Disease-causing Keratin Mutations and Cytoskeletal Dysfunction in Human Skin

In vitro Models and new Pharmacologic Strategies for Treating Epidermolytic Genodermatoses

JEAN CHRISTOPHER CHAMCHEU
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Abstract

Epidermolysis bullosa simplex (EBS) and epidermolytic ichthyosis (EI) are rare skin fragility diseases characterized by intra-epidermal blistering due to autosomal dominant-negative mutations in basal (KRT5 or KRT14) and suprabasal (KRT1 or KRT10) keratin genes, respectively. Despite vast knowledge in the disease pathogenesis, the pathomechanisms are not fully understood, and no effective remedies exist. The purpose of this work was to search for keratin gene mutations in EBS patients, to develop in vitro models for studying EBS and EI, and to investigate novel pharmacological approaches for both diseases.

We identified both novel and recurrent KRT5 mutations in all studied EBS patients but one which did not show any pathogenic keratin mutations. Using cultured primary keratinocytes from EBS patients, we reproduced a correlation between clinical severity and cytoskeletal instability in vitro. Immortalized keratinocyte cell lines were established from three EBS and three EI patients with different phenotypes using HPV16-E6E7. Only cell lines derived from severely affected patients exhibited spontaneous keratin aggregates under normal culture conditions. However, heat stress significantly induced keratin aggregates in all patient cell lines. This effect was more dramatic in cells from patients with a severe phenotype. In organotypic cultures, the immortalized cells were able to differentiate and form a multilayered epidermis reminiscent of those observed in vivo. Addition of two molecular chaperones, trimethylamine N-oxide dihydrate (TMAO) and sodium 4-phenylbutyrate (4-PBA), reduced the keratin aggregates in both stressed and unstressed EBS and EI keratinocytes, respectively. The mechanism of action of TMAO and 4-PBA was shown to involve the endogenous chaperone system (Heat shock proteins e.g. Hsp70). Besides, MAPK signaling pathways also seemed to be incriminated in the pathogenesis of EBS. Furthermore, depending on which type of keratin is mutated, 4-PBA up-regulated Hsp70 and KRT4 (possibly compensating for mutated KRT1/5), and down-regulated KRT1 and KRT10, which could further assist in protecting EBS and EI cells against stress.

In conclusion, novel and recurrent pathogenic keratin mutations have been identified in EBS. Immortalized EBS and EI cell lines that functionally reflect the disease phenotype were established. Two pharmacologic agents, TMAO and 4-PBA, were shown to be promising candidates as novel treatment of heritable keratinopathies in this in vitro model.

Keywords: epidermolysis bullosa simplex, epidermolytic ichthyosis, genodermatoses, keratin, keratin mutation, keratinocytes, gene therapy, pharmacological therapy, immortalization, gene regulation, trimethylamine N-oxide (TMAO), sodium 4-phenylbutyrate (4-PBA), tissue engineering, cell culture, heat shock proteins, MAP kinases

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urn:nbn:se:uu:diva-123071 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-123071)
In memory of my mother
Tienguep Josephine,
To my father
&
My Children

“An idea that is developed and put into action
is more important than an idea that exists only as an idea”
(Buddha)

“Judge your success by what you had to give up
in order to achieving (get) it”
(Dalai Lama)
List of papers included

This thesis is based on the following papers, herein referred to by their Roman numerals.


*Reprints were made with permission from the publishers.*
Introduction to Skin Biology ................................................................. 11
  Structure and Function of Human Skin ..................................................... 11
    The Epidermis and Epidermal Homeostasis .......................................... 12
Intermediate Filaments (IF) .................................................................. 14
  Keratin Intermediate Filaments (KIF) ..................................................... 15
Structure and Assembly of KIFs ............................................................ 15
  Keratin Expression in the Epidermis ..................................................... 17
Keratinocyte Culture in Dermatological Research ................................. 18
  Cellular Physiology and Pathopysiology linked to Cell Signaling, Molecular Chaperone and Protein Degradation Pathways ......................... 19
    Role of molecular chaperones and the ubiquitin-proteasome pathways in protein degradation ................................................................. 20
    Removal of misfolded proteins such as keratins in cells ..................... 21
    MAPK signal Transduction in the Epidermis ....................................... 22
Mutations in Epidermal Keratins and their Associated Pathologies .......... 26
  Epidermolysis Bullosa (EB) ................................................................. 27
  Epidermolysis Bullosa Simplex (EBS) .................................................. 27
  Epidermolytic Ichthyosis (EI) ............................................................... 31
Therapeutic approaches for EBS and EI ............................................... 32
Aim of the Research ............................................................................... 34
  The specific objectives are; ................................................................ 34
Materials and Methods ....................................................................... 35
  Patients characteristics (Paper I, II, IV) ................................................ 35
  Blood specimen and skin biopsies sampling (Paper I-IV) ....................... 35
    Isolation and establishment of primary keratinocyte cultures (Paper I, II, III, IV) ................................................................................ 36
  Immortalization of keratinocytes from EBS and EI patients ................. 37
  Heat stress assay and treatment with TMAO and 4-PBA ..................... 40
Results .................................................................................................... 44
  Paper I ................................................................................................ 44
    Effects of keratin mutations on EBS disease phenotype ..................... 44
    Effect of keratin mutation, heat stress and treatment with TMAO on cell fragility ................................................................. 46
Paper II .......................................................... 48
  Generation of immortalize keratinocytes ........................................ 48
  Effects of heat stress and TMAO on cytoskeleton resilience .......... 48
Paper III ........................................................................ 49
  Chemical chaperones reduced heat-induced keratin aggregation ...... 50
  Effects of heat stress and TMAO on the expression of Hsp70 and Hsp90 ................................................................. 51
  Heat stress and TMAO differentially modulate MAPK signaling ...... 52
Paper IV .......................................................................... 53
  Establishment and characterization of EI keratinocyte cell lines .... 54
  Keratin expression, differentiation and characteristics of heat stress on EI-keratinocytes ...................................................... 54
  Pretreatment with TMAO and 4-PBA suppresses keratin aggregation in EI keratinocytes ...................................................... 54
  TMAO and 4-PBA alter the mRNA expression of keratins 1, 4 and 10 and Hsp70 in unstressed keratinocytes ......................... 55
  The EI disease phenotype is reproduced in organotypic epidermis .... 55

Discussion and Conclusions ................................................. 57
Patient characteristics and genotype - phenotype correlations ........ 57
Establishment and characterization of EBS and EI keratinocyte lines 58
Effect of keratin mutations on cell fragility ................................. 59
Effect of heat stress on keratin cytoskeleton resilience in keratin-defective cells ................................................................. 59
Effects of treatment with TMAO and 4-PBA on keratin aggregation and involvement of molecular chaperones and MAP kinases ...... 60
Possible implications of 4-PBA and TMAO treatment on keratin regulation in EI ................................................................. 61
Tissue engineering of EBS and EI epidermis .................................. 61

Conclusions ........................................................................ 63

Future Perspectives ............................................................ 64

Summary in French .............................................................. 66

Summary in Swedish ............................................................ 68

Acknowledgements ............................................................. 70

References ........................................................................ 75
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>BMZ</td>
<td>Basement membrane zone</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DED</td>
<td>De-epidermized dermis</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EBS</td>
<td>Epidermolysis bullosa simplex</td>
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<td>EI</td>
<td>Epidermolytic ichthyosis</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>HS</td>
<td>Heat stress</td>
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<tr>
<td>K</td>
<td>Keratin</td>
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<tr>
<td>KRT</td>
<td>Keratin gene</td>
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<tr>
<td>IF</td>
<td>Intermediate Filament</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>4-PBA</td>
<td>Sodium 4-phenylbutyrate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEST</td>
<td>Penicillin + Streptomycin</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>Quantitative real-time RT-PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress activated protein kinases</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SB</td>
<td>Stratum basale</td>
</tr>
<tr>
<td>SC</td>
<td>Stratum corneum</td>
</tr>
<tr>
<td>SG</td>
<td>Stratum granulosum</td>
</tr>
<tr>
<td>SS</td>
<td>Stratum spinosum</td>
</tr>
<tr>
<td>TMAO</td>
<td>Trimethylamine N-oxide dihydrate</td>
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</table>
Introduction to Skin Biology

The skin is a dynamic, multilayered complex tissue covering the exterior of the body, and is strategically located at the interface between the external and internal environments, mediating a multiplicity of functions. The skin histology reveals a built-in synchrony in the epidermis, delineates the existence of a tremendous degree of communication and coordination between adjacent and non-adjacent cell layers in the tissue.

The skin provides physical permeability and protective barriers, which helps to regulate body temperature, fluid balance, sensation, stores water and fat, metabolizes vitamin D and against external damaging factors such as mechanical insults, heat, cold, ultraviolet radiation, and chemical and infectious microbial organisms. In addition, the skin plays an important role in the immune system, psychosocial and sociosexual communication, and is also able to repair wounds and self regeneration. Disturbances in these coordinated functions due to hereditary or acquired factors results in a wide range of skin diseases.

Structure and Function of Human Skin

The skin is the largest organ of the human body, accounting for approximately 15 percent of body weight and covers a surface area of ~1.5 -2.0 m² for an average adult. The skin is about 1–4 mm thick and consists of three distinctive tissues; from inside out, the innermost tissue subcutis, the dermis and the outermost skin tissue epidermis (Fig. 1a).

The subcutis (hypodermis or subcutaneous fat), mainly consists of adipose (fatty) tissue that connects the lower reticular dermis to the underlying skeletal components. The subcutis functions as an energy storage source, insulator and shock absorber thus protecting the inner organs. The subcutis permits skin mobility over underlying structures and also has a cosmetic effect in molding body contours with thickness that markedly varies with sex, age, and at different body sites between individuals.

Overlying the subcutis is the dermis which constitutes the principal tissue mass of the skin, and consists of a fibrous, filamentous and cellular connective tissue. Its main constituents are collagen and elastic fibers, which provide structural flexibility and tensile strength. The dermis consists of two major compartments: the upper papillary dermis that is tightly connected to
the uppermost layer of the skin, the epidermis, via the basement membrane, which is the main part of the dermal–epidermal junction and the lower reticular dermis which is bound to the subcutis \(^1,2\). Fibroblasts comprise the major dermal resident cells and are responsible for the metabolism of connective tissue matrix proteins and the secretion of signaling factors. Other resident dermal cells include myofibroblasts, macrophages, mast cells, and transiently circulating cells of the immune system. The dermis houses many functional elements of the skin, such as the complex networks of the vascular, neural and lymphatic systems, and embraces the epidermal appendages such as hair follicles, sebaceous, eccrine and apocrine glands (see Fig. 1a), and the nails. The dermis aids in the regulation of body temperature, skin repair, water storage, and protection of the body from mechanical insults. It interacts and supplies the epidermis and epidermal cells with diffusible nutrients and oxygen from its underlying vascular network.

The Epidermis and Epidermal Homeostasis

The epidermis is a non-vascularized, stratified keratinizing squamous epithelium of approximately 75-150 µm (up to 600 µm thick on load bearing skin-sites such as the palms and soles). The epidermal tissue is mainly composed of 90-95% keratinocytes, having functions that determine the condition of the epidermis. Based on the skin site, four (hairy skin) or five (palmo-plantar or glabrous skin) distinct cell layers (strata) can be recognized on histological cross-sections: stratum basale (germinativum; SB), stratum spinosum (prickle cell layer; SS), stratum granulosum (granular cell layer; SG), stratum lucidum (only in palms/soles; SL) and stratum corneum (horny layer, SC) (Fig. 1b) \(^1,2\). These layers correspond to increasingly different stages of keratinocyte maturation, i.e. the biochemical processes of terminal differentiation, in which keratinocytes lose their ability to proliferate, become enlarged, flattened, anucleated and increasingly dehydrated.

The SC consists of layers of flatten, anucleated keratinocytes called “corneocytes”, about 30 µm in diameter, and densely packed with keratin and embedded in extracellular lipid matrix. Corneocytes are connected to each other by cornodesmosomes and are gradually removed from the skin surface in a process called desquamation, making place for new cells coming from underneath. The epidermal turnover is a highly organized process both in space and time, such that an absolute balance between proliferation and differentiation exists \(^1,2\). The SC is approximately 10–20 µm thick and is essential in terms of its barrier properties and protection, both outside in and inside out, with a well organized structure built of corneocytes and intercellular lipids which together form an efficient water permeability barrier \(^5\). The flattened corneocytes are formed as a result of keratin filament aggregation by filaggrin, and by cross-linking of proteins, e.g. loricrin and involucrin, beneath the plasma membrane catalysed by the enzymes transglutaminase
(TGase)-1 and 3. The biochemical processes leading to the formation of the SC involves degradation of organelles, organization of keratin bundles inside the cell, and formation of a cornified envelope around it. This is accompanied by secretion of lamellar bodies; that is organelles containing a mixture of polar lipids, free sterols, and phospholipids, as well as catabolic enzymes. After extrusion at the SG–SC interface, these polar lipids are enzymatically converted into non-polar products, and assembled into lamellar structures surrounding the corneocytes.\textsuperscript{1,2}

Beneath the SC comes SG, composed of few layers of non-dividing, flattened keratinocytes that produce keratohyaline granules, which contain pro-filaggrin, a precursor of filaggrin and degradation enzymes. These cells flatten as cells below progressively push them to the skin surface and are connected to each other by desmosomes. At the same time there is degradation of cell organelles and nuclei and keratinocyte cell membranes become increasingly impermeable. SG overlies the SS containing 2–4 layers of keratinocytes with some limited capacity for cell division. Also found here are bone marrow-derived Langerhans’ cells, which are sentinel cells of the immune system which scour the skin for evidence of entry by foreign entities, i.e. presentation of antigens during T lymphocyte-mediated immune responses. The basal or dividing layer of the epidermis (stratum basale or germinativum) consists of cuboidal or columnar shaped proliferative (progenitor) keratinocytes closely adhered to components of the underlying basement membrane zone (BMZ) such as laminin 5 and collagen IV which separates the epidermis from the dermis. Hemidesmosomes connect these keratinocytes to the BMZ and are part of the dermal-epidermal junctional complex. In addition to the differentiating (maturing) keratinocytes, the stratum basale also houses other keratinocytes with stem cell properties. Other important epidermal cell types include Melanocytes, i.e. pigment-producing cells that protect the skin against the damaging effects of ultraviolet light (Fig. 1b), the enigmatic Merkel cell (with sensory functions) and various cells of the immune system.

The epidermis is renewed or regenerated and repaired by a population of stem cells, confined to the interfollicular epidermis and skin appendages (e.g. the hair follicles), capable of unlimited self-renewal.\textsuperscript{6} Beside these stem cells with high proliferative capacity, a second type of proliferating keratinocytes also termed transit-amplifying or progenitor cells, are located in the viable epidermis that divide a limited number of times and finally lose proliferative potential to terminal differentiation as they move upward to the cornified cell layer. These progenitor stem cells comprise approximately 10% of basal cells and provide the epidermis with its regenerative capacity.
Desmosomes and hemidesmosomes are important for cell-cell and basal cell attachment to underlying dermis. In the keratinocytes, keratin intermediate filaments (KIF; see below) extend and attach to sites of cell-cell adhesion (desmosomes) and cell-matrix attachment (hemidesmosomes), which contributes to cell integrity in stratified epithelia. Two hemidesmosomal proteins (plectin and the bullous pemphigoid 1 antigen, BPAG1), associate on the cytoplasmic surface of the integrin and anchor the KIF network to hemidesmosomes.

**Intermediate Filaments (IF)**

The cell cytoskeleton of all multicellular organism consists of three abundant filament systems which play important roles in the organization, mechanical integrity and strength of tissue cells: the actin microfilaments (MFs; 7-10nm diameter), intermediate filaments (IFs; 10-12 nm diameter), and interconnected microtubules (MTs; 25 nm diameter). Each filament system is built from a family of proteins with cell-tissue specific regulation of expression, with each protein family being encoded by the corresponding gene family. IFs are by far the most complex of the cytoskeletal proteins with at least 60 different IF proteins, subcategorized into six broad types based on tissue-specific expression, sequence similarity and protein structure see www.interfil.org.
Keratin Intermediate Filaments (KIF)

The keratin intermediate filaments (KIFs) are the most abundant structural IF protein constituent in the cytoplasm of epithelial tissue cells of which the epidermis forms a part. KIFs are encoded by large and conserved multigene family coding proteins that form a network of 10-to12-nm-wide KIFs. KIFs network represents about three-quarters of known IF in humans and builds into a dense, three-dimensional transcellular and highly dynamic cytoskeleton network structure spanning between the nucleus and extending to the cell periphery, where they anchor and interact with cell-cell (desmosomes) and cell-matrix (hemidesmosomes) adhesion complexes. This organization provides structural stability, flexibility, and ensures the mechanical integrity of the different epithelial cells and their specific tissues.

Keratins vary in sizes between 40-70 kDa and are divided into two sizes: the smaller or low molecular weight acidic type I (40-64 kDa, with PI: 4.7-6.1) and the larger or high molecular weight neutral-basic type II (52-70 kDa, with PI: 5.4-8.4) subgroups of IF proteins. A novel consensus nomenclature for mammalian keratin genes and proteins has been established and grouped into three categories: (1) epithelial keratins, (2) hair keratins, (3) keratin pseudogenes. The nomenclature for both genes and proteins includes 28 type I (K9, K10, K12–K20, K23–K28, K31–K40) and 26 type II (K1-K8; K71-K86) keratins, which form two clusters of 27 genes each. In the human genome, genes encoding type I and type II keratins are mainly clustered at two different loci on chromosomal regions 17q12-q21 and 12q11-q13, respectively. The epidermal type I keratin genes e.g. KRT1, KRT2, KRT4, and KRT5, each comprise 9 exons, whereas the genes coding for epidermal Type II keratins KRT10 and KRT14 each consist of 8 exons (see www.interfil.org).

Structure and Assembly of KIFs

Similar to all IFs, keratins share a head-rod-tail structural domain organization, and the basic polypeptide structure consists of a central α-helical coiled-coil rod domain of ≈310 to 315 amino acids in size interrupted by non-helical linker regions, and they are flanked by variable, non-helical amino-terminal head and carboxy-terminal tail domains (Fig. 2). The central alpha-helical rod domain is composed of four helical segments (1A, 1B, 2A and 2B) that are interrupted or flanked by three short non-helical flexible linkers (L1, L12 and L2). The rod domain is composed of repeats of seven amino acid residues (a-b-c-d-e-f-g)n termed “heptad repeats”. Positions “a” and “d” are occupied by hydrophobic residues that are considered crucial for the coiled-coil formation. In addition, near the middle of the 2B domain is a discontinuity in the heptad repeat (helix inversion), where the heptad pattern is interrupted, giving rise to the “stutter” region that might play specific roles.
in the elongation and rotational characteristics of keratins. This helical segment is highly conserved among IF and does not participate in the formation of the coiled-coil dimer. The start of the 1A rod domain and the end of the 2B rod domain, the so-called helix initiation (HIP) and helix termination peptides (HTP), respectively, comprise ~20 amino acid sequence motifs that are most highly conserved among the different keratins. These motifs play a pivotal or critical role in overlapping interactions in keratin intermediate filament (KIF) assembly and thus mutations in these motifs interfere with the early stages of filament elongation. The helix boundary motifs are mutational “hot spots” in almost all inherited keratin disorders. The terminal head domain regions consist of the subdomains (variable) V1 and (homologous) H1 and the terminal tail domain regions of subdomains H2 and V2, and the end (E) domains (Fig. 2). Variations in the head and tail domains account for much of the diversity among the individual keratin proteins within one group. This results in type II keratins being larger and more extensible than their type I counterpart. The end domains mediate interactions with other filaments and cellular proteins and serve as substrates for posttranslational modifications that regulate structure, organization and function.

**Figure 2.** Schematic representation of type I and II keratin polypeptide domain structure. Each keratin molecule consists of a central alpha helical rod domain which is composed of four helical segments, 1A, 1B, 2A and 2B that are interrupted by three flexible non-helical linker domains L1, L12 and L2. The rod domain begins and ends with highly conserved sequence motifs, helix initiation (HIP) and helix termination (HTP) peptides and is flanked by head and tail domains, respectively. (Courtesy of Mbeumi Sergette)

Keratins are obligate heterodimers, i.e. heteropolymeric pairing of one type I and one type II keratin molecule constitute their filamentous stage. Heterodimer formation is by coiled-coiled association of the corresponding rod domains where the two participating monomers exhibit a parallel, in-register alignment. The heterodimers align laterally in an overlapping and antiparallel fashion to form tetramers which polymerise to elongated chains and form KIFs through lateral packing (Fig. 3).
Figure 3. Keratin intermediate filaments assembly: Keratin polymerization obligatorily begins with the formation of coiled-coil obligate heterodimer structures involving winding around each other of the central rod domains of type I and type II polypeptides, a requirement underlying the pairwise transcriptional regulation of keratin genes in vivo. The heterodimers then associate (side-by-side) and assemble in an overlapping staggered and antiparallel fashion to form stable tetramers. Tetramers then associate end-to-end to form protofilaments and finally, four protofilaments laterally build keratin intermediate filaments. Each filament contains approximately eight protofilaments wound around each other in a rope-like structure. (Adapted from Virtanen M. 2001, with permission)

Keratin Expression in the Epidermis

In the epidermis, about two-thirds of known keratin genes are expressed, and in the interfollicular epidermis, progenitor basal keratinocytes express the K5/K14 pair, and to a lesser extent K15 in the SB. During commitment to terminal differentiation at the spinous layer, keratinocytes switch off K5/K14 transcription and rapidly turn on differentiation-specific keratin pair K1/K10 expression, and eventually K2 expression further up in the granular layer. Other site-specific suprabasal keratins include K9, which is predominantly expressed post-mitotically in the thick primary epidermal ridges of palmo-plantar epidermis, and K6, K16 and K17 which are induced by trauma to the skin or during specific diseases (e.g. psoriasis or infections) at the expense of K1/K10 and occurs in the thinner, secondary ridges connecting them. These deviations reflect regional or appendageal specialization.
Keratinocyte Culture in Dermatological Research

Initial attempts to grow skin and its cellular components centred on the use of organ cultures and explant cultures, where whole pieces of skin were kept alive and growth was confined to the epipole around the piece or onto the plastic around the explants. These cultures had a short life span and limited applications, as mixed cultures of keratinocytes, melanocytes, Langerhans cells, fibroblasts, Merkel’s cells, nerve cells and glandular cells are obtained. To understand the function and dysfunction of this complex structure, it was necessary to study partial aspects in models of manageable size and to reintegrate the results back into tissue context, i.e., establishment of pure cultures of keratinocyte compartment of the skin tissue. The selective cultivation of keratinocytes has considerably increased our understanding of keratinocyte biology in health and disease. Pure keratinocytes culture in monolayer and reconstructed human skin equivalents are alternatives to animal and human experimentation and they also provide a means to improve and extend our knowledge of biological and pathobiological processes in the skin.

Pure keratinocytes were successfully cultured by Rheinwald and Green in 1975 using a serum containing DMEM/Ham’s F12 medium and a feeder layer (irradiated mouse 3T3 cell lines). Ever since then, culture cell yield has been improved tremendously via the incorporation of mitogens, such as epidermal growth factor (EGF) and the cAMP-elevating agent cholera toxin. In culture, keratinocytes attach as single cells or small clusters and then grow at the periphery of the colony whilst stratifying in the centre, and forming intercellular desmosomes, growing as coherent colonies until confluent stratifying multilayered squames are obtained. The poorly formed squames do not present normal skin morphology, but cells are flattened, attenuated without the formation of granular or cornified layers, having sparse membrane coating granules and keratohyaline granules, but with cultures having similar phenotype as regenerating epidermis. Since then, ways of avoiding serum and presence of mouse feeder cells (3T3 cells) in keratinocyte cultures have been developed and defined serum-free media are now commercially available. In such media the cells fail to form desmosomal interconnections and are spaced out as a monolayer opening up a means to study not only cross-talks but also specified effects especially in disease models such as epidermolysis bullosa simplex (EBS) as opposed to epidermolytic ichthyosis (EI) which would require much higher calcium levels. In low Ca²⁺ medium keratinocytes expressed germinative basal epidermal keratins, such as K5 and 14, whereas in high Ca²⁺ medium keratins of differentiated epidermis, K1 and K10 are expressed. The use of proliferative patient’s keratinocytes for the study of EBS and EI keratinocytes depends on the longevity, and phenotypic stability of the keratinocytes that can be improved in long-term proliferative and reconstituted skin equivalent models. In order to ob-
tain such systems, keratinocytes can be transformed by replication incompetent virus exposure, e.g. Simian virus 40, human papilloma virus (HPV) and by oncogene transfections. Immortalized, keratinocyte culture models have improved considerably the biology of cutaneous keratin disorder in the areas of protein, cell and gene manipulation, and recently testing of pharmacological approaches for new remedies.

In skin equivalents, epidermal keratinocytes are grown exposed to air on different matrix; (i) type I collagen with either primary dermal fibroblasts or heterologous mouse fibroblast lines or possibly other cells embedded 29, or (ii) on de-epidermized dermis or cell culture inserts depending on the need 30,31 giving rise to almost normal tissue architecture. The quality of the human skin equivalents has reached a point that their suitability for skin toxicity testing will make great progress. Next to the field of toxicity and safety standards, skin equivalents offer a well-characterized model for studies of basic skin biology, pathogenesis of skin diseases, wound repair and regulation of melanogenesis 32.

We now know the keratinocyte is not just a simple passive cell awaiting terminal differentiation but is an active secretory cell with important biochemical and immunological functions with the ability to unravel pathological and therapeutic aspects of the skin.

**Cellular Physiology and Pathopysiology linked to Cell Signaling, Molecular Chaperone and Protein Degradation Pathways**

Human diseases and mouse genetics in recent years have provided strong experimental evidence in support of new functional roles of keratins beside their structural function. It is evident that keratins are involved in six broadly defined functions: structural support, cytoarchitecture, stress response, regulation of signaling pathways toward apoptosis, protein synthesis and distribution 33,34. Therefore, keratins form complex signalling cascades and interact with various kinases, adaptor and apoptotic proteins thereby affecting multiple cellular functions. Keratins may participate in the regulation of apoptosis through several mechanisms: binding of apoptosis-related molecules 35, acting as a phosphate “sponge” for stress-activated kinases to prevent potential activation of pro-apoptotic substrates 36 or to regulate the key effectors of the metabolic stress responses via phosphorylated epitopes on keratins 37.

Heat and other forms of stress that cause proteins to denature induce the synthesis of several classes of proteins known as heat shock proteins (Hsp), many of which act as molecular chaperones 38. A major role of these molecular chaperones is to catalyze the refolding of dena-
tured proteins after stress. However, certain molecular chaperones are constitutively produced, and have important functions under normal conditions. Such functions include the following: a) promotion of proper protein folding after synthesis, b) stimulation of assembly and disassembly of multimeric proteins, and c) facilitation of protein translocation across a variety of intracellular membranes. In addition, molecular chaperones also stimulate the breakdown of proteins, such that when molecular chaperones fail in their functions either naturally or from the effect of external stress such as heat, oxidative and osmotic stress, they then facilitate the degradation of the mishandled (aggregated) proteins. Characteristic protein aggregates are observed in a number of disorders, including neurodegenerative diseases, Alzheimer’s and Parkinson’s diseases, as well as heritable skin diseases such as EBS and EI. These protein aggregates are a characteristic clinical feature of misfolding disorder, though it remains unclear whether protein aggregation is a protective measure or pathogenic mechanism. Since little is understood regarding the formation, degradation, understanding the specificity of these aggregates is a question of further investigation. However, recent evidence distinguished aggregates of keratin as dynamic assemblies with short turnover time, compared to the static aggregates seen in other neurodegenerative disorders.

Role of Molecular Chaperones and the Ubiquitin-Proteasome Pathways in Protein Degradation

There are two main pathways of protein degradation (proteolysis) in the cells: lysosomal protease degradation and the ATP-dependent ubiquitin proteasome degradation pathways (UPP). Lysosomes are organelles that contain digestive enzymes surrounded by membrane used to digest and degrade the contents of vacuoles. Lysosomal degradation is used during phagocytosis, endocytosis and autophagy to digest proteins, and may also be used to break down misfolded (aggregated) proteins in protein misfolding disorders. The UPP is involved in regulating the cell cycle, signal transduction, differentiation, and stress response. A majority of these functions are mediated by the conditional turnover of regulatory and structural proteins. The UPP is the major mechanism used to degrade abnormal proteins or proteins at the ends of their life cycles. Proteins targeted for degradation are covalently modified by ubiquitin on lysine residues and the substrate-ubiquitin conjugate is then degraded by a very large proteolytic complex, 26 S proteasome in an ATP-dependent manner.
Removal of Misfolded Proteins such as Keratins in Cells

The tendency of a misfolded polypeptide to aggregate can severely affect cellular function and cause cytotoxicity reviewed in 47,57. Two strategic approaches are envisaged to account for the molecular mechanisms that prevent the accumulation of misfolded polypeptides and thus aggregate formation: (i) misfolded proteins are recognised by molecular chaperones and promote folding to the native state or (ii) may be removed by proteolytic machineries such as the ubiquitin/proteasome system. Keratins are degraded in a similar way as other soluble cytosolic proteins in an ATP dependent process that involves priming of the keratin for degradation and ligating to ubiquitin.

The assembly state of KIFs has been examined in human A549 cells, and stress disassembled and degraded keratin proteins via the ubiquitin-proteasome pathway 53. Inhibition of the proteasome prevented the degradation of keratins in shear-stressed A549 cells. Interestingly, recognition of the keratin protein for ubiquitination, and subsequent degradation, appears to be dependent upon the phosphorylation of K8 Ser-73 58. Thus, phosphorylated KIF proteins in cells are targeted for degradation via the ubiquitin-proteasome pathway in response to mechanical stress. The specific phosphorylation of K8 pSer-73, but not K8 pSer-23 or K8 pSer-43, was associated with the shear stress-mediated disassembly of the KIF network, thus acting as a signal recognition for ubiquitin conjugation 58,59.

Both the assembly and organization of IFs are regulated by post-translational modifications (glycosylation, serine / serine-threonine phosphorylation, acetylation and disulfide bonds formation), especially phosphorylation, which occurs within the head and tail domains, at certain phosphorylation sites. These phosphorylations are responsible for most of the structural heterogeneity and the presumed tissue specific functions of IF proteins 21. Mutation at this site, K8 S73A, prevented ubiquitin from being covalently attached to keratin protein. The events could signal the activation of ubiquitin by the E1 (ubiquitin activating enzyme), followed by the transfer of ubiquitin to the E2 (ubiquitin conjugating enzyme: UbcH5/Ubc3 families are specific E2s involved in keratin labeling for degradation by proteasome). The E2 shuttles the ubiquitin molecule to a keratin-specific ubiquitin ligase, E3, which has yet to be identified. However, recent findings suggest CHIP/STUB1 as keratin specific ubiquitin ligase 51. The E3 delivers the ubiquitin to the phosphorylated keratin, which has been targeted for degradation. The polyubiquitinated keratin protein is then degraded by the large 26 S proteasome 56, meanwhile short ubiquitinated chains are transported and degraded by the lysosomes.
Mode of Action and Cellular Functions of Molecular Chaperones

Through controlled binding and release cycles, chaperones facilitate the correct fate of the non-native polypeptide⁶⁰–⁶³. Misfolded proteins are delivered to the chaperonins by other molecular chaperones, notably members of the Hsp70 family⁶¹,⁶³. The two main mammalian cytosolic Hsp70 proteins are the constitutively expressed Hsc70 (70-kDa heat shock cognate protein) and the heat-inducible Hsp70 (sometimes referred to as Hsc/Hsp70), which act as to initially recognize and stabilise non-native polypeptides. Mammalian Hsc/Hsp70 binds nascent and newly synthesized polypeptides comprising about 15–20% of total protein⁶⁴, which further increased under stress conditions. Hsp70 proteins shield hydrophobic regions, apparently preventing protein aggregation and promoting proper folding. The fate of the chaperone-bound polypeptide is determined to a significant extent by the cofactors that associate with the chaperone/substrate complex and the intracellular balance of the competing and cooperating cofactors therefore set the threshold between folding and degradation. The protein, termed CHIP (for carboxyl terminus of Hsp70 interacting protein), is intimately involved in the regulation of molecular chaperones Hsp70s and Hsp90s, having close collaboration with the ubiquitin/proteasome system during protein quality control.

MAPK signal Transduction in the Epidermis

Signal transduction at the cellular level refers to the movement of signals from outside the cell to inside. Upon binding of their appropriate ligand the receptors activate specific signal transduction pathways and thereby control a number of cellular processes including cell cycle, proliferation, differentiation and apoptosis through regulation of the transcription of specific genes. Aberrant signalling leads to altered homeostasis and is believed to be part of the pathogenesis and pathophysiology in many skin diseases, including hyperproliferative and inflammatory skin diseases as well as neoplasms. Signal transduction can be mediated through small ion movements, which results in changes in the electrical potential of the cell, or through protein phosphorylation by different kinases. Protein phosphorylation changes the protein conformation and enzyme activities. This also suggests that the activation signal can be turned off by removal of the incorporated phosphates and therefore phosphatases also function as regulators of signal transduction. This excerpt briefly reviews the mitogen-activated protein kinase (MAPK) signal transduction pathways and the implications in the skin epithelium.

MAPK signalling pathways constitute a large kinase network that regulates a variety of physiological processes. To date, three MAPK pathways have been characterized in detail. The ERK pathway is activated by a large variety of mitogens and by phorbol esters and in the epidermal keratinocytes recent evidence shows their involvement in mechanical stress, whereas the c-Jun
NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 pathways are stimulated mainly by environmental stress and inflammatory cytokines (Fig. 4). MAPK cascades are organized as modular pathways in which activation of upstream kinases by cell surface receptors leads to sequential activation of an MAPK module (MAPKKK→MAPKK→MAPK) (See Fig. 4). After MAPKs (ERK1, 2, JNK1–3, and p38α, β, γ, δ) are activated either in the cytoplasm or in the nucleus, they bind and regulate transcription by modulating the function of a target transcription factor through serine/threonine (ser/thr) phosphorylation 65-67. In addition to the transcriptional effects of MAPK signaling, accumulating evidence indicates that MAPKs regulate cell behaviour also by phosphorylating cytoplasmic target proteins, such as apoptotic or cytoskeletal proteins (See Fig. 4).

Figure 4. MAPK signaling pathways. MAPK signaling pathways are organized in modular cascades in which activation of upstream kinases by cell surface receptors lead to sequential activation of a MAPK module (MAPKKK to MAPKK to MAPK). Shown are the major MAPK pathway components and examples of the MAPK pathway target proteins (Depicted from http://www.cellsignal.com/pathways/map-kinase.jsp).
Extracellular-regulated kinase 1, 2 (ERK) pathways

The ERKs were the first MAPK family to be characterized and two isoforms, ERK1 and 2, which are sometimes referred to as p44/p42 MAP kinases have been characterized. The ERK pathway is activated mainly in response to mitogens and growth factors, as well as cytokines, virus infection, transforming agents and carcinogens and ligands for heterotrimeric guanine nucleotide-binding protein (G-protein)-coupled receptors. Once ERK1 and 2 have been activated they can target cytoplasmic, membrane, cytoskeletal and nuclear proteins. Most of the signals activating the ERK pathway are initiated through receptor-mediated activation of the small G-protein, Ras. Activated Ras then recruits cytoplasmic Raf (MAPKKK) to the cell membrane for activation. All three Raf proteins share the same downstream MAPKK substrate mitogen-activated protein kinase kinases 1, 2 (MEK1, 2). An important role of ERK activation is modulation of gene expression, associated with cell growth, proliferation, differentiation and survival. ERK1 and ERK2 have both been demonstrated in the epidermis and in normal human keratinocytes in vitro. Increased ERK1 and ERK2 expression has been demonstrated in the basal and lower suprabasal layer of lesional psoriatic skin and ERK activation was demonstrated to play an important role in rescue of EBS cells.

c-Jun N-terminal kinase (JNK) pathway

The c-Jun N-terminal kinase (JNK) was originally identified as the UV-induced factor responsible for phosphorylating and thereby activating the proto-oncogene transcription factor c-Jun. Three highly related but distinct gene products, JNK1, JNK2 and JNK3, have been described which share 85% sequence identity and are expressed ubiquitously. The c-Jun N-terminal kinase (JNK) pathway is mainly activated by cellular stress and by cytokines. These stimuli activate JNKs through several upstream kinases (MAPKKKs). MAPKKs for JNKs are MKK4 and MKK7, which are both needed to fully activate JNK. Like all other MAPKs, JNKs are activated through phosphorylation of a tyrosine and a threonine residue, but specificity from the other MAPKs is ensured by the separating proline (TPY), they exert their effects only on transcription factors in the nucleus. This is in contrast to the other MAPKs which phosphorylate targets both inside and outside the nucleus. Activation of the JNK signaling pathway plays an important role in regulating apoptosis as well as tumourigenesis and sustained JNK activity is necessary for cellular homeostasis, whereas strong stress stimuli in non-transformed cells primarily lead to JNK-mediated apoptosis. In the skin JNK1 and JNK3 have been demonstrated in cultured normal human keratinocytes in vitro, and immunohistochemical analyses of normal human skin revealed that phosphorylated (active) JNK is expressed in the nuclei in the suprabasal-granular cell layer indicating that JNK participate in regulation.
of gene transcription in differentiating normal human keratinocytes. The most classical JNK substrate is the transcription factor c-Jun, from which JNK derived its name 67,77,79. JNK can activate other transcription factors, such as ATF-2, Elk-1, MEF-2c, p53, and c-Myc. JNK also has other non-transcriptional substrates, for example the antiapoptotic proteins, Bcl-2 and Bcl-xL 67,77,80.

**P38 pathway**
The p38 MAPK family has been shown to consist of four different isoforms, p38α, p38β, p38γ and p38δ 79,81,82. Different expression, activation and substrate specificity of each specific p38 isoform result in their different physiological functions. In the epidermis, p38α and p38β are expressed together with p38δ 83 which plays a key role in regulating epidermal differentiation and apoptosis 84. By contrast, p38γ expression has not been detected 83. The p38 MAPK pathway is predominantly activated in response to a plethora of inflammatory cytokines, and plays a key role in regulating the cellular responses to these cytokines. However the pathway is also activated by pathogens and by environmental stress such as osmotic stress, ultraviolet light, heat shock, and hypoxia, and can also be activated by mitogens. A number of downstream targets of p38 have been demonstrated; in the nucleus, p38 regulates the activity of a number of transcription factors including ATF-1/2, Elk-1, p53, NF-κB and AP-1 71. The expression and regulation of these downstream targets of p38 MAPK have not been investigated in human skin as much although it has been speculated that imbalance may be important in the pathogenesis of some skin diseases. This protein has been shown to be involved in the pathogenesis of epidermolysis bullosa simplex and correlates with disease severity and defective keratin aggregation 85. Activation of the p38 MAPK pathway is required for apoptosis induction in several different cellular models 86. Conversely, p38 MAPK pathway activity has been reported to promote cancer cell growth and survival. The molecular mechanisms that determine whether p38 signalling either promotes or inhibits cell proliferation and survival have not been elucidated. It has been suggested that in normal physiological contexts, MAPK cascades would function independently with no crosstalk between them, whereas interplay between pathways would be induced only during pathological situations when signal strength exceeds the capacity of the pathway 87.
Mutations in Epidermal Keratins and their Associated Pathologies

Several human disorders are reported to be caused by defects in genes which encode IF proteins. In the human epidermis, pathogenic mutations in the coding sequence of keratins and their associated linker proteins have been discerned. These account for a wide range of abnormal genetic skin and membrane fragility diseases commonly termed genodermatoses. To date genodermatoses are known to be caused by mutation in more than 100 different genes including about 21 different keratin genes (for details, see the intermediate filament database www.interfil.org; 13). The first indications that dominant-negative mutations in basal keratins K5 or K14 elicit human skin blistering disorder, EBS came in the early 1990’s (reviewed in 88-90). In most conditions, the associated pathology results from fragile keratinocytes expressing the mutated keratin protein. These diseases commonly termed epidermolytic keratinopathies are individually rare (typically, less than 1:25-50,000 live births), but can be devastating to affected patients, incurably affecting their quality of life, and are occasionally lethal in severe episodes. For most of these disorders, there exist a good correlation between the type of mutated keratin gene, the nature and position of the mutation in the polypeptide and the extent to which the mutation alters the properties of keratin assembly, thus the severity of the clinical phenotype.

The majority of mutations reported in keratins are missense mutations (~90%) with a small number of small in-frame insertion/deletion mutations and a few intronic splice site defects leading to larger in-frame deletions. The functional consequences at the protein level are expression of mutant polypeptides at normal or near-normal levels with amino acid substitutions, deletions or insertion of a foreign amino acid. The mutant proteins form heterodimers with the wild-type keratin partner and thereby integrate into the keratin network where they render the cytoskeleton susceptible to collapse when exposed to environmental stress 89,91. Mutations in KIF and their associated linker proteins have lead to a series of cutaneous and mucous membrane disorders.
Epidermolysis Bullosa (EB)

EB represents a large and heterogeneous group of genetically determined skin fragility disorders characterized by increased blistering or erosion of the skin and mucous membrane occurring in response to mild or no mechanical trauma. About 1 in 20,000 individuals is affected by one of the EB types. Electron microscopy and immunofluorescence antigen mapping have been fundamental in our understanding of these genodermatoses and a revised classification, distinguished four major subtypes. Based on the level of skin cleavage within the cutaneous basement membrane zone (BMZ) \(^92\), and they include (i) the intra-epidermal EBS where cytolysis and blister formation is located intra-epidermally within the basal keratinocytes above the BMZ and are caused by mutations in either the keratin 5 or keratin 14 gene, (ii) the intra-lamina lucida (junctional epidermolysis bullosa) with split at the level of the lamina lucida of the BMZ resulting in blister formation but with no obvious abnormality of tonofilament structure, caused by defects in laminin-332, collagen XVII, or \(a6\beta4\) integrin, (iii) the sub-lamina densa (dystrophic epidermolysis bullosa), where cleavage is at the superficial dermis just underneath the lamina densa of the BMZ at the level of the anchoring fibrils that attach the epidermis to dermis, caused by mutations in the gene coding for collagen VII, often with documented tonofilament abnormality and (iv) mixed types (Kindler syndrome). The intra-epidermal EB is further separated into two subgroups, the basal and suprabasal types, respectively, to include newly described entities, such as EBS caused by plakophilin or desmoplakin mutations \(^92\). Since the first descriptions of keratin mutations in EBS in the early 1990’s, all EB subtypes have been characterized at the ultra-structural and molecular levels with more than 1000 mutations being described in more than 21 genes that encode for structural proteins in the human skin and its appendages as well as involvement of mucous membranes, including more than 150 \(KRT5\) and \(KRT14\) mutations see (www.interfil.org, \(^{13}\), www.hgmd.cf.ac.uk/).

Epidermolysis Bullosa Simplex (EBS)

Epidermolysis bullosa simplex (EBS) is a group of rare predominantly autosomal dominant genetic skin diseases affecting approximately 1:25000–50 000 live births of the population \(^89,92\). EBS has become the prototype for understanding disease pathology and genotype–phenotype correlations within a broad spectrum of keratin disorders. In EBS, two major subtypes have been defined: suprabasal and basal EBS \(^92\). Within the scope of this thesis, only subtypes caused by keratin mutations will be described. The suprabasal EBS types lethal acantholytic EB and plakophilin deficiency caused by mutations in desmoplakin and plakophilin-1, respectively, includ-
ing the basal EBS types; EBS with muscular dystrophy (plectin mutations),
EBS with pyloric atresia (mutations in plectin and α6β4 integrin) and EBS
Ogna (plectin mutations) will not be further discussed (for a review of theses
subtypes (see www.interfil.org; 90). EBS is the most common subtype of EB
with clinical manifestations usually present at birth, characterized by intra-
epidermal blistering due to cell degeneration within the basal layer of the
epidermis with involvement of mucosal epithelia. Blistering is often associ-
ated with mechanical stress and the blisters tend to heal without scarring.
EBS is caused by mutations in keratin KRT5 or KRT14, and the pathogenic
mutations usually occur within regions of the keratin genes that encode “hot-
spots” in the protein structure, namely the H1 domain of the head region
(only for type II keratins), two segments (1A and 2B) of the rod domain, and
the central linker region L1213. Upon mild physical trauma, the keratin fila-
ment network is easily compromised, resulting in structural failure of the
affected epithelial keratinocytes and tissue integrity (reviewed in 93,94). The
degree of severity of the clinical phenotype has been directly linked to the
position of the pathogenic mutation in the keratin protein but other additional
factors may also exacerbate the disease 51,95. According to the recent classifi-
cation, EBS has been subdivided into four major phenotypic subtypes: a) the
generalized Dowling-Meara EBS (EBS-DM; OMIM 131760), b) other gen-
eralized non-DM EBS (gen non-DM EBS; OMIM 131900), c) the localized
EBS (EBS-Loc; OMIM 131800) and d) EBS with mottled pigmentation
(EBS-MP; OMIM 131960) 92.

In both generalized forms, the most severe Dowling-Meara subtype
(EBS-DM) and the milder non- Dowling-Meara subtype (gen-nonDM EBS),
also previously known as Koebner form, present at birth with generalized
and pronounced blistering, while localized EBS is milder with blistering
confined to palmar and plantar regions of the body. Nevertheless, other not
yet identified genetic or epigenetic modifiers and environmental factors,
such as patient lifestyle and climate condition, clearly influence the pheno-
typic expression as different subtypes of EBS have been associated with the
same mutation in several instances 96-98.

The generalized Dowling-Meara subtype (EBS-DM) is the most severe
form being manifested at birth with erythema, widespread blistering, ero-
sions and areas of denuded skin presenting spontaneous clusters of blisters
also called “herpetiform” at multiple sites of the body which improves with
age (Fig. 5a, b). Progressive palmpoplantar keratoderma becomes the chief
complaint in adulthood. Other hallmarks include callosite formation (Fig.
5c), secondary bacterial infections and sepsis, involvement of mucous mem-
branes, nail dystrophy, healing of lesions without scarring. Inflammation
especially of hemorrhagic blisters may be followed by transient milia forma-
tion, as well as healing of skin with hypo- and hyperpigmentation 89. Diag-
nostic criteria include ultrastructural examination of skin biopsies showing
the characteristic clumps or electron dense aggregates composed of K5 and
K14 KIFs protein in the cytoplasm of basal keratinocytes harbouring the mutation 99.

The pathogenic mutations in EBS-DM are usually missense mutations which reside in the highly conserved helix boundary domains (the HIP of the 1A segment and the HTP of the 2B segment). More than one-third of DM EBS cases are caused by a particular mutation in the \textit{KRT14} gene that affects a highly conserved arginine within the HIP of K14, Arg125. This genetic “hot spot” is most likely due to a hypermutable CpG dinucleotide that is conserved in all type I keratins. When mutated, the arginine codon (CGC) is either replaced by cysteine (TGC) or by histidine (CAC).

The generalized non-DM EBS (gen-non-DM EBS), is a more moderate subtype characterized at birth or in early infancy with generalized blistering, without clustering. The clinical presentation is moderate, shows no extracutaneous involvement. Moreover, palms, soles and extremities are mostly affected and often in response to minor trauma and induced by increased ambient temperature. The disease associated mutations in the generalized non-DM EBS are located more centrally in the rod domain and sometimes more widely distributed along both K5 and K14 genes, including the non-helical linker segments (reviewed in 94).

The localized EBS (EBS-loc) is a clinically mild phenotype and the commonest form previously known as EBS-Weber Cockayne (EBS-WC), characterized by late appearing skin blistering restricted to areas of greater friction or trauma such as hands and feet. Children tend not to be affected until they start to walk or crawl and the blistering tends to be worse in warm weather. Secondary infections of blistering lesions on the feet are the most common complication. Some affected individuals suffer from focal keratoderma (thickening of the skin of hands and feet). Mutations in EBS-loc are most frequently found in four mutation clustering sites often lying outside of the helix boundaries of K5 or K14, including the non-helical L12 linker motif, or in the the amino-terminal homologous domain (H1) of K5, or in the 2B segment of K14 (see www.interfil.org; 13). However, exceptions do exist and patients with mild phenotype (EBS-loc) have been identified with mutations in the conserved 1A and 2B helix hotspots 100-102.

Ultrastructural abnormalities of the cytoskeleton are far less severe than those seen in EBS-DM and some cases of gen-non-DM EBS. In EBS, genotype–phenotype correlations are quite well established. The clinical severity relates in most cases to the location of the mutations and the degree to which these mutations perturb keratin structural assembly. Generally, six mutation hotspots have emerged: mutations in EBS-DM are generally restricted to the helix boundary peptides of K5 and K14 which mark the importance of these structures for KIF assembly and elongation. In milder forms of EBS, the underlying mutations occur outside the helix boundary motifs, namely the H1 domain of K5, the second half of the 1A domain, the L12 domain and the central 2B domain of both proteins. Since such mutations do not interfere
with the elongation process during filament assembly, ultrastructural examination reveals apparently normal filament, but consists of structurally weakened filaments that break upon mild mechanical stress. Conservative amino acid changes in the helix boundary motifs as well as complete disintegration of the amino acid sequences by frame shift mutations may also result in milder disease phenotypes. Thus, based on the location of a mutation in K5 and K14 one can possibly predict the resulting phenotype.

**Autosomal recessive epidermolysis bullosa simplex, EBS-AR**

Although EBS is generally transmitted in an autosomal dominant mode, about 5% of EBS cases have been identified with inherited recessive mutations. More than 10 different KRT14 mutations have been associated with recessive EBS (EBS-AR) including nonsense mutations, missense mutations, splice site mutations, deletion and deletion/insertion mutations. In some cases of recessive EBS, compound heterozygous mutations have been described in K5. For further reading regarding other forms of EBS, see reviews.

![Image](image_url)

Figure 5. **Clinical features of Epidermolysis bullosa simplex and Epidermolytic ichthyosis** a, b), Child with severe generalized EBS (Dowling-Meara) blistering in the trunk and leg c) adult with severe EBS-DM and painful plantar callosites d), Epidermolytic Ichthyosis patient showing a diffuse hyperkeratosis of the hand, flexures and erythroderma e) with sharp hyperkeratosis of the lower back.
Epidermolytic Ichthyosis (EI)

Epidermolytic ichthyosis (EI; OMIM 113800) is a form of congenital ichthyosis with a prevalence of 1 in 200,000-300,000 people\textsuperscript{108}. EI, previously known as bullous congenital ichthyosiform erythroderma (BCIE) or Epidermolytic hyperkeratosis (EHK), is a relatively rare,autosomal dominant keratinization disorder which presents at birth with generalized erythroderma (redness of the skin), severe blistering and hyperkeratosis (thickening of the uppermost layer of the skin), erosions and peeling of the skin even with mild trauma (Fig. 5d-e). Superficial ulcerations develop on the flexural surfaces, and because of the disruption of the epithelial barrier, neonates with EI are at risk of developing severe infection, electrolyte imbalances, and sepsis. EI is often associated with rapid healing of denuded areas with recurrent episodes of blistering on the background of erythroderma but may persist throughout life. Later on, blistering becomes infrequent; hyperkeratotic plaques with verrucous scales, mainly involving flexural and intertriginous areas, develop but can also appear on the scalp, neck and infragluteal folds (See Fig. 5d-e). In the majority of patients palmoplantar hyperkeratosis is present and bacterial colonisation of the macerated scales causes a distinct foul odour\textsuperscript{109-113}. The cutaneous pathology in EI results from the expression of abnormal K1 or K10 proteins\textsuperscript{114}. Since these two keratins provide structural stability to keratin intermediate filaments in the suprabasal keratinocytes, the blistering is more superficial than in EBS, and there is increased proliferation of suprabasal keratinocytes leading to ichthyosiform lesions. Ultrastructurally, the basal cells are normal, but irregularly shaped pathognomonic KIFs clumps are identified in suprabasal keratinocytes, giving a dense peri-nuclear shell-like appearance as primary event with secondary suprabasal cytolysis, blister formation and hyperkeratosis\textsuperscript{106}.

Most of the pathogenic mutations are missense mutations that usually occur within highly conserved regions of the alpha-helical rod domains and the non-helical H1 domain of K1 and K10. Milder variants of the disease are associated with mutations in the L12 linker region or outside the helix boundary motifs, similar to mild EBS\textsuperscript{115}. Therefore, the positions of the mutations along the keratin polypeptides and the level of expression of the mutated genes,\textit{KRT1} and \textit{KRT10}, could as well explain the clinical features of this disorder\textsuperscript{116-118}. Rare dinucleotide alterations in \textit{KRT10} that lead to substitution of two adjacent amino acids\textsuperscript{116,119} and spontaneous de novo point mutation, deletion, deletion/insertion and splice site mutations in \textit{KRT1} and \textit{KRT10} have been described in about half of these patients\textsuperscript{118,120}. Similar to EBS-DM, a genetic “hot spot” has been identified in EI that affects an evolutionarily highly conserved arginine residue (p.Arg156). The nature of the mutations may predict the disease phenotype as it has been ascertained that \textit{KRT1} mutations are associated with palmoplantar keratoderma whereas \textit{KRT10} mutations lead to
the non-palmoplantar variants. Such an association appears to be true for $KRT1$ mutations; however, exceptions do exist and $KRT10$ mutations have been identified in patients with severe EI and palmoplantar keratoderma. An interesting missense mutation in the HTP of K1, p.Ile479Thr, is associated with a mild ichthyosis-like phenotype in some cases and epidermolytic palmoplantar keratoderma alone in other families.

Genotype–phenotype correlations have now clarify that a much more complex situation exists and that the genetic background may also modulate the phenotype, illustrated by the findings that the conserved codon 156 mutations in $KRT10$ mostly associated with severe phenotype may result in a mild form of EI. This is analogous to the EBS phenotypes where mutations in the same location are associated with different phenotypes for mutations affecting K5 p.Iso183 as well as the K5 p.Val186 mutations.

Recessive EI has been identified in a consanguineous family with a severe phenotype of EI caused by a nonsense mutation in $KRT10$ that leads to the loss of K10 expression, characterized by sparse keratin filaments with amorphous and homogenous-like keratin aggregates. The nevoid variant of EI, also termed epidermal nevus of the epidermolytic hyperkeratotic type, exists. Here, ichthyosiform lesions are often distributed along the Blaschko lines and alternate with normal skin. The discovery of heterozygous $KRT10$ missense mutations in skin lesions, and its absence in normal skin suggested the occurrence of postzygotic, spontaneous mutations during embryogenesis. Patients with the nevoid variant of EI having children with full-blown EI do have underlying gonadal as well as cutaneous mosaicism, and mosaicism has been described in EBS and in palmoplantar verrucous nevus (reviewed in ). Details of other forms of epidermolytic keratoderma are reviewed in.

**Therapeutic approaches for EBS and EI**

To date, no real therapy exists for keratin intermediate filaments disorders. Most, if not all, current therapies for keratin filament–related diseases are directed toward amelioration of specific tissue damage. In the clinic, the treatments of epidermolytic disorders due to keratin dysfunction is essentially symptomatic and palliative and are mostly based on individual clinicians’ experience including management of the symptoms and treatment of secondary skin infections on a case by case basis. Therapeutic approaches in EBS and EI include reducing sweating, avoiding heat and preventing secondary bacterial infections of the skin. Moreover, punctuating blisters with a needle and protecting the skin by bandages is important and represents the main clinical remedies for EBS. Apart from EI, which is often improved during therapy with vitamin A derivatives including tazarotene, acitretin and tretinoin, EBS therapy has made little progress in the last decades. How-
ever, protein-, cell- and gene-based therapies have recently made some progress in other types of EB. In dominant disorders where mutant keratins exert a dominant-negative effect, conventional gene replacement may not be effective as the effects of the mutant gene need to be removed without affecting the normal gene; however, strategies are being developed to selectively inhibit mutant alleles. Methods based on the use of therapeutic RNA molecules such as ribozymes or short inhibitory RNAs are currently being developed for clinical use, and they offer the possibility to inhibit expression of the mutant allele without affecting wild-type gene expression. This method was effective in down-regulating mutant keratin 14 allele expressions in cultured cells. In a cell culture model for pachyonychia congenita, addition of mutant-specific siRNAs allowed normal keratin filament formation, suggesting selective inhibition of mutant K6a. siRNA-based therapeutics are currently in clinical trials for pachyonychia congenita.

More recent studies suggest novel small molecules and chemical chaperone-based therapeutic approaches for EBS. The approach using compounds with chaperone potential such as TMAO and 4-PBA (Fig. 6) have been evaluated for the treatment of other protein instability disorders, some of which are approved for clinical treatment of human protein conformational diseases.

Improved understanding of the regulatory functions of keratins may reveal potential targets for the development of novel therapeutic strategies to overcome current treatment limitations. The existence of similar keratin mutations that leads to different phenotypes in different kindred suggests the existence of genetic and epigenetic modifiers of mutations in the KIF genes. The identification of such modifiers is currently being defined and it is hoped will assist in defining compensatory mechanisms that can be exploited to therapeutic advantage.

![Chemical structures of TMAO and 4-PBA](image)

*Figure 6. Chemical structures of a) TMAO and b) 4-PBA*
Aim of the Research

To investigate and characterize pathogenic keratin mutations in a group of EB and EI patients, to establish *in vitro* prognostic new tools in which to test the feasibility of novel approaches to understand the pathogenesis and to pharmacological treatments for EBS and EI.

The specific objectives are;

To investigate, elucidate and verify pathogenic keratin mutations in Swedish EB patients (Paper I).

To compare genotype-phenotype correlations in EBS and EI *in vivo* and *in vitro*, (Paper I, III and IV).

To generate and characterize primary and immortalize keratinocytes from patients with EBS (*KRT5* mutations), EI (*KRT1* and *KRT10* mutations), and from healthy volunteer (Paper I-IV).

To evaluate the ensuing effects of stress-induced destabilization of KIF cytoskeleton network in cultured patient-derived and normal healthy keratinocytes (Paper I - IV).

To establish *in vitro* organotypic models for EBS and EI (Paper II-IV).

To use *in vitro* models to explore novel therapies for epidermolytic keratin disorders by:

(i) Investigating the effects of treatment with TMAO (Paper I-IV) and 4-PBA (Paper III-IV).

(ii) Exploring the molecular mechanism of action of TMAO on cellular MAPK signaling and molecular chaperones in EBS and EI disorders (Paper III-IV).
Materials and Methods

In this section, a few methods from the work are summarized; the reader is referred to the individual papers (I-IV) for further details.

All patient sampling were approved by the Regional Ethical Review Board (EPN) in Uppsala and were conducted according to the Declaration of Helsinki Principles. All patients and healthy volunteers (between 17-48 years of age) gave informed and written consents.

Patients characteristics (Paper I, II, IV)

Patients from different families affected with different subtypes of the simplex form of EB (aged 36-47) and EI (aged 17-48 years) were recruited via the Uppsala Genodermatoses Centre (Dermatology Clinic outpatient unit), Uppsala University Hospital. In these studies, three patients with genetically undefined and one patient with defined EBS (Paper I, II, III), three patients with clinical and genetically defined EI (Paper IV) and one healthy adult volunteer were included. All patients were diagnosed as autosomal dominant based on long-term follow-up, clinical studies together with family history and molecular analyses. Most patients were untreated except for EI patients who were treated with emollients.

Blood specimen and skin biopsies sampling (Paper I-IV)

Blood samples for genomic DNA analyses were collected from all patients and available near relatives into EDTA vials and were stored at –20°C before use (Paper I, II, III, and IV).

Skin biopsies were obtained after infiltrating the skin with Xylocain-lidocain. Briefly, punch biopsies (Ø 3-4 mm) were obtained from a normal adult healthy volunteer (Paper IV) and from both clinically affected but uninvolved skin and lesional/mildly affected skin of EBS (Paper I, II, III) and EI (Paper IV) patients respectively. The punch biopsies consisted of epidermis and dermis with minor subcutis. They served for isolation and initiation of primary keratinocyte cultures, immunofluorescence staining and
for electron microscopy. The punch biopsies intended for isolation and establishment of primary cell cultures were transported in transport medium (TM; DMEM / 10% FBS / 6% antibiotic-antimycotic) while those for immunohistology were collected in Histocon solution and later snap frozen. Biopsies for electron microscopy were collected in 2.5% glutaraldehyde and stored at 4°C. In addition, shave biopsies consisting of the entire epidermis and a minor proportion (≤20%) of the papillary dermis were sampled from clinically mildly affected patients’ skin and were also from lesional skin sites (1.5 cm²) of some EBS (Paper I) and EI (Paper IV) patients. The biopsies were stored at –70°C before RNA extraction and analyses of mRNA expression.

Isolation and establishment of primary keratinocyte cultures (Paper I, II, III, IV)

Keratinocytes were established in two keratinocyte culture systems: (a) with serum-free growth medium and (b) with classical keratinocyte growth medium containing serum (with or without feeder fibroblasts). Briefly, punch biopsies were transported in TM and washed several times in PBS (Mg²⁺/Ca²⁺-free) supplemented with an antibiotic-antimycotic mix. Subcutaneous tissue and dermis were trimmed off and then incubated for 30 min at room temperature in 0.05% trypsin/0.01% EDTA (TE). The epidermis was carefully separated from the dermis and finely minced (0.5–1 mm²). The suspension was transferred into a Cellstir® flask (Wheaton Science Products) and moderately stirred for 45 min on a battery operated stirrer at 37°C in 5% CO₂ in a humidified incubator. The epidermal cell suspension was filtered through 70-µm cell strainers (BD Falcon) and the trypsin enzymatic digestion was inhibited with three volumes of a defined trypsin inhibitor or equal volumes of DMEM containing 10% FBS, for serum-free or serum-containing cultures, respectively. The trypsinization cycle was repeated four times and fresh TE was added to the flask each time. The released cells were pelleted at 180 × g for 7 min, re-suspended in growth medium, counted using a Bürker haemocytometer and cell viability was determined by trypan blue dye exclusion. From this point, the isolated primary keratinocytes were either seeded in EpiLife serum-free medium, supplemented with HKGS-V2 and antibiotics (Cascade Biologics) in pre-coated flasks (Coating matrix, Cascade Biologics) or on γ-irradiated Swiss 3T3 J2 fibroblast feeder layer in classical keratinocyte growth medium, consisting of a 3:1 (vol/vol) mixture of DMEM/F-12 (Ham), 5% FBS and antibiotic mix, supplemented with 5 µg/ml insulin, 0.45 µg/ml hydrocortisone, 2 × 10⁻¹¹ M lyo-thyronine, 5 µg/ml transferrin, 9 × 10⁻¹⁰ M cholera toxin, 0.1 U/ml non-essential amino acids and 1.8 × 10⁻⁴ M adenine (all from Sigma–Aldrich). From first medium replenishment, four days after cell seeding, 10 ng/ml of human recom-
binant epidermal growth factor was also included as supplement. These cells were maintained at 37°C in 5% CO2/air incubator and the media were replenished every second day until considerably large cell colonies were visualized without the aid of the microscopy. At this stage the cells were subcultured for the first time, subsequent subcultures were at 80–90% confluency and at a split ratio of 1:10. After the detachment of cells, DMEM containing 10% FBS was added. The cells were pelleted, suspended in growth medium and seeded as above. Cells from both culture systems were cryopreserved after each third-fourth passage and stored in liquid nitrogen. Cells frozen in liquid nitrogen were thawed, briefly spun down and directly cultured in respective fresh growth medium.

**Immortalization of keratinocytes from EBS and EI patients**

Keratinocytes established in two the different culture media system were constantly maintained in the same culture system before, during, and after generation and transduction. The cultures of normal and mutant keratinocytes were immortalized by transducing with replication-incompetent retrovirus encoding the (HPV16) E6 and E7 in tandem or transduced with the empty vector (pLXSN) as control as described elsewhere 48. Normal, EBS and EI keratinocytes initiated in serum-free condition were seeded on pre-coated plates in EpiLife medium. Briefly, 1–2 days later concentrated retroviral supernatants were mixed with equal volume of medium containing polybrene and equilibrated for 15 min at room temperature. The mix was then added to keratinocytes, spin infected (350× g at 32°C for 1 h), and the medium was replaced with fresh growth medium and allowed to culture for 2-3 days. Transduced cells were selected with Geneticin (G418) (100 – 200 μg/ml for 5-7 days) and subsequently pooled, expanded and characterized.

For cells established in serum-containing medium, E6E7-transductants were generated by co-plating keratinocytes with mitomycin C-treated PT67 retroviral producer cells. Three to four days later, the PT67 cells were selectively removed by incubation with EDTA and irradiated 3T3 J2 feeder cells were added prior to selection with G418. Feeder cell dependency was checked regularly after transduction and when cells became independent they were routinely cultured in classic medium and subcultured at a split ratio of 1:25–50. The replicative life span, immortalization and the cumulative population doubling level were determined and calculated as previously described 48.
Isolation of genomic deoxyribonucleic acids (gDNA)
Genomic DNA was extracted from peripheral blood leukocytes and from cultured keratinocytes (primary or immortalized) by E.Z.N.A blood and tissue isolation kits (Omega BioTek) according to the manufacturer's protocol. Concentration and purity were determined by spectrophotometric measurements of OD$_{260}$nm and OD$_{280}$nm.

RNA extraction and cDNA synthesis by RT-PCR (Paper I – IV)
Total RNA was extracted from subconfluent (Paper I, II, III) or Ca$^{2+}$-differentiated (Paper IV) cultured cells as well as from shave biopsies (Paper I, IV). TRIzol Reagent (Invitrogen) was added to the cultured cells and total RNA was isolated according to the manufacturer’s protocols. After resuspension in RNase-free water, the concentrations were determined at A$_{260}$nm and the samples subsequently stored in aliquots at -70°C pending use. Priming of first strand cDNA synthesis was performed by mixing total RNA and oligo d(T)$_{16}$ followed by an incubation for 10 min at 65°C. After centrifugation, a 20-μl reaction volume was made by adding DEPC-water to a master mix for a final concentration of 1X First Strand Buffer, 10 mM DTT, 500 μM of each dNTP, 140 U M-MLV reverse transcriptase (Invitrogen) and 12.5 ng/μl random hexamers (GE Healthcare, Uppsala, Sweden). After a second annealing step at 25°C for 10 min, synthesis of cDNA was at 37°C for 60 min, followed by enzyme inactivation at 70°C for 15 min, diluted in DEPC-treated water yielding a final concentration of ~10 ng cDNA/μl and stored at -70°C pending use.

PCR amplification of gDNA and cDNA fragments (Paper I-IV)
For the amplification of all studied keratins (K1, K4, K5, K10, K14), exons and exon/intron boundaries were sequenced. Several primer-pairs were used for the amplification of keratins with modifications$^{120,126,142}$. All primers were obtained from Applied Biosystems. The templates (gDNA) and (cDNA) were added to a standard reaction mixture consisting of 1× PCR buffer II, 1.5-2.5 mM MgCl$_2$, 0.4 μM primers, 0.2 mM of each dNTPs, 0.25U AmpliTaq Gold DNA polymerase (all from Applied Biosystems,) and DNase-free water. The thermal cycler was programmed as follows: 10 min at 95°C followed by 35-39 cycles of: 60s at 95°C, 60s at 56-59oC (depending on the primer-pair and gene of interest), 60-90s at 72°C, followed by final 7 min at 72°C. The cDNA synthesized were used to investigate alternative splice variants of the KRT5 and 14 gene regions by PCR amplification. These PCR reactions were carried out in a final volume of 20 μl with 2 μl cDNA template (~20 ng total RNA), 0.2 mM of each dNTP, 1×PCR buffer II, 1 mM MgCl$_2$, 0.4 μM each of the primers, enzymes and programmed as above.
Agarose gel electrophoresis and clean up of amplicons

After PCR amplification, a 10-μl aliquot of the amplicon was mixed with 2.5-μl of loading buffer and were resolved by electrophoresis in parallel with a DNA molecular weight marker on a 1% agarose gel stained with ethidium bromide. The products were visualized under UV light and documented with a digital CCD camera. The remaining aliquots of amplicons were purified using the ExoSAP-IT PCR Clean-up Kit for sequencing (GE Healthcare).

Automated DNA sequencing of PCR products and mutation analysis

The entire coding regions and the non-coding intron/exon boundaries of KRT5 and KRT14 as well as hotspot regions of KRT1 and KRT10 were sequenced in both directions as described \(^{120,126,142}\) and were partially modified (see Paper IV). The purified samples were sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI 3700 DNA genetic analyzer (Applied Biosystems). Digitally stored raw data and DNA electropherograms were analyzed with Chromas Pro software version 1.42 (Technelysium Pty Ltd). Sequences were compared to reference sequences from the NCBI Entrez Nucleotide database. De novo, recurrent KRT genes mutations were identified (Paper I) or verified (Paper II, III, IV).

Quantitative real-time polymerase chain reaction (qPCR)

The number of transcripts were studied by quantitative real-time PCR (qPCR) in a 20-μl reaction, using cDNA as template (≈20 ng total RNA) as described elsewhere \(^{143}\). Triplicate reactions were set up for each sample using 2×TaqMan Universal PCR buffer or Fast SYBR Green Master mix (Applied Biosystems, Stockholm, Sweden). The primers and probes were added at 400 nM and 200 nM, respectively, for probe detection, while the primer concentrations for SYBR-detection were 200 nM. The 96-well plates were placed in a 7500Fast (Applied Biosystems) programmed as follows: 9 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. All mRNA expressions were normalized to the amount of the reference gene TBP encoding TATA-binding protein.

Preparation of cell lysates and quantification of protein for protein analysis (Paper III)

Cells were harvested by washing in ice-cold PBS, and scrapped with a rubber policeman into lysis buffer (20mM Tris pH 7.5, 0.5% (v/w) Triton X-100, 0.5% (v/w) deoxycholate/deoxycholic acid, 10 mM EDTA, 30mM NaPyro-Phosphate, 150mM NaCl). Immediately before use phosphatase and protease inhibitors were added to the buffer. The cell suspensions were transferred to Eppendorf tubes and was repeatedly pipetted and extracted for 30min on ice. The supernatants were cleared by centrifugation at 15,000×g for 30min at 4°C. The protein concentrations of the lysates were determined
by the Bradford assay (Bio-Rad) using bovine serum albumin for the standard curve. Samples were subsequently stored at -70°C until further use.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot analyses (Paper IV)**

Protein extracts (2-5 μg) were mixed with sample buffer (containing 62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% (w/v) bromophenol blue), denatured for 5 min at 95°C and cleared by brief centrifugation. Proteins were separated on a 12% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes by semi-dry transfer. Prior to transfer, the components were pre-soaked in transfer solution (50 mM Tris, 40 mM glycine, 4% (w/v) SDS and 20% (v/v) methanol). The membranes were first blocked with 5% non-fat dry milk in TBS with 0.1% Tween-20 (TBS-T) for 1 h at room temperature and then incubated for 2 h at room temperature or overnight at 4°C with polyclonal rabbit or mouse monoclonal anti-human antibodies. After repeated washes with TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary IgG antibodies for 1 h. Antigen signals were visualized by chemiluminescence using ECL Plus™ and recorded using ECL Hyperfilm (GE Healthcare). Densitometric analysis was performed on scanned films after inverting the band signals and quantified with ImageJ software (NIH). For re-probing, blots were stripped with 0.4 M NaOH for 10 min, washed and re-blocked prior to the addition of the next primary antibody and the process repeated as above.

**Heat stress assay and treatment with TMAO and 4-PBA**

All cell lines, grown on glass cover slips, multiwell chambers or as organotypic epidermis in cell culture inserts were heat shocked at 43°C for 30 min, followed by recovery period performed as described 48,49. Briefly, the medium in the wells containing glass coverslips or inserts were replenished with a 45°C warm growth medium and the plates were placed at 43°C for 30 min in a closed water bath. In a subset of experiments cells were either (i) pre-treated with chemical chaperones (TMAO; Sigma-Aldrich) or (4-PBA; Calbiochem) for 24-48 h at subconfluency or (ii) induced to differentiate by addition of calcium or PD153035 and pre-treated with chemical chaperones for 24-48 h prior to thermal stress.

Subsequently, the cells and tissue sections obtained from stressed organotypic cultures were processed by immunocytochemistry and immunohistochemistry, respectively. EBS cells were heat stressed and harvested at subconfluent while EI cells were after Ca2+-induced differentiation. The total number of cells expressing cytoplasmic keratin aggregates and the number of detached cells (identified by remnants of keratin stainings and/or nuclear
stainings), were semi-quantitatively determined. The proportion of cells rescued by the individual drugs were determined and expressed as the extent of protection by the proportion \( P(\%) \) with the formula: \( P(\%) = \{100 - [(V_a /V_0) \times 100]\} \), expressed as percentage, where \( V_0 \) and \( V_a \) are the percentages of cells with aggregates without and with drug, respectively.

**Reconstruction of organotypic epidermal skin models**

The epidermal skin tissue was engineered in vitro using two organotypical model systems. A model in which serum-free established cells were seeded on polycarbonate filters (cell culture inserts) in Epi-Life serum free medium with addition of growth supplements \( 30,48 \), and then exposed to air-liquid interface. The second model is a skin equivalent model in which cells established in serum-containing medium are seeded on de-epidermized dermis (DED) and grown submerged on DED for 5 days before being lifted to the air-liquid interface. Organotypic cultures were incubated in 5% \( \text{CO}_2 \) in humidified air at 37°C and medium was replenished after every second day until harvest after 14-21 days.

**Reconstruction of organotypic epidermis on cell culture inserts.**

Subconfluent keratinocytes were harvested and re-suspended in EpiLife growth medium containing 1.5mM CaCl\(_2\), at the density of \( 1.5 \times 10^6 \) cells/ml as previously described \( 48 \). Subsequently, \( 0.3 \times 10^6 \) cells were seeded into Millicell-PCF cell culture inserts (Millipore Corporation) placed in 6-well plates containing 2.0 ml medium per well. After 24 h, the medium in the insert was removed and the cells placed at the air-liquid interface. The medium in the wells was replaced with 1.2 ml growth medium containing 1.5mM CaCl\(_2\), 50μg/ml L-ascorbic acid and 10ng/ml recombinant human keratinocyte growth factor.

**Reconstruction of the epidermis on de-epidermized dermis**

Skin equivalents were established using de-epidermized dermis (DED) as previously described \( 144 \). The DEDs were placed in 6-well culture plates and stainless steel rings were placed on the dermis and trypsinized subconfluent keratinocytes (\( 5 \times 10^5 \) cells/ml) established in classical complete keratinocyte growth medium were seeded inside the rings onto the papillary dermis on the DEDs. After 5 days in submerged culture, the dermis was raised to air-liquid interface on stainless steel grids and growth medium was added up to the lower reticular layer of the composite for 8-14 days.

**Harvest of organotypic epidermis**

Inserts were harvested after 8-14 days at air-liquid interface and punch biopsies for histology and immunofluorescence staining were obtained using a dermal biopsy punch (\( \varnothing \) 3-4 mm). The biopsies were embedded in O.C.T.\textsuperscript{TM} compound and quickly snap-frozen in liquid nitrogen and preserved at -70°C
until used. The biopsies were cryosectioned and 6-μm sections were carried on SuperFrost microscope slides, air-dried for 30 min, and stored at -70°C until later further processed as described below. Two pieces of 3-4 mm punch biopsies were obtained from each inserts.

DED composites were removed from the grids, fixed in 10% formalin and embedded in paraffin for histology and immunofluorescence while the remaining part was placed in Trizol (Invitrogen) and stored at -70°C for RNA isolation. Deparaffinized DED or punch sections were stained with H&E for histologic examination.

**Immunocytochemistry and immunofluorescence microscopy**

Cells were grown to 70–80% confluence or induced to differentiate by raising the Ca²⁺ concentration from 0.06 mM to 1.5 mM for 5 days, or grown in the presence of the epidermal growth factor receptor inhibitor PD153035 (Sigma-Aldrich) for 3 days. The cells were harvested by two brief washes with 1×PBS, fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.5% Triton X-100 in PBS. After blocking unspecific binding epitopes, probing with specific primary monoclonal antibodies recognizing keratins and differentiation markers were performed as described elsewhere. The stained slides were mounted with an antifade and DAPI-containing medium, which stains nuclei blue. The images were taken with a digital Zeiss Axio-phot (F1 imager) microscope, equipped with Axiocam MRm digital camera and AxioVision Software Rel.v4.6 (Carl Zeiss).

**Immunohistochemistry and immunostaining of skin and organotypic epidermis cryosections**

Histologic sections were stained with Mayers HTX and mounted with glycerol-gelatin. Sections were fixed in 100% ice-cold acetone for 5min, washed twice with PBS and unspecific epitopes were blocked with 10% normal goat or horse serum for 30 min at room temperature. Subsequently, slides were stained with mouse monoclonal for proliferative basal K5 and K14 and differentiation related suprabasal K1 and K10 as well as other differentiation-related markers as described elsewhere.

**Statistical analysis**

Data analysis was performed by one way ANOVA test with Bonferroni’s multiple comparison test or unpaired Student’s t-test where applicable using Prism 5.0 (GraphPad Software Inc.). Results were expressed as mean ± S.D. Statistical significance was assumed for p<0.05.
Table 1. Summary of analyses performed in Papers I–IV.

<table>
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<tr>
<th>Analyses performed</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
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<td>Immortalization</td>
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<td>Treatment with TMAO</td>
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<td>Treatment with 4-PBA</td>
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<td>3D epidermis on DED</td>
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<td>Molecular analyses of cultured cells and engineered skin tissues</td>
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<td>Western blot</td>
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cDNA = complementary deoxyribonucleic acid; qrt-pcr = quantitative real-time polymerase chain reaction; RNA = ribonucleic acid;
Results

In this section, the main results from the work are summarized and some additional unpublished data are presented. For further details, the reader is referred to the individual papers (I-IV).

Paper I

In this study, we clinically diagnosed three EBS patients with different clinical severities and discovered novel and recurrent pathogenic keratin 5 mutations in DNA from blood and primary keratinocytes. Keratinocytes were isolated from punch biopsies obtained from all patients and primary cultures were established in serum-free growth medium. The cells were used to investigate the functional and structural consequences of the different mutations in relation to the patients’ clinical phenotypes at resting and heat-stressed conditions, respectively. Using immunofluorescence analyses, we investigated keratin filament network aggregation and fragility (cell detachment) in the absence and presence of drugs. Since no established treatment exists for EBS, development of novel drug therapies is required, and the chemical chaperone trimethylamine N-oxide dihydrate (TMAO), a promising treatment rational for several protein misfolding disorders, was tested in this study. Therefore, pretreatment of the patient’s primary keratinocytes with TMAO on KIF aggregation was evaluated as a possible stabilizer of the cytoskeleton in heat-stressed EBS cells.

Effects of keratin mutations on EBS disease phenotype

The clinical and molecular findings of these patients are summarised in Paper I. Briefly, in addition to our previously reported KRT5 mutation (V186L) in one patient (EBS2), we discovered a novel I183M (EBS3) and a recurrent E475G (EBS1) substitution in KRT5 genes in two patients respectively, and both mutations caused the severe generalized EBS-DM phenotype. Neither pathogenic mutation nor any single nucleotide polymorphism (SNP) was identified in the entire KRT14 gene in the defined EBS patients (Paper I).
Additionally, in a fourth family, the index patient (EB4), a 36-year-old man, and his 30-year-old paternal female cousin were both affected with blisters on the lower extremities and on hands and feet that had been tentatively diagnosed as EBS. No parental siblings were available for examination, but reportedly some had a history of minor blistering on hands and feet. The index patient’s blisters on the legs typically healed without formation of scars (Fig. 7). Blisters were not herpetiform and occurred on the hands and feet; the disorder had a late onset with a seasonal variation of blistering. The cousin experienced exacerbation of blisters and itch triggered when visiting hot, humid climates and when bitten by mosquitoes. Immunohistochemical analysis showed normal expression of all adhesion proteins in and around the basal membrane. EM analysis of a skin biopsy from EB4 did not show keratin aggregates, nor cytolysis in basal keratinocytes as well as no separation between the epidermis and dermis. Thus, EB4 and his cousin were initially classified as localized, EBS-loc, although direct proof was lacking (Chamcheu JC, Törmä H and Vahlquist A unpublished data).

Meanwhile in EB4 and his cousin, molecular genetics analysis of genomic DNA, did not identify any disease-causing mutations in the entire KRT5 and KRT14 genes, apart from both patients being homozygous for the C->A exchanges at bp591 and 594 in KRT5 exon 2. Sequence analysis of the entire keratin 5 and 14 genes with primers for genomic and cDNA also revealed two SNPs exchanges at 591 bp and 594 bp in exon 2 of the KRT5 gene in the other EBS patients. However, a previously reported SNP in exon 1 (bp228 C->T leading to a synonymous amino acid change) and an unreported nucleotide substitution in exon 1 (bp280 G->A resulting in a pA94T amino acid substitution) were identified in KRT14 in the index patient 4 (EB4). These substitutions were present in 74% of examined healthy controls. Analysis of cDNA from primary cells of the index patient (EB4) did not detect any splice variants. The absence of pathogenic KRT5 and KRT14 mutations in this mild disease phenotype suggests the involvement of other genes. Therefore, the genetic defect in this family remains to be elucidated (Chamcheu JC, Törma H and Vahlquist, unpublished data).
Figure 7. Clinical feature of an EBS-Loc-Like patient without any keratin 5 or 14 mutation, a) showing blisters at sites of trauma the shin b) which heals without the formation of scars.

Effect of keratin mutation, heat stress and treatment with TMAO on cell fragility

Established primary EBS-keratinocytes cultured under standard growth conditions did not exhibit keratin filament aggregates or cell loss, except in the cells derived from a severely affected patient with the p.I183M mutation where 3% of stained keratinocytes exhibited keratin aggregates and 2% of cells detached from tissue culture vessel. After heat stress, the proportion of cells that displayed keratin filament aggregates increased to 21%, 27% and 13%, in the p.I183M (EBS3), p.E475G (EBS1) and p.V186L (EBS2) mutant cells respectively (Figure 8) 49.
Figure 8. Photomicrographs of keratinocytes showing a) normal KIF network of radiating polymers, b) heat stress KIF network showing aggregates (clumps), and c) remnant of KIF of cells detached from the tissue culture vessel as a result of heat-induced fragility of EBS cells.

Interestingly, in cells derived from patient EB4, patchy and spotty keratin filament aggregates were found in 2.5% of the cells despite the absence of detectable EBS associated pathogenic KRT5 and KRT14 mutations. The proportion of keratin aggregates in EB4 cells (with no identified pathogenic KRT5 or KRT14 mutations) were by far less than those evaluated in EBS1, EBS2 and EBS3 cells but were comparable to the control normal human epidermal keratinocytes (HEK) (Chamcheu JC, Vahlquist A and Törmä H, unpublished data).

Therefore, using heat stress model, we revealed a correlation between genotype and severity of the patients’ cells in vitro, in terms of cellular fragility (Fig. 9). TMAO treatment of cultured cells derived from the severe EBS3 reduced the percentage of cells with spontaneous KIF aggregates and cell remnants.

Figure 9. Effects of keratin mutations, heat stress and TMAO on primary keratinocytes, a) shows the proportion of keratin aggregates, b) and the proportion of detached cell remnants
In addition, pretreatment of cells with TMAO significantly and dose-dependently protected EBS-keratinocytes, and dramatically reduced the percentage of heat-induced keratin aggregate-containing cells and cell remnants, suggesting cytoprotection (see Fig. 9). This clearly implies improved stability of the keratin-mutant cytoskeleton. In summary, in this paper we revealed a genotype/phenotype correlation in vivo and in vitro in cultured primary keratinocytes from mild to severe phenotypes of EBS extending this observation to heat stress conditions.

Paper II

Due to the variable behavior of primary keratinocytes with higher passage numbers, these cells were deemed suboptimal for long-term functional and therapeutic assays. Therefore, we established immortalized human keratinocyte cell lines, from one EBS patient with a KRT5-mutation (V186L) (EBS2 in Paper I) and one healthy control (NKc1) respectively, using human papillomavirus 16 (HPV16-E6/E7). Cell lines were established in two cell culture systems, in serum-free (EB21 and NKc11) and serum-containing (EB22 and NKc12) media, respectively.

Generation of immortalize keratinocytes

The continuous cell lines exhibited a robust growth in both culture systems. The serum-free cultures, which were initially dependent on coating with collagen to enhance cell attachment and spreading, became coating-independent directly after infection with HPV16-E6/E7. In addition, the transduced cells established in serum-containing medium became feeder-independent thirteen passages after infection and crisis. All of them have long exceeded >160 population doublings (PD), having similar cellular characteristics in relation to their growth properties, keratin expression profiles, and ability to differentiate in organotypic cultures.

Effects of heat stress and TMAO on cytoskeleton resilience

Heat stress mostly induced significant keratin aggregates in both EB21 and EB22 cell lines, with comparatively more KIF network aggregation in serum-free established (EB21) cells. The effects in both cultures were by far higher than in the control (NKc11 and NKc12). Notably, in cultures established in serum-containing medium both NKc12 and EB22 cells were resistant to heat induced KIF network collapse. Moreover, the few aggregates present in the EBS cell lines were confined to the cell periphery or to the peri-nuclear regions. NKc11 and NKc12 cells exhibited very few or no aggregates. Pretreatment of cells with TMAO significantly and dose-
dependently reduced both the fraction of heat-induced keratin aggregate-containing cells and cell remnants in EBS\textsuperscript{48}. On cell culture inserts or de-epidermized dermis, immortalized cells were able to regenerate an almost normally differentiating epithelium.

**Paper III**

In this study, two new immortalized keratinocyte cell lines were established from a severely affected generalized EBS-DM patient (EBS1), with a p.Glu475Gly substitution in K5 (see Paper I). Similar to our previous immortalization described in Paper II, one cell line was established in serum-free medium (EB11) and the other in serum-containing medium (EB12). These newly established cell lines have now passed >160 PDs (Fig. 10a). The immunofluorescence staining patterns of K5 and K14 appeared as typical filamentous networks and the cells were able to differentiate in organotypic cultures (Fig. 10b-c).

![Figure 10.](image)

**Figure 10.** a) The growth curves (passage history) of EB11 and EB12 versus number of days in culture either as immortalized or primary (P) keratinocytes. Control, pLXSN transductants are represented by open symbols, while E6E7 transductants are represented by closed symbols. The downward arrow outlines the time of transduction, while the horizontal arrow denotes the finite replicative lifespan of pLXSN control cells. The E6E7-transduced cells have a prolonged life span (> 197 population doublings) compared to pLXSN-transduced cells, b) tissue morphology of Hematoxyline staining of EB21 organotypic epidermis generated on polycarbonate inserts; note the multilayered epithelium formed and the presence of keratohyaline granules at the granular layer, c) immunofluorescence (K10) staining of organotypic tissue section reconstructed with EB11 cells on de-epidermised dermis; note the formation of distinct cell layers apart from parakeratosis, dotted lines denotes BMZ.

The proportion of heat stress-induced keratin aggregates correlates with EBS phenotype No keratin aggregate was seen in any cell lines under normal culture conditions while heat stress significantly induced keratin aggregates in all immortalized EBS cells (EB11, EB12, EB21, and EB22). By
studying EB11 and EB12 together with EB21 and EB22, we found that the percentage of thermally-induced keratin aggregate-containing cells was highest in the cell lines from the most severely affected EBS patient (Fig. 11a-b). Only very few or no aggregates were seen in normal cells (NKc11 and NKc12). This observation allowed us to extend the genotype/phenotype correlation also to immortalized cells.

**Figure 11.** Effects of heat stress and pretreatment with chemical chaperones reduced the formation of heat-induced keratin aggregates in EBS cells. Cells were treated with TMAO in serum-free (a) or serum-containing conditions (b) or with 4-PBA in serum-free conditions (c) for 48 h, heat-stressed for 30 min and stained with a K5-antibody after 15 min recovery time. Keratin aggregates and total number of cells were semi-quantitatively scored and results expressed as percentage of cells presenting keratin-aggregates. A minimum of 1000 cells were counted per cover slip in duplicate coverslips. The results represent the mean +/- S.D. (n=3) and p-values are indicated (Student’s t-test; Mann–Whitney).

Chemical chaperones reduced heat-induced keratin aggregation

Treatment of all immortalized cells with TMAO prior to stress, dose dependently reduced the percentage of cells containing keratin aggregates in both serum-free and serum-containing cultures (Fig. 11a-b). Pretreatment of serum-free EB11 and EB21 cell lines with 4-PBA dramatically reduced the fractions of keratin aggregate-containing cells as well (Fig. 11c).
Effects of heat stress and TMAO on the expression of Hsp70 and Hsp90

The observations that keratin aggregation is a dynamic process in mutant cells, together with the recent findings that synthetic chaperones suppress aggregate formation, raised the question whether the expression of endogenous molecular chaperones is also altered in EBS cells in response to stress. We therefore compared the expression of stress proteins in keratin-defective versus control cells, with or without heat stress and in the presence or absence of TMAO, using qRT-PCR and western blot. Under normal culture conditions (unstressed and untreated cells), the transcripts of Hsp70 were high in EB11 and EB21 cells by 100% and 50%, respectively. After heat stress, Hsp70 expression increased in all cell lines, reaching a peak by 4 h of recovery. The Hsp70 mRNA values returned to pre-stress levels after 24 h of recovery in all cell lines, but an abnormally rapid drop was observed within 6 and 8 h of recovery in EB11 cells.

Protein analysis by western blot showed that unstressed EBS cells had higher levels of stress proteins (Fig. 12a(-) and b) and that heat stress induced an increase in Hsp70/Hsc70 protein levels in the two keratin mutants (EB11 and EB21), between 2 and 4 h of recovery time (Fig. 12a(0-60)). The levels remained practically constant in control cells NKc11 throughout the recovery time-course. In all unstressed cultures, the treatment with TMAO resulted in increased Hsp70/Hsc70 protein levels (Fig.12a (-) and d). The results suggest that pretreatment of EBS cells with TMAO tends to normalize the heat stress response, and this may be achieved through a transient increase in Hsp70/Hsc70 levels, causing a decrease in the number of keratin aggregates after heat stress (see Fig.12a and d). In contrast to 70-kDa proteins, mRNA and protein levels of Hsp90 were higher in EB11 than in EB21 and NKc11 cells at resting state and were minimally influenced by heat stress in the mutant cell lines (see Paper III). Hsp90 mRNA showed a minor peak at 4 h of recovery in EB11 cells while the levels were unaltered in the other two lines. By contrast, the control cells showed low levels at resting condition. The control cells showed a slight increase in Hsp90 protein after 1 h of recovery. However, the increase was much lower than in the two mutants (EB11 and EB21). Pretreatment with TMAO greatly increased Hsp90 levels in NKc11 cells and slightly in EB21 cells. This level starts decreasing after 15 min reaching the control baseline levels by 4 h of recovery. By contrast, TMAO pretreatment did not seem to affect Hsp90 levels in the severe mutant EB11.
Heat stress and TMAO differentially modulate MAPK signaling

Basal levels of native p38 protein were high in all cell lines. In TMAO-treated cells, reduced native p38 was observed in EB11 and EB21 in comparison to NKc11. Unstressed EB11 cells showed a higher level of activated p38 (phosphorylation) compared to EB21 and NKc11 cells (see Fig. 12a and c). After heat stress, p-p38 increased up to 60 min then slowly declined towards baseline levels at 4 h recovery. TMAO treatment clearly reduced the levels in both unstressed and heat-stressed severe mutant (See Fig. 12a and e) EB11 cells without recovery by 40% (See Fig. 12e). Before heat stress EB11 and EB21 cells showed a decrease in p-p38 levels even below base-line level, but apparently no effect on the NKc11 cells.

After heat stress, in TMAO treated cultures, a rapidly decreased p-p38 level in the keratin-defective cells was observed.

Native JNK1/2 protein levels were high in all EBS cells but lower in NKc11 under unstressed state. Heat shock transiently increased the levels only in EB cells. In the presence of TMAO the levels decreased with time in all cells. p-JNK1/2 were found present at resting state only in EB11 cells. In contrast, heat stress strongly induced p-JNK in all cells, but did so more dramatically in EB11 cells. TMAO reduced levels of p-JNK1/2 throughout recovery time both in unstressed and heat-stressed cells.

Native ERK1/2 levels were high in all cell lines and were not significantly affected by heat stress. The levels of activated ERK1/2 (p-ERK1/2) were higher in unstressed EB cells as compared to control cells. Upon heat stress, p-ERK1/2 showed a minor transient increase in EBS cells, whereas in NKc11, it appeared only after 4 h of recovery. TMAO gradually reduced native ERK2 levels throughout the recovery period in all cell lines. Besides, TMAO reduced p-ERK1/2 levels in both unstressed and stressed EBS cell lines (EB11 and EB21) while it was strongly increased in NKc11, already prior to stress.
Figure 12. western blot data a) for Hsp70/Hsc70 and activate p38 with and without heat-stress and TMAO b) The densitometric values of resting states samples for b) Hsp70/Hsc70, c) p-p38 in all unstressed cells d) Hsp70/Hsc70 and e) p-p38 in the presence and absence of TMAO for all cell lines. All values are normalized with corresponding beta actin, and both b and c are analysed under unstressed condition. Results in a-d are shown as mean ±SD (n=2.)

Paper IV

In this study we established and characterized immortalized keratinocytes from EI patients and tested their usefulness by studying the functional responses of the mutant cytoskeleton to heat stress and the rescuing effects of the chemical chaperones TMAO and 4-PBA. With a similar methodology as for EBS cells, we also tried to delineate the mechanism of action of these compounds at cellular and molecular levels of the disease.
Establishment and characterization of EI keratinocyte cell lines

Keratinocytes, isolated from biopsies of mildly affected skin with epidermolysis hyperkeratosis (EH) histologically, were established as primary cultures in low calcium, serum-free growth medium. They exhibited cobblestone morphology and displayed restricted replicative life span. The cells were transduced and immortalized with HPV16-E6/E7 generating the following cell lines with respective mutations, EH11/12 (K1_p.Val176_Lys197del), EH21/22 (K10_p.156Arg>Gly), EH31/32 (K10_p.Leu161_As p162del) and NKc21 (control). These cells have been successfully cultured for many generations and currently exceed >160 PDs. The corresponding cell lines established in serum-containing medium (EH12, EH22 and EH32), were not studied further.

Sequence analysis of immortalized EI keratinocytes (>140 PDs) exhibited the same mutations as in primary cell lines, and as reported in the patients’ blood DNA.

Keratin expression, differentiation and characteristics of heat stress on EI-keratinocytes

The staining pattern of basal keratins (K5 and K14) mostly appeared as a filamentous network. When exposed to high Ca\(^{2+}\) concentrations or an epidermal growth factor receptor tyrosine kinase inhibitor (PD153035) all cells differentiated and expressed the wild type and mutated suprabasal keratins side by side. EH31 cultures, from a patient with severe phenotype, were the only cells showing aggregates under resting condition upon differentiation. Heat stress disrupts KIF network in monolayer cultures of differentiated EH31 cells presenting KIF aggregates at the periphery of outer cells of stratified colonies, whereas identically treated PD153035-differentiated cells showed destruction of most of the K10 network. In contrast, 3% of EH11 cells presented few large, but punctate aggregates appearing mostly around the peri-nuclear region. Normal control keratinocyte line (NKc21) did not display any changes in the K1 and K10 cytoskeleton network when subjected to heat stress.

Pretreatment with TMAO and 4-PBA suppresses keratin aggregation in EI keratinocytes

TMAO or 4-PBA diminished the percentage of keratin aggregate-containing EH31 cells under resting condition, and also dramatically reduced the number of aggregates in these cells and EH11 upon heat stress. Interestingly, the aggregates present in chemical chaperone-treated heat-stressed cells were less coarse and mostly confined to the periphery. The expression of K1 was not altered by TMAO exposure but was clearly reduced by 4-PBA.
TMAO and 4-PBA alter the mRNA expression of keratins 1, 4 and 10 and Hsp70 in unstressed keratinocytes

In all cells, the magnitude of the expression of KRT1 and KRT10 was slightly induced by TMAO and greatly reduced by 4-PBA when pretreated for 24 h with either agent (Fig. 13a and c). The magnitude of the expressions of keratins and HSP70 in the different cell lines appeared as: EH31>>EH11>Nkc21. Moreover, 4-PBA also displayed opposite effects on HSP70 expression in different cell lines, with a significant increase in the control NKc21 and mild mutant EH11 cell lines, but a decrease in the severe K10 mutant (EH31) cells (Fig. 13a and 13b). Treatment with TMAO did not significantly affect HSP70 expression in severe EH31 cells (Figure 13d).

Figure 13. TMAO and 4-PBA alters the mRNA expression of KRT1 and KRT10 in an opposite manner in all cell lines. Immortalized EI and wildtype keratinocytes were Ca²⁺-differentiated and the mRNA expression of a) KRT1 b) KRT4 c) KRT10 and d) HSP72 was analysed by qPCR. TMAO increased the mRNA expression of KRT1 and KRT10 in all cell lines while 4-PBA reduced the expression (a and c). The two drugs have dissimilar effects on the expression of KRT4 and HSP72 in the cell lines (b and d). Results in a-d are shown as mean ±SD (n=3). Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s multiple comparison test.

The EI disease phenotype is reproduced in organotypic epidermis

EI cells differentiated in organotypic cultures within 8-14 days, forming a multilayered epidermis that exhibited slight parakeratosis (incomplete nuclear degradation in stratum corneum) and expressed markers of differentiation despite immortalization. Organotypic tissue also revealed histologic features with close resemblance to the graded severity of the clinical pheno-
type of the disease. Thus the severe mutant of EH31 and the moderate phenotype EH21 showed overt cellular fragility (cleft formation) in the suprabasal layer (with intact basal and cornified layers), while the mild mutant EH11 tissues appeared normal (Figure 14). Post heat shock, suprabasal fragility increased in EH31 and EH21 organotypic tissues. The EH31 tissues exhibited more extensive cell fragility in comparison to the EH21 tissues, whereas no clefts were seen in the EH11 tissues (data not shown). Using these immortalized EI cell lines, the phenotypes of the different patients were reproduced in this organotypic model; in the order EH31>EH21>EH11 as cleft formation.

Figure 14. Hematoxylin staining of organotypic epidermis generated using immortalized EI keratinocytes. EI organotypic epidermis was harvested on day 12 and all organotypic epidermis showed a well-defined basal layer, a mild hyperplasia, foci of parakeratosis and irregular cornification. EH31 showed least parakeratosis and more prominent cornification and fewer enlarged keratohyaline granules. The organotypic epidermis formed from the most severe EH31 cells showed cleft formation or cytolysis at the suprabasal layers leaving unperturbed basal and cornified layers upon heat stress (EH31). In the organotypic epidermis from EH21 a more subtle cleft was formed, whereas EH11 showed no clefts at all.
Discussion and Conclusions

As the KIF cytoskeleton network is instrumental in providing physical resilience to epithelial cells, mutations in the KIF genes may lead to heritable cell and tissue fragility disorders (reviewed in 90,94). Two studied examples include EBS and EI, which are mostly dominantly acting inherited epidermal skin tissue fragility disorders characterized by intra-epidermal blistering, due to mutations in genes encoding for basal epidermal keratins 5 or 14 and suprabasal keratins 1 or 10, respectively. This results in either basal cytolysis with extensive blistering in EBS or suprabasal cytolysis with superficial blistering and verrucous hyperkeratosis especially in flexural and intertriginous areas, i.e. in EI. A striking ultrastructural feature of both disorders is the compromised KIF network with clumping at the expression layer of the respective keratin mutations, i.e., pathognomonic K5 and K14 aggregates in basal keratinocytes in generalized EBS 49,114,145-147, and irregularly shaped K1 and K10 aggregates appearing suprabasally as dense peri-nuclear shell, in severe EI (reviewed in 148). Although most pathogenic mutations causing EBS and EI are missense mutations occurring within highly conserved regions of the alpha-helical rod domains and the non-helical H1 domain of basal or suprabasal keratins (see www.interfil.org; 13), exceptions now exist with mild disease phenotypes identified with mutations in the conserved helix hotspots 100-102.

Patient characteristics and genotype - phenotype correlations

In the present investigations, we screened three additional EB patients (EBS1, EBS3 and EB4) for KRT5 and KRT14 mutations, and discovered novel and recurrent disease-causing as well as non-disease-causing keratin gene mutations in two of them. One of the EBS patients (EBS2) was previously genetically defined 124. In another patient (EBS1), a missense point mutation was identified in K5 (p.E475G) in the 2B domain of K5 (HTP), which has consistently been associated with generalized EBS DM 149,150. Another missense mutation K5 (p.I183M) was identified in the 2A domain of the HIP in patient (EBS3), associated with generalized EBS DM. No pathogenic K5/K14 mutation was found in the fourth family (EB4 and
cousin) with less severe phenotypes, in spite of extensive sequencing which could reflect the well-known fact that approximately 25% of EBS patients do not show pathogenic keratin 5 or 14 mutation 98. Thus the identity of the putative disease-causing mutation in this family remains to be identified. The pathogenic mutations underlying the EI patients together with the functional effects of the mutations in this study have been described elsewhere 120,126.

The genotype/phenotype correlations of keratinopathies have been extensively studied before. For instance, in our study one patient with a point mutation in the 1A domain of K5 (p.I183M) 49, had a severe form of EBS, the same severe phenotype was reported with a different substitution K5 (p.I183F) 151,152. A different substitution at the same residue K5 (pI183V) 101, resulted in a mild phenotype (EBS-Loc), emphasizing that the hydrophobicity index (nature and size) of the substituted amino acids’ side-chains might influence keratin stability and/or assembly. The phenotypic difference between the patients with mutations at the same residue indicates or suggests that in addition to the mutation, the clinical expression of the genotype may be influenced by other factors e.g. additional SNPs, epigenetic or unknown pathomechanisms. Recent reports are currently revealing additional pathomechanism in EBS including the involvement of inflammatory cytokine profiles 95, and the molecular chaperone cellular stress burden 51.

Establishment and characterization of EBS and EI keratinocyte lines

Knowledge about the biology and pathophysiology of keratinocytes, especially in association with genodermatoses, has benefited tremendously from the use of knockout and transgenic mice models 153-155. However, it is important to complement such knowledge with biochemical studies and pharmacological assays using patient-derived keratinocytes. Primary keratinocyte cultures exhibit finite replicative lifespans, a limitation that makes them suboptimal for long-term reproducible evaluations, especially when testing new treatment rationales. Immortalized cells are invaluable tools required at early stage for devising therapeutic strategies, and phenotypic rescue of inherited skin disorders using genetically manipulated cultured cells have shown proofs of principle 156-158. While only few reports describe the establishment of human EI cells 159,160, several EBS cell lines and functional assays now exist 48,161,162, and they have been useful in exploring the effects of basal keratin mutations. We deemed it necessary to establish new models with which to study pathomechanisms and to test new treatment regimens for epidermolytic keratinopathic genodermatoses (EBS and EI). We therefore, established novel EI and EBS cell lines, stably expressing keratin mutations from patient origin, with the ability to reproduce in vitro the disease phenotype under appropriate conditions. Satisfactorily, all the EBS and EI
cell lines express normal and mutant KRT5 / 14 and KRT1 / KRT10 mRNA transcripts and proteins at levels comparable to primary keratinocytes grown in the same conditions, except for EH31 that showed more than 100 times higher values compared to the mild EH11.

Effect of keratin mutations on cell fragility
Analysis of primary and immortalized EBS and EI keratinocytes demonstrated some common features as well as certain divergence related to the phenotypic variation seen in the patients. Normal KIF organization was observed in cultures from most primary EI and EBS keratinocytes, except cells from the severely affected patients (EBS3; Paper I and EH3; Paper IV), where spontaneous keratin aggregates were observed already under normal growth conditions. This emphasizes the disruptive potential of mutations at these highly conserved residues of K5 and K10 for the disease severity. In addition, EB11 cells, derived from the most severely affected patient, and to some extent EB21 cells, also showed at resting state high basal levels of 70-kDa Hsp, p-p38, p-JNK and p-ERK correlating with graded severity of the patients which agrees with previous reports. These changes could presumably be due to an increased physiological stress burden on the severely mutant cells, incurred by the requirements of handling mutated keratins.

Effect of heat stress on keratin cytoskeleton resilience in keratin-defective cells
Since most patients with epidermolytic keratin disorders suffer from disease exacerbation during hot humid climate, heat shock, which has been used previously, seemed to us to be an ideal stress model for these investigations. When subjected to thermal stress, cultures derived from severely affected EBS (EB11, EB12, EBS1, EBS3) and EI (EH31) patients exhibited more keratin-aggregate containing cells than the moderate EBS (EBS2, EB21, EB22) and EI (EH21) cells, which were in turn more fragile than the mild EI (EH11) cells. The least fragility was observed in the normal control (NKc11p, NKc11, NKc12p, NKc12,) cells which exhibited a more resilient filament network. We also observed that when cells are grown in conditions favoring desmosomal connections (serum containing medium) as well as at increased confluency; they are more resistant to the effect of heat stress. This emphasizes the necessity of uniformity in cellular confluency when performing such experiments. Interestingly, the effect of heat stress on keratin aggregation in both EBS and EI cell lines were similar to those of their respective primary cells, indicating that immortalization per se did not interfere with the intrinsic properties of the patient’s keratinocytes.
Effects of treatment with TMAO and 4-PBA on keratin aggregation and involvement of Hsp and MAP kinases

In this study we repeatedly showed significant reduction of keratin aggregates when EBS and EI cells were pretreated with TMAO or 4-PBA compared to non-treated (± heat stress) cells (Paper I-IV). Furthermore, TMAO and 4-PBA altered the transcript and/or protein expression profiles of Hsp and keratins (Paper III and IV). Additionally, TMAO normalized these changes in Hsp and MAPK levels (Paper III), both at normal culture conditions and after heat stress, probably reflecting a stabilizing effect of these compounds on the keratinocyte cytoskeleton, in conformity with known effects of these chemicals in other heritable protein conformational disorders. Additionally, a strong tie was found between the reduction of keratin aggregates and the activation of some members of the MAPKs signaling pathways (especially p38 and JNK), as well as members of molecular chaperone machinery, Hsp70 and Hsp90 (both at transcript and protein levels). Heat stress rapidly induced keratin aggregates, and revealed an early, rapid and transient stress response such as activation of endogenous chaperones (70-kDa HSPs and HSP90) and MAPK (p38, ERK1/2 and SAPK) with direct correlation to the severity of the clinical phenotype, which were rapidly attenuated in the presence of TMAO. We found that heat-induced keratin aggregates were reduced by TMAO which correlated with reduction of activated p-p38 and p-JNK, as well as with Hsp reduction. The recent knowledge that p-p38 colocalizes with keratin aggregates, and that keratin disorders are putative protein folding disorder, combined with the fact that keratin aggregates are dynamic assemblies, assist in the interpretation of our data. Recently, it was found that Hsp70 is linked to the degradation of phosphorylated keratins by the ubiquitin proteasomal pathway, suggesting that the transient increase in Hsp70 levels in the EBS cell lines in the presence of TMAO is linked to the rapid clearance of mutant keratin aggregates. Moreover, studies in other disease models have shown specific effects of chemical chaperones on protein trafficking in conjunction to alteration of Hsp70 expression. Taken together, we therefore postulate that TMAO-mediated reduction of keratin aggregates involving p-p38 and p-JNK is proportional to cytoprotection and thus is directly related to increased cellular survival. Proof of principle is the demonstration that JNK activation rescued cells from apoptosis. This identifies a common link between keratinopathies and other protein folding disorders and also increase our understanding of the molecular mechanisms involved in the cytoprotective effects of TMAO.

The mechanism behind the reduction of keratin aggregates and stress activated signaling proteins in the presence of TMAO could be due to either of two ways or both: (i) the mutant keratins are physically protected from aggregating by TMAO due to unfavourable enthalpic interaction of peptide.
backbone and/or water-mediated stabilization as described or (ii) TMAO favours the priming between the mutated keratin and ubiquitin ligase followed by ubiquitin-proteasome mediated degradation.

Alteration of heat stress and MAPKs support that TMAO rescue is tightly involved with the chaperone machinery and ubiquitin-proteasome degradation, providing numerous new leads for investigations of pharmacological therapies of keratin-based skin disorders.

Possible implications of 4-PBA and TMAO treatment on keratin regulation in EI

In Paper IV, there was a clear difference in response to 4-PBA between the various immortalized EI cells, which may be related to either down-regulation of K1 and K10 mRNA or by compensatory up-regulation of K4, which is normally expressed in the buccal epithelium and after retinoid stimulation of normal epidermis. Retinoids are known to regulate many epidermal genes, including some keratins, and topical application to human skin, induces the expressions of K4, K6, K13, and K19, not normally present in interfollicular skin in vivo, but constitutively expressed in oral mucosa. Analogous to 4-PBA activity seen here, retinoid treatment of the skin down-regulates K1, K2, K10 and K14. Since retinoids affects keratin gene expression in a complex manner, it is not apparently clear if it is mediated by direct ligand binding and activation of retinoid receptors or indirectly by some other mechanisms.

However, the fact that 4-PBA down-regulates K1 and K10 may be a disadvantageous property which has to be examined more in detail in future investigations. If 4-PBA treatment in future investigations increases K4, it would act as a replacement of K1 in the K1 mutant patients as pair-wise partner for K10, speculatively reinforcing the diseased cytoskeleton or tissue stability. The decreased expression of K10 by 4-PBA treatment may aggravate the disease unless otherwise compensated by expression of other keratins, such as K13, or other proteins. In the case of K1 mutation, the down-regulation of K1 is not so critical because the remaining normal K4 would normalize and reinforce cytoskeleton resilience. We therefore seemingly proposed the evaluation of the effects of 4-PBA for such effects in view of clinical development.

Tissue engineering of EBS and EI epidermis

After analyzing the effect of drugs on monolayer cultures a natural step would be to test the effects on animal models or in organotypic disease models in vitro prior to clinical development. Since EBS and EI are both tissue fragility disorders, which may not be adequately reproduced in monolayer cultures, we also tend to mimic the disease phenotype in reconstructed epi-
dermis in vitro, where one could test the effects of drugs prior to clinical development. Despite immortalization, it was possible to induce EBS and EI cells to differentiate into tissue engineered epidermis on cell culture inserts or de-epidermized dermis. In fact EI cells were able to reproduce the histologic and phenotypic alterations reminiscent of the EI patients described elsewhere \(^{120, 126}\). Thus, intracellular vacuolation, blister formation and fragility of the generated epidermis were demonstrated \textit{in vitro}, a phenomenon that is consistent with a previous report in an \textit{in vitro} recessive dog model of EI \(^{175}\). In contrast, the severe EBS cell lines reproduced their phenotypes in organotypic models only when heat stressed (Fig. 15). Both models promise however to be useful in the future development of clinical remedies for EBS and EI.

![Figure 15. Tissue engineered epidermis using EB11 cells on cell culture insert showing a) unstressed insert and b) Heat-stressed inserts with basal cytolysis.](image)
Conclusions

The research in the present thesis specifically shows:

- Novel and recurrent disease-causing keratin 5 mutations including single nucleotide polymorphisms (SNPs) were identified in EBS patients.
- Keratinocyte cell lines were established from normal volunteers, and EBS and EI patients with mutations or deletions in keratin 5 and 1/10 alleles, respectively.
- All these cell lines express normal markers of proliferation and terminal differentiation in submerged cultures and can recapitulate the defective disease epithelium differentiation program in tissue engineered skin equivalent models.
- An *in vivo* / *in vitro* correlation has been demonstrated in cultured primary and immortalized keratinocytes using the heat stress model and by studying the appearance of keratin aggregates, cell loss and cell fragility.
- The expression of HSP and MAPK in stressed keratinocytes and their down regulation in the presence of TMAO present sensitive markers for studying pathomechanisms and novel treatment approaches in EBS.
- The inductive and repressive effects of 4-PBA on different cellular proteins point to new bioactivity of a chemical chaperone of interest for epidermolytic keratinopathies.
- The involvement of signaling pathways in EBS cells and the way in which TMAO exerts its cytoprotective effect together suggests that the chaperone protein machinery and stress-activated MAPKs may be new therapeutic targets in keratinopathies.
- These effects of chemical chaperones on EBS and EI keratinocytes were previously unknown.

The exact roles of the findings in this thesis are a subject of further investigation.
Future Perspectives

The past two decades have seen characterization of the molecular mechanisms and the identification of specific mutations in epidermolytic keratinizing genodermatoses. This provided the basis for improved diagnosis, re-classification, and prognostic implications and has formed the basis for prenatal testing and pre-implantation genetic diagnosis but progression towards therapies has been slow. Therefore to date, more than nine mutated keratin genes have been associated with diseases of the skin and its appendage. The phenotype of a given keratin–associated disorders may be genetically heterogeneous, with several mutations remaining to be defined in the filament genes. It seems that some of the biology and pathophysiology of keratin disorders is still unexplored and thus that future research might overcome the present limited treatment options in these disorders.

Gene therapy of monogenetic keratinopathies, which can involve silencing of the mutated or both (mutated and wild type) alleles to eliminate the effect of accumulation of mutant keratins, has been suggested for a long time. The major challenges are overcoming the dominant negative effects of K5 and K14 (EBS) and K1 and K10 (EI) mutations. By using the described cell lines different gene correction strategies could be tested, such as inhibition of the translation of mutated keratins by siRNA or spliceosome-mediated RNA trans-splicing (SMaRT) in order to replace certain mRNA exon segments. Another approach would be to substitute defects in the cell skeleton by other means, for instance by replacing keratins with desmin (a type III IF and could possibly reinforce the fragile keratin network) as shown in vitro. Despite many efforts, gene therapy has only entered the phase of clinical studies in pachyonychia congenita.

As our immortalized culture model systems have reproducible phenotype and have been found able to rescue EBS and EI cells by certain chaperones, the next stages in this research will involve the use of the cell lines in a larger scale to further develop additional translational tools and to test new therapies.

An advantage of devising general pharmacological approaches for therapy of EBS and EI is that candidate drugs may work not only in these disorders but also in other keratinopathies. Possible pharmacological utility of the cell lines include screening of chemical libraries for compounds that protect the filaments or rescue the cells by repressing the mutated keratin allele or by inducing the expression of functionally complementary keratins. An
advantage of these pharmacologic approaches is the possibility of alleviating personalized medicine and being able to use the treatment for all patients irrespective of knowledge regarding the specified mutation. As the structural and functional regulatory mechanisms of keratins unfold, mostly aided by relevant animal and patient-derived cell culture models, several well-tailored treatment strategies can be developed, to improve on current therapeutic hurdles or limitations. In the future it would be interesting to examine the putative protective effects of TMAO, 4-PBA and other compounds in the organotypic models of EBS and EI.

An essential goal of therapy is to alter the natural history of end-organ damage in keratin–related disorders, which should putatively increase the patients’ quality of life.
La peau est le plus large organe du corps. C’est un tissu dynamique, complexe, composé d’une multitude de couches. Sa position est stratégique, recouvrant l'extérieur du corps, à l'interface entre les milieux internes et externes, lui permettant de réguler un grand nombre de fonctions.

L'altération de ces fonctions intégrées, du fait de facteurs héréditaires ou bien acquis, compromet l'intégrité de cet organe vital et est à l'origine de plusieurs maladies de la peau. Dans l'épiderme, les filaments intermédiaires, dont la composante majeure est la kératine, travaillent en collaboration et forment une barrière de protection. Les mutations des gènes codant pour les kératinocytes empêchent la résilience des kératinocytes. Ceci provoque une instabilité des filaments intermédiaires, une cytolyse et une fragilité du tissu épithélial conduisant à une gamme de génodermatoses kératiniques épidermolytiques. Les keratinopathies épidermolytiques sont des maladies héréditaires rares caractérisées par une fragilité de la peau et des cloques intra-épidermiques basales ou supra-basales. Ces maladies sont transmises de façon autosomale dominante. Elles sont causées par des mutations au niveau des gènes de kératine de la basale épidermique (KRT5 ou KRT14) ou supra-basale (KRT1 ou KRT10), conduisant respectivement à une épidermolyse bulleuse simple (EBS) ou une ichthyose épidermolytique (EI). La caractérisation moléculaire des filaments intermédiaires de kératine épidermique et de leurs protéines associées sert de base pour identifier les mutations associées à des maladies distinctes de la peau chez les patients atteints de génodermatoses épidermolytiques kératinisantes.

Le phénotype de chaque maladie correspond au niveau d'expression des gènes mutés, ainsi qu’au niveau des types et positions des mutations et de leurs conséquences au niveau des ARN messagers et protéines. L'identification des mutations fournit une base pour améliorer le diagnostic, pour reclasser les maladies modifiant les pronostiques. Cette identification forme aussi la base du diagnostic prénatal et préimplantatoire. La connaissance précise de la pathogenèse est la condition préalable pour le développement de thérapies visant à neutraliser et potentiellement guérir ces maladies dévastatrices. Il faut pour cela de nouveaux modèles de tests étudiant la peau malade au niveau cellulaire et subcellulaire.
Malgré une connaissance étendue de l'étiologie des maladies, la pathogénèse de ces troubles est mal comprise et aucun remède efficace n’existe. L'objectif général de cette thèse était de chercher de nouvelles mutations du gène de kératine chez des patients EBS, de développer de nouveaux modèles in vitro pour étudier la pathogénèse de l'EBS et EI, et d'étudié de nouvelles approches pharmacologiques pour ces deux maladies.

Ces objectifs ont été réalisés dans les quatre manuscrits inclus dans cette thèse et résumés ci-dessous:

Nous avons identifié deux nouvelles mutations récurrentes de KRT5, même si un patient EBS ne présentait pas du tout de mutations pathogènes kératine. Grâce à des cultures de kératinocytes primaires, provenant de patients EBS, nous avons pu reproduire in vitro une corrélation entre d’une part la sévérité clinique et l'instabilité du cytosquelette d’autre part. Des lignées cellulaires de kératinocytes immortalisés venant de trois patients EBS et trois patients IE, avec différents degrés de gravité, ont été établies à l'aide de HPV16-E6E7. Seules les lignées cellulaires dérivées de patients gravement atteints ont montré des agrégats spontanés de kératine dans des conditions normales de culture. Le stress par la chaleur, qui perturbe le cytosquelette déf ectueux en kératine, a induit des agrégats de kératine dans toutes les lignées cellulaires de patients, mais de façon plus importante dans les cellules de patients présentant un phénotype sévère. Dans les cultures organotypiques, les cellules immortalisées d’EBS et d’EI ont été capables de se différencier et de former un épiderme avec plusieurs couches rappelant les observations in vivo.

L’addition de deux petites molécules, le triméthylamine N-oxyde dihydrate (TMAO) et le sodium 4-phénylbutyrate (4-PBA), a permis de réduire la proportion d’agrégats de kératinocytes EBS et EI, en condition normale et en condition de stress, en protégeant ou stabilisant le cytosquelette. Le mécanisme d'action du TMAO et du 4-PBA a été identifié : il modifie les composantes du système endogène chaperon (protéines Hsp) ainsi que la voie de signalisation MAPK, deux parties qui semblent être incriminées dans la pathogénie de l'EBS. De plus, selon le type de la kératine muté, le 4-PBA a induit les protéines Hsp70 et KRT4 (peut-être pour compenser le KRT5 muté), et inhibé les KRT1 et 10, ce qui pourrait aider à protéger les cellules EBS et EI contre le stress.

En conclusion, des mutations pathogènes nouvelles et récurrentes de kératine ont été identifiées dans les cas d’EBS. Des lignées cellulaires immortalisées d’EBS et d’EI ont montré qu’elles reflètent fonctionnellement le phénotype de ces maladies. Avec ce modèle in vitro, deux agents pharmacologiques, le TMAO et le 4-PBA, se sont révélés être des candidats prometteurs en tant que de nouveaux traitements de keratinopathies héréditaires.
Huden är kroppens största organ och fungerar som en barriär mot omgivningen. Den yttersta delen av huden, epidermis, nybildas kontinuerligt för att bibehålla barriärfunktionen. Fel i funktioner som är av betydelse för nybildningen av barriären, antingen ärftliga eller förvärvade, äventyrar integriteten för detta vitala organ och ligger till grund för flera hudsjukdomar. I den dominerande celltypen i epidermis, keratinocytterna, finns det speciella strukturfibrer som skapar ett nätverk i cellerna som gör att de kan motstå fysiska påfrestningar. Den viktigaste gruppen av cytoskelettproteiner i keratinocytterna är keratinfilament. Mutationer i gener som kodar för keratiner minskar cytoskelettets motståndskraft och orsakar cellcytolys och ökad instabilitet i epitelvävnaden. Två av dessa sällsynta, ärftliga hudsjukdomar är epidermolysis bullosa simplex (EBS) och epidermolytic iktyos (EI), är som kännetecknas av blåsbildning på olika nivåer i epidermis vilket drastiskt påverkar patienternas livskvalitet. Dessa sjukdomar orsakas av autosomalt dominanta mutationer i keratingenerna KRT5 eller KRT14 respektive KRT1 eller KRT10.

Sjukdomsbilden vid EBS och EI återspeglar mängden av muterat protein, samt typen och positionen av genmutationerna. Identifieringen av nya mutationer har lagt grunden för bättre diagnos, reklassificering med prognostiska konsekvenser och har utgjort grunden för prenatal diagnostik vid dessa åkommor. Dessa kunskaper är även en förutsättning för utveckling av nya behandlingar som potentiellt motverkar och botar dessa sjukdomar. För utvecklingen av nya läkemedel krävs även nya testmodeller för studier på cell- och subcellulär nivå. Trots stor kunskap vad beträffar sjukdomarnas etiologi finns ännu inga effektiva botemedel. 

patienter med svårare klinisk symptom uppvisade aggregat av muterade keratiner redan under normala odlingsbetingelser.


Sammanfattningsvis har nya sjukdomsframkallande keratinmutationer identifierats i EBS, och immortaliserade EBS och EI keratinocyter som återspeglar sjukdomarnas specifika fenotyp har etablerats. Cellinjerna kan användas för att studera nya behandlingsprinciper vid dessa hudsjukdomar. Med denna *in vitro* modell, har två farmakologiska substanser, TMAO och 4-PBA, visats vara lovande som nya tänkbara behandlingar.
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