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REGULATION OF EPIDERMAL TIGHT JUNCTIONS BY CALCIUM ATPases AND p38

by

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"Omnia mea mecum porto."

Simonides

To my family

Laura Raiko

Regulation of epidermal tight junctions by calcium ATPases and p38

Department of Dermatology and Venereology, and Department of Cell Biology and Anatomy, Faculty of Medicine, University of Turku and Turku University Central Hospital, Turku, Finland and The Turku Doctoral programme of Clinical Sciences (GSCS) and National Graduate School of Clinical Investigations (VKTK). Annales Universitatis Turkuensis, Medica-Odontologica, 2013

ABSTRACT

The epidermis is the upper layer of the skin and keratinocytes are its most abundant cells. Tight junctions are cell junctions located in the granular layer of the epidermis. They maintain the polarity of the cells and regulate the movement of water-soluble molecules. Epidermal tight junctions may lose their integrity when there are defects in intercellular calcium regulation. Hailey-Hailey and Darier´s disease are dominantly inherited, blistering skin diseases. Hailey-Hailey disease is caused by mutations in the ATP2C1 gene encoding a calcium/manganese ATPase SPCA1 of the Golgi apparatus. Darier´s disease is caused by mutations in the ATP2A2 gene encoding a calcium ATPase SERCA2 of the endoplasmic reticulum. p38 regulates the differentiation of keratinocytes. The overall regulation of epidermal tight junctions is not well understood. The present study examined the regulation of tight junctions in the human epidermis with a focus on calcium ATPases and p38. Skin from Hailey-Hailey and Darier´s disease patients was studied by using immunofluorescence labeling which targeted intercellular junction proteins. Transepidermal water loss was also measured. ATP2C1 gene expression was silenced in cultured keratinocytes, by siRNA, which modeled Hailey-Hailey disease. Expression of intercellular junction proteins was studied at the mRNA and protein levels. Squamous cell carcinoma and normal human keratinocytes were used as a model for impaired and normal keratinocyte differentiation, and the role of p38 isoforms alpha and delta in the regulation of intercellular junction proteins was studied. Both p38 isoforms were silenced by adenovirus cell transduction, chemical inhibitors or siRNA and keratinocyte differentiation was assessed.

The results of this thesis revealed that: i.) intercellular junction proteins are expressed normally in acantholytic skin areas of patients with Hailey-Hailey or Darier´s disease but the localization of ZO-1 expanded to the stratum spinosum; ii.) tight junction proteins, claudin-1 and -4, are regulated by ATP2C1 in non-differentiating keratinocytes; and iii.) p38 delta regulates the expression of tight junction protein ZO-1 in proliferating keratinocytes and in squamous cell carcinoma derived cells. ZO-1 silencing, however, did not affect the expression of other tight junction proteins, suggesting that they are differently regulated.

This thesis introduces new mechanisms involved in the regulation of tight junctions revealing new interactions. It provides novel evidence linking intracellular calcium regulation and tight junctions.

Keywords: Epidermis; tight junction; keratinocyte; p38; Darier’s disease; Hailey-Hailey disease; calcium; squamous cell carcinoma

Laura Raiko

Epidermaalisten tiivisliitosten säätely kalsium ATPaasien ja p38:n avulla

Iho- ja sukupuolitautioppi, Solubiologia ja Anatomia, Lääketieteellinen tiedekunta, Turun yliopisto ja Turun yliopistollinen keskussairaala, Turun Kliininen tutkijakoulu ja Valtakunnallinen Kliininen tutkijakoulu, Turku, Annales Universitatis Turkuensis, Medica Odontologica, 2013

TIIVISTELMÄ

Epidermis on ihon pinnallisin kerros ja sen yleisimpiä soluja ovat keratinosyytit. Tiivisliitokset ovat soluliitoksia, jotka sijaitsevat epidermiksen jyväissolukerroksessa. Ne ylläpitävät solujen polariteettia ja estävät vesiliukoisten molekyylien liikettä. Epänormaali solunsisäinen kalsiumsäätelyn vaikuttaa epidermiksen tiivisliitoksiin. Hailey-Hailey tauti ja Darierin tauti ovat vallitsevasti periytyviä rakkulatauteja. Hailey-Hailey tauti aiheutuu mutaatioista ATP2C1 geenissä, joka koodaa Golgin laitteen kalsium/mangaani ATPaasia nimeltä SPCA1. Darierin tauti aiheutuu mutaatioista ATP2A2 geenissä, joka koodaa endoplastisen limakalvoston ATPaasia nimelta SERCA2. p38 säätelee keratinosyyttien erilaistumista. Epidermiksen tiiviisliitosten säätely tunnetaan huonosti.

Tässä tutkimuksessa selvitettiin, säätelevätkö ATP2C1 ja ATP2A2 ATPaasit ja p38 epidermiksen tiivisliitoksia. Hailey-Hailey tautia ja Darierin tautia sairastavien potilaiden ihoa tutkittiin leimaamalla näytteitä soluliitosproteiinien vasta-aineilla. Lisäksi iholta mitattiin transepidermaalista veden haihtumista (TEWL). Hailey-Hailey tautia mallinnettiin vaimentamalla keratinosyyteistä ATP2C1 geenin ilmentyminen. Tämän jälkeen määritettiin muutoksia soluliitosproteiineissa. Levyepiteelisyöpäsoluja ja normaaleja ihmisen keratinosyyttejä käytettiin p38 alfa ja delta-välitteisen erilaistumisen tutkimiseen. p38 alfan ja deltan ilmentyminen estettiin adenoviraalisesti, kemiallisesti tai siRNAn avulla, jolloin voitiin arvioida muutoksia keratinosyyttien erilaistumisessa.

Tuloksista ilmeni, että i) Solujenväliset liitokset muodostuvat normaalisti akantolyyttisillä ihoalueilla Darierin tautia tai Hailey-Hailey tautia sairastavilla potilailla mutta tiivisliitosproteiini ZO-1:n ilmentyminen leviää myös okasolukerrokseen; ii) ATP2C1 säätelee tiivisliitosproteiinien claudin-1 ja -4 ilmentymistä jakaantuvissa keratinosyyteissä; iii) p38 säätelee tiivisliitosproteiini ZO-1:n ilmentymistä erilaistuvissa keratinosyyteissä ja levyepiteelisyöpäsoluissa. ZO-1:n ilmentymisen estäminen ei vaikuta muihin tiivisliitosproteiineihin, mikä viittaisi siihen että niiden ilmentyminen on erilailla säädeltyä.

Tämä väitöskirjatutkimus tuo uutta tietoa keratinosyyttien tiivisliitosten säätelystä. Tutkimus liittää ensimmäistä kertaa tiivisliitokset p38 signalointiin ja solunsisäiseen kalsiumsäätelyyn.

Avainsanat: Epidermis, tiivis liitos, keratinosyytti, p38, Darierin tauti, Hailey-Hailey tauti, kalsium, levyepiteelisyöpä

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ABBREVIATIONS

ATP	adenosine tri phosphate
ATP2A2	ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2
ATP2C1	ATPase, Ca^{2+} transporting, type 2C, member 1
BSA	bovine serum albumin
CAR	coxsackie and adenovirus receptor
CaR	G-protein coupled receptor Ca
cDNA	complementary DNA
CRB3	Crubs homolog 3
CREB	cAMP response element binding protein
CTX	xenopus thymocyte receptor
DD	Darier´s disease
DMEM	Dulbecco´s Modified Eagle´s Medium
DNA	deoxyribonucleic acid
IgG	immunoglobulin G
IgSF	immunoglobulin superfamily
IP3	inositol 1, 4, 5-triphosphate
ER	endoplasmic reticulum
FCS	fetal calf serum
GADPH	glyceraldehyde 3-phosphate dehydrogenase
HHD	Hailey-Hailey disease
IIF	indirect immunofluorescence
JAM-A	junctional adhesion molecule-A
kDa	Kilodalton
KGM	keratinocyte growth medium
MAPK	mitogen-activated protein kinase
NCX	Na^{+}/ Ca^{2+} exchanger
N-FAT	nuclear factor of activated T-cells
NFkB	nuclear factor kappaB
Par	partitioning-defective
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	Protein Kinase C
PMCA	plasma membrane Ca^{2+} -ATPase
P2Y2	purinergic receptor P2Y G-protein coupled, 2
P2X7	purinergic receptor P2X ligand-gated ion channel, 7
qPCR	quantitative PCR
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
SCC	squamous cellular carcinoma cells
SFM	serum free medium
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
siRNA	short interfering RNA
SPCA	secretory pathway Ca2+-ATPase
STED	stimulated emission depletion
TEER	transepidermal electrical resistance
TEWL	transepidermal water loss
TJ	tight junction
ZO	Zonula occludens

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-III:

I Laura Raiko, Pekka Leinonen, Päivi M. Hägg, Juha Peltonen, Aarne Oikarinen and Sirkku Peltonen. Tight junctions in Hailey-Hailey and Darier´s diseases. Dermatology Reports Vol. 1 (1) 1-5. (2009) http://www.pagepress.org/journals/index.php/dr/article/viewArticle/dr.2009.e1/1609

II Laura Raiko, Elina Siljamäki, Mỹ G. Mahoney, Heli Putaala, Erkki Suominen, Juha Peltonen and Sirkku Peltonen. Hailey-Hailey disease and tight junctions: Claudins 1 and 4 are regulated by ATP2C1 gene encoding Ca2+/Mn2+ ATPase SPCA1 in cultured keratinocytes. Experimental Dermatology Vol. 21 (8):586-591. (2012)

III Elina Siljamäki, Laura Raiko, Mervi Toriseva, Liisa Nissinen, Tuomas Näreoja, Juha Peltonen, Veli-Matti Kähäri and Sirkku Peltonen p38δ mitogen-activated protein kinase regulates the expression of tight junction protein ZO-1 in differentiating human epidermal keratinocytes.
Archives for Dermatological Research, 2013, DOI 10.1007/s00403-013-1391-0. In press.

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1. INTRODUCTION

The epidermis is the uppermost layer of the skin and keratinocytes are its most abundant cells (Underwood 2004). Intercellular junctions connect the keratinocytes with other keratinocytes mainly *via* desmosome junctions. Desmosomes provide the epidermis its mechanical strength (Alberts et al. 2003). Tight junctions are cell junctions located in the granular layer of the epidermis (Pummi et al. 2001; Morita & Miyachi 2003; Brandner et al. 2002). They maintain the polarity of the cells and inhibit the movement of water-soluble molecules and thus play a role in the barrier function of the skin (Alberts et al. 2003). Adherens junctions, that are present in every living layer of epidermis, regulate the formation of other intercellular junctions and support the tissue during mechanical stress (S. Burge 1994; Alberts et al. 2003).

Hailey-Hailey disease and Darier´s disease are dominantly inherited blistering skin diseases (Foggia & Hovnanian 2004; S. M. Burge 1992; Dobson-Stone et al. 2002). Hailey-Hailey disease is caused by mutations in the ATP2C1 gene. This gene encodes a calcium/manganese ATPase SPCA1 which is located in the Golgi apparatus. (Z. Hu et al. 2000; Sudbrak et al. 2000) Darier´s disease is caused by mutations in the ATP2A2 gene. The ATP2A2 gene encodes a calcium ATPase SERCA2 which is located in the endoplasmic reticulum (Sakuntabhai, Ruiz-Perez, et al. 1999). The mutations cause blistering and acantholysis in the epidermis when desmosome junctions open (Savignac et al. 2011; S. M. Burge 1992). In present study, these two genodermatoses were used as models of impaired calcium regulation.

p38 is an intracellular signaling molecule and present in keratinocytes (Junttila et al. 2007). p38 delta signaling mediates differentiation in keratinocytes and it also mediates apoptosis (Eckert et al. 2004; Kraft et al. 2007). p38 alpha is another isoform of p38 present in keratinocytes (Junttila et al. 2007). In this study, we evaluated the regulatory role of p38 signaling in the human keratinocytes. Squamous cell carcinoma, which is one of the most important human skin cancers, was used to model abnormal differentiation (Colmont et al. 2012).

Understanding the interactions of different junction proteins and their regulation in normal and abnormal situations can potentially improve the well-being of people who suffer from these abnormalities. Understanding the cellular mechanisms can also help to improve the treatment of these diseases.

2. REVIEW OF LITERATURE

2.1 The skin

The skin is the largest organ in human body (contributing to about 7% of adult weight with a surface area of 1.2-2.2-m^2) (Young et al. 2006; Marieb & Hoehn 2006). The thickness of the skin varies from 5-mm in the upper back to less than 1-mm in the eyelids (Young et al. 2006).

TEXT BOX 1.

The functions of the skin:

1. *Barrier* (mechanical, chemical and thermal threats): Bacteria or fungi cannot penetrate intact skin.
2. *The largest sensory organ in the body*: Receptors for touch, pain, temperature and pressure. These receptors are located in skin areas that have the most physical contact with the environment.
3. *Thermoregulation*: Subcutaneous fat insulates to some extent from cold. The dense vascular network in the skin helps the body to lose excess heat. Eccrine sweat glands secrete watery secretion that evaporates from the skin surface with extra heat. The nervous system controls the actions of the vascular network and the sweat glands.
4. *Metabolism*: The skin produces vitamin-D (cholecalciferol) from its precursor dehydrocholesterol by the ultraviolet light. Subcutaneous fat serves as an energy supply (triglycerides) and acts as a shock absorber. The sweat contains metabolic wastes and helps them to exit the body.
5. *Blood reservoir*: The rich vasculature of the skin enables the skin to function as a blood reservoir.
6. *Aesthetic role*: By decorating the skin, one can express their personality (*e.g.* tattoos and makeup).
7. *Immunologic role:* Langerhans cells (dendritic cells) and macrophages exist in the skin.

Sources: (Marieb & Hoehn 2006) and (Young et al. 2006).

2.1.1 The layers of the skin

Subcutaneous tissue

Subcutaneous tissue consists mostly of adipose tissue with triglyceride stores. Large blood vessels enter and exit the skin through subcutaneous tissue (Marieb & Hoehn 2006). The skin appendages (sebaceous glands, eccrine sweat glands, hair follicles and in some skin areas, also apocrine sweat glands) are located in subcutaneous tissue and dermis (Young et al. 2006). (Figure 1)

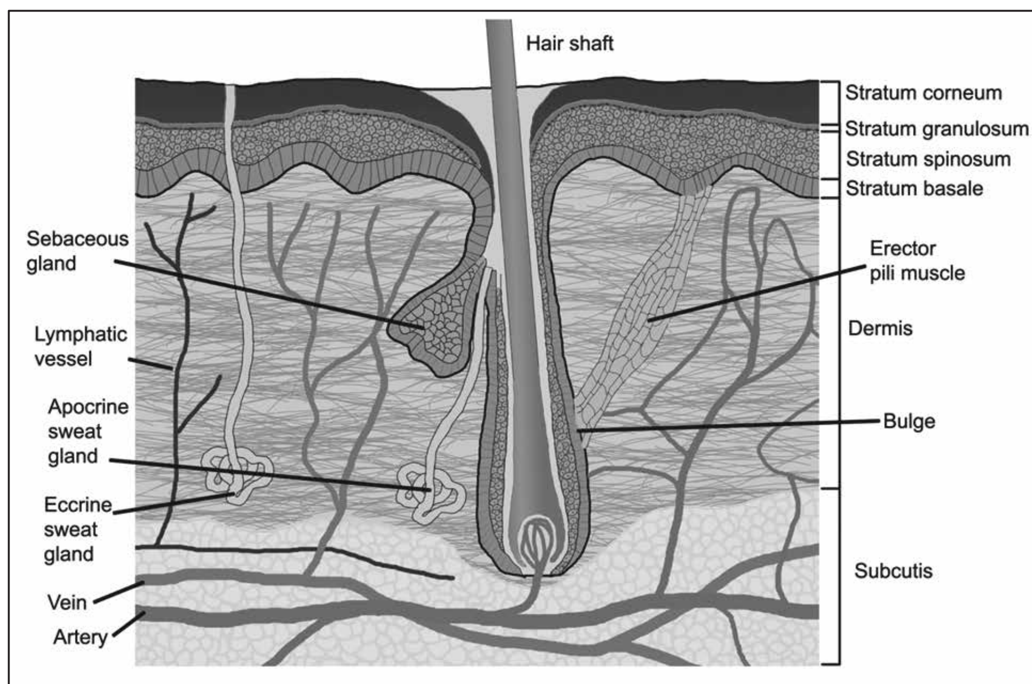

Figure 1 The structure of the human skin.
Modified from (Burns et al. 2010; Underwood 2004; Ohyama 2007).

Dermis

The dermis is connected to the upper layer, called the epidermis, *via* basement membrane and hemidesmosome junctions. Rete ridges formations, which are branches of epidermis that reach the upper part of dermis, protect the skin from mechanical tearing forces by allowing skin to stretch (Young et al. 2006). Dermis is fibrocollagenous and elastic connective tissue which is strong and flexible. The cell types in dermis consist of fibroblasts, macrophages and occasionally mast cells and white blood cells. The dermis contains blood vessels, nerves, sensory receptors and lymphatic vessels (Marieb & Hoehn 2006; Young et al. 2006). Most of the hair follicles, sweat glands and sebaceous glands are also located in the dermis however, they originate from the epidermal tissue (Marieb & Hoehn 2006).

The most abundant collagen type in the skin dermis is type I collagen (Burns et al. 2010). Type III collagen forms 10% of the total collagen mass in the adult dermis (Burns et al. 2010). The ratio of collagen I and collagen III is about 8:1 (Burns et al. 2010). Collagen V accounts for less than 5% of the total collagen in the dermis (Burns et al 2010). This collagen regulates the lateral growth of the large collagen fibers and it is located on their surface (Burns et al. 2010). If collagen V is absent, the fibers are irregular and their diameter is abnormal (Burns et al. 2010). Type VI collagen is located in thin microfibrils, independent of thicker collagen fibers (Burns et al. 2010). Collagen XVII is important in stabilizing the dermal-epidermal junctions (Burns et al. 2010). Another important component of dermis is elastin, which is abundant in the reticular dermis. Glycosaminoglycans are large space filling matrix molecules which are associated *e.g.* with elastin. (Pasquali-Ronchetti & Baccarani-Contri 1997). Hyaluronan is one of the most important glycosaminoglycans in skin. Hyaluronan participates in many regulatory tasks in the human body (Camenisch et al. 2000).

The dermis can be divided into two parts: the papillary layer and reticular layer. The papillary layer is the upper layer, which is composed of reticulate collagen and the elastin fiber structures containing many blood vessels (Marieb & Hoehn 2006). In this layer, there are needlelike spurs of dermis extending to the epidermis which are called dermal papillae. Many dermal papillae contain capillaries (Plexus subcapillaris) and some have pain receptors (free nerve endings) and touch receptors (Meissner's corpuscles). In the palms and soles of feet, these papillae are above larger bulges, named dermal ridges, which cause the epidermis above to be formed into epidermal ridges (or finger prints). The lower level of dermis is called the reticular dermis and it composes 80% of the thickness of dermis. The reticular dermis is irregular connective tissue. The plexus cutaneous, a vascular network, is located between this layer and subcutaneous tissue. The extracellular matrix of the reticular dermis contains thick braids of imbricated collagen fibers, of which, most are parallel to skin. Areas between these braids form lines in the skin. These lines are important when operating on skin because wounds heal better when they are cut parallel to these lines (the skin stretching is minimal). (Marieb & Hoehn 2006) (Figure 1)

Epidermis

Layers of epidermis

The epidermis forms the superior layer of the skin. It is a multilayered structure with keratinocytes of different characteristics. The keratinocytes differentiate during their journey to the uppermost layer (Kirschner & Brandner 2012). (Figure 1)

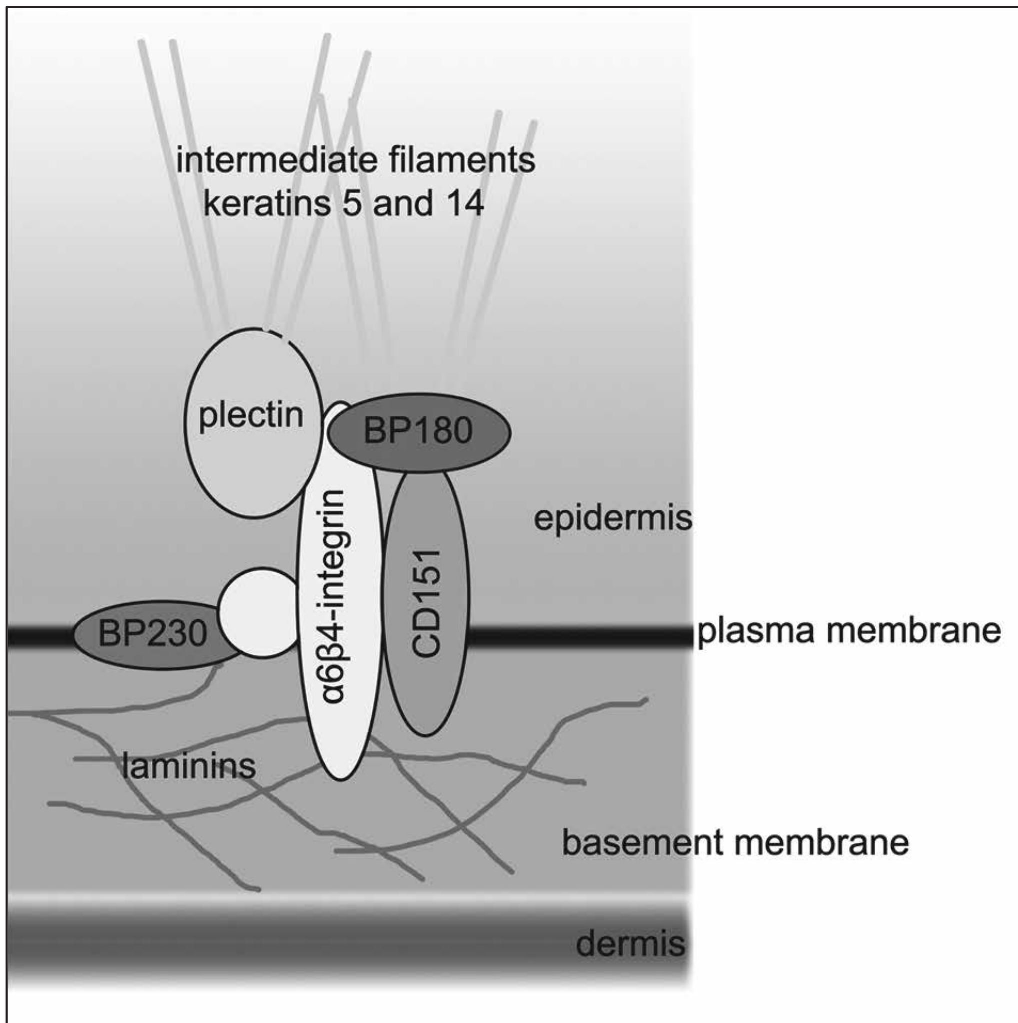

Figure 2 The structure of a hemidesmosome. Modified from (H. Zhang & Labouesse 2010).

Stratum basale

The basal layer consists of cuboidal or shallow columnar cells (Young et al. 2006). In this layer, the undifferentiated keratinocytes undergo continuous mitoses because the skin regenerates in stratum basale (Marieb & Hoehn 2006; Kirschner & Brandner 2012). The regenerating cells are the stem cells (L. Alonso & Fuchs 2003). In the keratinocyte culture, there are three different cell types: holoclones with greatest proliferation capacity; meroclones with intermediate proliferative potential; and paraclones with modest proliferation capacity (Barrandon & H. Green 1987). Holoclone cells might be stem cells *in vivo* (L. Alonso & Fuchs 2003). Studies using rat or human hair follicle tissues show that cells directly below the bulge (human) or the cells in the bulge (rat) had the highest proliferative potential (Kobayashi et al. 1993; Rochat et al. 1994; Taylor et al. 2000). In the interfollicular area of the adult

human skin deep rete ridges have shown to contain stem cells and transient amplifying cells, while differentiating cells are found in the shallow rete ridges (Webb et al. 2004).

The differentiation affects the types of keratins synthesized in the keratinocytes. The keratins synthesized in the basal layer are keratins 5 and 14 (Pekny & Lane 2007). During differentiation these keratins are replaced by keratins 1 and 10 (Bragulla & Homberger 2009).

The uneven, inferior part of the basal cells, is attached to the basement membrane with hemidesmosome junctions (Marieb & Hoehn 2006). Hemidesmosomes can be seen as electron-dense structures extending from the intracellular part of the basal keratinocytes to the upper part of the dermal-epidermal basement membrane (Burns et al. 2010). In the hemidesmosome junction, the intracellular plaque serves as an anchor site for the intermediate filaments of the hemidesmosome (Green & Jones 1996). These filaments consist of keratins 5 and 14 (Burns et al. 2010). Type VII collagen forms anchoring fibrils (Burns et al. 2010). The hemidesmosome has also type XVII collagen (BP180) which is an intracellular plaque protein (Burns et al. 2010; H. Zhang & Labouesse 2010). Integrins have an important role in stabilizing the hemidesmosome junctions (K. J. Green & J. C. Jones 1996). Alpha6beta4-integrin is a transmembrane protein and it links the hemidesmosome to the basal membrane *via* laminin 332, plectin, BP180, and BP230 (H. Zhang & Labouesse 2010). (Figure 2)

The basement membrane consists mainly of collagen IV which forms web like structures (Khoshnoodi et al. 2008). Collagen IV has many interactions with other molecules affecting cell migration, differentiation and cell adhesion (Khoshnoodi et al. 2008). Other important components of the basement membrane are proteins of the laminin family (Burns et al. 2010). The most common laminin in the basement membrane is laminin 332 (Burns et al. 2010). Other laminins in the basement membrane are laminin 311 and 511 (Burns et al. 2010). Integrins mediate the cell binding of laminins (Burns et al. 2010). The intercellular junctions linking keratinocytes are located in the basal layer are desmosomes and adherens junctions (Burns et al. 2010).

In the stratum basale, 10-25% of the cells are melanocytes (Marieb & Hoehn 2006). The melanocytes produce melanin which is transferred *via* melanosomes to the surrounding epidermis and into the growing hair shafts in hair follicles (Burns et al. 2010). Melanocytes have long dendritic processes reaching up to the stratum spinosum that transfer the melanin to the keratinocytes surrounding them (Bolognia et al. 2008; Marieb & Hoehn 2006). Keratinocytes intake melanin *via* phagocytosis and melanin is placed above the nucleus where it protects the DNA (Bolognia et al. 2008). The melanin provides protection from the sun by inhibiting the harmful effects of ultraviolet radiation (Burns et al. 2010). Production of different melanin types (brown/black eumelanin and yellow/red phaeomelanin) and localization in skin and hair cause different pigmentation (Burns et al. 2010). Merkel cells are also occasionally seen in the stratum basale (Marieb & Hoehn 2006). The disc-shaped

Merkel cells are attached to sensory nerve endings forming Merkel discs which serve as light touch receptors (Marieb & Hoehn 2006).

Stratum spinosum

When the keratinocytes move to stratum spinosum, the differentiation process begins. In the stratum spinosum, there are multiple layers of unevenly shaped keratinocytes with large lightly staining nuclei and easily distinguishable nucleoli which marks active protein synthesis (Marieb & Hoehn 2006; Young et al. 2006). The intermediate filaments of these keratinocytes consist of cytokeratins 1 and 10 (Burns et al. 2010; Kirschner & Brandner 2012). Cytokeratin 10 is also an early differentiation marker (Kirschner & Brandner 2012). The cytokeratin forms bundles, called tonofibrils, which anchor to intercellular junctions called desmosomes (Young et al). The desmosome is the most typical intercellular junction in this layer (Burns et al. 2010; Kirschner & Brandner 2012). Desmosomes and keratin intermediate filaments have a role in the formation of the spindle-shaped appearance of the keratinocytes in the stratum spinosum (Kirschner & Brandner 2012; Burns et al. 2010)). Furthermore, gap junctions exist mainly in the stratum spinosum (Salomon et al. 1994). Melanin granules produced by melanocytes and Langerhans cells inhabit this layer (Marieb & Hoehn 2006).

Type XXIX collagen is located in the epidermis and it is most abundant in suprabasal layers (Burns et al. 2010). Collagen XXIX is not visible in the stratum spinosum and stratum granulosum of the atopic dermatitis patients (Burns et al. 2010). The differentiation process proceeds from the stratum spinosum to the surface layers (Kirschner & Brandner 2012). This is observed when the intermediate differentiation marker involucrin is studied. It is present only in the upper layers of stratum spinosum (Kirschner & Brandner 2012).

The space between adjacent keratinocytes is filled with hyaluronan in the stratum basale and stratum spinosum (R. Tammi et al. 1988). Hyaluronan is a high molecular weight glycosaminoglycan which has many functions, for example, in differentiation, development and tissue repair (Camenisch et al 2000). For instance, trauma of the epidermis (*in vivo*) can cause up to a seven-fold increase in the concentration of hyaluronan (R. Tammi et al. 1988; R. Tammi et al. 2005).

Protein kinase C alpha has a role in the irreversible growth arrest during the differentiation of keratinocytes (Jerome-Morais et al. 2009). In a study using normal human keratinocytes and skin samples, protein kinase C alpha localizes in the first suprabasal layer of the human epidermis, suggesting that, *in vivo*, it may contribute to terminal differentiation-associated growth arrest in the lower layers of the stratum spinosum (Jerome-Morais et al. 2009). Down regulation of protein kinase C alpha, in an *in vitro* organotypic epidermis, results in an increase of proliferation marker expression in suprabasal and basal keratinocytes (Jerome-Morais et al. 2009). Differentiation and stratification were also reduced (Jerome-Morais et al. 2009).

Stratum granulosum

The differentiation process continues in the stratum granulosum. The keratinocytes lose their multi-angled shape and flatten when approaching the surface of the skin (Young et al. 2006). The stratum granulosum consists of 3-5 layers of flattened keratinocytes which express many differentiation associated proteins (Morita & Miyachi 2003). Filaggrin and loricrin are late differentiation markers which are expressed and processed by the keratinocytes of stratum granulosum (Kirschner & Brandner 2012). Filaggrin, the filament aggregating protein, has a role in this flattening process of the cells (Burns et al. 2010). The keratinocytes in the upper third of epidermis also contain involucrin (Murphy et al. 1984). The nuclei and cell organelles of the keratinocytes disappear in the superior part of stratum granulosum (Marieb & Hoehn 2006; Young et al. 2006). The plasma membranes of the cells become thicker when the intracellular proteins are attached on the inner surface of the cell membrane (Marieb & Hoehn 2006). These keratinocytes are called corneocytes (Morita & Miyachi 2003).

Two sorts of granules accumulate in the granular cells: keratohyaline granules and lamellated granules (Marieb & Hoehn 2006). The keratohyaline granules contain proteins rich in sulphur, such as cysteine and other proteins like involucrin and loricrin (Young et al. 2006; Hohl et al. 1993). Profilaggrin is also a component of keratohyaline granules (Burns et al. 2010; Fleckman et al. 1985; Presland et al. 1997). Keratin and profilaggrin compose 80-90% of the mass of epidermis (Burns et al. 2010). In the terminal differentiation of granular cells profilaggrin is proteolytically cleaved into filaggrin peptides and profilaggrin N-terminal domain (Presland et al. 1997). Filaggrin monomers bind to intermediate filaments (e.g. keratins 1 and 10) forming tight bundles in the outermost stratum corneum. This results in collapse and flattening of the keratinocytes (cornification). (Manabe et al. 1991) Cornification can also take place without the presence of filaggrin but the pattern is abnormal (R. Gruber et al. 2011). Filaggrin has also a role in the formation of cellular envelope (lipid-protein complex) which prevents water loss and the entry of harmful pathogens and allergens (Candi et al. 2005). On the surface of the skin filaggrin breaks down releasing its amino acids which are important for the hydration and barrier function of the skin (Candi et al. 2005; Rawlings & C. R. Harding 2004). The antimicrobial effect of the lipids has been documented (Miajlovic et al. 2010). The amino acids also participate in the maintenance of the skin pH gradient which is important for e.g. enzymes of ceramide metabolism (Jungersted et al. 2010; Fluhr et al. 2010). It has also been suggested that the amino acids may contribute to the UVB protection of the skin (Gilmour et al. 1993; McLoone et al. 2005; Mildner et al. 2010). Genetic and histologic studies have linked mutations in filaggrin to eczema and ichthyosis (Cookson et al. 2001; Irvine 2007; Palmer et al. 2006; Fleckman et al. 1985; Nirunsuksiri et al. 1995; Sybert et al. 1985)

Lamellated granules contain glycolipids that prevent water evaporation from the skin surface (Marieb & Hoehn 2006). The glycolipid spreads into the extracellular space and covers the outer surface of the cells and the intercellular spaces which

functions to prevent the invasion of foreign material into the skin (Marieb & Hoehn 2006; Morita & Miyachi 2003). Tight junctions are located in the stratum granulosum where they contribute to the barrier function of the skin (Morita & Miyachi 2003).

Stratum lucidum

The stratum lucidum is a thin, stripe-like and transparent layer above stratum granulosum (Marieb & Hoehn 2006). This layer can be observed only in the thickest patches of skin such as in palms and soles. This layer contains layers of flat, dead keratinocytes. In this layer or stratum corneum, above the stratum lucidum, the secretion of keratohyaline granules adhere to the keratin filaments causing a parallel organization of keratinocytes (Marieb & Hoehn 2006).

Stratum corneum

Formation of stratum corneum is the end product of the differentiation of keratinocytes and the keratinization process in the epidermis (Kurasawa et al. 2011). The stratum corneum contains 20–30 cell layers forming 2/3 of the total thickness of the epidermis (Marieb & Hoehn 2006). The stratum corneum is a very important part of the barrier function of the skin (Kirschner & Brandner 2012). Keratin and thickened plasma membranes of cells protect the skin from mechanical forces and glycolipids which makes skin waterproof (Young et al. 2006). The dead keratinocytes of this layer are called corneocytes. They are attached to each other *via* corneodesmosomes and the cross links formed by differentiation associated proteins (Morita & Miyachi 2003). Lipids fill the intercellular spaces around them (Kirschner et al. 2012). Inside the corneocytes, disulfide bonds connect the keratin filaments and keratohyaline granules to form a structure called a cornified envelope (Bragulla & Homberger 2009; Morita & Miyachi 2003). Millions of dead keratinocytes are lost every day and the skin regenerates every 25-45 days (Marieb & Hoehn 2006). This regeneration is accelerated in some diseases such as psoriasis (Burns et al 2010). The human skin is capable to accelerate the rate of regeneration in situations like inflammation or injury (Bolognia et al. 2008).

TEXT BOX 2.

Cytoskeleton:

The cytoskeleton is a network of filaments that maintain its shape and gives the cell its mechanical strength and drives and guides the movements of the cell. In the cytoskeleton, there are three different types of filaments. The thinnest fibers are actin filaments. These are extremely abundant in muscle cells where they have a role in cell contractions. The thickest filaments are named microtubules. They are tube-shaped structures which are often seen in the mitotic cells where they pull the duplicated chromosomes into two daughter cells. The third type of filament is called the intermediate filament. These filaments fortify the cells to withstand mechanical forces. (Alberts et al. 2003)

2.2 Cell junctions in epidermis

Keratinocytes in the epidermis are connected to each other by intercellular junctions. The most important junctions in the human epidermis are tight junctions, adherens junctions and desmosomes.

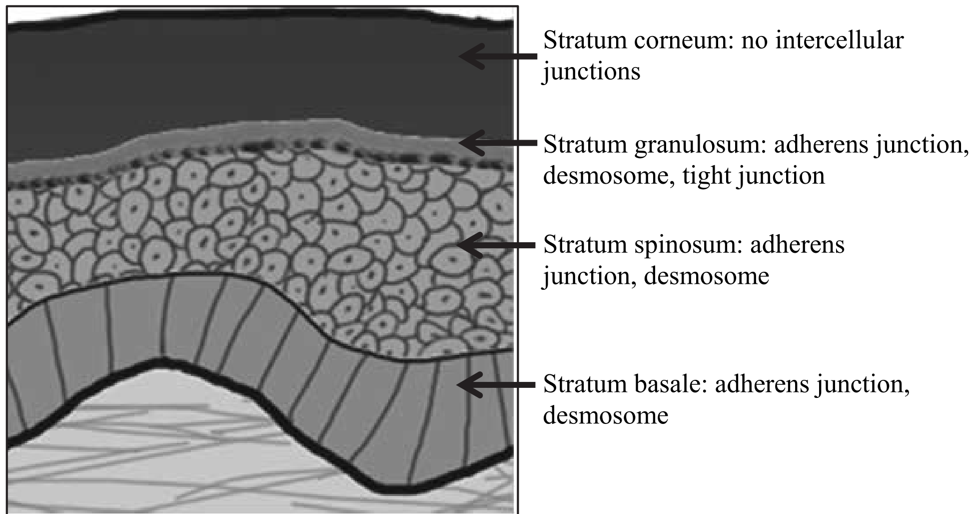

Figure 3 Cell junctions in different epidermal layers.

2.2.1 Tight junctions in the epidermis

In the granular layer, keratinocytes are surrounded by continuous tight junctions (Pummi et al. 2001; Morita & Miyachi 2003; Brandner et al. 2002). The presence of a dense network of continuous strands resembling tight junctions in the granular layer of the human epidermis were discovered by ultrastructural analysis (Schlüter et al. 2004). This suggested that tight junctions are important in the formation of a seal in the epidermal structure (Schlüter et al. 2004). When studied with an electron microscope, an apical region of two adjacent membranes between polarized epithelial cell areas are visible (Farkas et al. 2012). (Figure 3)

Many different mechanisms may explain how tight junctions arise in the granular layer (Michels et al. 2009). Keratin layers may give the initiative signal or tight junction formation can be inhibited by an overlying viable cell layer in the lower parts of the epidermis (Michels et al. 2009). On the other hand, the changes in the calcium level, in the epidermis, may have a role in the formation of tight junctions since, in the cell cultures, tight junctions are formed when the local calcium concentration is increased. At the same time, the permeability barrier is improved which emphasizes the role of tight junctions as a barrier (Yuki et al. 2007).

2.2.2 Adherens junctions in the epidermis

Adherens junctions are present in every living layer of the epidermis (S. Burge 1994). When studied with an electron microscope, an adherens junction resembles a narrowing of the intercellular space between adjacent keratinocytes (Ishiko et al. 2003). Desmosome junctions are often located near the adherens junctions in the human epidermis which suggests a functional relationship (Haftek et al. 1996). The maturation of adherens junctions depends on the presence of functional desmosomes (Desai et al. 2009). Betacatenin and vinculin are typical proteins for epidermal adherens junctions (Haftek et al. 1996). Actin and E-cadherin are not specific to adherens junctions but they are co-expressed in the human epidermal adherens junction unlike in other intercellular junctions (Haftek et al. 1996).

2.2.3 Desmosomes in the epidermis

Desmosomes are present in all living layers of epidermis (S. Burge 1994; D. Garrod & Chidgey 2008; K. J. Green & J. C. Jones 1996). They are the main cell junction that connects the keratinocytes with each other and they provide the epidermis mechanical strength (Foggia & Hovnanian 2004; Alberts et al. 2003). Desmosomes were the first junctions recognized by electron microscope analyses since they have a distinctive ultrastructural appearance (Hibbs & Clark 1959). In the human epidermis, desmosomal components, plakoglobin and plakophilin 1, are located in the outer plaque of the desmosome structure (Ishiko et al. 2003). E-cadherin and betacatenin are visible in areas between desmosomes, at sites where close membrane associations can take place (Ishiko et al. 2003). It is also known that the presence of adherens junction is needed for the formation of desmosomes (Desai et al. 2009). Therefore, a functional connection between adherens junctions and desmosomes might be possible.

2.3 Tight junctions

2.3.1 Functions of tight junctions

Tight junctions inhibit the movement of water-soluble molecules between the cells and maintain the polarity of the epithelial cell (Alberts et al. 2003). These junctions participate in tumor suppression, the regulation of gene transcription and cell proliferation (Farkas et al. 2012). A study using reconstructed human epidermis shows that when the tight junction barrier is compromised, epidermal homeostasis is lost and both hyperproliferation and precocious differentiation can be seen (Kurasawa et al. 2011). Calcium influx was also increased suggesting that tight junction may maintain the epidermal homeostasis by controlling the epidermal calcium gradient (Kurasawa et al. 2011).

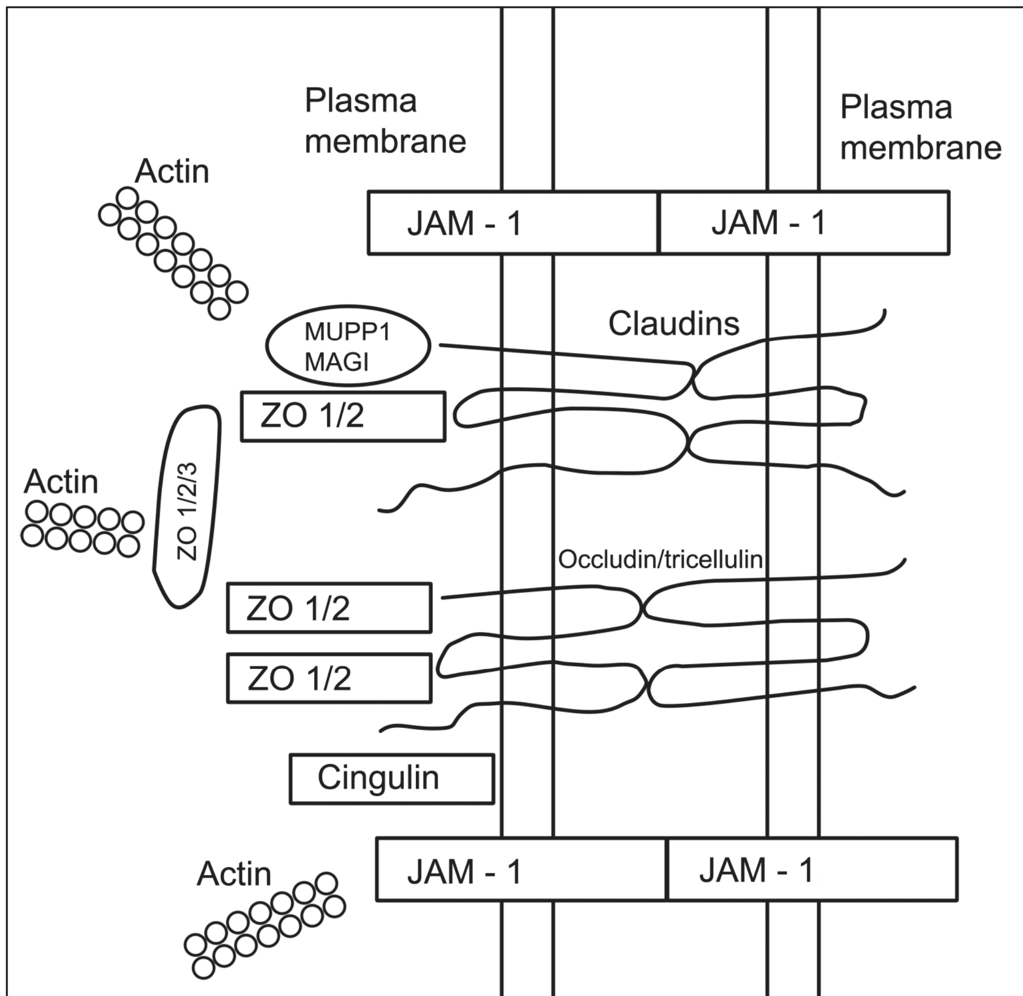

Figure 4 Schematic presentation of tight junction. Modified from (Niessen 2007).

2.3.2 The structural components of tight junctions

Tight junctions consist of integral membrane proteins, such as occludin and the Claudin family, which form permeability barriers (Schneeberger & Lynch 2004). Other key transmembrane proteins are junctional adhesion molecule-A (JAM-A) and other CTX-family members (*e.g.* coxsackie and adenovirus receptor (CAR)) (Farkas et al. 2012). Transmembrane proteins are attached to scaffolding components such as ZO proteins which are linked to actin cytoskeleton (Farkas et al. 2012). The scaffolding components also isolate regulatory proteins (Farkas et al. 2012). (Figure 4)

In the epidermis, the most abundant membrane proteins, in tight junctions, are the claudin family proteins, claudin-1 and claudin-4, which are expressed widely (Brandner et al. 2002; Morita & Miyachi 2003). Transmembrane proteins, occludin and the intracellular plaque protein ZO-1, are normally located on the granular layer

(Pummi et al. 2001; Brandner et al. 2002). The intracellular plaque protein, ZO-1, connects the integral proteins to the intracellular actin filament cytoskeleton (Schneeberger & Lynch 2004). The plaque molecules mediate signaling messages to the cytoplasmic proteins (Schneeberger & Lynch 2004). A diverse group of intracellular or nuclear proteins (*e.g.* regulation proteins, tumor suppressors, transcriptional or post-transcriptional factors) are also part of the tight junction structure (Schneeberger & Lynch 2004). (Figure 4)

2.3.3 The regulation of tight junctions

The regulation of tight junctions has not been studied in detail. However, some aspects are known. Each class of tight junction proteins is able to regulate other tight junction components either directly or indirectly through mediators (Farkas et al. 2012). Moreover, tight junctions have an important role in regulating epithelial homeostasis such as proliferation, migration and differentiation (Farkas et al. 2012). Several single pass transmembrane tight junction proteins that control epithelial homeostasis through signaling have been described. These proteins can be divided into two categories. The first group in the category is immunoglobulin superfamily (IgSF) such as JAMs and CAR. The second group consists of non-IgSF proteins such as crumbs homolog 3 (CRB3). (L. A. Williams et al. 1999; Raschperger et al. 2004; Makarova et al. 2003; Bergelson et al. 1997) JAM-A is the most widely studied IgSF protein. It has been shown to contribute to the regulation of barrier function, migration and proliferation in polarized epithelial cells (Laukoetter et al. 2007; Vetrano et al. 2008; Nava et al. 2011)

The regulating role of tight junction proteins is the most prominent during tissue renewal and wound healing where signaling cascades occur (Farkas et al. 2012). In cells with immature junctions tight junction proteins such as ZO family proteins and pro-mitogenic signaling molecules translocate to the nucleus where they affect cellular proliferation (Farkas et al. 2012; Gottardi et al. 1996; Jaramillo et al. 2004). In differentiated cells, these molecules are isolated by mature intercellular junctions which inhibit proliferation (Farkas et al. 2012).

ZO-1 associated nucleic acid binding protein (ZONAB) which is regulated by ZO-1 has also been shown to have a role in the regulation of epithelial cell proliferation, differentiation and cell density (Balda et al. 2003; Lima et al. 2010; Georgiadis et al. 2010). ZONAB has been suggested to sense the density of the epithelium and participate in the regulation of the switch between proliferation and differentiation (Balda et al. 2003; Georgiadis et al. 2010; Lima et al. 2010).

Claudins have also a role in the regulation of the permeability in epithelial cells (M. Furuse et al. 2002). Furthermore, claudin-1 suppression has been shown to inhibit the localization of occludin at the cell membranes in cultured human keratinocytes (Yamamoto, Saeki, et al. 2008). Inhibition of occludin does not affect the localization of claudin-1. This suggests that claudin-1 is crucial for the gathering of occludin to the tight junctions. Inhibition of claudin-1 or occludin also suppresses the transepidermal

electrical resistance and the number of tight junctions is decreased showing that these proteins are involved in the regulation of tight junctions (Yamamoto, Saeki, et al. 2008).

Calcium is important for the formation of tight junctions. When the concentration of extracellular calcium is increased the tight junction proteins move to continuous localization at the cellular borders (Yuki et al. 2007; Pummi et al. 2001; Brandner et al. 2002). Calcium also causes functional changes in the tight junctions such as the establishment of transepidermal electrical resistance (TEER) and reduced permeability for larger molecules (Yuki et al. 2007). Atypical PKC with its binding partners Par 3 and Par 6 are also needed for the functional barrier in tight junctions (Helfrich et al. 2007). Atypical PKC is regulated by Tiam1 (Mertens et al. 2005).

Acute UV exposure has been shown to impair the barrier function of tight junctions (Yuki et al. 2011; Yamamoto, Kurasawa, et al. 2008). Moreover, chronic UV exposure results in changes in the tight junction proteins: claudin-1 is downregulated in the lower layers of epidermis meanwhile claudin-4, occludin and ZO-1 have a broader localization (Rachow et al. 2013).

Proinflammatory cytokines also have an effect on the barrier function of the tight junctions. In the first 24h after the assembly of tight junction TNF-alpha and interleukin-1-beta improve the TEER but later TEER resistance is decreased. In fully developed tight junctions only TNF-alpha improves TEER. (Kirschner et al. 2009) IL-4 has also been shown to increase TEER in keratinocytes (De Benedetto et al. 2011).

2.3.4 Mice models demonstrating roles of tight junction proteins

Mouse models support the critical barrier function of tight junctions in the epidermis (M. Furuse et al. 2002). Tight junctions have a significant role in the inhibition of excess evaporation. Especially, claudins are important in the maintenance of the epidermal permeability barrier. Claudin-1 deficient mouse models show an increase of water evaporation through the epidermis and these mice do not survive past one day (M. Furuse et al. 2002).

A transgenic mouse model, overexpressing claudin-6, showed defects in tight junctions and stratum corneum. These mice died within 2 days after birth. They had an altered permeability barrier of the skin which was indicated by increased transepidermal water loss. (Turksen & Troy 2002). This result emphasizes the importance of a balance in expression of tight junction proteins.

Occludin deficient mouse models showed no detectable alteration in the epidermal barrier function (Saitou et al. 2000; Schulzke et al. 2005) but these mice, however, had alterations in transport functions especially in the gastric mucosa. This suggests that occludin may have a role in the cellular differentiation process and thereby its deficiency impairs the function of mucosal cells (Schulzke et al. 2005). These results

show that the presence of occludin is not crucial for the barrier function of epidermis. However, loss of occludin has consequences in other tissues.

E-cadherin may have an important role in ensuring the correct localization of the key tight junction components. The permeability of the mouse skin (*e.g.* water evaporation) increased when the expression of E-cadherin was inhibited and the mice experienced perinatal death (Tunggal et al. 2005).

2.3.5 Cell culture models and regulation of tight junctions

Cell culture models offer a useful tool to investigate the regulation of tight junctions at a cellular level. In these cultures, the expression of genes of interest can be deleted or altered.

Occludin was discovered to mediate cytokine (TNF alpha and interferon gamma) induced regulation of tight junctions in a MDCK cell line (a cell line derived from kidney cells of an adult female cocker spaniel). The knockdown of occludin decreased the sensitivity to these cytokines and occludin-overexpression increased the sensitivity to cytokines (Van Itallie et al. 2010). This highlights the importance of occludin in the formation of functional tight junctions, however, its presence is not crucial for proper barrier function in the epidermis.

In the human primary keratinocyte cultures, somatostatin has a role in the regulation of tight junctions (Vockel et al. 2010). The somatostatin receptor (subtype 3) and tight junction component, MUPP1, interact with high extracellular calcium concentration (Vockel et al. 2010). The permeability of tight junctions was also studied and the results showed that somatostatin has a role in the regulation of permeability (Vockel et al. 2010). This regulation occurs through somatostatin receptor (subtype 3) or MUPP1 (Vockel et al. 2010). Somatostatin elevates the expression of a tight junction protein claudin-4 (Vockel et al. 2010).

In HaCaT –keratinocytes, it is shown that claudin-4 is phosphorylated by atypical PKC during tight junction formation. The phosphorylated claudin-4 protein co-localizes with ZO-1 in tight junctions. Both the phosphorylation of claudin-4 and the formation of tight junction were dependent on the presence of atypical PKC activity in HaCaT cells which shows the complexity of the regulation of tight junctions (Aono & Hirai 2008).

2.3.6 The clinical significance of tight junctions

Mutations in claudin-16 are linked to familial autosomal and recessive renal disorder leading to hypomagnesemia with hypercalciuria and nephrocalcinosis (D. Simon et al. 1999; Weber et al. 2001; Konrad et al. 2008). Claudin-19 mutations lead to similar renal disorders. In addition, retinal abnormalities associate with claudin-19 mutations (Konrad et al. 2006). Claudin-14 mutations lead to an autosomal and recessive deafness due to impaired cochlear hair cells (Wilcox et al. 2001). Claudins -14, -16, -

19 are not expressed in the skin which explains the absence of skin symptoms (Krause et al. 2008). Mutations in ZO-2 cause hypercholanemia (Carlton et al. 2003). A rare congenital disease, Nance-Horan syndrome, which leads to impaired neurological development and cataracts, results from gene mutations which encodes the Nance-Horan syndrome protein (Burdon et al. 2003). This protein is co-localized with the tight junction protein, ZO-1, in intercellular junctions, however, mutations in Nance-Horan proteins have not been reported to cause skin symptoms (Sharma et al. 2006; Burdon et al. 2003). The absence of skin symptoms suggests that there may be compensatory mechanisms present in the skin.

In contrast, mutations in claudin-1 affect the phenotype of skin. A mutation leading to a premature stop codon in the claudin-1 gene and thus a total absence of claudin-1 protein leads to dry ichthyotic skin (Hadj-Rabia et al. 2004). The organization and morphology of the tight junctions of the epidermis were normal. The stratum corneum, however, showed a thicker and a more compact structure when compared to normal epidermis. Impaired ionic selectivity was suggested to explain the findings of the skin. The patients with this mutation also had neonatal sclerosing cholangitis (Hadj-Rabia et al. 2004).

Skin, with atopic dermatitis, shows abnormalities in the expression of claudin-1. A study, where the expression and function of claudin-1 was investigated, showed that there is a reduced expression of claudin-1 in the skin of persons with atopic dermatitis (De Benedetto et al. 2011). The levels of claudin-23 were also lower. These results were supported by measurements of mRNA levels and protein levels. Impairment of bioelectrical barrier was also seen in the epidermis of patients with atopic dermatitis. *In vitro*, the functions of tight junctions were impaired but the differentiation rate was enhanced when the expression of claudin-1 was inhibited (De Benedetto et al. 2011). These results emphasize the crucial role of claudin-1 in the maintenance of permeability barrier in the skin.

Psoriatic skin shows differences in the localization of certain tight junction proteins. In psoriatic skin plaques, the localization of occludin and ZO-1 spreads from the granular layer to the middle of stratum spinosum (S. Peltonen et al. 2007). This suggests that tight junctions may have a role in the phenotypic abdnormalities of psoriatic skin. In the perilesional skin area or in healed lesions, the localization of these tight junction proteins did not show any differences when compared to normal control epidermis (S. Peltonen et al. 2007).

2.4 Adherens junctions

2.4.1 Functions of adherens junctions

Adherens junctions function to aid the tissue withstand mechanical stress by forming supporting networks (Alberts et al. 2003). Moreover, they regulate cell junctions, differentiation and morphology (D’Souza-Schorey 2005). Adherens junctions

participate in cell signaling and they have an important role in the regulation of keratinocyte growth and in modification of tissues and organs during the embryonic development (Tsukita et al. 1992). Calcium is needed for adherens junctions to function properly and the lack of calcium ions weakens the functions of adherens junctions (Tsukita et al. 1992).

Adherens junctions are important in the formation and maintenance of other intercellular junctions (Niessen 2007). Adherens junctions are linked to the formation of tight junctions in the epidermis (Niessen 2007). Furthermore, there are interactions between key tight junction and adherens junction components (Niessen 2007). One example of these interactions is the connection between cadherins and ZO-1 in mouse fibroblasts (*in vitro*) (Itoh et al. 1997). When cadherin-containing intercellular junctions are assembled, the ZO-1 protein is synthesized near the newly forming junctions (Itoh et al. 1997). This provides the initial structural foundation for tight junctions (Itoh et al. 1997).

2.4.2 Components of adherens junctions

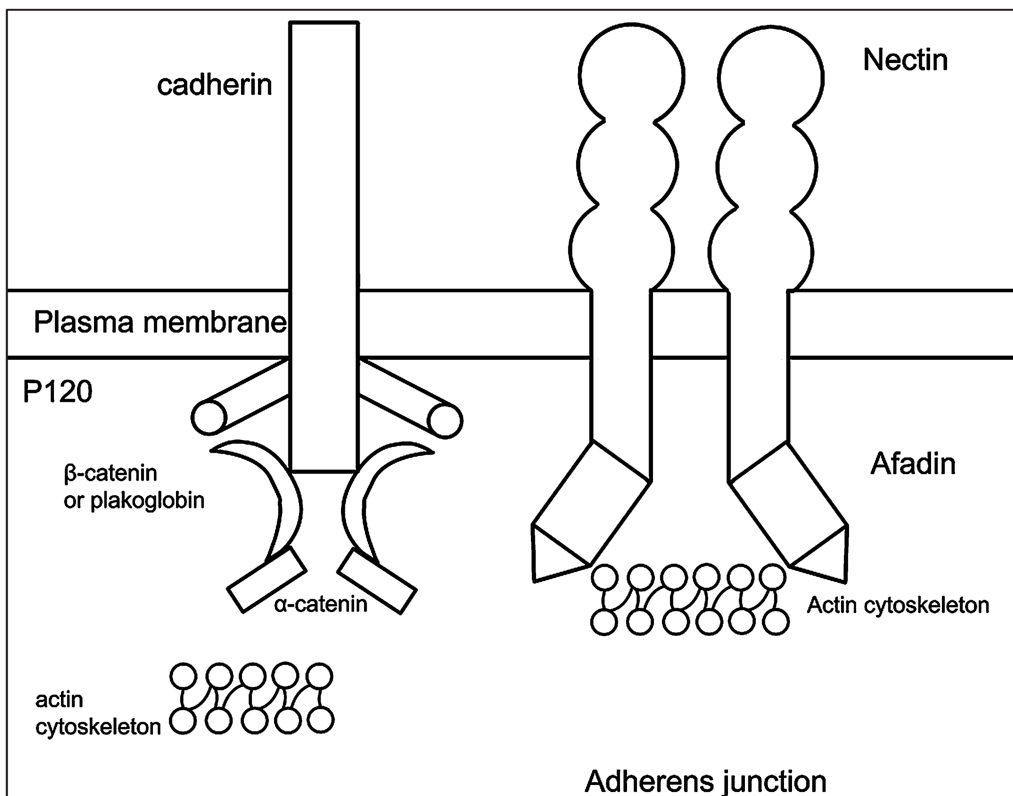

Figure 5 Adherens junction. Modified from (Niessen 2007; Burns et al. 2010).

Adherens junctions are attached to actin filaments inside the cell (Niessen 2007). Adherens junction cadherin molecules are attached *via* linker proteins (*e.g.* alpha-actinin and vinculin) to actin filaments (Lowe & A. Stevens 1997; Alberts et al. 2003). Adherens junctions have two key components: nectin-afadin complexes and classic cadherin complexes (Niessen 2007). Nectin is a transmembrane protein which is attached to the actin-binding protein, afadin, inside the cell, providing a structure that is linked to the actin cytoskeleton (Niessen 2007; D'Souza-Schorey 2005). Two different cadherins are present in the epidermis: E-cadherin and P-cadherin (P. J. Jensen et al. 1997). P-cadherin is only present in the basal cell layer but E-cadherin is expressed in all epidermal layers (P. J. Jensen et al. 1997). The E-cadherin-catenin complex is attached to the components of cytoskeleton and the regulator and signaling molecules form adherens junctions (Baum & Georgiou 2011). E-cadherin binds to betacatenin which in turn is attached to alphacatenin which have interactions with the proteins binding to actin (Bershadsky 2004; Yonemura et al. 2010). Those proteins binding to alphacatenin are vinculin, zyxin and afadin (D'Souza-Schorey 2005). (Figure 5)

2.4.3 Regulation of adherens junctions

Adherens junctions detect the changes in cell junctions and in mechanical stress (Baum & Georgiou 2011). The regulation of adherens junctions occurs either locally, by cytoskeletal dynamics, proteolytic cleavage or phosphorylation of the key components or at a higher level by transcriptional regulation (Niessen 2007).

2.4.4 The clinical significance of adherens junctions

E-cadherin is an additional target of IgG autoantibodies in pemphigus foliaceus and pemphigus vulgaris, which are blistering skin diseases in humans. In pemphigus foliaceus, the autoantibodies target desmoglein-1 and in pemphigus vulgaris autoantibodies target desmoglein-3 (Evangelista et al. 2008).

Mouse models show both *in vivo* and *in vitro* that the level of cadherins is important while the type of cadherin (P or E-cadherin) is not (Tinkle et al. 2008). Another cadherin type was able to substitute the absence of one cadherin type. When the both cadherins were absent, it was discovered that the epidermal barrier function and the regulation of intercellular junctions was impaired (Tinkle et al. 2008).

2.5 Desmosomes

2.5.1 The functions of desmosomes

Desmosomes provide epithelium mechanical strength (Alberts et al. 2003). Keratinocytes are linked mainly through desmosomes which make this junction type important in the epidermis (Foggia & Hovnanian 2004). Desmosomal components participate in the development and remodeling of tissues (Thomason et al. 2010).

2.5.2 The components of desmosomes

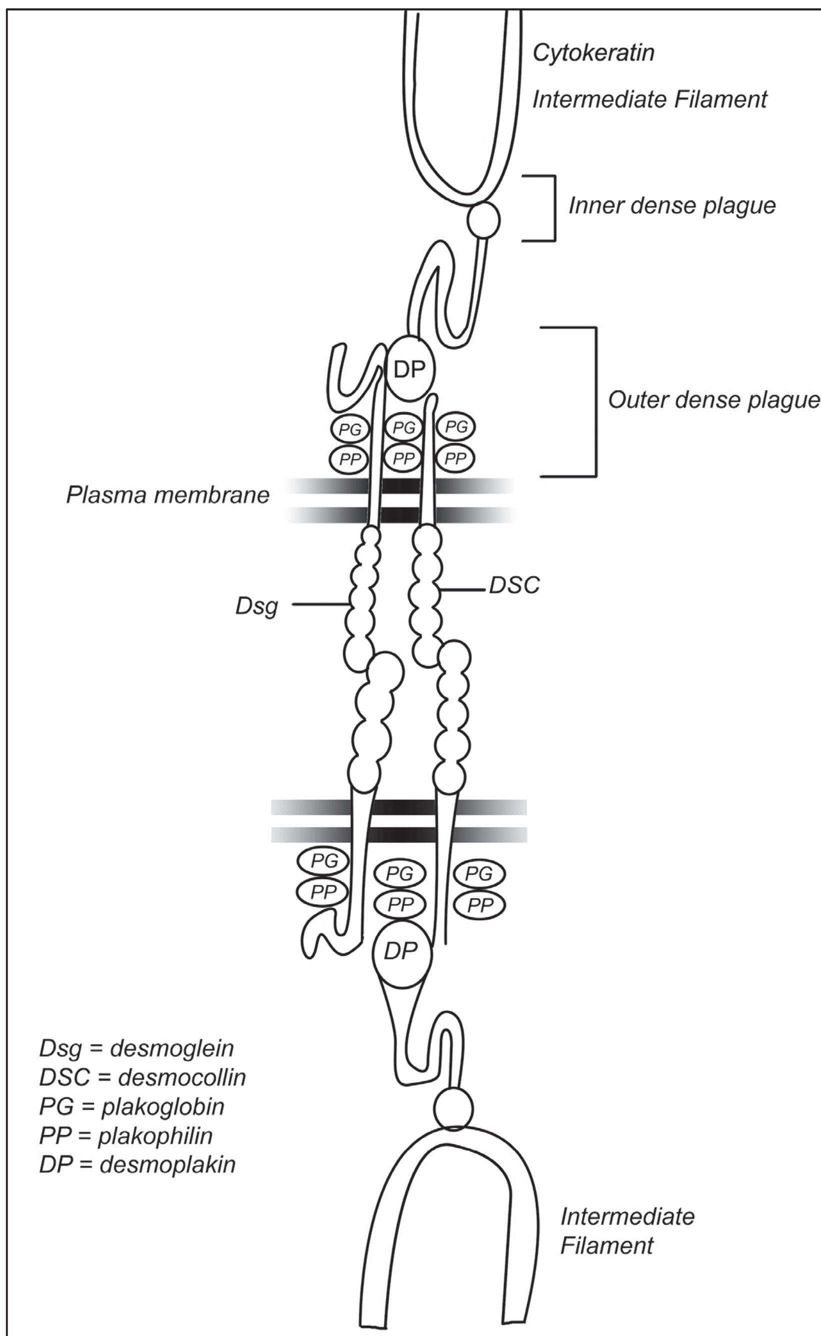

Figure 6 The structure of desmosome junction. Modified from (D. Garrod & Chidgey 2008).

Desmosomes have three key protein components: desmosomal cadherins (desmoglein and desmocollin); the plakin family (desmoplakin and to some extent

envoplakin, plectin and periplakin); and armadillo proteins (plakoglobin and plakophilin) (Thomason et al. 2010). The intracellular plaques of adjacent cells are linked together *via* transmembrane proteins desmoglein and desmocollin (Lowe & A. Stevens 1997; Alberts et al. 2003; Bektas & Rubenstein 2009). The cytoplasmic part of desmoglein binds to plakoglobin which in turn is bound to desmoplakin (Bektas & Rubenstein 2009). The intracellular plaque of desmosomes consist of several linking proteins such as desmoplakin, plakophilin and plakoglobin (Lowe & A. Stevens 1997; Hakuno et al. 2000). Plakophilins are bound to desmoplakin, plakoglobin, desmoglein and desmocollin (Bektas & Rubenstein 2009). Desmoplakin connects the desmosome to the keratin cytoskeleton (Bektas & Rubenstein 2009). (Figure 6)

The cadherins are calcium-dependent cellular adhesion molecules (Kovács et al. 2004). This is important in the pathogenesis of many skin diseases (*e.g.* Darier´s disease and Hailey-Hailey disease) (Kovács et al. 2004). Desmosomal adhesion requires about one micromolar concentration of extracellular calcium (Nagar et al. 1996). The calcium-dependent desmosomal formation takes place only during special physiological events, *in vivo,* such as formation or modification of a tissue (*e.g.* embryonal development and wound healing) (D. Garrod 2010).

Four different desmogleins and three different desmocollins are known (R. L. D. Vitae et al. 2004). The expression of desmoglein-2 is low in the epidermis and it is restricted to the basal layer. The expression of desmoglein-3 extends from the basal layer to the stratum spinosum. Desmoglein-1 and -4 are expressed during the cell differentiation (Brennan et al. 2010). A mouse model shows that desmoglein-2 upregulation in the upper dermis can compensate for the loss of function of desmoglein-1. This may have therapeutic applications in the treatment of pemphigus foliaceus in the future (Brennan et al. 2010).

2.5.3 Experimental models and formation of desmosomes

Cultured mouse epidermal cells show that desmosomal components (desmoplakin) are formed in the cytoplasm of the cells before the formation of the desmosome junction. These components are later reorganized with bundles of intermediate filaments to form desmosome junction (J. C. Jones & Goldman 1985).

Another cell study using mouse keratinocytes shows that bundles of intermediate filaments (containing keratin) are accumulated near the center of the cell surrounded by bundles of microfilaments in low calcium concentration. After an increase in the extracellular calcium concentration of the medium, the desmosomes begin to form and the filaments are redistributed to be part of the new desmosome. The intermediate filament-desmoplakin structures are often located near adherens junction associated with microfilament bundles. This close connection is still maintained after the desmosomes are completely formed (K. J. Green et al. 1987).

In a later study (both mouse and human keratinocyte cultures), desmoplakin molecules aggregated in keratinocytes cultured in low calcium concentrations. These aggregates were interpreted as remnants of transportation vesicles. There are many different desmosomal proteins synthesized in the keratinocytes when cultured in low calcium concentration (*e.g.* desmoplakins I and II, desmoglein and plakoglobin). These proteins form directly desmosomes without precursor aggregates containing intermediate filaments (Duden & Franke 1988).

Desmoglein-2 and -3 were studied in different cell lines of keratinocytes. It was discovered that siRNA mediated silencing of desmoglein-3 had more serious consequences to cellular cohesion than silencing of desmoglein-2. If desmoglein-3 was absent, the cells lost their cohesion but if desmoglein-2 was absent the cells were able to maintain the cohesion unless they were sheared (Hartlieb et al. 2013). This shows the different features of desmosomal proteins belonging to the same protein family.

2.5.4 Desmosomes in Hailey-Hailey disease and Darier´s disease

Desmosomes play a crucial role in the events leading to acantholysis in Darier´s disease and Hailey-Hailey disease. Studies show that in the skin of patients with Darier´s disease or Hailey-Hailey disease, there are variances in desmosomal properties.

Immunolabeling of the Darier disease or Hailey-Hailey disease skin samples for desmosomal proteins (desmoplakin 1 and 2, desmoglein-1, desmocollin 2 and 3) showed that that the expression of these proteins was reduced in the basal areas of the lesional sites (S. M. Burge & D. R. Garrod 1991). In the acantholytic keratinocytes, the staining was bright and diffuse (S. M. Burge & D. R. Garrod 1991). Focal intracellular staining was found in acantholytic Hailey-Hailey disease keratinocytes. Some Darier disease keratinocytes also showed similar staining. This suggests that acantholysis may antecede the intracellular accumulation of desmosomal proteins. The localization of the desmosomal proteins in the perilesional area was similar to that in the normal skin (S. M. Burge & D. R. Garrod 1991).

Three Darier patient skin samples and four Hailey-Hailey patient skin samples were analysed to elucidate the distribution of desmosomal and adherens junction proteins (Hakuno et al. 2000). It was discovered that there is a difference in staining patterns between the intracellular and extracellular domains of desmosomal cadherins and E-cadherin in the acantholytic cells. This was the case in both Hailey-Hailey disease and Darier’s disease. The same difference was not found from pemphigus vulgaris and pemphigus foliaceus samples which were used as control samples. In acantholytic Hailey-Hailey disease and Darier’s disease cells, a diffuse cytoplasmic staining was observed against attachment plaque proteins and intracellular epitopes of desmosomal cadherins. Considerably reduced staining against extracellular epitopes of desmogleins was detected. Furthermore, an intracellular epitope against E-cadherin was detected but the extracellular epitope against E-cadherin was lost (Hakuno et al. 2000).

Using epidermal cell lines, in which the expression of SERCA2 (a sarco/endoplasmic reticulum Ca^{2+}-ATPase) is silenced with pharmacological or siRNA methods, the keratinocytes showed desmosomal disruption and impaired adhesive strength. SERCA2-deficient keratinocytes showed up to 60% reduction in the border translocation of desmoplakin (Hobbs et al. 2011). Membrane translocation of PKC alpha, which is regulator of desmosome-intermediate filament assembly, was also impaired up to 70%. Exogenous activation of PKC alpha returned the reduced levels of desmosomes and cell adhesion back to levels comparable to normal. This suggests that SERCA2 is a regulator of PKC alpha (Hobbs et al. 2011). The impaired regulation of desmosome-intermediate filament assembly may thus play a role in the pathogenesis of Darier´s disease.

2.5.5 The clinical significance of desmosomes

The mutations in genes encoding desmosomal components cause various symptoms in humans. The symptoms include woolly hair, acantholysis and blistering of the skin, fragility of the skin, hyperkeratosis, alopecia, palmoplantar keratoderma and teeth enamel abnormalities (Mahoney et al. 2010; Norgett et al. 2000; Whittock et al. 2002). Moreover, desmosomal diseases consist of a group of ventriculopathies of heart potentially leading to sudden death due ventricular cardiomyopathy (Mahoney et al. 2010). None of the mutations described affect embryonic development. The symptoms are present in different combinations and the homozygosity with desmoplakin mutations leads to the most severe symptoms.

Some of the skin biopsies of patients with desmoplakin mutations show abnormalities in keratin. The skin biopsies revealed perinuclear keratin in the keratinocytes of the suprabasal layers. The reason for this finding could be the collapse of the intermediate filament network. These results suggest that desmoplakin has a role in the attachment of the intermediate filaments to the desmosomes (Norgett et al. 2000). Acantholysis in all skin layers with focal detachment of desmosomes into intercellular areas and perinuclear condensation in the suprabasal keratin intermediate filament network was also discovered in another study with different mutations (Whittock et al. 2002).

2.5.6 Mouse models and function of desmosomes

A desmoplakin knock-out mouse model shows that desmoplakin has an essential role in epidermal structure. These mice have a normal amount of desmosomes but the function of these desmosomes is impaired due to lack of keratin filaments. In the skin of these mice, mechanical stress causes blistering. The epidermal formation was impaired because actin reorganization and membrane sealing is abnormal. The amount of adherens junctions is also reduced (Vasioukhin et al. 2001).

Mice lacking desmocollin-1 have impaired epidermal barrier function with acantholysis of the granular layer. Their epidermis is more fragile and shows hyperproliferation and abnormal differentiation which is indicated by overexpression

of keratins 6 and 16. These results suggest that desmocollin-1 has an essential role in the epidermal barrier function, cell adhesion and differentiation (Chidgey et al. 2001).

In a mouse model in which the desmoglein-3 gene was target disrupted, the mice were born healthy but by the 18th day of life they were runted when compared to control mice (P. J. Koch et al. 1997). The runted mice also showed hair loss when weaned. It was discovered that the mice had oropharyngeal erosions and typical pemphigus vulgaris histology in their oropharyngeal biopsies. Electron microscopy revealed separation of desmosomes. Suprabasilar acantholysis and crusting existed on traumatized skin areas. These results show the important role of desmoglein-3 in the cellular adhesion of epidermis (P. J. Koch et al. 1997).

p63-deficient mouse keratinocytes had reduced protein and mRNA levels of desmocollin- 3, desmoplakin and desmoglein-1 suggesting that p63 regulates the expression of these desmosomal proteins (Ferone et al. 2012).

2.5.7 Pemphigus and desmosomal components

Pemphigus is a group of autoimmune diseases affecting skin and mucous membranes (Bolognia et al. 2008). Histologically, it is characterized by intraepidermal blistering and loss of cellular adhesion in keratinocytes. Circulating IgG autoantibodies attached to keratinocyte-rich surfaces can be detected. In pemphigus vulgaris, autoantibodies are against desmoglein-3 and pemphigus foliaceus autoantibodies are against desmoglein-1. The prevalence of these diseases is equal between women and men. The average age when the disease normally occurs is between 50 to 60 years old. The incidence of pemphigus vulgaris in Finland is 0.76 cases per million per year (Hietanen & Salo 1982). Pemphigus foliaceus is twice as common than pemphigus vulgaris (Hietanen & Salo 1982).

Blisters in the pemphigus vulgaris are located in the basal and lower suprabasal layers of epidermis, since desmoglein-3 is localized in the lower layers of epidermis (Amagai et al. 1996). Patients with pemphigus vulgaris often develop painful erosions in oral mucosa (Bolognia et al. 2008). More than half of the patients also have easily ruptured blisters and wide cutaneous erosions (Bolognia et al. 2008). Epidermal cohesion is lost and patients easily have the upper layers of epidermis moved laterally after a mild pressure (Nikolsky sign) (Bolognia et al. 2008). The intact bulla can also be moved using a light pressure (Asboe-Hansen sign) (Bolognia et al. 2008). Pemphigus vulgaris can be fatal without a proper treatment because a lot of fluids are lost after large areas of epidermal barrier are lost and secondary infections can occur (Bolognia et al. 2008).

2.6 Calcium signaling

2.6.1 Calcium as signaling molecule

Calcium is a crucial signaling molecule in cellular signaling. Intracellular calcium concentration is maintained at low level (about 100 nM) using different energy demanding calcium pumps (Alberts et al. 2003; Clapham 2007). The calcium gradient between the extracellular and intracellular space is 20,000-fold (Clapham 2007). Inside the cell, calcium is stored in the endoplasmic reticulum and Golgi apparatus (Clapham 2007). The calcium concentration in the endoplasmic reticulum of the epithelial cells is 400-550 µM (Miyawaki et al. 1997; M. T. Alonso et al. 1998). The calcium stores of Golgi apparatus are much smaller: 300 µM (Pinton et al. 1998). In the extracellular space, there also exists calcium (Alberts et al. 2003). Nearly all calcium signaling systems are based on short calcium impulses (Berridge et al. 2003). A traditional concept, capacitative calcium entry was previously used to explain the events during calcium signaling (Putney 2009). Capacitative calcium entry is defined such that the calcium stores and their channels and pumps are like a machine which is able to operate with calcium like a capacitator and resistor in series. After a stimulus the mechanism would release calcium and later store it again (Putney 2009). Today, the term capacitative calcium entry has been replaced with a new more accurate term, store-operated calcium entry (SOCE) (Putney 2009).

2.6.2 Intercellular calcium signaling

In addition to the intracellular calcium signaling, calcium signaling may occur between neighbor cells. In intercellular calcium signaling (also known as calcium waves), an increase in cytoplasmic calcium concentration spreads radially from the initiating cell to the neighbor cells (Leybaert & Sanderson 2012). The intercellular calcium signaling can be initiated by a stimulation of single cell, by chemical ligands or certain extracellular conditions that affect a bigger population of cells (Leybaert & Sanderson 2012). Calcium permeable receptor channels have an important role in the intracellular propagation of intercellular calcium signaling (Leybaert & Sanderson 2012). These channels include IP_3 receptor, ryanodine receptor, polycystin-2 receptor and two-pore channels (Leybaert & Sanderson 2012). Gap junctions propagate the intercellular calcium signal (Leybaert & Sanderson 2012). Gap junctions are channels between two cells connecting the cytoplasm of the adjacent cells (Leybaert & Sanderson 2012). Connexon structures (hemichannels) of adjacent cells interact to form a gap junction. Connexons are composed of six connexin proteins (Sáez et al. 2003; Perkins et al. 1997). Gap junctions can be open or close in response to extracellular signals in many cell types (Alberts et al. 2003). Gap junctions are considered to be open for calcium and second messenger IP_3 (Sáez et al. 1989). Calcium and IP_3 are both propagators of calcium signaling, however IP_3 can move more freely and quicker since it is not buffered by cytoplasmic proteins (Leybaert & Sanderson 2012). Therefore IP_3 can move longer distances. IP_3 also stimulates the release of calcium (Leybaert & Sanderson 2012).

Intercellular calcium signaling has a crucial role in coordination and synchronizing the functions of a large population of cells (Leybaert & Sanderson 2012). For instance, proliferation and differentiation of keratinocytes may be synchronized *via* intercellular calcium signaling (Tsutsumi et al. 2009). ATP-mediated extracellular calcium signaling communication is an important signaling mechanism in epithelial cells (Enomoto et al. 1994). Mechanical stress induces ATP-mediated calcium signaling in keratinocytes (Koizumi et al. 2004). Moreover, intercellular calcium signaling communication *via* gap junctions occurs in keratinocytes when the cells are stressed under mechanical forces (Tsutsumi et al. 2009). After creating mechanical stress stimuli, the calcium levels of keratinocytes were measured and it was discovered that in differentiating keratinocytes, the calcium signals are extended. Immunohistological studies revealed that gap junction proteins connexins 26 and 43 were present on the surfaces of differentiating keratinocytes. After blocking gap junctions, a significant decrease in calcium signaling occurs (Tsutsumi et al. 2009).

2.6.3 The interactions during intracellular calcium signaling

During calcium signaling, **(1)** the G-protein coupled receptor Ca (CaR) binds calcium from the extracellular space (Ambudkar 2007). **(2)** This activates phospholipase C creating inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG) from phospatidylinositol bisphosphate (Savignac et al. 2011). IP3 acts as a second messenger when binding to its receptors on the ER and Golgi apparatus (Savignac et al. 2011). **(3)** The binding causes a release of calcium from the intracellular stores into the cytoplasm (Savignac et al. 2011). **(4)** The release of intracellular calcium into the cytoplasm activates the inflow of extracellular calcium through the calcium channels of the plasma membrane (Savignac et al. 2011). **(5)** The increase of intracellular calcium activates calmodulin (a calcium binding protein) (Savignac et al. 2011). **(6)** As a consequence, a calmodulin-dependent phosphatase in cytosol, called calcineurin, is activated **(7)** and dephosphorylates N-FAT (a calcium-dependent transcription factor) (Savignac et al. 2011). **(8)** After this N-FAT moves to the nucleus and launches the transcription of the calcium-dependent genes (Savignac et al. 2011). Also other calcium-dependent transcription factors are activated (*e.g.* CREB and NFkB) (Savignac et al. 2011). **(9)** As a consequence of the elevation of cytosolic calcium level also the transportation systems that transport calcium away from the cytosol are launched in order to restore the calcium level back to the resting state (Savignac et al. 2011). **(10)** PMCA and NCX on the plasma membrane transport calcium from the cytosol to extracellular space (Savignac et al. 2011).

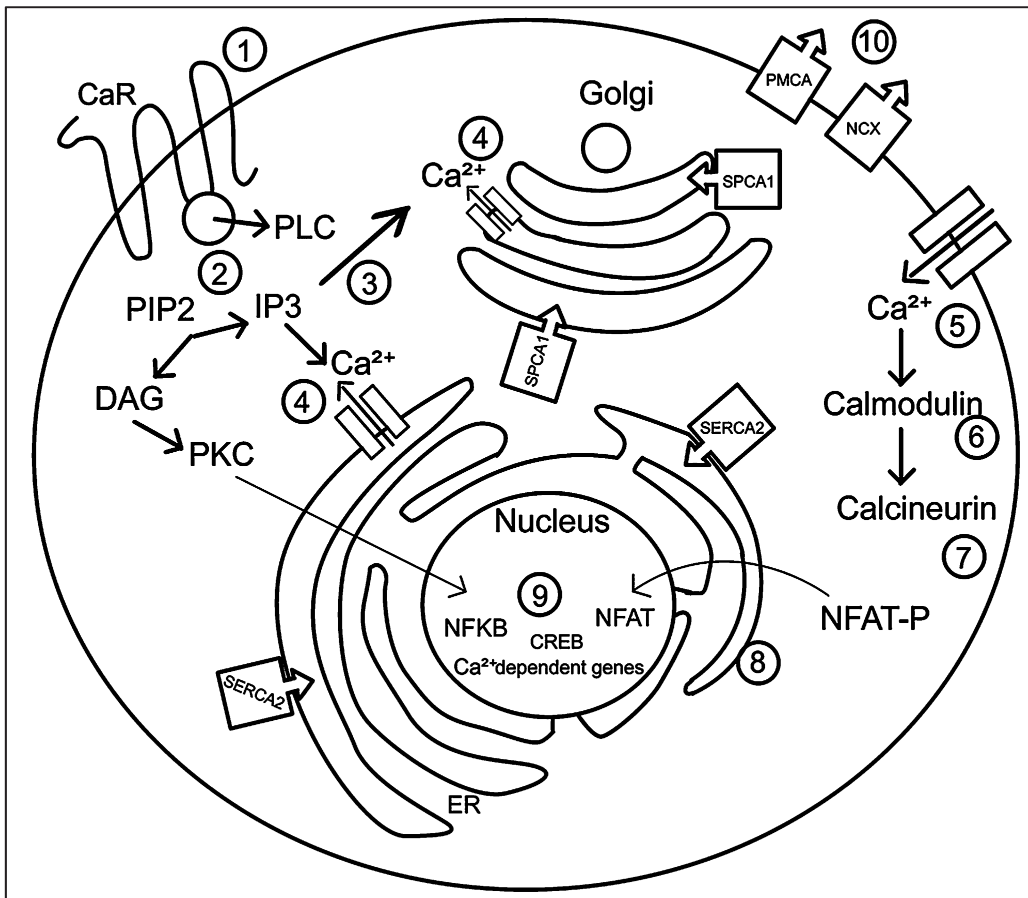

Figure 7 Calcium signaling. Modified from (Savignac et al. 2011). The numbers in the schematic represent the signaling process and are defined in the text below.

SERCA2 and SPCA1 restore the intracellular calcium stores back to normal (Savignac et al. 2011). SERCA2 is a calcium pump located on the endoplasmic reticulum which transports calcium from the cytosol to the luminal parts of endoplasmic reticulum using energy from ATP (Berridge et al. 2003; Sakuntabhai, S. Burge, et al. 1999). hSPCA1 is a Ca/Mn ATPase located on the Golgi membrane. It uses energy from ATP to transport calcium and manganese from the cytosol to the lumen of the Golgi apparatus (Z. Hu et al. 2000). (Figure 7)

2.6.4 Calcium and epidermis

Increased extracellular calcium concentration (over 0.1 mM) causes the differentiation of epithelial cells (Pillai & Bikle 1991; Elias et al. 2002). In calcium concentrations of less than 0.1 mM, the cultured keratinocytes stay in the proliferative phase. After the increase of calcium to 1.2 mM the differentiation in the culture begins (Hennings & K. A. Holbrook 1983). The increase of extracellular calcium also causes typical changes

in the morphology of epidermis (formation of desmosomes, stratification, and cornification) (Savignac et al. 2011).

The classical paradigm describing calcium and the epidermis was that in the epidermis, the calcium concentration is four times higher in surface of epidermis than in the basal layer (calcium gradient) which is considered to be important for differentiation (Z. Hu et al. 2000). The total calcium in the epidermis increases progressively when moving from the basal layer to the stratum granulosum in the epidermis. After this, the calcium level decreases when moving to the stratum corneum (Savignac et al. 2011; Menon et al. 1985). However, modern methods have increased the understanding of the epidermal calcium levels. In an electron probe microanalysis where the normal human epidermis was studied, it was detected that the calcium level in the basal layer was higher than the calcium level in the lowest level of the stratum spinosum (Leinonen et al. 2009).

2.7 Darier´s disease

2.7.1 Clinical features of Darier´s disease

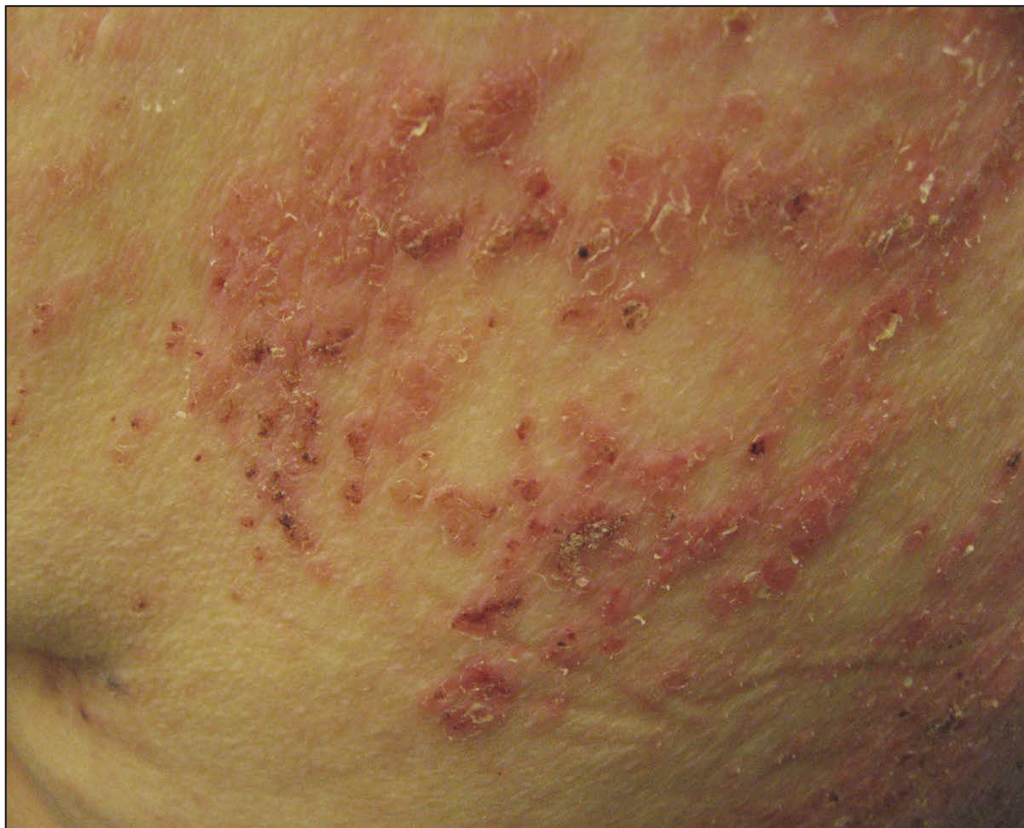

Figure 8 Skin of a Darier patient. Photo courtesy of Sirkku Peltonen

Darier´s disease (OMIM 124200) was first described independently by J. Darier (Saint- Louis, France) and by JC White (Harvard, USA) in 1889 (Darier J 1889; J. C. White 1889). The incidence is estimated to be between 1:30 000 and 1:55000 (S. M. Burge & Wilkinson 1992). This disease is inherited autosomally and dominantly (Foggia & Hovnanian 2004). Typical symptoms in Darier's disease are keratotic papules on seborrheic skin areas and flexural areas (Savignac et al. 2011). There are often secondary infections on these skin areas (Savignac et al. 2011). Finger and toe nail abnormalities are typical in Darier´s disease (Foggia & Hovnanian 2004). The nails have longitudinal white and red bands accompanied by a distal notch (Foggia & Hovnanian 2004). The palms of hands and feet are often covered with palmoplantar pits and/or keratotic papules (Foggia & Hovnanian 2004). Neuropsychiatric symptoms such as mental disorders, mental retardation, epilepsy and encephalopathy have also been described (N. J. Jacobsen et al. 1999; Ruiz-Perez et al. 1999). The symptoms appear often in teenage years and symptoms are chronic (Savignac et al. 2011). The

symptoms exacerbate during UVB-radiation, heat, abrasion and secondary infections (Savignac et al. 2011). Curative treatment does not exist but oral retinoids can be used as treatment (Savignac et al. 2011). This disease significantly impairs patient's quality of life (Savignac et al. 2011). (Figure 8)

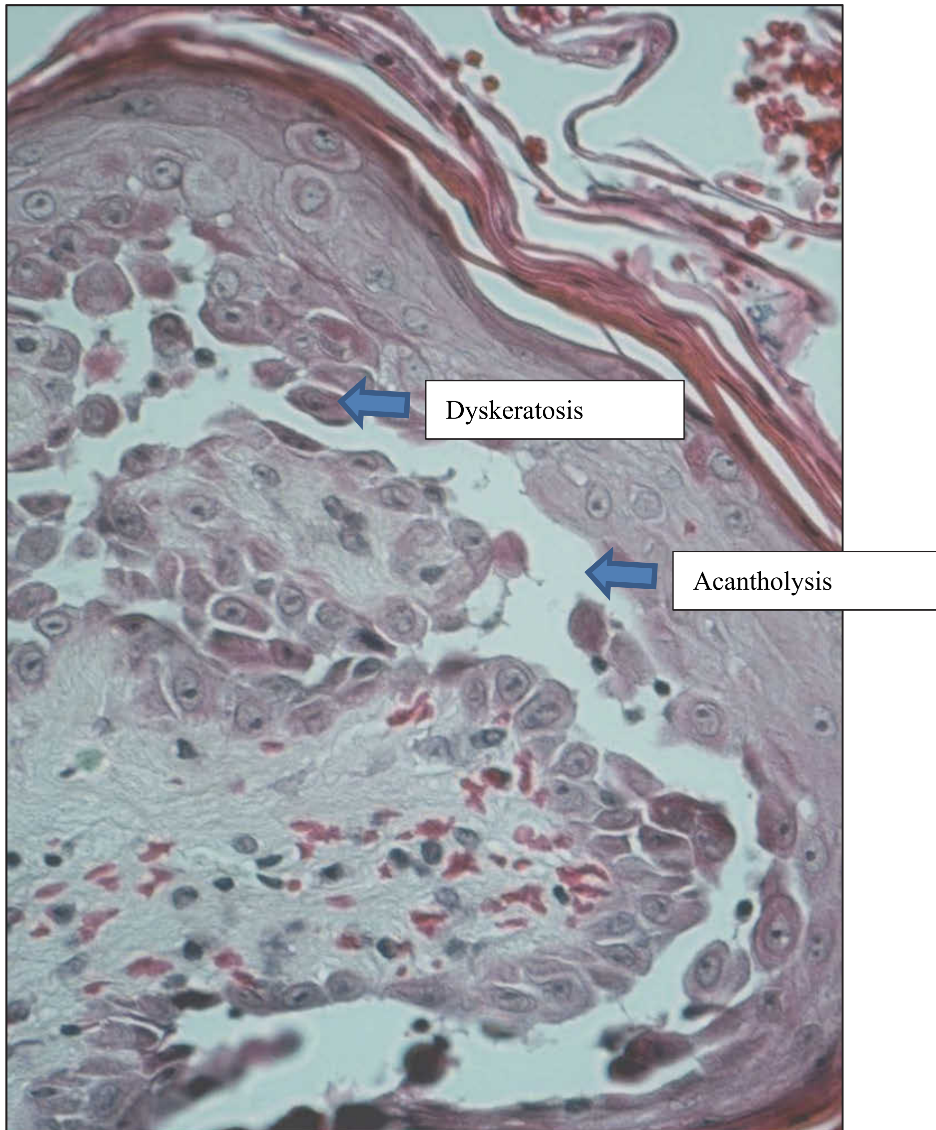

Figure 9 The histology of Darier's disease

2.7.2 The histological findings

Histologically, there is typical acantholysis (opening of cell-cell junctions) leading to suprabasal cleft formation (Savignac et al. 2011). Related to this, round, presumably apoptotic cells (corp rond) can be observed. There is also dyskeratosis (abnormal and

premature keratinization) (Savignac et al. 2011). Dyskeratosis is the most distinctive feature in Darier´s disease (Foggia & Hovnanian 2004). Electron microscopy reveals opening of desmosome junctions, perinuclear aggregation of keratin filaments and vacuolization of cytoplasm (Sakuntabhai, Ruiz-Perez, et al. 1999). (Figure 9)

2.7.3 ATP2A2

Darier´s disease is caused by mutations in ATP2A2 gene encoding SERCA2, a sarco/endoplastic reticulum calcium ATPase type 2 isoform in chromosome 12q23-24.1 (Sakuntabhai, Ruiz-Perez, et al. 1999). ATP2A2 gene is about 72 kb with 21 exons (Foggia & Hovnanian 2004). SERCA2 protein is 115 kDa in size and has three cytoplasmic domains and two calcium binding sites (Foggia & Hovnanian 2004; Spencer et al. 1991). SERCA2 pumps maintain the calcium stores of endoplasmic reticulum and create calcium oscillations into the cytosol after extracellular stimuli (Missiaen et al. 2000; Berridge et al. 2003). SERCA2 actively transports calcium back from the cytosol to the lumen of endoplasmic reticulum using the energy from the ATP hydrolysis (Foggia & Hovnanian 2004). SERCA2 has three isoforms: a, b and c. The isoforms a and b are expressed in the cytoplasm of cultured keratinocytes but in adult skin only the b isoform is predominantly expressed in the structures of epidermis (Foggia & Hovnanian 2004).

ATP2A2 gene is regulated by transcription factor Sp1 (Takagi et al. 2008). Takagi et al. (2008) have shown that the transcription factor Sp1 binds to the promoter region of ATP2A2 gene in keratinocytes *in vivo*. After siRNA inhibition of Sp1 expression, a marked reduction in the promoter activity of ATP2A2 gene was detected. The mRNA levels of ATP2A2 gene were also decreased. The results suggested that Sp1 affects the ATP2A2 gene expression (Takagi et al. 2008).

TEXT BOX 3.

Endoplasmic reticulum:

The endoplasmic reticulum (ER) forms more than a half of the cell´s intracellular membranes. It is a netlike structure consisting of interconnected tubules and sacs. Membrane-bound ribosomes are attached to the surface of ER. These ribosomes synthesize the proteins translocated into the ER. The ER is an entry point for proteins on their way to other organelles and also to endoplasmic reticulum itself. Most proteins that enter the ER are chemically modified there. These modifications include adding oligosaccharide side chains for various functions (transport signal, protection, and control). Also disulfide bonds can be formed to stabilize thew structure. The transmembrane proteins and water-soluble proteins are attached to the endoplasmic reticulum. Water-soluble proteins move through endoplasmic reticulum and are released to the lumen of the endoplasmic reticulum. Proteins are transported from one compartment to other or into other organelle using transport vesicles. Transmembrane proteins can stay in the ER or move forward into another organelle like the Golgi apparatus. (Alberts et al. 2002)

2.7.4 The mutations causing Darier´s disease

Diverse mutations in ATP2A2 gene exist in patients with Darier´s disease (Sakuntabhai, Ruiz-Perez, et al. 1999). In patients these variations in cutaneous phenotypes (atypical or severe symptoms) are linked to missense mutations (Ruiz-Perez et al. 1999). Neuropsychiatric symptoms, though were not associated with certain mutation classes (Ruiz-Perez et al. 1999). On the other hand, another group demonstrated that mutations causing neuropsychiatric symptoms had non-random clustering in the 3´end of the gene (N. J. Jacobsen et al. 1999). In this study, missense type mutations were more common in patients with neuropsychiatric symptoms than in other Darier patients (70% versus 38%). The mutations lead to potential alternative splicing and a potential premature stop codon. The results suggest that Darier gene ATP2A2 has various effects on the brain and it has a role in the pathogenesis of neuropsychiatric disorders (N. J. Jacobsen et al. 1999).

The most severe disease phenotype was often observed in patients with missense mutation or in-frame insertion (Sakuntabhai, S. Burge, et al. 1999). 24 different mutations were studied in a study by Sakuntabhai and his co-workers (Sakuntabhai, S. Burge, et al. 1999). These mutations were scattered all over the ATP2A2 gene (Sakuntabhai, S. Burge, et al. 1999). The majority of these mutations (54%) led to a premature stop codon (Sakuntabhai, S. Burge, et al. 1999). Eight percent of the mutations led to in-frame insertions or deletions and 38% resulted in an amino acid substitution (Sakuntabhai, S. Burge, et al. 1999). Variability in phenotypes between individuals carrying the same mutation was observed (Sakuntabhai, S. Burge, et al. 1999).

These studies demonstrate that genotype-phenotype correlations are controversial. However, one common feature can be stated, different mutations in the ATP2A2 gene can occur in different regions of the gene and still cause Darier´s disease.

2.7.5 The pathogenesis of Darier´s disease

The exact mechanisms of how SERCA2 deficiency leads to acantholysis and dyskeratosis are unclear. Abnormal functions of SERCA2 can cause alterations in gene expression and in the functions of the calcium stores of the endoplasmic reticulum (Foggia & Hovnanian 2004). Abnormal calcium oscillations may alter the phosphorylation of target proteins. Altered calcium oscillations can also affect the genes that participate in the regulation of growth and differentiation in keratinocytes (Dhitavat et al. 2004). It is possible that loss of function in one ATP2A2 allele causes disturbances in the transportation of plasma membrane proteins and/or plasma membrane associated proteins (*e.g.* desmosome proteins) (McMillan & Shimizu 2001; Kitajima 2002).

The mutated SERCA2 monomer can also have an influence on the healthy SERCA2 monomer. In a study where the effects of 12 different Darier´s disease mutations were analyzed, SERCA2b monomers affect the activity of each other (W.

Ahn et al. 2003). The keratinocytes of Darier patients were cultured and their features were analyzed. In the majority of samples, the proteasome-mediated degradation of SERCA2b was activated (W. Ahn et al. 2003). The mutant SERCA2b pumps were less active than the wild type pumps (W. Ahn et al. 2003). In many samples from the patients whose mutations cause severe symptoms, the abnormal SERCA2b pump also inhibited the activity of endogenous and co-expressed wild type SERCA2b (W. Ahn et al. 2003).

The human body often has compensatory mechanisms when its functions are impaired. Compensatory mechanisms exist in the skin of Darier´s patients.

The keratinocytes of Darier patients show compensatory SPCA1 (a calcium manganese ATPase of the Golgi apparatus) upregulation which enables keratinocytes to compensate the SERCA2 inactivation caused by mutations (Foggia et al. 2006). This compensation is necessary for the viability of keratinocytes and partial compensation of calcium signaling in Darier patients (Foggia et al. 2006).

Changes in the skin have been compensated by upregulating of some intercellular junction components. For instance, there is P-cadherin upregulation in the skin of Darier patients which is thought to be a compensation mechanism for the changes in epidermis caused by Darier´s disease (Kovács et al. 2004).

These compensatory mechanisms have a role in reducing the impact of Darier´s disease on the human skin.

2.7.6 SERCA2 mutations and calcium homeostasis

The abnormal calcium homeostasis has a crucial role on the pathogenesis of Darier´s disease. Therefore, several studies on SERCA2 mutations and calcium homeostasis exist.

Several mutations causing Darier´s disease were analyzed by a Japanese group. In the first study three different Darier disease mutations were analyzed (Sato et al. 2004). One of the mutations caused reduced affinity of SERCA2b for cytoplasmic calcium ions. In the second mutation, the affinity was normal. The third mutation caused alteration in the feedback inhibition mechanism for elevated luminal calcium concentration. The results suggest that the avoidance of haploinsuffiency is very important in keratinocytes since already one mutated SERCA2b can cause symptoms (Sato et al. 2004).

The same Japanese group later analyzed 51 Darier´s disease causing mutations and it was detected that even mild disturbances in the calcium homeostasis will lead to Darier´s disease (Miyauchi et al. 2006). Most of the mutations resulted in complete absence or significantly reduced SERCA2b activity. Some mutations caused significant reductions in the expression of SERCA2b. Seven mutations led to high or normal

calcium affinity with abolished or markedly reduced calcium transportation activity or in some cases abnormal kinetic features (Miyauchi et al. 2006).

The calcium level in resting state is elevated in the keratinocytes cultured from patients with Darier´s disease (Leinonen et al. 2005). The Darier keratinocytes also show abnormalities in the calcium metabolism. The keratinocytes of Darier patients show a milder increase in the intracellular calcium concentration than the control keratinocytes. After treatment with thapsigargin, the keratinocytes of Darier patients are unable to lower their calcium levels (Leinonen et al. 2005). This indicates that keratinocytes of patients with Darier´s disease have impaired calcium regulation which can play a role in the pathogenesis of Darier´s disease.

A study analyzing skin biopsies from the lesional areas of Darier patients showed that the calcium concentration in the basal layer of the lesional areas was lower than in normal skin (Leinonen et al. 2009). It was also observed that lesional cell surfaces did not have an ATP receptor P2Y2 (involved in calcium signaling) on their plasma membranes as control keratinocytes had. The acantholytic cells had intense P2X7 (causes opening of calcium channels) labeling in their plasma membranes. These findings emphasize the possible role of impaired calcium regulation in the pathogenesis of Darier´s disease. Moreover, the localization of keratins 10 and 14 was abnormal in lesional Darier patient skin samples. In some of the lesional Darier disease areas, keratin 10 was not expressed in suprabasal layers like in normal skin. Keratin 14 was intensively labeled in the cytosol of keratinocytes of all epidermal layers that were part of the lesion not only in the basal layer like in normal skin. The non-lesional skin of Darier patients did not show differences with normal control skin in these studied localizations (Leinonen et al. 2009).

A mouse model with the SERCA2+/- genotype has been used for analysis *in vivo* (X. S. Zhao et al. 2001). In these mice, one allele of ATP2A2 gene was deleted. The plasma membrane calcium pump isoforms in these mice were upregulated, which led to shorter free cytosolic calcium oscillation (after maximal agonist stimulation) than in controls. The alterations in the calcium transportation caused about 50% reduction in the free cytosolic calcium oscillation frequencies. Exocytosis in the SERCA-depleted mice was 10-fold more sensitive to calcium. Exocytosis was otherwise similar in controls and SERCA+/- mice. This adaptability gives one possible explanation in the normal functions of most tissues in the Darier patients (X. S. Zhao et al. 2001).

Calcium depletion in the ER has an effect on the regulation of cell cycle (*e.g.* impaired upregulation of $p21^{WAF1}$, a cyclin-dependent kinase inhibitor) in a study using tissue material from a canine family with inherited skin blistering disorder (Müller et al. 2006). The dogs had depleted SERCA2-gated calcium stores which led to impaired calcium homeostasis (Müller et al. 2006). The impaired upregulation of $p21^{WAF1}$ postpones the exit of keratinocytes into the quiescent stage during the cellular differentiation (Müller et al. 2006). The delayed upregulation of $p21^{WAF1}$ in stress situations is known to cause cell cycle progression without repair (Shapiro & J. W.

Harper 1999; M. H. Lee & H. Y. Yang 2001; Weinberg & Denning 2002). If the DNA of the cell is damaged this can cause the accumulation of secondary somatic mutations and finally persistent lesions, which can explain the emergence of lesions in Darier´s disease (Müller et al. 2006).

These studies demonstrate that the mutations in SERCA2 impair the calcium signaling and affect also cellular differentiation of keratinocytes and localization of receptors and keratins in the epidermis.

2.7.7 Desmosomes and Darier´s disease

Since opening of cell junctions is a typical feature in Darier´s disease, studies on intercellular junctions may explain the mechanism of the disease. Because desmosomes are the main junction linking keratinocytes together, studies on desmosomes in Darier´s disease have advanced knowledge about this disease.

The trafficking of desmoplakin to the cell surface is markedly inhibited in keratinocytes cultured from patients with Darier´s disease (Dhitavat et al. 2003). When normal human keratinocytes were treated with thapsigargin (an inhibitor of SERCA), it was discovered that the transport of desmoplakins, desmoglein and desmocollin to the cell surface was impaired (Dhitavat et al. 2003). These proteins are then located diffusely in the cytoplasm (Dhitavat et al. 2003). The study also suggested that SERCA and desmoplakin may have interactions during the differentiation (Dhitavat et al. 2003). The expressions of both desmoplakins and SERCA2 increases when the calcium concentration is elevated (Dhitavat et al. 2003).

A study using normal human keratinocytes and SCC9 (human oral squamous cell carcinoma cells) showed that modulation of PKC alpha signaling by SERCA2 has a role in desmoplakin translocation and regulation of intercellular adhesion (Hobbs et al. 2011). PKC alpha is a known regulator in desmoplakin-intermediate filament interactions and desmosomal assembly. The function of SERCA2 was inhibited using thapsigargin or siRNA mediated silencing. It was discovered that SERCA2 deficiency impaired the adhesive strength between the cells and the desmosomal assembly was disturbed. There was a 60% reduction in the translocation of desmoplakin to the cellular borders. The membrane translocation of PKC alpha was also reduced by 70%. It was discovered that exogenous activation of PKC alpha in the SERCA2 deficient cells was able to return the normal desmosomal assembly and desmoplakin localization and elevate the adhesive strength back to normal levels. The results suggest that there is SERCA2-mediated regulation of desmoplakin and that PKC alpha has a role in mediating the interaction (Hobbs et al. 2011). This suggests a possible mechanism for the opening of desmosomes in Darier´s disease.

2.8 Hailey-Hailey disease

2.8.1 The symptoms of Hailey-Hailey disease

Hailey-Hailey disease (OMIM 16960) was first described in 1939 by the Hailey brothers in in Atlanta, Georgia, USA (Hailey & Hailey 1939). The disease is autosomally and dominantly inherited but the phenotype varies even within the members of the same family (S. M. Burge 1992; Dobson-Stone et al. 2002). This suggests that modifying environmental factors have an effect on the phenotype of the patient (Dobson-Stone et al. 2002). The patients typically have plaques of bullous lesions in their neck, armpits, groins, genital and anal area, umbilical region and sometimes also disseminated to the scalp area. Painful cracking of skin (rhagades) in flexural areas and vesicles and erosion are common features. There are rarely lesions in mucosa but sometimes lesions can be seen in mouth, esophageal and vulvar areas. (Figure 10)

Figure 10 Skin of a Hailey-Hailey patient. Photo courtesy of Raimo Suhonen

Many patients have also light longitudinal lines in nails (S. M. Burge 1992). Patients suffer from itching, pains, secondary infections and declined quality of life

(Hailey & Hailey 1939; Palmer DD 1962; S. M. Burge 1992). Abrasion, heat and sweating worsen the symptoms. Usually the symptoms appear by 30-40 years of age. The incidence is about 1:50 000. Corticosteroids, oral retinoids and antibiotics are used to the symptoms (S. M. Burge 1992). Treatments with laser or liquid nitrogen can also be beneficial in refractory cases (D. Simon et al. 1999; Awadalla & Rosenbach 2011; Segal 1984; Thai & Sinclair 1999).

An autosomal dominant skin disease can also be segmental. The segmental forms are divided into two types. Type 1 segmental disease mutation occurs during the early embryogenesis and results in heterozygosity for this mutation. The lesions of the skin show similarities to the phenotype of non-segmental (germline) mutations. The nonlesional areas are both genetically and clinically normal. In type 2 segmental disease, a second mutation occurs in heterozygotic embryo. The patient develops, later, non-segmental, diffuse lesions of the skin (Happle 1996; Happle 1997). A patient with type 2 segmental Hailey-Hailey disease was described by Poblete-Guetierrez and her co-workers (Poblete-Gutiérrez et al. 2004). The patient developed the symptoms of nonsegmental Hailey-Hailey disease at 24 years of age. Earlier, since the age of 3 months, the unilateral symptoms appeared, persisting into adulthood with frequent

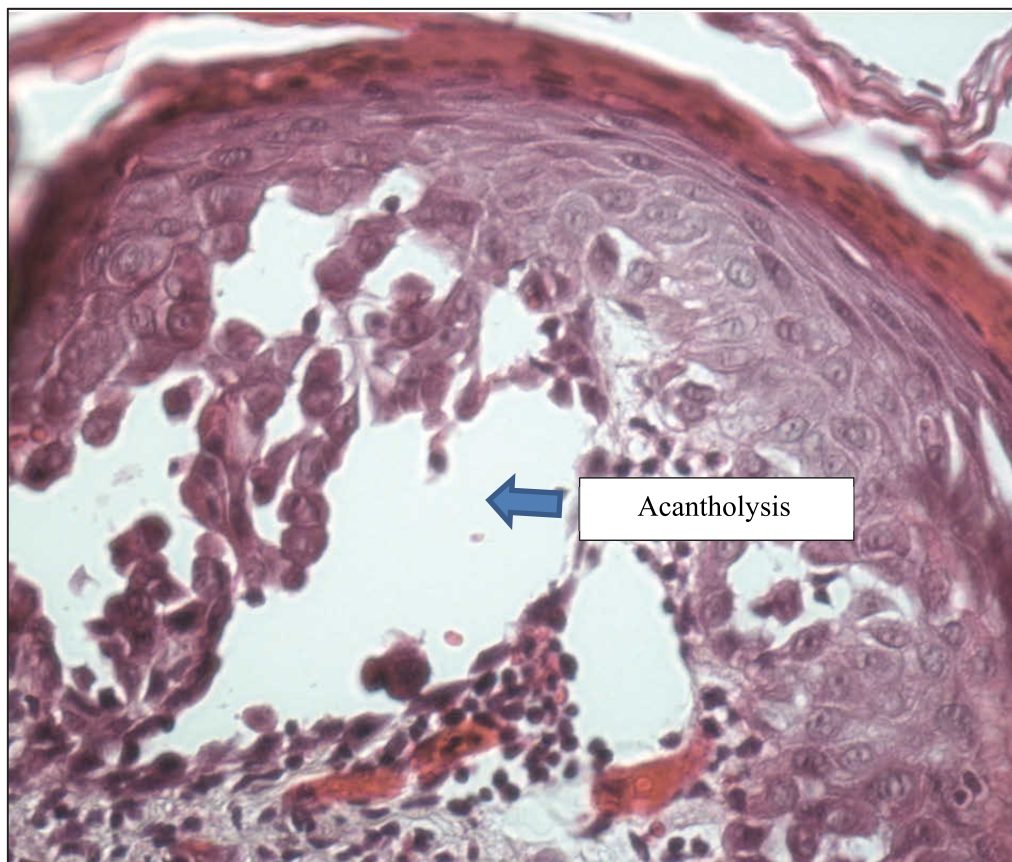

Figure 11 The histology of Hailey-Hailey disease

exacerbations. Her seven relatives in four generations were also examined and it was discovered that 4 of them had Hailey-Hailey disease. The mutation was analyzed using a control group of 150 healthy unrelated individuals. It was discovered that the patient had a mutation in the maternal allele of the Hailey-Hailey chromosomal region. In the most severely affected skin areas, the paternal healthy allele was lost which caused the segmental symptoms (Poblete-Gutiérrez et al. 2004)

Histologically, typical findings in the Hailey-Hailey disease include acantholysis in the suprabasal level, vesicle formation or cracking of epidermis (Norgett et al. 2000; Hashimoto et al. 1995). The keratinocytes show no consistent ultrastructural abnormalities *in vitro* (Cooley et al. 1996). (Figure 11)

2.8.2 ATP2C1

Hailey-Hailey disease is caused by mutations in ATP2C1 gene encoding a type 1 Golgi secretory pathway $Ca2^{+}/Mn2^{+}$ ATPase (hSPCA1) (Sudbrak et al. 2000; Z. Hu et al. 2000). This gene is located in chromosome 3q21-24 (Bolognia et al. 2008). The gene has two splice variants: ATP2C1a and ATP2C1b which have same expression patterns but differ in their carboxy termini (Z. Hu et al. 2000). The ATP2C1a gene encodes 919 amino acids and ATP2C1b encodes 888 amino acids (Z. Hu et al. 2000). The SPCA1 protein is 115 kDa in size (Foggia & Hovnanian 2004). SPCA1 has only one calcium binding site which can bind a manganese or calcium ion with high affinity (Foggia & Hovnanian 2004). In the Golgi apparatus, calcium and manganese are needed for the correct production, processing and maturation of proteins intended for membrane or secretion (Foggia & Hovnanian 2004). Calcium in the Golgi lumen also controls the transport of proteins and the processing of their precursors (Oda 1992; Foggia & Hovnanian 2004).

A study using normal human skin samples showed that ATP2C1 was localized in the basal cell layer in normal epidermis (M. Yoshida et al. 2006). This and studies on normal cultured human keratinocytes suggested that ATP2C1 may have a role in keeping the basal keratinocytes in an undifferentiated state. Detachment of keratinocytes from culture dish or addition of high calcium concentration medium did not alter the expression of ATP2C1. Instead the expression of differentiation markers keratin 10 and involucrin was elevated after these procedures. SiRNA–mediated knockdown of ATP2C1 gene caused an elevated expression of these differentiation markers (M. Yoshida et al. 2006). This suggests that the reduction of ATP2C1 induces differentiation in the epidermis. When keratinocytes were treated with calcium ionophore A23187, no change in differentiation markers was seen. Instead using more manganese selective ionophore Br-A23187, an increase of expression in differentiation markers was seen. This suggests that manganese starvation in the Golgi apparatus may have a role in ATP2C1-mediated regulation of the differentiation of keratinocytes (M. Yoshida et al. 2006).

When the endogenic expression of ATP2C1 pump was inhibited using siRNA method, the post-translational modification of the wild-type thyroglobulin (a secreted glycoprotein) was abnormal (Ramos-Castañeda et al. 2005). The degradation of the mutated thyroglobulin in the ER was also faulty. The cells were abnormally sensitive to the stress in the ER. Another finding was that the ATP2C1 protein in immunofluorescence was more distinct in low calcium concentration and when the extracellular calcium concentration increased the amount of ATP2C1 in immunofluorescence decreased. This suggests that the main function of the ATP2C1 is not the lowering of calcium from the cytosolic pools, but instead storing the calcium for the secretory pathway. These results also suggest that the differentiation of keratinocytes is associated with a strong upregulation of the ATP2C1 gene. The expression of ATP2C1 gene helps to deliver the adhesion molecules *via* the secretory pathways of the cell. Faulty expression could cause the symptoms of Hailey-Hailey disease (Ramos-Castañeda et al. 2005).

TEXT BOX 4.

Golgi apparatus:

The Golgi apparatus consists of flat membrane-enclosed stacks. These stacks have 4-6 cisternae. The Golgi apparatus is prominent in cells specialized to secretion. The Golgi apparatus has two faces: *cis*-face and *trans*-face. The *cis*-face is the one where proteins and lipids enter the Golgi apparatus and the *trans*-face is the one where they exit the Golgi apparatus (Alberts et al. 2002). It modifies, packs and concentrates the proteins and lipids that are made in the endoplasmic reticulum (Marieb & Hoehn 2006). There is forward and backward routes for proteins so that misplaced proteins can be returned to the correct sites also in the previous compartments (Alberts et al. 2002). Misfolded or incorrectly assembled proteins are returned to endoplasmic reticulum (Alberts et al. 2002). Inside the Golgi apparatus, oligosaccharide chains can be added or removed and sometimes also phosphate groups are added to the proteins or lipids (Marieb & Hoehn 2006; Alberts et al. 2002). The oligosaccharide side groups serve as address tags which direct the proteins and lipids to lysosomes or transports vesicles. The Golgi apparatus is an important location of carbohydrate and polysaccharide synthesis (*e.g.* glycosaminoglycans of the cell wall) (Alberts et al. 2002).

Ton and co-workers (2002) studied the expression of the human SPCA1 in yeast and Chinese hamster ovary cells. The human SPCA1 localized exclusively to Golgi apparatus also in these cell types and SPCA1 transported calcium and manganese with a high affinity to these ions (Ton et al. 2002). This finding established the function of the SPCA1 and its localization in the Golgi apparatus (Ton et al. 2002).

2.8.3 The pathogenesis of Hailey-Hailey disease

The affinity of SPCA1 protein for calcium and manganese ions is high and these ions are bound with equal affinities (R. J. Fairclough et al. 2003). It is believed that the mutation causes production of incorrectly folded or unstable SPCA1 proteins in Hailey-Hailey disease (R. J. Fairclough et al. 2003). The mutated SPCA1 proteins induce faster degradation than the wild type controls (R. J. Fairclough et al. 2003). Furthermore, SPCA1 may also lose its sensitivity for ion binding (R. J. Fairclough et al. 2003). On the other hand, it is also possible that the mutation can affect the interactions with the regulatory proteins in keratinocytes (R. J. Fairclough et al. 2003). Oxidative stress activation of miRNA 125b has also been suggested to play a role in the pathogenesis of Hailey-Hailey disease (Manca et al. 2011).

The pathogenesis of Hailey-Hailey disease can be variable. Fairclough and her co-workers studied the molecular and physiological effects of ATP2C1 mutations (R. J. Fairclough et al. 2003). Over 50% of the mutants showed low levels of protein expression although the mRNA levels and the localization of the proteins were normal (R. J. Fairclough et al. 2003). Some of the mutants showed lack of ion transport which was caused by altered partial reactions in the catalytic cycle such as impaired calcium and manganese binding or faulty conformational transition of the phosphoenzyme intermediate in energy transduction (R. J. Fairclough et al. 2003).

2.8.4 SPCA1 mutations and calcium homeostasis

Calcium signaling is an important signaling mechanism in keratinocytes and the mutations of SPCA1 impact this signaling. Therefore, studies on the effects of the mutations in SPCA1 on calcium signaling have been carried out.

There are two types of results in studies about calcium concentrations of Hailey-Hailey keratinocytes in resting state. Hu and co-workers measured elevated resting state calcium concentrations and subnormal ability to control excess cytosomal calcium (*in vitro*) (Z. Hu et al. 2000). In a study by Leinonen and co-workers it was demonstrated that the resting state calcium levels of control keratinocytes and Hailey-Hailey keratinocytes were approximately the same (Leinonen et al. 2005). There is a possibility that the type of mutation in ATP2C1 gene is different in these two studies and affects results.

Hailey-Hailey keratinocyte show changes in calcium metabolism. Addition of ATP to keratinocytes of Hailey-Hailey patients caused less pronounced elevation of intracellular calcium than in control keratinocytes. After treatment with thapsigargin, the keratinocytes of Hailey-Hailey patients did not lower their calcium concentration as efficiently as the control keratinocytes. (Leinonen et al. 2005) This indicates that calcium regulation in the keratinocytes of patients with Hailey-Hailey disease may be impaired.

Despite the increased intracellular calcium concentration, the keratinocytes of Hailey-Hailey patients expressed low levels of involucrin (a known differentiation

marker) in both high and low extracellular calcium concentrations. (K. M. Aberg et al. 2007) This suggests there might be a defect in differentiation of keratinocytes. The mRNA level of involucrin was lower in Hailey-Hailey keratinocytes than in the normal control keratinocytes (K. M. Aberg et al. 2007). The synthesis of involucrin mRNA was normal but the degradation rates were much higher in Hailey-Hailey keratinocytes. Upregulation of a compensatory promoter was detected. (K. M. Aberg et al. 2007) This shows the adaptability of the keratinocytes.

The apparently normal epidermis of Hailey-Hailey patients contains reduced calcium stores and the calcium gradient is abnormal (*in vivo*) (Behne et al. 2003). The abnormally high calcium concentration can affect the gene expression or alter the post-translational modification of the target proteins (activation of protein kinase C) (Foggia & Hovnanian 2004). Protein kinase C is a known regulator of desmosome assembly and desmoplakin – intermediate filament adhesion (Hobbs et al. 2011). It is also possible that the low calcium and manganese concentrations of the Golgi lumen can alter the post-translational modifications of membrane proteins which are important for epidermal cell adhesion (*e.g.* desmosomal proteins) (Foggia & Hovnanian 2004). Since the mutation in Hailey-Hailey disease affects primarily skin, the epidermal cells are more sensitive for the sufficient amount of SPCA1 proteins (Foggia & Hovnanian 2004).

Skin biopsies from the lesional areas of Hailey-Hailey patients show that the calcium concentration in the basal layer is lower than in normal skin (Leinonen et al. 2009). It was also observed that lesional cell surfaces did not have ATP receptor P2Y2 (involved in calcium signaling) on their plasma membranes like control keratinocytes had. The acantholytic cells had intense P2X7 (causes opening of calcium channels) labeling in their plasma membranes. These findings suggest that impaired calcium signaling may have a role in the pathogenesis of Hailey-Hailey disease. Furthermore, the localization of keratins 10 and 14 was abnormal in lesional Hailey-Hailey patient skin samples. In some of the lesional Hailey-Hailey disease skin areas keratin 10 was not expressed in the keratinocytes of the suprabasal layers like in normal skin. In the normal skin keratin 14 is located in basal layer but in the lesional Hailey-Hailey skin areas it was expressed in all epidermal layers. However, the non-lesional skin of Hailey-Hailey patients did not show differences with normal control skin in these studied areas (Leinonen et al. 2009).

These studies show that the mutations in SPCA1 lead to numerous changes in the calcium homeostasis and the localization of keratins.

2.9 Mitogen-activated protein kinases

2.9.1 Definition of MAPKs

Mitogen-activated protein kinase (MAPK) are a family of kinases that mediate many cellular responses to extracellular stimuli such as growth factors, hormones, stress and cytokines (S.-H. Yang et al. 2012). There are multiple MAPK pathways in every

eukaryotic cell (Krishna & Narang 2008). The major MAPK pathways in mammals are: the extracellular signal-regulated kinase (ERK), extracellular signal regulated kinase 5; p38 pathways; and c Jun N-terminal kinase (JNK) (Krishna & Narang 2008; Wang & Tournier 2006). ERK pathways respond to growth factor signals (S.-H. Yang et al. 2012) and extracellular stress signals activate JNK and p38 pathways (S.-H. Yang et al. 2012).

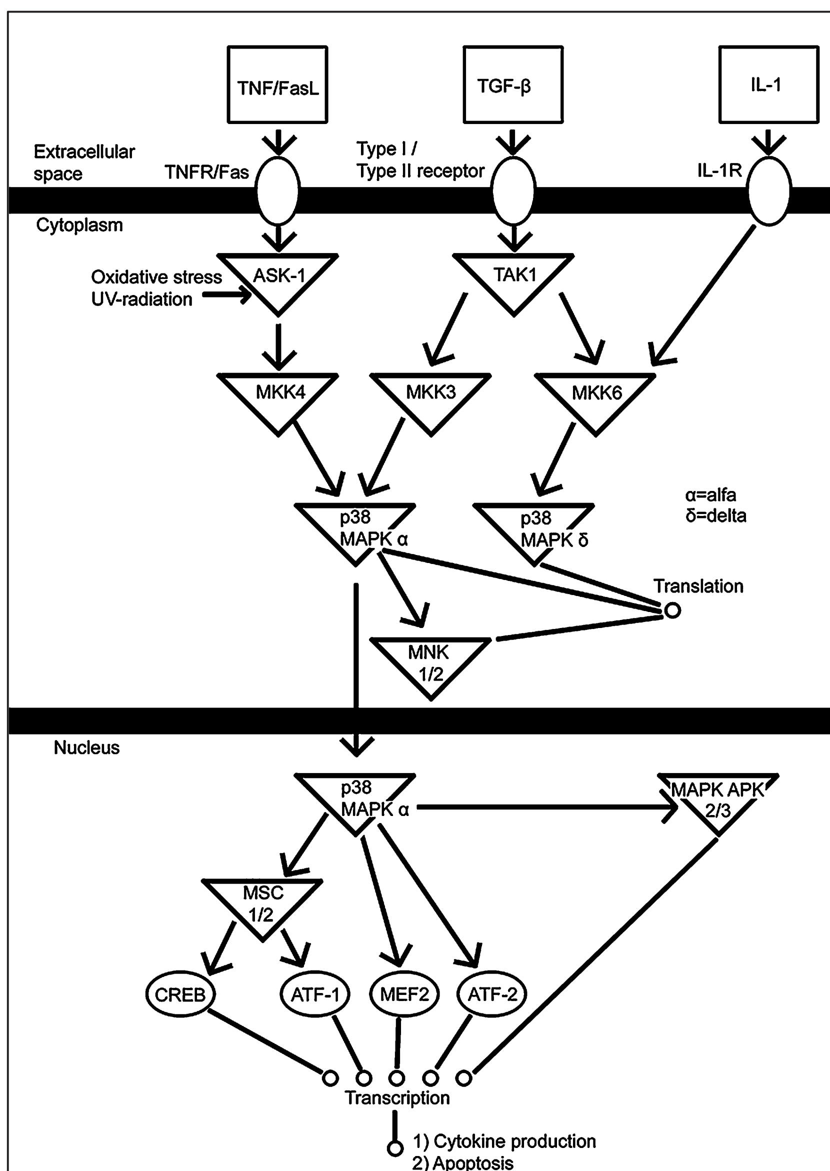

Figure 12 Schematic presentation of p38 signaling. Modified from ("The interactive pathway analysis of complex omics data were generated through the use of IPA (Ingenuity Systems, Www.ingenuity.com)" 2013)

2.9.2 p38

p38 has four isoforms: α, β, γ and δ (Krishna & Narang 2008). In normal human epidermal keratinocytes the isoforms α and δ are the most common (Junttila et al. 2007). p38δ has a role in the differentiation of keratinocytes since it regulates the expression of involucrin and mediates the apoptosis of keratinocytes (Eckert et al. 2004; Kraft et al. 2007). In head and neck squamous carcinoma cells p38α and p38δ isoforms have been shown to regulate invasion and growth (Junttila et al. 2007).

The p38 pathway is activated by various stress stimuli. These stimuli can be inflammatory cytokines such as interleukin-1 (IL-1), death ligands such as tumor necrosis factor (TNF) or type 2 transmembrane protein Fas-ligand (FasL), TGF-beta related polypeptides and numerous environmental factors such as UV-radiation or oxidative stress. The activation of p38 pathway causes the phosphorylation of several protein kinases. First, MAPKKKs e.g. apoptosis signal regulating kinase 1 (ASK1) and TGF-beta activated kinase 1 (TAK-1) activate the MAPK kinases (MKKs) that in turn activate p38 MAPK. The activation on p38 MAPK leads to phosphorylation of several MAPK-activated protein kinases including MAPK-interacting kinases (MNK) mitogen- and stress-activated protein kinases (MSKs), MAPK-activated protein kinases (MAPKAP –K2 and -3). These kinases amplify the signal. (Figure 12) ("The interactive pathway analysis of complex omics data were generated through the use of IPA (Ingenuity Systems, Www.ingenuity.com)" 2013)

When p38 MAPK is translocated to the nucleus it phosphorylates several transcription factors e.g. activates transcription factor (ATF-1 and -2), myocyte specific enhancer-binding factor-2 (MEF-2), Elk-1 and cAMP regulatory element binding protein (CREB). Some transcription factors can also be phosphorylated by MSKs and MAPKAPS. The activated regulatory factors induce transcription of several genes leading to increased transcriptional activity, cell death and protein synthesis. (Figure 12) ("The interactive pathway analysis of complex omics data were generated through the use of IPA (Ingenuity Systems, Www.ingenuity.com)" 2013)

2.10 Squamous cell carcinoma

2.10.1 Pathogenesis of squamous cell carcinoma

The most important risk factor for epithelial cancers is ultraviolet radiation (UV) exposure (Diepgen et al. 2012). UVB radiation (and also UVA radiation in lesser extent) causes formation of pyrimidine dimers in DNA and RNA (Diepgen et al. 2012). This leads to mutations in keratinocytes and neoplastic transformation (Diepgen et al. 2012). The type of skin affects the risk of developing skin cancer (fair skin type increases the risk) (Diepgen et al. 2012). UV radiation causes skin cancer by inducing DNA mutations and by suppressing T lymphocytes (Diepgen et al. 2012). UVA radiation reinforces the harmful effects of UVB radiation (Diepgen et al. 2012). It

penetrates deeper into the skin and causes premature ageing and immunosuppression (Diepgen et al. 2012).

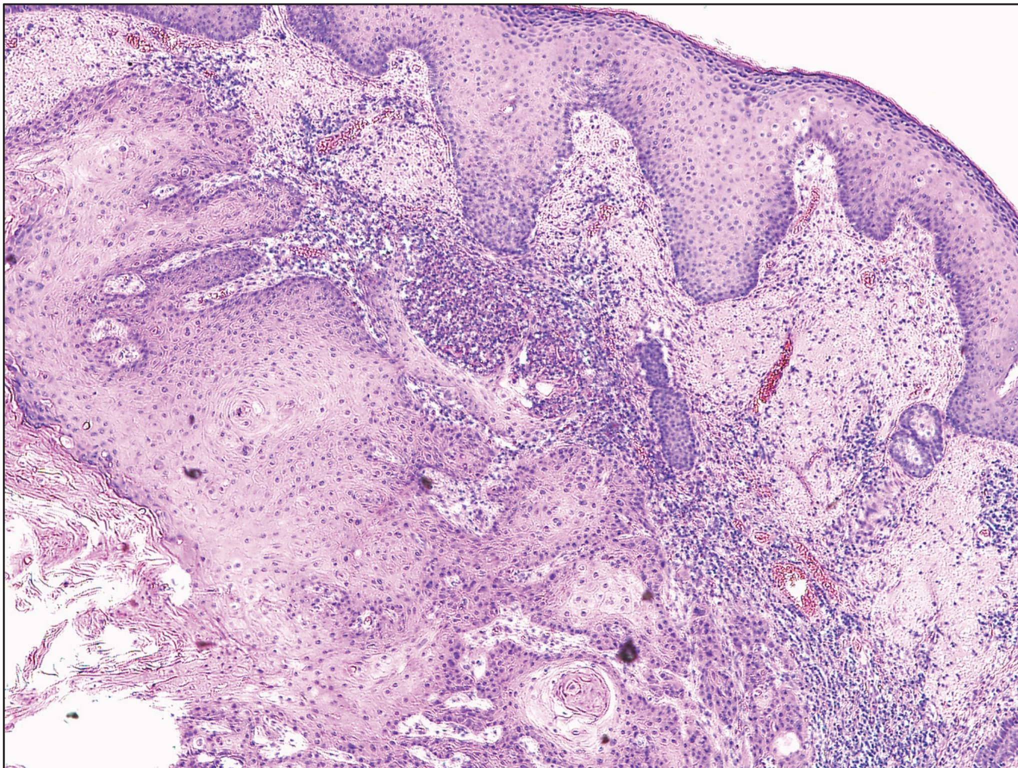

Figure 13 The histology of squamous cell cancer. The cells are organized in malignant and disordered way. Photo courtesy of Mehdi Farshchian and Department of Pathology, University of Turku

Squamous cell carcinoma may also develop into chronically ulcered and/or inflamed skin area (*e.g.* chronic wounds or skin of a lupus patient) (Alam & Ratner 2001). Some inflammatory skin diseases, such as lichen sclerosus et atrophicus, also increases the risk of carcinoma (R. W. Jones et al. 2004). In the infected areas, the cells of the innate and acquired immune system compete for the site creating an environment that can either prevent or initiate carcinogenesis (Shiao et al. 2011). Other predisposing factors include carcinogens (*e.g.* arsenic), immune suppression and viruses (*e.g.* HPV) (N. H. Cox et al. 1999).

2.10.2 Cellular events leading to carcinoma

UVB radiation can cause development of skin cancer through changes in oncogenes and in p53 tumor suppressor genes (Diepgen et al. 2012; Alam & Ratner 2001). These events lead to initiation and progression of skin cancer (Diepgen et al. 2012; Alam & Ratner 2001). UVB radiation can cause a mutation in p53 tumor-suppressor gene and as a result the cell undergoes apoptosis. If the cell already has one premalignant change

(a dysfunctional p53), a mutation in other p53 allele causes the cell to escape apoptosis. Instead, the abnormal cell population continues dividing and actinic keratosis develops. Uncontrolled cellular proliferation can lead to squamous cell carcinoma *in situ* and invasive squamous cell carcinoma (Alam & Ratner 2001).

The majority of human squamous cell carcinoma tumors express increased levels of activated Ras and MAPK (Dajee et al. 2003). However, H, N or K-RAS genes do not carry mutations (Dajee et al. 2003).

The stem cells of cancer tissue typically self-renew and have the ability to produce differentiated daughter cells, re-create heterogeneity of the tumor tissue, and have long-term ability to cell division (Patel et al. 2012).

2.10.3 The histology and the clinical picture of squamous cell carcinoma

Histologically squamous cell carcinoma shows disorganized keratinocytes with a typical malignant cytology (Underwood 2004). Acantholysis, dyskeratosis and pseudoglands can also sometimes be seen (Rapini 2012). Foci of keratinization can be seen within the tumor resembling the normal upper epidermal structure but the organization is malignant and disordered (Underwood 2004). Cornification and also keratin pearls exist within the tumor (Petter & Haustein 1998). The tumor is often accompanied by chronic inflammatory cell infiltrate which mainly consists of T-lymphocytes. Natural killer cells, mast cells, B-lymphocytes, plasma cells, macrophages and Langerhans cells are often present. (Kwa et al. 1992) Squamous cell cancer is rarely metastatic and usually if it metastasizes this occurs very late. (Underwood 2004) (Figure 13)

The more differentiated the tissue is, the better is the prognosis of the patient. If the cancer tissue is poorly differentiated and the normal tissue structure is lost, the prognosis of the patient is poor (Brantsch et al. 2008). The location of the tumor also affects the behavior of the cancer since tumors of the lip or ear often have higher recurrens and metastasis rates (Rowe et al. 1992). The risk of metastasis is also higher in squamous cell carcinomas of penis, scrotum and anus (Kwa et al. 1992). Furthermore the etiology of the cancer also affects the risk of metastasis. Carcinomas that develop after burns, radiation, scarring or complicated chronic ulcers metastasize more often. (T. M. Johnson et al. 1992; McGrath et al. 1992) Also cases where squamous cell carcinoma arises in a patient with Darier´s disease or Hailey-Hailey disease have been described in literature (Holst et al. 2000; Cockayne et al. 2000; Chun et al. 1988; Vázquez et al. 2002; von Felbert et al. 2010). In these cases the possible disease mechanism is due to persistent lesions. The metastasis often develop into regional lymph nodes, the liver, lungs, bones and brain (Preston & Stern 1992).

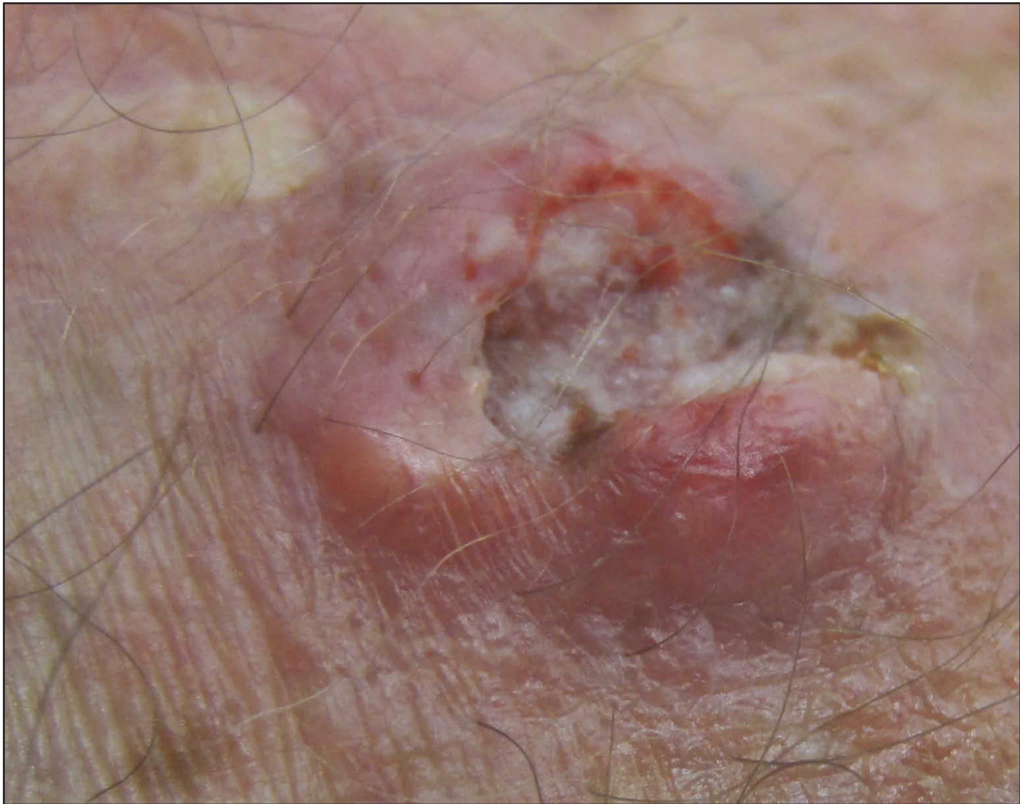

Figure 14 Squamous carcinoma of leg. The tumor is ulcerated lesion with hard, raised edges. Photo courtesy of Atte Kivisaari.

Clinically squamous cell carcinoma is often seen as roughened keratotic areas, papules, nodules, verrucous areas, ulcers that do not heal or horns (Underwood 2004; McKee et al. 2005). It can be indurated tumor that grows slowly and is often painless (McKee et al. 2005) The most common treatment is surgical excision. (Underwood 2004) (Figure 14)

2.10.4 Classification of squamous cell carcinoma

Squamous cell carcinomas can be classified according their level of differentiation using categories well, moderately or poorly differentiated. (Weedon 1998; McKee et al. 2005).Also numerical classification according to Broder´s system can be used. In this system grade 1 means that 75% or more of the lesion is well differentiated. Grade 2 means that 50% or more is well differentiated. Grade 4 is the most malignant stage where less than 25% of the tumor is well differentiated. (McKee et al. 2005) An invasive squamous cell carcinoma can be classified using clinicopathological TNM staging. Stage 1 means that the diameter of the tumor is under 20 mm. Stage 2 tumors have a diameter over 20 mm but they are not metastatic yet. Stage 3 tumors have a lymph node involvement. At stage 4 the cancer has distant metastasis. (Underwood 2004)

2.10.5 Squamous cell cancer in Finland

The average incidence of squamous cell carcinomas in Finland is 1201 patients per year, of which 596 are women and 605 are men (years 2006-2010). Age-standardized incidence rate of non-melanoma skin cancer in Finland is about 11.0 /100 000 in men and 6.4/100 000 in women (years 2006-2010). The incidence has risen in the last 10 years by 3.6% in men and by 2.8% in women. The proportion of new non-melanoma skin cancers of all cancers in Finland is 4.5% in men and 4.7% in women. The prognosis of the patients is quite good since the risk of dying from this cancer before age 75 is 0.0%. Age-standardized rate of dying from non-melanoma skin carcinoma is 0.5/100 000 in men and 0.2/100 000. The proportion of non-melanoma skin cancer of all cancer deaths is 0.5% in men and 0.4% in women. (Engholm et al. 2012)

2.10.6 Intercellular junctions, extracellular matrix and cancer

Increased expression of certain claudins is associated with tumorigenesis in many cancer types (*e.g*. claudins-1 and -7 in cervical neoplasia) (Turksen & Troy 2011; J.-W. Lee et al. 2005). Some claudins are also down regulated during tumorigenesis (Turksen & Troy 2011). Mutations in claudins can initiate the tumorigenesis (Turksen & Troy 2011). Other possible explanations for claudin downregulation in certain tumors might be hypermethylation of promoter region with gene silencing (Turksen & Troy 2011). This feature is very common in human cancers and DNA hypermethylation is linked to the process of carcinogenesis (S. Gopalakrishnan et al. 2008; Turksen & Troy 2011).

The expression of tight junction proteins, occludin, ZO-1 and claudin-4 is associated with keratinization in squamous cell carcinoma (Morita et al. 2004). Claudin-1, instead, has a different patterns of expression in squamous cell carcinoma cells (Morita et al. 2004). The changes in claudin-1 expression are supposed to be a consequence of faulty cell junctions in malignant keratinocytes (Morita et al. 2004). Furthermore, complete loss of occludin is a frequent phenomenon in squamous cell carcinoma (Rachow et al. 2013). This has been linked to decreased intercellular adhesion and reduced susceptibility to apoptosis (Rachow et al. 2013).

Matrix metalloproteinases have also a role in the carcinogenesis. Claudin-1 enhances the invasiveness of oral squamous cell carcinoma by activating matrix metalloproteinase 2 and membrane-type matrix metalloproteinase 1 and these events would then promote the cleavage of laminin-5 and result in invasive phonotype (Oku et al. 2006).

Moreover, hyaluronan concentration of the squamous cell carcinoma of skin changes when the tumor turns more malignant: high grade tumors express decreased amounts of hyaluronan when compared to less malignant squamous cell carcinoma or normal control skin (Karvinen et al. 2003). The same observation has also been made with melanoma cells suggesting this phenomenon might be typical in the carcinogenesis (Siiskonen et al. 2013). This is reasonable since hyaluronan is associated with regulation of differentiation (Camenisch et al. 2000).

Involucrin, a differentiation marker, is absent in malignant epidermal tissues (Murphy et al. 1984). In squamous cell carcinoma, *in situ* labeling is diffuse (Murphy et al. 1984). In a study where an organotypic skin model of squamous cell carcinoma was used, this showed that inhibition of type VII collagen (a component of anchoring fibril of the basement membrane) led to increased invasiveness and migration (Martins et al. 2009). A tissue array of sporadic squamous cell carcinoma and immunostainings of recessive dystrophic epidermosis bullosa skin showed that loss of collagen VII leads to decreased expression of involucrin (Martins et al. 2009).

The downregulation or functional mutation of E-cadherin is linked to carcinogenesis in many sorts of cancers: breast, bladder, nasopharyngeal and lung cancer (Berx et al. 1998; Bremnes et al. 2002; Rakha et al. 2005; Syrigos et al. 1995; Wijnhoven et al. 2005; Zheng et al. 1999). The role of desmosomal cadherins in cancer progression is unclear. A study with invasive mouse fibroblast cell line showed that transfection with desmosomal proteins inhibits the invasiveness. This suggests that desmosomes have a potential role in the inhibition of tumorigenesis (Tselepis et al. 1998). Laterone group investigated the role using keratinocyte cultures and mouse model. The results were however contradictory: in the allograft assays, loss of desmoglein-3 inhibited carcinogenesis but in the autochthonous model, the loss did not show any effect on carcinogenesis (S. Baron et al. 2012).

3. AIMS OF THE STUDY

The purpose of the present study was to define the regulation of epidermal tight junctions by calcium ATPases and p38. The specific aims of the study were:

1. To elucidate the role of tight junction proteins in the process leading to acantholysis in Hailey-Hailey disease and Darier´s disease
2. To investigate how ATP2C1 regulates intercellular junction proteins in normal human keratinocytes
3. To evaluate the regulation of tight junctions in human keratinocytes
4. To determine the role of p38 signaling in the regulation of intercellular junctions in normal human keratinocytes and in squamous cell carcinoma

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Tissue samples (I-III)

All human tissue material was obtained from Turku University Hospital, Turku, Finland or Oulu University Hospital, Oulu, Finland with appropriate permissions from the University Hospitals and approvals of the Ethical Committee of the Hospital District of Southwest Finland and the Joint Ethical Committee of the Oulu University Hospital, respectively. Normal human skin samples were obtained from healthy persons undergoing plastic surgery. All patients gave their written consent. Paraffin-embedded skin biopsies of four Hailey-Hailey disease patients and five Darier disease patients were obtained from the Department of Pathology, Turku University Hospital. Four-millimeter skin biopsies were obtained in apparently healthy and lesional skin areas of eight Darier patients (aged 35-69 years) and nine Hailey-Hailey patients (aged 45-80 years).

4.1.2 Cell lines (II, III)

Normal human keratinocytes
Squamous cell carcinoma derived keratinocytes

4.1.3 Keratinocyte cell cultures (II, III)

Keratinocyte cell cultures were established from surgically removed normal skins of people undergoing plastic surgery at Turku University Hospital.

4.1.4 Squamous cell carcinoma (III)

Human cutaneous SCC cell lines were established from surgically removed skin SCCs as described previously (Johansson et al. 1997). SCC cell were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with glutamine, penicillin-streptomycin, non-essential amino acids (Gibco) and 10% fetal calf serum (Biowest).

4.1.5 Cell culture media (II, III)

Normal human keratinocytes were cultured in keratinocyte growth medium (KGM-2) SFM (PromoCell GmbH, Heidelberg, Germany) supplemented with Supplement Mix and 0.06mM $CaCl_2$ (both from PromoCell) and penicillin-streptomycin mixture (Gibco, Paisley, UK)

SCC cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with glutamine, penicillin-streptomycin, non-essential amino acids (Gibco) and 10% fetal calf serum (Biowest)

4.1.6 Antibodies (Table)

Table 4.1 List of primary antibodies

Antibody	Cat no	Source	Used in
Antibody against p38δ/SAPK4 (C19)	sc7585	Santa Cruz Biotechnology Inc, CA, USA	III
Antibody for plakoglobin (11E4)		Dr. James Wahl, University of Nebraska Medical Center, Omaha NE, USA	II
Desmoglein-2 (6D8)		Dr. James Wahl, University of Nebraska Medical Center, Omaha NE, USA	II
Mouse mab[1] to ATP2C1 (Clone 2G1)	WH0027032M1	Sigma-Aldrich, St. Louis, MO, USA	II
Mouse mab to beta-actin (Clone AC-15)	A5441	Sigma-Aldrich, St. Louis, MO, USA	II-III
Mouse mab to betacatenin	M3539	Dako, Glostrup, Denmark	I, III
Mouse mab to beta-Catenin (clone 15B8)	C7207	Sigma-Aldrich, St. Louis, MO, USA	II
Mouse mab to claudin-4 (clone 3E2C1)	32-9400	Invitrogen, Carlsbad, CA, USA	I-III
Mouse mab to desmocollin-2/3 (clone 7G6)	32-6200	Invitrogen, Carlsbad, CA, USA	II
Mouse mab to desmoglein-1	32-6000	Invitrogen, Carlsbad, CA, USA	II
Mouse mab to desmoglein-3	32-6300	Invitrogen, Carlsbad, CA, USA	II
Mouse mab to desmoplakin	Ab 71690	Abcam, Cambridge, UK	I-III
Mouse mab to E-cadherin (clone 4A2C7)	33-4000	Invitrogen, Carlsbad, CA, USA	I-III
Mouse mab to ZO-1 (clone ZO1-1A12)	33-9100	Invitrogen, Carlsbad, CA, USA	I-III
Nonimmunized Mouse IgG	sc2025	Santa Cruz Biotechnology Inc, CA, USA	I
Nonimmunized rabbit IgG	sc2027	Santa Cruz Biotechnology Inc, CA, USA	I
Rabbit pab[2] to involucrin	ab53112	Abcam, Cambridge, UK	III
Rabbit pab to MEK1	#9124	Cell Signaling Technology, Danvers, MA, USA	III
Rabbit pab to p38 MAPK	#9212	Cell Signaling Technology, Danvers, MA, USA	III
Rabbit pab to p44/42 MAPK	#9102	Cell Signaling Technology, Danvers, MA, USA	III
Rabbit pab to phosphoCREB	#9191	Cell Signaling Technology, Danvers, MA, USA	III
Rabbit pab to phosphoMEK1/2	#9121	Cell Signaling Technology, Danvers, MA, USA	III
Rabbit pab to phosphop38 MAPK	#9211	Cell Signaling Technology, Danvers, MA, USA	III
Rabbit pab to phosphop44/42 MAPK	#9101	Cell Signaling Technology, Danvers, MA, USA	III
Rabbit pab to claudin-1	51-9000	Invitrogen, Carlsbad, CA, USA	I-III
Rabbit pab to occludin	71-1500	Invitrogen, Carlsbad, CA, USA	I-III
Rabbit pab to ZO-1	61-7300	Invitrogen, Carlsbad, CA, USA	I

[1] mab=monoclonal antibody

[2] pab=polyclonal antibody

Table 4.2 List of secondary antibodies

Antibody	Cat no	Source	Used in
Alexa Fluor 488-conjugated goat anti-mouse IgG	A11029	Molecular Probes Inc. Eugene, OR, USA	I-III
Alexa Fluor 568-conjugated goat anti-rabbit IgG	A11011	Molecular Probes Inc. Eugene, OR, USA	I-III
ATTO 647N	AD 647N	ATTO-TEC GmbH, Siegen, Germany	II-III
Hoechst 33342	H3570	Invitrogen, Eugene, OR, USA	I-III
Horseradish peroxidase-conjugated secondary antibody for mouse IgG	7076	Cell Signaling Technology, Danvers, MA, USA	II-III
Horseradish peroxidase-conjugated secondary antibody for rabbit IgG	7074	Cell Signaling Technology, Danvers, MA, USA	II-III

4.2 Methods

4.2.1 Cell culture protocol

The skin samples were harvested to common salt solution or EBSS, both containing 1% penicillin-streptomycin solution and fungizone 2 µl/ml (amphotericin-B). Skin samples were first cleared from dermis and subcutis with a sharp scalpel. Then the skin was cut into small pieces (1-cm x 2-cm). The skin was incubated overnight in trypsin 0.25%-dispase solution in room temperature. The solution contained, in addition to trypsin 0.25%, 1% penicillin-streptomycin solution, dispase 200 µl/ml (stock 10 mg/ml), amphotericin-B 2 µl/ml. Skin pieces were put into the solution epidermis downwards. The following day soybean trypsin inhibitor (SBTI, 0,25mg/ml Thermo Fisher Scientific/ Finnzymes, Vantaa, Finland cat no 17075029) or Dulbeccoś Modified Eagleś medium (DMEM) +10% fetal calf serum (FCS) was added to stop the trypsination. Similar volumes of SBTI solution and trypsin-dispase solution were used. Twice as much DMEM+10% FCS was used as trypsin-dispase solution.

The keratinocytes were scraped off from the tissue using a sharp scalpel. The scalpel loses its sharpness easily when vigorously scraping skin, so it has to be changed quite often. The cells and the solution were transferred into a test tube after scraping. 2-4 skin pieces were scratched per test tube. Cell solution was put into a centrifuge at a speed of 1000 rpm for 10 minutes. The supernatant was poured away, and the cells in the bottom of the tube were mixed with the normal keratinocyte growth medium (KGM-2) with the addition of 2% inactivated, decalcified FCS. The solution was then transferred to a culture plate. Cells were incubated in normal culture conditions for at least three days. Next, the medium was changed with the addition of 2% inactivated decalcified FCS. After the first medium change, the medium was changed at most

every second day (depending on the density of the culture). Inactivated decalcified FCS was no longer used after the first medium change.

In addition to keratinocytes, the primary cell culture contains several other cell types, such as fibroblasts and melanocytes. However, these cells disappear during passaging. Keratinocytes can grow in clusters or in tight and sharply demarcated sheets on the bottom of the culture plate. When cultured in low calcium concentration, the keratinocytes stay in undifferentiated stage and can be passaged. The differentiation and formation of intercellular junctions begin when the calcium concentration of the culture medium is elevated. In this study, calcium concentration in low calcium medium was 1.2 mM and in the high calcium medium the concentration was 1.8 mM.

4.2.2 Methods of protein analyses

- Indirect immunofluorescence labeling used in I-III
- Avidin-biotin labeling used in I
- Western analysis used in II, III

4.2.3 Microscopy

- Zeiss AxioImager M1 microscope equipped with AxioCam ICc3 and Axio Vision Release 4.8 software (used in II-III)
- Zeiss LSM 510 META confocal microscope and LSM 3.0 software (used in I-II)
- Leica TCS SP5 stimulated emission depletion (STED) (used in II-III)

4.2.4 SiRNA-transfection (II, III)

Normal human keratinocytes were transfected with two different SPCA1 siRNAs: SASI_Hs01_00044646 (siRNA 46) (sense 5′ GUGAAUUACCAGUCAGUGA, antisense 5‘UCACUGACUGGUAAUUCAC), SASI_Hs01_00044645 (siRNA 45) (sense 5′ GGUAUAAUAGGAAUCAUCA, antisense 5‘UGAUGAUUCCUAUUAUACC) (Sigma, St Louis, MO, USA) (used in II) and for ZO-1 Hs_TJP1_7 (Qiagen, Venlo, Netherlands) (used in III). p38 alpha and delta isoforms were inhibited in normal keratinocytes and squamous cell carcinoma cells using siRNAs Hs_MAPK13_5 (sense 5’-GGAGUGGCAUGAAGCUGUATT-3’, antisense 5’-UACAGCUUCAUGCCACUCCGG-3’) and Hs_MAPK14_6 (sense 5’-GAGAACUGCGGUUACUUAATT-3’, antisense 5’-UUAAGUAACCGCAGUUCUCTG-3’) (HP Genome wide siRNA, Qiagen, Venlo, Netherlands) (used in III). Negative control siRNA (cat no 1027310; Qiagen, Venlo Netherlands) was used as control (Used in II-III). The transfection was performed using SiLentFect Lipid reagent (Bio-Rad, Hercules, CA), and the transfection medium contained siRNA at concentration of 75 nM.

4.2.5 Adenoviral cell transductions (III)

Recombinant adenoviruses for flag-tagged p38δ (RAdp38δ), p38α (RAdp38α), dominant-negative mutants of p38δ (RAdp38δAF), p38α (RAdp38αAF), and constitutively active MKK3b (RadMKK3bE) were kindly provided by Dr. Jiahuai Han (Scripps Research Institute, La Jolla, CA). Recombinant adenovirus RAdLacZ, which contains the *Escherichia coli* β-galactosidase gene under control of the CMV IE promoter was kindly provided by Dr. Gavin W. G. Wilkinson (University of Cardiff, UK).

4.2.6 p38 MAPK inhibitors (III)

Normal human keratinocytes and squamous cell carcinoma keratinocytes were treated with p38 MAPK inhibitors SB203580 (Calbiochem, Darmstadt, Germany) and BIRB796 (Axion Medchem, Groningen, The Netherlands).

4.2.7 RNA analyses (qPCR) (II)

RNA was isolated from parallel cultures to those grown for protein analyses using RNeasy Mini Kit (cat no 74104, Qiagen). Complementary DNA (cDNA) was synthesized using RT-PCR Kit (Thermo Fisher Scientific/Finnzymes Vantaa, Finland). KAPA Probe fast qPCR Kit (KK4706, Kapa Biosystems, Boston, MA, USA) was used for qPCR. Oligonucleotides were purchased from Oligomer, Helsinki, Finland. Primers used were Universal ProbeLibrary primers number 4, 56, 21, 66, 47, 77. The PCR reactions were carried out at the core facility of Turku Centre for Biotechnology, Turku, Finland.

4.2.8 Microarray analyses (II)

Keratinocytes of third to fifth passages were used for microarray expression analyses. Cells grown to 40–60% confluency were harvested for RNA isolation at time points of 0, 0.5, 4, 12, 24 and 48 h after addition of high calcium medium. Agilent whole human genome microarray (G4112A; Agilent Technologies, Palo Alto, CA, USA) was used as the microarray platform. The Agilent platform used yielded two-channel data, in which the expression in high calcium medium in each time point was compared with the mRNA levels in the low calcium medium at the same time point. In log2-transformed data, value 1 thus corresponds to twofold change.

4.2.9 Measurements of epidermal barrier

TEWL measurement (I)

The VapoMeter with a closed cylindrical chamber (Delfin Technologies Ltd, Kuopio, Finland) (used in I). TEWL was measured from lesional skin of nine patients with Hailey-Hailey disease and eight patients with Darier´s disease.

TEER (II)

EVOM voltohmmeter (World Precision Instruments, Hertfordshire, UK) (used in II). Six to nine replicates of each condition in two different keratinocyte lines were measured in 24 hours after transfection and up to 3 days.

4.2.10 Statistical analyses (II, III)

The statistical analysis for western blots was made using two-tailed, paired Student´s t-test. P-values less of 0.05 or less were considered as statistically significant. All the tests and calculations were made using IBM SPSS Statistics 21 (Chicago, IL).

5. RESULTS

5.1 The localization of intercellular junction proteins in the perilesional epidermis of Darier´s disease and Hailey-Hailey disease patients shows normal distribution (I)

We studied the distribution of selected tight junction, adherens junction and desmosomal components in order to see how the diseases affect them. The distribution of intercellular junction proteins was studied in the skin samples of eight Darier´s disease patients and nine Hailey-Hailey disease patients. The paraffin embedded skin biopsies were immunolabeled using Avidin-Biotin method for paraffin-embedded tissue samples and the frozen specimens were immunolabeled using indirect immunofluorescence method. Immunolabeling of the skin samples showed that the localization of tight junction proteins: claudin-1; claudin-4; ZO-1; and occludin in the perilesional Hailey-Hailey disease and Darier´s disease epidermis did not differ from the localization of these proteins in normal human epidermis. We also studied the localization of desmosomal component desmoplakin and adherens junctions components E-cadherin and betacatenin. Their localization was also similar to normal epidermis. (I, Fig. 2)

5.2 The tissue distribution of intercellular junction proteins in the acantholytic areas of Hailey-Hailey disease patients (I)

The tissue distribution of intercellular junction proteins was studied in detail using Hailey-Hailey disease samples on lesional skin areas. In the lesional Hailey-Hailey skin areas, the tissue distribution of ZO-1 expanded to the acantholytic cells of spinous cell layer in the epidermis. Desmoplakin was located inside the acantholytic keratinocytes suggesting that the formation of desmosomes was impaired or the desmosomes were dissociating. In the stratum granulosum, claudin-1 and desmoplakin were co-localized. E-cadherin was also present in the intercellular contact sites of the stratum granulosum and some acantholytic keratinocytes had E-cadherin in their intercellular contact sites. Tight junction proteins, claudin-1 and ZO-1, persisted in the cell-cell contact sites of acantholytic keratinocytes. Betacatenin was located inside the keratinocytes in the acantholytic cells but it was also discovered on cellular periphery. (I, Fig. 3)

5.3 The transepidermal water loss in Darier´s disease and Hailey-Hailey disease patients is increased in the lesional skin areas (I)

Transepidermal water loss (TEWL) was measured in order to study the functions of the intercellular junctions and epidermal permeability barrier *in vivo*. Nine patients with Hailey-Hailey disease and eight patients with Darier´s disease were included. TEWL was measured from lesional skin area and compared to the results from apparently healthy abdominal skin areas of the same patient. In Hailey-Hailey patients, the

lesional areas were axillary (6 patients), groin (1 patient), leg (1 patient) and chest (1 patient). In Darier´s disease patients, the lesional areas were in chest skin. Transepidermal water loss was increased in the lesional skin areas up to three and four-fold in both Darier´s disease and Hailey-Hailey disease patients as a sign of an impaired epidermal barrier function. In apparently normal skin areas, the transepidermal water loss was comparable to the transepidermal water loss of the normal control skin. (I, Fig. 1)

5.4 The optimization of keratinocyte culture method (II)

We used normal keratinocytes so that we could create a disease model and employ siRNA silencing. Normal keratinocytes have limited numbers of divisions and are expensive, so we cultured the cells ourselves. When initiating the study using keratinocytes, we had to optimize the culture method. Our method was based on a serum-free cultivation method previously described (Boyce & Ham 1983; Boyce & Ham 1985). After series of trials, we optimized their method. Vigorous scraping and having a scalpel sharp enough were the most crucial improvements to the basic method. The stem cells cannot be separated from the basement membrane and neighboring cells without optimal enzyme digestion combined with enough mechanical force. Finally, every skin sample obtained from the patients gave rise to 5-7 passages of keratinocytes.

5.5 The mRNA levels of the junctional proteins in differentiating keratinocytes (II)

Increased extracellular calcium concentration causes differentiation in cultured keratinocytes (Hennings et al. 1980; Yuspa et al. 1989). A whole genome array was used in order to identify the changes in gene expression during the differentiation of keratinocytes. The mRNA levels of intercellular junction proteins were analyzed in order to detect changes related to the differentiation process. The mRNA levels of selected intercellular junction components in normal human keratinocytes of third to fifth passage were studied using Agilent whole genome cDNA microarrays. Keratinocytes with 40-60% confluent cultures were harvested at time points 0, 0.5, 4, 12, 24, 48 hours after addition of calcium. The mRNA levels of tight junction components claudin-1, ZO-1 and occludin showed a rise during the first 4-24 hours when switched to high calcium concentration after which the levels decreased. Desmoplakin and E-cadherin followed the same pattern. Betacatenin had its own expression pattern: its levels decreased during 48 hours. (II, Fig. 3)

5.6 Claudins 1 and 4 are regulated by ATP2C1 in cultured keratinocytes (II)

The keratinocytes of patients with Hailey-Hailey disease are hard to obtain because the disease is rare and the keratinocytes do not grow well in cell cultures. Therefore, other ways were needed in order to model this disease. We decided to create a model of

Hailey-Hailey keratinocytes using normal human keratinocytes. The expression of SPCA1 which is a calcium and manganese pump located in the Golgi apparatus, was inhibited using siRNA method. The inhibition of ATP2C1 gene was demonstrated using the Western analysis method. (II, Fig. 1) The effects of the inhibition were also analyzed at the mRNA level using qPCR method. The inhibition by siRNA was almost complete. A clear decrease of SPCA1 protein was shown by Western analysis. The mRNA levels of ATP2C1 gene in SPCA1 inhibited cells were significantly decreased as shown by qPCR. After the successful inhibition of ATP2C1 gene, the normal human keratinocytes were used as a model of the keratinocytes of patients with Hailey-Hailey disease. Expression of selected intercellular junction proteins ZO-1, occludin, betacatenin, E-cadherin, desmoplakin, desmocollin 2/3, and desmogleins -1, -2 and -3 and plakoglobin was then studied using Western analysis. Beta-actin and GADPH were used as loading controls. (II, Fig. 2)

A main finding was that the silencing of the ATP2C1 in normal human keratinocytes leads to overexpression of tight junction proteins claudins-1 and -4 already in low extracellular calcium concentration. We also detected a rise in occludin, ZO-1 and betacatenin concentrations in some of the keratinocytes cultured in low calcium concentration however the result was not statistically significant. In other studies of intercellular junction proteins, the increase in expression was detected only after addition of calcium. The control keratinocytes showed a similar expression pattern: the amounts of intercellular junction proteins elevated after increases in calcium concentrations. (Figure 15)

These results showed that inhibition of ATP2C1 did not have a significant effect on studied desmosomal proteins desmoplakin, desmogleins -1, -2 and -3, plakoglobin or tight junction proteins ZO-1, or occludin or adherens junction proteins E-cadherin or betacatenin (Figure 15). We also used qPCR to study mRNA levels of certain junction proteins. The mRNA levels of claudin-1 increased 145% and 247% in low calcium cultures and 229% and 244% in high calcium concentration cultures when using two different siRNAs. In contrast, the levels of ZO-1 decreased 73% and 46% in low calcium cultures and 51% and 23% in high calcium cultures with two siRNAs. Other studied proteins (betacatenin, desmogleins -1 and -2 and desmoplakin) did not show any changes when compared to controls.

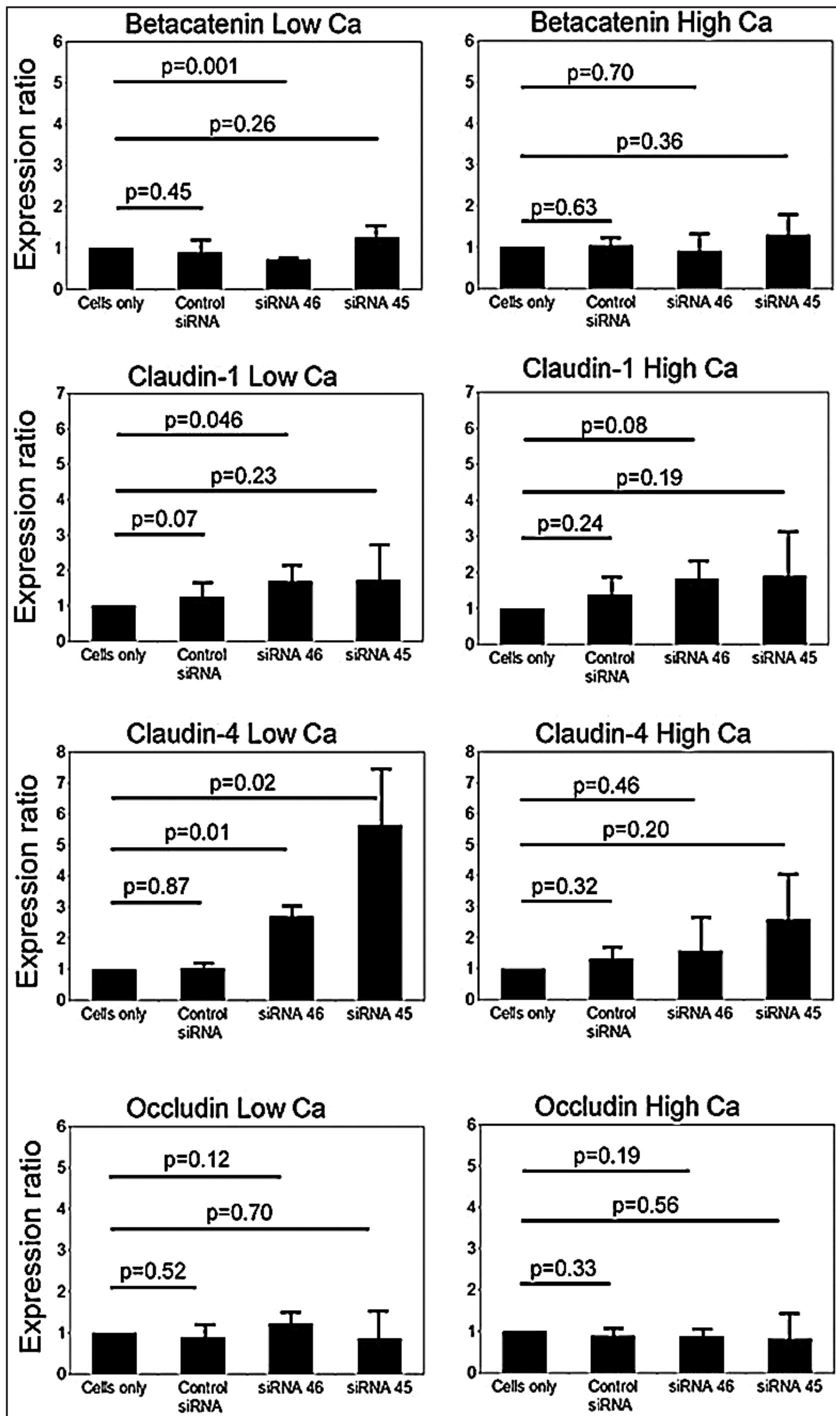

Figure 15 Protein levels of selected intercellular junction proteins after siRNA inhibition of ATP2C1. A significant increase in the expression of claudin-1 and -4 was seen in keratinocytes cultured in low calcium concentration.

5.7 The changes in the formation of intercellular junctions in SPCA1-inhibited keratinocytes (II)

Keratinocyte cultures were also studied to detect the changes in intercellular junctions after SPCA1 inhibition. Indirect immuno-fluorescence labelings were visualized using confocal and STED microscopy. (II, Fig. 4) Using a STED microscope, which selectively reduces fluorescence, provides better resolution than can be obtained than using confocal microscope. Microscopy supplements the results from Western analyses since the Western analyses only show the average level of proteins in the cell culture. The indirect immuno-fluorescence method shows the distribution of proteins in the cells and also the differences between single cells.

The results showed that there are differences between the SPCA1 inhibited keratinocytes and normal control keratinocytes. All studied junctions (tight junctions, adherens junction and desmosomes) were formed normally (Figure 16). The localization of desmoplakin and desmoglein-3 in the desmosomes was, however, delayed in the inhibited cells during the first 2-4 h (II, Fig. 4 (a)-(d′)). Normal looking desmosomes were formed by 24-hours and SPCA1-inhibited keratinocytes showed no difference when compared to control keratinocytes (Figure 16).

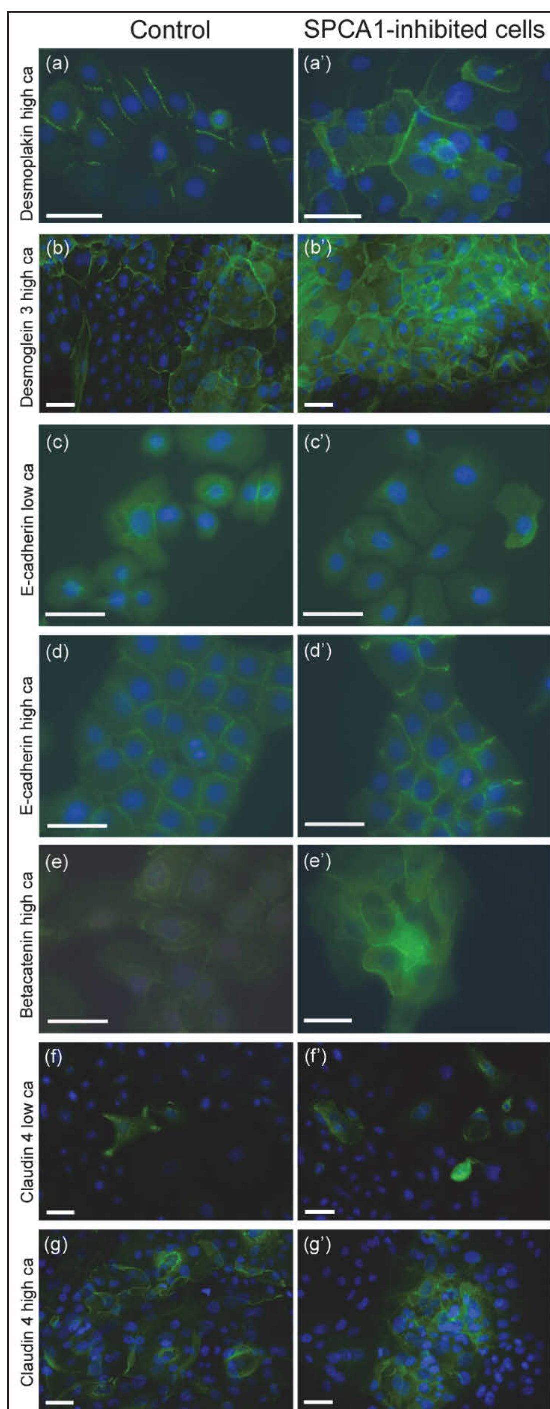

Figure 16 Intercellular junction proteins in control keratinocytes and SPCA1 inhibited cells cultured for 72h.

More intracellular adherens junction protein, betacatenin, was observed in SPCA1 inhibited cells as shown in Figure 16 (e) and (e´). The changes were only in the localization, not in the expression of betacatenin, since according to Western analyses, its protein level did not differ from those of control cells. This suggests that its translocation to the cellular borders might be impaired. More of the tight junction protein occludin was seen inside the keratinocytes in SPCA1 inhibited cells cultured in low calcium concentration. SPCA1 inhibited keratinocyte cultures showed more claudin-4 positive cells than control cultures as seen in Figure 16 (f). Combined with the increased expression of claudin-4, as demonstrated by Western analyses, this suggests that SPCA1-inhibited keratinocytes might have intracellular pools of claudin-4. ZO-1 and occludin formed zipper-like structures when observed with STED or a confocal microscope.

5.8 Transepithelial electrical resistance in the SPCA1-inhibited keratinocytes (II)

After the analyses of protein levels and the localization of intercellular junction proteins, we formed a new hypothesis: what happens to the epidermal permeability barrier after SPCA1 inhibition? The epidermal permeability barrier is improved when tight junctions are formed in the keratinocyte cultures. This also increases the electrical resistance in cell cultures (Yuki et al. 2007). Therefore, measurements of transepithelial electrical resistance can be used to measure the epidermal permeability barrier. We expected that transepithelial electrical resistance would rise in SPCA1-inhibited keratinocytes since the expression of two tight junction protein claudins-1 and -4 was increased. Measurements of transepithelial electrical resistance (TEER) were carried out using two different cell lines and six to nine replicates of each condition in order to see the effects of SPCA1 inhibition on the epidermal barrier function. The measurements were made in 24 hours after transfection and once a day up to three days. The measurements showed a 152% increase in the electrical resistance in SPCA1-inhibited cells cultured in low calcium concentration medium for 24 hours. No significant change was observed in keratinocytes cultured in high calcium concentration medium.

5.9 p38 delta Mitogen-Activated Protein Kinase signaling regulates the expression of tight junction protein ZO-1 in normal human keratinocytes (III)

p38 alpha and delta are active isoforms of p38 in the human keratinocytes (Junttila et al. 2007). When this study was initiated, the effects of p38 on the intercellular junctions had not been widely studied. The regulation of intercellular junction proteins by p38 signaling in normal human keratinocytes was studied using cultured keratinocytes. The expression of p38 delta or alpha was inhibited using siRNA method, adenovirally delivered mutants or chemical inhibitors (SB203580 or BIRB796). In the high extracellular calcium concentration, the inhibition of p38 delta reduced the amount of tight junction protein ZO-1 as demonstrated by Western analyses. The

expression of other studied junction proteins (claudin-1, occludin, betacatenin, E-cadherin and desmoplakin) remained comparable to that of control cells in Western analyses. Indirect immunofluorescence staining of cell cultures showed similar results as Western analyses: only ZO-1 showed changes with its amounts being reduced in p38 delta inhibited keratinocytes. The activation of p38 signaling pathway was indicated by p-creb upregulation. (III, Fig. 1 2, 3, 4)

5.10 The inhibition of tight junction protein ZO-1 does not affect the other tight junction proteins in normal human keratinocytes (III)

After the observation that p38 delta regulates the expression of ZO-1, the next step was to study how the inhibition of ZO-1 affects other tight junction components. (III, Fig. 5) The inhibition of tight junction protein ZO-1 did not affect the levels of other tight junction proteins studied (occludin, claudin-1 and claudin-4) in normal human keratinocytes. This was demonstrated by Western analyses and indirect immunofluorescence stainings. These results suggest that these tight junction proteins are differently regulated which is in line with our previous findings with SPCA1 inhibited keratinocytes and immunolabeling of skin biopsies (I and II).

5.11 The p38 signaling regulates the expression of tight junction protein ZO-1 and ZO-2 in keratinocytes derived from human squamous cell carcinoma (III)

The regulation of intercellular junction proteins by p38 signaling was studied in cultured squamous cell carcinoma derived keratinocytes in order to study the effects of abnormal differentiation on the junctions. The expression of p38 alpha or delta was silenced using siRNA method in squamous cell carcinoma derived keratinocytes cultured in medium with high calcium concentration. The method was the same as the one used with normal keratinocytes previously. It was discovered that the expression levels of ZO-1 were decreased with p38 alpha and delta inhibition in Western analyses. The levels of claudin-1, claudin-4 and ZO-2 in the inhibited cells showed only a slight reduction when compared to control cells. (III, Fig. 6 b)

5.12 The distribution of tight junction proteins in squamous cell carcinoma derived keratinocytes (III)

The distribution of intercellular junction proteins was also studied in cell cultures. It was discovered that ZO-1 and ZO-2 were located in sparse and less continuous tight junctions in cultures where the expression of p38 delta was inhibited. The inhibition of p38 delta or alpha did not affect the distribution of other intercellular junction proteins studied (desmoplakin, E-cadherin, claudin-1, claudin-4 and betacatenin). (III, Fig. 6 a)

6. DISCUSSION

When we started this study, our vision was to investigate the regulation of intercellular junctions by calcium ATPases and p38 signaling. The results showed that the main changes induced were in tight junctions. The dynamics of tight junctions in the human epidermis during acantholytic process are unclear. This doctoral thesis provides new information and insights about the interactions between junction proteins - with the focus on tight junctions in normal human epidermis. Our results suggest that the tight junction protein, ZO-1, is differently regulated than other tight junction proteins.

6.1 The localization of tight junction proteins in the perilesional epidermis of patients with Hailey-Hailey disease or Darier´s disease (I)

When this study was initiated, we expected to find changes in the distribution of intercellular junction proteins in the perilesional skin areas of the patients with Darier's or Hailey-Hailey diseases.

The localization of tight junction proteins in normal human epidermis is previously described. Tight junctions are located in the granular layer (Pummi et al. 2001; Morita & Miyachi 2003; Brandner et al. 2002). The studies on cultured keratinocytes show that the formation of tight junctions is linked to the elevation of extracellular calcium concentration (Yuki et al. 2007). Furthermore, the measurement of the calcium concentration in the perilesional epidermis of Darier's disease patients shows that the calcium concentration is decreased (Leinonen et al. 2009). These findings led us to study if the perilesional, apparently, healthy skin of patients with Darier´s or Hailey-Hailey disease has abnormalities in the intercellular junction proteins, expecially in tight junctions. The hypothesis was that tight junction proteins might locate differently in the apparently normal skin areas since impaired differentiation causes changes in distribution of tight junction proteins (S. Peltonen et al. 2007).

The localization of the key tight junction proteins was studied using Darier´s disease and Hailey-Hailey disease skin biopsies from perilesional areas. Surprisingly, the localization of tight junction proteins claudin-1, claudin-4, occludin and ZO-1 was similar to their localization in the normal human epidermis. Thus, the changes in calcium concentration and epidermal differentiation in these diseases did not affect the expression and localization of these tight junction proteins on tissue level in epidermis. The treatments of Darier´s disease and Hailey-Hailey disease usually aim at the regeneration of skin. The normal localization of the key junction proteins in the skin of patients with these diseases suggests that the healing skin can form normal junctions at places where junctions previously were missing.

6.2 The localization of tight junction proteins in the lesional epidermis of Hailey-Hailey disease and Darier´s disease patients (I)

The localization of adherens junction proteins and desmosomal proteins in the lesional skin areas of patients with Darier´s disease or Hailey-Hailey disease is described (Hakuno et al. 2000). However, the localization of tight junction proteins in the lesional skin is unclear. In a study where the calcium concentration of lesional skin areas of Hailey-Hailey disease patients and Darier´s disease patients was measured, it was observed that the calcium concentration in the basal layer is lower than in normal human epidermis (Leinonen et al. 2009). In order to study the consequences of this abnormally low calcium concentration to the epidermal tight junctions we immunolabeled skin samples of patients with Darier´s disease or Hailey-Hailey disease with antibodies against several intercellular junction proteins. Some samples were double labeled to check for possible co-localizations. It was discovered that the localization of ZO-1 was expanded to the keratinocytes of the acantholytic stratum spinosum in Hailey-Hailey disease. A similar finding was described in the edges of healing blisters and psoriatic epidermis (Pummi et al. 2001; S. Peltonen et al. 2007; Malminen et al. 2003). The reason for this spreading of ZO-1 to the stratum spinosum in these conditions might be due to the abnormal differentiation of keratinocytes or disturbances in calcium metabolism since calcium concentration is linked to the formation of tight junctions. It is also possible that combined effect of these two factors is the reason for the abnormal distribution of ZO-1. Other tight junction proteins studied (occludin and claudins -1 and 4) did not show any changes in their localization when compared to normal skin. This suggests that ZO-1 is differently regulated than other tight junction proteins. The finding was later reinforced in the second and third studies of this thesis.

6.3 The transepidermal water loss in the lesional skin areas of patients with Darier´s disease or Hailey-Hailey disease (I)

Tight junctions are important in maintaining the normal epidermal permeability barrier and restricting excess water evaporation as shown in mouse models where different tight junction proteins are abnormal (M. Furuse et al. 2002; Turksen & Troy 2002; Troy et al. 2005). In these mice models, the abnormal tight junctions cause dysfunction in the permeability barrier and the water evaporation from inside the skin is increased (M. Furuse et al. 2002; Turksen & Troy 2002; Troy et al. 2005). These results prompted us to investigate the consequences of impaired intercellular junctions in the lesional skin of patients with Darier´s disease or Hailey-Hailey disease using measurements of transepidermal water loss. This method gives information of the function of the permeability barrier of the skin *in vivo*. These results showed that transepidermal water loss was increased three- to four-fold in the lesional skin areas of the patients when compared to healthy skin area of the same patients. The skin biopsies, however, showed that the tight junction components needed for functional tight junction were still present in the granular layers of these patients. This suggests that the increased water evaporation is due to breaking of the blisters in the lesional

skin not due to loss of tight junction proteins. The impaired barrier function of epidermis explains why secondary infections are common in patients with Darier´s disease or Hailey-Hailey disease. The impaired barrier function also provides a possibility to deliver medical subtances to the blistering area easier. In future this possibility could be used to find new solutions that affect the pathogenesis of these diseases.

6.4 ATP2C1 gene regulates the expression of tight junction proteins claudin-1 and claudin-4 (II)

Mutations in the ATP2C1 gene encoding calcium/manganese pump SPCA1 cause Hailey-Hailey disease (Sudbrak et al. 2000; Z. Hu et al. 2000). Acantholysis in the suprabasal level is a typical feature, vesicle formation or cracking of epidermis are also seen (Norgett et al. 2000; Hashimoto et al. 1995). The acantholysis is thought to be a result of disintegration of intercellular junctions (S. M. Burge & D. R. Garrod 1991; Hakuno et al. 2000; Leinonen et al. 2009). In the first study of this thesis, we demonstrated that all key junctional proteins are present at the tissue level. After that, we were interested to study what happens at the cellular and protein level. We expected to find changes in the protein amounts that might underscore the pathogenesis of the disease. We decided to focus on Hailey-Hailey disease since acantholysis occurs in Hailey-Hailey disease, while in Darier´s disease, the abnormal differentiation causes more complex changes at the tissue level. We used a keratinocyte culture with ATP2C1 gene knocked out to model keratinocytes of Hailey-Hailey disease patients. The inhibition level was significant. We observed that in the SPCA-inhibited keratinocytes, the levels of tight junction proteins, claudin-1 and claudin-4, were elevated already at low calcium concentration. Other intercellular junction proteins, studied in the SPCA1 deficient keratinocytes, protein amounts increased only after the increase of calcium concentration. These studied proteins were: ZO-1, occludin, betacatenin, E-cadherin, desmoplakin, desmocollin 2/3, and desmogleins -1, -2 and -3 and plakoglobin. In the control keratinocytes (untreated and negative siRNA), the amounts of every protein studied were elevated only after addition of calcium. This suggests that abnormal calcium concentration in the SPCA1 inhibited keratinocytes might be an explanation for our observation since in the cell cultures, tight junctions are only formed after addition of calcium (Yuki et al. 2007). These results also demonstrated that other tight junction proteins are differently regulated because the inhibition of SPCA1 only affects claudins -1 and- 4. This is a novel discovery.

6.5 The formation of desmosomes is delayed in SPCA1 inhibited keratinocytes (II)

The exact mechanism leading to acantholysis in Hailey-Hailey disease is not known. Desmosomes are the main junctions linking keratinocytes to each other (Foggia & Hovnanian 2004). There are known interactions between different intercellular junction types, for example, formation of desmosomes needs the presence of adherens junctions (Desai et al. 2009). We investigated the localization of desmosomal components and

components of adherens and tight junctions in differentiating SPCA1-inhibited keratinocytes in culture using confocal laser microscopy and STED microscopy. The differentiation was initiated using elevations in calcium concentrations. A delayed formation of desmosomes was observed in SPCA1-inhibited keratinocytes. The translocation of desmoglein-3 and desmoplakin to desmosomes was delayed but eventually the desmosomes are formed and by 24 hours, there was no difference when compared to control keratinocytes. The formation of adherens junctions or tight junctions was not altered in SPCA1-inhibited keratinocytes. This observation suggests that the loss of function in the ATP2C1 gene leads to a delay in the formation of desmosomes in keratinocytes. This may be due to abnormal calcium concentration in keratinocytes, which can disturb the normal formation of desmosomes. There was also more intracellular desmoplakin, desmoglein-3, occludin, claudin-4 and betacatenin in SPCA1-inhibited cells that suggests that these proteins are not transported to their normal locations in the same manner as in control cells.

6.6 The regulation of junction proteins in differentiating keratinocytes (II)

The keratinocytes undergo continuous mitoses in the basal layer of epidermis where the skin regenerates (Marieb & Hoehn 2006). The differentiation process of keratinocytes proceeds in the stratum spinosum to the surface layers (Kirschner & Brandner 2012). This differentiation can be replicated by addition of calcium in cultures.

Whole human genome DNA microarrays were used to study the changes in the expression of genes for junction proteins during the differentiation of keratinocytes. The expression profiles of desmoplakin, claudin-1, occludin, ZO-1 and E-cadherin followed the same pattern: the mRNA levels increased during the first 24 hours and after that the levels started to decrease. These findings were in agreement with previous findings in cell cultures where keratinocytes differentiate and form junctions soon after addition of calcium (Hennings et al. 1980; Yuspa et al. 1989). Betacatenin followed its own expression pattern which differed from other studied junction proteins. It showed a decrease in its expression during 48 hours. This suggests that adherens junctions may follow different pattern than other intercellular junction types studied here. It is also possible that already available betacatenin protein is used to form adherens junctions. The result that the expression of adherens junction component E-cadherin did not differ from the expression patterns of other studied junction proteins also indicates to that.

6.7 p38 delta signaling regulates tight junction protein ZO-1 in normal human keratinocytes (III)

In the human keratinocytes α and δ are the most common isoforms of p38 (Junttila et al. 2007). p38 delta regulates the expression of involucrin and thus has a role in differentiation of keratinocytes and it has also been suggested to mediate the apoptosis in keratinocytes (Eckert et al. 2004; Kraft et al. 2007). Since differentiation includes

formation of intercellular junctions and studies on p38's role in intercellular junctions have not been made, we decided to study their connections. We studied the p38 signaling in normal keratinocytes using siRNA or adenoviral mediated inhibition or pharmacological inhibition of p38 isoforms alpha and delta. After p38 delta inhibition, in differentiating keratinocytes, the levels of ZO-1 also decreased. This observation suggests that p38 delta regulates the expression of ZO-1, which links together the regulation of differentiation in keratinocytes and tight junctions.

6.8 The inhibition of ZO-1 does not affect other tight junction components (III)

After the observation that ZO-1 is regulated by p38 delta in normal keratinocytes, we studied how ZO-1 regulates other tight junction components. The intracellular plaque protein ZO-1 connects the integral membrane proteins to the intracellular actin filament cytoskeleton (Pummi et al. 2001; Schneeberger & Lynch 2004). Each class of tight junction proteins is able to regulate other tight junction components (directly or indirectly through mediators) (Farkas et al. 2012).

The expression of ZO-1 was silenced using the siRNA method and the expression of occludin, claudin-1 and claudin-4 was studied. The result was that the expression of these tight junction components did not change after the inhibition. This suggests that the tight junction component, ZO-1, is differently regulated than other tight junction proteins.

6.9 p38 signaling regulates tight junction proteins ZO-1 and ZO-2 in squamous cell carcinoma derived keratinocytes (III)

The findings in normal human keratinocytes prompted us to study the effects of p38 signaling on the intercellular junction proteins of squamous cellular carcinoma derived keratinocytes. p38 signaling has various effects in abnormal keratinocytes. For instance, p38 signaling has been shown to participate in induction of apoptosis in UVB treated keratinocytes in culture (Nys et al. 2010). The effects of p38 signaling for the intercellular junctions have, however, not been elucidated. The effects of p38 delta inhibition in squamous cellular carcinoma derived keratinocytes were similar to the effects detected in normal keratinocytes.

The amount of ZO-1 was decreased in cells transfected with p38 alpha or delta specific siRNA. It was also discovered that the amounts of ZO-2 and claudins 1 and 4 were slightly decreased after p38 inhibition. These results demonstrate that the regulation of tight junctions by p38 signaling in squamous cell carcinoma differs from the p38 regulation in normal human keratinocytes. In the squamous cell carcinoma, p38 alpha signaling played a key role in the regulation of tight junctions.

7. SUMMARY AND CONCLUSIONS

The present study investigated the regulation of epidermal tight junctions by ATPases and p38 signaling. Tight junctions of epidermis were studied during acantholysis and in apparently healthy skin in Darier´s disease and Hailey-Hailey disease. Skin biopsies from patients were labeled using antibodies recognizing intercellular junctions proteins. A keratinocyte culture model of Hailey-Hailey disease was developed by silencing the expression of ATP2C1 gene with siRNA method from normal human primary keratinocytes. Using this model, changes in protein and mRNA expression levels and localizations of intercellular junction proteins were studied. The regulation of intercellular junctions by p38 signaling was also studied using siRNA method and pharmacological inhibitors. After the inhibition, the protein levels and localization of intercellular proteins were studied in normal human primary keratinocytes or in human squamous cell carcinoma cell lines. The following conclusions were made on the basis of the results of the present study:

1. The tissue distribution of tight junction proteins claudin-1, claudin-4, ZO-1 and occludin in the perilesional epidermis of Hailey-Hailey patients and Darier´s disease patients did not differ from the distribution in the normal human epidermis. In the lesional Hailey-Hailey disease skin, the distribution of ZO-1 was spread into the acanthotic stratum spinosum. In the acantholytic keratinocytes, intracellular desmoplakin occurred while claudin-1 and ZO-1 persisted in the plasma membranes even when there were no neighboring cells. This indicates that these junction types are differently regulated.

2. The ATP2C1 gene regulates the tight junction proteins claudin-1 and claudin-4 in non-differentiating keratinocytes, *in vitro*.

3. p38 delta affects the tight junction protein ZO-1 in differentiating normal human keratinocytes, *in vitro.*

4. p38 modulates the tight junction protein ZO-1 in squamous cell carcinoma cells *in vitro*.

5. Junctional proteins are differently regulated. Even junction proteins related to the same junction type can have different regulation.

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