted in Hoxb13 knockout mice. Hox genes encode a highly conserved family of transcriptional regulators that function as critical regulators of axial patterning in the embryo (Manak and Scott 1994). While 39 of these genes are expressed in both adult and fetal human skin, very little is known about what roles Hox gene might be playing in skin development, renewal or repair. Hoxb13, a factor expressed in both adult and fetal human skin, is interesting because its expression is selectively downregulated in fetal wounds that heal without a scar (but not downregulated in adult wounds that heal with scarring). This observation led Mack et al. (Mack, Abramson et al. 2003) to hypothesize that loss of Hoxb13 function in adult mouse skin would result in improved wound healing. Indeed, they determined that Hoxb13 knockout wounds closed faster, had greater tensile strength, and reconstituted more normal collagen architecture than their wild type (WT) counterparts (similar to fetal scarless healing). Intriguingly, levels of HA were significantly higher in both the dermal and epidermal layers of Hoxb13 knockout adult skin as compared to WT skin. These combined observations suggest that

HA levels are persistently higher in fetal skin and in fetal wounds that heal in a scarless fashion.

HA might contribute to enhanced wound healing in several ways. It could allow for faster wound closure by increasing the proliferation and/or migration rates of keratinocytes. Alternatively, HA is thought to inhibit differentiation so that high levels of HA could confer upon skin a less differentiated state, allowing for "fetal-like" healing.

2.2.9. Role of HA in responses to epidermal barrier injury

To ask whether and how HA may be important in epidermal responses to wounding, researchers have begun to ask the targeted question of whether HA is altered following limited superficial wounding (stratum corneum disruption), using several approaches. For example, in a study from our laboratory (Maytin, Chung et al. 2004), barrier disruption was achieved by tape-stripping, or by application of an organic solvent. After twice-daily applications of acetone for 5 days to the skin of mice, the epidermis responded by undergoing hyperplasia (3-fold thickening), and HA increased in the epidermal compartment by 6-fold. To investigate the functional significance of this additional HA in the tissue, an HA-degrading enzyme (hyaluronidase) was applied topically for 90 minutes. This regimen had been shown to effectively remove HA from the pericellular spaces. In areas of skin treated with hyaluronidase following barrier disruption, epidermal thickening was largely prevented (reduced by 50%), implying that HA plays a functional role in mediating the hyperplastic response (Maytin, Chung et al. 2004).

Other studies have examined a link between CD44 (the principal HA receptor) and barrier recovery after injury. In a study, a significant delay in early barrier recovery

after acute barrier disruption was shown to occur in CD44 k/o versus wild-type mouse skin. That work suggested that hyaluronan and its cell-surface receptor (CD44) can stimulate lamellar body (LB) formation and secretion. Slower barrier recovery in CD44 k/o mice could be further attributed to reduced LB formation, loss of apical polarization of LB secretion, and down-regulation of cholesterol synthesis (Bourguignon, Ramez et al. 2006).

In summary, studies with experimental barrier disruption have revealed a number of changes that appear to play a role in barrier activity. However, the relative importance for each of these factors in stimulating and coordinating the restoration of stratum corneum function is an important and unresolved question.

2.3. Growth factor signaling and HA metabolism following injury

This section discusses the role of Epidermal Growth Factor Receptor (EGFR) in epidermal injury and the relationship of this class of receptors to changes in hyaluronan that occur in epidermal disease and injury.

2.3.1. The Epidermal Growth Factor Receptor (EGFR/ErbB) family of receptors, and its ligands

The ErbB (erythroblastic leukemia virus (v-erb-b) oncogene homologue (Erb-B)) receptor family has four members, EGF receptor (EGFR)/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. These receptors are expressed on most human cell types, with the possible exception of mature hematopoietic cells. ErB signaling pathways regulate fundamental functions in epidermal cells including survival, proliferation and migration. Importantly, it has been observed that diverse skin disorders, such as xerosis, eczema, psoriasis, ichthyosis and dermatitis may be associated with the

altered expression of these growth factor receptors or their ligands, as well as abnormal lipid levels in the stratum corneum.

As shown in Fig. 2-10, all members of the ErbB family of receptors have a common extracellular ligand-binding domain, a single membrane-spanning region, and a cytoplasmic protein tyrosine kinase domain. Each ErbB receptor type has a preferred set of ErbB partners, and associated ligands (Fig. 2-10). ErbB-1 and ErbB-4 are complete and independent receptors; when they are bound to a ligand, they undergo dimerization and can activate signalling whether as homodimers or as heterodimers. The other two receptors, ErbB-2 and -3, are not independent. ErbB-2 (HER2 in humans) cannot bind to any soluble ligand, but does act as a preferred partner in heterodimeric complexes with other, ligand-bound ErbBs. The fact that ErbB2/HER2 requires no ligand and can activate pathways that stimulation growth and proliferation is a major problem in HER2-overexpressing human tumors (e.g., breast cancer). ErbB-3, on the other hand, has a deficient kinase function; it cannot generate signals in isolation as opposed to the other receptors. Nevertheless in the context of a heterodimer, primarily with ErbB-2, ErbB-3 can mount potent intracellular signals. Interestingly, ErbB- 1, -3 and -4 can each bind several distinct ligand molecules, which, according to genetic evidence, are partially redundant in their function (Hubert 2007).

EGF (epidermal growth factor)-like proteins are ligands for the ErbB receptors. They comprise a group of structurally similar growth factors, which contain a conserved six-cysteine residue motif called the EGF-domain. The EGF family is divided into two
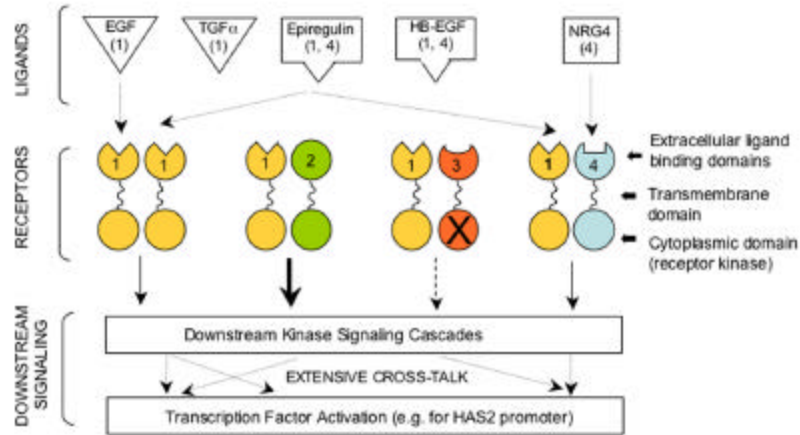
Figure 2-10: EGF/ErbB family of receptors and their ligands.
Ligands and receptors of the ErbB family that involve the founding member, EGFR/ErbB1. The ErbB1/ErbB1 homodimer, and all possible heterodimer combinations with ErbB2, ErbB3, and ErbB4, are shown. For ligands along the *top*, the particular receptors that can bind to each ligand are indicated in parentheses. Note that the ErbB2 receptor has no ligand-binding site, and ErbB3 has a nonfunctional kinase domain (indicated by the "X"). (Yarden Y, Sliwkowski, 2001)

groups based on their affinity for receptors. One group is EGF, HB-EGF (heparin bound epidermal growth factor), TGF-a (Transforming growth factor-alpha), AR (Amphiregulin), BTC (betacellulin) and ER (epiregulin), which bind to ErbB1. The other group is neuregulins, which are ligands for ErbB3 and ErbB4. The interactions between the varied growth factors and their four receptors allow a considerable variety of effects according to the cell type and the message received by the cell.

2.3.2. Details of EGFR structure and signalling

EGFR, a 170-kd glycoprotein, is composed of three regions corresponding to microanatomical locations (extracellular, transmembrane, and cytoplasmic; see Fig. 2-11). Single-molecule fluorescence-resonance energy transfer shows that EGF-EGFR complexes indeed form dimers at the molecular level. The activation of ErbB receptors is dependent upon receptor dimerization, which induces a change of conformation leading to the unveiling of an intrinsic tyrosine kinase activity. Receptor dimerization results in

phosphorylation of specific tyrosine residues within the receptor's cytoplasmic tail. These autophosphorylated residues serve as docking sites for a variety of signaling molecules, some of them being substrates of the receptor; recruitment and docking leads to the activation of intracellular signaling pathways. An ATP binding pocket, located between the two lobes of the kinase domain, is the focus of specific inhibitor design that exploits differences in kinase structure to achieve selectivity.

The monomeric EGFR has a much reduced kinase activity compared with the dimerized receptor; it is assumed that in the absence of dimerization, the kinase is in an inactive conformation. Ligand binding increases the proportion of dimerized EGFR and reorients the kinase domains in a way that increases their affinity for ATP binding, probably as a result of conformational change, thereby enhancing kinase activity. Use of a monoclonal antibody specific to the phosphorylated EGFR confirms that the EGFR becomes phosphorylated after dimerization (Sako, Minoghchi et al. 2000).

EGFR is an important channel for relaying multiple signaling inputs. This statement is supported by at least five lines of evidence, as follows. (i) A cascade of biochemical events that leads from EGFR to the activation of the proto-oncogene Ras, and eventually to the MAPK serine/threonine kinases, have been analyzed (Fig. 2-12). The key player in EGF-dependent Ras activation is the adaptor protein Grb2. Following activation of the EGFR kinase and autophosphorylation, Grb2/Sos complex migrates to the receptor at the plasma membrane. This facilitates the interaction of membrane-associated Ras with Sos, resulting in the exchange of Ras-bound GDP for GTP, and, hence, Ras activation (Lowenstein, Daly et al. 1992). Activated Ras, in turn, activates the serine/threonine kinase Raf-1. Raf-1 activation, through a series of intermediate kinases,
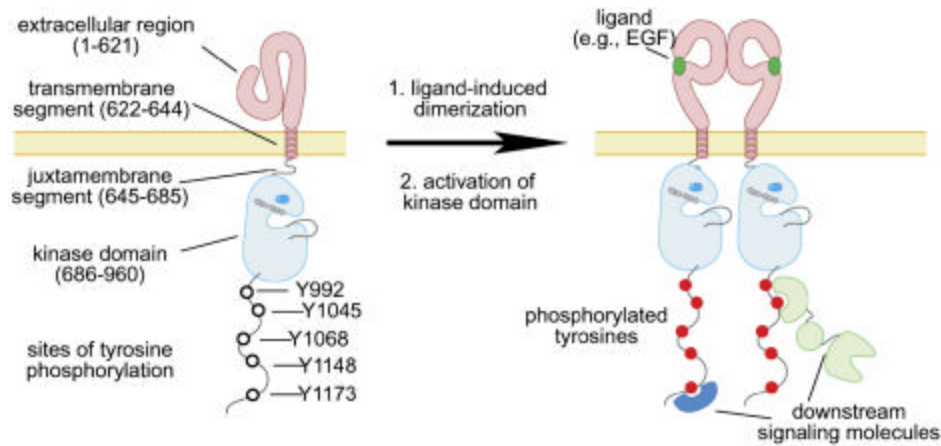
Figure 2-11: Ligand-induced dimerization of EGFR and active/inactive states of its kinase domain. General view of the EGFR and the ligand-induced dimerization and activation of the receptor. (Zhang, Gureasko et al. 2006).

leads to the phosphorylation, activation, and nuclear translocation of Erk-1 and Erk-2, which catalyzes the phosphorylation of nuclear transcription factors.

(ii) Members of the Src family of kinases are important in another pathway involving EGFR (Osherov and Levitzki 1994). Overexpression of Src proteins strongly enhances EGF-mediated proliferation and transformation in epithelial cells. Conversely, inhibition of Src activity blocks EGF-dependent DNA synthesis and reverses the transformed phenotype of EGFR- or ErbB2-overexpressing cells (Maa, Leu et al. 1995). (iii) STAT proteins, in particular STAT-1, -3, and -5, also have been implicated in EGFR signaling. (iv) EGF stimulation of a cell has marked effects on its phospholipid metabolism, including phosphatidylinositol turnover and production of phosphatidic acid and arachidonic acid. Of the enzymes involved in these pathways, at least three can be activated directly by EGFR (ie, PLC? (Kamat and Carpenter 1997), PI3-K (Verbeek, Adriaansen-Slot et al. 1998; Laffargue, Raynal et al. 1999), and phospholipase D [PLD] (Li and Malik 2005)), whereas others (eg, phospholipase A2) are regulated indirectly by EGF-mediated activation of other pathways. (v) EGFR can activate Ca-dependent
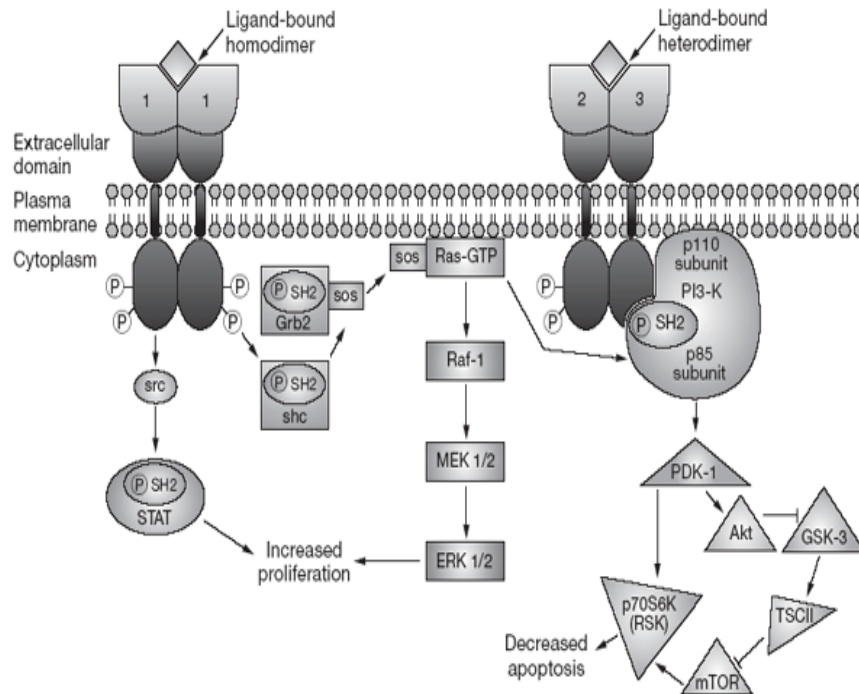
Figure 2-12: EGFR signal transduction pathways implicated in cellular proliferation.
Tyrosine phosphorylation on key intracellular residues of EGFR recruits docking proteins, including the SH2 domain-containing proteins Shc and Grb, which in turn couple to Ras. The guanine nucleotide exchange factor SOS, then activates Ras, which in turn promotes a cascade of kinases that result in ERK 1/2 phosphorylation. STAT proteins also become activated in response to EGFR phosphorylation. The combined activation of ERK 1/2 and STAT leads to increased proliferation. Heterodimerization with HER3 recruits PI3-K, which activates a cascade that results in Akt phosphorylation. Activation of downstream components of Akt leads to decreased apoptosis. (Sartor 2004).

pathways, such as Raf1 and nuclear factor-kappa B (NF-?B), and, through PKC, can activate multiple signalling components, including the MAPK (Rosen and Greenberg 1996) and JNK pathways (Chan and Wong 2004). PI3-K is activated in response to EGFR ligands through formation of ErbB1/ErbB3 heterodimers (Frolov, Schuller et al. 2007), and probably also through Src phosphorylation of EGFR.

2.3.3. Ectodomain shedding of pro-ligands

EGF-like factors are synthesized as transmembrane precursors, which can undergo proteolytic cleavage at the cell surface to release a mature soluble ectodomain;

this process is often referred to as "ectodomain shedding". However, until recently, there was a paucity of information regarding regulation and the identity of the proteases that are critical to stimulate posttranslational proteolytic "ectodomain shedding" of the ErbB ligands.

Ectodomain shedding of EGF-like factors has been linked to multiple zinc-binding metalloproteases of the MMP (matrix metalloprotease) and ADAM (a disintegrin and metalloprotease) families (Kenny 2007). The ectodomain shedding of EGFR ligands has emerged as a critical component in the functional activation of EGFR and interreceptor cross-talk. Identification of the sheddases for EGFR ligands using mouse embryonic cells lacking candidate sheddases has revealed that ADAM 10, -12 and -17 are major sheddases of the EGFR ligands in response to various stimulants such as GPCR agonists, growth factors, cytokines, osmotic stress, wounding and phorbol ester (Sahin, Weskamp et al. 2004). Interestingly, activity of ADAM 10 is noted in HB-EGF release from certain cancer cells. ADAM 10 dependent shedding of the EGFR ligands and CD44 is stimulated by calcium ionophores (Nagano, Murakami et al. 2004). There is data to suggest that shedding of HB-EGF by ADAM 12 plays an important role in cardiac hypertrophy, and that inhibition of HB-EGF shedding could be a potent therapeutic strategy for cardiac hypertrophy (Liao 2002). ADAM 17 is critical for the cleavage of ligands and subsequent EGFR activation in a variety of epithelial cancers, like head and neck (Gschwind, Hart et al. 2003), ovarian (Rosso, Piazza et al. 2007), colorectal (Merchant, Voskresensky et al. 2008), breast (Kenny and Bissell 2007)and gastric (Tanida, Joh et al. 2004). ADAM 17 knock out mice have developmental defects similar to the ones lacking the EGFR or the ligands HB-EGF or Amphiregulin.

Recently there has been an increasing focus on MMPs as sheddases and regulators of EGFR dependent signaling pathways. Gastrin peptides increase the levels of MMP-2, MMP-3, and MMP-9 as well as HB-EGF gene expression/protein secretion and EGFR activation in endothelial cells (Clarke, Dickson et al. 2006). MMP-2 and -9 are reported to be independent prognostic markers for tumor growth, invasion and metastasis in head and neck squamous cell cancer (Do, Lim et al. 2004). Recent studies show that MMP-9 (linked to EGFR activation in bladder cancer cell lines) has a significantly higher activity in patients with invasive bladder cancer (Nutt, Durkan et al. 2003). MMP-7 (matrilysin) cleaves proHB-EGF, is frequently over-expressed in human cancer tissues, and is linked with cancer progression (Ii, Yamamoto et al. 2006).

2.3.4. EGFR ligand-independent transactivation

Extracellular stimuli, independent from ligands of the EGF family, can activate the EGFR. These diverse stimuli include numerous agonists for heptahelical G-protein-coupled receptors (GPCR), cytokines and integrins. For example, there is evidence to suggest that IL-6 upregulates cellular signalling via the epidermal growth factor receptor pathway. IL-6 is known to induce EGFR expression in normal human keratinocytes in a time and dose-dependent manner (Oyama, Sekimata et al. 1998). On the other hand, mutant EGFR can activate the gp130/JAK/STAT3 pathway by means of IL-6 upregulation in primary human lung adenocarcinomas, making this pathway a potential target for cancer treatment (Gao, Mark et al. 2007).

In addition to direct activation by EGFR-family ligands, the large family of GPCRs has been reported to transactivate EGFR via both ligand-dependent and independent mechanisms. GPCR agonists such as lysophosphatidic acid, bradykinin,

thrombin, and carbachol cause rapid tyrosine phosphorylation of the EGFR. GPCRs can induce the cleavage of membrane-bound EGFR ligand precursors, or directly activate the juxta-membrane tyrosine kinase domain of EGFR (Schafer, Marg et al. 2004). GPCR ligands can also induce signalling events downstream of the EGFR, such as recruitment of the adapter protein Shc and activation of the mitogen-activated protein kinases (MAPK) ERK1/2, JNK and p38. The progression of colon, lung, breast, head and neck, prostate and ovarian cancers have all been reported to be mediated, at least in part, by GPCR-EGFR crosstalk (Bhola and Grandis 2008).

Of some further interest is the insulin-like growth factor receptor (IGFR), which induces EGFR activation via metalloprotease-dependent release of HB-EGF in several tumour cell types. IGFR-EGFR cross-talk is certainly apparent in mammary epithelial cells, since EGFR blockade abrogates IGFR-driven MAP kinase signalling and primes apoptosis.

Another interesting theory is a proposal that EGF like repeats, present in certain matrix molecules, may directly activate the EGFR. LPA and several other proinflammatory cytokines can induce thrombomodulin to shed its lectin-like domain, thereby exposing its EGF-like domain and activating the EGF receptor (Wu, Lin et al. 2008).

Of special interest for hyaluronan biology, the CD44 receptor can affect ErbB receptor function. CD44 interacts with ErbB proteins; it forms an active signaling complex with ErbB2 and anchors MMP-7 to the membrane. This then leads to the activation of ErbB4 by liberating heparin-binding EGF from its precursor (Yu, Woessner et al. 2002). CD44 shedding can be stimulated by hyaluronan oligosaccharides (Sugahara,

Hirata et al. 2006) and by EGF, which induces ADAM 10 -dependent CD44 cleavage and cell migration which may be attributable to the increased expression of EGFR on these cells (Murai, Miyauchi et al. 2006).

2.3.5. Inhibitors of EGFR

Two classes of anti-EGFR agents have shown clinical activity and achieved regulatory approval for the treatment of cancer. The most common pharmacologic approaches to inhibiting EGFR have been to develop monoclonal antibodies and small-molecule inhibitors. Tyrosine kinase inhibitors such as gefitinib (ZD1839), erlotinib (OSI-774), or AG1478 (4-3-chloroanilino-6, 7-dimethoxyquinazoline) competitively bind to the ATP pocket of EGFR to inhibit its activity. For example, AG1478 has been observed to potently inhibit the proliferation of keratinocytes in psoriasis skin (Ben-Bassat and Klein 2000; Ben-Bassat 2001) (Powell, Ben-Bassat et al. 1999).

Whereas the small-molecule inhibitors affect the intracellular portion of the receptor to prevent tyrosine kinase phosphorylation and subsequent activation of signal transduction pathways, monoclonal antibodies to EGFR block ligand binding to the extracellular domain. Monoclonal antibodies exhibit greater specificity for the EGFR compared with some of the small-molecule compounds (Herbst 2004). A number of antibodies with different functions have been designed in this regard. Cetuximab works against the extracellular domain of the EGF receptor and prevents binding of the ligand to the receptor (Robert, Ezekiel et al. 2001). Pertuxumab, on the other hand, is designed to interfere with the dimerization of the ErbB2 and the ErbB3 members of the EGF receptor family, thereby preventing the downstream signal transduction. The signals generated from this dimerization determine many phenotypic properties of cancer cells (Groner,

Name of Agent	Target
I. Tyrosine-kinase inhibitors	
AG1478 (Tyrphostin)	EGFR
OSI-774 (Reversible inhibitor, EGFR specific)	EGFR
ZD1839	EGFR
GW2016,	EGFR and ErbB2
CI1033	Pan ErbB
II. Monoclonal Antibodies	
IMC-C225 (Cetuximab)	EGFR
ABX-EGF (Fully humanized antibody)	EGFR
ICR-62 (Rat)	EGFR
EMD-72000 (Human)	
Pertuxumab	ErbB2 and -3
Trastuzumab	ErbB2
Cetuximab	EGFR

Table 2-1: Tyrosine Kinase Inhibitors and Monoclonal Antibodies to ErbB receptors

Hartmann et al. 2004). Trastuzumab, a monoclonal antibody directed against ErbB2, is successful both clinically and commercially. This antibody interferes with signals generated by the receptor and causes the arrest of the cell cycle in tumour cells. In addition, it recruits immune effector cells as cytotoxic agents and has been extensively studied in human breast cancer (Albanell and Baselga 1999; Baselga 2000; Behr, Behe et al. 2001; McKeage and Perry 2002; Aird, Ding et al. 2008).

2.3.6. EGFR- an essential regulator of multiple epidermal functions

Most of the current knowledge about the function of the EGFR and its family members during normal development derives from the analysis of mutant mice.

Inactivation of the EGFR results in death of the EGFR-null mice, either at midgestation (129/Sv), birth (C57BL/6) or postnatal day 20 (MF1, C3H and CD1) depending upon their genetic background (Sibilia and Wagner 1995). EGFR-deficient mice exhibit epithelial and neural phenotypes as well as craniofacial malformations (Miettinen, Chin et al. 1999). These mice are born with open eyes and show impaired epidermal and hair follicle growth (failure to develop a hairy coat) most likely because EGFR signalling is necessary for maintenance of hair follicle integrity (Threadgill, Dlugosz et al. 1995; Sibilia, Steinbach et al. 1998).

Current understanding of autocrine functions in keratinocytes assigns a principal function to EGFR and its ligands. In very early studies, EGF was thought to come principally from submaxillary glands, so that sialoadenectomy was used as a tool to investigate the effects of reduced EGF levels *in vivo*. It was reported that the organs most affected were the mammary gland and the epidermis (Tsutsumi, Kubota et al. 1987). Later, EGFR immunoreactivity was found throughout the epidermis, although more accentuated in the basal layer (Misumi and Akiyoshi 1990; Zambruno, Girolomoni et al. 1990). Human keratinocytes appear to require the binding of EGF or other family member ligands to the EGFR to maintain sustained proliferation in culture (see Fig.2-13). Keratinocytes synthesize four EGF family growth factors (TGF-alpha, amphiregulin, HB-EGF and epiregulin), which efficiently regulate keratinocyte growth and differentiation via auto- and cross-induction pathways (Hashimoto 2000). Interestingly, keratinocytes do
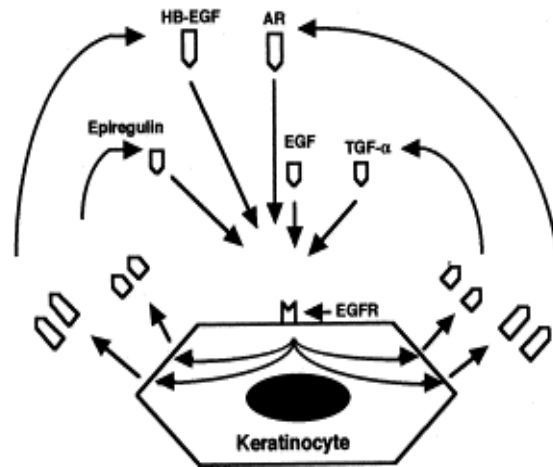
Figure 2-13: Auto- and cross-induction mechanism of TGF-a, Amphiregulin, HB-EGF and Epiregulin in normal human keratinocytes. (Hashimoto 2000).

not synthesize EGF under normal circumstances but are able to signal upon addition of the ligand. In the skin *in vivo*, EGF is provided by the underlying dermis fibroblasts (Marques, Martinez et al. 1999).

2.3.7. EGFR and keratinocyte proliferation

Although EGFR stimulation can lead both to keratinocyte proliferation and to keratinocyte migration, these responses are separable and mediated via different signalling pathways. We will first discuss EGFR and proliferation, and later describe EGFR and cell motility (Section 2.3.8).

EGF/EGFRs have an important role in cell proliferation; evidence is accumulating that overexpression of ErbB ligands or receptors, as well as ligand-independent receptor activation, occurs in many epithelial cancers. In wound healing and repair, EGFR has a documented role in corneal epithelial wound healing (Xu, Ding et al. 2004) and cutaneous wound closure (Kurten, Chowdhury et al. 2005), as well as a more widespread role in tissue remodelling, including cardiac hypertrophy (Chan, Smith et al. 2006), intestinal regeneration (Buffin-Meyer, Crassous et al. 2007), and bronchial epithelium

regeneration (Zhao, He et al. 2006). Upregulated expression of the EGFR and its ligands in primary keratinocytes appears to contribute to the autocrine growth and survival of these cells and prevents their differentiation (Gibbs, Silva Pinto et al. 2000) (Hansen, Woodson et al. 2000) (Pasonen-Seppanen, Karvinen et al. 2003) (Ponec, Gibbs et al. 1997).

A hallmark of increased proliferation *in vivo* is epidermal hyperplasia. Retinoids such as all-trans retinoic acid (tRA) strongly induce epidermal hyperplasia, and the mechansm of action of tRA is now thought to be linked to EGFR pathways. In mice the expression of HB-EGF and generation of its soluble form activates EGFR and ErbB2, which are pivotal in tRA-induced epidermal hyperplasia (Kimura, Iwamoto et al. 2005). Likewise in human skin, topical treatment with (tRA) induces EGFR ligands HB-EGF and AR. The increase in HB-EGF mRNA throughout the epidermis, whereas AR induction is limited to basal keratinocytes scaling (Rittie, Varani et al. 2006). Topical tRA activates extracellular signal-regulated kinase 1/2 (Erk1/2), the downstream EGFR effectors in human skin *in vivo*. Similar findings were also shown in human skin organ culture (Rittie, Varani et al. 2006). These data demonstrate a central role of EGFR activation in retinoid-induced epidermal hyperplasia, and suggest that EGFR inhibitors can mitigate retinoid-induced scaling (Rittie, Varani et al. 2006).

Another cause of hyperproliferation in the skin is acute exposure to UV light; recent research demonstrates that EGFR is also a major regulator in this response. The UV-induced activation of EGFR promotes skin tumorigenesis by suppressing cell death, augmenting cell proliferation, and accelerating epidermal hyperplasia in response to UV. Interestingly, a physiologic dose of UV exposure to human skin enhances the constitutive

level of EGFR by 2.5+/-0.5-fold at 6 and 24h after UV exposure in comparison to normal skin (Katiyar 2001). UV light can augment keratinocyte proliferation and suppress apoptosis, leading to epidermal hyperplasia via induction of EGFR (El-Abaseri, Putta et al. 2006). A study showed that UV irradiation can induce ectodomain shedding of HB-EGF in keratinocytes and leads to EGFR phosphorylation (Wang, Turlington et al. 2005). Also, UV-induced MMPs in human keratinocytes appear to trigger the activation of EGFR and subsequent activation of the MAPK cascade, leading to transcriptional upregulation of the MMPs (Wan, Belt et al. 2001).

Mechanical stretch is another positive regulator of proliferation in skin keratinocytes and other cell systems. Mechanical stretch can stimulate phosphorylation of EGFR and the formation of EGFR membrane clusters. Functional blocking of EGFR phosphorylation by either selective inhibitors (AG1478 and PD168393) or dominant-negative expression suppresses this phosphorylation. Stretch-induced activation of EGFR and downstream signalling protects cells against induced apoptosis (Kippenberger, Loitsch et al. 2005).

In psoriasis, thick plaques result principally from epidermal hyperproliferation; several lines of evidence suggest a role for the EGFR system in this process. In human psoriatic skin, EGFR is over expressed in the basal and first suprabasal layers, as observed by immunohistochemistry (Sergi, Kahl et al. 2000). HB-EGF and TGF alpha are identified as key molecules involved in epidermal proliferation in psoriasis (Romanowska, al Yacoub et al. 2008). The PTK inhibitor from the tyrphostin family--AG-1571 (SU-5271), potently inhibits proliferation of psoriatic keratinocytes in excellent

correlation with its EGFR kinase inhibitory activity, and was recently in clinical trials by SUGEN Inc (Ben-Bassat 2001).

An interesting study used EGF nanofibers to improve in wound healing in diabetic animals. The expression of keratinocyte-specific genes and proliferation was significantly increased with application of EGF-conjugated nanofibers. The EGF-nanofibers exerted superior activity for *in vivo* wound healing, along with an increased immunohistochemical-staining for EGFR (Choi, Leong et al. 2008).

2.3.8. EGFR and keratinocyte motility

The migration of epidermal keratinocytes is an important step in skin wound healing, and there are many instances which document the role of EGFR in wound closure. A few studies have shown that directional migration of keratinocytes involve redistribution of the EGF receptor at the leading edge, resulting in asymmetric polymerization of actin in migrating cells (Zhao, Pu et al. 2002). Specific inhibition of the EGF receptor kinase activity using low concentrations of PD158780 blocks directional migration (Fang, Ionides et al. 1999).

ATP, released upon epithelial injury, acts as an early signal to trigger cell responses including an increase in HB-EGF shedding, subsequent EGFR transactivation and downstream signaling, and ultimately more rapid wound closure (Yin, Xu et al. 2007). Similarly, catecholamines released from keratinocytes can stimulate keratinocyte migration and wound reepithelialization through a mechanism that requires both EGFR and ERK activation (Pullar, Rizzo et al. 2006). Hsp60 released from bacteria or inflammatory cells may promote epithelial cell migration through activation of EGFR and MAP kinases (Zhang, Koivisto et al. 2004). Elevations of epidermal growth factor

(EGF) and Ca^{++} concentrations in the wound site are associated with reepithelialization during wound healing. In addition, Ca^{++} and EGF can both induce increases in matrix metalloproteinase-9 (MMP-9) synthesis. Using primary keratinocytes, it has been shown that the combined action of Ca^{++}, EGF, and MMP-9 regulates the contributions of extracellular-regulated kinase and phosphatidylinositol-3 kinase and favors chemokinetic and chemotactic migration of keratinocytes (Morris and Chan 2007).

In addition to the ADAMs and MMPs, reactive oxygen species generated during injury and inflammation are potent inducers of HB-EGF shedding from the cell surface, which in turn phosphorylates EGFR (Kim, Lin et al. 2005). Cellular stress with methyl-beta-cyclodextrin, a molecule that extracts membrane cholesterol and thereby disrupts the structure of lipid rafts, strongly induces the synthesis of HB-EGF in keratinocytes through the activation of p38 mitogen-activated protein kinase. In addition, *in vitro* sectioning of skin samples also induces the expression of HB-EGF at the border of the incisions (Mathay, Giltaire et al. 2008).

Besides their microbicidal functions, human beta-defensins (hBD) and LL-37 activate different immune and inflammatory cells, and their expression is enhanced in inflamed skin and cutaneous wound sites. In addition these peptides induce phosphorylation of EGFR, STAT1 and STAT3, which are intracellular signaling molecules involved in keratinocyte migration and proliferation (Niyonsaba, Ushio et al. 2007).

The expression of collagenase-1, an enzyme essential for keratinocyte migration, is induced upon contact between the integrin and native type I collagen. Sustained migration during re-epithelialization of epidermal wounds is dependent upon production

of this enzyme, and requires autocrine EGFR activation by HB-EGF as an obligatory intermediate step (Pilcher, Dumin et al. 1999).

Soluble EGFR-immunoglobulin G-Fcgamma (Fcr) fusion protein, which is able to neutralize all EGFR ligands, suppresses keratinocyte migration *in vitro*. The application of OSU8-1 to wound sites in mice greatly retards reepithelialization as the result of a failure in keratinocyte migration. These findings indicate that the shedding of EGFR ligands represents a critical event in keratinocyte migration (Tokumaru, Higashiyama et al. 2000). EGFR overexpression in primary keratinocytes results in the biochemical activation of Akt and STAT pathways and induced enhances cell migration and cell aggregation. This modulation of cell migration via EGFR occurs through the up-regulation of matrix metalloproteinase 1 (Andl, Mizushima et al. 2003).

In order to directly evaluate the role of endogenous EGFR in cutaneous incisional wound healing, one study examined EGFR null- and wild-type skin after injury. By 5d after wounding, re-epithelialization was complete in all EGFR wild-type wounds, as opposed to only 40% of EGFR null wounds. Delayed wound closure in EGFR null skin was accompanied by an increase in edema, longer lasting and more prominent eschar, and increased distance between opposing wound edges (Repertinger, Campagnaro et al. 2004). In summary, increasing evidence suggests that the EGFR pathway has a major impact on epidermal proliferation, migration and the inflammatory/immune reactions of the skin.

2.3.9. EGFR and HA dynamics

The effect of EGFR or its ligands on the synthesis of hyaluronan has been studied in epithelial cells (Yamada, Itano et al. 2004), keratinocytes (Pappinen, Tikkinen et al.

2007) (Pienimaki, Rilla et al. 2001), fibroblasts (Heldin, Laurent et al. 1989) and pericardial cells (Honda, Noguchi et al. 1991). EGFR differentially modifies the expression of a number of HA-related genes associated with cellular invasion and proliferation. Most interestingly and as discussed previously, the human HAS2 gene responds dramatically to EGF, and is under complex regulation (Saavalainen, Pasonen-Seppanen et al. 2005). On the other hand, HA is shown to activate several receptor tyrosine kinases (RTKs) especially EGFR in colon, prostate, and breast carcinoma cells (Misra, Toole et al. 2006). Evidence supports the role of CD44 in forming stable complexes with RTKs, which may provide a versatile system for the regulation of cellular invasion and proliferation.

All-trans retinoic acid (tRA) is known to compromise epidermal differentiation and causes keratinocyte hyperproliferation through mechanisms not completely understood, but may involve HA. There are data to suggest that the effects of tRA on keratinocyte proliferation and HA syntheses are partly mediated through EGFR signaling (also see section 2.3.7). In work on REK (Rat Epidermal Keratinocyte) cultures (Pasonen-Seppanen, Maytin et al. 2007), our collaborators studied the influence of tRA on epidermal morphology and HA metabolism Their work showed that tRA upregulates HB-EGF mRNA expression and increases the phosphorylation of EGFR and extracellular signal-regulated kinase 1/2 (ERK1/2). Interestingly, the activation of EGFR and ERK1/2 is seen within 30 minutes of tRA treatment, suggesting that the activation of this signalling pathway is a primary response to tRA.

Another study suggests the role of HA in the reversal of skin atrophy. Kaya et al. have discovered that HA fragments cause marked epidermal hyperplasia and significant

skin thickening in patients with age- or corticosteroid-related skin atrophy. The effect of these fragments on keratinocyte proliferation is abrogated by antibodies against HB-EGF and its receptor, EGFR, which form a complex with a particular isoform of CD44 (CD44v3), and by tissue inhibitor of metalloproteinase-3 (TIMP-3) (Kaya, Tran et al. 2006). In particular, the upregulation of the expression of HA synthases Has2 and Has3 and CD44 induced by EGF in basal keratinocytes seems to contribute to maintaining their proliferative and undifferentiated state of the cells. Interestingly, epidermal HA synthesis, controlled by EGF and TGF-beta through changes in the expression of Has2 and Has3, correlates with epidermal proliferation, thickness, and differentiation (Pasonen-Seppanen, Karvinen et al. 2003).

Adhesion and migration of cells through extracellular matrices (ECM) are critical events in tumor invasion and metastasis. The pericellular matrices of many malignant human tumors are rich in hyaluronan, and it is known that manipulation of HA has strong effects on tumor progression in animal models. Increased HA production stimulates ErbB2 activation, leading to increased cell survival activities and several malignant cell properties. Recently CD44 was implicated in cell-ECM adhesion, which is crucial for tumor cell migration, signal transduction and metastasis. A study group investigated the role of EGF in regulating cellular interactions with ECM components, and in particular, hyaluronan, by modulating CD44 expression. They utilized monoclonal antibody blocking assays and showed that functional EGFR signaling is required for upregulation of CD44 expression. Thus, there is evidence that EGF stimulates cell binding to HA *in vitro* by regulating CD44 expression via the EGFR (Zhang, Singh et al. 1996).

Monaghan et al. have shown that increased CD44 expression in response to EGF stimulation plays a significant role in tumor invasion (Monaghan, Mulligan et al. 2000). Mitogenic activation by EGF requires the concomitant functionality of EGFR and the hyaluronan receptor CD44. Also, the increase in migration requires the receptor CD44 and its respective ligand HA (Ellis, Schor et al. 2007). CD44 co-immunoprecipitates with EGFR and ErbB2, and HA promotes CD44/EGFR interaction, EGFR-mediated oncogenic signaling, and resistance to chemotherapy in squamous cell cancer (Wang and Bourguignon 2006). CD44 interaction with EGFR plays a pivotal role in Rho/Ras co-activation, PLC epsilon-Ca^{++} signaling, and Raf/ERK upregulation that are all required for CaMKII-mediated cytoskeleton function in head and neck squamous cell carcinoma progression (Bourguignon, Gilad et al. 2006).

From the standpoint of a clinician, the functional interaction between ErbB proteins, CD44 and hyaluronan has the potential to modify the therapeutic efficacy of ErbB1- and ErbB2-directed therapies. However, much work needs to be done before these experimental observations will make their way into clinical practice.

2.4. Rat Epidermal Keratinocyte (REK) organotypic model

A 3-D Rat Epidermal Keratinocytes (REKs) model is an epidermal organotypic system (MacCallum and Lillie 1990). These REKs form a confluent basal cell layer when cultured over a reconstituted native collagen substratum and then undergo differentiation and stratification when grown lifted at an air liquid interface to form an epidermis.

Initial experiments showed that the basal cell interface with the open collagen substrata exhibited numerous, small cytoplasmic processes extending down into the collagen meshwork. This indicated that the basal keratinocyte cells were unable to
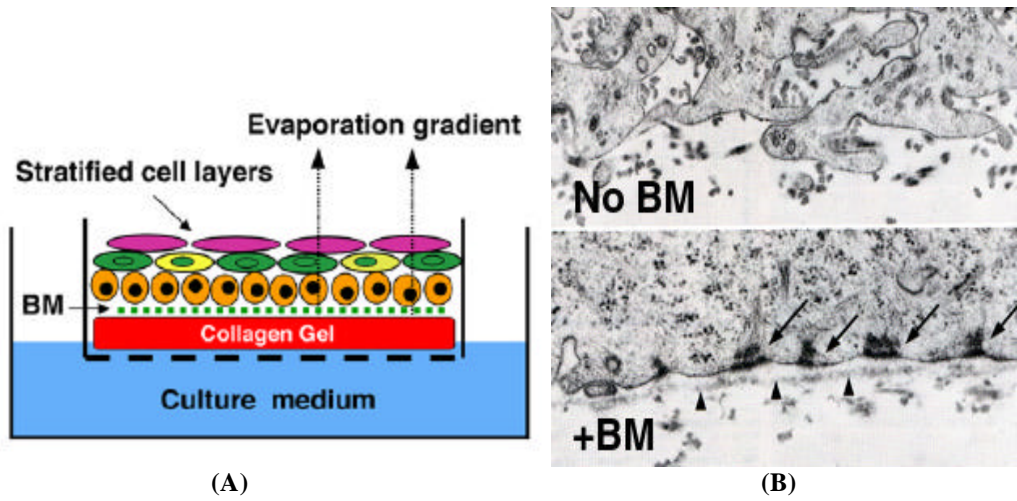
Figure 2-14: A three dimensional organotypic model of the epidermis using rat epidermal keratinocytes grown on a collagen matrix.
(A) Production of the model epithelium involves a two-step process. First, MDCK cells are grown on a polymerized collagen gel (no dermal cells/components) in a Transwell insert for 3 weeks and are then lysed, leaving behind a fully-functional basement membrane (BM, dotted line). Second, immortalized rat epidermal keratinocytes (REKs) are cultured on the BM and allowed to stratify to produce a stratified epidermis including the stratum corneum (see Materials and Methods). (B) Electron Microscopy showing cytoplasmic processes extending into the collagen (No BM) as opposed to the hemidesmosomes and a functional basal lamina (+BM). Adapted from http://www.glycoforum.gr.jp/science/hyaluronan/HA04/HA04E.html.

reconstitute a functional basal lamina and hence to reconstitute a normal subjacent epidermal barrier.

To provide the basement membrane (BM) for the REK model, a renal epithelial cell line named Maden Darby canine kidney (MDCK) cells which is highly-specialized to produce abundant BM components, is grown on the collagen gels for 18 days, then lysed, and the REKs seeded on top of the BM. REK cells cultured on this basal lamina now form a smooth basal cell surface with numerous hemidesmosome attachments typical of an epidermal interface with underlying dermis *in vivo*. These cultures, then, form a true epidermal compartment in culture, bounded by the cornified layer on the surface and the basal lamina beneath the basal cell layer.
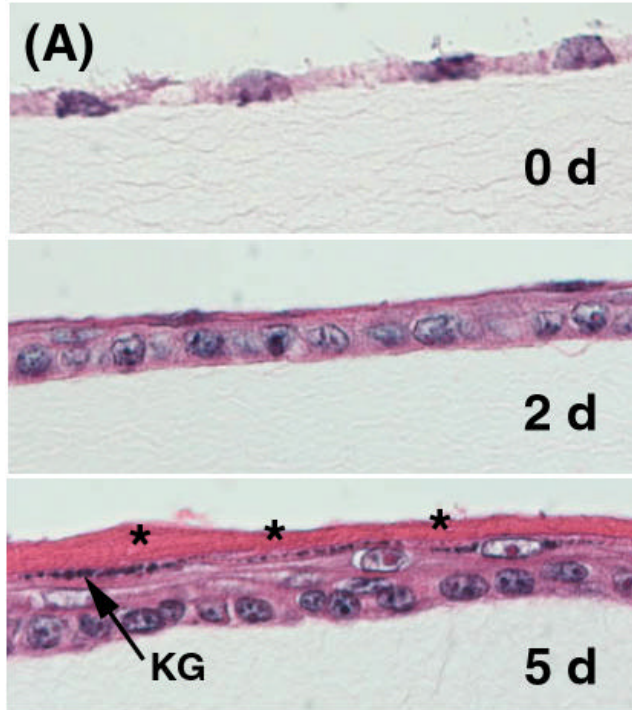
Figure 2-15: Air-lifted REKs stratify on collagen for 5 days and express the specific differentiation markers, like the KG (Keratohyaline granules) and a thick stratum corneum (*). Adapted from our laboratory (Maytin et.al.).

Another seminal feature of the REK system is that keratinocytes can grow and stratify in the complete absence of any paracrine support from fibroblasts. After seeding the REK cells onto a basement membrane previously laid down on collagen by MDCK cells, the REKs are grown to confluence, and then lifted to the air-medium interface. By 2 days the cultures begin to stratify and by 5 days, a number of features associated with terminal differentiation become fully evident. These include the presence of one or two layers of flattened suprabasal cells, abundant keratohyaline granules (KG), and a well-developed stratum corneum (asterisks) (Fig. 2-15). Immunostaining reveals expression of keratin 10 in the suprabasal layers, starting at two days and increasing at 5 days. Another epidermal-specific differentiation marker expressed by the REK cells is filaggrin, a very late differentiation marker that is synthesized as a large precursor protein

profilaggrin, stored in keratohyaline granules, and eventually cleaved into the small peptide subunits as the cells prepare to die and enter the stratum corneum. Immunofluorescence staining with a rat filaggrin antibody shows appropriate expression in the granular layer.

Permeability barrier characteristics of REK organotypic cultures correspond to those in a human skin equivalent model and are similar to normal cadaver skin, as reported in a study, in which the investigators measured transepidermal water loss (TEWL) and corticosterone permeation in REK organotypic cultures (Pasonen-Seppanen, Suhonen et al. 2001). Another study compared the stratum corneum lipid content of the REK system with the corresponding material from human skin. The lipid composition was determined by thin-layer chromatography and mass-spectrometry, and the thermal phase transitions of stratum corneum were studied by differential scanning calorimetry (DSC). All major lipid classes of the stratum corneum were present in REK system in a similar ratio as found in human stratum corneum. Compared to human skin, the level of non-hydroxyacid-sphingosine ceramide was increased in the REKs, while alpha-hydroxyacid-phytosphingosine ceramide and non-hydroxyacid-phytosphingosine ceramides were absent. Also some alterations in fatty acid profiles of REK ceramides were noted, e.g., esterified omega-hydroxyacid-sphingosine contained increased levels of oleic acid instead of linoleic acid. The fraction of lipids covalently bound to corneocyte proteins was distinctly lower in REK compared to human skin, in agreement with the results from DSC. These differences in stratum corneum lipid composition and the thermal phase transitions may explain the minor differences previously observed in drug permeation between REK and human skin (Pappinen, Hermansson et al. 2008).

The potential of REK cultures with a proper stratum corneum barrier as a model for screening skin irritants has been evaluated (Pappinen, Tikkinen et al. 2007). Hence the REK model has barrier properties comparable to human skin, and represents a powerful tool to study cellular responses to epidermal barrier disruption in a well-controlled environment *in vitro*.

Chapter III
Common Methods

3.1. Cell culture

3.1.1. REK (Rat Epidermal Keratinocyte) cell line

The REK clone used in this study was isolated by Donald MacCallum (MacCallum and Lillie 1990) from neonatal rat epidermal keratinocytes (a gift from Howard Baden). The cells were grown in Dulbecco's MEM (1 g glucose/l, 10% fetal bovine serum, 50 mg/ml gentamycin sulfate) at 37 °C in a 5% humidified CO_2 incubator. REKs in monolayer culture were passaged after trypsin release (0.05% trypsin in Ca^{++}, Mg^{++}-free Earle's balanced salt solution plus 0.02% EDTA, buffered to pH 7.4 with 20 mM HEPES); cells were seeded at a 1:6 dilution.

3.1.2. MDCK (Madan Darby Canine Kidney) cell line

MDCK cells (gift of Donald MacCallum, University of Michigan) were grown in Dulbecco's MEM (glucose/l, 10% fetal bovine serum) at 37 °C in a 5% humidified CO_2

incubator. MDCKs in monolayer culture were passaged after trypsin release (0.05%
trypsin in Ca^{++}, Mg^{++}-free Earle's balanced salt solution plus 0.02% EDTA, buffered to
pH 7.4 with 20 mM HEPES); cells were seeded at a 1:5 dilution.

3.1.3. Cell freezing protocol

3.1.3.1. Freezing

One 100 mm dish per Nunc cryovial, 0.5 ml of 10% DMSO+50% serum in
media, which are then sandwiched between styrofoam (to provide insulaion), and later
placed in -70 °C freezer overnight. Next day, these are placed in liquid nitrogen storage.

3.1.3.2. Thawing

The tube is placed directly in warm water, stirring gently, to thaw within 1 min. 1
ml of complete media is added to the tube, which is then to a 25 ml flask containing pre-
warmed media in the incubator.

3.2. REK lift cultures

3.2.1. Time line: To create a model of barrier injury in REK cultures
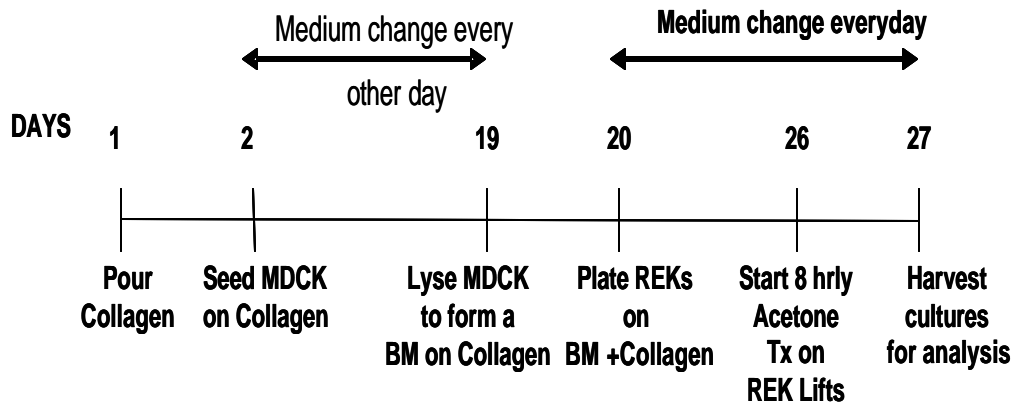
Figure 3-1: Timeline for creating a model of barrier injury in REK cultures.

3.2.2. Preparation of collagen gels

All procedures were carried out on ice, to prevent premature polymerization of the collagen. Commercially available rat tail collagen I (BD Biosciences, Bedford, MA; 3.9 mg/ml) was mixed with Hank's salt solution containing phenol red, and buffered with 20 mM HEPES. The pH was adjusted by adding small (25 ml) aliquots of 1 N sodium hydroxide until a pale orange (approximately pH 7), homogeneous solution was formed, being careful to avoid bubble formation during mixing. The homogeneous solution (800 ml/insert) was added to plastic Transwell inserts (Costar; diameter 2.5 cm; pore size 3.0 mm) housed in 6-well cluster plates. Polymerized collagen fibrils were formed after incubation at 37 °C for 2 h in the 5% CO_2 incubator. Collagen gels, once formed, were stored immersed in PBS with 50 mg/ml gentamycin sulfate at 4 °C until use.

3.2.3. Preparation of basement membranes

Before use, the gels were soaked thrice in PBS at room temperature over one hour, followed by three washes in complete MDCK medium for 20 min each, at 37 °C in the incubator. MDCK cells (gift of Donald MacCallum, University of Michigan) were seeded onto the collagen gels at a high density (200,000 cells/2.5 cm insert) in DMEM/10% FBS. The cells were allowed to grow for 21 days and the media changed thrice weekly. The cells were removed by detergent lysis and washed. Briefly, cells were immersed in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, with 0.1% bovine serum albumin and 0.1 mM $CaCl_2$) for 10 min at 37 °C in a humidified atmosphere. They were then treated with 0.2% deoxycholate in hypotonic lysis buffer twice for 5 min, after which complete removal of adherent MDCK cells was monitored by light microscopy.

Collagen gels with denuded basement membranes on their surface were stored at 4°C immersed in PBS with 50 mg/ml gentamycin sulphate until use. Before use, the gels were soaked in PBS thrice for 20 minutes at room temperature followed by a soak in DMEM thrice for 20 min, at 37 °C in the incubator.

3.2.4. Establishment of organotypic cultures

Before use, the gels were soaked thrice in PBS at room temperature over one hour, followed by three washes in complete REK medium for 20 min each, at 37 °C in the incubator. REKs growing in subconfluent monolayers without signs of stratification were trypsinized and seeded onto inserts containing a collagen-basement membrane at a density of 200,000 to 250,000 cells per 2.5 cm insert. The seeded REKs were then immersed for 48 h in DMEM/10% FBS present both in the upper and lower chambers of the Transwell. When the cells reached confluence, the medium was removed from the upper chamber to expose the cells to air. The medium in the lower chamber was changed daily (3.2 ml) for the duration of the experiment. Typically, studies were carried out for 5 days at 37 °C in the 5% CO_2 incubator, with Transwells harvested on the 5th day post lift.

3.2.5. Acetone treatment of REK lifts

Acetone (99.7 %, Fischer Scientific) was filtered through a 0.22 um filter and used for creating barrier injury in REK lift cultures. Acetone was applied directly to the surface of the air lifted REK cultures as 470 µl aliquots, left on for 30 seconds. Beginning on day 4 post lift, the first treatment was applied, and then at 8 h intervals after that, a second, third, and fourth aliquot was applied. Cultures were harvested on the 5th day.

3.2.6. Harvesting REK cultures

3.2.6.1. Immunochemistry

The entire REK culture (epithelium, collagen and 3µm membrane) was cut from a single Transwell of a six well plate. This culture was rolled upon itself such that the epithelium faced the inner side of the roll. This roll was further processed as mentioned in section 3.3.1.

3.2.6.2. Flurophore Assisted Carbohydrate Electrophoresis (FACE) analysis

As shown in this Fig. 3-2, individual REK culture was first cut from the Transwell along with the 3 µm polyester membrane. The entire culture was then placed face down on a blotting paper. Another blotting paper was placed on the top of the inverted culture, making a sandwich. A pair of tweezers was used to gently remove the collagen from the epithelium. The epithelium which was stuck to the blotting paper was then cut into smaller pieces and digested with proteinase K for further analysis.
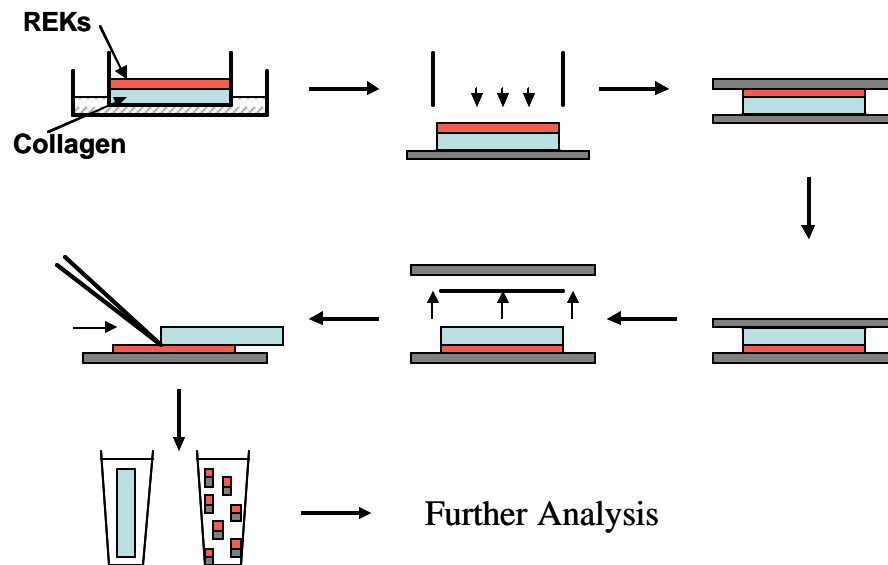
Figure 3-2: Schematic of the separation of epithelium from collagen used for FACE analysis.

3.3. Tissue fixation and stains

3.3.1. Paraffin fixation

5-day old REK lift cultures were fixed in Histochoice Tissue Fixative MB (Amresco, code H120-1L) overnight at 4 °C, dehydrated in isoparaffinic alcohols (Richard-Allan Scientific) in this order; twice in Flex 95 (30 mins each), thrice in Flex 100 (30 mins each) and in Clear-rite3 for an hr. The specimens were then cut from the insert and rolled like a sushi roll, and the roll was held in place by placing a plastic "napkin ring" around the rolled sample. The specimens were placed in a cassette and left in paraffin overnight. Paraffin blocks were made the next day and microtome was used to section specimens (usually 5 microns thick). These sections were placed on the slides and left overnight to dry on a slide warmer at   40 °C.

3.3.2. Frozen sections

REK lift cultures were harvested and prepared for frozen sections using OCT. 5µm sections were cut, and stored at 4 deg C for 10mins and allowed to dry with the use of a hair dryer set at cool air. These sections were then fixed in cold methanol for a total of ten minutes, followed by PBS wash and mounted in Vectashield.

3.3.3. H&E staining

H&E staining was done as per the standard protocols followed in the laboratory.

3.4. Immunohistochemistry for HA

3.4.1.1.　Timeline: Protocol for immuno-histochemistry in the REK organotypic model of barrier injury

DAYS 1   2   19   20   26   27

Pour   Seed MDCK   Lyse MDCK   Plate REKs   Start 8   Harvest
Collagen on Collagen to form a   on BM   hrly   cultures
                       basement   +Collagen   Acetone   for analysis
                       membrane   Tx on
                       on Collagen   REK Lifts

DAYS 28   30   31   32   33   34   35   36-38

Tissue   Processing   Embedding   Sectioning H&E   Immuno-   Microscopy   Image
fixation in of   the   of   staining staining   Analysis
Histochoice specimens   specimens   specimens   with
            for   in paraffin at the lab   IPLAB
            embedding   blocks   microtome

Figure 3-3: Time line for H&E or Immunostaining for the work done in this thesis.

3.4.2. Protocol for HA immunohistochemistry in the REK organotypic model of barrier injury

Primary antibody utilized for HA staining was a biotinylated HA-binding protein, from bovine cartilage (1:10, Seikagaku Corp). Sections were mounted in Vectashield (Vector, Inc.), viewed with an Olympus BX50 microscope with epifluorescence attachments, and images were captured digitally using a Polaroid DMC-2 camera. The digital TIFF files were analyzed using IPLab Spectrum Software (Signal Analytics, Vienna, Virginia). To adjust for small differences in background staining between slides from different experiments, a region from the dermis in each slide was traced out, and its mean integrated density was used as a correction factor to normalize the epidermal intensity values across all experiments.

3.5. Flurophore Assisted Carbohydrate Electrophoresis (FACE) analysis

3.5.1. Timeline: FACE analysis in the REK organotypic model of barrier injury

DAYS 1   2   19   20   26   27

Pour Collagen   **Seed MDCK on Collagen**   **Lyse MDCK to form a BM on Collagen**   **Plate REKs on BM +Collagen**   **Start 8 hrly Acetone Tx on REK Lifts**   **Harvest cultures for overnight Proteinase K digestion**

DAYS 28   31   32

FACE Analysis Day 1-4   **Cast gels and run the samples**
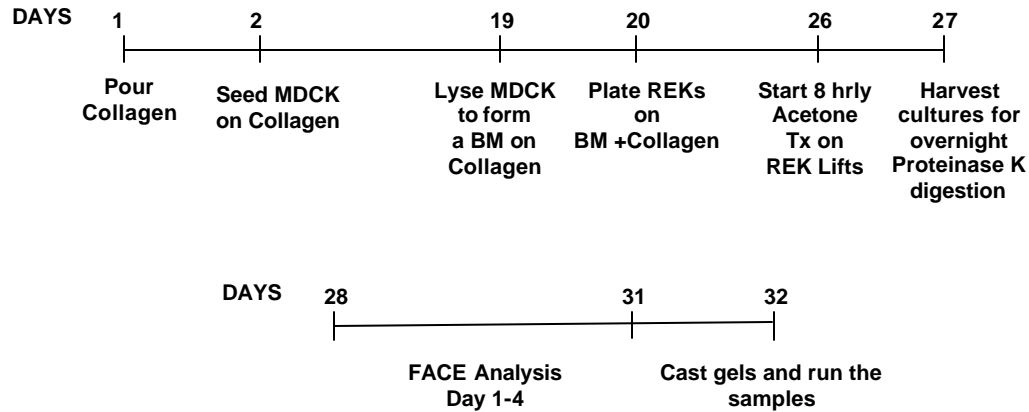
Figure 3-4: Time line: FACE analysis for the work done in this thesis.

3.5.2. Protocol for FACE analysis in the REK organotypic model of barrier injury

For analysis of REK cell layers, the entire polyester membrane and overlying gel was cut from the plastic frame, the cell layers were either carefully separated from the collagen gel, and the cell layer and collagen gels were each digested separately using protease K, (3 U per sample) for 3 h at 60 °C or the entire culture (epithelium and collagen) was used together and digested in proteinase K.

After inactivation of the protease K (100° C for 5 min), samples were precipitated with cold ethanol (1:4 ratio) at minus 20 °C overnight. The resulting pellet was resuspended in ammonium acetate (100 mM, pH 7), digested with hyaluronidase SD from *Streptococcus dysgalactiae* (100 mU/ml, Seikagaku) for 1 h at 37 °C, followed by the addition of chondroitinase ABC from *Proteus vulgaris* (100 mU/ml, Seikagaku) and incubation of 3 h at 37 °C. Samples were then frozen on dry ice, and lyophilized.

End-labelling of the HA digestion products with the fluorescent probe, 2 aminoacridone (AMAC), was performed as follows. To each freeze-dried sample, 40 µl of AMAC (500 nmol) in 85% DMSO/15% acetic acid was added, and the sample

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incubated for 15 min at room temperature. Then, 40 µl of sodium cyanoborohydride (1.25M) in ultrapure water was added, and the mixture incubated for 16 h at 37 °C. At completion of this derivatization reaction, 20 µl of glycerol (20% final conc.) was added to each sample prior to electrophoresis. For stroage, samples were kept in the dark at minus 80 °C. If samples formed a precipitate during storage, they were heated to 60 °C for 5-10 min. For electrophoretic analysis, samples were run on high-percentage acrylamide gels (Monogel, from GLYKO; 1 h at 500 V, cooled) and the resulting bands illuminated under UV light (365 nm) for image-capture and analysis via a Quantix CCD camera, as described in Calabro et al. (20000, 273-281).

Ref. Calabro, Hascall, & Midura. Glycobiology 10(3): 283-293, 1000. (for tissue extraction). *Ref*. Calabro et al, Glycobiology, 10 (3): 273-281, 2000. (for the AMAC derivatization).

3.6.   Data Analysis

3.6.1. Image processing using IPLab[TM]

The immunostained sections were viewed with an Olympus BX50 microscope with epifluorescence attachments, and images were captured digitally using a Polaroid DMC-2 camera. The sections were digitally captured along the length of the REK cultures in a random sequence. The digital TIFF files were analyzed using IPLab Spectrum Software (Signal Analytics, Vienna, Virginia). IPLab was used to count the total signal intensity as a sum of pixel units through out the region of interest (epidermis on the basement membrane) per each section/digital image. Since all epidermal sections in the TIFF file were of a different length, a free form tool was used to measure the basement membrane length of each section. Per individual experiment (a set of control

and experimental samples), one length (largest basement membrane length) was used to normalize the signal intensity across the specimens. To adjust for small differences in background staining between slides from different experiments, a region from the dermis in each slide was traced out, and its mean integrated density was used as a correction factor to normalize the epidermal intensity values across all experiments.

3.6.2. Statistical Tests

For most of the experiments analyses were done by using students T-test. In those experiments we wanted to compare the means of two independent groups (uninjured *vs* acetone injured cultures) and assess whether they were statistically different from each other.

One way Analysis Of Variance (ANOVA- Minitab statistical software from http://www.minitab.com/) was used for the data when it was required to compare the means across more than two independent groups (uninjured *vs* graded form of acetone injury).

Chapter IV
In Vitro 3D Model Of Epidermal Barrier Injury

4.1.   Introduction

In the skin, epidermal barrier function is maintained by the stratum corneum that represents the end result of programmed keratinocyte differentiation, and is composed of terminally-differentiated keratinocytes interspersed with intercellular lipids (mainly ceramides and sphingolipids) (Lampe, Williams et al. 1983). The barrier function is absolutely essential for protecting the body from external environmental pathogens, UV radiation, and noxious chemicals, as well as for preventing water loss and aiding in thermoregulation. Barrier function can be perturbed in a number of ways, through direct physical injury and indirect physiological responses. For example, severe *xerosis* (dryness) is characterized by redness, fissuring, scaling, and itching, and results from an inflammatory cascade triggered by cytokines released from the epidermis in response to barrier disruption (Elias, Wood et al. 1999). In atopic dermatitis, skin barrier disruption alone may set up a chronic inflammatory condition which leads to the pathological

manifestations of dermatitis (Proksch, Holleran et al. 1993). Barrier function can be impaired by stress-induced changes in hormonal profiles, for example by adrenal glucocorticoids that reduce the production of lipids and intercellular lamellae in the stratum corneum (Denda, Tsuchiya et al. 2000).

In the case of acute or subacute perturbations of the skin, e.g., by surfactants, tape stripping, or organic solvents, a rapid recovery phase occurs over the first few hours, which is followed by a period of slower recovery that requires several hours in order to complete barrier restoration. Studies with experimental barrier disruption have revealed a number of changes that appear to play a role in barrier activity including: (i) Release of cytokines that mediate the inflammatory response and may be implicated in lipid synthesis and DNA repair (Lampe, Williams et al. 1983; Elias and Feingold 2001) (ii) Changes in the concentrations of calcium and potassium ions that regulate the formation of lamellar bodies and keratinocyte differentiation (Menon and Elias 1991; Elias, Wood et al. 1999) (iii) Increases in hyaluronan in the matrix that help to regulate the hyperplastic response seen *in vivo* (Maytin, Chung et al. 2004). However, the relative importance for each of these factors in stimulating and coordinating the restoration of stratum corneum function, is an important and unresolved question.

Studies to understand the molecular mechanisms that are involved in the response to barrier injury can be inherently difficult in the intact skin, since interactions between epidermis, dermis, vascular elements, and nerve endings are difficult to dissect. Hence, a simplified model to study the response of the epidermis to barrier injury, in a well controlled setting *in vitro*, would be highly desirable. As discussed before, permeability

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barrier characteristics of REK organotypic cultures correspond to those in a human skin equivalent model and are similar to normal cadaver skin, as reported in a study by Pasonen et. al, in which the investigators measured transepidermal water loss (TEWL) and corticosterone permeation in REK organotypic cultures (Pasonen-Seppanen, Suhonen et al. 2001). Hence the REK model has barrier properties comparable to human skin, and represents a powerful tool to study responses to epidermal barrier disruption in a well-controlled environment *in vitro*. Another seminal feature of the REK system is that keratinocytes can grow and stratify in the complete absence of any paracrine support from fibroblasts.

Studies by us (Maytin, Chung et al. 2004) and others (Man, Feingold et al. 1993; Denda, Wood et al. 1996) showed that acetone, when applied to the skin of mice, disrupts the barrier (stratum corneum) and causes an epidermal hyperplastic response. The goal of the current study was to test whether superficial barrier disruption of the REK model would induce an epidermal hyperplasia response *in vitro*, in the absence of any dermal involvement (since the system has no fibroblasts, only keratinocytes). To ask this question, the integrity of the stratum corneum in REK organotypic cultures was disrupted by the repeated application of an organic solvent (acetone). The results show that even in a purely epidermal system, barrier disruption induces a hyperplastic thickening, accompanied by moderate increases in keratinocyte proliferation. Unexpectedly, at higher levels of damage to the barrier a strong increase in keratinocyte differentiation and cornification was observed. These findings imply that the ability of skin to mount a restorative response to barrier injury is an intrinsic property of the epidermal compartment.

4.2. Methods

4.2.1. Immunohistochemistry

5-day old REK lift cultures were fixed in Histochoice Tissue Fixative MB (Amresco, code H120-1L) overnight at 4 °C, dehydrated in isoparaffinic alcohols (Richard-Allan Scientific), and embedded in paraffin. 5-µm sections were cut and then dried overnight at 40 °C. H&E staining and immunohistochemistry were done using standard protocols. Primary antibodies utilized in this study were polyclonal antibodies including rabbit anti-mouse K10 (1:500, BABCO), goat anti-rat filaggrin (1:2000, a generous gift from Dr. Beverly Dale-Crunk) and rabbit monoclonal Ki67 (1:200, NeoMarker, Inc.). Sections were mounted in Vectashield (Vector, Inc.), viewed with an Olympus BX50 microscope with epifluorescence attachments, and images were captured digitally using a Polaroid DMC-2 camera. The digital TIFF files were analyzed using IPLab Spectrum Software (Signal Analytics, Vienna, Virginia). To adjust for small differences in background staining between slides from different experiments, a region from the dermis in each slide was traced out, and its mean integrated density was used as a correction factor to normalize the epidermal intensity values across all experiments.

4.2.2. BrdU uptake studies

At 5-day post lift, medium containing 1:1000 dilution of Bromo-deoxyuridine (BrdU, Amersham Biosciences) was added to the bottom well of the cultures, which were then incubated an additional 2 h and mounted in paraffin. 5 µm sections were cut and dried overnight at 40 °C. The primary antibody utilized in this study was monoclonal anti-mouse NCL-BrdU (1:100, Novocastra Labs). Sections were mounted in Vectamount (Vector Inc.), viewed with an Olympus BX50 microscope, and images captured digitally

using a Polaroid DMC-2 camera system. Identical exposure times were used for all images captured in a given series.

4.2.3. Fluorescein-Cadaverine uptake studies

At 5-days post-lift, medium containing 100 µM fluorescein-cadaverine (CadavF, Molecular Probes) or an equimolar amount of fluorescein (Molecular Probes) was added to the bottom well of the cultures, which were then incubated an additional 4 h and mounted in OTC (Electron Microscopy Sciences). 5 µm frozen sections were air-dried and fixed in methanol. Sections were mounted in Vectashield, viewed with an Olympus BX50 microscope, and images captured using a Polaroid DMC-2 camera system.

4.2.4. Western blot

The REK layer was peeled away from the collagen matrix and stored at -20 deg C. Cells were then centrifuged at 5000 rpm at 4 °C for 1 min followed by a wash with ice-cold PBS. Cells in lysis buffer (7 M urea, 2% IGEPAL, 5% ß-mercaptoethanol) were lysed with three 4 s bursts using an ultrasonic probe. Equal quantities of protein, along with prestained molecular size markers (BioRad), were resolved on a 12% SDS-PAGE gel and transferred to PVDF membranes (Immobilon, Millipore Corp). Western blotting with rabbit polyclonal antisera specific for K10 (1:10,000) and filaggrin (1:10,000) was done as described by Sinha, et.al. (Sinha, Anand et al. 2006). Blots were incubated with peroxidase-conjugated goat anti-rabbit IgG and developed using enhanced chemiluminescence reagents (ECL kit, Amersham) followed by exposure to X-ray film (Biomax, Kodak).

4.3. Results

4.3.1. Q-Tip method of acetone application to create barrier injury

REK cultures were air lifted for 5 days in a six well Transwell plate. Beginning on day 3, a Q tip soaked in acetone was gently swabbed across the entire culture twice a day for three days. These cultures were harvested at day 5; paraffin embedded and sectioned for H&E. Image analysis was done to check for changes in the REK tissue. Surprisingly, this method of injury did not show any significant change in tissue thickness, cell number or the overall morphology of injured *vs* uninjured REK cultures. The experiment was repeated twice with similar results.

4.3.2. Direct application of acetone to create barrier injury

Acetone (99.7 %, Fischer Scientific) was filtered through a 0.22 μm filter and applied directly to the surface of the air lifted REK cultures as 470 µl aliquots, left on for 30 seconds. Beginning on day 4 post lift, the first treatment was applied, and then at 8 h intervals after that, a second, third, and fourth aliquot was variably applied. Cultures were harvested on the 5th day.

The tissue was paraffin embedded and sectioned for H&E stain. Image analysis was done to check for any changes in the REK tissue. Interestingly, this method of injury showed significant change in the tissue thickness, cell number as well as the overall morphology of the injured *vs* uninjured REK cultures. The experiment was repeated thrice with similar results.

4.3.3. Acetone treatment affects tissue in a temporally- and spatially-dependent fashion in REK lift cultures

In order to measure the effect of acetone on cell viability, 470 µl of acetone was added to REK cultures for successive time intervals of 30, 90 and 180 seconds and harvested the cultures at 24 hours. Results are shown in Fig. 4-1. H&E stained sections that were exposed to acetone for 30 seconds showed a slight increase in tissue thickness (data not shown) whereas increasing time spans of acetone exposure (90, and 180 seconds) caused greater thickening at the centre of the cultures, but also a marked
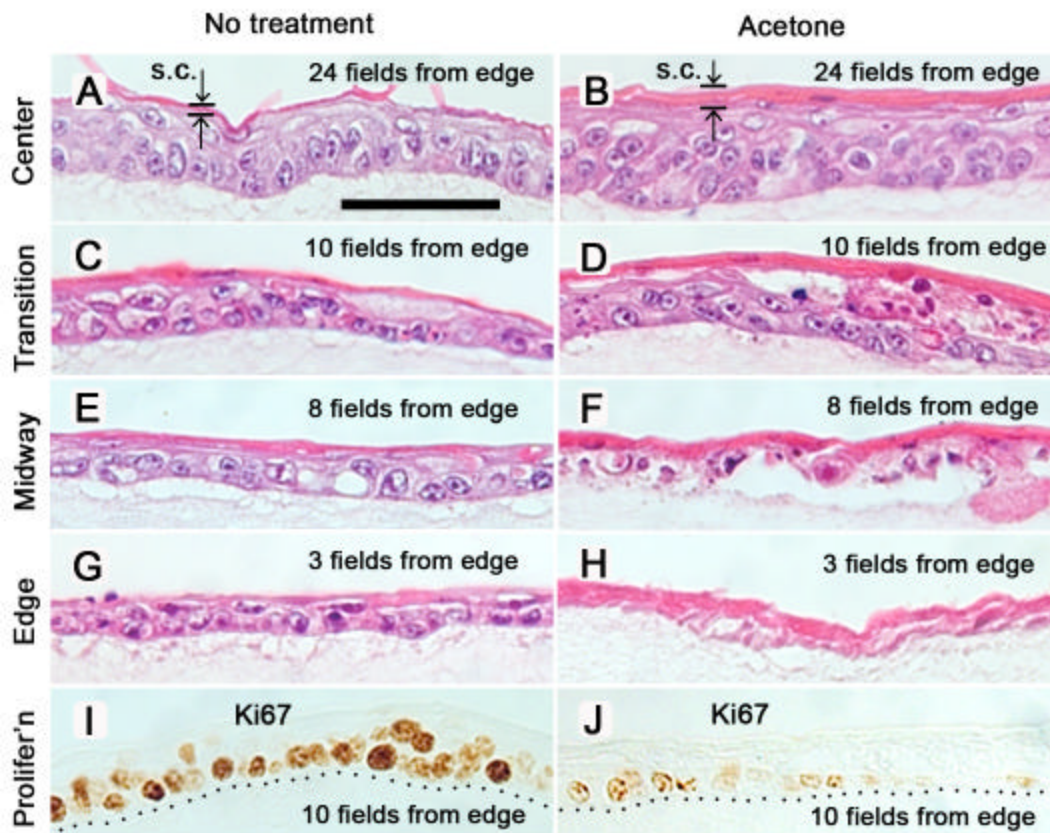
Figure 4-1: Epidermal hyperplasia in response to acetone varies significantly along the length of the specimen.
Representative Hematoxylin and eosin stained sections from untreated REK cultures on the *left* (A, C, E, and G), and acetone treated on the *right* (B, D, F, and H). The vertical arrows with horizontal bars denote the stratum corneum (*s.c.*). Note the thicker stratum corneum as well as the epidermal thickness in acetone treated samples (cumulative exposure of 90 seconds) as opposed to controls (A and B). The sections are from the center of the specimen (A and B), transitional zone (C and D), midway between the edge and transition zone (E and F), and from the edge (G and H). Ki67 stain (as described in Methods) are from REK cultures without treatment (I) and following treatment with acetone (J). *Dotted lines,* dermal‑epidermal junction. *Scale bar,* 50 µm.

decrease in viable cell numbers at the edge (Fig. 4-2A). In order to check for cell

proliferation, the sections were stained with an antibody to Ki67. At 30 sec of acetone

exposure, Ki67-positive cell numbers were comparable to control, but at 90 and 180 sec,

the Ki67 staining was reduced, indicating an increase in acetone toxicity and a decrease

in cell viability (Fig. 4-2B).In the acetone-treated sample illustrated in Fig. 4-1(A to H),

an overall increase in tissue thickness relative to controls was observed, but the response

differed along the length of the specimen. The maximal effect was seen in the central

region which contained the healthiest cells (approx. 24 +/- 3 microscopic fields from the

edge). A transitional zone was evident in the acetone samples at ~10 +/- 3 fields from the

edge; cell death appeared in acetone-treated samples but not in controls. As one
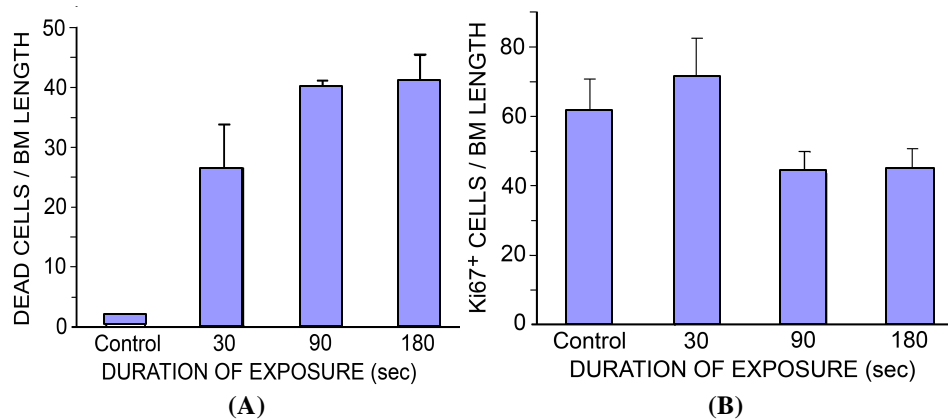
Figure 4-2: Analysis of acetone induced injury and cell death in REK organotypic cultures.

(A) Analysis of cell death in acetone-treated REK organotypic cultures. REK cultures were treated with successive exposures to acetone of 30, 90 and 180 seconds as a form of graded injury over time. Paraffin sections stained with hematoxylin and eosin were randomly selected from control and acetone-treated samples, and dead cells were counted across the entire length of the specimen and normalized to the basement membrane (*BM*) length. Each bar represents the mean +/- SD of eight high power fields (magnification, 200x).

(**B**), **Analysis of viable cell numbers in acetone-treated REK organotypic cultures.** REK cultures were treated with successive exposures to acetone as a form of graded injury and stained with anti-Ki67 antibody. Ki67-positive cell counts were counted and normalized to the basement membrane length. Each bar represents the mean $^+$/- SD of eight high power fields.

approached the edge of the specimen, acetone-treated cultures exhibited complete cell death whereas untreated controls retained a countable number of viable cells (Fig. 4-2A, 4-2B). By Ki67 staining, a suppression of proliferation in acetone-treated specimens began at ~10 fields from the edge, roughly corresponding to the transition zone seen by H&E staining. Hence, for quantification purposes in subsequent analyses, only the viable tissue that began approximately 10 fields from the edge of the specimens for both untreated and acetone-treated samples.

4.3.4. Acetone treatment results in epidermal hyperplasia with stratum corneum thickening

Acetone exposures longer than 30 sec caused unacceptable amounts of cell death (Fig. 4-2A). Hence, for subsequent experiments it was decided to look at the graded effect of acetone, delivered as multiple applications of acetone but with each application limited to the relatively nontoxic condition of 30 sec. This design yielded two benefits: (i) it avoided extensive cell damage (especially at the center of the cultures), and (ii) it mimicked the *in vivo* work, wherein acetone was applied twice daily over a one-week period to induce barrier injury in mouse skin (Maytin, Chung et al. 2004). Acetone was applied at different frequencies to air lifted cultures on day 4 and 5 post lift (as described in Common Methods), and then the samples were analyzed as frozen or paraffin- fixed sections to assess changes in epidermal parameters. In H&E stained sections, a significant epidermal hyperplastic response following two and three acetone exposures was observed (*compare* Figs 4-3A, C, and D, respectively). A marked increase in the total cell numbers was observed in the acetone-treated samples, and was significantly different from control for two-acetone and three-acetone exposures as determined by
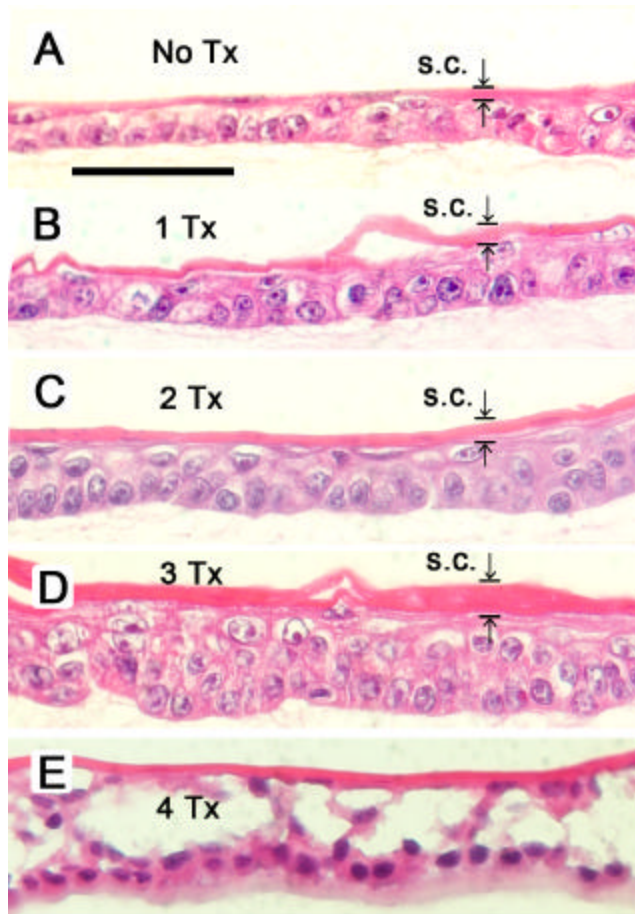
Figure 4-3: Hyperplastic response of 3-D organotypic raft cultures to acetone injury.
(A-E) Appearance of the model tissue in the absence of acetone (A), or in response to 1Tx, 2Tx, 3Tx, and 4Tx-treatments (B-E, respectively) (*Scale bar*, 50 μm). Samples were fixed in paraffin and the sections were cut and stained with the routine H&E stains. The stratum corneum became markedly thickened in response to acetone injury, as observed in (D).

Student's t-test (Fig. 4-4A). A few apoptotic cells after three-acetone treatments were observed, whereas nearly-complete cell death was observed after four treatments (Fig. 4-3E). The stratum corneum was significantly thickened in response to acetone injury, as observed on the H&E stains (Fig. 4-3C and D) and in the plotted data (Fig. 4-4B) of three different experiments.
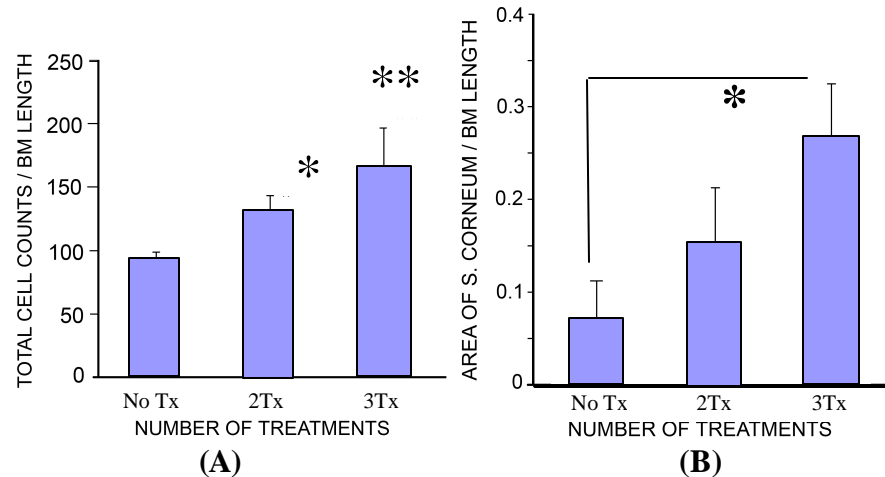
Figure 4-4: Quantitative analysis of viable cell counts and stratum corneum thickness from three different experiments.
(A) Total cell count. Paraffin sections were stained with Hematoxylin and eosin, microscopic images photographed, and viable cell counts measured using IPLab. No Tx=Uninjured, 2Tx= two acetone and 3Tx= three acetone treatments. Data are pooled from three different experiments on REK lift cultures. *Asterisks,* difference with respect to control, significant by student's t-test, P<0.05 (**) and P<0.025 (*).

(B) Stratum corneum thickness. Paraffin sections were stained with Hematoxylin and eosin, microscopic images photographed, and stratum corneum thickness evaluated using the image processing technique IPLab. No Tx=Uninjured, 2Tx= two acetone and 3Tx= three acetone treatments. The epidermal thickness is normalized to the basement membrane length in arbitrary pixel units. *Asterisk,* significance compared to control by student's t-test, P<0.025 (*).

Also, the thickened stratum corneum appeared more compact in comparison to the wavy and lacy consistency seen in control cultures.

4.3.5. Acetone treatment increases cellular proliferation only at intermediate levels of barrier injury

Given the apparent overall thickening of the acetone treated lift cultures, it was questioned whether increased cellular proliferation *versus* decreased cellular loss (via retention of cornified keratinocytes) might be responsible for the observed epidermal thickening. To evaluate the effect of acetone on cell division, 5-day lift tissue was stained with the proliferation marker, Ki67. A significant increase in the number of proliferating
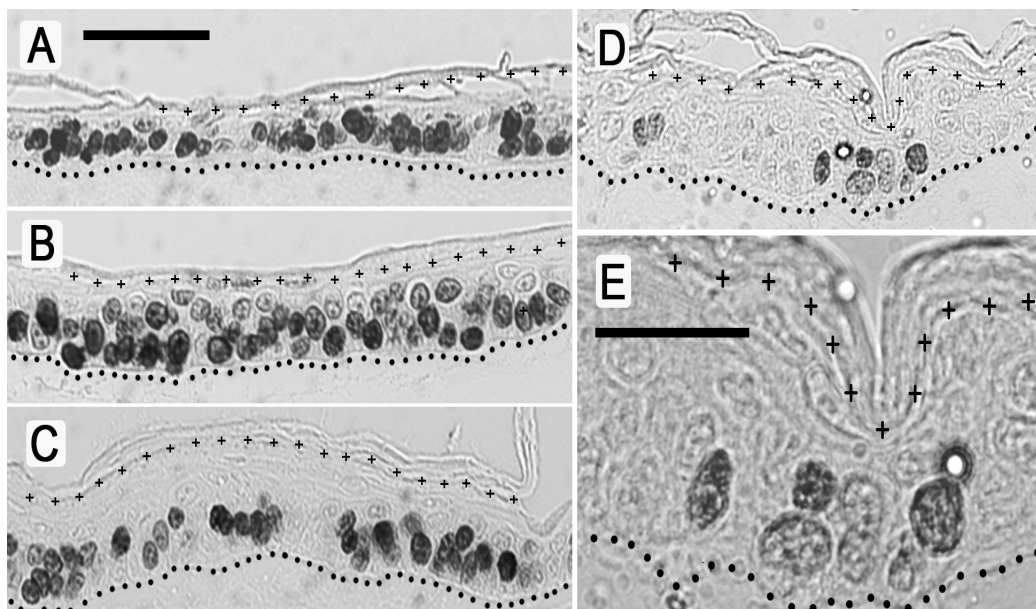
Figure 4-5: Enhanced cellular proliferation, as revealed by increased Ki67 and BrdU stains in REK organotypic cultures following treatment with acetone.
Acetone-treated samples were sectioned and stained with Ki67 stain (A-C) and BrdU (D-E): (performed as described in Common Methods). Representative Images from the center of each culture are shown. (A), No acetone treatment. (B), REK cultures that received two acetone treatments, or (C), three acetone treatments. (D), REK cultures given three acetone treatments and stained with BrdU. (E), High power view (magnification, x400) to illustrate BrdU-stained cell clusters as described in Results. *Dotted lines,* dermal-epidermal junction. *Crosses,* stratum corneum.

cells was observed after two acetone exposures, as quantified by counts of Ki67-positive cells (Fig. 4-6A) which was significant at P < 0.01 by Student's t-test. Another interesting finding was an increase in Ki67 stained cells in the suprabasal layers of these cultures (Fig. 4-5B) as opposed to the untreated samples.

Ki67 is a marker that is present in cells during essentially all phases (G1-, S-, M- and G2) of the cell cycle (Borue, Lee et al. 2004). For a more stringent study to detect newly proliferating cells, it was decided to perform BrdU labeling, with BrdU added 2 hrs prior to the harvest of the REK lift cultures. BRDU is a marker of proliferating cells, such that the cells stain with BRDU only during the 'S- synthetic' phase of their cell cycle. In two-acetone samples, a significant increase in the number of proliferating cells

was observed (Fig. 4-6B). This data corresponded well with the proliferation seen with Ki67 staining. In three-exposure samples, however, the result was different than at two exposures (and confirmed the Ki67 data). Namely, despite tissue thickening and a measurable increase in total cell numbers, the relative number of BrdU stained cells was decreased in the three-acetone specimens (Fig. 4-6B). The data from Ki-67 and BRDU study showed that the two-acetone treated samples showed a significant increase in cellular proliferation as compared to untreated cultures.
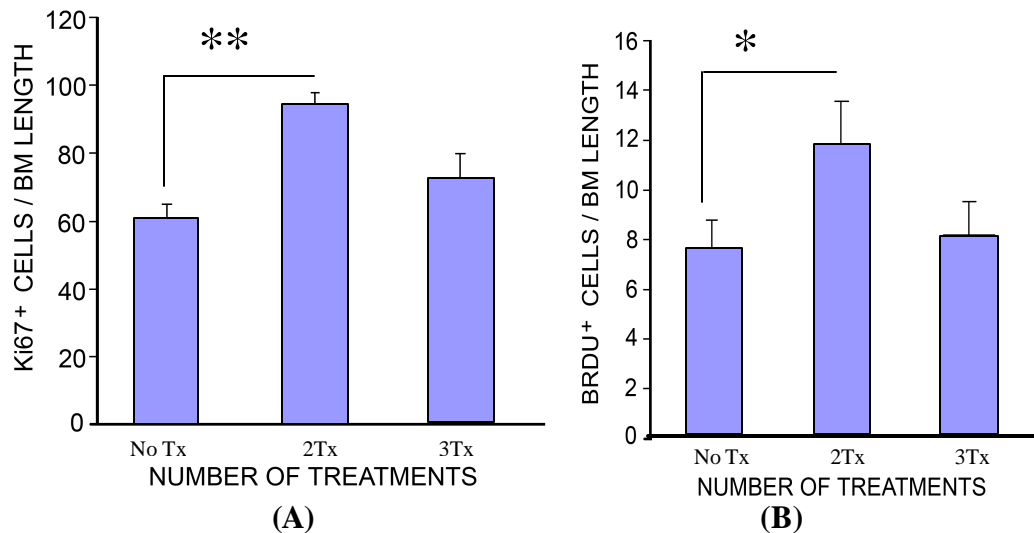
Figure 4-6: Analysis of cells stained with Ki67 and BRDU in organotypic REK cultures.

(A), Analysis of cells stained with Ki67 following treatment with acetone. Paraffin sections were stained with Ki67, microscopic images photographed, and positive cell counts evaluated using the image processing technique IPLab. No Tx=Uninjured, 2Tx= two acetone and 3Tx= three acetone treatments. Data are pooled from three different sets of experiments on REK lift cultures. Asterisks, an increase in proliferation in the two-acetone treated samples is significant at P < 0.01 (**) by Student's t test.

(B) Analysis of cells stained with BrdU in organotypic REK cultures following treatment with acetone. BrdU uptake was studied in REK organotypic cultures treated with acetone to quantify the changes in cell proliferation. Samples were fixed in paraffin and images were captured for further analysis IPLab as described in common methods. No Tx =Uninjured, 2Tx= two acetone and 3Tx= three acetone treatments. Data are pooled from three different sets of experiments on REK lift cultures. Asterisk, an increase in proliferation in the two-acetone treated samples is significant at P < 0.025 (*) by Student's t test

4.3.6. Acetone treatment leads to increases in early (K10) and late (Filaggrin) markers of epidermal differentiation

Terminal differentiation were significantly increased in a murine model of acetone induced epidermal hyperplasia (Maytin, Chung et al. 2004). To examine these markers in our organotypic system, REK lift cultures were immunostained with the early differentiation marker keratin 10 (Fig. 4-7A, B and C) and with the late differentiation marker filaggrin (Fig. 4-7D, E and F). In the untreated samples, K10 was seen in the suprabasal and granular layers (Fig. 4-7A) whereas filaggrin staining was weak and confined to the granular layer (Fig. 4-7D).
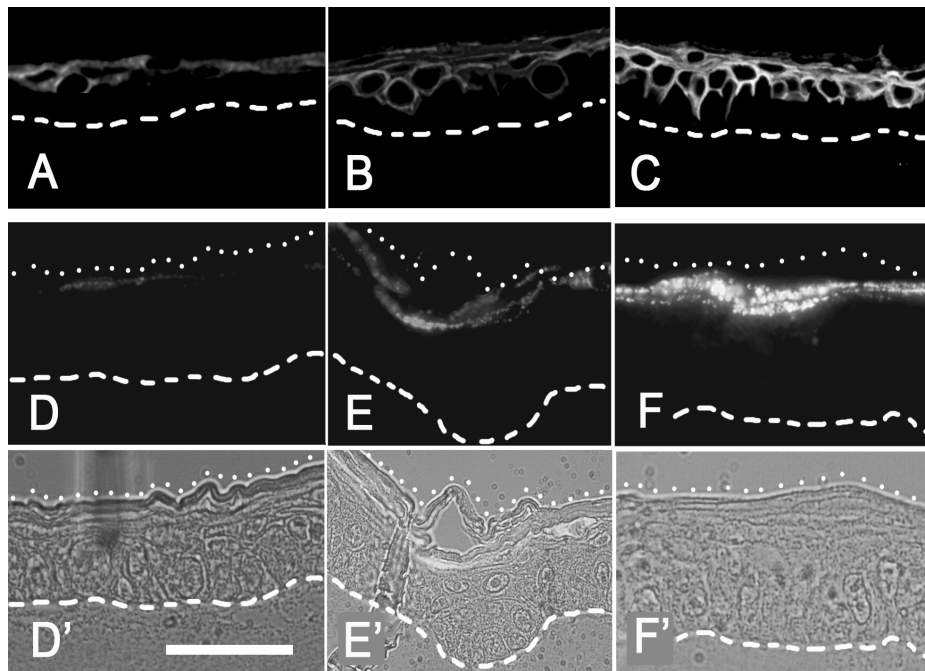
Figure 4-7: Expression of epidermal differentiation markers is significantly increased in REK lift cultures treated with acetone.
Representative sections immunostained for K10 (A-C), Filaggrin (D-F), or visualized by phase contrast (D'-F'). Panels show REK cultures given (A), no acetone treatment. (B), two-acetone treatments, or (C), three-acetone treatments. Filaggrin stained samples (second row) are paired with corresponding phase images (third row). Dashed lines, dermal-epidermal junction. Dotted lines, top of stratum corneum. Scale bar, 50 μm

NUMBER OF ACETONE EXPOSURES

| None | 2x | 3x | None | 2x | 3x | *MW* (kD) |

A Coomassie Blue

B *Keratin 10*
1.0 1.2 1.7
60
50
40

C *Filaggrin*
1.0 0.9 11.0
220
100
60
50
40
30
20
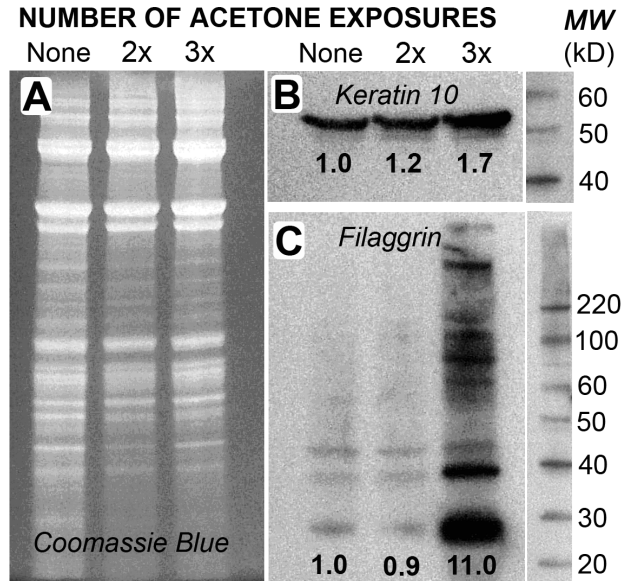
Figure 4-8: Increases in the differentiation markers K10 and filaggrin are confirmed by western blots in acetone-treated REK lift cultures.
Western analysis performed on untreated and acetone-treated samples as described in Materials and Methods. (A), Coomassie stain for the control (None), two (2x), and three (3x) acetone treated REK cultures. (B), Expression of K10, an early marker of differentiation, (C), Filaggrin, a late marker of differentiation, expressed in untreated and acetone treated REK cultures. *MW,* molecular weight of protein standards in kilodaltons. *Numbers* beneath protein bands, relative induction (-fold) over untreated controls, determined by densitometry. Each experiment was repeated twice.

This overall expression pattern agrees with the one observed in mouse skin *in vivo* (Maytin, Lin et al. 1999; Maytin, Chung et al. 2004).

After two acetone exposures minor increases were noted, but after three-acetone exposures the expression levels of K10 (Fig. 4-7C) and filaggrin (Fig. 4-7F) were significantly increased. These changes were confirmed biochemically by western blot experiments (Fig. 4-8), that showed a 1.7-fold increase in K10 (Fig. 4-8B) and an 11-fold increase in filaggrin (Fig. 4-8C). Filaggrin is secreted as a propeptide with identical units each of 29 kDa joined together by linkage sequences which can be clipped by a protease. Thus, on the Western blot we see a ladder of filaggrin protein.

4.3.7. Acetone treatment increases transglutaminase enzyme activity and stratum corneum cross-linking

To determine whether acetone treatment influences transglutaminase (TGase) enzymatic activity, the culture medium was supplemented with Cadaverine fluorescein (CadavF) which serves as a TGase substrate (Mack, Li et al. 2005).

In the REK system, it has been shown that incorporation and cross-linking of CadavF into the tissue is highly specific because an equimolar amount of fluorescein
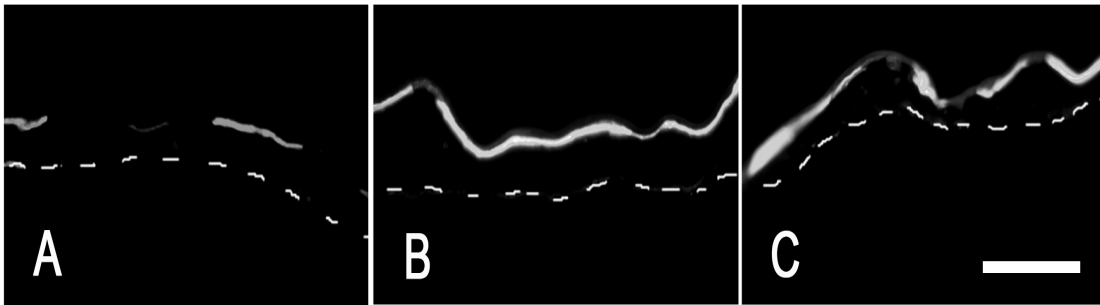
Figure 4-9: Acetone-treated REK cultures show heightened transglutaminase activity. **Representative** immunostained sections for fluorescein-cadaverine, a substrate of transglutaminase that becomes incorporated into the stratum corneum. (A), Control. (B), two-acetone, and (C) three-acetone treated REK cultures. Lift cultures treated with fluorescein alone were negative for incorporation (data not shown). *Dashed lines*, dermal-epidermal junction. The experiment was performed twice with similar results.

alone, added 4h prior to harvest of the lift tissue, gives a negative signal (Mack, Li et al. 2005). For the current experiment, control and acetone-treated samples were evaluated for CadavF incorporation.

4.4. Discussion

This work has shown that it is possible to induce a hyperplastic epidermal response in an organotypic culture system in response to barrier injury. This data suggests that acanthosis (thickening) is an inherent property of the epidermal compartment, and that

repeated barrier injury is required to develop hyperplasia of the epidermis. Perhaps most surprisingly, high levels of barrier injury, instead of promoting a more proliferative phenotype, appears to advance the keratinocyte differentiation program as evident by an increase in transglutaminase-mediated cross-linking and formation of a thickened stratum corneum. This data demonstrate a continuum of response. Thus, after moderate injury (two acetone exposures), the tissue prepares for increased proliferation and activates pathways to replace the dead or injured cells. This includes an observed increase in Ki67 and BrdU labeling (heightened mitotic activity) in the basal and suprabasal layers of the skin equivalents. In response to higher levels of injury (three acetone exposures), a relative suppression of this proliferative phenotype is observed as the tissue seems to move towards differentiation.

Others have presented evidence to support the notion that cells (in this case, keratinocytes) must undergo cell-cycle withdrawal and growth-arrest, before differentiation can proceed and the expression of differentiation-related genes can occur (Saunders and Jetten 1994; Mathay, Giltaire et al. 2008). After low levels of injury (up to two acetone exposures), no appreciable change in the expression of common differentiation markers like K10 and filaggrin was noted in our system at a time when proliferation was maximal in the model epidermis. However, at three exposures, when proliferation was inhibited, there was an induction of K10 and filaggrin protein expression in the cultures (as measured by immunohistochemistry and western blots).

Transglutaminase activity may be a more sensitive indicator of early changes leading to growth-arrest and differentiation. In control cultures, CadavF uptake was discontinuous and discernible only at low levels, whereas after only two acetone

treatments, CadavF incorporation was maximally observed along the full length of the acetone-treated tissue. Overall, the changes in proliferation and differentiation observed in the REK system seem to relate very well to physiological changes that are expected in the intact epidermis.

Another interesting finding is the presence of 'cell clusters' that stain positive for BrdU in the basal layer of our REK cultures after three-exposures to acetone. These small groups of cells may represent transit-amplifying proliferative clones that are adding to the overall thickness of these cultures in the absence of increased overall cellular proliferation. This might help to explain the increase in total thickness of the epidermal equivalents at a time when the rest of the tissue is seen to exhibit pronounced differentiation. There is also a possibility that these cells start rapidly proliferating only in the presence of a sub-maximal injury, which is seen with three-acetone exposures but is not evident at lower doses. If these turn out to be transient amplifying cells or stem cells that are positive for p63 (Pellegrini, Dellambra et al. 2001), exciting possibilities for using the REK/acetone system for improving clinical application of epithelial cultures for cell therapy, and for studying epithelial tumorigenesis, could emerge.

Another study (Koria, Brazeau et al. 2003) has reported changes in gene expression after acute barrier disruption in a tissue-engineered human skin equivalent 'Epiderm', following a single 10-minute exposure to acetone. Using cDNA microarrays, they reported a biphasic epidermal response to barrier injury that featured an up-regulation of a number of genes involved in signal transduction and inflammation early on, followed by genes related to growth and lipid synthesis at a later time. In their experiments, the emphasis was on the development of global gene expression profiles that could be used

as molecular 'signatures' in engineered tissues, to develop criteria for assessing suitability of these tissues for transplantation. Thus, the emphasis was on mRNA levels. This work, in contrast, explores the changes in morphology (measured by histology, immunochemistry and Western analysis) that occur as a result of acetone injury in REK cultures. There is focus upon protein expression, the final endpoint of gene expression. This model differs in another notable way from the previously-mentioned study with 'Epiderm', because the REK model has a well-developed basement membrane with junctional complexes that connect to the basal epidermal layer via hemidesmosomes, anchoring fibrils and filaments. For optimum stability and authentic cell signaling, keratinocytes need to be firmly attached to the basement membrane.

Perhaps the most notable conclusion that is discerned from this study is that the epidermis, by itself, is capable of mounting an appropriate response to barrier injury. The REK 3-D system contains no cellular dermal component, blood vessels, nor nerve endings. Hence, any changes in the proliferation and differentiation program observed after acetone injury in these cultures occurs in the absence of any paracrine or endocrine input. Autocrine influences from the epidermal cells themselves, of course, are likely to play a role in these proliferative and differentiation responses. Understanding the latter response is of particular interest, since terminal differentiation of keratinocytes to build the stratum corneum represents the main contributor to barrier formation in the skin. In summary, this model is an elegantly simple system to observe epidermal changes following barrier disruption in a clearly-defined environment in which dermal influences need not be considered. The presence of a well-developed basement membrane (a requirement of nearly all epithelial tissues *in vivo*) provides an added advantage. The

latter feature of the REK system gives some confidence that the observed responses to superficial injury in these skin equivalents closely mimic the events that occur in the intact epidermis *in vivo*. The REK system should provide a useful, physiologically-relevant *in vitro* model of barrier injury for researchers interested in understanding the responses of keratinocytes to acute or chronic forms of epidermal injury.

Chapter V
Epidermal Barrier Injury Affects Hyaluronan Metabolism

5.1.   Introduction

Hyaluronan (HA), a high molecular weight glycosaminoglycan, is generally regarded as a component of the connective tissue matrix. Recently the importance of HA in epithelial tissues such as the retina of the eye and the epidermis of the skin is increasingly recognized. HA is synthesized by keratinocytes of the basal and spinous layers of the epidermis, where it modulates the micro- and macro environment around these cells. Evidence that HA plays a regulatory role in the epidermis includes the fact that retinoic acid, known to enhance epidermal proliferation and promote a thicker epidermis, also leads to HA accumulation in the epidermis as opposed to hydrocortisone which decreases HA levels and results in a thinner epidermis (Agren, Tammi et al. 1995).

The fundamental observation that drives the work in this thesis is the fact that HA increases dramatically in injured skin (in particular at the epidermal wound edge) (Oksala, Salo et al. 1995) within the first 12-14 h after injury *in vivo*. High amounts of

HA were noted in wound fluids from experimental sheep many years ago (Longaker, Chiu et al. 1991), but our laboratory (Mack, Li et al. 2005) and our collaborators (Saavalainen, Pasonen-Seppanen et al. 2005) have detailed this phenomenon more recently in mouse skin, using newer techniques that can visualize HA histologically in the tissue. High levels of HA in the wound edge epithelium (Oksala, Salo et al. 1995) imply an important function for HA in wound repair and re-epithelialization. The hyaluronan-dependent pericellular coat is known to have multiple important roles, from serving structural and mechanochemical functions (Toole 2004; Lewthwaite, Bastow et al. 2006), to the regulation of cell division and motility, as well as cancer progression and metastasis (Boregowda, Appaiah et al. 2006; Ricciardelli, Russell et al. 2007). Time-lapse studies show that pericellular matrix formation facilitates cell detachment and cell rounding during mitosis supporting tissue hyperplasia/growth (Evanko, Tammi et al. 2007).

Barrier disruption (disruption of the stratum corneum) via repeated applications of acetone, or detergent or via repeated tape stripping is a well-established model for eliciting an epidermal repair response characterized by increased DNA synthesis and epidermal thickening. In the previous studies of superficial barrier injury in mouse skin (twice-daily applications of acetone for 5 days) in our laboratory, a 6-fold increase in hyaluronan that was associated with a 3-fold thickening of the epidermis was detected (Maytin, Chung et al. 2004). Treatment with hyaluronidase led to an increase in differentiation, implying that HA functionally participates in injury-induced hyperplasia. To facilitate further study of the HA molecule and its role in epidermal hyperplasia, a

model of epithelial barrier injury using an organotypic keratinocyte (3-D) model of the epidermis was developed in our laboratory (Ajani, Sato et al. 2007).

The new aspect described here discloses the fact that changes in the physical state of the extracellular matrix, specifically in the status of hyaluronan, can have a profound affect on the behaviour of keratinocytes. The overall goal is to characterize the effect of barrier injury on hyaluronan metabolism, as well as on the quantity and size of the molecule in the tissue engineered organotypic skin model.

5.2. Methods

5.2.1. Schema depicting the method of acetone application to cause barrier injury

1Tx (mild), 2Tx (moderate), 3Tx (severe) and 4Tx (toxic) represent different time-dependent doses of acetone application required to produce a graded form of barrier injury in REK organotypic cultures. The acetone was gently poured on the cultures, allowed to sit on top of the cultures for 30 secs, after which it was manually removed
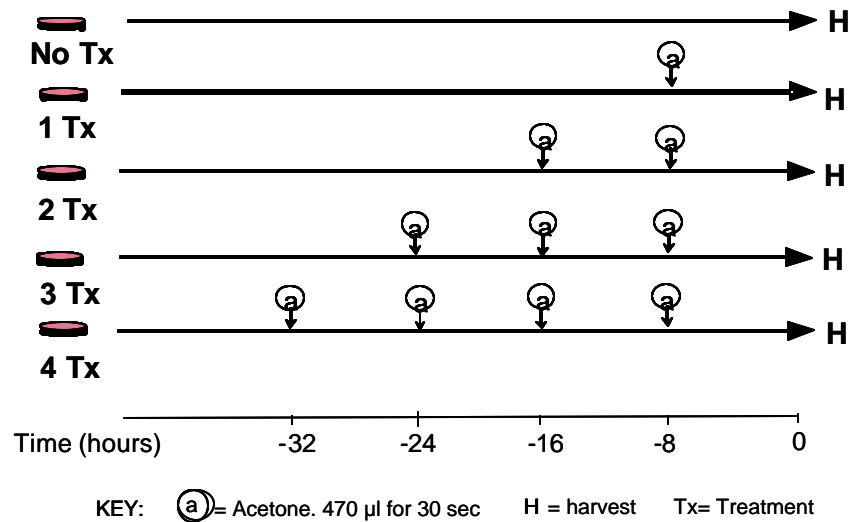
Figure 5-1: Schematic of the acetone treatment used in graded barrier injury experiments.
REK lifts were allowed to grow and stratify for 5 days at an air-liquid interface. On day 4 (32 hrs before harvest), and then every 8 hourly until harvest, a single pulse of 470 µl of acetone was applied for 30 seconds.The medium was changed an hour before the first acetone application i.e., 33 hrs before harvest.

from the cultures with a 500 µl pipette tip. Any residual acetone was allowed to evaporate from the cultures. As these were air-lifted cultures; no washes were given at any time.

5.2.2. HA-polymer molecular size distribution

The entire culture (REK epithelium, collagen and the 3 µm porous membrane) was cut from a Transwell of a six well plate. For each experimental condition, cultures from three Transwells were required to obtain the final signal. The epithelium along with the collagen gel was digested using protease K (3 U per sample) overnight at 60 °C.

After inactivation of the protease K (100° C for 5 min), samples were precipitated with cold ethanol (1:4 ratio) at minus 20 °C overnight. The resulting pellet was resuspended in ammonium acetate (100 mM, pH 7), followed by heat inactivation of proteinase K. Nucleic acids present in the sample were digested by adding DNAse and RNAse at 37 °C overnight. This was followed by ethanol precipitation and resuspension of the samples in ammonium acetate. At this step the total sample (three Transwell cultures) was divided into half, (one and a half Transwell culture), and one half was digested with hyaluronidase SD from Streptococcus dysgalactiae (100 mU/ml, Seikagaku) at 37 °C overnight. The enzyme was heat inactivated by single 5 min incubation in boiling water bath. The samples were resuspended in 1x TAE and left overnight at 4 °C.

A 1% Agarose gel was cast and the gel was pre-run for 6 hrs at 80 V. 2µl of loading buffer was added to the samples, and the entire sample was loaded in one well i.e., one and a half culture (from a six well Transwell plate) was loaded in each well. High and low molecular weight defined HA samples for use as molecular weight

calibration standards (Select HA^{TM}) were obtained from Hyalose L.L.C. (Ohlahoma City, OK). Seakem High gelling temperature (HGT) agarose was obtained from FMC Bioproducts. Stains-All^{TM} dye (3,3'-dimethyl-4,5,4',5'-dibenzothiacarbocyanine) was purchased from Sigma laboratories.

Immediately after the run, the gel was placed in a solution containing 0.005% Stains-All^{TM} in 50% ethanol overnight at room temperature. The stain solution must be protected from light; hence the gel was stained under a light protective cover. For destaining, the gel was transferred to water for an hour. Final destaining occurred upon exposure to ambient room light for 30 mins.

The gels were photographed using EPSON 4850 with a transparency adaptor at a resolution of 600 dpi.

Reference:(Lee and Cowman 1994).

5.2.3. RT-PCR

5.2.3.1. RNA extraction and quantification

REK cultures were harvested as described in section 2.2.8. Total RNA was isolated from cell cultures using TRI reagent (Sigma) by adding 1 ml of Trizol per one sample. 200 µl chloroform (Sigma) was added to each aliquot before mixing by inversion for 15 sec. Samples were incubated for 5 min at RT, then centrifuged with the brake off at 13,000 rpm for 15 min at 4 °C. The aqueous layer (500 µl) was collected and an equal volume of isopropanol (Sigma) was added. Samples were then incubated at –20 °C overnight then centrifuged at 13,000 rpm for 15 min at 4 °C. The precipitated RNA was then washed with 1.5 ml 100% ethanol (Sigma), centrifuged (conditions as above), and the supernatant removed. This wash step was then repeated using 1.5 ml 70% ethanol.

Samples were then air-dried for 1 h at RT before being resuspended in 20 µl double distilled H_2O by vortexing.

5.2.3.2.   Determination of RNA concentration

The concentration of RNA was determined by spectrophotometric analysis using a single beam spectrophotometer (BioRad). 6 µl RNA was diluted in 294 µl H_2O. Absorbance readings at 260 nm were taken. The concentration was then calculated using the following equation:

RNA conc (µg/ml) = Abs_{260} x Molar extinction coefficient x Dilution factor

Molar extinction coefficient for RNA = 40

Dilution factor = 50

5.2.3.3.   cDNA synthesis

Reverse Transcription was carried out with 1 µg total RNA in a total volume of 13 µl comprising 250 ng of random primers (Invitrogen) and 10 mM dNTP mix. The mixture was heated to 65 ℃ for 5 mins then incubated on ice for 2 mins. 1x First Strand Buffer, 0.1 M DTT, 1 µl RNase OUT Recombinant RNAse Inhibitor and 200 units of Superscript III Rnase H reverse transcriptase (all Invitrogen) were then added to the sample. The solution was heated at 25 ℃ for 5 mins followed by 50 ℃ for 60 mins. cDNA was stored at -20 ℃ until needed.

1 µl of cDNA generated in the above method was used per 25 µl of PCR reaction comprising of 5 µl of 5x Go Taq colourless buffer containing 1.5 mM $MgCl_2$ (Promega), 10 mM dNTPs, Go Taq polymerase and 10 µM of each primer. Cycling conditions were as follows.
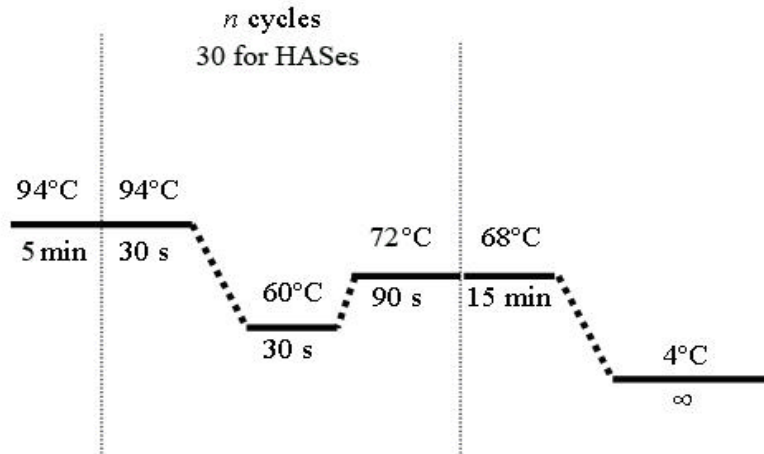
Figure 5-2: Schematic of the PCR cycling program used in RT-PCR experiments.
The number of cycles (n) for HAS 1, 2 and 3 are given

A denaturation step at 94 ℃ for 5 min was followed by n cycles (Table. 5-1), comprising

94 deg C for 30 s, TM ℃ for 30 s (Table. 5-1) and 72 ℃ for 90 s, where n = the number

of amplification cycles that yielded a visible product in the linear range.

Primers	n Cycles	TM ºC
Beta-Actin	30	60
HAS1,2,3	30	60
HYAL 1	35	60
HYAL 2	40	65
HYAL 3	30	65

Table 5-1 Data regarding the primers, n cycles and the annealing temperature (TM) used for the RT-PCR experiments.

RT-PCR primers	Sequence 5'-3'
beta actin-F	TTC TAC AAT GAG CTG CGT GTG
beta actin-R	ATC ACA ATG CCA GTG GTA CG
HAS1- F	GAG GTG GAG GCT GAG GAC
HAS1-R	AGC TGA CCC AAG AGT CCA GA
HAS2-F	CTG GAA GAA CAA CTT CCA TGA
HAS2-R	ATA AGA CTG GCA GGC GGT TT
HAS3-F	CTT CTT TGT GTG GCG TAG CA
HAS3-R	GCA CTT GCT GCC TAC GAA CT
HYAL1-F	CCG TTT GGC TTT AGT TTC CA
HYAL1-R	AGC TTC GCT GCA CAA AAG AG
HYAL2-F	ACA GCC GCA ACT TTG TCA G
HYAL2-R	GTA CTG GCG CTG GGA TTG
HYAL3-F	TGG GGC TTT TAT CGA TAC CC
HYAL3-R	CAG CCT TGG TCA CGT TGA T

Table 5-2: 5'-3' sequence for the RT-PCR primers used for the experiments. F= forward primer; R= reverse primer.

Reaction products were analysed on 2.5% Agarose gels by flat-bed electrophoresis.

5.3. Results

5.3.1. Barrier injury leads to an accumulation of epidermal HA in the 3-D REK organotypic system

Barrier injury of the native skin causes a robust epidermal HA response, with highly elevated levels of HA supporting keratinocyte proliferation (Maytin, Chung et al. 2004). To replicate this phenomenon in the REK model, lift cultures were injured by short-contact acetone treatments as described in section 5.2.1.

5.3.1.1. Increase in epidermal HA by immunohistochemistry in acetone-treated REK lift cultures

To examine HA in the organotypic system, REK lift cultures of barrier injury were immunostained with the biotinylated HA binding protein. In the untreated samples, HA was seen in the basal and suprabasal layers as a very fine stain around the keratinocytes (Fig. 5-3A), as opposed to a marked increase in HA throughout the acetone-injured cultures that was more pronounced in the suprabasal layers (Fig. 5-3B). This overall expression pattern agrees with the one observed in mouse skin *in vivo* (Maytin, Lin et al. 1999; Maytin, Chung et al. 2004). Increases in HA were noted after one and two acetone exposures, but after three-acetone exposures the expression levels of HA were significantly decreased (data not shown).

The specificity of HA staining was confirmed by treating the cultures with
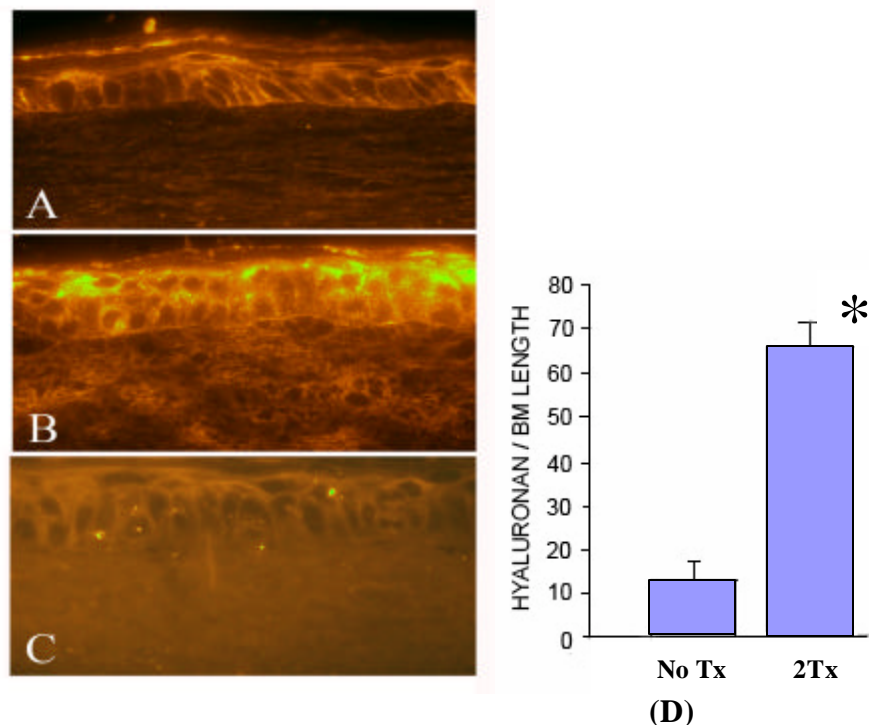
Figure 5-3: Expression of epidermal hyaluronan is significantly increased in REK lift cultures treated with acetone.
Panels show REK cultures given (A), no acetone treatment, (B), two-acetone treatments and (C), two acetone treatments followed by strepH. Paraffin sections were stained with hyaluronan, microscopic images photographed, and quantitative analysis of the HA signal intensity stain was performed using the image processing technique IPLab. No Tx= uninjured and 2Tx= two acetone treamtment. Data are pooled from three different sets of experiments on REK lift cultures. (D) *Asterisks,* an increase in hyaluronan in the two-acetone treated samples is significant at $P < 0.005$ by Student's t test.

Streptomyces Hyaluronidase (Fig. 5-3C), an enzyme which decreased the HA signal to background levels.

Quantification of staining intensity using the IPLab image analysis software, showed a statistically significant increase in HA, as seen in Fig. 5-3D.

5.3.2. An increase in epidermal hyaluronan in acetone-treated REK lift cultures is confirmed by FACE

Biochemical analysis with FACE (described in section 3.5.2) was performed to confirm the increase in HA observed with the immunostaining in REK organotypic cultures. In these samples, the entire culture (epithelium and collagen) was used for HA analysis. The one and two acetone treatments showed a detectable increase in HA as opposed to the uninjured (control) cultures (Fig. 5-4). Three and four treatments showed a decrease in HA, at levels similar or lower than the levels in the uninjured epithelium (Fig. 5-4). The plotted graph in (Fig.5-5) shows a trend in HA levels across different amounts of barrier injury, with a significant increase observed with two acetone treatments.
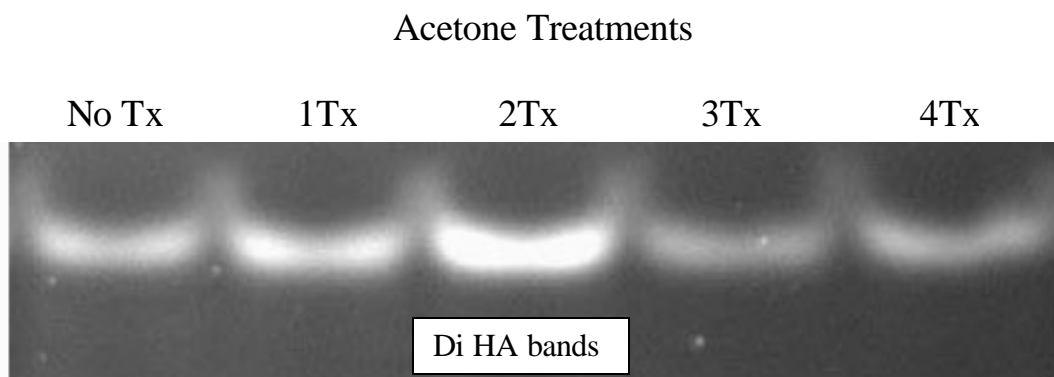
Figure 5-4: Analysis of total HA levels in the acetone treated REK lift cultures.
FACE analysis on REK lifts shows di-HA band for the control/uninjured (None), one (1Tx), two (2Tx), three (3Tx) and four (4Tx) acetone treated cultures. Each experiment was repeated thrice with similar results.

Acetone Dose Response for HA accumulation
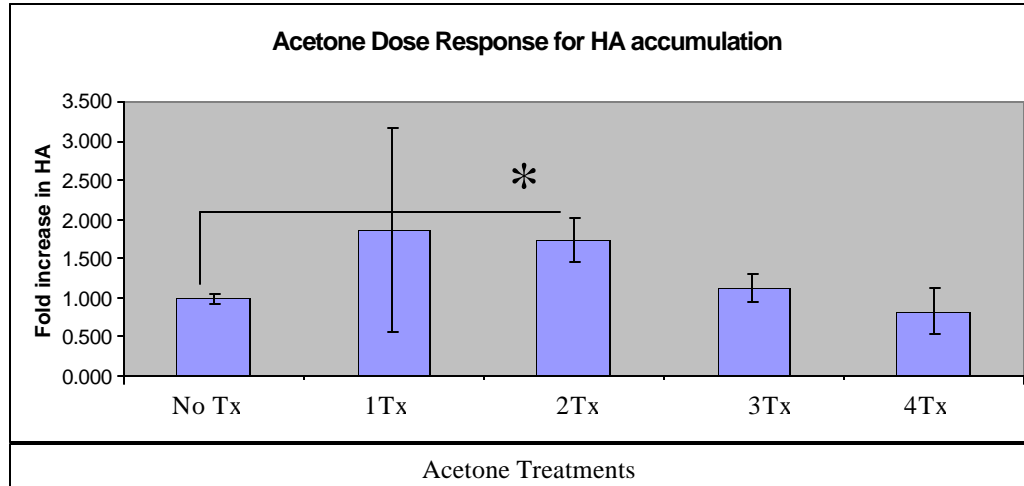
Figure 5-5: Analysis of acetone induced HA accumulation in REK organotypic cultures. REK cultures treated with successive exposures to acetone as a form of graded injury over time. FACE analysis was performed and plotted as a fold increase in HA levels over uninjured cultures. *Asterisk,* an increase in hyaluronan in the 2Tx compared with No Tx is significant at $P < 0.005$ by Student's t test in data pooled from three experiments. No Tx= No Acetone; 1Tx, 2Tx, 3Tx, 4Tx= One, two, three and four 470 µl acetone treatments spaced 8h apart. ANOVA analysis was done and showed significance between No Tx and 2Tx. The difference between 2Tx and 3Tx was also significant.

5.3.3. HABP *vs.* FACE analysis to detect HA levels in the collagen matrix of

acetone treated REK lift cultures

A significant increase in the bHABP staining in the collagen matrix was observed in the acetone treated REK cultures as compared to uninjured REKs. To confirm this increase in HA levels in the collagen layer, the epithelium was separated from collagen as described in section 3.2.6. Individually the epithelium and collagen were processed for FACE as described in section 3.5.2. Interestingly, little or no HA was detected in the collagen in the injured cultures as compared to a significant increase in epithelial HA which was expected after acetone mediated barrier injury.

IPLab analysis revealed a 25-fold increase in epithelial HA as compared to collagen after one-acetone treatment and a 45-fold increase in epithelial HA as compared to collagen in two-acetone treated REK cultures.

5.3.4. Acetone-treated REK cultures show a change in the HA molecular weight distribution

The size of the HA polysaccharide dictates its biological effects in many cellular and tissue systems, based upon many reports in the literature (2.2.5 and 2.2.6). Also, the long and short HA polymers appear to have antagonistic effects in some biological systems. Given the apparent overall increase in tissue thickness as well as an increase in HA levels in REK cultures treated with one and two acetone exposures, these cultures were processed to look for the molecular size distribution of HA. The HA molecular weight distribution was measured by IPLab. A significant increase in the higher molecular weight HA was observed, as shown in Fig.5-6.

Interestingly, along with the increase in the high molecular weight HA sizes, there was a decrease in the relative amounts of HA in the low molecular weight range in acetone treated REK cultures (Fig. 5-6 and 5-7). Specificity of the stain for HA was shown by treating half of the processed cultures with Streptomyces Hyaluronidase (an enzyme known to specifically degrade HA) and running these cultures on parallel lanes on the gel which showed the disappearance of the smear indicating that it was HA (Fig. 5-7B and 5-7D). Another indication of the specificity of these assays was obtained by an examination of HA in skin. Murine skin was processed to look for HA molecular weight distribution, which showed a robust staining and a distribution of HA on the gel that was entirely eliminated by treatment with hyaluronidase. Thus, this technique worked on murine skin, and indicates a high specificity for hyaluronan (Fig. 5-7E and 5-7F).
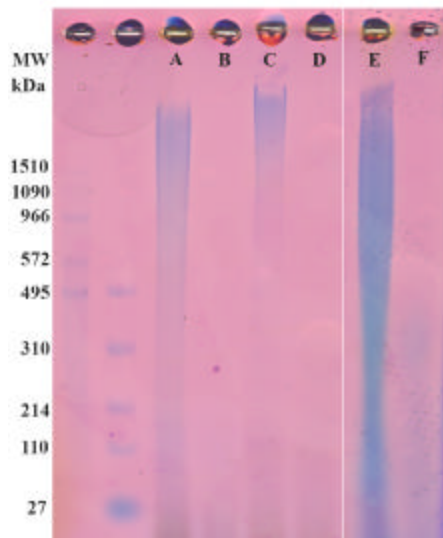
Figure 5-6: Analysis of HA molecular size distribution in the acetone treated REK lift cultures.
(One and half well epithelium harvested from a six well plate in each lane) A, Control. B, An equal amount of sample treated with Strep. Hyaluronidase. C, Two-acetone treated sample. D, An equal amount of acetone treated sample as in lane C but also treated with Hyaluronidase. E, Murine skin. F, an equal amount of the skin sample treated with Strep. Hyaluronidase. Molecular weight distribution shows the markers for high and low molecular weight distribution in kDa. Each experiment was repeated thrice with similar results.

(A)   HA Molecular weight (1x Acetone)   **(B)**   HA Molecular weight (2x Acetone)
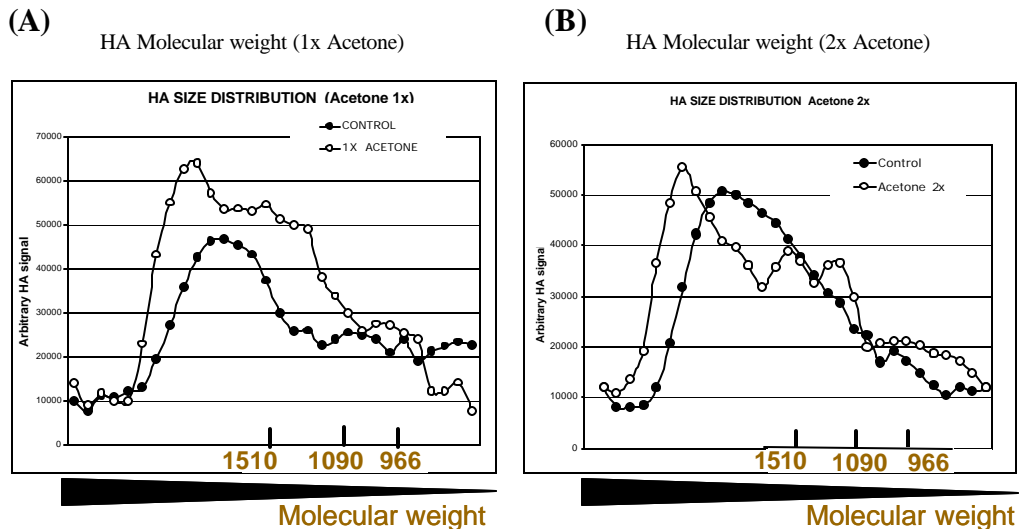
Figure 5-7: Analysis of HA molecular size distribution in the acetone treated REK lift cultures.
The 'Y' axis shows the HA signal intensity as measured by IPLab. The 'X' axis shows the position of the Molecular weight markers in kDa, corresponding to the HA signal on the gel.(A) One acetone treatment, (B) Two acetone treatments.

5.3.5. Effect of acetone induced barrier injury on the genes involved in HA metabolism

To explore the mechanism behind the increase in total HA levels in the acetone treated REK lift cultures, expression of the major mammalian HA synthetic and HYAL (catabolic) enzymes was measured by RT-PCR. The experimental protocol is described in section 5.2.3. The mRNA levels of Has1, Has2 and Has3 and HYAL 1, HYAL2 and HYAL3 were measured in acetone treated cultures using Rat specific primers designed to yield amplification products that can be displayed on a single gel (Fig. 5-8). The results show that the acetone mediated barrier injury induces levels of Has 2 and Has 3 but not of Has 1 (which is not detected in REKs even in the non-injured state). Levels of HYAL 1 were unchanged and those of HYAL 2 and 3 were decreased after acetone treatment. These changes suggest that the increase in the high molecular weight HA is mediated by increased HA-synthetic activity or a decrease in the activity of hyaluronidases.

This observation would need to be further confirmed by techniques like the real time PCR or Northern blot for RNA analysis. Real time PCR will be a direct technique to quantify the fold increase in the message as well as a sensitive technique to document the relatively smaller increases in the Has expression in the injured REK cultures. Also, we need to consider that the increase in the message does not directly correlate with the total amount or the activity of these enzymes. As a next step, experiments need to be designed to study the enzyme activity which is another critical factor that dictates the HA levels in these cultures. It would be possible to study the Has and Hyal enzyme activity since these experiments are performed in a cell line. Inhibition of these enzymes by substrate starvation with methyl-umbelliferone, can be used for these studies.

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(A) Analysis of HA Synthases by RT-PCR: Exp 1, 2 and 3
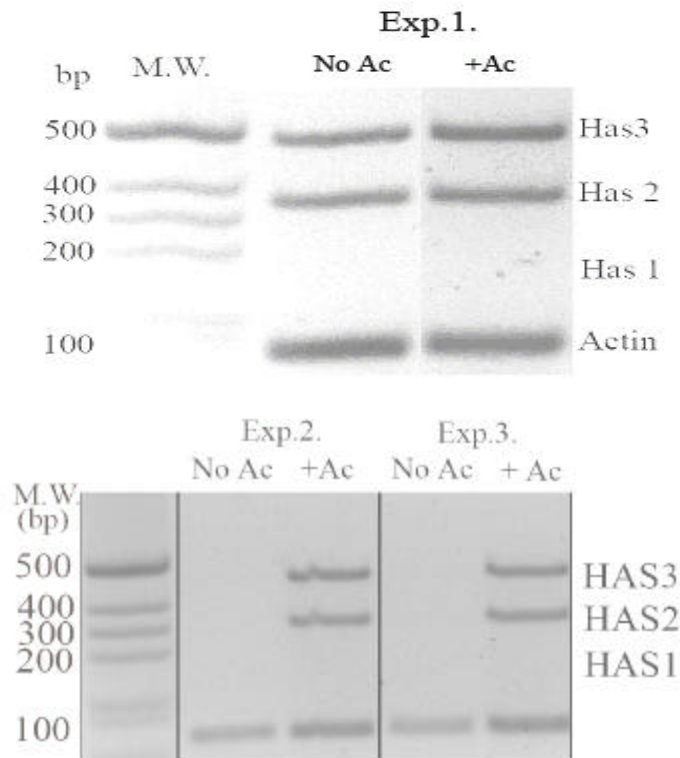
(B) Analysis of HA degrading enzymes by RT-PCR: Representative sample from Exp.1.
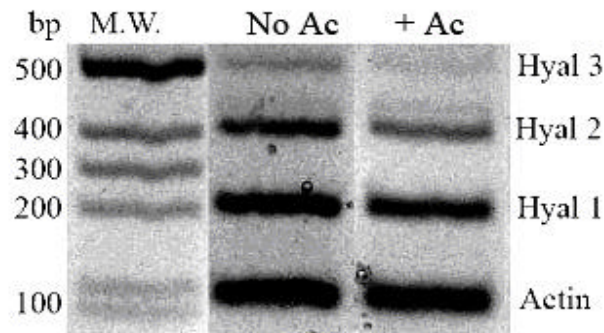
Figure 5-8: Multiplex PCR to show expression of HA metabolic enzymes after barrier injury in REK 3-D lift cultures.
Cells were harvested 16 hr after acetone injury, RNA was obtained and cDNA amplified using rat specific primers for three Has and three HYAL enzymes, whose PCR products can be combined on a single ethidium-stained agarose gel. (A), REK cultures either untreated (No Ac) or treated with 2 applications of acetone (+Ac), and analyzed for Has1, Has2, and Has3 expression. (B) Representative sample showing lysates from Exp.1. analyzed for HYAL1, HYAL2, and HYAL3 expression.

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5.4. Discussion

This study indicates that it is possible to study hyaluronan metabolism in relation to barrier injury in the 3-D REK model. The data suggest that epithelial keratinocytes can respond to barrier injury by modulating the levels of HA in the extracellular matrix. After mild (one acetone exposure) and moderate (two acetone exposures) injury, the epithelium responds by increasing the hyaluronan levels within the matrix. In contrast, more severe barrier injury (three and four acetone exposures) leads to a decrease in HA levels. From the previous work (chapter IV), it is known that two acetone exposures promote a more proliferative phenotype and lead to epidermal hyperplasia as compared to three acetone exposures that accelerates the keratinocyte differentiation program. Thus, it appears that a moderate injury, sufficient to stimulate epidermal tissue to prepare for increased proliferation, also activates pathways to increase hyaluronan, a matrix molecule known to support cellular proliferation.

Interestingly, although HA is present in both the basal and suprabasal layers after moderate injury, it is more pronounced in suprabasal locations (close to where acetone was applied). Also, HA in the suprabasal cells appears to be located intracellularly, suggesting that either HA is degraded and taken up and internalized, or synthesized from intracellular sites. Both are theoretically possible (Hascall, Majors et al. 2004).

As of now, two techniques are available in a few laboratories to measure and quantify this amazingly large molecule (HA). Immunochemistry with HA binding protein helps to visualize the location of HA in the 3-D cultures, and FACE helps to measure the total quantity of HA in tissue. It is important to compare the data obtained by these two techniques to get a better understanding of HA biology in the 3-D epithelium.

Interestingly, HABP reveals a relatively higher increase in staining for HA in the injured cultures as compared to the total HA levels documented by FACE in our organotypic cultures. Several possibilities, can explain these discrepancies. For example, the HA binding protein might be recognizing a different structural form of HA that is present only in the acetone-injured tissue. The possibility that smaller molecular sizes of HA might stain better with HABP was suggested by increased HABP staining of collagen in acetone-treated cultures. To examine this, the epithelium was carefully separated from the collagen, and subjected to HA analysis by FACE. However, this study documented that HA was not present in significant amounts in the collagen as compared to the marked increase observed in the epithelial matrix.

There is evidence in the literature to support the notion that an increase in matrix hyaluronan supports cell proliferation and inhibits differentiation *in vivo* (Maytin, Chung et al. 2004). It is therefore interesting to observe and report similar findings in an organotypic model. As one step further to the findings reported in the literature, the molecular weight distribution of HA in the epithelial compartment has been measured. After mild to moderate barrier injury, an increase in the higher molecular weight HA polymer is observed in the REK epithelium. Since only one cell type is present in the REK system, it is noteworthy that increases in HA along with observed changes in its molecular weight distribution must be principally mediated by the keratinocytes. This increase in the high molecular weight HA in the matrix after moderate injury might directly facilitate cell mitosis and increased proliferation, documented as increased Ki67 and BRDU staining and epidermal hyperplasia in the REK system (chapter IV). The

mechanisms linking the increase in high molecular weight HA polymer and cellular proliferation would be interesting to further study and characterize in the future.

The increase in high molecular weight structures of HA observed here can be attributed to the increase in the activity of the Has2 and Has3 enzymes. It is known that epidermal keratinocytes abundantly express Has3 from the basal to the granular layers, and extrude HA through the plasma membrane into the ECM while it is being synthesized as an unmodified polysaccharide. Thus, an increase in Has2 expression in the system after barrier injury as well as Has3 represents an interesting new finding. As a next step, it would be interesting to study the response of the tissue in terms of Has2 message at successive time intervals beginning from the point of injury.

The fact that an increase in the Has2 message is observed at the same time while the finished product (HA molecule) level is increased in the tissue after moderate injury (two acetone) whereas the total HA levels decrease markedly after severe (three acetone) injury, suggests a strong correlation between the Has expression and HA production. It would be interesting to study the message at different times following injury, and observe exactly how the tissue behaves in terms of Has activity across the spectrum of acetone mediated barrier injury, ranging from mild (1Tx) to its severest (3Tx) form.

On the other hand, in the setting of inflammation or tissue destruction HA is broken down into its lower-molecular-weight components. Two acetone treatments do not seem to affect the HYAL levels, supporting the observations from the literature that this form of moderate injury pushes the tissue towards growth and proliferation and away from an inflammation-supporting environment. There is evidence from our previous work that barrier injury, seemingly restricted to the epithelium, does not elicit any potent

inflammatory response in murine skin *in vivo* (Maytin, per, communication). It is possible that higher levels of injury (three acetone exposures), might elicit an increase in the low molecular weight polymers of this molecule. It would be interesting to check activity of the HYAL enzymes across the spectrum of acetone treatment, ranging from mild (1Tx) to the severe (3Tx) type of barrier injury.

Thus, a tissue engineered model of barrier injury has been developed in which HA production is greatly enhanced. HA accumulation is more pronounced in the suprabasal location as opposed to the basal layer (where the most HA is seen under physiological circumstances). This model offers a distinct advantage for studying and characterizing the HA molecule and the enzymes that synthesize and degrade this polymer, all within a clean epithelial environment. Therefore, this simple yet robust model will be an elegant tool to further study the mechanistic aspects of HA biology after epithelial injury.

Chapter VI
Epithelial Barrier Injury Induces HA Synthesis Via Release Of A Soluble Factor And EGFR Activation

6.1.   Introduction

Barrier injury leads to a significant increase in hyaluronan (HA) levels throughout the epidermis, which correlates with epidermal hyperplasia and enhanced keratinocyte proliferation (see chapter V). Another form of cutaneous injury, which causes a robust epidermal HA response, is a full thickness scalpel injury of native skin (Maytin, Chung et al. 2004; Tammi, Pasonen-Seppanen et al. 2005). Interestingly, in the latter form of injury, it is observed that the increase in HA is not limited to the site of wounding, but extends well beyond the localized area of injury. Multiple needle punctures of REK lift cultures collaborate this finding. After needle injury, HA induction is observed throughout the culture, even when the punctures are delivered only to the centre of the epithelium (Maytin, Mack et al., 2005).

Careful consideration of the experimental data and of the recent literature, has led to the specific hypothesis that EGF-family receptors may be an essential part of the

mechanistic pathways through which epithelial injury leads to up-regulation of HA. The initial hint towards this hypothesis is that the epidermal growth factor (EGF) family of ligands, its principal receptor EGFR, and hyaluronan all appear to be linked both to cell proliferation and migration in the epidermis. As reviewed in detail in the Background chapter, EGF/EGFRs have a documented role in epidermal cell proliferation (section 1.4.7.) and keratinocyte motility (section 1.4.8), both of which are critical for wound healing. In fact, EGFR has a crucial role in tissue remodeling across a wide spectrum of epithelial tissues including the intestine (Buffin-Meyer, Crassous et al. 2007), bronchium (Zhao, He et al. 2006), cornea (Xu, Ding et al. 2004), and epidermis (Kurten, Chowdhury et al. 2005).

Related to injury it is noted that mechanical stretch regulates keratinocyte proliferation partly by stimulating the formation of EGFR membrane clusters and EGFR phosphorylation in the skin (Kippenberger, Loitsch et al. 2005). Regarding the apparent correlation between EGFR activation and HA expression, the effect of EGF ligands and the EGFR pathway upon the synthesis of HA has gained attention in recent years (Yamada, Itano et al. 2004) (Pappinen, Tikkinen et al. 2007) (Pienimaki, Rilla et al. 2001). As discussed previously (section 2.3.9), the human Has2 gene is known to respond dramatically to epidermal growth factor (Pienimaki, Rilla et al. 2001; Karvinen, Kosma et al. 2003; Saavalainen, Pasonen-Seppanen et al. 2005). Interestingly, epidermal HA synthesis, controlled by EGF and TGF-beta through changes in the expression of HAS2 and HAS3, correlates with epidermal proliferation, thickness, and differentiation (Pasonen-Seppanen, Karvinen et al. 2003). There are also data to suggest that the effects of trans Retinoic acid (tRA) on keratinocyte proliferation and HA synthesis are partly

mediated through EGFR signaling (Pasonen-Seppanen, Maytin et al. 2007). Interestingly, our experiments using pinprick injury of organotypic cultures demonstrate that physical wounding leads to rapid increases in HA levels, which can be blocked by EGFR inhibition (Monslow J. and Maytin E., communication). These data, and multiple considerations from the work of others, therefore suggest the overall hypothesis that wounding causes the release of growth factors and activation of growth factor receptors (EGFR), thereby activating pathways that increase HA through the activation of hyaluronan synthases and that is critical for normal wound healing.

The evidence discussed above suggests that EGFR might be potentially involved in the HA response not only after deep wounding, but also after shallow abrasion wounding of the epidermis. This study proposes to test whether epidermal barrier injury leads to a release of one or more soluble factor, through which activation of EGFR might mediate the observed increase in HA. By contrasting the pathways utilized to increase HA after acetone-induced barrier injury with those pathways utilized after a full thickness (pinprick) injury of the epidermis, this work expects to gain mechanistic insights into these phenomena.

6.2. Methods

6.2.1. Schema for medium transfer experiments

For each experiment, REK cultures were grown in a six well Transwell plate and airlifted for 5 days. (Row 1) of Fig. 6-1 shows pair of donor (C) and recipient cultures (CT) to illustrate how media were transferred. After 5 days of air-lift, the medium from the donor culture was transferred to the recipient culture and then this recipient culture
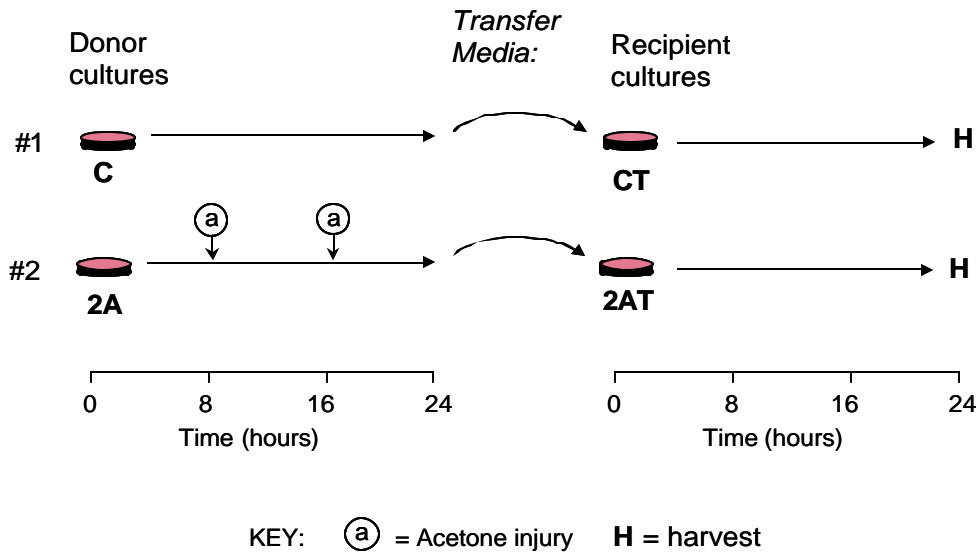
Figure 6-1: Schematic model of the medium transfer experiments.
On day 4 i.e., 24 hrs before the point of transfer, medium was changed for all cultures. (#1) REK cultures were air lifted for five days. REK medium from the donor culture (C) was transferred to a recipient/uninjured culture (CT), and further incubated in that medium for additional 24 hrs. (#2) REK cultures were air lifted for five days. On day 5 the donor culture (2A) was treated with a single pulse of 470 µl of acetone repeated twice at 8 hr intervals. The medium was transferred to the recipient, uninjured culture (2AT), with further incubation in that medium for another 24 hrs.

(CT) was further incubated for an additional 24 hrs. (Row 2) of Fig. 6-1 shows another pair of donor (2A) and recipient cultures (2AT). The donor culture (2A) was treated twice with 470 µl of acetone applied as a pulse for 30 s at 8hr intervals. After day 5th of air-lift, the medium from the donor culture (2A) was transferred to the recipient culture (2AT), and likewise incubated for an additional 24 hrs. The recipient cultures were then harvested for further analysis.

Fig.6-2 depicts the layout of the Transwell plates, and how the donor and recipient cultures were physically arranged. Note that in some experiments, a REK donor culture was injured by pinpricking (100 punctures with a 28 gauge needle) rather than with acetone injury, to serve as a positive control.
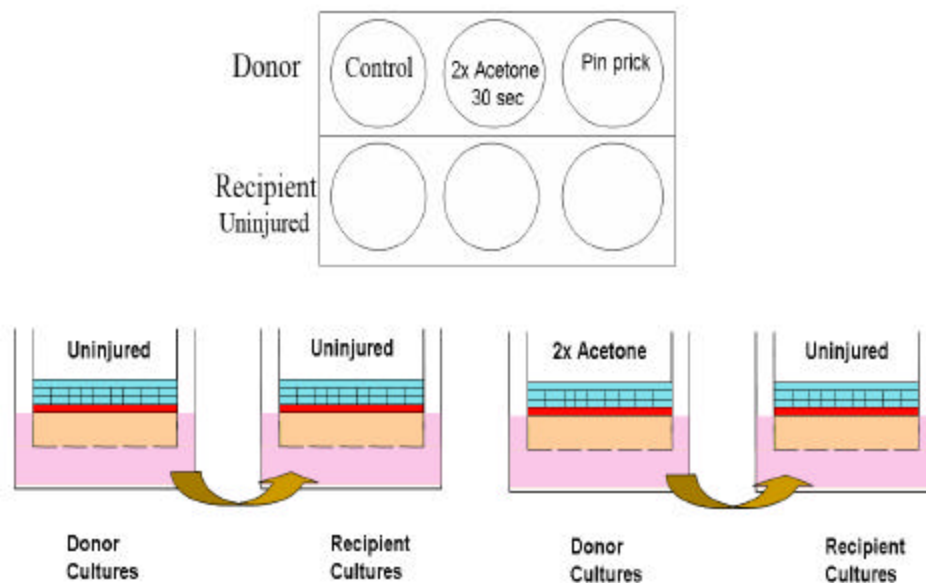
Figure 6-2: Transwells in a six well plate for the medium transfer experiments in this thesis.
The six well Transwell plate shows a set of donor cultures in the top row (control, 2x Acetone and pinprick) depending upon the method of injury and the recipient cultures in the bottom row.
 Bottom: Transwell cultures in pairs depict the medium transfer from a Donor culture to its corresponding Recipient culture. The recipient cultures were further incubated in that medium for 24 hrs.This schema was followed for all the transfer experiments in this thesis.

6.2.2. Protein arrays for detection of cytokines

Mouse cytokine arrays (membranes upon which 20 different antibodies were affixed at known locations) were purchased from Panomics Inc. To prepare conditioned media, REK lift cultures were injured with acetone (470 µl for 30 seconds), 100 pin-pricks or 2 ng/ml of TNF a and further incubated for 6 hrs from the point of injury. The medium was collected and stored at -80°C until testing. Then either, conditioned media (1.5 ml) or various controls such as 10-fold-diluted sera or DMEM media alone were incubated with the cytokine array membranes.
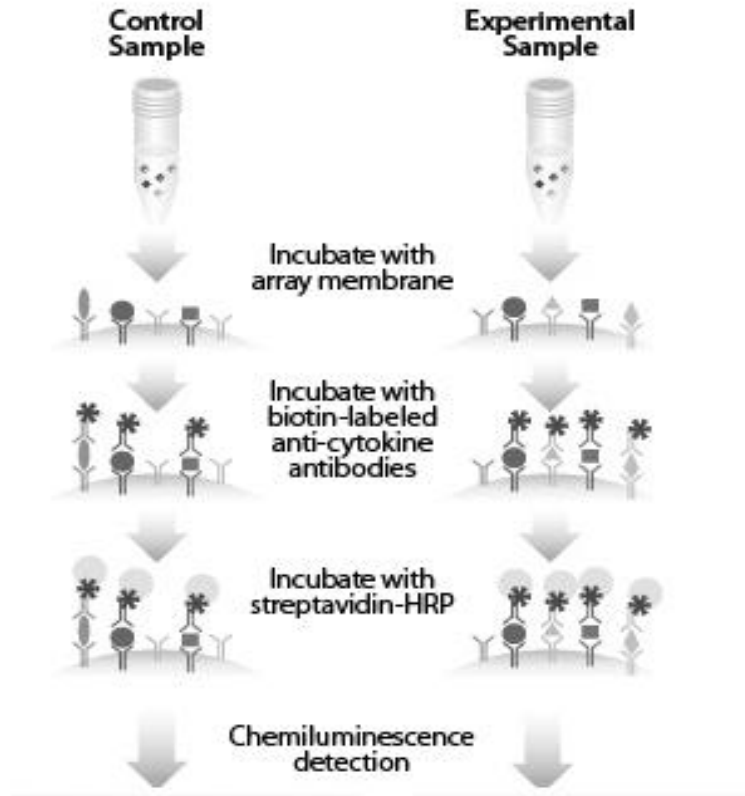
Figure 6-3: Schema for analysis of cytokines in the REK medium.
Adapted from http://www.panomics.com/product.php?product_id=39

Each membrane was first incubated with a blocking buffer for 2 hrs at RT. Membranes were then washed and incubated with 1.5 ml of the conditioned medium for 2 hrs. After washing, a biotin conjugated anti-cytokine mix was added to the membrane and further incubated for 2 hrs. 1x Streptavidin-HRP solution was prepared and the membrane was soaked in this solution for 1 hr. Detection solution was prepared immediately before use by mixing equal amounts of detection buffers A and B for each membrane. The membranes were exposed to Hyperfilm ECL^{TM} and several different exposures were taken of the array blot.

6.3.   Results

6.3.1.   HA inductions in response to barrier injury in the REK culture model are mediated via a paracrine factor

As discussed before in section 6.1, HA induction is reported to occur far from the site of injury. To test the hypothesis that HA is induced by a soluble factor, experiments were designed as described in Fig.6-1. Two methods of injury were employed, pinprick (100 pricks through out the REK culture with a 28 gauge needle) and two acetone treatments as depicted in 6-1. The donor and recipient cultures were harvested for immunohistochemistry with HABP as described in section 3.4.2. Interestingly, the recipient cultures showed a significant increase in HA levels.

The IPLab quantification of the images (Fig. 6-5) showed a 2.5-fold increase in HA levels in recipient wells (2AT) receiving medium from acetone-injured donor cultures (2A) as compared to recipient cultures (CT) receiving medium from uninjured donor cultures (C). This difference was statistically significant with respect to control transfer.
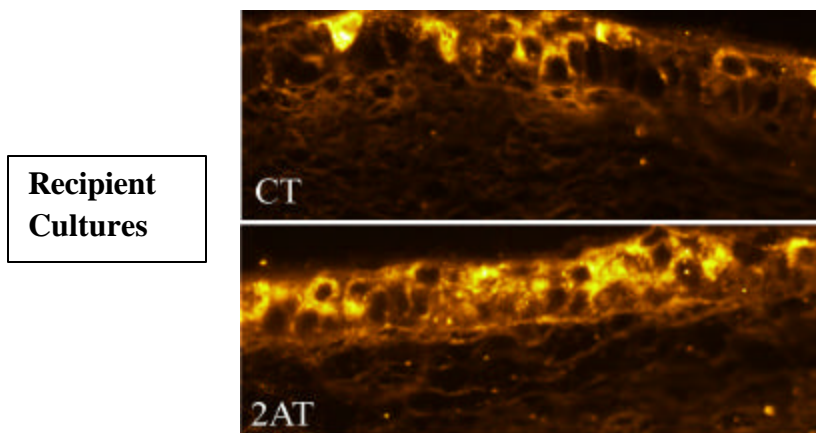
Figure 6-4: HABP staining is significantly increased in recipient cultures after medium transfer from acetone treated REK cultures.
CT= Control Transfer; 2AT= Two acetone transfer.

HA levels in recipient cultures
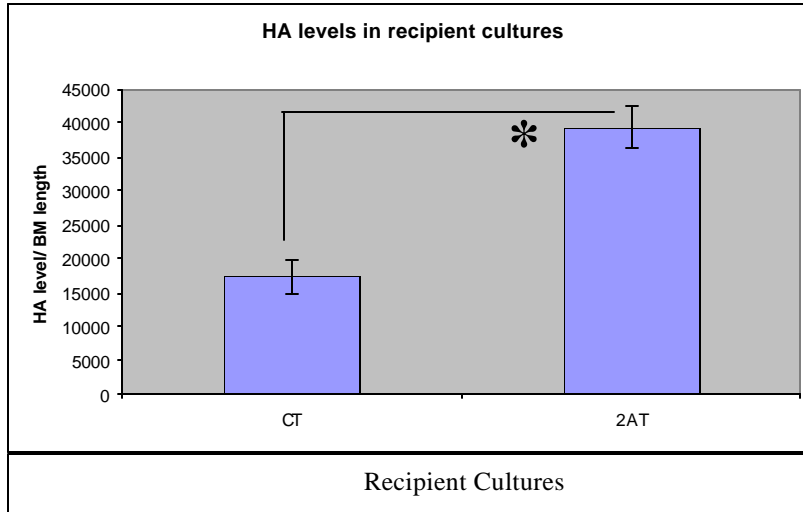
Figure 6-5: Quantification of hyaluronan in the recipient cultures. Data pooled from three experiments.
Paraffin sections were stained with HABP, microscopic images photographed, and HA levels evaluated using the image processing technique IPLab (see common methods for details). CT= Control Transfer; 2AT= Two acetone transfer.
The epidermal hyaluronan is normalized to the basement membrane length in arbitrary pixel units. Data are pooled from three different sets of experiments on REK lift cultures. Asterisk, difference with respect to control transfer is significant by student's t-test, $P<0.0005$ (*).

6.3.2. Increases in epidermal hyaluronan in the recipient cultures is confirmed by the FACE technique

FACE analysis was performed (see Methods 3.5.2 for details) as an independent confirmation of the increase in HA observed in immunostained REK organotypic cultures. In these samples the entire culture (epithelium and collagen) was used for FACE analysis. The recipient cultures that had received medium from injured donor cultures two acetone transfer (2AT) and pin prick transfer (PPT) showed a significant increase in HA as compared to the uninjured (CT) cultures. The recipient culture (2AT) that received the medium from the donor injured with acetone (2A) showed the maximal increase in HA (Fig. 6-6).
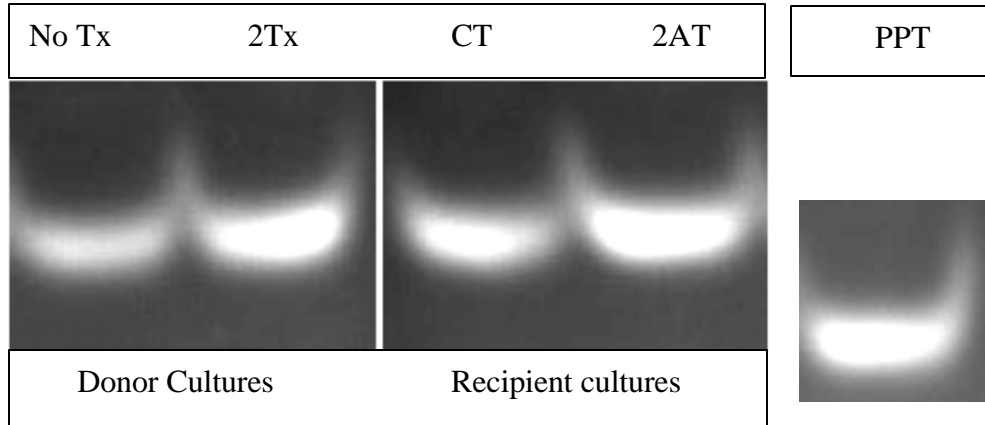
Figure 6-6: FACE shows an increase in HA in recipient culture receiving medium from injured donor culture.
The donor cultures (C and 2A); the recipient cultures (CT, 2AT) receiving medium from (C, 2A) respectively. No Tx= No acetone treatment, 2Tx: Two acetone treatments. PPT represents the positive control, recipient culture receiving medium from a pinpricked culture.

IPLab analysis was used to quantify HA. There was a 2-fold increase in HA in the recipient cultures receiving medium from the injured donor cultures, and this result was statistically significant when compared with CT (Fig. 6-7).
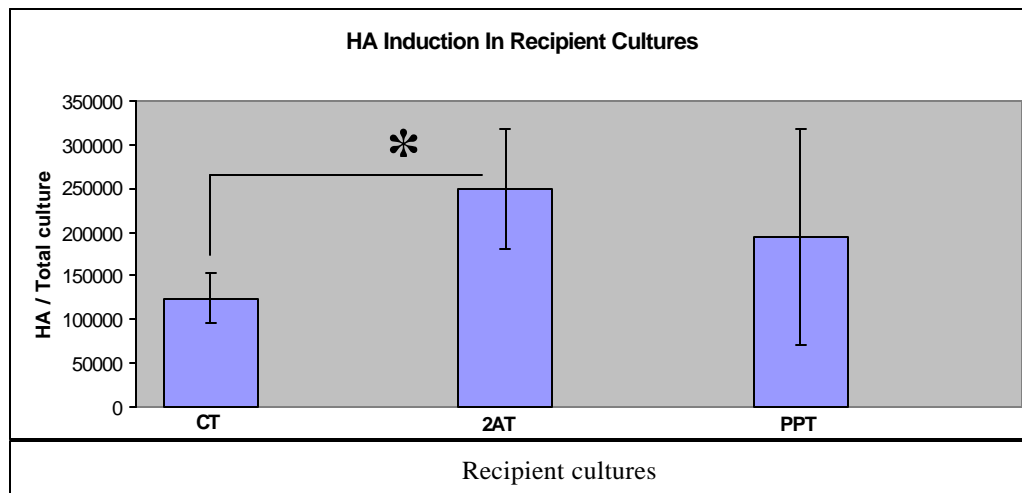
Figure 6-7: Media transfer experiments to demonstrate induction of HA expression by a soluble factor in 3-D REK lift cultures.
HA levels in recipient (uninjured) cultures were measured by FACE and quantitative image analysis (IPlab software, signal analytics Inc; arbitrary units). Asterisk, difference with respect to control transfer is significant by student's t-test, $P < 0.05$ (*).Data pooled from three different experiments for controls and two acetone treatments. The pinprick experiment was repeated twice. The donor cultures (C and 2A); the recipient cultures (CT, 2AT) receiving medium from (C, 2A) respectively. No Tx=No acetone treatment, 2Tx: Two acetone treatments. PPT represents the positive control, recipient culture receiving medium from a pinpricked culture.

6.3.3. Antibody array to screen for cytokines as possible paracrine factor(s) responsible for EGFR activation and HA production

Before addressing the question of whether the soluble factor is an EGF-family ligand, we sought to ask whether a large number of soluble molecules, including potential non-EGF effector molecules such as cytokines, might be released as a downstream consequence of barrier injury. A few pro-inflammatory cytokines such as TNFa, IL-1 and IL-6 are implicated in the injury response observed after barrier injury in the intact skin. Also, certain cytokines are known to activate the EGFR via sheddases especially IL-6 (further discussed in section 2.3.3).

To determine the changes in these proinflammatory cytokines, the medium was collected from cultures injured with acetone, pin-prick or TNFa exposure and screened for cytokine levels as described in section 6.2.2.

As seen in Fig 6-8C, injury resulted in a decrease in the pro-inflammatory cytokines which are known to be released after barrier injury in the *in vivo* skin like TNFa, IL-1 and IL-6. The addition of a positive control, TNFa, indeed increased the levels of TNFa as well as RANTES (see Fig. 6-8C, far right panel) indicating that this was a functional system.

For negative controls, one might consider that the medium collected for this particular experiment was obtained from REK cultures growing in DMEM containing 10% serum. To check the possibility that cytokines might already be present in the serum, another set of blots were used and were treated with only DMEM (Fig. 6-8A) or only 10% serum (Fig. 6-8B). These blots showed cytokine signal much lower than the REKs grow in DMEM and 10% serum (compare blots A, B with C in Fig. 6-8) suggesting that

uninjured REKs secrete many cytokines, including IFN?, IL-1, RANTES, IL-5 and VEGF under normal conditions.

As another experimental condition REK lifts were allowed to grow and stratify in reduced (1%) serum for 5 days and then injured with acetone treatments as described in 6-1. These cultures grown in 1% serum looked histologically healthy on H&E stains but the cytokine blots inexplicably showed a significant amount of background smear (data not shown). We believe that this may be related to a decrease in blocking of non-specific sites on the membrane. At any rate, medium with 1% serum could not be used further. To rule out the possibility that high background was simply not the effect of the reagents used from Panomics, similar experiments were carried out with the array kit from another company (Chemicon). These blots were captured at the same 1 min incubation, and further increases in exposure time led to a proportional increase in the background signal.

Overall, the findings obtained by performing these experiments (three times with Panomics kit and twice with the Chemicon membranes), confirmed that there is a decrease in the cytokines as a result of injury in the REK lift cultures. This decrease could be due to the fact that may be these cytokines were released but acted in a paracrine fashion in the REK epithelium and were not secreted in the medium. Another explaination is that there could have been an earlier peak in the levels of these cytokines but this was missed as the medium for our experiments was collected at a later time (8h post injury).

Also the results indicate that the REKs under normal conditions secrete a high concentration of cytokines, as evident by comparing the blots from REKs that were grown in medium with 10% serum versus blots from medium/10% serum alone.
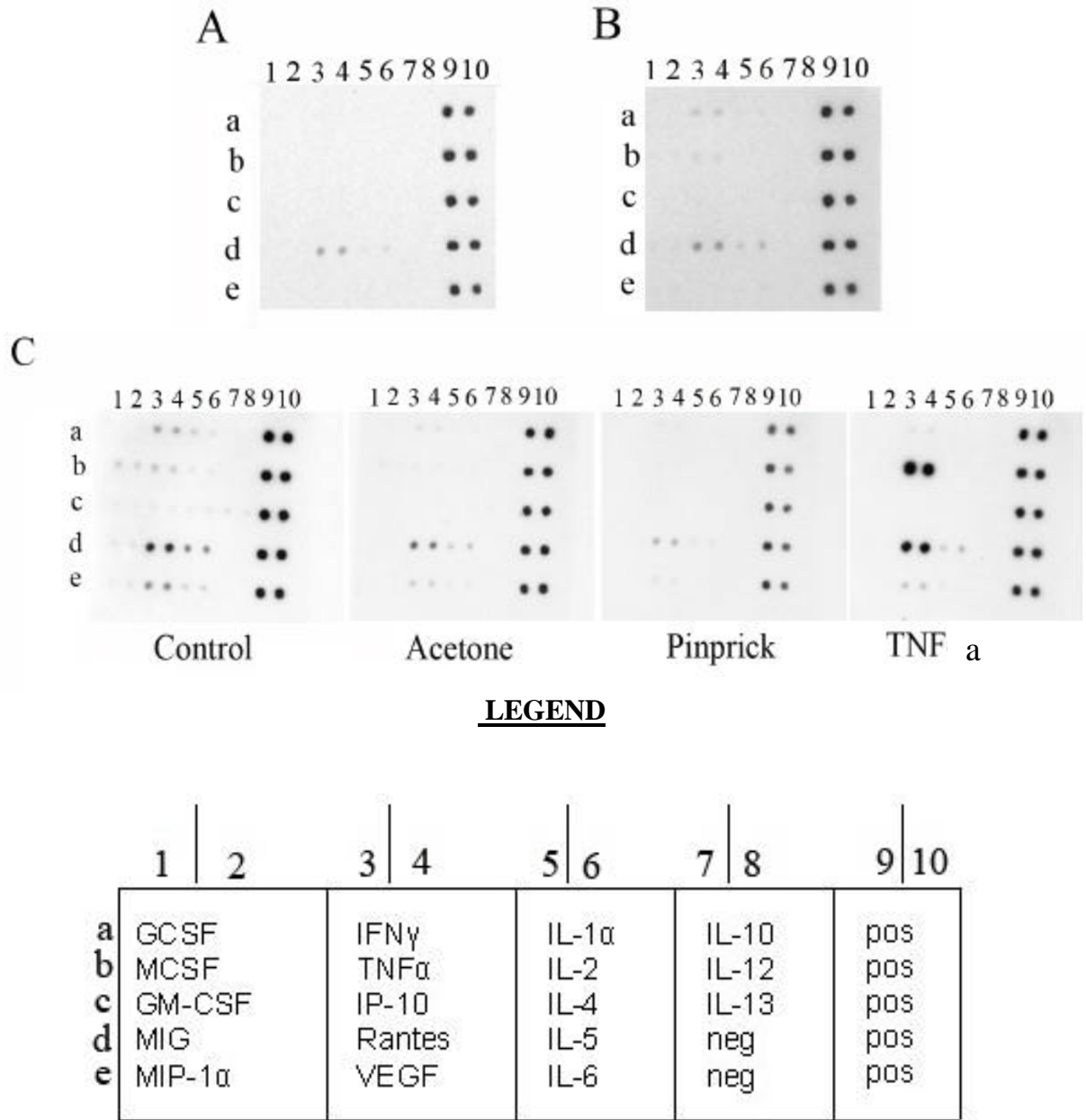
Figure 6-8: Cytokine Array blots at 1 min exposure. The first and second panels show cytokine array blots from only DMEM (A) and DMEM with 10% serum (B).

The third panel (C) shows the blots from the medium (DMEM with 10% serum) obtained from uninjured/Control, Acetone, Pinprick and TNFa stimulated REK lift cultures. The legend shows the location of different cytokines on the membrane blot. Note all antibodies spotted in duplicate.

6.3.4. The induction of epidermal HA synthesis after barrier injury requires activation of the EGFR pathway

Based upon evidence from the literature and from pinprick injury (section 6.1), this study was designed to test for putative involvement of EGF-like growth factors or their receptors in HA induction after barrier injury in REK cultures.

Experiments were performed to ask whether AG1478 (an inhibitor of the kinase activity of EGFR; Calbiochem Inc.), may block EGF-mediated induction of HA when
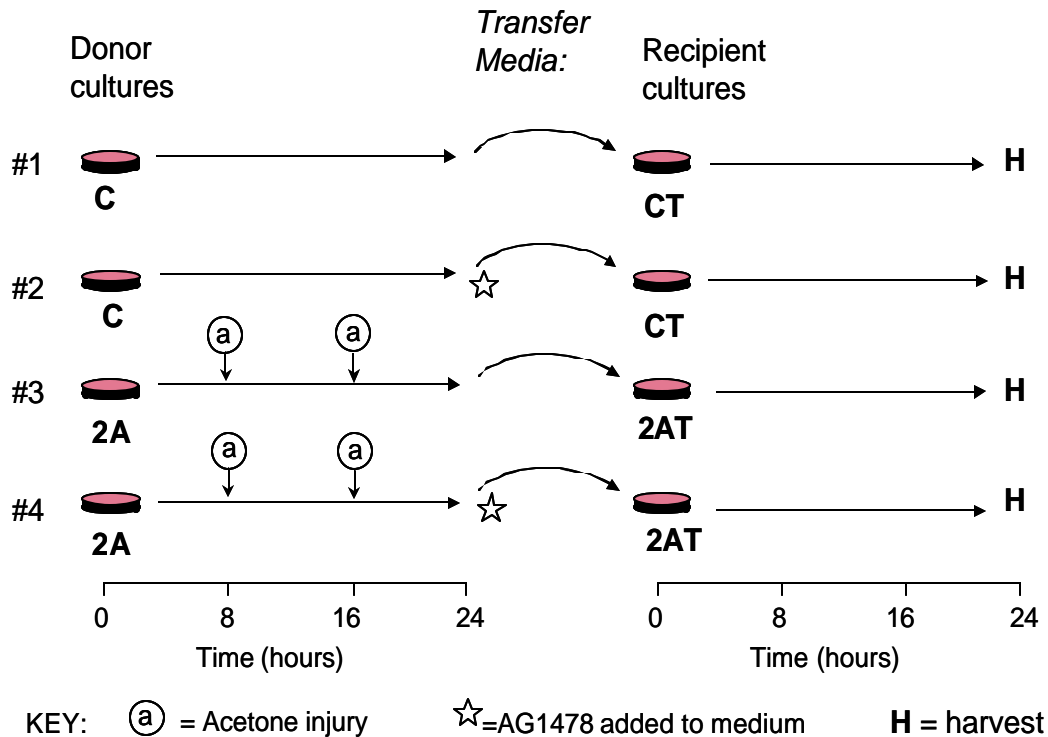
Figure 6-9: Schema of the medium transfer experiment with AG1478 in 3-D REK lift cultures. REK cultures were airlifted for 5 days. On day 4 i.e. 24 hrs before the point of transfer, medium was changed for all cultures. (1) REK donor culture (C) without any treatment for all 5 days of REK lift. Afterwards the medium was transferred to a different uninjured culture (CT). (2) REK donor culture without any treatment for all 5 days of REK lift (C). Culture medium was transferred to the recipient uninjured culture (CT) but with addition of AG1478 at the time of medium transfer, and further incubated for 24 hrs. (3) REK donor culture (2AT) treated with a single pulse of 470 μl of acetone for 30 seconds at 16h and 8h before medium transfer. Afterwards the medium was transferred to an uninjured culture (2AT) with further incubation of this culture in the donor medium for 24 hrs. (4) REK donor culture (2AT) treated with a single pulse of 470 μl of acetone for 30 seconds at 16h and 8h before medium transfer. The medium was then transferred with the addition of AG1478 to an uninjured REK culture (2AT), with further incubation of this culture in the donor medium for 24 hrs.

used at a dose of 1 or 10 μm. These doses were chosen because in our lab previously these were shown to block EGF mediated increases in HA, without causing toxicity. As depicted in the schema (Fig. 6-9), the inhibitor was added to the medium at these two concentrations at the time of medium transfer. The recipient cultures were harvested for FACE analysis as described in section 2.5. As a positive control, addition of EGF (1 ng/ml) showed a marked increase in HA in the uninjured REK cultures, which was significantly abrogated by the addition of AG1478 at the concentration of 10 μm (Fig 6-10). This confirmed the activity of AG1478 in the REK cultures.

For experimental purposes, the inhibitor was tested at 10 μm for its ability, to block the injury-mediated induction of HA. As shown in Fig.6-11., AG1478 significantly abrogated the induction of HA as seen with FACE analysis of REK cultures. When quantified by densitometry, the AG1478 mediated blocking of EGFR was shown to be complete, with a full return of HA to control levels (Fig.6-12). This study showed that the soluble factor appears to trigger HA induction through the activation of the EGFR pathway, but there could be other pathways involved in this phenomenon. To confirm these experiments one can use a different chemical inhibitor of the kinase, inhibitor of the tyrosine moieties or specific antibodies directed towards the ligand binding site of EGFR.
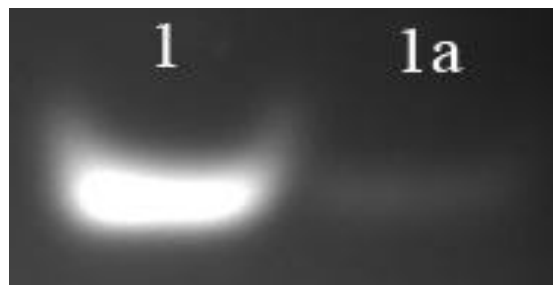
Figure 6-10: HA in REK cultures by FACE analysis shows a marked increase with EGF treatment (1) and a significant decrease following AG1478 exposure before EGF treatment (1a).

Recipient Cultures
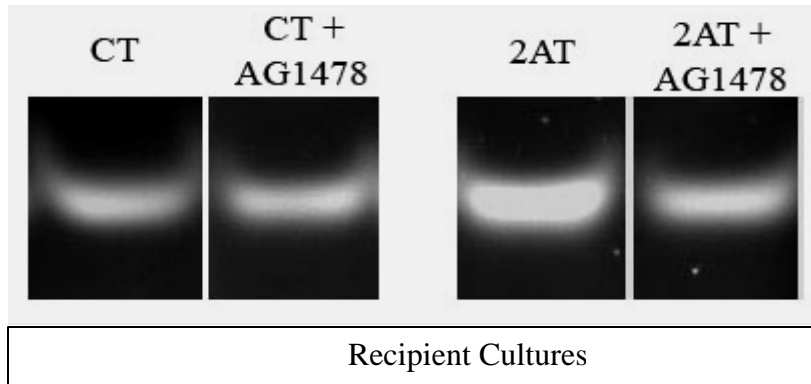
Figure 6-11: Results of an EGFR-blocking experiment that shows that EGFR pathway activation is partly responsible for HA induction.
REK 3-D cultures were injured with two acetone exposures, in the presence or absence of the EGFR kinase inhibitor AG1478, and then harvested for FACE analysis. The full experiment was performed twice as outlined in the protocol of Fig. 6-9. Recipient cultures receiving medium from uninjured cultures (CT), AG1478 added to the medium (CT+ AG1478), medium transferred from acetone injured cultures (2AT), AG 1478 added to the medium.
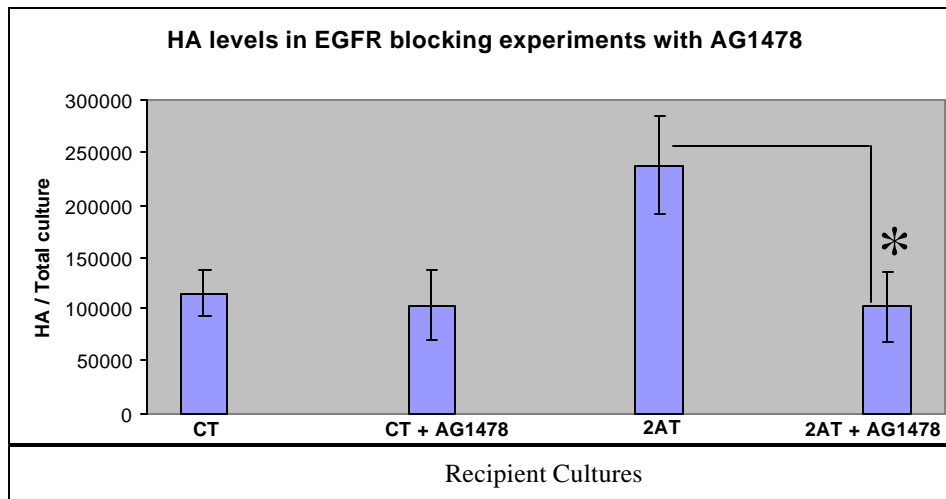
Figure 6-12: Quantification of Hyaluronan in EGFR blocking experiments with AG1478. Data pooled from two different experiments.
HA levels in recipient (uninjured) cultures were measured by FACE and quantitative image analysis (IP lab software, signal analytics Inc; arbitrary units). Recipient cultures receiving medium from uninjured cultures (CT), AG1478 added to the medium (CT+ AG1478), medium transferred from acetone injured cultures (2AT), AG 1478 added to the medium (2AT+ AG1478). Asterisk, difference with respect to 2AT is significant by student's t-test, $P < 0.001$ (*).

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6.3.5. The induction of epidermal HA synthesis after barrier injury requires Epidermal Growth Factor

To support the previous work with the EGFR kinase inhibitor AG1478, which showed that the injury-mediated induction of HA partially required EGFR activity, this study focused upon the role of EGF, one of the principal ligands of the EGFR, in this phenomena. As depicted in the schema (Fig. 6-13), an EGF neutralizing antibody (EGF N Ab) (Upstate Technology) was added to the medium at a concentration of 5µg/ml at the time of medium transfer.
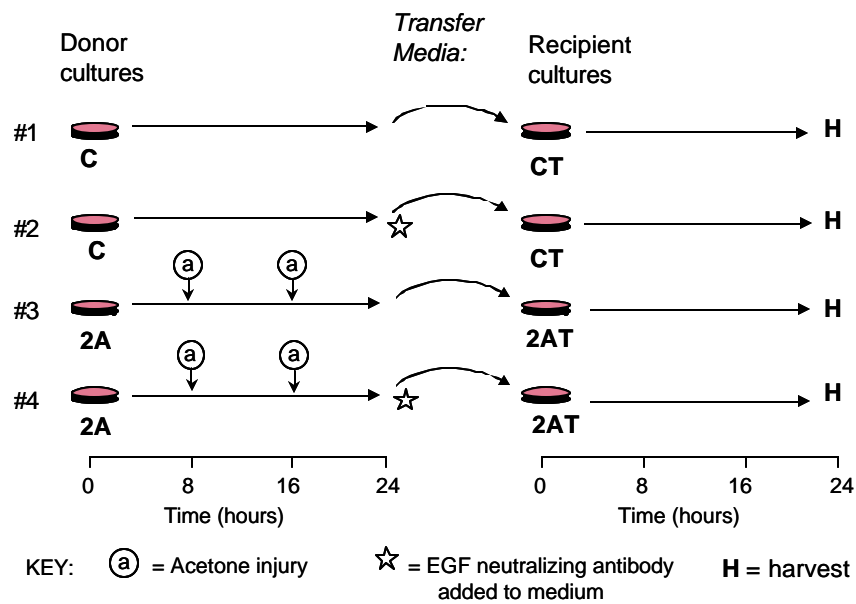
Figure 6-13: Schema of the medium transfer experiment with EGF-N Ab in 3-D REK lift cultures.
REK cultures were airlifted for 5 days. On day 4 i.e. 24 hrs before the point of transfer, medium was changed for all cultures. (1) REK donor culture (C) without any treatment for all 5 days of REK lift. Afterwards the medium was transferred to a different uninjured culture (CT). (2) REK donor culture without any treatment for all 5 days of REK lift (C). Culture medium was transferred to the recipient uninjured culture (CT) but with addition of EGF neutralizing antibody at the time of medium transfer, and further incubated for 24 hrs. (3) REK donor culture (2AT) treated with a single pulse of 470 µl of acetone for 30 seconds at 16h and 8h before medium transfer. Afterwards the medium was transferred to an uninjured culture (2AT) with further incubation of this culture in the donor medium for 24 hrs. (4) REK donor culture (2AT) treated with a single pulse of 470 µl of acetone for 30 seconds at 16h and 8h before medium transfer. The medium was then transferred with the addition of EGF neutralizing antibody to an uninjured REK culture (2AT), with further incubation of this culture in the donor medium for 24 hrs.
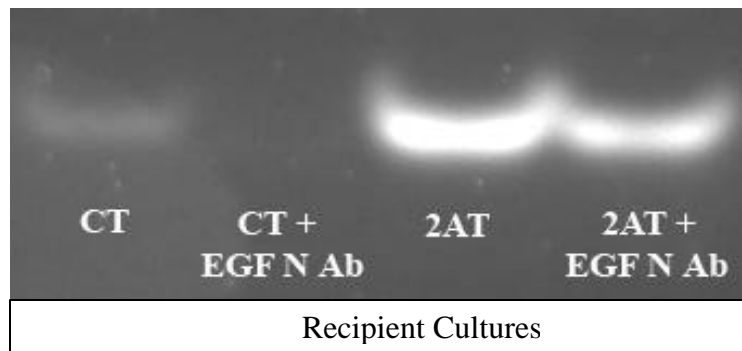
Figure 6-14: Results of an EGF neutralizing experiment that shows that EGF activity is necessary for HA induction after barrier injury.
Recipient cultures receiving medium from uninjured cultures (CT), EGF Neutralizing Antibody added to the medium (CT+ EGF N Ab), medium transferred from acetone injured cultures (2AT), EGF Neutralizing Antibody added to the medium (2AT+ EGF N Ab).
FACE analysis of the full experiment, performed as outlined in the protocol.

This concentration had been shown previously to block pinprick induced abrogation of HA in the REK system (Monslow, J, pers. communication). The recipient cultures were harvested for FACE analysis as described in section 3.5.

The results showed that EGF neutralizing antibody decreased the HA signal in both injured and uninjured REK cultures, as illustrated in Fig. 6-14. These findings were confirmed by HABP staining of REK lift cultures after performing the experiments depicted in Fig. 6-15 and quantified in Fig. 6-16. Interestingly, this antibody decreased the HA signal to a lower intensity (Fig. 6-15 and 6-16), than in controls and lower than the decrease observed after blocking with AG1478 (section 6.3.4), perhaps indicating a role for EGF ligand in basal maintenance as well as injury-induced synthesis of HA.

Together these data confirm that a major EGFR ligand i.e. EGF is important in HA synthesis after barrier injury.

Recipient Cultures

Figure 6-15: HABP staining is significantly decreased in recipient cultures after medium transfer from acetone treated REK cultures containing EGF- N Ab. The experiment was performed as in Fig. 6-13. Recipient cultures receiving medium from uninjured cultures (CT), EGF Neutralizing Antibody added to the medium (CT+ EGF N Ab), medium transferred from acetone injured cultures (2AT), EGF Neutralizing Antibody added to the medium (2AT+ EGF N Ab).
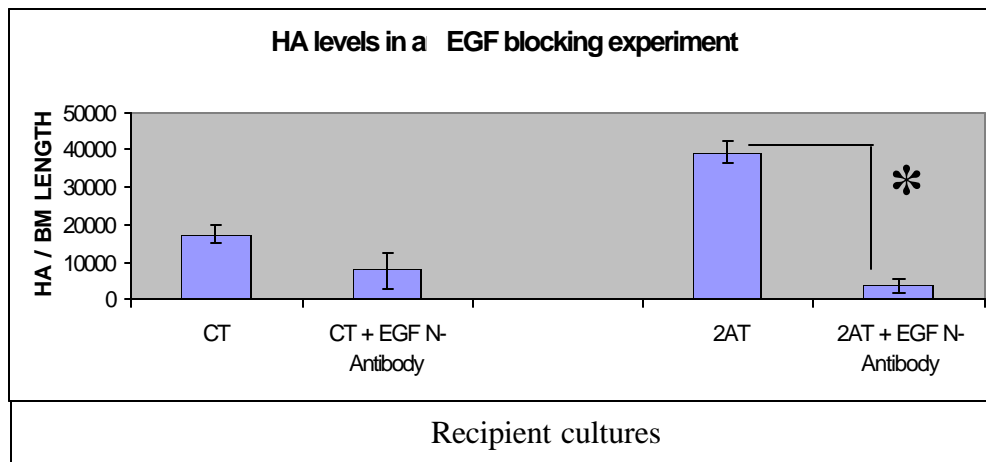
Figure 6-16: Quantification of hyaluronan in an EGF blocking experiment, Data pooled from eight sections per condition.
Paraffin sections were stained with HABP, microscopic images photographed, and HA levels evaluated using the image processing technique IPLab. The epidermal hyaluronan is normalized to the basement membrane length in arbitrary pixel units. Asterisk, difference with respect to 2AT is significant by student's t-test, $P<0.0005$ (*). Recipient cultures receiving medium from uninjured cultures (CT), EGF Neutralizing Antibody added to the medium (CT+ EGF N Ab), medium transferred from acetone injured cultures (2AT), EGF Neutralizing Antibody added to the medium (2AT+ EGF N Ab).

6.3.6. EGF family ligands are important regulators of HA induction via EGFR activation after barrier injury

After confirming the involvement of EGFR and one of its principal ligands-(EGF) in the HA response after barrier injury, this particular study was designed to corraborate the previous work and to test the involvement of other EGFR- ligands in this phenomenon.

To attack this question a ligand-scavenging reagent was used consisting of a soluble form of EGFR (an EGFR-Fc fusion protein) that can bind not only EGF, but also
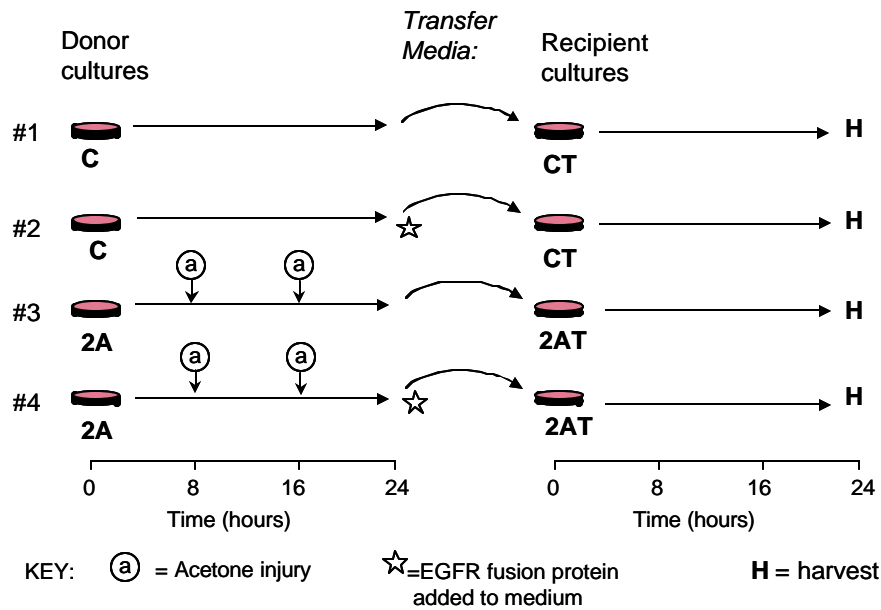
Figure 6-17: Schema of the medium transfer experiment with EGFR-Fusion protein in 3-D REK lift cultures.
REK cultures were airlifted for 5 days. On day 4 i.e. 24 hrs before the point of transfer, medium was changed for all cultures. (1) REK donor culture (C) without any treatment for all 5 days of REK lift. Afterwards the medium was transferred to a different uninjured culture (CT). (2) REK donor culture without any treatment for all 5 days of REK lift (C). Culture medium was transferred to the recipient uninjured culture (CT) but with addition of EGFR-Fusion protein at the time of medium transfer, and further incubated for 24 hrs. (3) REK donor culture (2AT) treated with a single pulse of 470 µl of acetone for 30 seconds at 16h and 8h before medium transfer. Afterwards the medium was transferred to an uninjured culture (2AT) with further incubation of this culture in the donor medium for 24 hrs. (4) REK donor culture (2AT) treated with a single pulse of 470 µl of acetone for 30 seconds at 16h and 8h before medium transfer. The medium was then transferred with the addition of EGFR-Fusion protein to an uninjured REK culture (2AT), with further incubation of this culture in the donor medium for 24 hrs.

HB-EGF, amphiregulin, epiregulin, and TNF-a (i.e., all EGF-family ligands). The source of the EGFR-Fc fusion protein (Apollo Inc.) was a DNA sequence which encodes the signal peptide and extracellular domain of EGFR (aa 1-525) and is fused to the Fc region of IgG1 (aa 99-330). This fusion protein was used in the working concentration of 0.05 and 0.5 µg/ml for the experiments.

Results are illustrated in Fig. 6-18 and analyzed quantitatively in Fig. 6-19. The presence of the EGF scavenging reagent increased the HA signal in the REK cultures, as observed in Fig. 6-18. This induction in HA levels following treatment with the EGFR fusion protein was statistically significant (Fig. 6-19).
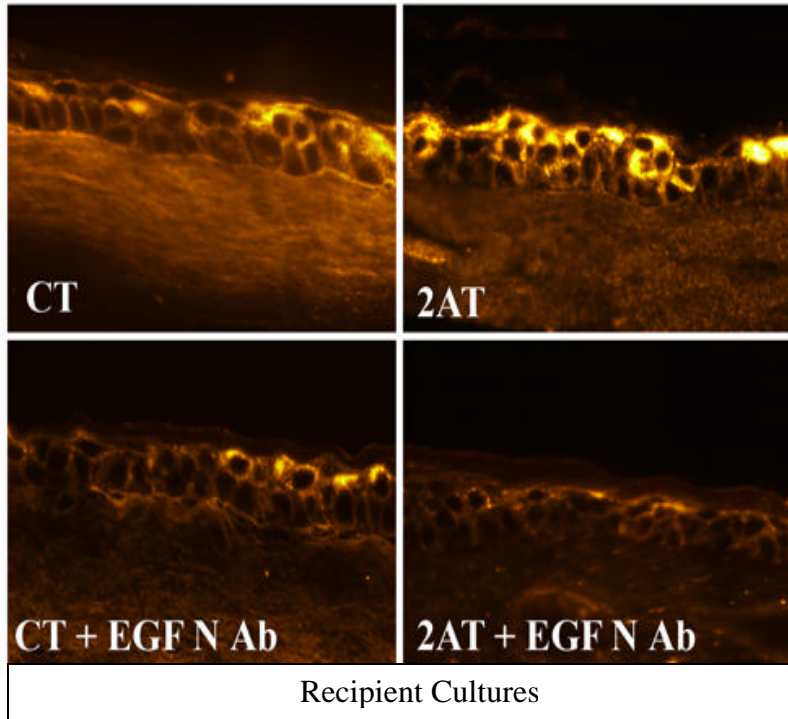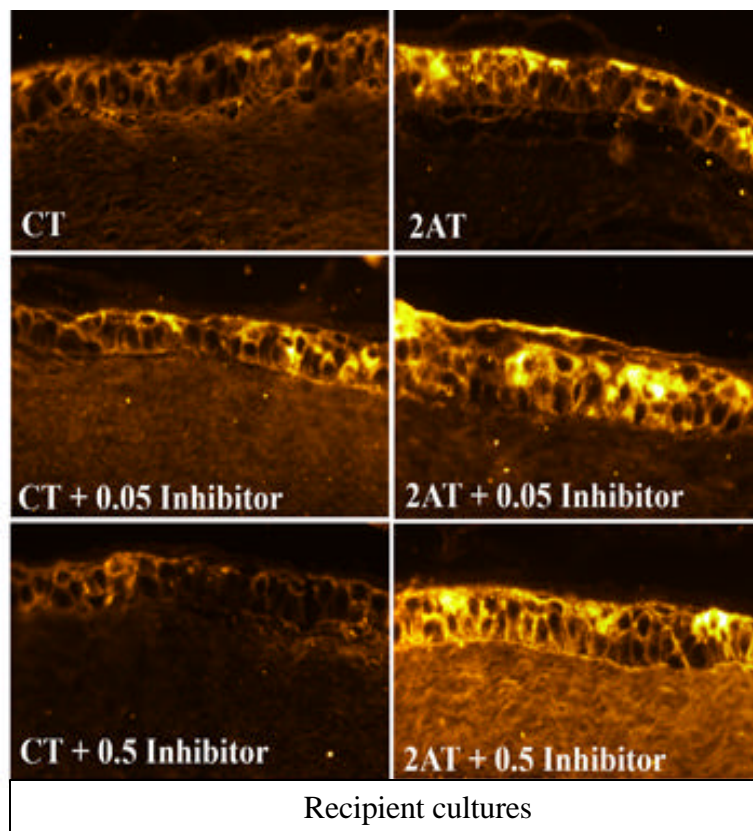

Figure 6-18: HABP staining is significantly increased in recipient cultures after medium transfer from acetone treated REK cultures containing EGFR-Fusion protein. Concentrations of 0.05 or 0.5 µg/ml EGFR Fc-fusion protein ("inhibitor") were added to the culture media at the time of transfer.
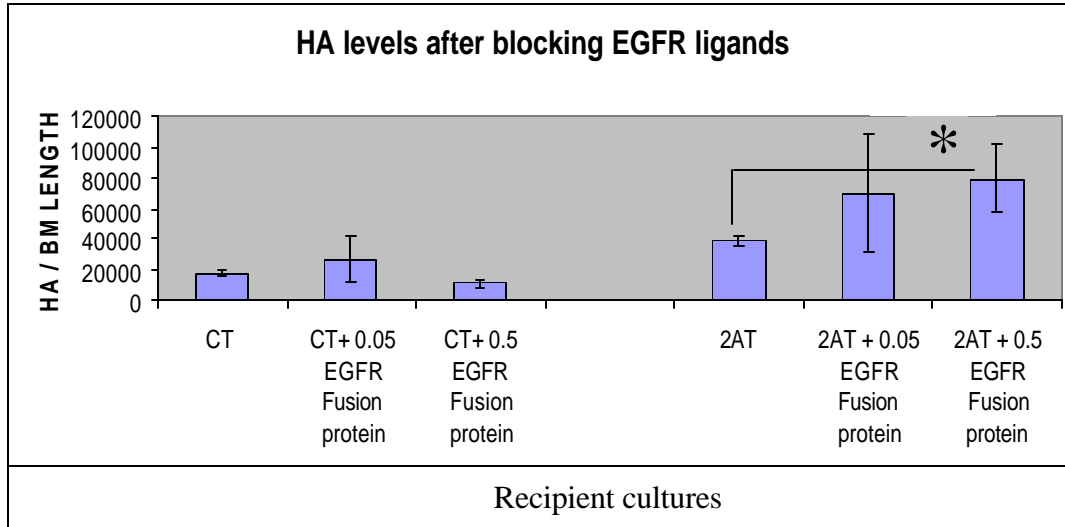
Figure 6-19: Quantification of Hyaluronan in the recipient cultures. Data for CT and 2AT pooled from three experiments whereas the inhibition was performed as a single experiment. Data pooled from eight sections per condition.
Paraffin sections were stained with HABP, microscopic images photographed, and HA levels evaluated using the image processing technique IPLab. The epidermal hyaluronan is normalized to the basement membrane length in arbitrary pixel units. Asterisk, difference with respect to two acetone treatment is significant by student's t-test, $P< 0.0005$ (*). Recipient cultures receiving medium from uninjured cultures (CT), EGFR fusion protein added to the medium in two different concentrations (CT+ 0.05 and 0.5 EGFR fusion protein), medium transferred from acetone injured cultures (2AT), EGFR fusion protein added to the medium (2AT+ 0.05 and 0.5 EGFR fusion protein).

6.4.   Discussion

In this study, experiments were designed to test the hypothesis that barrier injury causes the production and/or release of soluble factor(s), that act via a paracrine mechanism to increase HA production. Data from medium transfer experiments support the hypothesis and show that there is a significant increase in HA in the recipient cultures that have received medium transferred from acetone-injured cultures. This finding was confirmed by immunohistochemistry as well as by FACE analysis. In some experiments, there was an increase in HA levels in cultures receiving medium transfer from uninjured cultures (Fig. 6-6, and data not shown). Those increases were generally small in magnitude and could be due to a depletion of necessary nutrients in the medium and

resultant physiological stress during the 24 hr incubation (since nutrient depletion and ER stress have been reported to induce HA under certain conditions).

In the search for the paracrine factor, first and foremost a global cytokine array was designed to identify any changes in the cytokine profile after barrier injury. It has been reported in the literature that there is an increase in certain inflammatory cytokines such as IL1, TNFa and IL-6 immediately after barrier injury (Section 2.1.13). Cytokine arrays were used to detect any change in these cytokines within the medium which was used for the transfer experiments. Surprisingly, the medium collected from the uninjured cultures showed a stronger cytokine signal as compared with the injured REKs. To confirm this finding and to further compare it with the pinprick injury, REK tissue was pricked with a 20 gauge needle (100 times) across the entire epithelium. This method of full thickness injury also showed a decrease in cytokine signal on the array. External addition of TNFa caused an increase in RANTES (known to be induced by TNFa), and the levels of both molecules were significantly higher on the array blot. These findings confirmed that the REK system was functional and the injury response (barrier as well as pinprick) resulted in a global decrease in cytokines in the REK system.

To try to clarify the question of increased cytokine signals in the uninjured cultures (in case serum was responsible for some of these signals), the serum concentration in the medium was reduced to 1%. On histology the REKs looked healthy, but the cytokine blot from the low-serum medium showed an even stronger signal than before, suggesting that serum starvation results in an increased cytokine release in the REK cultures. Further control experiments, examining DMEM alone or 10% serum in DMEM, showed only a very faint cytokine signal on the blots. All these results point to the fact that the REK

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system inherently secretes a high load of cytokines which, interestingly, is reduced after injury.

To further the search for the paracrine factor, interest was shifted towards growth factors, and in particular the epidermal growth factor family of receptors and ligands. There is strong evidence in the literature to support the role of EGFR and its ligands in keratinocyte proliferation (section 2.3.7) and HA biology (2.3.9). A study was designed to test for the involvement of EGFR, by blocking the receptor kinase activity. The results showed a significantly blunted HA response to injury in the presence of AG1478, suggesting that the soluble factor (s) released after injury indeed function via the EGFR signaling pathway.

To further ascertain the role of EGFR ligands in the HA-induction pheomena, emphasis was put on EGF. EGF is an abundant and well-characterized ligand of EGFR, and is known to affect HA biology (section 2.3.9). An EGF neutralizing antibody was used to soak up this particular ligand in the medium, and assess its effect upon HA levels in the recipient cultures. Interestingly, this study showed a marked decrease in HA in recipient cultures, confirming without a doubt that EGF is an important effector of the HA response seen after barrier injury.

To ask a more global question, and to block the activity of all the EGFR ligands, a soluble EGFR fusion protein was identified and used as a ligand scavenging reagent to soak up all the different EGFR ligands present in the medium. Surprisingly, this study showed an increase in HA levels in the recipient cultures after the medium was transferred from acetone injured cultures. The extent of HA induction appeared to be roughly proportional to the concentration of the scavenging reagent. While this result was

unexpected, based upon the EGF neutralizing antibody study, it is a fascinating and potentially important finding. It suggests that there is a fine balance amongst the ErbB receptors, principally EGFR and its ligands, in terms of HA biology after injury.

It is important here to understand that there are four ErbB receptors and five principal ligands to EGFR/ErbB1. While we have shown that EGFR and EGF are crucial to HA synthesis after barrier injury, the involvement of more than one EGF ligand and/or ErbB receptor could help to explain the discrepancy in results observed with EGFR scavenging receptor (refer to Fig. 6-20). Three possibilities are as follows: 1) There exists a fine balance between the ErbB family of receptors regarding HA synthesis. Depending upon the type of receptor activation, there could be induction or inhibition of the HA synthetic pathway. For example Amphiregulin/'Y' might increase HA synthesis via EGFR signaling but inhibit HA synthesis via signalling through ErbB3. Hence, when the fine balance between these ligands is disturbed (removal of Amphiregulin/'Y' by the EGFR-Fc protein), the inhibition mediated by ErbB3 is lost, resulting in an increased HA signal (Fig. 6-20). 2) Depletion of all EGFR ligands could increase the signal transduction and activation of other ErbB receptors via another family of ligands like heregulins, known to bind ErbB2/ErbB3 and increase HA synthesis (Bourguignon, Gilad et al. 2007). 3) In the absence of sufficient signalling through EGFR, ErbB2 might form homodimers (in the absence of ligand) and lead to increased signal transduction resulting in an increase in HA synthesis (since ErbB2 is very active in downstream transduction).

The previous work (see chapter V) has shown the involvement of HAS synthetic enzymes as an effector of the HA increase observed after barrier injury. Experiments were designed to check for these enzymes in the recipient cultures. Surprisingly, there
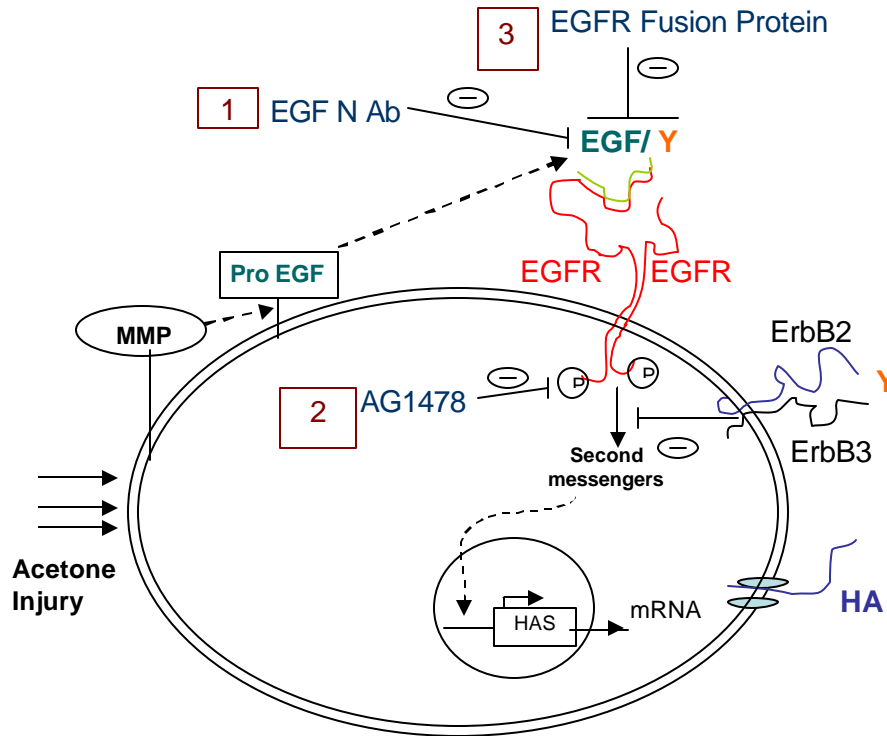
Figure 6-20: Summary of the role of EGFR in HA synthesis following barrier injury in the REK model. Soluble factor could be EGF or Y (all EGF-like ligands).
Elimination of soluble EGF itself from the medium (1), or blockade of EGFR receptor kinase (2), blocks HA synthesis and accumulation. Elimination of other EGF- family ligands (Y) on the other hand, leads to hyperinduction of HA levels (3). For example, Ligand 'Y' a EGF family ligand is able to bind EGFR, but can also bind to ErbB3, which following injury has a degree of control over ligand dependent signalling through ErbB1. Therefore when ligand 'Y' is also removed via the EGFR-Fc (ligand scavenging receptor), changes in signalling occur, which then has a downstream effect on HA production.

was no significant change in the HA synthases (data not shown). This could be due to the fact that the cultures were harvested at the time when the endproduct (HA) was examined in the cultures i.e. 24 hrs after medium transfer. Hence in the future it will be important to perform a time course study and check for HA synthetic enzymes as a function of time, starting at 2hrs after medium transfer all the way up to 24 hrs. Regarding HA synthases, the data collected from this study suggest the model illustrated in Fig. 6-20.

Looking to the future, it will be important to analyze the levels of the ErbB ligands particularly those of EGF in the transfer medium. At the same time it is important to note

that there could be EGFR receptor transactivation via release of a soluble factor e.g., ATP (further discussed in section 2.3.4). Thus, it could be a soluble factor independent of the ErbB ligand, which in turn activates another pathway, may be the MMPs (discussed in section 2.3.3) to release an effector of EGFR in the recipient cultures (Fig. 6-20). It could be a two or a three step process before the final funnelling of the signal i.e. induction of HA synthesis via homo-heterodimerization of EGFR. Essentially, the REK model of barrier injury represents a simplified tool to help dissect the very complicated link between HA induction and ErbB family signalling.

In summary, this study has shown that injury to the REK lift cultures leads to a release of a soluble factor(s) that mediates increased HA synthesis in uninjured cells exposed to the soluble factor. Also, the soluble factor seems to require activation of the EGFR pathway to bring about HA induction, wherein EGF seems to be critically involved. Whether the factor is EGF or alternatively, a non-EGF effector molecule which in turn modulates EGF like ligands and ErbB receptor activation, is a question to be addressed in the future work.

Chapter VII
Conclusion and Recommendations

7.1.   Summary

The work in this thesis focused upon developing, and then using a 3-D organotypic epidermal model (REK air-lifted cultures) to characterize the changes that occur in cellular proliferation and differentiation following barrier injury. Cutaneous barrier injury is a superficial but very important form of injury to the skin that occurs in daily life, and is also responsible for a number of highly prevalent diseases such as atopic dermatitis, hand eczema, and asteatotic dermatitis which remain poorly understood and hard to treat. Once the injury model was characterized, it was then used to examine the role of the matrix molecule hyaluronan (HA). Throughout this study, the relatively simple bioengineered system proved able to mimic many of the changes observed following barrier injury *in vivo*, and can be considered a strong tool to dissect the role of the epidermis in response to barrier injury, and also to help simplify and clarify the study of some complex phenomena related to hyaluronan biology.

Acetone-induced injury to the upper cornified layer of the model epidermis resulted in epidermal responses similar in some ways to those observed with a full thickness injury, including hyperplasia of the injured epidermis and strong inductions in HA levels. The epithelial response was dependent upon the amount of stratum corneum injury. "Mild injury" (1Tx acetone treatment) did not affect the tissue phenotype, whereas a "moderate injury" (2Tx acetone treatment) led to cellular proliferation and "severe injury" (3x acetone treatment) enhanced epidermal differentiation. Acetone injury that led to tissue hyperplasia also induced HA synthase enzyme levels and increased HA production. The synthesized HA was of a high molecular weight, an interesting finding because others have shown that high molecular weight HA favors cellular proliferation. The exact mechanisms that link HA to cellular proliferative responses following epidermal injury are still unknown, but the bioengineered barrier injury model may prove a useful tool in this area in the future.

The current work also highlighted the role of EGF and ErbB signalling in the wound healing response. The data showed a predominant role for ErbB1 and its major ligand EGF in HA synthesis following barrier injury. The partial blockage in HA levels observed after EGFR inhibition and the increase in HA levels after treating the cultures with the EGFR fusion protein, opens up possibilities for roles for additional ligands in addition to EGF, and receptors in addition to EGFR. While complex, the data do confirm that there is a fine balance and interplay between the ErbB receptors and their ligands in the HA response after barrier injury.

Overall, this study has achieved the methodological endpoints of (1) characterizing a new *in vitro* 3D model of barrier injury, and (2) standardizing methods to

study hyaluronan in terms of the molecular weight distribution, the enzymes which create and degrade HA, and the overall quantity and tissue localization of HA in the model both before and after tissue injury. Many of the techniques used in this study are not commercially available and will need to be further standardized over time, but already have excellent potential to answer some complex questions regarding injury and hyaluronan. Finally, we have helped to advance the idea that regeneration of epithelial function is a fundamental part of the healing response to injury, and HA is an important effector of this response.

7.2. Significance and Recommendations

The main impact and importance of this thesis has been the development of a new tissue-engineered model of stratum corneum barrier injury. With the creation of this model, initial studies have concentrated upon characterizing morphological changes in the epithelial tissue and changes in the matrix molecule hyaluronan after barrier injury, in a controlled *in vitro* setting. This novel bioengineered model of barrier injury has proven to be a powerful tool to answer some focused questions about HA biology after wounding. Table 7-1 summarizes the specific questions that were addressed in this thesis, and how our experiments have advanced the state of knowledge about barrier injury and role of HA. For example, moderate amounts of barrier injury in the model were shown to stimulate cellular proliferation and to increase HA synthesis, responses which closely mirror the responses observed after barrier injury *in vivo*. The importance of this is that the bioengineered model will now be useful to study mechanistic pathways related to these phenomena in the future, perhaps answering some vital questions regarding the role of HA in proliferation and differentiation in normal and injured epidermis. An example

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of possible work of this type would be to expand upon our interesting findings that under certain conditions, barrier injury causes epidermal differentiation. Enhanced differentiation was only observed with the severest form of barrier injury, along with an accompanying decrease in HA levels. A more mechanistic approach towards studying this phenomenon might give insight into the pathways that govern epithelial differentiation and HA. It will be interesting in the future to compare the changes in HA, in terms of its molecular size and its metabolizing enzymes in the setting of tissue differentiation versus proliferation.

Regarding the paracrine factor that is released after barrier injury and mediates the increase in HA at locations far from the site of actual injury, this work has provided strong but indirect evidence that EGFR activation as well as its principal ligand EGF are involved in the HA response. Further experiments are needed to directly document the release of EGF or other ErbB ligands after barrier injury. Changes in EGF and other known EGFR ligands could be assayed using ELISA to detect the levels of these ligands in the medium. As a complementary approach, experiments could be designed using blocking antibodies to receptors other than EGFR (especially ErbB2, since ErbB2 has no specific ligand of its own). In the search for the paracrine factor responsible for EGFR pathway activation and increased HA synthesis, it is important to keep in mind that a few molecules outside the EGFR-ligand family are known to be released after injury, which might in turn facilitate the release/shedding of EGFR pro-ligands and subsequent EGFR activation in the effector tissue.

Another area for future work will be to clarify the roles of HA synthases and degrading enzymes in the barrier injury response. Our initial experiments were directed

towards measuring HAS and Hyal enzyme levels via PCR did not show significant changes in the recipient cultures at 24 h after media transfer. It is possible that some changes in expression of Has or Hyal enzyme might have in fact occurred, but peaked earlier than 24 h. Therefore, it will be important to perform a time course study and check for HA metabolizing enzymes as a function of time, beginning at the time immediately after wounding and then at regular intervals. .

Though our newly-described *in vitro* model of barrier injury has behaved very similarly to preliminary studies performed with barrier injury in murine skin, it will be essential to test some of the new findings further *in vivo*. Many of the tools and methods that have been developed and characterized in our *in vitro* system and can be applied in the *in vivo* environment. For example, the changes we have described regarding changes in HA molecular weight distribution and in HA metabolizing enzymes can and should be examined and confirmed in acetone-treated mouse skin. Another very important area for further work will be to test the effects of barrier injury upon EGFR activity and its link to HA synthesis *in vivo*. A variety of knockout mice are available, including HAS, EGFR and CD44 knockouts, which can be used to perform wounding experiment and to compare and extend the data available from this thesis.

The significance of the work in this thesis and the findings are listed in a summary table on the next page.

Summary and Implications of findings in this thesis (Table. 7-1):

AIM	Known before about epidermal barrier injury *in vivo*	Shown by my work/ REK model of barrier injury *in vitro*	Not known/ REK model of barrier injury can be used to further study these parameters
1	Epidermal Hypertrophy occurs	Hypertrophy [*in vitro* REK model]	
1	Increased DNA synthesis occurs *in vivo*. Increase in anti apoptotic genes and proliferation markers occur in an *in vitro* model (Koria et.al. 2003)	Increased keratinocyte proliferation occurs in the *in vitro* model (Ki67 and BRDU)- No dermal input required for enhanced keratinocyte proliferation after barrier injury	What signals governs this increased proliferation? Are the BRDU stained cells (3 Tx) actually stem cells or Transient amplifying cells?
1	Increased expression of differentiation related genes occurs	Increase in the early differentiation marker (K10) and late (Filaggrin, Transglutaminase and thickened stratum corneum)- No dermal input required for enhanced keratinocyte differentiation	Why does the tissue respond with increased differentiation after severe (3 Tx) barrier injury?
1	Repeated barrier injury enhances the hyperplastic response	Data expands and supports current knowledge: Repeated acetone treatment is required to develop maximal hyperplasia It appears that cells need to undergo growth arrest before they begin to differentiate	What molecular mechanisms are triggered after repeated barrier injury?
2	Hyaluronan is increased after barrier injury *in vivo* (only HABP data available)	Increase in hyaluronan is observed in an *in vitro* system (no dermal components). Confirmed by two different techniques (HABP and FACE)	Which molecules govern this phenomenon? (We believe that Has enzymes play a role) Reason for difference between the magnitudes of changes observed after HABP *vs* FACE.-Why?

2	Loss of HA by hyaluronidase treatment leads to a significant decrease in the hypertrophic response seen after barrier injury *in vivo* (Maytin et.al. 2004)	Increase in HA supports proliferation (2 Tx); with a more severe form of barrier injury a decrease in HA to base line level leads to growth arrest and supports differentiation (3Tx)	What is the mechanism by which extracellular HA regulates cell proliferation?
2	Mol. Wt distribution of HA after injury- No reports	An increase in the high molecular wt HA occurs after 1 Tx and 2 Tx; Murine skin shows a broad HA signal across high and low mol wts	Few other tissues support this finding e.g. cancer cells. Also monolayer REKs have a pericellular HA coat. Are the same mechanisms at play after barrier injury?
2	Status of HA metabolizing enzymes after barrier injury- No reports	An increase in HA synthases Has 2 and 3 occurs along with a decrease in HA degrading enzymes Hyal 2 and 3 by RT-PCR	How specific are these results? What happens to the mRNA at different time points after injury? Need to do Real-time PCR/ Northern blot to confirm. Also what happens to the enzyme activity? Could use enzyme inhibitors, siRNA to block the message or HAS and HYAL knockouts/knock downs. PLC-gamma is known to phosphorylate Has2 directly; could try to inhibit PLC gamma
3	Widespread increase in HA after focal barrier injury- Not reported	The injury-mediated increase in HA requires a paracrine soluble factor(s) after barrier injury. Confirmed by 2 different techniques	Does barrier *vs* full thickness injury exhibit similar features in regards to HA response after injury? Which mechanisms are involved in these phenomena?
3	Cytokines are increased after barrier injury *in vivo*- IL1, TNFalpha, IL6	No increases are noted in these cytokines by antibody arrays on the medium collected after barrier injury	Might need to look at different time points after injury? Could do arrays on cell lysates to look for changes in cellular cytokines pre- and post-injury
3	Link between EGFR and HA after barrier injury- No reports	EGFR is involved in the HA increase observed after barrier injury	Another inhibitor to confirm; Cytoplasmic pathways? Transcription factors? Role of ErbB2 and ErbB3?

3	Link between EGF and HA response after barrier injury- No reports	EGF is an important mediator of HA increase after barrier injury	Is EGF secreted by REKs (check with ELISA)? Are MMPs involved? Does EGF act via receptors other than ErbB1 in the *in vitro* model?
3	ErbB family ligands in HA response after barrier injury- No reports	EGFR fusion protein upsets the HA response after barrier injury	What is the relative role of each of the ErbB ligands in HA response after barrier injury?

Table. 7-1: Summary and Implications of findings in this thesis.

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