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LEENA RAUHALA

EFFECTS OF UVB-EXPOSURE, EXTRACELLULAR NUCLEOTIDES AND BETAINE ON KERATINOCYTE BIOLOGY AND HYALURONAN METABOLISM

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ABSTRACT

Skin is constantly subjected to the adverse effects of ultraviolet (UV) radiation. The outermost surface of the epidermis, which contains layers of tightly packed keratinocytes, is crucial in protecting an individual against not only UV radiation but also various chemical, physical and biological insults. In mammals, the extracellular matrix glycosaminoglycan hyaluronan is abundantly synthesized between the epidermal cells by three hyaluronan synthases (HAS1-3). Its significance for normal physiology as well as neoplastic changes has slowly emerged.

Various chemical and physical signals regulate the expression of the HAS-enzymes. Of these, the role of ultraviolet radiation has been studied previously, but detailed data on the intracellular signaling cascades involved have been scarce. The role of extracellular nucleotides, which may act as local danger signals, in regulating hyaluronan accumulation is also mostly unexplored. Additionally, the major practical challenge of effectively protecting and moisturizing skin has not been adequately solved. The organic osmolyte betaine has been applied in various formulations, but its specific impacts either alone or in combination with UV or other stressors have not been analyzed comprehensively. In this thesis, the effects of acute UVB exposure, extracellular nucleotides and betaine were tested in different *in vitro* models to study hyaluronan synthesis in stressed keratinocytes. Simultaneously, the regulatory capacity of betaine on keratinocyte metabolism and gene expression was tested.

The results clearly show that hyaluronan synthesis is highly responsive to acute UVB as well as the nucleotides UTP, ATP and their degradation products. Common signaling proteins activated by UVB and the nucleotides include the Ca^{2+}-associated Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) and the mitogen-activated protein kinase p38. Protein kinase C (PKC), extracellular signal regulated kinase (ERK), signal transducer and activator of transcription 3 (STAT3) and cyclic AMP-responsive element-binding protein (CREB) are also involved in regulating *HAS2* expression in response to the nucleotides. Interestingly, betaine is able to modulate some of these effects, although it most clearly influences keratinocyte maturation, with a distinct upregulation of the late differentiation marker keratin 2.

The work provides novel insights on how increased hyaluronan synthesis after exposure to common stressors might function as an adaptive, protective mechanism in epidermal keratinocytes. The results also highlight the modulatory potential of betaine. Understanding these changes at the molecular level may help to develop tools that attenuate potentially pathological changes at the onset.

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Medical Subject Headings: Betaine; Epidermis; Extracellular Matrix; Glycosaminoglycans; Hyaluronic Acid; Keratin-2; Keratinocytes; Nucleotides; Protein Kinases; Signal Transduction; Skin; Ultraviolet Rays

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TIIVISTELMÄ

Iho ja erityisesti sen ylin kerros tiukasti pakkautuneine keratinosyytteineen altistuu jatkuvasti mm. ultraviolettisäteilylle (UV). Tämä kudos suojaa yksilöä paitsi UV:ltä myös erilaisilta kemiallisilta, fysikaalisilta ja biologisilta uhkatekijöiltä. Nisäkkäillä kolme HA-syntaasia (HAS1-3) tuottaa keratinosyyttien väliseen tilaan runsaasti glykosaminoglykaani hyaluronaania (HA), joka vaikuttaa monin tavoin epidermiksen normaaliin fysiologiaan. HA:n roolia myös kudosten pahanlaatuistumisessa on alettu ymmärtää.

Monet kemialliset ja fysikaaliset tekijät säätelevät HAS-entsyymien ilmentymistä. UV-säteilyn osuutta on aiemmin tutkittu, mutta yksityiskohtaista tietoa mm. solunsisäisistä signalointireiteistä on silti niukasti. Solun ulkopuolelle vapautuvien, mm. vaarasignaaleina toimivien nukleotidien roolia HA:n kertymisessä ei myöskään ole juuri aiemmin selvitetty. Lisäksi keskeinen käytännön kysymys ihon kosteuttamisesta ja suojaamisesta on osittain ratkaisematon haaste. Betaiini on kosteutta sitova orgaaninen osmolyytti, jota lisätään jo nyt moniin valmisteisiin. Sen molekyylitason vaikutusmekanismeista geenien ilmentymiseen keratinosyyteissä joko yksin tai yhdessä solustressiä välittävien tekijöiden kanssa ei kuitenkaan ole aiempia kattavia tuloksia. Tässä väitöskirjatyössä tutkittiin betaiinin lisäksi akuutin UVB-säteilyn ja solunulkoisten nukleotidien vaikutuksia keratinosyyttien metaboliaan ja HA-synteesiin. Kattavissa *in vitro* -kokeissa selvitettiin, miten hyaluronaanin synteesi muuttuu stressitekijöille altistuneissa keratinosyyteissä.

Tulokset osoittavat, että HA-metabolia aktivoituu sekä UVB-säteilyn että uridiinitrifosfaatin (UTP), adenosiinitrifosfaatin (ATP) ja niiden hajoamistuotteiden vaikutuksesta. Kiinnostavaa on, että UVB ja nukleotidit aktivoivat osittain samoja signalointiproteiineja. Näihin kuuluvat kalsiumvälitteisesti toimiva CaMKII ja stressikinaasi p38. Lisäksi proteiinikinaasit PKC ja ERK ja transkriptiotekijät STAT3 ja CREB säätelevät *HAS2*-geenin ilmentymistä akuutin nukleotidialtistuksen jälkeen. Betaiini pystyy osittain muokkaamaan näitä vaikutuksia, mutta sen selkein rooli on keratinosyyttien erilaistumisen säätelyssä. Kerrostuville keratinosyyttiviljelmille annettu betaiini lisää huomattavasti oka- ja jyväissoluissa ilmentyvän keratiini 2 -proteiinin määrää.

Kokonaisuudessaan työ auttaa ymmärtämään, kuinka lisääntynyt HA-synteesi voi toimia adaptiivisena, suojaavana mekanismina epidermaalisissa keratinosyyteissä. Näiden muutosten molekyylitason ymmärtäminen voi auttaa kehittämään työkaluja, joilla mahdollisesti patologiset muutokset voidaan pysäyttää jo alkuvaiheessa.

Yleinen Suomalainen asiasanasto: betaiini; hyaluronaani; iho; keratiinit; kinaasit; nukleotidit; soluväliaine; transkriptio (biologia); ultraviolettisäteily

I can do all this through Him who gives me strength.
Phil. 4:13 (NIV)

x

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Kuopio, March 2017

Leena Rauhala

List of the original publications

This dissertation is based on the following original publications:

I **Rauhala L**, Hämäläinen L, Salonen P, Bart G, Tammi M, Pasonen-Seppänen S, Tammi R. Low dose ultraviolet B irradiation increases hyaluronan synthesis in epidermal keratinocytes via sequential induction of hyaluronan synthases *Has1-3* mediated by p38 and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) signaling. *J Biol Chem 288(25):17999-18012, 2013.* **doi: 10.1074/jbc.M113.472530**

II **Rauhala L***, Hämäläinen L*, Dunlop TW, Pehkonen P, Bart G, Kokkonen M, Tammi M, Tammi R, Pasonen-Seppänen S. The organic osmolyte betaine induces keratin 2 expression in rat epidermal keratinocytes – A genome-wide study in UVB irradiated organotypic 3D cultures. *Toxicol In Vitro 30:462-75, 2015.* **doi: 10.1016/j.tiv.2015.09.015**

III **Rauhala L**, Jokela T, Kärnä R, Bart G, Takabe P, Oikari S, Tammi MI, Pasonen-Seppänen S, Tammi RH. Extracellular ATP activates hyaluronan synthase 2 (*HAS2*) in epidermal keratinocytes via $P2Y_2$, Ca^{2+}-signaling, and MAPK pathways. *Submitted.*

IV Jokela T, Kärnä R, **Rauhala L**, Bart G, Pasonen-Seppänen S, Oikari S, Tammi MI, Tammi RH. Human keratinocytes respond to extracellular UTP by induction of hyaluronan synthase 2 expression and increased hyaluronan synthesis. *In press. J Biol Chem, 2017.* [Epub ahead of print]. **doi: 10.1074/jbc.M116.760322**

*Equal contribution

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Contents

Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
ATRA	*All-trans* retinoic acid
BCC	Basal cell carcinoma
BGT-1	Betaine/GABA transporter 1
bHABC	Biotinylated HA binding complex
CaMKII	Ca^{2+}/calmodulin dependent protein kinase II
CD44	Cluster of differentiation 44
CE	Cornified envelope
CREB	cAMP responsive element binding protein
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELSA	Enzyme-linked sorbent assay
ERK	Extracellular signal regulated kinase
GAG	Glycosaminoglycan
GlcNAc	Glucosamine
GlcUA	Glucuronic acid
HA	Hyaluronan
HABC	HA binding complex (formed by HA binding region of aggrecan and link protein)
HaCaT	a spontaneously immortalized, aneuploid human keratinocyte cell line
HAS	Hyaluronan synthase
HE	Hematoxylin and eosin
HPLC	High-performance liquid chromatography
HYAL	Hyaluronidase
IL	Interleukin
JNK	c-Jun N-terminal kinase
K or *KRT*	Keratin
KGF	Keratinocyte growth factor
KO	Knockout
LB	Lamellar body
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor kappa B
NHEK	Normal human epidermal keratinocyte
P1	Purinergic receptors for adenosine
P2X	Purinergic receptors for ATP
P2Y	Purinergic receptors for adenosine and uridine nucleotides
PBS	Phosphate-buffered saline
PKC	Protein kinase C
qRT-PCR	Quantitative real-time PCR
REK	Rat epidermal keratinocyte
RHAMM	Receptor for hyaluronan-mediated motility
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SC	Stratum corneum
SG	Stratum granulosum
SCC	Squamous cell carcinoma
SHAP	Serum-derived hyaluronan-associated protein
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TEWL	Transepidermal water loss
TGase	Transglutaminase
TGF	Transforming growth factor
TJ	Tight junction
UDP	Uridine diphosphate
UTP	Uridine triphosphate
UVA	Ultraviolet radiation A (315-400 nm)
UVB	Ultraviolet radiation B (280-315 nm)
UVR	Ultraviolet radiation

1 Introduction

Skin is our largest organ, comprising up to 5% of body weight in adults. Its outermost layer, the epidermis, functions at the interface of the external and internal environments as a sensory organ and permeability barrier, protecting from outside-in threats such as pathogens, physical and chemical irritants and mechanical strains as well as dehydration (the inside-out barrier).

One of the most physiologically-relevant environmental risk factors for skin is solar radiation; especially the short, high-energy wavelengths in the ultraviolet (UV) region. Of these, ultraviolet radiation B (UVB, 280-315 nm) and ultraviolet radiation A (UVA, 315-400 nm) not only cause direct mutagenesis but they also generate change in the extracellular matrix of the keratinocyte-rich epidermis by altering various signaling pathways (Muthusamy & Piva, 2010). The involvement of ultraviolet radiation (UVR) in the etiology and occurrence of skin cancers is evident and has been delineated in a recent meta-analysis (Xiang et al., 2014). UV can thus be considered an important stressor that activates the protective machinery of the cell, including DNA repair pathways, as well as apoptosis. Another factor affecting the stress responses of keratinocytes is the release of nucleotides such as adenosine triphosphate (ATP) and uridine triphosphate (UTP) and their degradation products into the extracellular space. The extracellular nucleotides act as important messengers or ”danger signals” to neighboring cells, inducing specific purinergic receptors on the plasma membrane. This occurs after processes such as mechanical stimulation and tissue wounding (Yin et al., 2007; Tsutsumi et al., 2009) as well as UVR (Inoue et al., 2007; Takai et al., 2011).

The relatively scant extracellular material of the epidermis is enriched in the linear, non-sulfated, high molecular-weight glycosaminoglycan hyaluronan (HA) that gives mechanical support and regulates cell signaling via various plasma membrane receptors (Toole, 2004; Vigetti et al., 2014b). HA is synthesized in mammals by three different enzymes, the hyaluronan synthases 1-3 (HAS1-3), which reside at the plasma membrane (Weigel & DeAngelis, 2007). It has become increasingly evident that HA synthesis and HAS expression are regulated by a multitude of cues, including growth factors, cytokines, and the precursor sugar balance but also environmental stress signals (Averbeck et al., 2007; Tammi et al., 2011). Specifically, UV radiation is known to affect HA metabolism in keratinocytes (e.g. Averbeck et al., 2007; Kakizaki et al., 2008; Tobiishi et al., 2011).

Notably, the extracellular nucleoside adenosine has been implicated in the regulation of HA metabolism (Grandoch et al., 2013). This establishes a possible link between the UVB-induced release of nucleotides and hyaluronan synthesis responding to cellular trauma. The role of HA metabolism in the early phases of malignant changes has also proven interesting (e.g. Karvinen et al., 2003a; Siiskonen et al., 2013). The processes affected by hyaluronan in the epidermis include proliferation, migration, differentiation and wound healing (Maytin, 2016). The two main degrading enzymes, hyaluronidases 1 and 2 (HYAL1-2), may also be regulated by environmental factors (Kurdykowski et al., 2011), although these enzymes are still fairly enigmatic.

Keratinocytes are also constantly subject to a harsh, dry environment. Betaine (trimethylglycine) is a small, organic metabolite used to regulate osmotic balance by various organisms. The potential of betaine to maintain and improve skin hydration as well as oral mucosal health (Rigano, et al., 2000; Rantanen et al., 2002) has lead to its widespread use as an additive in cosmetics (Rigano et al., 2000). Betaine may also protect against apoptosis in e.g. hyperosmotically challenged corneal epithelial cells (Garrett et al., 2013). Additionally, there is a connection between UV radiation and regulation of osmotic balance, as the uptake of osmolytes as well as the expression of their transporters is increased in keratinocytes in response to UV exposure (Warskulat et al., 2004; 2007).

The existing data prompted the present analyses of the effects of UVB, extracellular nucleotides and betaine in epidermal keratinocytes cultured *in vitro* either as monolayers or a more physiological (3D) epidermal equivalent. The main aim was to clarify, how these agents affect hyaluronan metabolism in keratinocytes of either rat (REK) or human (a spontaneously immortalized, aneuploid human keratinocyte cell line; HaCaT) origin. Additionally, the genome-wide effects of betaine on gene expression and its potential to modulate stress responses in these cells were explored.

The results indicate that acute UVB irradiation rapidly induces *Has* expression and HA synthesis in the REK cells. The effect is not as evident in the epidermal equivalent but both models confirm that UVB is a potent regulator of *Has2* and *Has3* transcription. This response depends on the kinases p38 and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), respectively. Acute treatment of HaCaT cells with UTP and ATP similarly induces *HAS1-3*, although with varying profiles. UTP solely upregulates *HAS2*, but ATP and its degradation products cause a range of stimulatory as well as inhibiting effects with a strong dependence on time. Both nucleotide treatments are also able to induce hyaluronan synthesis. The signaling pathways induced by UVB and the nucleotides have interesting parallels; both Ca^{2+}-related effectors (CaMKII, protein kinase C (PKC), cAMP responsive element binding protein (CREB)) and MAP kinases (p38, extracellular signal regulated kinase (ERK)) are activated by UTP as well as ATP.

Betaine may modulate the effects of ATP on *HAS2* expression, but its main contribution appears to be in the regulation of differentiation, ion channels and certain stress cues evoked by UV. This is exemplified by the distinct induction of keratin 2 and the downmodulation of *Atp2a3*, an endoplasmic reticulum (ER) Ca^{2+} pump that is intensely upregulated by UVB.

In conclusion, this work reveals novel regulatory mechanisms of HA synthesis and *HAS* expression. The data underline the general theme of the involvement of Ca^{2+}-associated pathways and common keratinocyte stress responses with the treatments applied. Tying UVB exposure and nucleotide release together in this setting and delineating their long-term effects on HA synthesis would be an interesting future approach. The results also highlight the potential of betaine as a modulator of keratinocyte differentiation and its ability to reverse the effects of certain environmental stressors. This provides a promising tool to combat the detrimental impacts of UVR.

2 *Review of the literature*

2.1 SKIN, KERATINOCYTES AND EPIDERMAL HOMEOSTASIS

2.1.1 Skin structure and function

Skin structure

Skin is our largest organ both by weight and by area. It consists of three specialized tissue layers: epidermis, dermis and subcutis (reviewed in Young et al., 2014). The epidermis, as the most superficial layer, protects the underlying tissues against dehydration, chemical threats, physical insults (radiation, mechanical stress/friction) and pathogens. The underlying dermis connects the epidermis to the rest of the body via a subcutis, mainly composed of adipose tissue, which is important both as a cushion and as a thermal insulator. Structurally, dermis is a fibroelastic supporting tissue that contains most of the sensory organs and nerves and the dense vasculature and lymphatics of skin, as well as immune cells. The skin also contains various appendages, located mainly in the dermis and subcutis. Hair follicles, sebaceous glands and eccrine and apocrine glands contribute to the function of the organ as a whole. To simplify, the following discussion concentrates on the interfollicular epidermis.

The epidermis itself is composed of several layers of highly specialized epithelial cells, called keratinocytes, at various stages of differentiation (Fig. 1; reviewed by Matsui & Amagai, 2015). In the course of their lives, keratinocytes proliferate, migrate and differentiate according to a strictly regulated program to produce a functionally unique, stratified squamous epithelium. During this time, the keratinocytes make their way from the deepest, basal layer of the epidermis to the surface. There they desquamate to produce a constantly renewing barrier.

The deepest basal layer (*stratum basale*; SB) usually consists of a single layer of cells with high proliferative capacity. According to the traditional view, there are stem cells responsible for the continuous renewal of the tissue as well as transit amplifying (TA) cells that eventually move to the upper layers and commence differentiation (Fuchs, 2008). Beneath the SB, there is a basement membrane, which is a well-developed interface found beneath all epithelia. It links the basal cell keratins via hemidesmosomes and integrins to the underlying dermis (Young et al., 2014). The three layers of the basement membrane (lamina lucida, lamina densa and lamina fibroreticularis) are composed of several proteins including collagens type IV and VII, laminins and nidogen as well as the glycosaminoglycan heparan sulfate (e.g. in perlecan). Disruption of these structures may lead to severe skin blistering diseases such as junctional and dystrophic epidermolysis bullosa (Hashmi & Marinkovich, 2011).

The next layer (*stratum spinosum*; SS) is 3-10 cells thick. Basally, it contains keratinocytes with limited proliferative capacity, but mainly the cells are at early stages of differentiation. The keratinocytes move gradually into the stratum granulosum (SG), where the cells start modifying their plasma membrane and further express the distinctive keratins, the keratin-binding filaggrin and other proteins that become cross-linked and form the outer proteinaceous layer of the cornifying cell. At the same time keratohyalin granules are being

formed. They contain several compounds, including the differentiation-associated keratins, filaggrin and loricrin. The SG can be further divided into three distinctive layers from its upper border towards more basally located strata. SG1, SG2 and SG3 differ in their state of differentiation, such that the gradually changing structure and functions can be assigned to a specific sublayer (Matsui & Amagai, 2015).

At the top, the cells are terminally differentiated corneocytes. They form 15-30 cornified layers embedded in a lipid-rich matrix. This stratum corneum (SC) ultimately sheds off the dead cells with continuous replacement from the deeper strata. The physiological, protective functions of the skin depend heavily on the correct architecture, maintenance and turnover of the stratum corneum (Elias, 2005).

Besides keratinocytes, epidermis harbors antigen-presenting, dendritic immune cells (Langerhans cells), pigment producing melanocytes and free terminal endings of sensory nerves (Young et al., 2014). Together, they form an elaborate unit capable of protecting the underlying tissues and relaying information from the outside in. The epidermis is also particularly well-suited for biological defense; its low pH and secreted antimicrobial peptides act as an integral part of innate immunity (Elias, 2005). Filaggrin also adds to these protective functions as its degradation products are used for hydration as well as absorption of UV radiation (Elias, 2005).

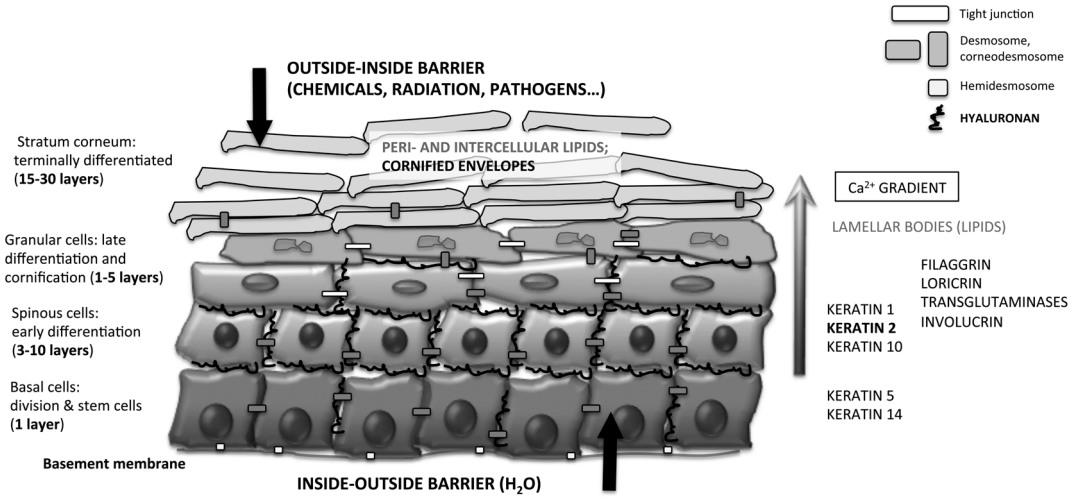

Figure 1. Epidermal keratinocytes and differentiation-related structural components (reviewed in the text). Other cell types (Langerhans cells, melanocytes and nerve endings) have been omitted for simplicity. The exact number of the layers (modified from Tobin, 2006) varies in different areas of the body (thin vs. thick skin).

Markers of epidermal differentiation: coordinated expression of cytoskeletal proteins

Keratinocyte function and differentiation involve the coordinated expression of specialized intermediate filaments called cytokeratins (Moll et al., 2008) and adhesion/signaling molecules such as integrins (Margadant et al., 2010). These bring about the typical rigid, highly cohesive phenotype necessary for a protective epithelium. Keratinocytes are particularly rich in specialized plasma membrane junctional complexes including tight junctions, gap junctions, adherens junctions, desmosomes, hemidesmosomes and corneodesmosomes (Cozzani et al., 2001; Matsui & Amagai 2015). Thereby the cells are able to communicate with and attach to one another and the underlying basement membrane,

participating in the formation of an efficient permeability barrier. Eventually, the keratinocyte loses its organelles and forms a firm, proteinaceous outer shell. This process is coordinately regulated by a set of transcription factors and signaling pathways, including the tumor protein p63, nuclear factor kappa B (NF-κB), CCAAT/enhancer-binding protein (C/EBP) and the homeobox protein Hoxb13 (Mack et al., 2005).

Keratins are typical structural components of epithelial cells. They belong to the intermediate filament class of cytoskeletal proteins that are differentially expressed in various cell types. Epidermal keratinocytes have a unique panel of keratins, whose expression is spatially and temporally tightly regulated. The proliferating basal keratinocytes express keratins 5 and 14 (K5 and K14) as well as K15 (Moll et al., 2008), whereas the more differentiated spinous cells start to produce K1 and K10. The last keratin to be expressed by the most differentiated cells in the upper spinous and granular layers is K2, previously designated as K2e (Collin et al., 1992). The newest addition to the list of differentiation associated keratins is K80, which is found in suprabasal cells as well as in hair follicles and eccrine sweat glands (Langbein et al., 2010).

Interestingly, specialized skin areas seem to have a unique keratin expression profile. In addition to the ubiquitous members of the family, K9 is found in the epidermis of palms and soles (i.e. glabrous skin without hair), and K2 also has a distinctive expression pattern in different regions in mouse skin (Fischer et al., 2014). Additionally, activated or fetal epidermal keratinocytes may express several other keratins, including K6 and K16 (Moll et al., 2008; Sano et al., 2009), K77 (Langbein et al., 2013) as well as K17 (Bernerd & Asselineau, 1997; Moll et al., 2008).

Filaggrin has traditionally been considered an important protein for the aggregation of keratin filaments in the granular layers (McGrath & Uitto, 2008). A long precursor protein, profilaggrin, with 10-12 repeating polypeptide units is hydrolyzed and dephosphorylated to produce the functional filaggrin monomers. However, knockdown of filaggrin with a specific small interfering RNA (siRNA) in an organotypic human keratinocyte model indicates that the protein may be redundant for bundling K1, K10 and K2 in the upper epidermis (Mildner et al., 2010). Filaggrin is nonetheless important for proper differentiation, barrier formation and protection of skin as indicated by the penetration of a tracer across the stratum corneum and increased susceptibility to UVB damage in the knockdown cultures (Mildner et al., 2010). Lack of filaggrin clearly alters the normal differentiation program of the cells and impairs the morphology of epidermal equivalents resulting in e.g. fewer keratohyalin granules and a thinner SC (Pendaries et al., 2014).

Stratum corneum proteins, cornified envelope and cornified lipid envelope

During terminal differentiation the corneocytes become surrounded by a tough, 15-nm-thick proteinaceous structure called the cornified envelope (CE). The CE is linked both to the intracellular keratin bundles and to an abundant extracellular lipid matrix, the (cornified) lipid envelope (CLE; Madison, 2003; Elias, 2005). The CE consists of several specialized proteins, which are also useful in monitoring the normal course of terminal differentiation.

In the upper SS, where Ca^{2+} concentration increases rapidly, envoplakin, periplakin and involucrin are synthesized and assembled at the inner face of the plasma membrane, crosslinked by the enzyme transglutaminase 1 (for a graphical representation, see Kalinin et al., 2001). In the granular layer, further proteins are added. Loricrin is the most abundant of these. It is further complexed with the so-called small proline-rich proteins (SPR) and

linked to other auxiliary proteins (e.g. repetin, cystatins α and M/E, elafin) and the existing protein scaffold by transglutaminases 3 and 1 (Kalinin et al., 2001; Elias, 2005). Additionally, the keratins K1, K10 and K2 are minor cross-linked components of the CE (Steinert & Marekov, 1995; Kalinin et al., 2001).

As in the lower layers of the epidermis, the cytoplasmic keratins are linked to specialized junctional complexes, corneodesmosomes, between the corneocytes. These protein assemblies resemble the desmosomes of the living layers, creating connections between the terminally differentiated keratinocytes. Normal cohesion between cells depends on the corneodesmosomes, and is essential for controlled desquamation of the stratum corneum (Ishida-Yamamoto & Igawa, 2015). Corneocyte detachment also depends heavily on the pH, which decreases from 7 to ~ 5 in the uppermost SC (Elias, 2005).

The extracellular lipids of the SC are secreted in lamellar bodies (LB), along with lipid processing enzymes as well as proteases and anti-proteases to control desquamation. The LB also contain corneodesmosin, an integral part of the corneodesmosomes, which protects the junctional complexes from proteolysis. Additionally, there are antimicrobial peptides, which are part of the innate immune system in skin (Elias, 2005). Synthesis of the LBs begins in the spinous layer with the contents being released to the intercellular space at the interface between the SG and the SC (Matsui & Amagai, 2015).

The main lipid constituents of the SC include cholesterol, free fatty acids and ceramides. They form a lamellar structure which also contains aqueous phases (Imokawa et al., 1991; van Hal, et al., 1996). At the junction of the CE and the CLE, ω-hydroxyceramides are extruded into the intercellular space as the LB merges with the plasma membrane. Ultimately, ω-hydroxyceramides become the sole constituent of this part of the membrane, linking to the CE proteins on one hand and to the extracellular lipids on the other to produce the wear and tear resistant, selectively permeable outermost layer of skin (Kalinin et al., 2001; Elias, 2005).

2.1.2 Regulation of keratinocyte proliferation and differentiation

Epidermal keratinocytes respond to a vast array of growth factors and cytokines. These are produced by the keratinocytes themselves or by other cells of the skin, namely dermal fibroblasts, immune cells, endothelial cells or melanocytes (Shirakata, 2010; Seeger & Paller, 2015). The systemic circulation also transports hormones and other compounds that reach the keratinocytes from the dermis via diffusion. The most important growth factor families, cytokines and signaling factors affecting keratinocyte proliferation, differentiation, migration and stress responses are summarized in Table 1. Some of them are discussed in further detail below.

The balance between proliferation and differentiation is carefully regulated both under normal physiological conditions and particularly during wound healing. Several skin diseases also manifest with perturbations in the proliferative potential of keratinocytes. These include psoriasis, acanthosis nigricans and the dysplastic conditions actinic (solar) keratosis and Bowen's disease. The most extreme examples of such imbalance are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), the latter of which also frequently forms metastases at later stages of the disease.

Table 1. Growth factors, cytokines and signaling factors affecting keratinocyte proliferation, migration, differentiation and stress responses

Growth factor etc.	**Produced by***	**Effects on keratinocytes**	**References**
Acetylcholine	Keratinocytes	Induction of epidermal cohesion/adhesion and regulation of motility	Grando, 2006; Seeger & Paller, 2015
Calcium (Ca^{2+}) > 0.1 mM	NA	Induction of differentiation	Elias et al., 2002; Bikle et al., 2012
EGF	Fibroblasts	Induction of proliferation	Seeger & Paller, 2015
EGF family (HB-EGF, TGFα, amphiregulin, betacellulin, epiregulin)	Keratinocytes	Induction of proliferation and migration	Shirakata, 2010; Seeger & Paller, 2015
EGF family (neuregulin)	Fibroblasts	Induction of migration	Seeger & Paller, 2015
Endothelin-1	Keratinocytes	Induction of proliferation	Shirakata, 2010
Glucocorticoids/HPA-axis	Keratinocytes, fibroblasts	Regulation of local/systemic stress responses and epidermal homeostasis	Slominski et al., 2012
GM-CSF	Keratinocytes, fibroblasts	Induction of proliferation	Seeger & Paller, 2015
HGF	Keratinocytes, fibroblasts	Induction of proliferation and migration	Seeger & Paller, 2015
IFNγ	T-lymphocytes, NK cells	Regulation of proliferation and differentiation; antiviral defence	Banno et al., 2003; Shirakata, 2010
IGF-1	Fibroblasts	Protection against UV damage and induction of DNA repair; induction of proliferation and migration	Shirakata, 2010; Fernandez et al., 2015; Seeger & Paller, 2015
Interleukins (IL-1α and IL-1β) and chemokines (e.g. CXCL and CCL)	Keratinocytes	Highly variable: e.g. regulation of dermal cells by keratinocytes; immune responses; regulation of keratinocyte proliferation and differentiation	O'Shaughnessy et al., 2010; Shirakata, 2010; Wu et al., 2012
KGF-1 (FGF7) KGF-2 (FGF10)	Fibroblasts	Induction of proliferation and migration; protection against oxidative stress and ROS formation by UVB; re-epithelialization after wounding (also FGF22 from keratinocytes contributes)	Auf dem Keller et al., 2004; Braun et al., 2006; Kovacs et al., 2009; Seeger & Paller, 2015

Table 1. (contd.) Growth factors, cytokines and signaling factors affecting keratinocyte proliferation, migration, differentiation and stress responses

Growth factor etc.	Produced by*	Effects on keratinocytes	References
NGF	Keratinocytes	Induction of proliferation; protection against UVB-induced apoptosis	Marconi et al., 2003; Shirakata, 2010
PDGF	Keratinocytes (esp. follicular), fibroblasts, platelets	Induction of proliferation and migration; activation of other cell types (fibroblasts, endothelial cells, macrophages) during e.g. wound healing	Kamp et al., 2003; Sutherland et al., 2005; Kaltalioglu & Coskun-Cevher, 2015
Retinoic acid	NA (metabolized by keratinocytes)	Regulation of proliferation and differentiation	Fisher & Voorhees, 1996
TGFβ (TGFβ1, TGFβ2 and TGFβ3)	Keratinocytes, fibroblasts	Inhibition of proliferation	Amjad et al., 2007; Shirakata, 2010
Thyroid hormone/HPT-axis (TSH, TRH)	Keratinocytes, fibroblasts	Regulation of proliferation, differentiation and response to growth factors; regulation of (energy) metabolism and immune activity	Slominski et al., 2012
TNFα	Keratinocytes, fibroblasts	Induction of inflammatory responses (activation of immune cells)	Bashir et al., 2009; Shirakata, 2010
VEGF-A	Keratinocytes	Induction of proliferation and migration (induction of angiogenesis)	Seeger & Paller, 2015
Vitamin C	NA	Induction of differentiation *in vitro*; protection against oxidative damage caused by e.g. UVR	Pasonen-Seppänen et al., 2001; Catani et al., 2005
1α,25-dihydroxyvitamin D_3	Keratinocytes	Inhibition of proliferation/induction of differentiation; regulation of calcium homeostasis	Bikle et al., 2004; Shirakata, 2010

* Many other cells also produce these factors, but for simplification only the main cell types of epidermis and dermis, keratinocytes and fibroblasts, respectively, are listed here. This also highlights the crosstalk between the two tissue compartments and indicates whether keratinocytes are capable of autoregulation by the given factor. Where keratinocytes and fibroblasts don't synthesize a specific factor, other specialized cell types are indicated. For small molecule effectors and ions, an exogenous source may also be assumed (not applicable = NA). Several hormones, including the steroid sex hormones, also regulate cutaneous biology and hyaluronan metabolism, but for simplicity's sake they are omitted here.

EGF = epidermal growth factor; FGF = fibroblast growth factor; GM-CSF = granulocyte macrophage-colony stimulating factor; HB-EGF = heparin-binding EGF; HGF = hepatocyte growth factor; HPA = hypothalamus-pituitary-adrenal gland; HPT = hypothalamus-pituitary-thyroid; IFNγ = interferon γ; IGF = insulin-like growth factor; IL = interleukin; KGF = keratinocyte growth factor; NA = not applicable; NGF = nerve growth factor; PDGF = platelet derived growth factor; ROS = reactive oxygen species; TGFα = transforming growth factor α; TGFβ = transforming growth factor β; TNFα = tumor necrosis factor α; TRH = thyroid-releasing hormone; TSH = thyroid-stimulating hormone; VEGF = vascular endothelial growth factor

Factors affecting keratinocyte proliferation

As the name suggests, the epidermal growth factor (EGF) family is probably the best example of factors that induce keratinocyte proliferation as well as migration and wound healing (Pastore et al., 2008). The members of this family (indicated in Table 1) exert their effects in keratinocytes via three related receptors: Epidermal growth factor receptor (EGFR; also known as ErbB1 or HER1), ErbB2 (HER2) and ErbB3 (HER3). Activation results in homo- or heterodimerization and trans-autophosphorylation of the tyrosine kinase domain and subsequent recruitment of intracellular adaptors and effectors (Bogdan & Klämbt, 2001).

EGFR can also be activated or kept in a phosphorylated state via other, interacting pathways (including UVB radiation, G-protein coupled receptor activation or interaction with integrins). These may lead to further downstream events such as shedding of the membrane-bound HB-EGF precursor or inhibition of specific phosphatase activity. Downstream, ERK plays a central role in the regulation of keratinocyte biology, including proliferation (Pastore et al., 2008). Interestingly, retinoids exert their hyperproliferative effects at least partly via EGFR-ERK signaling (Pasonen-Seppänen et al., 2008). Activation of the PI3K/Akt (phosphatidylinositol 4,5-bisphosphate 3-kinase/protein kinase B) pathway, which contributes to cell survival, is also important (Pastore et al., 2008).

Keratinocyte growth factor (KGF or KGF-1) belongs to the fibroblast growth factor family. Consequently, it is also known as FGF7. KGF in the skin is produced by dermal fibroblasts after induction with IL-1α, IL-1β or TNF-α, all of which are secreted by keratinocytes (Tang & Gilchrest, 1996; Maas-Szabowski et al., 1999). KGF also potently induces proliferation and migration, and may help protect the epidermis against external stress cues such as UVR (Braun et al., 2006; Kovacs et al., 2009).

Transforming growth factor β (TGFβ) appears to counteract the proliferative pathways in keratinocytes, although the different isoforms have differing functions and spatio-temporal expression patterns (Cho et al., 2004). For example, the TGFβ1 mRNA and protein clearly increase in HaCaT cell cultures during Ca^{2+}-induced differentiation. These phenomena are most evident when normal and psoriatic skin samples are compared: particularly TGFβ2 and TGFβ3 are lost in hyperproliferating epidermis of psoriasis vulgaris patients (Doi et al., 2003). This indicates their importance for normal epidermal homeostasis.

Factors affecting keratinocyte differentiation

A critical factor in regulating differentiation-related processes is Ca^{2+} (Elias et al., 2002; Bikle et al., 2012). Increasing the Ca^{2+}-concentration of the growth medium is also routinely utilized to differentiate keratinocytes *in vitro*. There is a distinct gradient of Ca^{2+} in the epidermis *in vivo* (Adams et al., 2015), although its exact nature has been dissected only fairly recently (Celli et al., 2010; Behne et al., 2011). Ca^{2+} concentration is higher in the differentiating keratinocytes and thus increases towards the upper SG. In the basal layer, the concentration appears variable and sometimes even higher, at least locally, than in the stratum spinosum (Leinonen et al., 2009; Celli et al., 2010). Finally, in the upper SC an abrupt decline towards the skin surface occurs (Adams et al., 2015).

Most of the epidermal Ca^{2+} appears to be intracellular (Celli et al., 2010). This suggests that its uptake from the extracellular fluid and/or the subcellular distribution and responsiveness of the cells to this ion are carefully controlled in the different layers (Celli et al., 2010; Adams et al., 2015). The Ca^{2+}-gradient regulates the secretion of lamellar bodies

and also the mRNA expression of loricrin, filaggrin and involucrin (Elias et al., 2002). The changes induced are opposite, however, as high Ca^{2+} (> 0.1 mM) upregulates the differentiation-related proteins and decreases LB secretion and extracellular lipid deposition (Elias et al., 2002).

One factor modulating and augmenting the Ca^{2+}-induced effects is 1α,25-dihydroxyvitamin D_3 (calcitriol). It upregulates the expression of proteins in the Ca^{2+} signaling pathways as well as induces the late differentiation markers involucrin and the transglutaminase TGase1 via its nuclear receptor/transcription factor VDR (Lehmann, 2005). Additionally, vitamin D is produced in keratinocytes in response to UV exposure. These cells appear to have the full machinery for converting the precursors 7-dehydrocholesterol, pre-D_3, D_3 and 25-hydroxyvitamin D_3 into the active form. Calcitriol is thus a potent, intrinsic mediator of photoresponses in skin (Lehmann, 2005). It may also help protect keratinocytes against UV radiation by boosting the permeability barrier, inducing antioxidative mechanisms and regulating the stress-activated protein kinases p38 and c-Jun N-terminal kinase (JNK) (Ravid et al., 2002; Hong et al., 2008).

Highlighting the stratified organization and gradual differentiation of epidermal keratinocytes, the vitamin A derivative *all-trans* retinoic acid (ATRA) also forms a gradient in the epidermis, but in a direction opposite to that of Ca^{2+} (Cheepala et al., 2007). Binding of ATRA or other retinoids to the nuclear retinoid acid and retinoid X receptors (RAR and RXR, respectively) induces changes in gene expression. This commonly causes increased proliferation or more rapid turnover of the basal keratinocytes (Fisher & Voorhees, 1996).

In psoriatic keratinocytes, however, retinoic acid treatment decreases the abnormally high proliferative capacity of the cells. This leads to an overall thinner substituted epidermis *in vitro* as compared to the psoriatic counterparts that are treated with vehicle only (Jean et al., 2011). Thus, the regulation of the epidermal proliferation-differentiation axis is complex, and retinoids act in concert with several other factors to maintain a correct balance. Moreover, the responses in normal skin seem to differ from those in pathological states.

The third vitamin known to affect terminal differentiation is ascorbic acid or vitamin C. Treatment of either normal human epidermal keratinocytes or HaCaT cells with L-ascorbate 2-phosphate has revealed that this extracellularly stable ascorbic acid derivative is as potent as 1.2 mM Ca^{2+} in inducing CE formation and K1 as well as TGase1 and loricrin expression; this occurs via a PKC- and AP-1-dependent pathway (Savini et al., 2002).

Moreover, vitamin C supplementation in 3D cultures of REK cells leads to improved stratum corneum structure, barrier properties as well as enhanced lipid deposition and expression of filaggrin (Pasonen-Seppänen et al., 2001). This demonstrates the importance of ascorbic acid in multiple aspects of terminal differentiation. Similarly, vitamin C enhances the synthesis and deposition of barrier lipids in human skin equivalents (Ponec et al., 1997). *In vivo*, hairless mice with a knockout (KO) mutation in an essential ascorbic acid synthesizing enzyme (SMP30/GNL KOs) developed a pronounced epidermal atrophy without significant changes in the classical differentiation markers (Sato et al., 2012). Nevertheless, this indicates that vitamin C has a role in epidermal homeostasis. Finally, vitamin C is an important antioxidant in skin, protecting keratinocytes against the detrimental effects of UV-induced free radical formation (Catani et al., 2005).

Beyond growth factors: intracellular effectors in proliferation and differentiation

Considering the intracellular signaling cascades that are activated in response to the factors discussed above, several ubiquitous kinase cascades have been implicated in the regulation of keratinocyte differentiation. Of special note in the context of epidermis, which is constantly subjected to external stressors including UV radiation, are the stress- and mitogen-activated protein kinases JNK and p38.

JNK is present as three isoforms (JNK1-3) in mammals. JNK (JNK1) may function downstream from EGF/EGFR as well as stressors such as UVB both in HaCaT cells and normal human epidermal keratinocytes (Assefa et al., 1997), possibly mediated by reactive oxygen species (ROS). JNK is also activated in HGF-stimulated keratinocytes, where it is co-regulated with the MEK/ERK pathway that induces matrix metalloproteinase 9 (MMP9) production and cell motility (Zeigler et al., 1999). Moreover, TGFβ has been implicated in modulating the JNK-pathway in HaCaT cells specifically when activated by UV stress (Merryman et al., 1998). As far as epidermal homeostasis is concerned, the inhibition of JNK induces neonatal epidermal keratinocyte differentiation and suppresses proliferation and migration (Gazel et al., 2006). This highlights the importance of balanced signaling pathways in epidermal maturation. Inhibiting JNK also promotes the formation of tight junctions (see below) and a differentiated phenotype in HaCaT keratinocytes (Kitagawa et al., 2014).

The MAP kinase p38 is known to regulate both keratinocyte differentiation and stress responses. It is expressed as four major isoforms p38α, p38β, p38γ and p38δ, and activated in response to many growth factors and stress signals (Eckert et al., 2003). In keratinocytes, the main isoforms are the ubiquitously expressed p38α and the differentiation associated p38δ (Junttila et al., 2007; Adhikary et al., 2010). In organotypic skin cultures treated with SB203580, the commonly used inhibitor for p38 isoforms α and β, keratin 10 expression is attenuated in the most suprabasal layers (Jonak et al., 2011). Part of the regulation of differentiation by p38 appears to occur via Hsp27 phosphorylation and modulation of its chaperone activity, which affects keratin aggregation and cornified cell envelope formation (Jonak et al., 2011). p38δ also regulates the tight junction component ZO-1 in differentiating keratinocytes (Siljamäki et al., 2014). In the differentiation responses, protein kinase C is a central upstream regulator of p38δ (Adhikary et al., 2010).

Additionally, p38δ is involved in keratinocyte apoptosis, functioning downstream of PKCδ in cultured normal human keratinocytes (Efimova et al., 2004). The mechanism involves complex formation between p38 and the MAP kinase ERK1/2 and suppression of ERK activity. Interestingly, p38δ activation may also lead to the opposite outcome: studies *in vivo* in p38δ$^{-/-}$ KO mice show that both ERK1/2 and signal transducer and activator of transcription 3 (STAT3) phosphorylation are inhibited in the absence of p38δ (Schindler et al., 2009). At the same time, the KO animals are more resistant to chemically induced tumor formation. This supports data from other studies, which indicate a role for p38δ in tumorigenesis, including in squamous cell carcinomas of the skin (reviewed by Efimova, 2010).

2.1.3 Epidermal tight junctions and the permeability barrier

The permeability barrier of the skin is highly dependent on the lipid matrix between the terminally differentiated and keratinized corneocytes of the outermost stratum corneum. There are, however, other components whose role in maintaining epidermal integrity has

begun to unravel only recently. Tight junctions (TJ) are multi-protein complexes extensively found in endothelia and simple epithelia. TJs effectively seal the intercellular spaces, limiting the paracellular movement of water, ions and small molecules. Their transmembrane constituents occludin, tricellulin and claudins have apposing and interweaving domains in the intercellular space of adjacent cells. These components are connected to the actin cytoskeleton via peripheral membrane proteins and linkers that include the zonula occludens 1-3 (ZO-1, ZO-2 and ZO-3), multi-PDZ domain protein 1 (MUPP1) and cingulin (Niessen, 2007).

The presence of tight junctions in mammalian skin was long open to debate (Hashimoto, 1971; Elias & Friend, 1975). It is now known that occludin, ZO-1 and ZO-2 are found co-localized in the stratum granulosum in rodent skin (Morita et al., 1998). The significance of tight junctions for epidermal development, function and pathophysiology was also demonstrated in human skin (Pummi et al., 2001). Additionally, lack of claudin-1 in KO mouse was shown to result in premature death due to excessive water loss through the epidermis that appeared structurally normal (Furuse et al., 2002). Today it is widely recognized that tight junctions are an essential component contributing to epidermal structure and function (Svoboda et al., 2016), including neoplastic changes (Rachow et al., 2013).

In this context it needs to be emphasized that the SC contains abundant lipids, but also some intercellular water (Imokawa et al., 1991; van Hal, et al., 1996). However, from the viewpoint of permeability, most of the water in the SC is bound intracellularly by small molecules and ions, collectively known as the natural moisturizing factor (NMF). There are also polar, pore-like structures that allow the absorption/penetration of hydrophilic compounds through the SC, despite the high lipid content of the cornified layers (Pouillot et al., 2008). In addition, TJs are not altogether exclusive for small molecules and ions. The complex and multifunctional tight junctions of the upper epidermis, which appear to be assembled very coordinately (Ishida-Yamamoto et al., 2012), thus provide a functional barrier that can be fine-tuned according to internal and external stimuli.

2.1.4 Environmental cues regulating epidermal homeostasis

One of the best characterized responses of skin to environmental irritation is the one that happens after exposure to UVB. The hyperproliferation of epidermis is a common phenomenon seen in native skin, although it is not always reproduced *in vitro* (Pearse & Marks, 1983; Del Bino et al., 2004; Bart et al., 2014). In fact, UVB has been shown to cause parakeratosis, apparent thinning of the viable cell layers as well as downregulation of K10 expression in a reconstructed skin model (Bernerd & Asselineau, 1997). Concomitantly, reduced amounts of loricrin, filaggrin and transglutaminase type I were identified 2-3 days after an acute irradiation with 50 mJ/cm^2 of UVB (Bernerd & Asselineau, 1997). In subchronically (for 3 days) exposed mouse skin, the expression of both involucrin and loricrin were increased (Hong et al., 2008). No hyperproliferation was observed in this case, but enhanced recovery from permeability barrier disruption was indicated.

It isn't clear why these apparently contradictory changes occur, but adaptive mechanisms with repeated exposures probably contribute. Interplay between various cell types and tissue compartments *in vivo* is also a likely explanation. All of the pathways can't be reconstituted even in elaborate 3D models, even though they provide simplified and well-controlled platforms for studying epidermal responses.

UVB also affects epidermal homeostasis by, among other things, disrupting the permeability barrier. This may take place through impaired extracellular lipid processing, which is due to decreased TGase1 expression. This is most notable 4 days post-irradiation in mouse skin (Tagaki et al., 2004). In this setting, hyperplasia is also conspicuous and concomitant with the increase in transepidermal water loss (TEWL). A relationship between an altered Ca^{2+} gradient and radiation insult has also been demonstrated (Jiang et al., 2007). 96 h after an acute UVB-exposure, Ca^{2+} concentration increases both in the lower layers (stratum spinosum and basale) and stratum corneum. These changes are accompanied by altered water movement/increased TEWL and epidermal proliferation, which are most pronounced 4 days after the irradiation.

In monolayers of keratinocytes, the responses depend heavily on the dose. In HaCaT cells a biphasic induction of cell death occurs: smaller doses of up to 10 mJ/cm^2 cause the controlled process of apoptosis whereas higher energy irradiation leads to necrosis (Mammone et al., 2000). In these cells, all the doses tested also downregulated markers of differentiation, including keratins K1 and K10 and involucrin (Mammone et al., 2000).

The effects of UV on epidermal homeostasis are not entirely negative. In mouse skin, the expression of antimicrobial β-defensins in response to UVB have been observed (Hong et al., 2008). UVR also induces the production of an endogenous sunscreen, urocanic acid (UCA), which is derived mainly from the histidine residues of filaggrin in the upper stratum corneum (Mildner et al., 2010). UCA can then absorb the harmful, short UV wavelengths of solar radiation. The role of endogenous UCA has been verified in a spontaneous mutant mouse line lacking the UCA-producing enzyme histidase (Barresi et al., 2011). The mutant animals are clearly more prone to DNA fragmentation and apoptosis caused by exposure to UVB.

Epidermis also strongly responds to changes in relative humidity (RH). Lamellar body secretion as well as barrier function are enhanced after exposure of hairless mice to low RH (Denda et al., 1998). TEWL at basal conditions (without injury) is also significantly lower in animals exposed to dry vs. humid conditions, indicating adaptive modifications in the SC. These changes are highlighted by the increased thickness of the epidermis and SC at low RH (< 10%) as well as an increased number of keratohyalin granules (Denda et al., 1998). Functional alterations have also been implicated in humans, as TEWL is significantly decreased in workers exposed to ultra-low humidity (Chou et al., 2005). Similar adaptations have recently been characterized in an *in vitro* model of human skin (Sun et al., 2015).

2.1.5 Modeling skin biology: monolayer and 3D cultures vs. studies *in vivo*

There are several possibilities for choosing a cell line or a model system for studying skin biology. Normal human epidermal keratinocytes (NHEK) are primary cells that are often isolated as a by-product of surgical procedures e.g. from foreskin, breasts or abdomen. They are also suitable for preparing 3D cultures, where they are often co-cultured with fibroblasts in an underlying collagen gel or de-epidermized dermis (see e.g. Rikimaru et al., 1997).

One of the most commonly used keratinocyte cell lines is HaCaT, a spontaneously immortalized human keratinocyte cell line (Boukamp et al., 1988). It has a functional retinoblastoma protein (pRb; Bates et al., 1994), but contains a mutation in the p53 gene (Lehman et al., 1993). HaCaT are nevertheless often used for studying e.g. stress responses (Mammone et al., 2000; Ravid et al., 2002; Warskulat et al., 2007; Graf et al., 2009). Notably,

the growth dependence of commonly cultured (keratinocyte) cell lines *in vitro* differs substantially from that seen in normal, untransformed cells (see e.g. Assefa et al., 1997). With this distinction in mind, however, simple monolayer cultures as well as 3D platforms provide a useful tool for modeling keratinocyte and epidermal biology, including cell signaling. HaCaT cells are not, however, optimal for producing stratified epidermal equivalents (Boelsma et al., 1999; Schoop et al., 1999).

Useful cells from other species include rat epidermal keratinocytes (REK; Baden & Kubilus, 1983), which are particularly practical and inexpensive in preparing organotypic cultures without feeder cells (Pasonen-Seppänen et al., 2001). This model reproduces all of the essential features of epidermal differentiation, both structurally and functionally (Suhonen et al., 2003). Mouse keratinocytes have also been utilized in a 3D setting for studying epidermal differentiation (Madison et al., 1989).

The preparation of stratified, differentiated 3D epidermal equivalents has been in use for several decades (Pruniéras et al., 1983). These models, many of which are also available commercially (e.g. EpiSkin, EpiDerm), reproduce most of the essential features of a stratified and differentiated epidermis. However, there are often more or less pronounced differences in the expression of e.g. keratins, indicating an imperfect switch from proliferation to differentiation (Boelsma et al., 2000).

The keratinocytes are usually plated either directly on a porous membrane support made of an artificial polymer (e.g. polycarbonate, polyester, polyethylene terephthalate) or a membrane coated with collagen, with or without embedded fibroblasts. The cultures are first grown immersed in medium. After a few days they are lifted to the air-medium interface and subsequently fed from beneath to achieve full stratification and differentiation. This results in the formation of a physiologically relevant 3D epidermis in 1-2 weeks (Pruniéras et al., 1983; Boelsma et al., 2000). The models may or may not incorporate a distinct basement membrane zone between the keratinocytes and the underlying matrix compartment (Tammi et al., 2000). Some models utilize the natural dermal equivalent, de-epidermized dermis (DED), that can be derived from cadaver skin (e.g. Pruniéras et al., 1983).

Full-thickness skin can also be excised and cultured *ex vivo*, with varying details (e.g. growth media, supporting matrix, maintenance submerged vs. at the air-liquid interface). This produces a system that is relatively self-sustained in the short term i.e. not necessarily requiring exogenous addition of serum-derived factors (Tammi et al., 1979; Kleszczyński & Fischer, 2012). One more possibility in producing a model that directly confers the conditions *in vivo*, is to make xenografts on athymic nude mice. This approach has been successfully employed in studying wound healing and exposure of epidermis to UVB (Demarchez et al., 1986; Del Bino et al., 2004).

Choosing the platform for studies on epidermal keratinocytes depends on the process studied. Effects of growth factors or stress stimuli on the activation of signaling pathways can be studied even in monolayer cultures, but the more physiological or functional the outcome desired, the need for a model that replicates differentiation and especially the cross-talk between different cell types increases. The possible superiority of an isolated, easily controlled and reproducible cell culture system, whether monolayer or a more complex 3D model, over an *in vivo* setting always needs to be carefully assessed. This is even more fundamental today, as the level of sophistication of the *in vitro* models continues

to increase. Most recent systems can even incorporate vascular-like structures (Matsusaki et al., 2015).

The presence of an intact and complete extracellular matrix (ECM) should be considered another important factor of the models, particularly when studying migration and invasion. The composition of the ECM also critically affects the metabolism of the embedded cells via binding to plasma membrane receptors and activating various intracellular signaling cascades. As in all epithelia, the ECM in epidermis is naturally scanty. It does contain glycosaminoglycans (GAGs), glycoproteins and matrix remodeling molecules such as matrix metalloproteinases (MMPs). In dermis, the amount of the ground substance and the embedded structural proteins (mostly type I collagen, type III collagen and elastin) is much higher. This matrix is deposited and modified by fibroblasts, which also extensively communicate with the epidermal keratinocytes in producing a coherent tissue.

Despite their inherent limitations, the models presented here are suitable for studying the metabolism of keratinocytes also in terms of their ECM components. Of these, the simplest GAG, hyaluronan, can reach high concentrations between the otherwise tightly packed keratinocytes (Tammi et al., 1988). Hyaluronan metabolism can be modeled in simple monolayers as well as 3D cultures.

2.2 HYALURONAN

2.2.1 The structure and physico-chemical properties of hyaluronan

Hyaluronan or hyaluronic acid (HA) is a ubiquitous GAG of the ECM of various tissues in mammals and other vertebrates such as *Xenopus laevis*, zebrafish and chicken (Spicer & McDonald, 1998; Müllegger & Lepperdinger, 2002b). Hyaluronan is also important for other organisms, including some bacteria (DeAngelis, 2002), invertebrates (Volpi & Maccari, 2003), and also algal viruses (Graves et al., 1999), widening the scope of this intriguing macromolecule even outside the animal kingdom.

The physical and biochemical properties of hyaluronan render it suitable for binding water and forming malleable intercellular matrices or outer coats and capsules. These structures can be important in e.g. the protection of tumor cells from cytotoxic attack (McBride & Bard, 1979). Pathogenic bacteria also utilize HA in evading host immune system (Cress et al., 2014). The efficiency of bacterial hyaluronan production is also utilized in producing commercial HA for various purposes (Liu et al., 2011).

Hyaluronan is composed of repeating disaccharide units of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcUA) joined by β-linkages (Fig. 2). The linear, unbranched molecule occupies a large volume in solution and is highly viscoelastic, making it an excellent space filler and gelling agent, particularly in association with other ECM components. Hyaluronan has intricate biophysical and biological characteristics, and it is unique among GAGs in several respects:

1. HA is synthesized at the plasma membrane, not in Golgi, by specific glycosyltransferases (EC 2.4.1.212) called hyaluronan synthases (in mammals: HAS1-3), with addition of each monomer at the reducing end of the chain (Vigetti et al., 2014b; Weigel, 2015).

2. HA is not covalently attached to a core protein, and does not need a protein primer for synthesis (Weigel, 2015; Theocharis et al., 2016). Covalent links do occur extracellularly with, for example, the heavy chain of inter-α-trypsin inhibitor (IαI) under specific conditions such as inflammation. This produces so-called serum-derived HA-associated protein-HA (SHAP-HA) complexes (Zhuo et al., 2004). HA also associates non-covalently with four major extracellular proteoglycans or hyalectans via their N-termini: aggrecan, versican, neurocan and brevican (Theocharis et al., 2016).

3. HA is not modified by sulfation or other functional groups, unlike the other common GAGs: chondroitin-4-sulfate, chondroitin-6-sulfate, heparin, heparan sulfate, keratan sulfate and dermatan sulfate (Theocharis et al., 2016).

4. The ability of HA to produce specific signaling and intra- or extracellular responses appears to be high (Toole, 2004). This ability depends on fragment size, as high molecular weight molecules, low molecular weight polymers and smaller oligomers behave differently (Stern et al., 2006; Misra et al., 2015). Roughly, their effects are the following:

- native, high molecular-weight HA (HMW-HA): > 10^6 Da → homeostatic balance
- intermediate fragments: 30-500 kDa → stimulation of proliferation, pro-inflammatory
- low molecular-weight HA (LMW-HA): < 50 kDa → pro-migratory, danger signal
- HA-oligosaccharides: 8-16 disaccharides → pro-angiogenic

Finally, as opposed to most other GAGs (except for serglycin) hyaluronan also accumulates intracellularly in addition to the usual peri- and extracellular localization. This is most evident in the perinuclear area, particularly in dividing cells (Evanko & Wight, 1999; Tammi et al., 2001; Hascall et al., 2004). This probably occurs mostly by uptake of existing extracellular molecules.

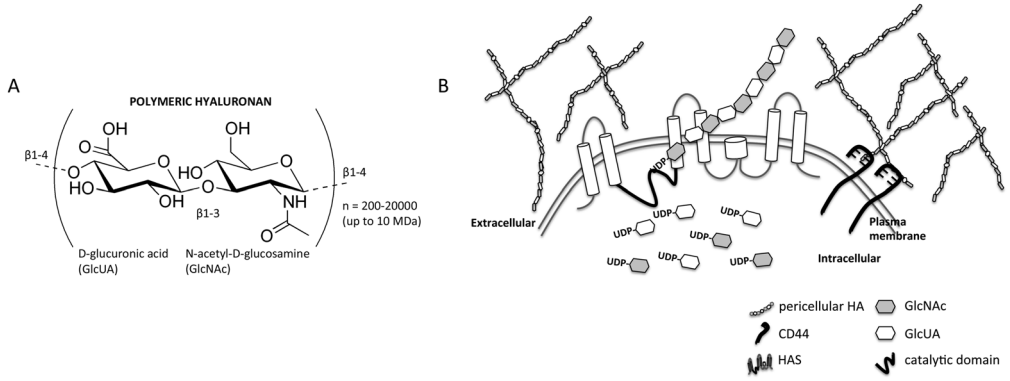

Figure 2. Structure of polymeric hyaluronan (A) and mechanism of its synthesis (B; reviewed in Tammi et al., 2011). CD44 = cluster of differentiation 44 (HA receptor); HAS = hyaluronan synthase; UDP-GlcNAc and UDP-GlcUA = the nucleotide sugar precursors of HA.

Intracellular HA was thought to help in efficient separation of the daughter nuclei as well as the following cytokinesis. However, it later appeared that these responses principally apply to cells undergoing simultaneous hyperglycemic stress. The accumulation of intracellular HA is accompanied by formation of a monocyte-binding extracellular matrix and autophagy (Wang & Hascall, 2005). This would occur, for example, during culturing in the routinely used Dulbecco's modified Eagle's medium (DMEM), which contains high glucose (25 mM). The stress-induced accrual of hyaluronan should probably be regarded as

a discrete case, where intracellular HA becomes particularly prominent. Interestingly, in addition to pericellular hyaluronan, an intracellular pool is evident in epidermal keratinocytes, particularly in the proliferative basal layer (Stern & Maibach, 2008).

Additionally, HA may accumulate via direct intracellular synthesis, which is related to more generalized cell stress. Chemicals that perturb the normal function of the ER or factors that inhibit global protein synthesis have been shown to induce the formation of cable-like hyaluronan strands, which seem to originate intracellularly (Hascall et al., 2004). *In vivo*, cable formation has been demonstrated in a diabetic context in rats (Wang A et al., 2015). This pathophysiologically common situation of excess glucose exposure can lead to inflammation and fibrosis, particularly in the kidney. Excess HA synthesis and the ensuing autophagic response can be prevented by treating the diabetic test animals with heparin, which redirects the metabolic machinery to decrease the intracellular accumulation of HA (Wang A et al., 2015). Cables of HA have also been described under other inflammatory settings (de la Motte et al., 2003; Jokela et al., 2008b). Based on these data, it is clear that HA synthesis and degradation and the respective enzymes are highly responsive to external cues.

2.2.2 Hyaluronan biosynthesis and regulation of hyaluronan synthases

The biosynthesis of hyaluronan takes place at the plasma membrane. Export of the nascent chain has been suggested to happen either via a pore made by the synthesizing enzymes themselves or by separate proteins such as the cystic fibrosis transmembrane conductance regulator (CFTR) or the ABC transporter MRP5 (Schulz et al., 2007; Schulz et al., 2010; Hubbard et al., 2012). Transport may require a simultaneous outward flux of cations to preserve electrical neutrality (Hagenfeld et al., 2012), although the actual mechanism has persistently eluded characterization.

Hyaluronan synthases

There are three related hyaluronan synthases in mammals, HAS1-3 (Itano & Kimata, 1996; Spicer et al., 1996; Watanabe & Yamaguchi, 1996; Shyjan et al., 1996; Spicer et al., 1997a; Spicer et al., 1997b). HAS1-3 differ somewhat in their activity and the length of the end product (Itano et al., 1999). HAS1 yields long chains (0.2-2 MDa), but is slow and highly sensitive to substrate concentration, particularly uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) (Rilla et al., 2013). HAS2 also produces HA of high molecular weight (0.2-2 MDa), but has faster synthesis rates. HAS3 seems to synthesize the smallest HA-chains (0.1-1 MDa) with an intermediate velocity (Itano et al., 1999). HAS3 has also been shown to generate larger HA molecules, but the measured size distribution may depend on the experimental setting. At least for HAS3-transfectants, the HA synthesized by intact cells appears larger than that isolated from membrane fractions (Brinck & Heldin, 1999; Takabe et al., 2015). Additionally, very high molecular weight HA (6-12 MDa), synthesized mainly by mutated HAS2, has recently been demonstrated in naked mole rats (Tian et al., 2013). This unsually large HA seems to explain both the long lifespan as well as the obvious cancer resistance of these animals.

Human *HAS1* (RefSeq Gene ID: 3036) contains 5 exons producing two slightly different transcripts and proteins, whereas *HAS2* (RefSeq Gene ID: 3037) is composed of 4 exons (Yamada et al., 1998; Monslow et al., 2003). *HAS3* (RefSeq Gene ID: 3038) is composed of 8 exons, 4 of which are protein coding. In total, human *HAS3* appears to produce three distinct transcripts (Liu et al., 2001; Monslow et al., 2003; Wang S et al., 2015), which

highlights the plasticity in this gene lacking a conventional TATA-box. Two of these code for the same protein variant (isoform a), and one (GenBank NM_138612.2) produces a shorter protein (isoform b), which also contains a unique C-terminus as compared to the longer isoform.

The proximal promoter sequences of each of the *HAS*-genes have also been examined; they contain binding sites (actual or potential) for several common transcription factors (Table 2). However, the experimentally confirmed transcriptional regulation of these genes and the significance of differential splicing, whether physiological or pathological, is only slowly beginning to unravel.

HAS1 KO animals are viable and fertile, and they don't have any obvious structural or functional abnormalities (Spicer & Nguyen, 1999; Kobayashi et al., 2010). HAS1 also seems to be differentially spliced under pathological conditions (Adamia et al., 2014). These splice variants (HAS1Va, HAS1Vb and HAS1Vc) are found in at least two separate hematological malignancies (multiple myeloma and Waldenström macroglobulinemia), where they also hold some diagnostic/prognostic value. The aberrant proteins produce both extracellular and intracellular HA. It appears likely, that in multiple myeloma particularly HAS1Va confers motility on B cells via the generation of a robust HA matrix (Adamia et al., 2014). Moreover, the splice variants act in a dominant-negative fashion to outplay the full-length HAS1 by translocating it in the cell as well as changing its half-life (Ghosh et al., 2009).

HAS2 appears to be the obligate isoform, as *Has2*$^{-/-}$ KO mice are not viable; the embryos have severe defects in cardiac development (Camenisch et al., 2000). HAS2 is the most abundant isoform in many mammalian tissues or cell types derived thereof (Tien & Spicer, 2005; Törrönen et al., 2014). Additionally, *Has2*$^{+/-}$/*Has3*$^{-/-}$ mice present with abnormal tooth development, impaired fertility and apparently altered skin physiology (Spicer et al., 2002). The constitutive expression of *HAS2* appears to depend on a rather short proximal promoter sequence (121 bp) under the control of the transcription factors Sp1 and Sp3 (Monslow et al., 2006), at least in human cells. Sp1 is also activated on the *HAS2* promoter in response to TNFα and ATRA (Saavalainen et al., 2007).

In addition, the natural antisense transcript of *HAS2*, *HAS2-AS1* (originally termed *HASNT*; Chao & Spicer, 2005), appears to be an important regulator of *HAS2*, mediating chromatin remodeling and increased accessibility at the human *HAS2* promoter (Vigetti et al., 2014a). This would require increased O-GlcNAcylation of NFκB, which first induces the *HAS2-AS1* transcript. The proposed mechanism highlights the regulatory potential of GlcNAc in *HAS* expression, making it a convenient feedback regulator of hyaluronan precursor sugar levels or – where pathological mechanisms come into play – hyperglycemia.

The *HAS2-AS1* transcript, which is partially complementary to exon 1 of *HAS2*, can also increase the stability of *HAS2* mRNA when the two form a duplex (Michael et al., 2011). In their original study Chao and Spicer reported, that ectopic expression of *HASNT* in stably transfected osteosarcoma cell clones downregulated both *HAS2* levels and HA production (Chao & Spicer, 2005). At the same time, a significant reduction in cell proliferation was observed, which could be counteracted by introducing *HAS3* into the *HASNT*-transfectants. Whether or not such overlap/substitution in HAS function exists *in vivo* hasn't been studied comprehensively, although *HAS2* may partially substitute for the other isoforms (Spicer et al., 2002). The tissue and cell type dependency of this type of compensation is also virtually unexplored. Most recent data argues that there are three separate opposite side transcripts

for murine *Has2* (*Has2os1-3*), which also respond differentially to stimuli. This may elicit distinct regulation on the sense *Has2* (Kretschmer et al., 2016).

Table 2. Transcription factors/promoter elements involved in the regulation of mammalian *HAS* expression. Data have been obtained under basal and/or stimulated conditions. A more or less direct functional verification was performed in different studies using gel shift assays, reporter gene assays, ChIP or functional inhibition with siRNAs/chemical inhibitors or overexpression of negative regulators.

Transcription factor	Binding to HAS1 promoter	Binding to HAS2 promoter	Binding to HAS3 promoter	Reference
AP-2	x			Yamada et al., 1998[2]
C/EPB or CCAAT element	x	x	x	Yamada et al., 1998[2]; Monslow et al., 2004; Wang S et al., 2015
c-Maf	x[1]			Dhiman et al., 2011
CREB	x[1]	x[1]		Yamada et al., 1998[2]; Makkonen et al., 2009; Maeda-Sano et al., 2014
GAGA	x			Yamada et al., 1998[2]
GATA	x			Yamada et al., 1998[2]
H4TF-2	x			Yamada et al., 1998[2]
IRF-1/IRF-2	x			Yamada et al., 1998[2]
LBP-1	x			Yamada et al., 1998[2]
LEF1		x[1]		Kretschmer et al., 2016[2]
MTE			x[1]	Wang S et al., 2015
MyoD	x			Yamada et al., 1998[2]
MZF1			x	Wang S et al., 2015
NFκB	x[1]	x[1]	x	Jones et al., 2001; Monslow et al., 2004; Stuhlmeier & Pollaschek, 2005; Kao, 2006; Saavalainen et al., 2007; Wang S et al., 2015
NF-Y/CCAAT		x		Monslow et al., 2004
p53	x			Yamada et al., 1998[2]
RAR/RXR		x[1]		Saavalainen et al., 2005; Saavalainen et al., 2007; Makkonen et al., 2009
Smad2/3/4/7	x[1]	x[1]		Usui et al., 2000[3]; Michael et al., 2011; Chen et al., 2012
SOX5	x		x	Yamada et al., 1998[2]; Wang S et al., 2015
Sp1	x[1]	x[1]	x[1]	Monslow et al., 2004; 2006; Saavalainen et al., 2007; Jokela et al., 2011; Michael et al., 2011; Tsui et al., 2011; Wang S et al., 2015
Sp3	x[1]	x[1]		Monslow et al., 2006; Michael et al., 2011; Chen et al., 2012
SREBP	x	x		Sakr et al., 2008
SRY	x			Yamada et al., 1998[2]
STAT1		x[1]		Saavalainen et al., 2005; Hosui et al., 2012
STAT3		x[1]		Saavalainen et al., 2005; Ohara et al., 2010; Bohrer et al., 2014; Jokela et al., 2014
YY1		x[1]		Jokela et al., 2011

[1] confirmed functionally; [2] in mouse; [3] bovine *HAS2*

As with *HAS1*, *HAS3* KO animals are viable, but the *HAS3*-knockouts are particularly prone to spontaneous epileptic seizures due to decreased extracellular space and hippocampal HA content and a resulting tighter packing of neurons (Arranz et al., 2014). *HAS3* KO animals are, however, much less susceptible to the inflammatory manifestations of colitis, highlighting the vast potential of HA in regulating immunological functions and tissue homeostasis (Kessler et al., 2015). Similarly to *HAS2*, the ubiquitous Sp1 transcription factor appears necessary for basal activity at the *HAS3*-promoter (Wang S et al., 2015).

HAS3 seems very responsive to various stimuli and it is also active under pathological conditions such as wounding and cancer (Tammi et al., 2005; Tobiishi et al., 2011; Kultti et al., 2014). A role for HAS3 has been implicated in cultured keratinocytes, as well as intact epidermis (Sayo et al., 2002; Tammi et al., 2005). In double KO animals (*Has1*$^{-/-}$/*Has3*$^{-/-}$) excisional wounds close faster despite lower epidermal and dermal HA levels (Mack et al., 2012). At the same time, a stronger inflammatory response and increased fibrosis may take place (Mack et al., 2012). Increased *Has2* mRNA expression and HA synthesis are observed in fibroblasts derived from the dermis of these mice, which may explain some of the physiological outcomes (Wang et al., 2014). These cells are also less susceptible to environmentally induced apoptosis via a *Has2*-mediated mechanism, as *Has2* siRNA is able to revert the stress resistant phenotype (Wang et al., 2014). These data might also indicate that *Has1* and/or *Has3* can act as negative regulators of *Has2* expression.

Growth factors and signaling cascades regulating HAS expression

Considering further the physiological regulation of the three *HAS*-isoforms, specifically in keratinocytes, the synthases have been shown to respond to several growth factors or other extracellular ligands (Table 3). The varying results in some studies may depend on the differentiation status of the cells, which is crucially important for their physiological responses. This is particularly true for TGF-β, which has been suggested to modulate the UVB-induced changes in *HAS* expression in chronically irradiated mouse skin (Dai et al., 2007). Similarly, the release of KGF and IL-1β from UVB-irradiated NHEK appears to contribute to the upregulation of both *HAS2* and *HAS3* (Kakizaki et al., 2008). Exploring these pathways is crucial, as it may enable therapeutic interventions in pathologies where hyaluronan production is disturbed, including cancers of the skin (Karjalainen et al., 2000; Karvinen et al., 2003a; Siiskonen et al., 2013).

Less is known about the intracellular signaling pathways and the various kinases that are activated. The classical signaling cascades and effectors downstream from the receptors for the cytokines and growth factors presented in Table 3 are most likely involved. These include the mitogen-activated protein kinase (MAPK) pathways, Janus kinase-STAT (JAK-STAT), cyclic adenosine monophosphate (cAMP), CREB and NF-κB. The involvement of Src kinase, ERK and Akt in the upregulation of *HAS2* in human keratinocytes after treatment with a ginsenoside has also recently been proposed (Lim et al., 2015). Additionally, in rat epidermal keratinocytes the involvement of the MEK/ERK cascade downstream from EGFR, possibly in direct response to ATRA, has been suggested in the upregulation of *HAS2* and perhaps also *HAS3* (Pasonen-Seppänen et al., 2008).

Table 3. Growth factors and cytokines involved in the regulation of *HAS* expression in keratinocytes

Growth factor/ligand etc.	Regulation of *HAS1* mRNA	Regulation of *HAS2* mRNA	Regulation of *HAS3* mRNA	Reference
all-trans retinoic acid (ATRA), retinoic acid, retinol		↑	↑	Sayo et al., 2004; Saavalainen et al., 2005; Pasonen-Seppänen et al., 2008
EGF/HB-EGF		↑	↑	Pienimäki et al., 2001; Pasonen-Seppänen et al., 2003; Saavalainen et al., 2005; Monslow et al., 2009
IFN-γ			↑	Sayo et al., 2002; Ohtani et al., 2009
IL-1β	↓[1]		↓[1]	Jokela et al., 2008b
IL-4			↑	Ohtani et al., 2009
IL-13			↑	Ohtani et al., 2009
KGF		↑	↑	Karvinen et al., 2003b
TGF-β	↑	↓	↓	Sugiyama et al., 1998; Sayo et al., 2002; Pasonen-Seppänen et al., 2003
TNF-α	↑	↑		Jokela et al., 2008b

[1] Tendency, not statistically significant

Many more inducing or inhibiting factors have been recognized in other cell types. In the epithelial breast cancer cell line MCF-7, the cyclic oligosaccharide methyl-β-cyclodextrin (MβCD), which depletes cholesterol from lipid rafts in the plasma membrane, dramatically inhibits *HAS2* expression as well as HA synthesis (Kultti et al., 2010). The signaling leading to *HAS2* transcription in these cells appears to go through the PI3K-Akt-mTOR-pathway. Of note, the residence of the HAS-enzymes in specific regions of the plasma membrane, as well as lipid composition, may play a role in their activity. This is implicated by *HAS3*-transfected MCF-7 cells where lipid raft microdomains partially colocalize with HAS3-rich microvilli. These protrusions, which are induced by the *HAS3*-overexpression, are efficiently disrupted after cholesterol depletion (Kultti et al., 2006). Moreover, aortic smooth muscle cells (ASMC) from the hyperlipidemic Watanabe rabbits (WHHL) as well as fibroblasts from patients with familial hypercholesterolemia exhibit abnormal accumulation of hyaluronan, which can be reversed by cholesterol depletion or lovastatin treatment (Sakr et al., 2008).

4-methylumbelliferone (4-MU) has been applied as a specific inhibitor of HA synthesis (Nakamura et al., 1995; Kakizaki et al., 2004), although its effects on cellular metabolism and ECM modification are likely to be of a more general nature (Nakamura et al., 2007; García-Vilas et al., 2013). With this approach, it has been shown that 4-MU depletes the intracellular precursor pool of UDP-GlcUA by being glucuronidated itself (Kakizaki et al., 2004). It also downregulates both *HAS2* and *HAS3* transcription (Kultti et al., 2009).

HAS2 expression and hyaluronan synthesis is also strongly downregulated by the therapeutically used glucocorticoids (dexamethasone, hydrocortisone) in fibroblasts (Zhang et al., 2000), keratinocytes (Gebhardt et al., 2010) and skin organ culture explants (Ågren et al., 1995). This may eventually result in atrophy in susceptible organs, including skin. These data need to be kept in mind, as hydrocortisone is often added to *in vitro* cultures, where it may inadvertently interfere with analyses of HA metabolism.

Regulation of HA synthesis via UDP-sugar balance

HA synthesis appears to be heavily dependent on the *de novo* production of the enzymes, as indicated by the frequently observed close relationship between *HAS* mRNA expression and HA accumulation both under normal conditions (Jacobson et al., 2000) and after a

stimulus (e.g. Tobiishi et al., 2011). However, the capacity to synthesize HA can also be regulated posttranslationally.

Most importantly, HAS activity depends on the cellular content of the simple precursor sugars and their metabolic pathways (Jokela et al., 2008a; Rilla et al., 2013). For instance, mannose significantly inhibits hyaluronan synthesis in REK keratinocytes by depleting the cellular pool of UDP-N-acetylhexosamines (Jokela et al., 2008a). This inhibition can be counteracted by supplying the cells with glucosamine. HA synthesis in human keratinocytes also increases in response to GlcNAc without changes in *HAS* transcription (Sayo et al., 2004). UDP-GlcNAc also seems to have a feedback mechanism on the expression of *HAS2* via the transcription factors SP1 and YY1 (Jokela et al., 2011). The O-GlcNAc modification (O-GlcNAcylation) of these regulatory proteins is an effective means to couple the metabolic status of the cell to *HAS* expression and hyaluronan synthesis.

The dependence of HA synthesis on sugar availability also creates an important pathobiological mechanism, as excess glucose could be converted to HA in diabetic individuals, overriding the normal homeostatic regulation. Accumulation of HA has indeed been observed in the aortic tunica media of diabetic patients (Heickendorff et al., 1994). Similarly, nephropathic complications in diabetes involve the accumulation of HA in the cortical interstitium (Lewis et al., 2008).

Posttranslational modifications of the HAS-enzymes

O-GlcNAcylation has been suggested to directly control HAS activity (Vigetti et al., 2012). Treatment of aortic smooth muscle cells with modifiers of this post-translational pathway indicated that HAS2 activity and half-life of the enzyme are increased as a result of GlcNAc addition to Ser^{221}. This serine residue resides in a cytoplasmic loop that is necessary for the glycosyltransferase activities. It was further demonstrated that O-GlcNAcylation inhibits the proteasomal degradation of HAS2. Interestingly, HAS3 was unaffected by the treatments.

Regulation of the HAS-enzymes by phosphorylation has also been suggested. In the ectopically expressed human HAS3 a clear serine phosphorylation could be seen in COS-7 cells. EGF was one of the factors that induced this post-translational modification, possibly indirectly via affecting cAMP-dependent pathways (Goentzel et al., 2006). The modified residue wasn't identified, however. Similarly, in the SK-OV-3.ipl human ovarian tumor cells serine phosphorylation was evident in all of the HAS-isoforms in response to a heregulin treatment, which activates the ErbB2-ERK-pathway and significantly augments HA production (Bourguignon et al., 2007).

Moreover, the involvement of AMP activated protein kinase (AMPK), which functions as an intracellular energy sensor, has been implicated (Vigetti et al., 2011). In aortic smooth muscle cells AMPK phosphorylates HAS2 at Thr^{110} efficiently inhibiting normal enzymatic activity. In dermal fibroblasts, however, AMPK has been linked to increased *HAS2* mRNA expression and HA production in response to adiponectin (Yamane et al., 2011). This may be another way by which cells adjust their HA synthesis to changing metabolic conditions.

Ubiquitination (Karousou et al., 2010) as well as the trafficking of the enzymes to the plasma membrane, where they are activated (Rilla et al., 2005; Deen et al., 2014), also provides a means to regulate HA production. Mono-ubiquitination of HAS2 at Lys^{190} appears critical for its activity, whereas poly-ubiquitinated molecules are targeted to degradation (Karousou et al., 2010). Lys^{190} is conserved among the HAS-enzymes, pointing to its potential role in regulating HA synthesis more widely (Spicer & McDonald, 1998).

The homo- and hetero-oligomerization HAS-isoforms in various combinations is a fairly recent finding, but seems important for their synthetic capacity (Karousou et al., 2010; Bart et al., 2015). One rationale for this could be the need for a bigger complex through which the nascent HA chain can be extruded into the extracellular space (Karousou et al., 2010). The formation of these complexes, which was demonstrated *in situ* as well as transfected cells, could also regulate the synthesis rate of HA (Bart et al., 2015). This becomes even more significant with the finding that all combinations of HAS homomers and heteromers are possible. The interaction seems to depend on a fairly short stretch in the N-termini of the enzymes, with possible additional binding domains at the C-termini, leaving the catalytic central fully functional but regulatable.

Furthermore, the residence of HAS3 at the plasma membrane and its synthetic activity have been shown to depend on its recycling to early endosomes. This retrograde transport is controlled by Rab10, one of the key regulators of intracellular vesicular traffic, and it provides an important, novel level of fine-tunable regulation in HA synthesis (Deen et al., 2014). HAS3 has been an apt target for these experiments, as it is easily detected at the plasma membrane. This isoform also produces prominent pericellular coats and microvilli-like structures when overexpressed and functional at the cell surface (Rilla et al., 2008).

Physical regulators of HA synthesis

The external, environmental factors affecting HA accumulation/retention and *HAS* expression have also been characterized. Hyaluronan metabolism is highly responsive to various physical signals, as evidenced by wounding or loss of epidermal permeability barrier integrity (Oksala et al., 1995; Maytin et al., 2004; Tammi et al., 2005; Monslow et al., 2009). For example, conditioned media from wounded organotypic keratinocyte cultures induces both HA accumulation and *Has3* expression in controls (Monslow et al., 2009). This further indicates that the effect depends on a soluble mediator. This factor was recognized as HB-EGF, which is rapidly cleaved at the plasma membrane after wounding. It induces downstream signaling from EGFR with increased *HAS* mRNA expression and HA deposition (Monslow et al., 2009). Disruption of the epidermal permeability barrier by stripping the stratum corneum with acetone also leads to intense HA accumulation in mouse skin (Maytin et al., 2004). The same is true for tape-stripped mouse skin, where *Has2* and *Has3* as well as *Cd44* (cluster of differentiation 44; a hyaluronan receptor) expression levels rise concomitantly 3 days after injury (Tammi et al., 2005).

In the context of skin, UVR is one of the most relevant and frequent stressors encountered in everyday life. Interestingly, it appears to be a highly potent regulator of hyaluronan synthesis both acutely and chronically. Furthermore, cell type-specific and temporal patterns in the responses have been observed (e.g. Averbeck et al., 2007; Dai et al., 2007; Kakizaki et al., 2008).

2.2.3 Hyaluronan degradation in mammals

The newly synthesized HA chains are rapidly degraded with half-lifes ranging from a few minutes in circulation to a few weeks in cartilage (Stern et al., 2007). The dynamic, stepwise catabolism of the molecule highlights its importance in maintaining normal homeostasis in the extracellular environment. For biochemical and bioengineering purposes, sonication, heating, irradiation, physical shearing and stirring as well as treatments with acids or alkali can be employed (Stern et al, 2007). These conditions also need to be minded, when

handling HA samples for qualitative or quantitative analyses to avoid unwanted fragmentation of the native molecules.

Biologically, the most relevant degradative mechanisms are enzymatic depolymerization and non-selective chain breakage by free radicals/ROS after e.g. UV irradiation or during inflammation (Greenwald & Moy, 1980; Ågren et al., 1997; Soltés et al., 2006; Hakozaki et al., 2008). The enzymatic degradation can occur either locally in the original tissue of hyaluronan production, as in the epidermis, or systemically after removal of the interstitial or synovial fluid to lymph nodes and further to liver, kidney and spleen (Stern et al., 2006; Jadin et al., 2012). This process is aided by several different HA receptors, including CD44, LYVE-1 and HARE (see chapter 2.2.4.).

A total of 6 genes encoding hyaluronidases exist in the human genome, many of which have homologs in other animals and bacteria (Stern & Jedrzejas, 2006). These include *HYAL1*, *HYAL2*, *HYAL3*, *HYAL4*, *PH-20*/*SPAM1* and *PHYAL1*, the latter being a probable pseudogene. Similar to the HAS-enzymes, their activity may be controlled by alternative splicing (Lokeshwar et al., 2002). Mammalian hyaluronidases are hydrolytic endo-β-N-acetyl-D-hexosaminidases that produce tetra- and hexasaccharides. These end products are further degraded to the simple precursor sugars by exoglycosidases. Hyaluronan is not the exclusive substrate of the HYALs, but more or less limited degradation of chondroitin and chondroitin sulfates also takes place (Stern et al., 2007; Honda et al., 2012).

The somatic degradation of hyaluronan in mammals is performed mainly by HYAL1 and HYAL2. HYAL1 seems to be an intracellular enzyme with an acidic pH optimum and a lysosomal localization (Triggs-Raine et al., 1999), as indicated by colocalization with cathepsins B and D (Malaisse et al., 2015). HYAL1 is also found in plasma, where its specific activity is high (Frost et al., 1997). Yet, the physiological role of the enzyme is not clearly revealed even by KO animals, which are viable and fertile. *Hyal1*$^{-/-}$ mice don't exhibit any obvious anatomical deformities either (Martin et al., 2008). However, the *Hyal1* KO animals do exhibit abnormal accumulation of HA in articular cartilage. This leads to osteoarthritis, similarly to the human lysosomal GAG storage disease mucopolysaccharidosis (MPS) IX (Martin et al., 2008). The condition in humans is due to a recessive mutation in *HYAL1* and it is characterized by periarticular masses rich in hyaluronan as well as alterations in axial growth (Natowicz et al., 1996; Triggs-Raine et al., 1999). At the same time, HYAL3 expression is increased, pointing to a partial redundancy between the isoforms.

In contrast to the human disease, HYAL1-deficient mice have normal levels of HA in circulation and elsewhere in the body. This also pertains to skin, where dermis is one of the most active sites of local HA degradation. However, marked accumulation of HMW-HA (up to 3 MDa) in the terminally differentiated and desquamating stratum corneum has been suggested to occur in *Hyal1*$^{-/-}$ mice (Malaisse et al., 2015). Even then, epidermal stratification appears normal, as the classical markers of differentiation and permeability barrier/TJ formation (K10, loricrin, claudin-1, occludin and ZO-1) are present and normally localized (Malaisse et al., 2015). The pH and permeability characteristics of the skin in the *Hyal1*$^{-/-}$ mice are also comparable to wild-type. The findings could in theory be explained by the fact that β-hexosaminidases may in part substitute for HYAL1, at least in the mouse (Gushulak et al., 2012).

In normal human keratinocytes, HYAL1 expression is increased in confluent monolayers (Malaisse et al., 2015). In maturing 3D reconstituted human epidermis (RHE) cultures it localizes very specifically to the granular layer, where HA content is low or

absent, and the enzyme is also observably functional in normal human epidermis as analyzed by zymography (Malaisse et al., 2015). Experiments in REK cells have also indicated that the degradative pathways in keratinocytes are active, even for newly synthesized HA (Tammi et al., 2000; 2001). Furthermore, the residence of internalized HA in lysosomes as opposed to endosomal vesicles appears fairly inconspicuous suggesting that this step of the pathway is fast (Tammi et al., 2001). Thus, intracellular degradative enzymes, whether HYALs or possibly exoglycosidases, must be active in proliferating undifferentiated keratinocytes.

HYAL2 was first characterized as a lysosomally active enzyme (Lepperdinger et al., 1998). Later, it was shown to reside at the plasma membrane as an extracellular, GPI-anchored protein (Rai et al., 2001; Müllegger & Lepperdinger 2002a). There, it appears to act in the first steps of HA catabolism. HYAL2 knockout hasn't been explicitly described in humans, but Hyal2$^{-/-}$ KO mice show a clear phenotype with skeletal deformities and changes in the circulatory and lymphatic systems (Jadin et al., 2008; Bourguignon & Flamion, 2016). More specifically, there are lower numbers of thrombocytes and mature red blood cells in the circulation, whereas plasma HA levels are highly elevated. HA also appears to accumulate in the liver sinusoidal system. The normal homeostatic regulation would thus be overwhelmed in Hyal2$^{-/-}$ mice, even with the balancing act of augmented HYAL1 activity (2-fold in comparison to the wild-type) in the plasma as well as increased *Hyal1* expression in the kidney (Jadin et al., 2008).

HYAL2 also appears to have another function at the plasma membrane; it regulates the formation of the HA-rich glycocalyx and the signaling mediated by CD44 (Duterme et al., 2009). In *Hyal2*-overexpressing stable transfectants the pericellular coat is effectively lost without any significant hyaluronidase activity conferred by the construct. This indicates that HYAL2 may have other, non-enzymatic properties that are important in maintaining a homeostatic balance in peri-/extracellular HA (Duterme et al., 2009). CD44 expression also seems indispensable for the normal catabolic pathways by HYAL1 and HYAL2 (Harada & Takahashi, 2007), strengthening the functional link between these HA-associated proteins.

HYAL3 is supposedly an enzyme capable of degrading hyaluronan with a low activity in an acidic environment, but its role seems to be a minor one and rather supporting the expression and activity of HYAL1 (Lokeshwar et al., 2002; Hemming et al., 2008). Hyal3$^{-/-}$ KO mice do not have a gross phenotype of HA accumulation, indicating that HYAL3 is probably not needed constitutively (Atmuri et al., 2008). However, a role for HYAL3 in both human and mouse sperm has been described (Reese et al., 2010). It may thus function in concert with the traditionally recognized sperm-specific hyaluronidase PH-20/SPAM1 (Gmachl et al., 1993). HYAL4 degrades chondroitin sulfate, despite its sequence similarity with e.g. HYAL1 (Kaneiwa et al., 2010), and HYAL5 has only been described in mouse, where it may participate in reproductional events (Reitinger et al., 2007; Kimura et al., 2009).

The latest addition to the group of hyaluronan-processing proteins is KIAA1199 (also known as CEMIP or HYBID). It is also expressed in dermal fibroblasts, where it responds to treatment with various growth factors (Yoshida et al., 2013; Nagaoka et al., 2015). This degradative pathway for hyaluronan appears to utilize clathrin-coated pits, but whether KIAA1199 is an actual enzyme or an auxiliary protein in HA degradation is still unclear (Yoshida et al., 2013). Functionally, overexpression of KIAA1199 has been implicated in EMT and cancer progression (Zhang et al., 2014).

To summarize (according to Stern, 2004), extracellular HA is first cleaved into medium-sized fragments of around 20 kDa (50-60 disaccharides) by HYAL2, either at the plasma membrane and/or in early endosomes. These fragments are internalized and further degraded into smaller oligosaccharides (up to tetrasaccharides) by HYAL1. β-N-acetylglucosaminidase and β-glucuronidase then complete the process in late endosomes and lysosomes. This is supported by data from the Hyal2$^{-/-}$ mice which have highly increased levels of HA in the plasma (Jadin et al., 2008). HYAL2 would thus function in the first steps of HA internalization and clearance from the lymph, extracellular fluid and blood, and HYAL1 could then continue the process intracellularly. It is noteworthy, that degradation of hyaluronan extends beyond mere disposal of excess ECM molecules. Hyaluronan fragments as well as the native, high molecular-weight polymers signal via various HA-binding proteins. This results in complex biological outcomes as discussed next.

2.2.4 The signaling properties of hyaluronan: hyaluronan receptors and hyaluronan binding proteins

Link module and general considerations for HA binding – Association of HA with various receptors affects its stability, conformation and degradation (Stern & Maibach, 2008). The hyaluronan binding domains in the various proteins, dubbed collectively as hyaladherins, often include one or two Link modules, with possible additional flanking amino acid residues. They may also lack the Link module, but contain basic amino acid motifs (BX$_7$B; as in receptor for hyaluronan-mediated motility, or RHAMM). The Link protein itself consists of an immunoglobulin domain and two Link modules, also known as proteoglycan tandem repeats. This combination as such is found in several HA-binding proteins (Day & Prestwich, 2002). Moreover, the simple Link protein crosslinks hyaluronan with other proteins, such as aggrecan, producing large, multimolecular complexes in HA-rich tissues, such as articular cartilage.

The most well-known members of the Link module superfamily are CD44, LYVE-1 and TSG-6 as well as the proteoglycans aggrecan and versican. Other proteins that lack the Link module, but are essential for HA binding and signaling, include RHAMM and IαI. In addition to the classical extracellular receptors, hyaladherins may also exist intracellularly, although this part of HA processing is not currently well understood.

CD44 – CD44 is probably the best-characterized example of integral plasma membrane proteins that function as receptors for peri- and extracellular hyaluronan. It also binds other molecules of the extracellular matrix including collagen, fibronectin and laminin. CD44 is a single-pass membrane protein that binds HA with its extracellular domains (Link module and BX$_7$B/basic residues). CD44 interacts with the cytoskeleton and the signaling machinery of the cell with a short intracellular domain regulating multiple cellular functions.

The CD44 gene contains 20 exons. It produces various different splice variants (CD44v) in addition to the standard receptor (CD44s or CD44H), which contains exons 1-5, 16-18 and 20 or 19, the inclusion of which results in a shorter cytoplasmic tail (Marhaba & Zöller, 2004; Prochazka et al., 2014). Exons 6-15 (v1-v10) are differentially spliced together with the standard exons to produce the variant forms. Exon 6 (v1) can be included in murine CD44, but in humans it contains a stop codon (Prochazka et al., 2014). The intracellular domain (exon 19 or 20) is short, connecting via a single transmembrane domain (exon 18) to the long extracellular part (Marhaba & Zöller, 2004). Besides splicing, variability in CD44

molecular weight and properties are produced by N- and O-linked glycosylation, phosphorylation and substitution with GAG chains, such as chondroitin and heparan sulfate (Tuhkanen et al., 1997; Zhou et al., 1999; Marhaba & Zöller, 2004).

The expression of the different variants depends mainly on cell/tissue type (Fox et al., 1994), although the physiological context also has an effect. Thus, cancer cells express a different set of CD44 proteins than normal, untransformed cells (Misra et al., 2011). The variants specific for keratinocytes also depend on the differentiation status of the cells (Zhou et al., 1999). Total CD44 expression decreases in confluent and differentiated keratinocytes, and the expression of some forms (CD44H and CD44E, also known as the epithelial variant CD44v8-v10) ceases. The CD44 variant specific for keratinocytes appears to be longer still (CD44v3-v10 or epican; Zhou et al., 1999; Marhaba & Zöller, 2004).

CD44 function can be regulated by changing its level of clustering, which is in turn controlled by splicing and glycosylation (Sleeman et al., 1996) as well as the size of the HA molecules available (Yang et al, 2012). For instance, CD44 oligomerization enhances HMW-HA binding, but not HA oligosaccharide tethering, in mouse T-cell lymphoma cells transfected with different CD44-constructs (Lesley et al., 2000). Available data indicate that polymer length is one of the most critical determinants in receptor-HA interaction. The conformational requirements of the HA-chain and the optimal orientation of the binding proteins are less well understood. The actual length of the HA chain required for binding appears to vary between cell types, even with the same receptor. In the case of CD44, endogenous HA is displaced with decasaccharides in keratinocytes (Tammi et al., 1998), whereas in chondrocytes a shorter chain (HA_6) is enough (Knudson, 1993).

The contribution of CD44 to specific recognition of GAGs in skin was long unresolved, but data from keratinocyte monolayers as well as skin *in vivo* has corroborated its role as the main receptor for HA in this tissue (Tammi et al., 1998; Tammi et al., 2001; Pasonen-Seppänen et al., 2012a). In mouse epidermis, CD44 appears to be a central molecule in organizing the HA-rich matrix, as hyaluronan in $CD44^{-/-}$ animals tends to aggregate diffusely in the suprabasal layers under conditions where HA synthesis is strongly stimulated (Pasonen-Seppänen et al., 2012a). In REK keratinocytes, about 50% of pericellular HA appears to be bound to CD44, while the remaining half is most likely still attached to the HAS-enzymes (Tammi et al., 1998). The role of the other HA receptors in these cells seems small.

RHAMM – Receptor for HA-mediated motility (RHAMM or CD168) was originally cloned and characterized in the early 1990's (Turley et al., 1991; Hardwick et al., 1992). It exists both at the plasma membrane as a non-integral, CD44- and growth factor receptor-associating and a free cytosolic or nuclear protein. Intracellularly, it binds to the cytoskeleton, particularly tubulin, affecting many aspects of cell morphology and migration. RHAMM expression under normal, physiological conditions is low, but increases during wound repair, tissue remodeling and in several cancers. RHAMM can be alternatively spliced in exons 4, 5 and 13, and N-terminally truncated variants have also been detected. These various isoforms are differentially expressed in cultured cells at various times after plating, as well as under pathological conditions, including wounded skin and neoplastic tissues. This structural variation and additional posttranslational modifications probably regulate the trafficking and localization of RHAMM to its multiple functional locations in the cell or at the plasma membrane (Cheung et al., 1999; Tolg et al., 2014).

Considering the association with HA, RHAMM binds both large, native polymers and smaller fragments; this binding is mediated by the C-terminal BX7B-domains (Day & Mascarenhas, 2004). The association with HA, CD44 and additional proteins results in a controlled regulation of adhesion, migration and transformation, including EMT (Misra et al., 2015), as well as proliferation (Day & Mascarenhas, 2004). Moreover, it seems that RHAMM not only regulates HA binding and cell fate but also the expression CD44, at least in cells of mesenchymal origin (Veiseh et al., 2015). This also highlights the co-operation between the proteins binding and processing hyaluronan.

LYVE-1 and HARE – The degradation of systemic hyaluronan by HYALs in the liver, spleen and lymph nodes is aided by two plasma membrane receptors that bind to and help internalize circulating HWM-HA in these organs (reviewed in Day & Mascarenhas, 2004). The integral plasma membrane protein LYVE-1 (lymphatic vessel endothelial receptor-1) is expressed on endothelial cells in lymphatic vessels as well as the sinusoids of the liver and the spleen. LYVE-1 was recently shown to require either receptor clustering or a specific configuration of the HA substrate to enable binding to either the matrix-derived HA-molecules or those on the surface of migrating macrophages (Lawrance et al., 2016). Thus, LYVE-1 participates in immunological functions as well as clearance of ECM components.

HARE (hyaluronan receptor for endocytosis) is also expressed on vascular endothelial cells in liver sinusoids, in the loose venous sinuses of the spleen and on lymph node sinusoids. It participates in the uptake of HA either from the circulation (liver and spleen) or lymph draining the tissues. The HARE protein is proteolytically processed from stabilin-2 (Day & Mascarenhas, 2004). Aside from HA, HARE binds to and internalizes other components of the ECM, including heparin, dermatan sulfate and chondroitin sulfates. Moreover, the binding of these ligands may activate intracellular signaling cascades that appear dependent on a specific endocytic motif within the HARE protein (Pandey et al., 2016). However, the functional complexity and signaling prospects of this scavenger receptor are only slowly starting to unravel.

Layilin – Layilin is one of the least explored HA-binding proteins, even though its binding properties appear specific for HA (Day & Mascarenhas, 2004). It is a single-pass transmembrane protein that interacts intracellularly with the cytoskeleton. Layilin localizes in several cell types, including the leading edges of migrating lung carcinoma cells (Chen et al., 2008). It also mediates loss of cell-cell contacts and barrier function in bronchial epithelial cells by disruption of E-cadherin expression in response to HA fragments generated by cigarette smoke (Forteza et al., 2012). This process activates the RhoA/ROCK pathway. Layilin may thus mediate information about the state of the extracellular milieu and HA matrix to the intracellular signaling machinery.

Inter-α-trypsin inhibitor and TSG-6 – IαI is a protein-GAG-protein complex found in plasma and urine. It acts as a protease inhibitor and a cross-linker of HA chains in tissues. IαI is composed of a light chain, the bikunin proteoglycan with an attached chondroitin sulfate chain, and two heavy protein chains which attach to the CS-moiety. The heavy chains are encoded by at least four different genes. They are responsible for the covalent cross-links between HA molecules in an ovulatory follicle and under inflammatory conditions in various tissues (Zhuo et al., 2004). This cross-linking is mediated by another HA-binding protein called TSG-6 (tumor necrosis factor-stimulated gene-6), which interacts with HA via a Link module. The TSG-6 protein is also associated with inflammatory processes, and it's able to bind and cross-link HA on its own (Baranova et al., 2011).

Intracellular HA receptors – In addition to the classical cell surface receptors, HA also appears to be bound intracellularly by several proteins. These include CDC37 (Grammatikakis et al., 1995), P32 (Deb & Datta, 1996) and IHABP4 (Huang et al., 2000). These proteins may have roles in regulating cell division (CDC37, IHABP4) as well as RNA splicing (P32; also known as HABP1 or gC1qR), but they are, however, still poorly understood.

Other hyaluronan-associated proteins: Emmprin (CD147) and TLR – HA has also been conceptually and experimentally linked to other plasma membrane receptors. CD147 (also known as emmprin or basigin) is an integral membrane glycoprotein which exists as four splice variants (Grass et al., 2014). It interacts with the other HA receptors, including CD44, but does not directly bind HA. Upregulated expression of CD147 may induce HA synthesis, simultaneously promoting anchorage-independent growth and cell survival (Marieb et al., 2004), although the mechanism is unclear. CD147, like many other HA-binding molecules, participates in inflammatory processes as well as wound healing. It also has a central role in various cancers where HA and CD147, in conjunction with CD44 and EGFR, may actually have a positive feedback loop promoting invasiveness (Grass et al., 2014).

Additionally, hyaluronan belongs to a group of naturally occurring danger signals (Schaefer, 2014). Particularly LMW-HA appears to have an important role in mediating information about a disintegrated ECM. One mechanism for this is signaling via the toll-like receptors (TLR), such as TLR2 and TLR4, to induce expression of inflammation-associated genes (Scheibner et al., 2006; Voelcker et al., 2008). This cascade also plays a role in cancer progression, as the TLR4-mediated signals from hyaluronan fragments may increase the invasive potential of human melanoma cells (Voelcker et al., 2008).

2.2.5 Hyaluronan in cancers

Retaining or abolishing HA in the peri- and extracellular matrix in tumors has many interesting features and implications. A case in point is the varying concentration of HA in the tumor parenchyma vs. the surrounding stroma, depending on the cancer type, stage and grade (Tammi et al., 2008). Stromal HA plays a distinct role in cancer development, participating in e.g. recruitment of tumor-associated macrophages (Kobayashi et al., 2010). Its synthesis is also responsive to signals from the tumor cells (Pasonen-Seppänen et al., 2012b).

Considering the cancer parenchyma, malignancies originating from simple epithelia tend to accumulate HA, whereas stratified tissues often end up losing the HA-rich pericellular matrix (Tammi et al., 2008). The different HAS-enzymes may be expressed differentially, opening up possibilities for diagnostic procedures or therapeutic interventions (e.g. Auvinen et al., 2014; Poukka et al., 2016). Differential splicing is also not uncommon in the HA-associated genes in tumor tissues: this applies to at least *HAS1* (Ghosh et al., 2009) and *CD44* (Misra et al., 2011). Examples of cancers, where increased HA levels and/or *HAS* expression associate with more aggressive tumor behavior or poor prognosis, include breast (Auvinen et al., 2014), ovarian (Hiltunen et al., 2002) and colorectal cancers (Ropponen et al., 1998). On the other hand, hyaluronan synthesis may decrease in cancers arising from stratified epithelia, including those of the oral epithelium (Kosunen et al., 2004).

Hyaluronan is strongly implicated in skin cancers, including epidermal SCC and melanoma (Karvinen et al., 2003a; Siiskonen et al., 2013). Increased HA synthesis may simply enable the cells to better detach from their surrounding matrix and the desmosomal contacts between adjacent keratinocytes. Abundant stromal hyaluronan may then enable efficient growth, invasion and metastasis. In this regard, the decreased HA staining observed in advanced melanomas and SCC (Karvinen et al., 2003a; Siiskonen et al., 2013) appears counterintuitive. However, the loss of a constraining matrix and contacts mediated by CD44 may help cells escape their immediate environment, once a transformed phenotype is established. Decreased HA content is actually recognized as a prognostic factor in advanced oral squamous cell carcinomas and melanoma (Karjalainen et al., 2000; Kosunen et al., 2004).

With respect to signaling, long HA-chains can induce the aggregation and interaction of CD44 and other plasma membrane proteins (receptors, transporters, proteases). This mechanism regulates processes and intracellular kinase cascades that inhibit apoptosis and favor proliferation or migration and invasion as well as chemoresistance (Toole, 2009; Yang et al., 2012). The specific binding of HA to its receptors, such as CD44 (particularly the tumor-associated variant forms) and RHAMM, have also rendered it an interesting molecule to be used in targeting drugs to tumor tissues. This could be done by conjugating the therapeutic molecule to a backbone of HA or encapsulating the drug within a domain of HA (Misra et al., 2015). Blocking CD44 by various techniques, such as isoform-specific antibodies, also seems a promising approach, as does degradation of the peritumoral HA matrix or inhibition of HA synthesis to enable drug and immune cell penetration and to attenuate the HA-mediated signaling (Tammi et al., 2008; Kultti et al., 2012).

Hyaluronidases are also involved in cancer progression and metastasis, particularly in neoplasias of the genito-urinary tract. Elevated expressions of HYAL1-3 and PH-20 have been observed in more than one cancer type (Lokeshwar & Selzer, 2008). Increased expression of HYAL2 is also evident in advanced melanoma (Siiskonen et al., 2013). Interestingly, the stretch in chromosome 3 bearing the first three *HYAL*-genes as well as a tumor suppressor (RASSF1) is often deleted in lung and breast cancers (Lokeshwar & Selzer, 2008). Changes in this chromosomal region have also been observed in ovarian cancer, even though the genomic imbalance in this case was not directly related to disease parameters or HA content (Tuhkanen et al., 2004). Ovarian cancers and endometrial carcinomas have later been shown to exhibit lowered levels of HYAL1 (Nykopp et al., 2009; Nykopp et al., 2015).

Interestingly, shorter HA-chains can promote neo-vascularization of the malignant tissue (Chanmee et al., 2016), but HA-fragments can also block signaling via CD44 by displacing native HMW-HA (Toole, 2009). However, very long HA-molecules have been proposed to induce longevity and cancer resistance in some species (Tian et al., 2013). These data further highlight the delicate balance of hyaluronan processing in tumors. Whether delivery of exogenous hyaluronan-carrying therapeutic agents or perturbation of the metabolic machinery with drugs changes this homeostasis in cancer, for better or for worse, is carefully being investigated. For now, it seems that high enough concentrations of hyaluronidase may indeed have tumor suppressive effects (Lokeshwar & Selzer, 2008).

2.2.6 Hyaluronan in epidermis

Localization and metabolic processing of epidermal hyaluronan

Early studies of the distribution of HA in human epidermis showed that it stains specifically in the lower, living layers (stratum basale and stratum spinosum) with biotinylated hyaluronan binding complex (bHABC) (Tammi et al., 1988; Wang et al., 1992). HA is also quite abundant in the sebaceous gland and in the hair follicle. In the sweat glands HA is present to a lesser extent. The dermal compartment of skin contains even larger amounts of hyaluronan, and the bulk of total HA in the body is found in these two outermost layers of skin (Anderegg et al., 2014). The concentration in developing epidermis is particularly high. This HA is most likely important for the proliferative capacity of the basal cells. HA also helps to maintain a proper water balance and provides a matrix for metabolite diffusion and migration of cells, including those of the immune system (Anderegg et al., 2014). This is particularly important in the epidermis, which contains no vasculature.

A study from 2000 (Sakai et al., 2000) reported that HA is found to a substantial degree in the stratum corneum as measured by biochemical methods. A similar observation was later made by Maytin et al. (2004) in mouse skin after disrupting the permeability barrier with a series of acetone treatments. In their immunohistochemical stainings, particles containing HA appeared different from the typical keratohyalin granules in the upper, differentiated layers of the epidermis. The stratum corneum in *Hyal1*$^{-/-}$ KO mice also appears to retain substantial amounts of HA, in contrast to the wild-type animals where detectable but low levels of HA are present (Malaisse et al., 2015). As there are no further data on the issue, the possible significance of retaining these small amounts of HA in terminally differentiated keratinocytes cannot be established at present. Theoretically, they could participate in the hydration of the outermost layers of skin, particularly after injury.

HA is actively synthesized in the epidermis, and the molecular weight of the molecules is high, up to 5 MDa (Tammi et al., 1991). Degradation into smaller fragments is also fast, as shown by 4 days of chase in human skin explants (Tammi et al., 1991). In this system, the half-life of metabolically labeled HA was around 1 day. The half-life of pericellular HA in keratinocyte monolayers is also short, approximately 8 h (Tammi et al., 1998). This probably reflects transfer of the newly synthesized molecules into the extracellular environment, more so than internalization and degradation. This is highlighted by the continued accumulation of labeled HA in the medium. The half-life of intracellular HA is estimated to be even shorter, around 2-3 h (Tammi et al, 2001). These data indicate an active, local turnover of HA in keratinocytes, which probably has important biological consequences.

Nevertheless, the majority of HA in keratinocytes (REK, HaCaT) is peri- or extracellular. Only a small proportion (4-7%) seems to be found within the cells under steady-state conditions i.e. after a 24-h labeling period (Tammi et al., 1998; Pasonen-Seppänen et al., 2012a). Internalization occurs by at least two mechanisms. The CD44-dependent pathway appears dominant in keratinocytes, but nonspecific bulk phase endocytosis may also play a role (Tammi et al., 2001).

Hyaluronan regulates proliferation and migration of epidermal cells

Epidermis is a tissue with limited extracellular material: nevertheless, the ability of the keratinocytes to divide and migrate during the tightly regulated differentiation program is high. The abundant pericellular HA, in conjunction with CD44, appears very important in sustaining mitoses (Tammi & Tammi, 1991) and proliferation (Kaya et al., 1997). *De novo*

HA synthesis is also related to the adhesion and migration of keratinocytes (Rilla et al., 2002). The resident Langerhans cells may benefit from the looser extracellular spaces created by hyaluronan as well. Specifically, increased deposition of HA appears to support cell proliferation and migration after wounding (reviewed by Aya & Stern, 2014).

With aging, epidermal hyaluronan has been reported to be lost with no drastic alterations to the dermal pool, even though the interactions of HA with other dermal components undergo modifications (Stern & Maibach, 2008). These changes result, in part, in the less than optimal functional properties of aging skin. There is, however, little data on epidermal hyaluronan metabolism in the elderly.

HA and CD44 regulate keratinocyte differentiation and permeability barrier formation

The role of CD44 in retaining pericellular HA has been highlighted in siRNA-treated HaCaT keratinocytes and CD44 KO mouse keratinocytes. There, the lack of the functional plasma membrane receptor reduced both peri- and intracellular HA and also changed its localization (Pasonen-Seppänen et al., 2012a). *In vivo*, the effects under normal physiological conditions were less dramatic. When epidermal remodeling was induced in $CD44^{-/-}$ mice (either by tape stripping or retinoic acid) hyaluronan accumulation was induced. However, its localization changed and very strongly staining, scattered spots were seen in the upper epidermal layers (Pasonen-Seppänen et al., 2012a). These could represent unattached hyaluronan deposits, which may affect permeability barrier function by altering the water balance of the tissue.

CD44 and CD44-HA interaction have also been shown to participate in the formation of an intact permeability barrier in skin more directly. CD44 KO mice exhibit a slightly delayed barrier formation during embryonic development, changing from the normal E17.5 to E18.5 (Kirschner et al., 2011). In addition, unusual localization and/or expression of lamellar bodies, tight junction components and the cell polarity complex protein Par3 is observed. In adult CD44 KO mice, skin recovery was delayed 1-3 h after tape stripping or acetone treatment as measured by TEWL (Bourguignon et al., 2006b). These animals also exhibited reduced epidermal staining of involucrin, profilaggrin and K10 as well as abnormalities in differentiation-related lipid metabolism after injury. Interestingly, this mouse strain showcased profound skin changes such as reduced epidermal proliferation and epidermal thinning even under basal, homeostatic conditions. Moreover, exogenous HA induced the expression of differentiation markers in organotypic cultures of human keratinocytes. This effect was blocked by an anti-CD44 antibody as well as *Cd44*-siRNA (Bourguignon et al., 2006b).

HA also appears to regulate differentiation in a 3D REK model (Passi et al., 2004). These cultures exhibit normal stratification and differentiation with a net accumulation of HA at the expected location in the basal and spinous layers. Removing HA further enhances the expression of K10 and filaggrin protein. Contrary to these findings, it was recently shown in normal human keratinocytes, that stripping HA either enzymatically or by addition of 4-MU causes no differences in *KRT10*, involucrin or filaggrin mRNA expression (Malaisse et al., 2014). Again, in these stratifying cultures a net accumulation of HA is observed, with *HAS1* being upregulated and *HAS3* downregulated during differentiation (Malaisse et al., 2014). The discrepancy between studies may be due to different culture conditions and media supplements, particularly hydrocortisone, which in high concentrations is a potent suppressor of epidermal hyaluronan metabolism (Ågren et al., 1995; Gebhardt et al., 2010).

The connection between hyaluronan and differentiation has also been shown the other way round, as hyaluronan synthesis and degradation in epidermis appear dependent on Ca^{2+} concentration and thus the maturation status of the cells. Isolated basal keratinocytes cultured in high, differentiation-inducing Ca^{2+} (1.2 mM) synthesize less HA than the same cells under low (0.05 mM) Ca^{2+} (Lamberg et al., 1986). The regulation thus seems to be a complex one, and probably also involves controlling the molecular weight distribution of the HA molecules present.

HMW-HA promotes cell survival and epidermal homeostasis

High molecular weight HA is known to act as a survival signal, which maintains normal homeostasis of the intact tissue. In contrast, fragmentation of HA may induce proliferation, migration and inflammatory responses. Balancing these processes may in part contribute to the equilibrium between proliferation and terminal cornification. Recently, Symonette and colleagues (2014) reported that an intermediate-sized hyaluronan (500 kDa) attached to a lipophilic phosphatidylethanolamine moiety could efficiently be delivered to mouse skin, particularly epidermis. This treatment induced keratinocyte proliferation and epidermal thickening as well as HA coat formation in fibroblasts *in vivo*. These data highlight the potential of HA in regulating and restoring epidermal homeostasis after pathological or environmental insults such as UVR, a major causative agent of neoplastic changes in skin.

2.3 UV AS AN ENVIRONMENTAL IRRITANT IN SKIN

2.3.1 Types of UV radiation and measurement conventions

Ultraviolet radiation (reviewed in Laihia et al., 2009) is part of the electromagnetic spectrum comprising everything from gamma rays to visible light and radio waves. It can be subdivided further into three regions in decreasing order of energy, all of which are produced by the sun: UVC (100-280 nm), UVB (280-315 nm; in some sources: 290-320 nm) and UVA (315-400 nm; in some sources: 320-400 nm). UVC and most of UVB are filtered out by the ozone layer, so that 1-10 % of UVR reaching the earth's surface is UVB and the remaining portion UVA; both of these have potent biological effects (Laihia et al., 2009).

The signaling pathways and biological effects elicited depend heavily on wavelength. This is manifested as separate UVA- and UVB-responses as well as a unique interaction between these radiation types (Krutmann, 2006). The outcome is additionally determined by the exposure type, whether acute or chronic (Weill et al., 2011), the irradiance or dose (energy input per unit area) and dose rate (Adler et al., 1996; Miller et al., 2008). The tissue compartment (epidermis vs. dermis) and cell type (Averbeck et al., 2007; Cho et al., 2008) and possible (partial) photoadaptation or pretreatment of the irradiated area with protective compounds (Seité et al., 2010) are also decisive. *In vitro*, the outcome is also somewhat influenced by the cell line used (Muthusamy & Piva, 2013). These considerations are particularly important when planning either clinical (e.g. phototherapy for psoriasis or atopic dermatitis) or cosmetic (tanning) treatment regimes as well as preparing broad-spectrum chemical sunscreens and physical filters such as sunglasses and fabrics.

Indicating exposures in humans is based mainly on two conventions: minimal erythemal dose (MED) or standard erythemal dose (SED) of erythemally weighted radiation, which takes into account the more damaging nature of the UVB wavelengths. MED is a variable always dependent on the individual's skin type and the degree of

previous solar exposures and tanning. It equals to approximately 200 J/m^2 (20 mJ/cm^2) in fair, unexposed skin. This dose causes a very light, but detectable erythemal reaction (Laihia et al., 2009). SED is an absolute measure, where 1 SED is equal to 100 J/m^2. This corresponds to approximately 10 min of direct, whole-body solar exposure at noon on a summer day in Northern Europe (Bogh et al., 2012). Such a dose attained every other week would also be enough to maintain adequate vitamin D levels during summertime. Thus, minimal erythema in light skin is produced after exposure to 2-3 SED (Table 4).

Table 4. Skin types and sensitivity to solar radiation and neoplasia; 1 SED = 100 J/m^2. Modified from Laihia et al., 2009.

Skin type	Skin color (prior to tanning)	Sensitivity	Dose (SED) required for erythema	Skin cancer risk
I	Extremely fair (freckled)	Burns easily (always); no tanning	2-3	Extremely high
II	Fair	Burns easily, tans a little	2-3	High
III	Fair of fair brown	Burns fairly easily, tans gradually (light tan)	3-7	Moderate
IV	Fair brown or olive	Burns occasionally, tans easily (moderately dark tan)	3-7	Low
V	Brown	Burns rarely, tans strongly (very dark tan)	7-12	
VI	Dark brown or black	Does not burn	7-12	

2.3.2 Effects of UV in skin

General points

UVB is the more energetic of the two types of UV irradiation penetrating the skin. Due to its properties and the inherent characteristics of skin, most of its absorbtion occurs in the epidermis, while UVA exerts many of its effects deeper, in the dermis (Laihia et al., 2009; Dupont et al., 2013). Besides melanin (absorption maximum: 300-370 nm) and urocanic acid (absorption maximum: 260-280 nm), which belong to the protective mechanisms of skin against radiation, many biomolecules essential for normal cellular functions and extracellular matrix integrity can be degraded or modified by UV light (Laihia et al., 2009; Watson et al., 2014). These include hyaluronan, whose synthesis changes in fibroblasts and keratinocytes in response to UVR *in vitro* as well as *in vivo* (Calikoglu et al., 2006; Averbeck et al., 2007; Tobiishi et al., 2011).

Repeated UV exposure induces hyperplastic and hyperkeratotic changes in the epidermis. This protects the skin from further damage by thickening the outermost layers, which can then better absorb or reflect the incident light. This adaptive mechanism seems particularly important in naturally more light-skinned (Caucasian) individuals with less constitutive pigment (Hennessy et al., 2005). Otherwise, accumulation of new melanin by UVB and the resulting pigmentation acts as an important protective mechanism. This delayed tanning, which may last up to several months, prevents sunburn to a much greater degree than the immediate reaction caused by UVA, whereby existing pigment is modified to produce a very transient (hours to days) change in skin tone (Laihia et a., 2009; Dupont et al., 2013).

However, neither of these adaptive changes are enough to protect skin entirely from the carcinogenic effects of solar radiation, particularly as UVB has been shown to disrupt the epidermal permeability barrier. This occurs by altering both the intercellular lipid

lamellae of the stratum corneum (Jiang et al., 2007) and the TJs in the granular layer (Yuki et al., 2011). Simultaneously, changes in the distribution of epidermal Ca^{2+} occur (Jiang et al., 2007).

DNA is at a particularly high risk of accumulating lesions when subjected to (repeated) UVB irradiation. The cyclobutane pyrimidine dimers (CPD) and (6-4) pyrimidinone photoproducts may establish mutations in the original sequence (Marrot & Meunier, 2008). These changes, when occurring in the renewing and proliferating stratum basale or the upper cell layers, may lead to malignancy originating from various cell types (see e.g. Boukamp, 2005). SCC and BCC originate from keratinocytes, whereas the resident melanocytes may transform to bring about malignant melanoma, and the neuronal Merkel cells can introduce Merkel cell carcinoma.

UVA produces more indirect effects via the generation of energetic metabolites such as ROS and reactive nitrogen species (RNS; Dupont et al., 2013). ROS may then create signature DNA lesions such as 8-oxo-deoxyguanosine (Marrot & Meunier, 2008). The potential for oxidative damage or direct genomic modifications can be exacerbated after topical or systemic exposure to chemicals or drugs that are themselves photoreactive under UV light (Marrot & Meunier, 2008). However, photosensitizers that are activated by less energetic wavelengths (visible light) can be utilized in photodynamic therapy for the treatment of various tumors, including those originating from keratinocytes (Anand et al., 2012).

The latest addition to the detrimental environmental exposures in skin include visible light as well as infrared radiation, which exert their effects via oxidative and heat damage (Dupont et al., 2013). Thus, some caution must be taken in using these parts of the electromagnetic spectrum for cosmetic or clinical purposes. Skin does possess effective anti-oxidative mechanisms to combat these threats; however, UV radiation is able to disrupt these protective systems (Sander et al., 2004). Keratinocytes are also highly effective in inducing apoptosis, when the environmental stress load becomes overwhelming. In this case, typical sunburn cells with pyknotic nuclei and scanty cytoplasm can be detected in the basal and suprabasal layers of the epidermis (Van Laethem et al., 2005).

Excessive exposure to UV radiation, particularly UVA, results in both local and systemic immunosuppression. This intensifies the carcinogenic potential together with the tendency of UVR to initiate and promote malignant changes (Norval & Halliday, 2011). On the other hand, this kind of immunosuppression may potentially modulate/control autoimmune conditions and inflammatory diseases, such as multiple sclerosis and asthma (Norval & Halliday, 2011). Likewise, on the positive side, UVB irradiation induces cutaneous vitamin D synthesis (Laihia et al., 2009). These high energetic wavelengths have been shown to enhance the permeability barrier and induce the production of antimicrobial peptides in the epidermis (Hong et al., 2008). UVA also contributes to nitric oxide formation in the skin, potentially affecting cardiovascular health as well as skin physiology (Paunel et al., 2005).

UV irradiation also generates alterations in the dermal compartment. Degradation of collagen and qualitative or quantitative changes to elastin make the tissue mechanically less resilient, reduce elasticity and cause visible wrinkles (Watson et al., 2014). These changes are closely related to increased ROS. Furthermore, HA in the lower dermis increases which can be reversed with hydrocortisone treatment, as previously discussed (Mitani et al., 1999).

When the protective mechanisms of the epidermis fail, neoplastic lesions may occur. In the case of SCC, well-characterized precancerous (dysplastic) stages exist. These include actinic or solar keratosis (AK) and Bowen's disease or SCC in situ (SCCIS). Both conditions exhibit morphological changes, abnormal proliferation and keratinization as well as atypical nuclei and karyotypic changes (Boukamp, 2005; Müller, 2014). Interestingly, in Bowen's disease the basal keratinocytes are not affected. This indicates that there are multiple pathways by which intraepidermal neoplasia may take place, although etiologically both AK and Bowen's disease are related to solar exposure. In BCC the epithelial stem cells in the basal layer are perturbed. Unlike SCC, this cancer is rarely metastatic, and it develops without any apparent precancerous forms (Boukamp, 2005; Müller, 2014).

Specific effects: activation of common signal transduction pathways in response to UVR

Due to recurrent exposure to UV radiation, epidermal keratinocytes should presumably show specific ways of responding to this stress factor (Adachi et al., 2003). Indeed, it seems that distinct intracellular signaling events take place in response to the various forms of UVR. As could be expected, the different kinase pathways also interact and the net output of sequential UVA + UVB may differ markedly from the effects induced by either exposure alone (Schieke et al., 2005; Muthusamy & Piva, 2013).

It has been unequivocally established that high enough doses of UVR cause extensive changes in gene expression and metabolism; these include activation of the DNA repair machinery, regulation of transcripts involved in cell cycle control and apoptosis as well as modulation of the oxidative status of the cell (Enk et al., 2006). There are, however, disparities depending on the experimental platform used, as keratinocytes *in vitro* may exhibit significant differences from an intact epidermis (Enk et al., 2006). Besides these adaptive alterations, the involvement of specific kinase cascades has been demonstrated (Fig. 3). Principal players are found in the MAPK family and particularly in the stress-activated protein kinase (SAPK) branch of it (including both JNK and p38).

The contribution of JNK as a major stress-activated kinase in modulating keratinocyte responses (proliferation, apoptosis, cell cycle arrest, cytokine production etc.) after UV exposure has been extensively characterized over the years. One of the first, comprehensive studies utilizing UVB was made by Assefa and colleagues (Assefa et al., 1997). Their results demonstrated that UVB activates JNK1 in a dose-dependent manner in both NHEKs and HaCaT cells. Maximal activation in HaCaT occurs already at 30 min post-irradiation. There appears to be substantial cross-talk between the EGF- and UVB-induced responses, possibly via involvement of EGFR. This is exemplified by the clear decrease of JNK1 activation by UVB after a pre-treatment with EGF.

It also seems that UVB and UVC specifically activate JNK in normal human keratinocytes (Adachi et al., 2003), whereas UVA is ineffective. Consistent with previous studies, the response is rapid, as phosphorylation of JNK occurs within 10 minutes of the UVB-exposure. This leads to activation of the transcription factor Elk1, which is not attained with stimuli effective in other cell types, including EGF. Thus, growth factor vs. stress-induced pathways appear unique in keratinocytes. The involvement of JNK in UVB-responses has been corroborated in more recent studies (Muthusamy & Piva, 2013).

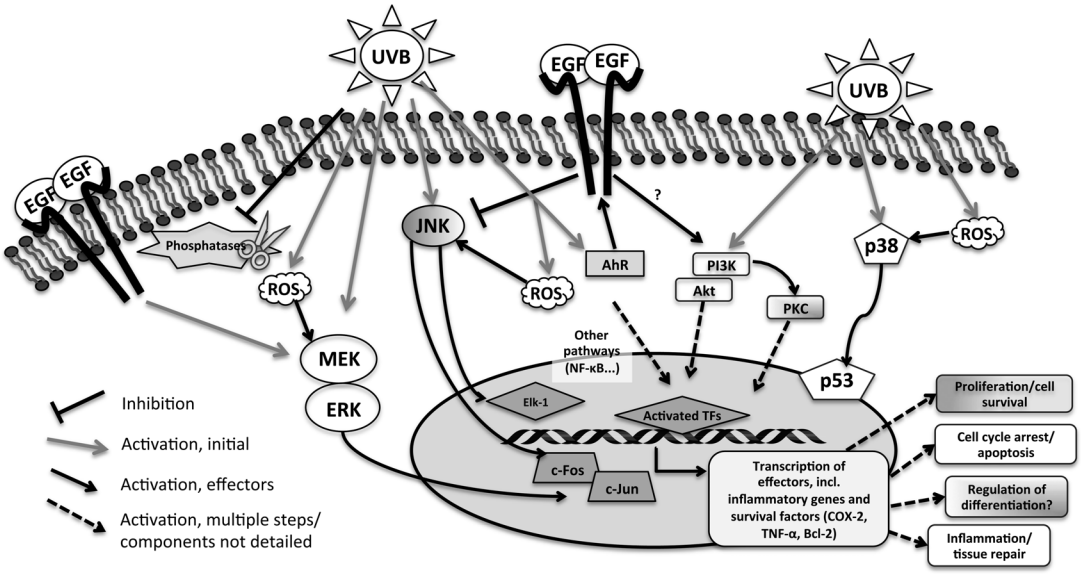

Figure 3. Common signaling cascades initiated by UVB in keratinocytes. More detailed description of the pathways, the abbreviations used and the references are given in the text. Figure has been drawn based on the respective articles cited. MEK = MAPK/ERK kinase; TF = transcription factor (including Elk-1, c-Fos and c-Jun).

The activation of p38 by UVB is also evident in several keratinocyte-derived cell lines. The response appears fast in HaCaT as well as in NHEK and the SCC line Colo16 (Muthusamy & Piva, 2013). These data and previous studies indicate that the effects relayed by p38 are dependent on increased activity rather than upregulation of mRNA synthesis. Active phospho-p38 may influence cell survival and apoptosis as well as inflammatory cascades. The outcome depends on the context of activation and the resulting changes in proteins such as p53, proteins of the Bcl-2 family as well as cyclooxygenase-2 (COX-2) and TNFα (Muthusamy & Piva, 2010). UVA is also able to activate p38, making this kinase a signaling target in both the epidermis and the dermis. Moreover, UVA administered pre- or postirradiation potentiates the effects of UVB on p38 activation in primary human keratinocytes (Schieke et al., 2005).

As previously discussed, p38 expression is related to keratinocyte differentiation. Interestingly, loricrin, filaggrin and involucrin exhibit changes after acute UVB exposure, as their staining intensities in human epidermis *in vivo* increase 24-48 h after 2 MED of UVB Lee et al., 2002). These transient alterations probably affect the protective or adaptive properties of skin, but as proliferation also increases, the mechanisms and the prospective roles played by p38 are obviously complex.

The third MAP kinase involved in the UVB-response is ERK1/2, which may mediate cell survival (Peus et al., 1999). Although UVB was incapable of activating ERK signaling in NHEK in the study of Adachi and coworkers (Adachi et al., 2003), ERK has been shown to be moderately or strongly activated by others (Assefa et al., 1997; Peus et al., 1999; Schieke et al., 2005). It is, however, probably not the main contributor to UVB-induced signaling, despite the fact that its usual upstream receptor EGFR is strongly implicated in the UVB-response in the epidermis (Xu et al., 2006; Van Laethem et al., 2009). The effects of EGFR are most likely mediated by other intracellular effectors, including the PI3K/Akt survival pathway (Wan et al., 2001).

Notably, EGFR, p38, JNK and ERK are activated by low doses of UVB in normal human skin, showcasing important parallels with the in vitro models (Fisher et al., 1998; Pfundt et al., 2001). Corresponding pathways and acute responses have been demonstrated in other studies *in vivo*, and it appears that mammalian systems also resemble one another (Einspahr et al., 2008).

One fairly recent addition to the intracellular effectors of UV is the aryl hydrocarbon receptor (AhR), a cytosolic protein and transcription factor regulating xenobiotic metabolism. After being activated by a photoproduct of tryptophan, it induces the internalization of EGFR and subsequent signaling via ERK1/2 (Fritsche et al., 2007). Another mechanism affecting the activation levels of receptors and their downstream effectors is the suppression of phosphatases such as PTEN. This inhibition appears in turn dependent on kinases that are induced by UVB, including pERK and pAkt (Ming et al., 2010).

The UVB signaling response further depends on ROS, as the antioxidant N-acetylcysteine, which is converted in cells to the active free radical scavenger glutathione, efficiently attenuates the UVB-induced JNK1 activity in HaCaT cells (Assefa et al., 1997). p38 and ERK1/2 also respond to ROS concentration and the antioxidant status in UVB-irradiated NHEK (Peus et al., 1999). The secretion of inflammatory cytokines is another well-known response to UV radiation, and these processes are also related to ROS production. TNFα in particular responds to UVB wavelengths and simultaneous stimulation with IL-1α (Muthusamy & Piva, 2013).

Cell-type specific responses

The overall survival rate of human keratinocytes after broadband UVB exposure is higher than that of fibroblasts in a long-term (12-14 days) clonal analysis. On the other hand, when the damage is irreparable, keratinocytes readily shift from a proliferating to a differentiating, or presumably apoptotic, phenotype (Otto et al., 1999). Keratinocytes are also more resistant to oxidative stress and contain fewer ROS, even after induction. Again, they readily respond by the highly controlled process of apoptosis, when the anti-oxidative/DNA repair capacity is exceeded (D'Errico et al., 2007). In a short-term follow-up (24 h), keratinocytes seem considerably more sensitive to lower doses of broadband UVB than fibroblasts (Cho et al., 2008).

The differences between studies may be due not only to the follow-up time, but also the method employed in evaluating the viability as well the exact characteristics of the radiation source and the cells used. Particular caution should be taken on analyzing cytotoxicity and signaling in the different keratinocyte types commonly used. Transformed and malignant cell lines may exhibit very different responses from primary, freshly isolated keratinocytes (e.g. Muthusamy & Piva, 2013).

To summarize, keratinocytes apparently utilize one of three ways to combat the mutagenic UVR stimuli:

1. The enzymatic DNA repair machinery may remove any damaged bases or longer lesions during cell cycle arrest. This first line of defense should be particularly efficient in the basal layer, which holds the renewing stem cells. Many studies analyzing global gene expression have shown activation of these pathways by UVR (nucleotide and base excision repair).

2. Less severely damaged keratinocytes could be lost through terminal differentiation and passage from the basal and spinous layers into the keratinized stratum corneum.

3. Irreparable cells undergo apoptosis. This produces the sunburn cells often encountered in the basal layer shortly after exposure to a physiologically relevant dose of UV.

In addition, keratinocytes produce growth factors and cytokines to recruit other cell types, including those of the immune system. These processes aim to minimize the detrimental effects of UVR both locally and systemically. How these responses are regulated still needs to be further characterized. The next section reviews one natural compound which holds promise as an agent that could modify keratinocyte responses to stressful conditions such as osmotic shock and UVR.

2.4 BETAINE AND OSMOTIC BALANCE

2.4.1 Compatible organic osmolytes

Compatible organic osmolytes (reviewed by Yancey, 2005) are usually considered small organic molecules that 1) do not disrupt the normal structure or function of cellular proteins and other macromolecules even at rather high concentrations, 2) are mostly electrically neutral at physiological pH and 3) are interchangeable, at least to some degree. These compounds include small carbohydrates, polyols, amino acids, methylamines and methylsulfonium solutes. Besides functioning as regulators of water balance, they may have specialized protective properties that render them suitable for combating various environmental imbalances such as redox stress, changes in temperature (e.g. cryoprotection) and energy depletion as well as stabilizing macromolecules.

2.4.2 Betaine: structure and function

Betaine or glycine betaine (N,N,N-trimethylglycine or TMG; Fig. 4) is a naturally occurring amino acid derivative (methylamine). It is found in many nutritional sources, particularly in sugar beets (*Beta vulgaris* var. *altissima*), spinach and marine invertebrates (Sakamoto et al., 2002; Zeisel et al., 2003). Most of the betaine in our diet comes from cereals (Ross et al., 2014). An integral plasma membrane protein, the betaine/GABA transporter 1 (BGT-1 or SLC6A12) is the main regulator of betaine import in many tissues, including liver and kidney, although amino and imino acid transporters also participate (Zhou et al., 2012).

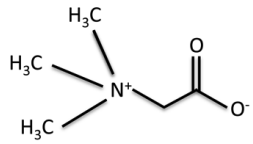

Figure 4. The chemical structure of betaine.

Betaine in different tissues may reach millimolar concentrations (Slow et al., 2009), most often in the constantly osmotically challenged medulla of the kidney, where betaine levels may exceed 100 mM (Lever & Slow, 2010). In rat skin the concentration is 300-400 μM in both sexes, although betaine levels are generally higher in males (Slow et al., 2009). Betaine is also found in low amounts in normal hair, and it could apparently be supplemented within the fiber by using betaine-containing hair products (Pulliainen et al., 2010). The concentration of betaine in plasma is lower than that in tissues, but varies with nutrition.

The typically low excretion in urine may increase in certain diseases, including diabetes. Betaine is also lost in sweat, particularly during heavy exercise (Lever & Slow, 2010).

The metabolic pathways of betaine are diverse (Lever & Slow, 2010). Its synthesis and degradation play essential roles in one-carbon metabolism, especially in the homocysteine-methionine and folic acid cycles (Fig. 5). Betaine also affects the systemic lipid balance. The metabolic conversion of choline to betaine occurs mainly in the liver and the kidneys, which influences the overall concentrations in the body (Lever & Slow, 2010). At the cellular level, betaine seems to protect plasma membranes (Kanbak et al., 2001, 2007).

More specifically, the main functions of betaine can be divided in two; it acts both as an organic, compatible osmolyte under hyperosmotic stress as well as a biochemical methyl group donor. In practice, cells efficiently accumulate betaine to increase their osmotic strength and to maintain water balance under high salinity. This accumulation does not compromise protein folding or enzyme function, which could otherwise be perturbed by excessive intracellular ion concentrations. However, the effects of betaine on protein stability and aggregation are complex, and the outcome depends on its concentration as well as pH (Natalello et al., 2009; Singh et al., 2009).

Downstream, betaine is able to modulate gene expression relating to inflammatory responses. In Kupffer cells, induction of cyclooxygenase-2 and activation of prostaglandin synthesis by hyperosmolarity and lipopolysaccharide can be reverted by adding 1 mM betaine (Zhang et al., 1996). Betaine also protects against apoptosis or growth inhibition in various cell types such as hyperosmotically challenged fibroblasts (Petronini et al., 1992), corneal epithelial cells (Garrett et al., 2013) and HaCaT keratinocytes (Graf et al., 2009).

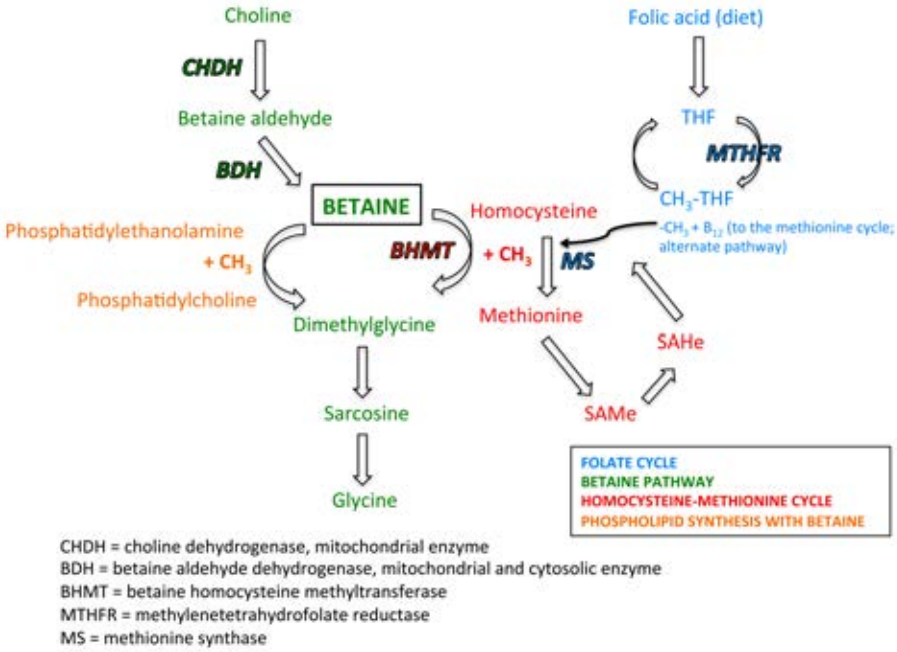

Figure 5. The metabolic pathways of betaine. CHDH = choline dehydrogenase; BDH = betaine aldehyde dehydrogenase; BHMT = betaine homocysteine methyltransferase; MTHFR = methylenetetrahydrofolate reductase; MS = methionine synthase; SAMe = S-adenosylmethionine; SAHe = S-adenosylhomocysteine; THF = tetrahydrofolate; B_{12} = cobalamin. Modified from Neuschwander-Tetri, 2001 and Craig, 2004.

As a methyl group donor, betaine enhances the bioavailability of methionine and S-adenosylmethionine (SAMe; Fig. 5). Thus, betaine lowers excessive concentrations of homocysteine, which increase the risk of cardiovascular and neurological disorders (Maron & Loscalzo, 2009). Furthermore, betaine can substitute for SAMe as a methyl donor in the synthesis of phosphatidylcholine, which explains its effects on lipid metabolism and liver function (Neuschwander-Tetri, 2001). Overall, betaine has a diverse palette of regulatory roles.

2.4.3 Betaine as a modulator of energy metabolism

The general effects of betaine have been investigated, focusing mostly on one-carbon and lipid metabolism as well as energy balance. All the enzymes required for the conversion of choline to betaine (choline dehydrogenase and betaine aldehyde dehydrogenase; Fig. 5) are present in the liver, which highlights the importance of these metabolic pathways in this organ. Furthermore, several animal studies have suggested that betaine has an important role in improving liver health. This can occur by alleviating hepatic steatosis and by increasing the circulation of cholesterol and phospholipids (Craig, 2004). Similar favorable changes have been indicated in humans, although the results are somewhat conflicting (Abdelmalek et al., 2009).

Betaine also appears to have beneficial effects on insulin sensitivity in the liver (Kathirvel et al., 2010) and other parameters of energy homeostasis (Zeisel, 2013). These effects may be relayed partially via mitochondria. Specifically, a lack of the betaine-synthesizing enzyme CHDH, as well as gene polymorphisms, result in defects in mitochondrial structure and function as well as abnormal ATP production and cell motility (Johnson et al., 2010). Betaine has also been added to animal feeds to improve growth performance (Eklund et al., 2005). The potential of using betaine to treat metabolic disorders remains open, however.

2.4.4 Betaine as a modulator of stress

As already mentioned, betaine acts as a hydrophilic osmoprotectant. Regulated by hypertonicity (Yamauchi et al., 1992; Miyai et al., 1996) BGT-1 expression as well as betaine intake are increased after a hyperosmotic shock in human monocytes and macrophages (Denkert et al., 1998). BGT-1 is similarly upregulated in mouse macrophages (Warskulat et al., 1995), where betaine seems to be a central osmolyte. Furthermore, betaine helps maintain osmotic balance in intestinal epithelial cells subjected to high salinity (Kettunen et al., 2001).

Changes in signaling and cytokine secretion in response to betaine have been observed when the cornea is subjected to drought and salinity of the tear film. In human corneal epithelial cells *in vitro*, betaine hydrochloride (10 mM) modulated MAPK signaling by reducing JNK and p38 phosphorylation after a hyperosmotic (400 mOsm) shock (Corrales et al., 2008). The ratio of *p*-p38/p38 also decreased with 10 mM betaine as compared to physiologic osmolarity (300 mOsm). Interestingly, the p38 inhibitor SB203580 blocks the hyperosmotically induced expression of BGT-1 and the myoinositol transporter SMIT in human peripheral blood cells (Denkert et al., 1998). These data suggest that p38 is a central kinase not only under irradiation stress (discussed previously) but also in regulating cell volume and composition. Furthermore, a negative feedback mechanism seems to downregulate the influx of betaine after homeostasis has been achieved.

Betaine has also been shown to be involved in NF-κB signaling, which is closely related to proinflammatory processes as well as aging. In 21-month-old rats betaine attenuated the increase in NF-κB activity even after a relatively short administration period of 10 days (Go et al., 2005). The increased phosphorylation and activation of pERK1/2 after stimulating endothelial YPEN-1 cells with *tert*-butyl hydroperoxide, an oxidative stress inducing compound, was also clearly reduced when the cells were pretreated with betaine (Go et al., 2005). Moreover, the downmodulating effect of betaine on cytokine and chemokine (TNFα, IL-1β, IL-6, IL-8 and CCL2) expression in hyperosmotically challenged primary human corneal epithelial cells has recently been described (Hua et al., 2015).

Besides curbing signaling via proinflammatory mediators and MAP kinases, betaine normalizes cell volume and downregulates apoptosis in cultured human corneal-limbal epithelial cells (HCLE) subjected to hypertonic stress (500 mOsm). A concomitant decrease in caspase activation (caspases 8, 9 and 3/7) and TNF-α secretion was observed as the stressed cells were treated with 5-10 mM betaine, whose uptake significantly increased under conditions of increasing osmolarity (Garrett et al., 2013).

Interestingly, the expression of BGT-1 and betaine uptake are increased by UVB in HaCaT keratinocytes (Warskulat et al., 2007) and by UVA in dermal fibroblasts (Warskulat et al., 2008). Additionally, normal human keratinocytes increase their BGT-1 expression after UVA/UVB exposure and betaine uptake after UVA irradiation (Warskulat et al., 2004). These observations indicate that betaine may function in the adaptation of keratinocytes to radiation injury.

Finally, betaine uptake seems to depend on the energy status and metabolic requirements of the cell. When the AMP/ATP sensing kinase AMPK is introduced into *Xenopus* oocytes together with BGT-1, electrogenic activation of the transporter by GABA is significantly reduced (Munoz et al., 2012). The function of the Na^+/K^+ ATPase, which maintains cell volume and plasma membrane potential, may become compromised in energy-depleted cells, leading to influx of ions and water. Suppression of osmolyte transporter activity by AMPK would ensure that cells low on energy avoid swelling caused by the intake of excessive osmolytes and water. Betaine itself activates AMPK to regulate lipid metabolism, at least in the liver (Song et al., 2007).

2.4.5 Betaine in skin and personal care formulations

In skin and keratinocytes, the actions of betaine are less well characterized. However, there are studies where a protective action against irritation caused by detergents commonly added to personal care products has been implicated. The effects have been analyzed on both keratinized and non-keratinized stratified epithelia. Rantanen et al. (2002) first tested the effects of betaine on oral mucosa. There, a mixture of 4% betaine combined with 1% sodium lauryl sulfate (SLS) in solution, mimicking a mouth wash, reduced irritation compared to the detergent (1% SLS) alone as measured by electrical impedance.

Betaine also seems to protect skin against the commonly used surfactants SLS and cocoamidopropylbetaine (CAPB). In addition, it may modify skin water balance by itself as indicated by significant changes in certain indices of electrical impedance (Nicander et al., 2003b). Skin hydration, on the other hand, may relate to the properties of the permeability barrier. Betaine alone (4% w/v in distilled water) did not cause adverse histological changes in the test subjects (Nicander et al., 2003b). When betaine was added to soaps containing

detergents, the protective properties were less clear, as all products tested caused visually observable irritation and increased TEWL (Nicander et al., 2003a).

The effects of osmolytes, as pure compounds or modified to become more lipophilic for better penetration into the epidermis, have also been tested in HaCaT keratinocytes (Graf et al., 2009). There, betaine alone was able to reverse the strongly depressed proliferation rate of the hyperosmotically challenged cells when used in 10 μM-10 mM concentrations. The lipophilic D,L-α-tocopheryl-(mono-) derivative of betaine (TMB) was ineffective, and actually reduced proliferation even further at the highest concentrations tested. This data was corroborated by Scheel and Keller (2012) who concluded that TMB is likely to be a skin sensitizer.

Long-term skin pre-treatment for 1 month with a multi-component cream (Physiogel AI) containing 0.36% betaine was shown to reduce erythema as well as thymine dimer formation in test subjects irradiated acutely with a light source emitting both UVA (80%) and UVB (Kemeny et al., 2007). Additionally, an antibacterial wound cleaning solution containing 0.1% polyhexanide and 0.1% betaine seems to enhance wound healing in several clinical studies (Wilkins & Unverdorben, 2013). Unfortunately, in many of these reports betaine is added to a component mixture, often in conjunction with known irritants. This leaves open the question of its exact role in the beneficial effects observed.

2.5 EXTRACELLULAR NUCLEOTIDES IN THE REGULATION OF CELLULAR METABOLISM AND HA SYNTHESIS

Adenine, guanine, cytosine, thymine and uracil are the nitrogenous bases which together with a pentose sugar (ribose/deoxyribose) form nucleosides. These can be further mono-, di- and triphosphated to form the corresponding nucleotides. They are involved in various aspects of intracellular events, including energy production (ATP) and signaling (GTP) as well as modification of metabolic intermediates (e.g. UDP-glucose). When present extracellularly, the nucleotides commonly contain either adenine or uracil.

Extracellular nucleotides have distinct signaling functions, which is highlighted by specific purinergic receptors residing at the plasma membrane. These are divided into three groups (reviewed by Burnstock, 2007). The four known P1 receptors (A_1, A_{2A}, A_{2B} and A_3) bind adenosine and are coupled to G-proteins, whereas members of the purinergic receptors for ATP (P2X) family of ligand-gated ion channels ($P2X_1$-$P2X_7$) bind ATP in high concentrations. The purinergic receptors for adenosine and uridine nucleotides (P2Y-receptors) have multiple ligands, as presented in Table 5. They are also coupled to G-proteins, which have the classical seven-pass transmembrane structure.

The presence of multiple different purinergic receptor types with the same ligands but possibly opposing effects, depending on e.g. the concentration, gives cells much needed physiologic plasticity. However, it also complicates dissecting their specific roles, especially when their expression patterns overlap in a particular cell type. Of note, expression of all of the P2Y-receptors has been detected in human keratinocytes, but the levels depend on the origin (Table 5). Moreover, specific receptors are found in distinct layers of the epidermis. $P2Y_1$ and $P2Y_2$ are expressed mainly in the proliferating basal cells, whereas $P2X_5$ localizes in the basal or the differentiating suprabasal keratinocytes. $P2X_7$ is found in the terminally differentiated/apoptotic cells at the border of the stratum corneum (Greig et al., 2003a).

Table 5. The purinergic P2Y-receptors. Modified from Burrell et al., 2003; Yoshida et al., 2006; Inoue et al., 2007; Burnstock, 2007; Jokela et al., 2014; Nagakura et al., 2014; von Kügelgen & Hoffmann, 2016.

Receptor	Main ligand	Examples of tissue and cell type distribution
$P2Y_1$	ADP	**Keratinocytes**, platelets, epithelial and endothelial cells, immune cells; brain, prostate, placenta
$P2Y_2$	ATP/UTP	**Keratinocytes**, epithelial cells, immune cells, kidney tubule cells; lung, heart, skeletal muscle, kidney
$P2Y_4$	UTP	**Keratinocytes**, cardiac endothelial cells; intestine, brain, pituitary
$P2Y_6$	UDP	**Keratinocytes**, epithelial cells, T cells, adipocytes, skeletal muscle cells, immune cells; placenta, kidney, intestine, brain
$P2Y_{11}$	ATP	**Keratinocytes**, granulocytes/immune cells; spleen, intestine, liver, brain
$P2Y_{12}$	ADP	**Keratinocytes**, platelets, glial cells, microglia; neural tissues
$P2Y_{13}$	ADP	**Keratinocytes**, leukocytes, neurons, glial cells; spleen, brain, lymph nodes
$P2Y_{14}$	UDP-glucose	**Keratinocytes**, immune cells, glial cells; placenta, adipose tissue, intestine

It is evident, that several stress stimuli or tissue trauma induce the release of nucleotides such as ATP and UTP from keratinocytes. Activation of the purinergic signaling pathways at large is associated with many physiological and pathological processes. These include skin inflammation, wound healing and permeability barrier repair (Burnstock et al., 2012).

Specifically, extracellular nucleotides appear to act as crucial signaling mediators or danger cues in skin or keratinocytes that have been compromised by insults such as mechanical stimulation (Yoshida et al., 2006), heating (Mandadi et al., 2009), chemical irritants (Mizumoto et al., 2003), UV radiation (Inoue et al., 2007; Takai et al., 2011) and barrier disruption (Denda et al., 2002). Even unstimulated cells may discharge nucleotides producing a steady-state condition. In such cases, as well as often in stimulated cells, the process of nucleotide release is controlled and seems to occur either via various plasma membrane channels and transporters or exocytosis (Lazarowski et al., 2003). Lysis of necrotic cells is thus not the main mechanism of releasing nucleotides as extracellular signaling mediators.

The triphosphated nucleotides are quickly degraded by a group of extracellular ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), also in keratinocytes (Ho et al., 2013). Adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine thus produced signal either through the P2Y-receptors (ADP; Table 5) or the P1-family (adenosine and possibly AMP; Rittiner et al., 2012). Of the adenosine receptors, the A_{2B}-subtype is the most common in human keratinocytes (Brown et al., 2000). The uptake of nucleosides may also occur via equilibrative nucleoside transporters such as ENT1. This pathway may explain some of the observed effects of the degradation products, including inhibition of proliferation by adenosine in primary human keratinocytes (Brown et al., 2000). Additionally, the adenosine metabolite adenine exerts its effects through the AdeR- or P0-receptors (Knospe et al., 2013), whereas the other common metabolite inosine may utilize the P1-receptors, including A_{2A} (Welihinda et al., 2016).

Functionally, ATP and UTP in low concentrations have been shown to increase proliferation of both HaCaT (Lee et al., 2001) and primary human keratinocytes (Dixon et al., 1999; Greig et al., 2003a). They also cause a rise in cytosolic Ca^{2+}, linking their release to epidermal differentiation (Burnstock et al., 2012). This would implicate that not only $P2Y_2$,

and probably $P2Y_4$ and $P2Y_{11}$, but also members of the P2X-family are involved. In addition to keratinocytes, purinergic signaling affects Langerhans cells, melanocytes and dermal fibroblasts as well as regulates physiological functions such as vascular flow and nerve signaling (Burnstock et al., 2012). P2X- and P2Y-receptors are also expressed in BCC and SCC, and their activation affects proliferation of neoplastic cells. For instance, low concentrations cause an increase in A431 squamous carcinoma cell numbers whereas high doses reduce them (Greig et al., 2003b). However, the effects of nucleotides on proliferation and malignancy are less than straightforward (Burnstock & Di Virgilio, 2013).

With respect to solar exposure, ATP has been shown to be released from HaCaT cells by UVB (Takai et al., 2011). This leads to activation of inflammatory processes via p38-mediated COX-2 expression. The UDP-activated $P2Y_6$-receptor appears particularly important in this context. UV radiation also increases IL-6 production by activating P2Y-receptors in NHEK (Inoue et al., 2007). These data strongly indicate that purinergic signaling is involved in mediating the pro-inflammatory effects of UV radiation in keratinocytes/skin.

Adenosine analogs have been shown to increase *HAS1* expression as well as accumulation of pericellular HA and binding of monocytes to this matrix in vascular smooth muscle cells (Grandoch et al., 2013). Adenosine slightly increased migration of these cells, which was blocked by knockdown of *HAS1*. These effects may be related to the regulation of inflammatory processes during atherosclerotic changes. *HAS1* transcription was also upregulated by adenosine or its analog 2-CADO in gingival fibroblasts, and synergistically induced in IL-1β-treated cells with 2-CADO (Murakami et al., 2001). This highlights the link between increased ECM production and inflammatory mediators/danger signals in these cells.

In HaCaT keratinocytes, the $P2Y_{14}$ receptor was recently shown to mediate increased *HAS2* expression in response UDP-Glc (Jokela et al., 2014). The enhanced transcription depends partially on the binding of $Tyr(P)^{705}$-STAT3 to several promoter regions containing STAT3 response elements. UDP-Glc also induces proliferation and migration. This may indicate activation of an inflammatory response, as also suggested by the increased expression of IL-8 after treatment with UDP-Glc (Jokela et al., 2014). However, as regards hyaluronan metabolism, data concerning extracellular nucleotides are still relatively scarce.

3 Aims of the study

Hyaluronan has an indispensable role in modulating cell and tissue behavior. Its central role during embryonic development and significance for the correct function of adult organs has also raised questions about its responsiveness to detrimental environmental cues and relevance for pathological processes. In the context of skin, activation of hyaluronan synthesis during wound healing, inflammatory conditions and malignancy has been elaborately described. However, much remains to be learned, particularly regarding acute or chronic environmental stressors, including UV radiation. The effector molecules and possible antagonists of these processes also need to be investigated.

To add to the growing knowledgebase, this study was carried out using both rat and human epidermal keratinocytes to:

1. Elucidate and compare the effects of acute UVB exposure on hyaluronan metabolism in rat epidermal keratinocytes grown both as monolayers and in a 3D culture system, and explore the signaling mechanisms evoking the changes

2. a) Explore, whether the organic osmolyte betaine, which is known to be involved in the modulation of heat or cold shock, hyperosmotic conditions and oxidative challenges, has significant modulatory effects of its own on rat epidermal keratinocyte gene expression, and **b)** investigate whether betaine is able to modify UVB-induced changes in global gene expression in rat epidermal keratinocytes

3. a) Study the effects of the extracellular nucleotides UTP, ATP and their degradation products on hyaluronan synthesis, purinergic receptor activation and intracellular signaling in the human keratinocyte cell line HaCaT and **b)** analyze whether betaine has any regulatory potential in cells subjected to the nucleotides (mimicking a danger signal)

4 Materials and methods

4.1 MATERIALS

4.1.1 Cell lines and monolayer cultures

This thesis utilizes two continuous keratinocyte cell lines: rat epidermal keratinocytes (REK; Baden & Kubilus, 1983) and a keratinocyte cell line of human origin, HaCaT (Boukamp et al., 1988). REK cells (original publications I and II) were sustained as monolayers in minimum essential medium (MEM; Thermo Fisher Scientific/Gibco, Waltham, MA) with the following supplements: 10% fetal bovine serum (FBS; GE Healthcare Life Sciences/HyClone, Logan, UT), 4 mM L-glutamine (EuroClone, Milan, Italy) and penicillin/streptomycin (50 μg/ml streptomycin, 50 U/ml penicillin; EuroClone). The cells were passaged three times a week with 0.05% trypsin and 0.02% EDTA (w/v) in phosphate-buffered saline (PBS) (Biochrom GmbH, Berlin, Germany) at a ratio of 1:6 to 1:12 and used for experiments as detailed in Table 6.

HaCaT cells (original publications III and IV) were cultured as monolayers in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Inc., Saint Louis, MO) without phenol red containing 10% FBS (HyClone), 2 mM L-glutamine (Euroclone) and penicillin/streptomycin (50 μg/ml streptomycin, 50 U/ml penicillin; Euroclone). The cells were passaged twice a week at a ratio of 1:6-1:7 similarly to the REK cells, with an additional treatment with 0.02% EDTA (Sigma-Aldrich) before trypsinization and used for experiments as detailed in Table 6.

4.1.2 Organotypic cultures

The REK cells are able to stratify and differentiate when cultured at the air-liquid interface on a type I collagen matrix cast on a porous, semipermeable membrane, where they form an epidermal equivalent in 10-14 days (Fig. 6). These organotypic 3D cultures were used in original publications I and II. Briefly, 8 ml of type I collagen (4 mg/ml in 0.1% CH_3COOH; prepared in-house from rat tail tendons) was mixed with 1 ml of 10x EBSS (Earl's Balanced Salt Solution; Sigma-Aldrich), 200 μl NaOH and 300 μl sodium bicarbonate (7.5%; Thermo Fisher Scientific/Gibco) buffer on ice, poured on commercial inserts (3.0 μm pore size, 6-well format; Costar® Transwell, Corning Inc., Tewksbury, MA) and let polymerize for at least an hour at 37°C.

300,000 REK cells suspended in the culture medium containing DMEM (high glucose; Thermo Fisher Scientific/Gibco) supplemented with 10% FBS (HyClone), 4 mM L-glutamine (Euroclone), 50 units/ml of penicillin and 50 μg/ml streptomycin (Euroclone) were plated on the collagen matrix and grown submerged for 3 days. Subsequently, the medium on top of the cell layers was removed and the cultures were maintained at the air-liquid interface until the end of the experiment. To facilitate normal differentiation, L-ascorbic acid (40 μg/ml; Sigma-Aldrich) was added with each medium change (every 1-2 days). This supplementation was started one day after exposing the cultures to air, and continued until 1 day before treating the cultures with UVB or betaine. The cultures were used for experiments as detailed in Table 6.

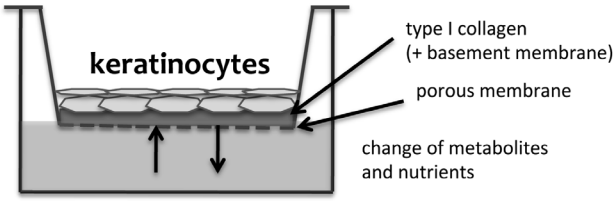

Figure 6. The organotypic 3D culture model.

4.1.3 Betaine, nucleotide and UVB treatment of the cultures

Betaine (trimethylglycine; Betafin® BP 20) used in the original publications (II-III) was kindly supplied by Finnfeeds Finland Ltd., Naantali, Finland. The UV exposures were performed using a portable lamp (UVM-57; UVP, Upland, CA) emitting midrange UV at a nominal wavelength of 302 nm. The spectral characteristics of the light source are presented as a supplement in original publication I. The nucleotides UTP, UDP, UMP, ATP, ADP, AMP, ATPγS, βγ-methylene-ATP and adenosine (Sigma-Aldrich) were used at concentrations varying between 0.01-100 μM. After concentration testing, 100 μM was chosen for most experiments. All other chemicals (including inhibitors and siRNAs), materials and equipment are detailed in the respective publications.

4.2 METHODS

The methods used to study the quality and quantity of hyaluronan, the expression of genes and proteins in response to acute UVB exposure, betaine and extracellular nucleotides, and the techniques to stain and analyze tissue sections and cell cultures are outlined in Tables 6-8. The specifics of each procedure, along with the commercial reagents used, are described in detail in the original publications (I-IV). Table 6 shows the treatments imposed on cells to study hyaluronan synthesis, gene/protein expression and metabolic/stress responses. Table 7 lists the methods used to analyze hyaluronan and its precursors, and Table 8 describes the procedures used to study gene and protein expression as well as the growth characteristics of the cultures.

Table 6. Treatments used to induce or repress HA synthesis, gene/protein expression and metabolic/stress responses

Treatment	Purpose	Culture model	Original publication	Reference
UVB irradiation 2.5-40 mJ/cm^2	Characterization of HA synthesis and stress responses	Monolayer REK; 3D REK	I, II	Optimized in original publication I
siRNA transfection	Knockdown of gene expression	Monolayer REK, monolayer HaCaT	I, III, IV	Optimized in original publications I, III, IV
Chemical inhibitors	Signaling protein inhibition	Monolayer REK; monolayer HaCaT	I, III, IV	Optimized in original publications I, III, IV
Betaine treatment	Characterization of metabolic/stress responses	Monolayer REK; 3D REK; monolayer HaCaT	II, III	Optimized in original publications II, III
Nucleotide treatments	Characterization of stress responses	Monolayer HaCaT	III, IV	Optimized in original publication III, IV

Table 7. Methods used to analyze hyaluronan and its precursors

Method	Purpose	Culture model	Original publication	Reference
HA-ELSA (sandwich-type)	Quantification of HA	Monolayer REK; monolayer HaCaT	I, III, IV	Hiltunen et al., 2002
Competitive HA-ELISA	Quantification of HA (smaller fragments down to 10 kDa)	Monolayer HaCaT	III	A commercial kit (K-1200; Echelon Biosciences Incorporated)
Size exclusion chromatography (gel filtration (HPLC)	Determination of molecular mass distribution of native HA	Monolayer REK; 3D REK; monolayer HaCaT	I, III	Tammi et al., 2000
Metabolic labeling ([^{3}H]glucosamine and [^{35}S]Na_2SO_4)	Quantification of newly synthesized HA and chondroitin sulfates	3D REK	I	Tammi et al., 2000
Histochemistry (bHABC-probe)	Analysis of HA distribution in fixed cultures (light and fluorescence microscopy)	3D REK; monolayer HaCaT	I, III, IV	Tammi et al., 1998
Anion-exchange high-performance liquid chromatography (HPLC)	Quantification of nucleotide sugar precursors	Monolayer HaCaT	III, IV	Rilla et al., 2013; Oikari et al., 2014

Table 8. Methods used to study gene and protein expression and the growth characteristics of the keratinocyte cultures

Method	Purpose	Culture model	Original publication	Reference
Quantitative real-time PCR (qRT-PCR)	Quantification of mRNA expression	Monolayer REK; 3D REK; monolayer HaCaT	I, II, III, IV	Optimized in original publications I-IV
Western blotting	Quantification of protein expression	Monolayer REK; 3D REK; monolayer HaCaT	I, II, III, IV	Optimized in original publications I-IV
Immunohistochemistry	Protein distribution (CD44, K2, K10, CaMKII)	3D REK; monolayer HaCaT	I, II, IV	Tammi et al., 2000; optimized in original publications II, IV
Hematoxylin & eosin staining (HE)	Epidermal morphology	3D REK	II	Routine protocol
Measurement of epidermal thickness (HE)	Proliferation responses to UVB and betaine	3D REK	II	Optimized in original publication II
Proliferation assay/cell counting	Proliferation responses to UVB and betaine	Monolayer REK	I, II	Optimized in original publications I, II
Genome-wide gene expression analysis (microarray)	Quantification of mRNA expression	3D REK	II	Optimized in original publication II
Bioinformatics	Determination of differential gene expression and pathway analyses	3D REK	II	Optimized in original publication II

In addition to the methods used in the published works, the Ca^{2+}-signaling associated PCP4 (PEP-19) protein was analyzed by immunohistochemistry as follows. The organotypic cultures were fixed in Histochoice® MB (Amresco, Solon, OH) overnight at 4°C and

embedded in paraffin. Later on, deparaffinized sections (3 μm) were blocked with 0.1% Triton X-100-1% BSA in 0.1 M sodium phosphate buffer (pH 7.4).

The primary antibody (sc-74816, Santa Cruz Biotechnology, Inc., Dallas, TX) raised against rat PCP4 (diluted 1:100 in 1% BSA) was applied overnight at 4°C. This was followed by incubation with a biotinylated secondary antibody (anti-rabbit, diluted 1:300 in 1% BSA; Vector Laboratories, Burlingame, CA) at room temperature for 1 h. The PCP4 signal was detected using the avidin-biotin peroxidase method (ABC reagent/Vectastain Kit, diluted 1:200; Vector Laboratories) with diaminobenzidine (0.05% DAB; Sigma-Aldrich). Nuclei were counterstained with Mayer's hematoxylin. The stained cultures were viewed and photographed as described for keratin 2 in original article II.

5 Results

5.1 UVB INDUCES HYALURONAN SYNTHASES AND HA ACCUMULATION IN RAT EPIDERMAL KERATINOCYTES (I)

5.1.1 Validation of the irradiation dose and biological effectiveness

Rat epidermal keratinocytes (REK) cultured as monolayers exhibited a clear response to UVB at doses above 5 mJ/cm^2 as analyzed by the proportion of dead cells and diminished proliferative capacity. With 10 mJ/cm^2 UVB approximately 10% of all calculated cells were considered dead. Additionally, the growth curves of control vs. UVB-treated cells indicated that the irradiated keratinocytes were significantly lagging behind in total numbers for the entire observation period (12-48 h post-UVB; I, Supplemental Fig. 2).

For the organotypic model, previous experiments (for examples, see Bart et al., 2014) had indicated that doses up to 20 mJ/cm^2 had little effect on the morphology of the 3D REK cultures. With 30 mJ/cm^2 typical sunburn cells with pyknotic nuclei began to appear among the basal and lower spinous cells. Cell debris below the basal layer was also detected. These effects could be attributed rather specifically to the UVB portion of the spectrum, as the spectral curve of the light source exhibited a major irradiance peak around 310 nm. Minor proportions from UVA and UVC were identified (I, Supplemental Fig. 1).

5.1.2 Changes in HA synthesis after an acute UVB exposure in REK cultures

REK keratinocytes proved to be highly responsive to acute UVB irradiation with respect to HA metabolism. In monolayers, hyaluronan accumulation in the culture medium increased in a dose-responsive manner. UVB at 2.5 to 5 mJ/cm^2 was ineffective but 10 to 20 mJ/cm^2 more than doubled the secretion of HA. The effect was evident already 12 h after the exposure (I, Fig. 1). Of these doses, the least cytotoxic 10 mJ/cm^2 was selected for further studies. For the organotypic cultures, our previous analyses had suggested an optimal dose of 30 mJ/cm^2, which was applied for most experiments. Histology of the 3D epidermis was also studied with lower and higher exposures (20 and 40 mJ/cm^2).

Accordingly, REK monolayers exhibited changes in the mRNA expression of all the major HA metabolizing enzymes in a highly coordinated fashion (I, Fig. 2). *Has1* was the first gene to be activated within 4 hours of the UVB insult, followed by *Has2* and *Has3* with very similar profiles and a biphasic upregulation, first at 12 h and later at 36 h. *Hyal1* and *Hyal2* were also activated maximally at 8-12 h, whereas *Cd44* was first strongly downregulated (2-8 h), returned to control levels by 12 h and was then slightly induced at 24-36 h. The molecular mass distribution of HA did not differ between the control and the UVB-treated cultures (I, Fig. 1), despite the upregulation of *Hyal1-2*. Additionally, there were no significant differences in the protein levels of the major isoform of CD44 between the UVB-treated samples and controls up to 36 h post-UVB (I, Supplemental Fig. 3).

Both *Has2* and *Has3* were clearly involved in the UVB-response in the monolayers. Treating the cells with specific siRNAs blocked the UVB-induced increase in the expression of the respective genes at 8 h post-UVB as well as HA accumulation in the culture medium at 24 h. This was particularly true for *Has3*, whose knockdown attenuated the increase in HA almost entirely (I, Fig. 3).

UVB exposure also modulated hyaluronan metabolism, particularly degradation, in the 3D REK cultures. Upregulation was seen in the mRNA expression of *Hyal1* and *Hyal2* as well as the synthetic enzymes *Has2* and *Has3* (I, Fig. 6). These changes were reflected as a modest increase in total HA (I, Fig. 6). Here, gel filtration analyses of the newly synthesized HA molecules did suggest enhanced fragmentation after the UVB treatment (I, Fig. 7). The general morphology of the epidermis remained normal in the irradiated cultures, but both hyaluronan and CD44 exhibited an irregular, patchy staining pattern with the highest dose (40 mJ/cm^2) tested (I, Fig. 7).

5.1.3 Cell signaling pathways responsible for HA accumulation after UVB

To further dissect the UVB-induced changes in keratinocyte hyaluronan metabolism, the involvement of the common intracellular signaling pathways were investigated in the monolayer cultures. The use of specific chemical inhibitors against EGFR, MEK1/2, PI3K, Akt1/2, p38 MAPK, JNK, STAT3 and CaMKII strongly indicated the involvement of MAP kinase pathways and Ca^{2+} signaling in the UVB-induced HA-response.

Specifically, p38 and CaMKII were involved in regulating the UVB-induced increase in HA synthesis. Inhibiting p38 signaling in the irradiated cultures with BIRB796 decreased HA secretion by 46% in comparison to the UVB-stimulus alone. Blocking CaMKII signaling with KN93 attenuated the response by 71% (I, Fig. 4). A minor effect was also seen with AG1478, the inhibitor against EGFR, as the UVB-induced hyaluronan response was reduced by 28% (I, Fig. 4). Each of these inhibitors also had some effects on basal HA secretion.

The activation of p38 by UVB was also verified by western blotting. 6 h after the UVB-exposure, p38 phosphorylation was increased about 3-fold, supporting the contribution of this stress-activated kinase (I, Fig. 4). The level of pJNK was also increased by about 1.5-fold at 1 h post-UVB (I, Supplemental Fig. 5), but as the specific inhibitor didn't affect HA accumulation, this pathway was not considered further.

The effects of BIRB796, KN93 and AG1478 on the expression of *Has1-3* mRNA defined the relative contributions of the different *Has* isoforms. Inhibiting p38 signaling almost completely abolished the UVB-induced upregulation in *Has2* measured at 8 h post-UVB (I, Fig. 5). The same was true for *Has1*, but there BIRB796 also significantly reduced the basal expression level, indicating a more general effect on *Has1* transcription. For *Has3*, CaMKII signaling was strongly implicated, as KN93 efficiently suppressed the UVB-induced upregulation at 24 h post-UVB (I, Fig. 5). When AG1478 was used, no such modulation of *Has* transcription could be seen; in fact, the inhibitor more than doubled the basal expression level of *Has1* (I, Supplemental Fig. 6). The other inhibitors did not significantly modulate the UVB-induced response (I, Supplemental Fig. 4).

Together, these data indicate that exposure to acute UVB is able to specifically activate hyaluronan synthesis in keratinocytes, and reveals novel regulatory pathways therein. The findings from original publication I are briefly summarized below (Fig. 7).

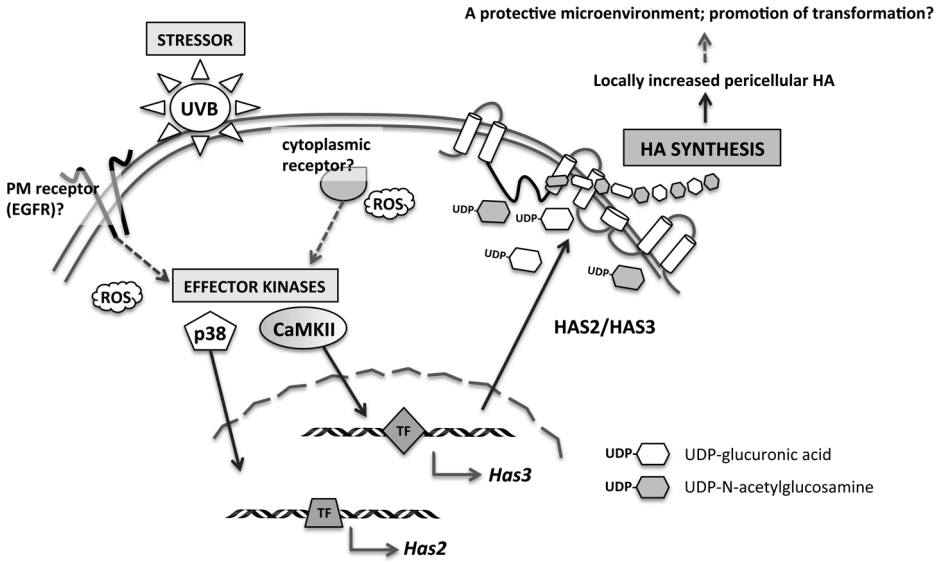

Figure 7. Summary of UVB-induced changes in REK cells (based on data in original article I; Rauhala et al., 2013). CaMKII = Ca^{2+}/calmodulin dependent protein kinase II; PM receptor = plasma membrane receptor; ROS = reactive oxygen species; TF = transcription factor.

5.2 UVB AND BETAINE DIFFERENTIALLY REGULATE KERATINOCYTE GENE EXPRESSION AND DIFFERENTIATION OF 3D REK CULTURES (II)

5.2.1 Global gene expression changes in the 3D epidermis

UVB exposure of the cultures caused a robust upregulation in the expression of genes regulating DNA repair, DNA replication, cell cycle and nucleotide metabolism (II, Table 3a). The UVB treated cultures also contained clear sunburn cells in the basal layer, indicating solid radiation damage (II, Fig. 4). Overall, UVB either alone or in combination with betaine significantly upregulated the expression of 362 genes and downregulated 260 genes (II, Fig. 1). Additionally, 5 genes were downregulated by all of the treatments.

UVB also exhibited exclusive upregulation in 140 genes and downregulation in 153 genes (II, Fig. 1 and Supplemental Table S3). These changes can be considered to be prevented by betaine as they were no longer significant when analyzed after the combination treatment. In this group, the most significantly upregulated genes included keratin 16 (*Krt16*; 2.54-fold increase), a marker often seen in hyperproliferative/activated epidermis, and the inflammatory interleukin 1α (2.22-fold increase). Downregulation was evident in *Sectm1a* (0.60-fold downregulation), which may exhibit immunomodulatory functions, and *Nbl1* (0.68-fold downregulation), a potential tumor suppressor.

Betaine alone (treatment with 10 mM for 11 days) significantly changed gene expression in the cultures, even though the total number of genes regulated was rather modest. Overall, betaine upregulated the expression of 33 genes and downregulated 56 genes (II, Fig. 1, Tables 1 and 2). Results with the combination treatment (betaine + UVB) were similar to UVB in many respects, but there were also truly unique effects (II, Table 6, Supplemental Table S5). Most interestingly, betaine modulated the UVB-response having both enhancing and reversing effects (II, Fig. 3, Supplemental Table S4).

UVB also caused a mild inflammatory response as indicated by the increased IL-1α mRNA expression (II, Fig. 3). None of the treatments elicited changes in epidermal thickness, however, a feature that is often seen in irradiated epidermis *in vivo* (II, Fig. 4). The dose chosen for the experiments (30 mJ/cm^2) thus appears optimal to induce metabolic changes without excessively disrupting the overall structure of the 3D epidermis.

5.2.2 Modulation of epidermal metabolism and growth by betaine

The biological processes most significantly affected by betaine were those relating to cell cycle, DNA replication and repair, nucleotide metabolism and metabolic pathways in general, which were all downregulated (II, Table 3b). Among others, the DNA replication licensing factors *Mcm4* and *Mcm7* were suppressed (II, Table 2). Some signaling cascades appeared to be upregulated at the level of biological processes, including the TGF-β pathway (II, Table 3a). There were, however, no clear changes in the Ki67-positive, proliferative cells in the 3D cultures. Betaine did not cause visible thinning of the epidermal equivalents either.

The inhibitory effects of betaine on DNA replication and cell cycle were also seen in the combination treatment, where these processes were less significantly upregulated (higher p-values) than in the UVB-treated group (II, Table 3a). Accordingly, when the enrichment of genes that are regulated by particular transcription factors were analyzed, it could be seen that betaine significantly affected MYC-, E2F1-, SOX2- and NANOG-responsive coding regions (II, Table 4). These proteins can be pinpointed in several aspects of the proliferation-differentiation-axis in the epidermis.

The possible slowing down of keratinocyte metabolism by betaine was functionally evaluated in REK monolayers as well. There, 10 mM betaine exhibited a slight, although not statistically significant, trend towards decreased growth in proliferating cultures followed for 3 days (II, Fig. 4). Typical for the betaine treatments used here, the overall effects were comparatively small, including the fold changes measured for up- and downregulated genes (II, Tables 1 and 2).

5.2.3 Changes in differentiation markers

In contrast with the subtle changes observed with most parameters in the betaine and UVB-treated 3D cultures, keratin 2 (*Krt2*) mRNA was induced over 2-fold in the original array and over 5-fold when verified with qRT-PCR (II, Table 1, Fig. 2). The protein exhibited an almost 10-fold increase by western blotting, and the change was also evident in histological stainings (II, Fig. 5). A strong K2 signal was seen in the upper spinous and granular layers, as expected. When the relative mRNA levels of *Krt2* in REK monolayers and the 3D epidermal equivalents were compared, the transcript was evidently much more abundant in the stratified model (II, Fig. 5). The change in the keratin-associated maturation pattern appeared specific for keratin 2, as the amount and localization of K10 protein were unchanged with betaine (II, Fig. 6). With UVB and the combination treatment, K10 was slightly downregulated.

In concert with these changes, some transcription factors and signaling molecules that are known to be important for keratinocyte differentiation, were upregulated with the betaine treatment. These included *Tgfβ2*, *Klf10* and *Egr1* (II, Table 1, Fig. 2). *Egr1* was the second most significantly upregulated gene by betaine, although it was also induced after UVB. Accordingly, *Egr1* has many functions in balancing the proliferation-differentiation-

axis. The targets of EGR1 were actually enriched among the genes unique for the UVB treatment, as were those of KLF4 (II, Table 5). These data indicate that UVB alone may also influence the maturation process.

The third highest upregulation with betaine was seen in Purkinje cell protein 4 (*Pcp4*), which in part regulates Ca^{2+}-metabolism. This gene product has not previously been characterized in epidermis, but its function in neurons, also of ectodermal origin, seems critical (e.g. Wei et al., 2011). In immunohistochemical stainings of the 3D REK cultures, the granular layer showed an intense signal at the plasma membrane as well as a granular, cytoplasmic pattern (white arrows and arrowheads, respectively, in Fig. 8; unpublished observations).

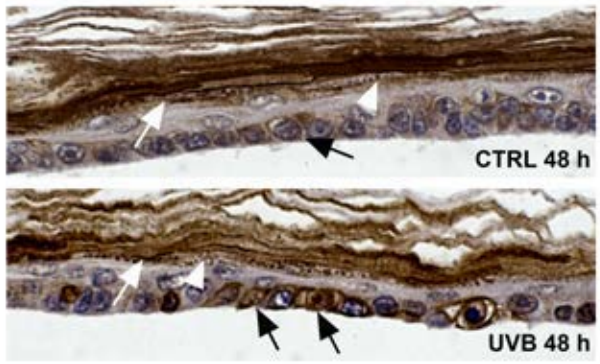

Figure 8. PCP4 protein expression in the 3D REK. The cultures were grown and irradiated with 30 mJ/cm^2 as indicated in original publication I. 48 h after an acute dose of UVB, PCP4 was stained as described under the *Materials and methods* -section of this thesis.

Some basal cells also stained positively (black arrows). To verify specificity, the positive signal could be abolished by using a blocking peptide (data not shown). Moreover, UVB treatment of the 3D epidermis significantly decreased the mRNA level of *Pcp4* (II, Supplemental Table S2), and may also have slightly reduced the staining intensity in the suprabasal layers (Fig. 8; unpublished observations).

5.2.4 Modulation of the protective mechanisms and osmotic balance

In addition to the substantial effect on keratin 2 expression, betaine appeared to reverse or modulate some of the UVB-induced effects. The strong downregulation of both variants of the *Fxyd2* ion transport regulator (both 0.3-fold by qRT-PCR) with betaine suggests a feedback mechanism under conditions where adequate osmoregulation has already been reached. On the other hand, when the epidermal equivalents are subjected to UVB, osmotic balance is disturbed and expression of transport channel components, including *Fxyd2*, is activated (1.39- and 2.16-fold for *Fxyd2* variants a and b by qRT-PCR, respectively). Betaine effectively abrogates this upregulation by UVB (II, Fig. 3).

In addition to *Fxyd2*, several solute carrier family members or enzymes involved in the formation of compatible organic osmolytes were significantly regulated by betaine. These included the monocarboxylic acid transporter 7 (*Slc16a6*; 1.2-fold upregulation in the array), the sorbitol producing aldo-keto reductase *Akr1b1* (0.6-fold downregulation in the array) and the glycine transporter *Slc6a9* (0.7-fold downregulation in the array). Of these, UVB alone significantly upregulated *Akr1b1*, indicating that the combination treatment returned its mRNA expression close to the control levels. Additionally, UVB upregulated the neutral

amino acid transporter *Slc1a5*; this effect was also partially reversed with the combination treatment (II, Supplemental Table 4).

Betaine, like other osmolytes, also participates in the maintenance of redox balance. Here, transcription of the anti-oxidative genes glutamate-cysteine ligase catalytic subunit (*Gclc*) and metallothionein 1a (*Mt1a*) were enhanced after the combination treatment. Similarly, downregulation of the glutathione transferases *Gsta2*, *Gsta4* and *Gsta5*/*LOC494499* and upregulation of *Gstm1* were observed uniquely by UVB + betaine (II, Supplemental Tables 4 and 5 and data not shown). The involvement of betaine in the metabolism of toxic metabolites as well as vitamin D was also suggested by the downmodulation of *Cyp2f4* and *Cyp24a1* expressions (II, Supplemental Tables 4 and 5).

One interesting facet of the regulation by betaine was the significant upregulation of the potential tumor suppressor *Nbl1* by betaine (1.56-fold by qRT-PCR) and the almost as significant suppression by UVB (II, Fig. 2 and Supplemental Table 2). Betaine was not able to inhibit this downregulation, although there appeared to be a trend towards a reversal (0.61-fold in UVB vs. 0.75-fold in UVB + betaine by qRT-PCR). Finally, *Atp2a3*, which regulates the levels of cytosolic Ca^{2+}, was strongly induced by UVB, but attenuated in the combination treatment. This indicates another possibly relevant regulatory point for betaine in cell stress reactions and intracellular ion composition.

5.3 EXTRACELLULAR ATP AND ITS DEGRADATION PRODUCTS MODULATE *HAS* EXPRESSION VIA ACTIVATION OF STRESS KINASE AND CA^{2+} SIGNALING IN HACAT KERATINOCYTES (III)

The final, parallel works of the thesis (III, IV) sought to clarify the effects of the extracellular nucleotides ATP, UTP and their degradation products on HaCaT keratinocytes. Such nucleotides have previously been shown to be released from different cell types after various insults, including mechanical irritation and UV radiation. As cited above, UVB and disruption of the permeability barrier strongly induce hyaluronan metabolism in REK cultures as well as native epidermis. This background prompted the study of the possible contribution of nucleotides in regulating the HA-response depicted in previous studies (e.g. Maytin et al., 2004; Tammi et al., 2005; Monslow et al., 2009) and here in original article I. For this purpose, the human cell line HaCaT was used. There are no previous data concerning the regulation of HA metabolism with ATP (original article III) or UTP (original article IV).

5.3.1 Extracellular ATP and its degradation products regulate *HAS* expression

When the HaCaT monolayers were treated with varying concentrations of ATP, there was a clear dose-response in *HAS2* mRNA expression. Treatment with 1 μM for 2 h caused an approximately 2-fold upregulation. 10 μM and 100 μM ATP further enhanced the effect (approximately 5-fold and 6-fold increase, respectively), and a plateau was reached with 100 μM (III, Fig. 1). The highest dose was used in all subsequent experiments, which often resulted in over a 10-fold stimulation of *HAS2*. The same concentration was applied for ADP, AMP, adenosine and their chemically modified analogs throughout the study.

The time-response was also tested. *HAS2* was moderately induced with ATP already after 30 min, peaked at 90 min and returned to the control levels by 4 h. A clear decrease (0.48-fold) was seen at 6 h, but then ATP appeared to produce another stimulatory peak

(2.3-fold) at 24 h (III, Fig. 1). *HAS2* was not the only hyaluronan processing enzyme responsive to ATP. *HAS3* was also induced around 2-fold with this nucleotide at 2 h (III, Fig. 1). *HAS1* did not change at this time point, but there was a later induction of about 4.5-fold at 6 h. Sustained levels of *HAS3* activation were seen at 4-6 h (1.7-fold; III, Fig. 1). Similar to *HAS2*, both *HAS1* and *HAS3* were upregulated at 24 h (about 3-fold). *HYAL1-2* and *CD44* were also slightly upregulated at 2 h (1.4-fold, 1.1-fold and 1.3-fold, respectively; III, Fig. 1 and Fig. 2). Interestingly, the increase in *HAS2* mRNA could be attenuated by a 48-h pretreatment with betaine (III, Fig. 1; the change at 2 h shown).

The degradation products of ATP (ADP, AMP and adenosine) also exhibited unique effects on *HAS* transcription. With ADP, a similar upregulation in *HAS2* could be seen as with ATP (III, Fig. 2), whereas AMP seemed to downregulate *HAS2* at the early time points (2-6 h), and have a later upregulation at 24 h (III, Fig. 2). Adenosine similarly suppressed *HAS2* at 2-6 h returning to control by 24 h (III, Fig. 2). Interestingly, *HAS1* was significantly upregulated by both AMP and adenosine at 4 h and by AMP at 6 h (III, Fig. 2). *HAS3* was largely unaffected by adenosine, but AMP again induced its expression at 24 h (III, Fig. 2).

5.3.2 The purinergic receptor $P2Y_2$ is a major regulator of the increased *HAS2* expression

The involvement of the purinergic receptors in relaying the nucleotide response was extensively tested with chemical inhibitors. Major involvement of the ATP-receptor $P2Y_{11}$ was excluded, as pretreatment with the specific antagonist NF340 did not greatly reduce the induction of *HAS2* with ATP (III, Supplemental Fig. 1). The G_i-coupled ADP-receptors $P2Y_{12}$ and $P2Y_{13}$ were indicated, however, since the G_i-inhibitor PTX significantly suppressed the ATP-induced upsurge in *HAS2* (III, Fig. 3). The ADP-receptor $P2Y_1$ may also contribute to the *HAS2*-response to some degree, as its antagonist MRS2179 modestly attenuated the ATP-induced upregulation of *HAS2* (III, Fig. 3). Dominating these minor effects, a specific siRNA against $P2Y_2$ almost entirely abolished the *HAS2*-response with ATP (III, Fig. 3). The contribution of a *bona fide* ATP-receptor is also supported by the fact that the stable ATP-analog ATPγS induced a response similar to the native, hydrolyzable ATP (III, Fig. 3).

5.3.3 Ca^{2+} and stress kinase signaling regulate the response of *HAS2* to ATP

The intracellular signaling cascades were also tested. Inhibitors of several protein kinases or intracellular effector molecules clearly attenuated the response of *HAS2* to ATP (III, Fig. 3). These included the Ca^{2+} metabolism associated CREB (42% inhibition with naphthol AS-BI phosphate and 46% with KG501), PKC (75% inhibition with bisindolylmaleimide I, BIM) and CaMKII (96% inhibition with KN93). Significant modulation of the response could also be attributed to p38 (46% inhibition with BIRB796) and MEK1/2 (44% inhibition with PD98059). Nevertheless, the JAK2/EGFR-inhibitor AG490 and STAT3-inhibitor IX (Cpd188) failed to modify the response (III, Supplemental Fig. 1).

The same pathways were also probed by western blotting. ATP clearly activated CREB, ERK, p38 and STAT3 (III, Fig. 4). In STAT3, the two common phosphorylation sites Tyr^{705} and Ser^{727} behaved differently. Ser^{727} showed a similar early induction (15 min) as the kinases and CREB, whereas Tyr^{705} was significantly phosphorylated only at 1-2 h. Interestingly, betaine pretreatment appeared to slightly inhibit the ATP-induced phosphorylation of STAT3 at Tyr^{705} as well as pCREB at 2 h (III, Fig. 4). Together, these data indicate that both Ca^2 signaling, which is so central to epidermal physiology, and MAP

kinases respond to an extracellular nucleotide stimulus in HaCaT keratinocytes by mediating enhanced *HAS2* transcription.

5.3.4 Adenosinergic control of HA synthesis

Next, the effects of the nucleotides on the accumulation of peri- and extracellular HA were tested both by using cytochemical stainings and biochemically (III, Fig. 5 and Fig. 6, respectively). Light microscopy revealed that total hyaluronan associated with the cell layer was moderately increased in the HaCaT cells treated with ATP for 2-4 h. The signal was mainly localized on the apical plasma membrane (III, Fig. 5). Intracellular HA was also evident and it had a similar tendency to increase in the ATP-treated cultures. When HA accumulation was measured biochemically in cultures containing 10% FBS, pericellular HA was increased by ATP at 2-4 h and the extracellular pool was slightly augmented at 4 h. At 6 h, pericellular HA was in fact diminished (data not shown).

These data prompted an alternative approach, where the HaCaT cells were cultured in medium containing minimum serum (1% FBS) to remove the potentially masking effects of unspecified growth factors, cytokines and other small molecules. Under these conditions, ATP induced a rapid and strong accumulation of both pericellular (measured in the trypsinates) and extracellular (medium) HA at 4-6 h (III, Fig. 6). These differences leveled off by 24 h. Adding 1 mM glucosamine further enhanced the effect of ATP at 6 h (III, Fig. 6). These data are in line with the HA-stainings, where ATP had a tendency to increase pericellular HA at the early time points (III, Fig. 5). It thus seems that the pericellular pool reflects the changes in the expression levels of the enzymes first, whereas extracellular/medium HA responds with a lag.

Referring to the HA-stainings of the cultures, no major changes after treatment with either AMP or adenosine were observed at 2-4 h (III, Fig. 5). Under the serum-deprived conditions, both AMP and adenosine clearly inhibited HA accumulation in the medium at 24 h, and adenosine also decreased pericellular HA at 6 h (III, Fig. 6).

Next we tested whether ATP altered the pools of the intracellular nucleotide precursor sugars. There were only minor changes to the content of UDP-GlcUA (III, Fig. 6), but UDP-GlcNAc was decreased at 3 h and 4.5 h (92% and 82% of the control, respectively). This could help to explain the augmenting effects of glucosamine, an additive that increases the intracellular supply of UDP-GlcNAc (Rilla et al., 2013). Exposing the keratinocytes to extracellular ATP also appeared to shift the molecular mass distribution of the newly synthesized HA towards lower molecular weight fragments. This suggests that degradative mechanisms were activated (original article III, Fig. 6). Adenosine was ineffective in this respect (III, Fig. 6).

5.4 EXTRACELLULAR UTP MODULATES *HAS2* EXPRESSION AND HA SYNTHESIS IN HACAT KERATINOCYTES (IV)

5.4.1 Extracellular UTP and UDP upregulate *HAS2* expression and HA synthesis

UTP behaved very similarly to ATP, exhibiting a mean 9.2-fold induction in *HAS2* mRNA expression (original article IV, Fig. 2). Unlike ATP, it did not significantly upregulate *HAS3*, and it was also ineffective with *HAS1* and *HYAL1-2*. Additionally, the dose response and time curve of induction of *HAS2* by UTP were almost identical to those seen with ATP (original article IV, Fig. 2). UTP did not decrease *HAS2* expression at 6 h, though, unlike ATP, where the suppression at this time point was clear. 100 μM UDP also induced *HAS2* mRNA, whereas UMP was ineffective or even slightly inhibitory (original article IV, Fig. 2). When tested with a smaller effective concentration, 10 μM UDP only marginally stimulated *HAS2*, clearly differing in its effect from the triphosphated form (original article IV, Fig. 2).

In the UTP-treated cells, HA increased both pericellularly, as shown by cytochemistry, and in the culture medium, although the response was slower in the latter (a clear induction by 2 h vs. 6 h, respectively; original article IV, Fig. 1). This is similar to what was seen with ATP: the changes in *HAS* expression are first reflected in the hyaluronan molecules, which are still tightly associated with the synthesizing cell. After this, release of the molecules into the extracellular matrix (medium) and/or uptake back into the cells may predominate. Unlike ATP, the UTP treatment did not affect the levels of the precursor sugars of HA (original article IV, Fig. 2), indicating that the nucleotides differ somewhat in their metabolic and signaling pathways.

5.4.2 The purinergic receptor $P2Y_2$ is a major regulator of the increased *HAS2* expression by UTP

Interestingly, UTP also appeared to regulate *HAS2* via $P2Y_2$, although the dependence was not as strong as with ATP (original article IV, Fig. 3). With UDP, $P2Y_6$ and $P2Y_{14}$ are also involved, as inhibition with MRS2578 and particularly with PTX reduce the upregulation in *HAS2* transcription by UDP. Their contribution to the UTP-induced effects seems less substantial (original article IV, Fig. 3). Thus, the receptor responsible for the high induction of *HAS2* by the triphosphated nucleotides in keratinocytes appears to be the dual ATP/UTP-receptor $P2Y_2$.

5.4.3 Ca^{2+} and stress kinase signaling regulate the response of *HAS2* to UTP

With UTP, much the same signaling pathways were involved as with ATP. p38 (52% inhibition with BIRB796), MEK/ERK (48% inhibition with PD98059), CaMKII (80% inhibition with KN93), PKC (48% inhibition with bisindolylmaleimide I) and CREB (31% inhibition with naphthol AS-BI phosphate) all contributed to the *HAS2*-response (original article IV, Fig. 5). Contrary to the ATP-treated cells, where the induction of *HAS2* was unaffected by the STAT3 inhibitor IX (Cpd188), the UTP-induced response was attenuated by 87% (original article IV, Fig. 5). The EGFR/JAK2 inhibitor AG490 was, however, ineffective with both ATP (original article III, Supplemental Fig. 1) and UTP (original article IV, Fig. 5), indicating that STAT3 is activated independently of the canonical JAK-STAT-cascade, most likely by p38 and/or pERK (original article IV, Fig. 6).

The phosphorylation of STAT3 on Ser^{727} was also evident with western blotting. It coincided with a strong activation of p38 after 15 min of UTP treatment (original article IV,

Fig. 4), whereas Tyr^{705} of STAT3 was unresponsive. Further corroborating the inhibitor data, CREB and ERK were rapidly (15-30 min) phosphorylated by the UTP treatment. Additionally, the nuclear translocation of pCaMKII was observed (original article IV, Fig. 4).

The findings from original publications III and IV are summarized in Figure 9. Together, these results establish for the first time that UTP and UDP as well as ATP and its degradation products potently regulate *HAS* transcription and HA metabolism in keratinocytes. This is also the first time when the effects of the phosphorylated forms of adenosine and uracil (ATP, ADP, AMP, UTP and UDP) on *HAS* expression have been demonstrated. The activation of distinct intracellular signaling pathways by ATP and UTP also adds to the existing knowledge of stress-activated pathways and endorses future research efforts.

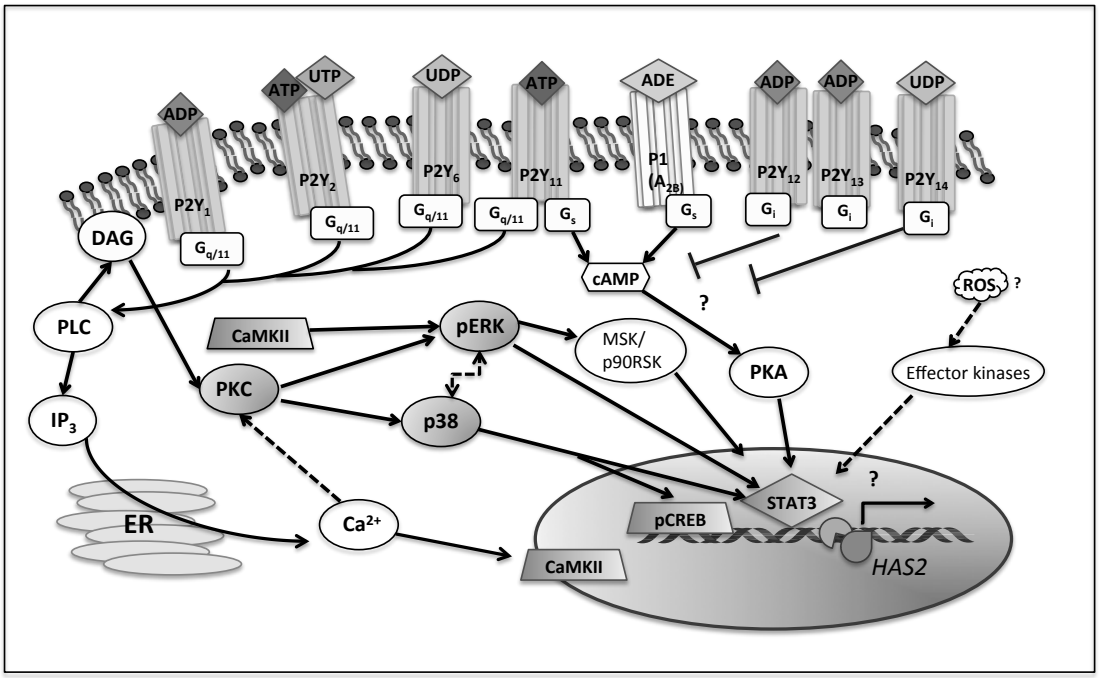

Figure 9. Summary of the signaling pathways regulating *HAS2* expression and HA synthesis activated by the extracellular nucleotides ATP, UTP and their degradation products. The figure is based on data in original articles III and IV and literature references therein (Rauhala et al., manuscript; Jokela et al., 2017). Shaded symbols in the cytoplasm and nucleus represent effectors whose contribution was tested in this study (solid arrows = canonical pathway components; dashed arrows = alternative connections and interactions). cAMP = cyclic AMP; DAG = diacylglycerol; G_i, $G_{q/11}$ and G_s = G proteins; IP_3 = inositol trisphosphate; MSK/p90RSK = mitogen and stress activated protein kinase/90 kDa ribosomal S6 kinase; P2Y and P1 = purinergic receptors; PKA = protein kinase A; PLC = phospholipase C; ROS = reactive oxygen species.

6 Discussion

6.1 STUDYING EPIDERMAL METABOLISM *IN VITRO*: VALIDITY OF THE MODELS USED

The 3D REK model has long been utilized in studying the metabolism of HA as well as penetration of chemicals and metabolism in the epidermis (Tammi et al., 2000; Pasonen-Seppänen et al., 2001; Pietilä et al., 2005; Pappinen et al., 2007). The REK cells stratify and differentiate efficiently and similarly to normal skin to produce an epidermal equivalent that is devoid of any confounding effects from other cell types. Thus, the model augments the traditional monolayer cultures of keratinocytes (REK, HaCaT). Using a human cell line in original articles III and IV further widens the scope of this thesis.

In the 3D system, REK cells actively synthesize HA and other glycosaminoglycans (Tammi et al., 2000, original article I). In control cultures, HA accumulation in the epidermal layers during differentiation is clear (Passi et al., 2004), and its synthesis can further be induced or inhibited by EGF and KGF or TGF-β, respectively (Karvinen et al., 2003b; Pasonen-Seppänen et al., 2003). The overall metabolism of HA in this culture model appears quite fast (Tammi et al., 2000; original article I).

Here, the 3D cultures were treated with UVB when there was already a thick stratum corneum present, mimicking the physiological situation. Although the dose (30 mJ/cm^2) chosen for most experiments causes a clear increase in the number of sunburn cells (Bart et al., 2014), its influence on HA accumulation and global gene expression was relatively modest. This may be due to the UV-absorbing stratum corneum. However, this protection was not enough to substitute for the lack of melanin, which is the major shield against UV-irradiation *in vivo*. The lack of such a barrier was indicated by excessive necrosis with the higher doses, which are well tolerated in animal models (e.g. Tobiishi et al., 2011). It is also likely that the lack of cross-talk between dermal fibroblasts and melanocytes, Merkel cells and Langerhans cells normally present in skin blunts the effect. This monoculture nature is thus probably the biggest disadvantage of the 3D REK model, as well as its great asset.

The need for growth factor receptor and cytokine signaling in the UVB-response is apparently high, particularly when considering the inflammatory cascades and erythema as well as neoplastic changes activated in intact skin (e.g. Madson et al., 2006; Laihia et al., 2009; Muthusamy and Piva, 2010). These signals (growth factors, interleukins, TNFα) also induce HA synthesis (e.g. Jokela et al., 2008b; Ohtani et al., 2009; Meran et al., 2013). In our 3D-cultures, the upregulation of IL-1α and IL-6 by UVB was modest at the mRNA-level, and TNFα was not significantly regulated. Generally, the microarray data was supported by qRT-PCR and/or western blotting data as well as immunohistochemistry, validating the experimental setup for studying global gene expression. The model also demonstrated low interexperimental variation. Importantly, UVB activated many of the expected pathways and processes, including formation of the typical sunburn cells.

Considering the effects of betaine in the culture systems used, it is important to note that this osmolyte is a natural substance whose catabolic machinery appears very efficient. In this light, it is probably not surprising that betaine alone does not induce very drastic changes in keratinocyte physiology. However, as betaine concentrations as high as 400 μM

have previously been reported in skin (Slow et al., 2009), it is reasonable to conclude that the epidermis actively accumulates and utilizes this osmolyte. Treating keratinocytes with a constant 10 mM concentration of betaine in the growth medium thus seems rational. Similar doses have also been employed in other studies for epithelial cells grown *in vitro* (Kettunen et al., 2001; Garrett et al., 2013; Hua et al., 2015). Furthermore, our preliminary tests indicated that betaine was not toxic to the REK cells even at millimolar concentrations.

6.2 HYALURONAN ACCUMULATION IN RESPONSE TO UVB AND EXTRACELLULAR NUCLEOTIDES

6.2.1 General considerations

In this work, a modest increase in total epidermal HA was observed in the 3D REK culture system in response to acute UVB-exposure. In addition, distinct accumulation of HA was demonstrated in the UVB-treated REK-monolayers (original article I). Interestingly, increased GAG accumulation in chronically UV-irradiated rat skin was suggested as early as 1968 (Nakamura & Johnson, 1968). After that, several groups readdressed the topic using different irradiation schemes and species (Schwartz, 1988; Longas et al., 1993; Takahashi et al., 1995; Mitani et al., 1999; Werth et al., 2011). Notably, UV exposure didn't lead to large or persistent hyaluronan accumulation in all cases, particularly after long-lasting irradiation schemes (Takahashi et al., 1995). In fact, loss of dermal hyaluronan and downregulation of *Has1-3* has later been observed in chronically irradiated mouse skin (Dai et al., 2007).

As most of these studies concentrated either on whole skin or the dermis, the specifics concerning epidermal hyaluronan metabolism were left open. Additionally, none of the earliest investigations could ascertain the roles of the respective HAS-enzymes. The role of the various wavelengths or different doses in inducing the effects were also not dissected in most of these works. Altered HA synthesis or *HAS* expression in response to UVR in an epidermal context was evident in some of the more recent reports (Averbeck et al., 2007; Kakizaki et al., 2008; Tobiishi et al., 2011). These data are also well in line with the effects seen in this thesis work. In most cases, UVR is stimulatory on HA synthesis, but the effects appear to be highly time-dependent, and may include a simultaneous activation of the degradative mechanisms (e.g. Tobiishi et al., 2011). For instance, a transient loss of epidermal hyaluronan 2 h after an acute UVA or UVB exposure was demonstrated in mouse skin (Calikoglu et al., 2006).

With these studies, it appears, that keratinocytes (HaCaT or NHEK), human dermal fibroblasts as well as intact mouse or human skin respond specifically to acute UVB in a temporally distinct, dose-responsive and cell/tissue type specific manner in their *HAS* and *HYAL* expression and HA production (Averbeck et al., 2007; Kakizaki et al., 2008; Tobiishi et al., 2011). Yet, the intracellular regulatory pathways remain elusive; although it has been shown that neutralizing antibodies against KGF and IL-1β dramatically attenuate the UVB-induced increases in *HAS2* and *HAS3* expression in NHEK (Kakizaki et al., 2008). The signaling components need to be studied with particular caution, as the source of growth factors or cytokines may be dermal as well as epidermal.

6.2.2 Differences between the models in response to UVB

Considering these existing data, the aim of the first original publication of this thesis (I) was to investigate more carefully the temporal effects of acute UVB irradiation on hyaluronan metabolism, and to characterize the intracellular signaling pathways involved. Experiments performed both on monolayer cultures and in a 3D epidermal equivalent of REK cells was an effective way to compare the effects of the culture platform, which notably relates to the differentiation status of the keratinocytes and the responses observed.

In the REK-monolayers, HA accumulated dose-dependently in response to UVB, with a maximum reached at 10-15 mJ/cm^2. In human keratinocytes (NHEK and HaCaT) more intense exposures (20-30 mJ/cm^2) are tolerated and most effective in inducing hyaluronan synthesis (e.g. Averbeck et al., 2007; Kakizaki et al., 2008). Most of the previous studies have actually utilized much higher doses than were employed here. With the highest dose tested in the monolayer REK (20 mJ/cm^2), HA accumulation was in fact reversed, so the less toxic 10 mJ/cm^2 was applied.

Underlying the accumulation of HA in the REK cells, *Has* induction was very fast, with *Has1* peaking already 4 h post-UVB. *Has2* and *Has3* responded with two waves of induction, first at 8-12 h and again at 36 h. Similar to our observations, *HAS1* was upregulated 3 h post-UVB in HaCaT cells (Averbeck et al., 2007), and *HAS2* and *HAS3* were induced 12-24 h after irradiation in NHEK (Kakizaki et al., 2008). The later induction of *Has2* and *Has3* in the REK monolayers at 36 h is also in line with the changes seen in UVB-irradiated mouse epidermis (Tobiishi et al., 2011). Interestingly, the initial downregulation of *Has2* evident in the REK cells has previously been observed in HaCaT (Averbeck et al., 2007; Hašová et al., 2011).

The time course of the response with *Has1* was different from those of *Has2* and *Has3*, underlining the uniqueness and elusive nature of this isoenzyme (Siiskonen et al., 2015). Of the isoforms in the REK monolayers, *Has3* appeared to be the most responsive to UVB. Based on the knockdown experiments, *Has3*-specific siRNA halved the basal HA production and significantly inhibited the UVB-induced increase. This novel finding emphasizes the role of *Has3* in contributing to stress-induced HA synthesis.

The more physiologically relevant 3D model reproduced the upregulation of *Has2* and *Has3* mRNA, but showed a more modest accumulation of HA. This may be partially due to the differences seen in the transcript levels of the principal HA receptor *Cd44* between the two systems. In the organotypic model, the *Cd44* transcript was upregulated at 8 h, returning to control by 24 h. This early induction together with increased fragmentation of HA may facilitate HA endocytosis in the 3D cultures dampening the accumulation of extracellular HA. In contrast, in the monolayers a significant downregulation of *Cd44* was seen at the early time points (2-8 h). Thus, it may be unavailable for binding newly synthesized HA for quite some time until catching up with its expression

The suppression of CD44 expression at the early time points in the REK-monolayers is in agreement with previous observations in HaCaT and intact mouse epidermis (Calikoglu et al., 2006; Hašová et al., 2011). Interestingly, UVB also increases the shedding of CD44 from the cell surface (Hašová et al., 2011). In our organotypic cultures a patchy and locally reduced expression pattern of CD44 was evident in histological sections 24 h post-UVB despite increased expression at 8 h. The dynamic alterations in CD44 expression after UVB are likely to affect cell signaling and HA metabolism/retention on multiple levels. The changes could also be reflected in CD44 transcription through feedback mechanisms.

Indeed, the later upregulation of *Cd44* in the REK-monolayers may represent increased signaling for the second wave of HA metabolism clearly observed after 24 h.

The time course of *Hyal1* and *Hyal2* expression also differed between the models used here. In the monolayers both were maximally upregulated at 8-12 h, similar to their early upregulation in HaCaT (Averbeck et al., 2007), whereas the induction in the 3D model was clearest at 24 h. In contrast, Hašová et al. (2011) observed a decrease in the *HYAL2* transcript in HaCaT 6 h after irradiation with 10 mJ/cm^2 UVB. In NHEK, measured at 12 h and 24 h post-UVB, a downregulation was seen in both genes, but only with the highest doses tested (50-120 mJ/cm^2; Kakizaki et al., 2008).

It is interesting that both the synthesizing and degrading enzymes are upregulated by UVB in both REK culture systems at the mRNA level. Yet, the potential activation of HYALs or ROS is evident only in the organotypic cultures as analyzed by the molecular mass distribution of newly synthesized HA. It is possible that the half-life of HA is shorter in the organotypic cultures due to more efficient binding and internalization (Tammi et al., 2000; 2001). As recently suggested, the activity of the degradative enzymes may increase with differentiation as well (Malaisse et al., 2015). The need for a more stringent homeostatic regulation in the stratified and differentiated system may also help to explain the data.

In contrast to our model, Tobiishi et al. (2011) observed epidermal hyperproliferation in mouse skin in response to a single dose of UVB. The hyperplastic response was most pronounced after 2-3 days, and it coincided with maximal epidermal HA synthesis. Repeated exposures, which would also be physiologically more relevant, might increase HA fragmentation needed for downstream inflammatory cascades and hyperproliferation even in our 3D REK model. This approach could also overcome the limited capacity of the cultures to withstand high UVB-doses.

A proper balance of hyaluronan synthesis and degradation is crucial for epidermal homeostasis. This is evidenced by the fact that depleting HA in the 3D REK cultures results in strong upregulation of the terminal differentiation markers keratin 10 and filaggrin (Passi et al., 2004). Interestingly, filaggrin mRNA expression was also moderately stimulated by UVB in our organotypic cultures, but the functional significance of this change wasn't established. Additionally, blocking EGF-induced HA synthesis with 4-methylumbelliferone in 3D REK cultures results in a much thinner epidermis with fewer vital cell layers (Rilla et al., 2004).

Overall, the data suggest that keratinocytes respond to acute UVB rapidly and specifically with accelerated HA metabolism. The effects vary according to the exact setting, where several factors, such as the dose, play a major part. These results also highlight the importance of CD44 regulation in skin by UVB, a process which may have many roles in modulating cell-ECM-interactions, HA retention in the pericellular environment and downstream signaling.

6.2.3 Extracellular nucleotides in the regulation of HA accumulation: convergence with the UVB-induced changes?

The effects of adenosine have previously been investigated in arterial smooth muscle cells and gingival fibroblasts, where it increases HA accumulation and/or *HAS1* expression (Murakami et al., 2001; Grandoch et al., 2013). However, there are no previously published data concerning the influence of the phosphated forms of adenosine or uridine on HA

metabolism. Similarly to UVB, the extracellular nucleotides ATP and UTP and their degradation products cause dynamic changes in HA metabolism. Mechanistically the effects are more complex, as the tri- and diphosphated forms often cause contrasting outcomes as compared to the corresponding monophosphates and nucleosides.

Both ATP and UTP and their diphosphated metabolites ADP and UDP significantly upregulate *HAS2* transcription in HaCaT. The time and dose response curves were very similar with both nucleotides, except for the inhibition of *HAS2* expression with ATP at 6 h. Contrary to UTP, ATP also induced the expression of *HAS1* and *HAS3*. These changes were accompanied by increased peri- and extracellular HA with both ATP and UTP.

Different methods give slightly different snapshots of the dynamic changes in HA accumulation. The increase was seen histochemically at 2-4 h with both ATP and UTP. In the trypsinate, also reflecting the pericellular HA pool, ATP caused an induction at 4-6 h. However, the increased HA synthesis was not reflected in the medium until after 6 h with both nucleotides. The newly synthesized HA appears to be efficiently retained in the vicinity of the cells before being taken up for degradation or released further into the ECM.

Increased internalization and catabolism are supported by the fact that ATP increased intracellular HA staining at 2 h as well as shifted the molecular mass distribution of HA towards smaller fragments at 6 h. Additionally, the expression of *CD44* tends to be elevated with both ATP and AMP at all the time points checked. As ATP is able to influence ROS production (e.g. Cheng et al., 2013), it is possible that this leads to increased fragmentation of HA (Soltés et al., 2006).

Here, the effects of AMP and adenosine on the expression of the HAS-enzymes also need to be considered, as they strongly oppose the induction in *HAS2* seen with ATP. However, in addition to the suppression of *HAS2*, both upregulate *HAS1* expression, and their net effect on HA metabolism thus appears complex. The extent of the inhibition of the ATP-induced effects through the conversion of ATP to AMP and adenosine cannot be established here. When added alone, the suppressive effect of each on HA synthesis seems dominating.

It is intriguing that both UVB and ATP/UTP cause a rapid net accumulation of HA in keratinocytes. This raises the possibility that the UVB-induced changes may partially depend on the release of nucleotides from the stressed cells (e.g. Takai et al., 2011). Both the REK- and the HaCaT-models show activation of all the HAS-isoforms, but the exact temporal pattern of the changes differ. Furthermore, the initial influences on *HAS2* and *CD44* were totally opposite, and the hyaluronidases failed to respond to the nucleotides. The downregulatory potential of AMP and adenosine on *HAS2* is also different from that seen with UVB: the suppression caused by acute radiation rebounds quickly but is sustained with the nucleotides. However, the second wave of activation in *HAS2* and *HAS3* seen in both the ATP-treated HaCaT and the UVB-exposed REK around 24-36 h could well be explained by sustained release of ATP.

6.3 MODULATION OF KERATINOCYTE INTRACELLULAR SIGNALING PATHWAYS BY UVB AND ADENOSINE NUCLEOTIDES: ROLE OF CA^{2+} AND MAP KINASES

6.3.1 Signaling cascades activated by UVB

The effector kinases responsible for the UVB-induced changes in HA synthesis (I) were unique, particularly with respect to CaMKII, whose role in HA synthesis has not been suggested previously. The effect of the CaMKII inhibitor KN93 on *Has3* induction in REK cells appeared quite specific. The inhibition was most pronounced 36 h post-exposure, which may point to an adaptive mechanism or a second wave of signaling activated. As the effect was also reflected in hyaluronan secretion, this kinase probably plays a truly significant role in HA metabolism. The MAPK p38 has been linked to *HAS3* activation in SSR-treated skin (Mouchet et al., 2010), but its connection with *Has2* (and *Has1*) with respect to UV-activated processes is novel.

We also saw activation of genes regulating intracellular Ca^{2+} stores in the microarray data set, where *Atp2a3* (SERCA3), a Ca^{2+} pump on the ER, was significantly upregulated by UVB (II). This mechanism most likely relates to returning excess cytosolic Ca^{2+} to the ER after a UVB insult. Interestingly, this stress response can be modulated by betaine. A couple of other genes known to regulate or be regulated by Ca^{2+}-metabolism, namely *Pcp4* (Wang et al., 2013), *ClCa2* (Bart et al., 2014) and *Pten* (Bononi & Pinton, 2015), were strongly suppressed by UVB. In addition to directly regulating Ca^{2+} stores and calmodulin signaling, UVB can control the release of ATP from irradiated cells (Takai et al., 2011), thereby potently enhancing the Ca^{2+}-associated branches of signal transduction.

The MEK-ERK cascade is central in UVB-stimulated cells. Its role in HA metabolism has also been characterized, but the connection between UVB/stress, ERK activation and HA synthesis has been largely unexplored. It is known that ERK is activated in mechanosensitive cells after exposure to strain. This response is modulated by an interaction with p38, which is itself constitutively active at the articular surface and regulates pericellular HA accumulation (Lewthwaite et al., 2006). In ovarian tumor cells ERK activation results in increased HA synthesis, possibly via HAS phosphorylation (Bourguignon et al., 2007), and in human dermal fibroblasts ERK regulates HA synthesis as well as *HAS2* and *HYAL1* transcription (Li et al., 2007; Röck et al., 2011).

Here, the route from UVB to MEK/ERK to HA could not be established, as experiments with inhibitors of this signaling branch did not influence HA-synthesis in the UVB-treated REK-cells. Yet, the results with ATP and UTP strengthen the notion that stress-activated pathways flow through MEK/ERK to induce HA synthesis, a cascade that may well be initiated by exposure to irritating doses of UVB.

6.3.2 Signaling pathways involved in the nucleotide-induced *HAS2* upregulation

Treatment of the HaCaT keratinocytes with ATP and UTP activated several distinct signaling pathways that affected *HAS2* transcription. According to the inhibitor data, the most influential kinases were PKC and CaMKII, but p38 and MEK/ERK also contributed. Downstream, the transcription factors CREB and STAT3 appeared to be central for increased *HAS2* expression.

CaMKII – As with UVB, nucleotide signaling to *HAS2* appears to depend on Ca^{2+}-associated pathways. In HaCaT cells treated with ATP and UTP the CaMKII inhibitor KN93

caused a near complete blockade of *HAS2* induction. This result points to a massive and physiologically relevant effect; indeed, CaMKII was recently shown to regulate the basal level of HA in both the medium and the pericellular fraction in HaCaT (Jokela et al., 2015).

PKC –PKC also regulated *HAS2* expression in response to both ATP and UTP. The 75% and 48% reductions, respectively, in the induction level of *HAS2* with the inhibitor BIM indicate a biologically significant process. PKC isoforms may be activated directly by cytosolic Ca^{2+} in addition to the classical PLC- and DAG-controlled pathway. Adenosine has also been shown to activate distinct PKC isoenzymes (Mochly-Rosen et al., 2012). It is thus possible that ATP triggers PKC by increasing the levels of intracellular Ca^{2+} or through conversion to adenosine. However, the opposing effects of ATP (upregulation) and adenosine (downregulation) on *HAS2* mRNA at 2 h would argue for a direct effect by ATP.

MAPKs – Another major group of intracellular signaling regulators activated in HaCaT by ATP and UTP were the MAP kinases. The MEK1/2-inhibitor PD98059 and the p38 inhibitor BIRB769 both significantly modulated the ATP/UTP-response in *HAS2*. The MEK-targets ERK1/2 and p38 were also activated at the protein level. The responses of p38 to both ATP and UTP are well in line with previous data (Takai et al., 2011). Here, a connection between PKC and MEK/ERK is possible, as PKCα has been shown to function upstream of ERK1/2 in the regulation of HA synthesis (Momberger et al., 2006). Furthermore, PKC and CaMKII may co-operatively induce EGFR/ERK-activation in ATP-stimulated vascular smooth muscle cells (Ginnan et al., 2004), and p38 is activated by PKC in differentiating keratinocytes (Efimova et al., 2004). These data highlight some of the potential points of cross-talk between the different signaling cascades activated here by ATP and UTP.

CREB and STATs – Both CREB and STAT3 are recognized regulators of *HAS2* transcription (Table 2 of this thesis). The CREB inhibitors AS-BI-phosphate and AS-E-phosphate were slightly more effective in blocking *HAS2* transcription in response to ATP than UTP. The effects of the two nucleotides on STAT3 were more divergent. Ser^{727} was phosphorylated by both with a similar, early time course (maximum at 15 min), whereas Tyr^{705} only responded to ATP, with a later activation (1-2 h). Moreover, the phosphorylation of Tyr^{705} by ATP was partially inhibited by a pretreatment with betaine. Interestingly, the STAT3 inhibitor IX was only effective in blocking the UTP-induced *HAS2* transcription. These data highlight the subtle but probably functionally significant differences between ATP and UTP.

Activation of purinergic receptors – To further dissect the mechanisms involved in the nucleotide responses, the receptors usually considered most specific for UTP and ATP were probed in the HaCaT keratinocytes. Based on knockdown with specific siRNAs and functional blocking with chemical inhibitors, the purinergic $P2Y_2$ was singled out as the main contributor to increased *HAS2* expression with both ATP and UTP. $P2Y_{14}$ relayed a smaller effect of UTP (via UDP), whereas $P2Y_4$ and $P2Y_6$ were considered to have negligible roles. With ATP, $P2Y_1$ as well as the PTX-responsive $P2Y_{12}$ and $P2Y_{13}$ showed a minor contribution to the responses.

The P2X-family proteins were possible candidates, but their involvement is challenged by the fact that 10 μM ATP was almost as effective as the higher concentration. $P2X_7$ in particular usually requires very high effective concentrations (EC_{50} = 300-400 μM; Burnstock et al., 2012). Moreover, ADP, which is not a standard ligand for the P2X-receptors, produced a similar response to ATP. If the P2X-receptors were involved, the most likely

members could be narrowed down to $P2X_5$ and $P2X_7$, which have previously been characterized in proliferating/differentiating and apoptotic keratinocytes in intact skin, respectively (Greig et al., 2003a).

We cannot exclude the P1-proteins in the adenosinergic response of the HAS-enzymes, particularly, when considering the high induction of *HAS1* with adenosine and AMP. Additionally, pharmacology of the purinergic receptors is complex; their function could be affected not only by the ligand and its concentration but also by the oligomerization of receptors (Burnstock, 2007). The potential interconversion of extracellular nucleotides at the cell surface further complicates the situation, as ADP can be converted to ATP + AMP by ectokinases, also in HaCaT cells (Burrell et al., 2005).

6.3.3 Common pathways induced by UVB and the extracellular nucleotides

UVB exposure has previously been shown to release ATP in HaCaT (e.g. Takai et al., 2011). This release resulted in the activation of p38 via the $P2Y_6$-receptor. In the microarray data set, activation of *P2y2* transcription by UVB was obvious (also see Bart et al., 2014), whereas in HaCaT a reduction in *P2Y2* mRNA in response to acute UVB has been demonstrated (Ruzsnavszky et al., 2011). These data lend support to the idea that the purinergic pathways are truly active and responsive to irradiation stress in keratinocytes.

CaMKII as a regulator of HA synthesis/*HAS* expression in response to nucleotides as well as UVB is one of the most interesting and pioneering findings of this work. CaMKII exists as four distinct isoforms (α, β, δ and γ), which regulate several aspects of cellular metabolism in response to binding Ca^{2+}/calmodulin or stimulation by ROS (Anderson et al., 2015). The kinase may activate several targets, including MAP kinases and AMPK. CaMKII is also able to phosphorylate CD44 to induce cell migration (Lewis et al., 2001), and its role in cancers relying on active glycolysis has started to emerge (Anderson et al., 2015). Interestingly, CREB, a potential downstream target of CaMKII, was activated by both ATP and UVB, as shown by western blotting and enrichment of target genes of this transcription factor (III and II, respectively). The data thus further links these otherwise distinct stressors, and highlights the significance of Ca^{2+}-dependent pathways in the responses observed.

Involvement of the p38 branch of stress kinase pathways in regulating *HAS* mRNA expression was in line with previous data: *HAS3* was shown to be a target of the kinase *ex vivo* in whole skin samples exposed to simulated solar radiation (Mouchet et al., 2010). Additionally, *Has2* is induced in mouse mammary epithelial cells by TGFβ1 in a p38-dependent manner (Porsch et al., 2013), and *HAS1* is similarly regulated in human synoviocytes (Stuhlmeier & Pollaschek, 2004). The potential interplay between the MEK-ERK and p38 pathways should not be ruled out either. The role and regulation of *Has1* by UVB remained open in these studies, however, as the inhibitors tested did not have a clear effect. As with *Has2*, p38 might be involved, but since the inhibitor BIRB796 also lowered the basal level of *Has1* expression, caution must be taken.

p38δ may function downstream from PKC during keratinocyte differentiation as well as apoptosis. Moreover, HA synthesis and CD44 expression have been shown to be regulated by Ca^{2+} balance in mouse skin (Lee et al., 2010). As both PKC and p38 were involved in the upregulation of *HAS2* by ATP and UTP, the physiological meaning of these changes in the context of the epidermis becomes ever more intriguing. However, since BIRB796 blocks the entire p38 family, the specific isoform was not identified.

The regulation with p38 also works in reverse fashion, as stimulation of inflammatory microglia and macrophages with extracellular HA (500-800 kDa) results in activation of p38. This induces the production of TNFα via increased phosphorylation of proteins of the translation machinery (Wang et al., 2006). As p38 is an inflammatory mediator in UV-irradiated skin (see e.g. Hildesheim et al., 2004), it is interesting to speculate, if a self-sustaining regulatory loop is possible. There UVB would first induce HA synthesis and also partial fragmentation of the newly synthesized molecules. This could then result in a renewed activation of p38 signaling and reinforcement of an inflammatory state as well as further HA production.

Perturbed ROS- and Ca^{2+}-signaling as well as ATP/UTP release in response to UV radiation are thus factors that need to be carefully considered in the (patho)physiological responses in HA synthesis. Overall, the involvement of p38 and CaMKII in the induction of *HAS* expression physically (by UVB) as well as biochemically (by ATP and UTP) extends and refines their roles as stress-activated kinases in keratinocytes.

6.4 REGULATING KERATINOCYTE STRESS RESPONSES AND CELL SURVIVAL BY UVB, EXTRACELLULAR NUCLEOTIDES AND BETAINE

6.4.1 Hyaluronan modulates cell survival and apoptosis

In the present work, acutely irradiated REK-cells showed a clear, systematic 2-3-fold increase of hyaluronan in the culture medium. This raises the question of its functional significance. It has recently been shown that the increased expression of *Has2* in *Has1/3* null murine fibroblasts leads to elevated hyaluronan levels. Functionally, an apoptosis resistant phenotype against UV exposure is observed (Wang et al., 2014). HMW-HA has also been demonstrated to protect epithelial cells against radiation damage. In HaCaTs, 970 kDa HA prevented the release of IL-6, IL-8 and TGF-β1 as well as slightly improved cell viability after acute UVB irradiation (10 mJ/cm^2; Hašová et al., 2011). In corneal epithelial cells, IL-6 and IL-8 secretion was similarly suppressed in UVB irradiated cultures pretreated with 1.5 MDa HA. Activation of caspases 3, 8 and 9 was also inhibited. At the same time, cell viability remained higher in the HA-treated cells with most of the UVB-doses tested (Pauloin et al., 2009).

These data emphasize the intriguing possibility that elevated HA levels and *Has* expression in UVB-treated REK cells (I) are a mechanism by which the keratinocytes try to counteract the environmental insult and regulate apoptosis. However, the UVB treatments clearly increased the proportion of dead cells, so the mechanism is not all-inclusive, and shouldn't be, as potentially mutated cells still need to be efficiently removed. In the 3D cultures, it was evident that DNA damage control and repair mechanisms as well as inflammatory genes were activated by UVB (II). Increased *Has* expression and HA synthesis are likely one part of a machinery that serves to maintain homeostasis and increase cell survival in the epidermis. If this is the case, the balance could also be disrupted, particularly after prolonged or repeated exposures, potentially leading to hyperproliferation, excessive migration and malignant changes.

Additionally, when UVB-treated skin fibroblasts are treated with GlcNAc, ROS generation decreases and cell viability increases, indicating that this HA precursor sugar has protective effects (Hwang et al., 2011). GlcNAc has other important functions in cells,

including O-GlcNAcylation, which regulates many cytoplasmic and nuclear proteins in response to nutrient balance and cellular stress (Hart et al., 2011). The different, competing pathways for its usage are central in regulating cellular physiology (Hascall, et al., 2014). Do the cells commit to producing a thick, protective HA coat or do they direct the component sugar for other metabolic and/or signaling functions? And which are the extrinsic or intrinsic factors that determine this balance?

ATP and UTP also have a large impact on the transcription of *HAS2* in HaCaT cells, which shows that the HA synthesizing machinery responds to various stressors (III, IV). The fact that this change is transient indicates a relatively tight control of nucleotide processing and the subsequent signaling steps. This moderation would in fact be vital, since ATP is easily released from mechanically stimulated cells. Consequently, simply rubbing the skin could induce the accumulation of HA and swelling of the tissue, if not kept in check.

The induction of HA synthesis with ATP was more obvious in serum-deprived (1% FBS) cultures. This suggests that growth factors or other serum components modulate the nucleotide-induced HA-response in keratinocytes. Other factors could also augment HA accumulation after a nucleotide 'shock'. This is supported by the fact that ATP induced an explicit increase in peri- and extracellular HA in cultures supplemented with glucosamine. The data further suggested that degradative pathways and internalization of HA are activated by ATP. The size of the nucleotide precursor sugar pool was also reduced. These effects may have influenced the net accumulation of HA in the ATP-treated cultures.

Subsequently, the increased extracellular HA might serve as a cushion or a scaffold for growth factors to protect the perturbed cells and normalize their metabolism after an insult. Interestingly, *HAS2* mRNA might have other functions in cells beyond the production of HA, possibly modulating signaling pathways and mediating cell survival, as previously suggested (Porsch et al., 2013; Wang et al., 2014).

6.4.2 The signaling effects of betaine

The potential reversal of the detrimental effects of UVB by betaine was of special interest in this thesis work. The mechanism by which betaine could achieve this, relates to the fact that UV irradiation, as well as high salinity, causes oxidative damage, generating reactive oxygen and nitrogen species. ROS and RNS, in turn, may activate several signaling pathways (e.g. Peus et al., 1999), and modulate the function of tonicity-responsive transcription factors (Zhou et al., 2005). The detrimental effects of these extracellular stimuli (i.e. cell volume changes and possible shrinkage) can then be reduced by the regulated uptake and/or synthesis of osmolytes, including betaine.

For instance, the mRNA levels of the betaine transporter *BGT-1* appear responsive to high NaCl-induced ROS, at least in human embryonic kidney cells, where efficient osmo- and redox-regulation is vital (Zhou et al., 2005). The microarray data (II) supports a regulatory role for betaine in these processes. Significant changes were found with UVB in the anti-oxidative and detoxifying enzymes of the glutathione transferase family as well as in *Mt1a* and *Gclc*, which possess similar functions (Lu et al., 2002; Mougiakakos et al., 2012). Moreover, betaine appeared to modulate their expression in the UVB-exposed cultures.

Betaine also directly influences the osmoregulatory machinery and ion transport, as indicated by the decreased expression levels of *Fxyd2* and *Akr1b1* in the 3D REK model. FXYD2-protein is the γ-subunit of the ubiquitous Na,K-ATPase. Its induction by

hypertonicity, heat shock or redox stress negatively regulates the sodium-potassium pump. Simultaneously, cell growth is suppressed. This links ion balance, cellular stress and proliferation through FXYD2 (Wetzel et al., 2004). As betaine apparently acts as a brake for *Fxyd2* expression, both under basal conditions and after UVB-irradiation, it may present a novel agent to modulate the growth and survival potential of keratinocytes. Interestingly, *Akr1b1* may stimulate proliferative and inflammatory processes as well as cancer (Srivastava et al., 2011). The enzyme thus not only metabolizes glucose and ROS-derived lipid aldehydes but also shows promise as a pharmacological target. The downregulation of *Akr1b1* with betaine may simply prevent excessive accumulation of organic osmolytes such as sorbitol. Nevertheless, inhibition of the UVB-induced *Akr1b1* expression by betaine suggests additional metabolic pathways it can modify.

The modulatory potential of betaine is reinforced by the attenuation of the ATP-induced increase in *HAS2* in HaCaT cells. Betaine reversed the phosphorylation of CREB and STAT3-Tyr^{705}, but did not inhibit the other kinases activated by ATP. However, STAT3 did not significantly contribute to the activation of *HAS2* transcription by ATP, and there were no modulating effects of betaine on the phosphorylated kinases by UTP (unpublished data). Yet, the recurring theme of betaine as a stress-regulating compound cannot be overlooked. Another recent study also points to such effects, as oral supplementation of betaine suppressed the expression of MEK and ERK in chronically UVB-irradiated hairless mouse skin (Im et al., 2016).

6.5 IMPLICATIONS FOR KERATINOCYTE PROLIFERATION AND DIFFERENTATION BY UVB AND BETAINE

The data from UVB-treated REK cells (I; supplementary data) indicated a strong inhibitory effect of the radiation on keratinocyte proliferation. Simultaneously, HA synthesis consistently doubled or even tripled. Even though the follow-up on the recovery of the monolayers lasted for 48 h, the irradiated cultures never caught up with the numbers of the control cells. In the organotypic cultures, where HA increased more moderately, there were no obvious differences in cell proliferation or epidermal thickness between the UVB-treated and control cultures (II). It is still interesting to speculate that the increased accumulation of hyaluronan could be a protective or compensatory mechanism, which could return the homeostatic balance and the proliferative capacity of UVB-exposed keratinocytes. Hyaluronan synthesis may support keratinocyte (hyper)proliferation (e.g. Rilla et al., 2004; Maytin et al., 2004), but its true significance for cell division or differentiation remains a controversial issue in existing literature.

As noted previously, FXYD2 may inhibit cell proliferation in response to stress. Since betaine significantly downregulated the expression of both *Fxyd2a* and *Fxyd2b*, both alone and in combination with UVB, it is very important to consider whether this alleviation of growth suppression is favorable or detrimental in sun-exposed skin. In any case, the microarray data and proliferation assays in the REK monolayers (II) indicated that betaine might restrict keratinocyte growth and induce differentiation. The downregulation of the replication licensing factors *Mcm4* and *Mcm7* as well as *Mcm3_predicted* highlighted this capacity. Similarly, betaine downregulated *Cxcl16*, a CXC chemokine ligand, which contributes to proliferation and metastasis in several cancers (Deng et al., 2010). Betaine also slightly inhibited migration in HaCaT cells in another set of experiments (Rauhala,

unpublished observations), pointing further to a slowing down of keratinocyte metabolism. There were, however no obvious changes in epidermal thickness or the fraction of Ki67-positive cells in the betaine-treated 3D cultures.

The effects of betaine on keratinocyte differentiation were highlighted by the robust upregulation of keratin 2 both at the mRNA and protein levels. This structural protein has recently been verified as a natural and important binding partner of K10 in selected skin areas, and may thus play a specific structural role (Fischer et al., 2014). Specifically, K2 is highly expressed in mouse plantar skin, where it is subjected to relatively high mechanical/abrasive forces of the inter-footpad regions (Fischer et al., 2016). Deletion of *Krt2* in these areas, particularly together with *Krt10*, causes thickening, hyperkeratosis and flaking, indicating abnormal differentiation and barrier formation. The double KO mice thus reproduce some features of palmoplantar keratodermas in humans.

Decreased K2 expression in circumscribed plantar hypokeratosis causes apparent corneocyte fragility (Ishiko et al., 2007), and mutations in the gene manifest as the skin blistering disease ichthyosis bullosa of Siemens, also with a defective stratum corneum (Akiyama et al., 2005). Therefore, the upregulation of keratin 2 with betaine may become relevant in a clinical context. It is an intriguing idea that betaine could modulate epidermal differentiation and mechanical properties in skin areas experiencing frictional forces and mechanical stress. The strong induction of keratin 2 expression in the betaine-treated 3D REK cultures also adds an interesting facet to the research of epidermal keratins and their physiological regulation. This is particularly noteworthy, as no significant changes were seen in the other classical differentiation markers filaggrin and K10.

Pcp4 was another gene, whose transcription was strongly induced by betaine. The localization of PCP4 in the granular layer, and occasionally in the stratum basale, suggests a new role for the protein in epidermis. Moreover, its strong downregulation by UVB points to a function in stress reactions. The protein might contribute to cornification in normal epidermis and participate in returning homeostasis after an insult. The role of Ca^{2+} is again highlighted, as PCP4 is a crucial upstream regulator of Ca^{2+}-signaling. PCP4 is able to bind the ion by itself and modulate the association of calmodulin with Ca^{2+}, also in response to Ca^{2+} releasing signals such as ATP (Wang et al., 2013). Ultimately, these cascades are vital in controlling epidermal differentiation.

One more interesting aspect to the regulation of keratinocyte proliferation and differentiation in the context of this work comes from the study by Elias and colleagues (Elias et al., 2002), where relative humidity was shown to induce changes in epidermal Ca^{2+}. Concomitantly with decreased Ca^{2+} and a less clear Ca^{2+} gradient under high relative humidity, the expression levels of the differentiation markers loricrin, profilaggrin and involucrin were suppressed. How betaine, which is also utilized in personal care products due to its supposed moisturizing properties, fits into these observations and participates in the overall Ca^{2+}-signaling, still needs characterization. Increases in pericellular hyaluronan, also known for its water-binding properties, after UVB-irradiation may be another crucial factor regulating Ca^{2+}-balance and acting in favor of proliferation rather than differentiation.

How these distinct processes add up in normal as well as stressed epidermis, probably depends on the existing homeostatic balance and potential underlying pathologies. Nevertheless, the potential of betaine to modulate the proliferation-differentiation axis in the epidermis is fascinating, if not yet unambiguous.

6.6 IMPLICATIONS FOR NEOPLASTIC CHANGES AND INFLAMMATION

The connection between increased hyaluronan production and malignancy has been suggested in the epidermis, as HA staining intensity is increased in *in situ* carcinomas and early stages of squamous cell carcinomas and melanomas (Karvinen et al., 2003a; Siiskonen et al., 2013). There is no such increase in the infrequently metastasizing BCCs, opening up further possible functional links between HA content and invasive capability. Indeed, a role for hyaluronan in EMT has been shown in epithelial cells (Zoltan-Jones et al., 2003) and cardiac endothelial cells in *Has2*$^{-/-}$ mice (Camenisch et al., 2000). Hyaluronan staining is also increased in hyperplastic areas of chronically UV-irradiated mouse skin, and this pattern is sustained in neoplastic specimens (Siiskonen et al., 2011).

Interestingly, the increase in the irregularity of HA and CD44 staining with a higher tumor grade observed by both Karvinen et al. (2003a) and Siiskonen et al. (2011) had a parallel in the 3D REK cultures, which showed a patchy pattern of pericellular hyaluronan and CD44 in the cultures having received the highest UVB-doses (I). Such local changes may be important for the occasional mutation-prone or transformed cell that can arise as a result of UV damage. Whether such cells then enter apoptosis or differentiation or continue to proliferate, will significantly affect epidermal homeostasis. The patchy HA-/CD44-staining might also indicate a disruption in the cell-matrix and cell-cell interactions and existence of keratinocytes that can escape from their normal tissue context. Increased hyaluronan synthesis could be particularly detrimental in situations where UVB also activates MMP expression (Lee et al., 2009), as both can contribute to a promigratory microenvironment.

Therefore, the observed increase in hyaluronan after a UVB insult might serve as one of the first signaling steps guiding cells towards increased proliferation or impaired differentiation, transformation and invasion. Keratinocytes are constantly subjected to variable amounts of this environmental irritant, which is a key agent in inducing epidermal neoplasias. Thus, the data presented in this thesis give further reason to believe that there is a link between stress responses, hyaluronan accumulation and malignant transformation. The knowledge of the specific alterations could be used in either biochemically targeting the adversely activated metabolic pathways or monitoring early changes in a lesion. It would also be relevant to explore whether the increased synthesis of hyaluronan in this setting also leads to stimulation of markers of EMT.

The high induction of *Has3* in the UVB-irradiated REK monolayers (I) becomes particularly interesting as this enzyme has been shown to be central to epithelial cell homeostasis in a HAS3-overexpressing MDCK model. These highly polarized cells normally form tubular structures *in vivo* and simple spheres in 3D-cultures. However, when stably transfected with GFP-HAS3, they start producing vast amounts of HA which disrupts the normal polarity, mitotic spindle orientation and cell-cell contacts (Rilla et al., 2012). This results in disturbed barrier formation and production of disorganized cysts with multiple lumina. The results are striking in showing how forced expression of HA may change the morphology, organization and behavior of epithelial tissues. The orientation of epidermal keratinocytes is particularly important in the basal layer, which contains the coordinately dividing stem cell population.

Considering the possible downstream pathways from the increased HA in the UVB-treated REK cells, changes in the hyaluronan receptor CD44 need to be examined. REK cells express the standard isoform (about 90 kDa) as well as longer variants, both in monolayers

and in the stratified, differentiated 3D model (Rauhala, unpublished data). There were no apparent changes in the western blot profiles of samples treated with UVB as compared with controls. It would be interesting to characterize the receptor profiles more fully, though, as the isoforms of CD44 are known to respond to pathophysiological signals. This is particularly true in cancers, many of which are found to contain splice variants not encountered in normal tissues (Marhaba & Zöller, 2004; Toole, 2009).

The isoform CD44S confers a survival advantage in human mammary epithelial cells, as its interaction with pericellular HA protects the cells against anoikis-induced apoptosis. At the same time, the cells undergo EMT, making them more prone to invade the surrounding tissues (Cieply et al., 2015). CD44 as the homing receptor of lymphocytes also binds to tissue HA (Aruffo et al., 1990), contributing to inflammation in various contexts. Prolonged inflammation accompanied with increased HA retention and fragmentation might also contribute to malignant transformation.

The effects of ATP and UTP in different malignancies appear complex, but as low concentrations of $P2Y_2$-agonists may mediate proliferation in the epidermis, and UVB also increased expression of this dual ATP/UTP receptor, there is a potential regulatory cascade. There, UVB would cause release of ATP/UTP that might signal through the $P2Y_2$-receptor to induce proliferation as well as *P2Y2* expression through a feedback loop. In our preliminary experiments, the concentration of ATP used (100 μM) was probably too high to induce proliferation (unpublished observations).

UTP, ATP and ADP also mediate inflammatory responses in keratinocytes contributing to the release of IL-6 (Inoue et al., 2007; Nagakura et al., 2014). Synthesis of this cytokine is activated by UVB as well, and the effect of irradiation can be blocked by antagonizing the P2Y-receptors with suramin, PPADS or RB2. These effects have some parallels in our data, where mRNA expression of the chemotactic IL-8 was increased with both ATP and UTP (unpublished observations).

The upregulation of *HAS2* and *HAS3* expression and the following increases in HA synthesis by ATP and UTP in the HaCaT keratinocytes reveal a new regulatory mechanism by which keratinocytes may modify their microenvironment. Indeed, several of the signaling pathways activated with both UVB and the nucleotides are known mediators in malignancies. In addition to its role in inflammation, the p38 pathway is involved in skin cancer development (e.g. Schindler et al., 2009). CaMKII can also induce tumor cell migration initiated by HA-CD44-interaction in HSC-3 squamous carcinoma cells (Bourguignon et al., 2006a). As HA binding also activates EGFR and ERK signaling to promote cell growth, tumor formation and invasion are both supported.

As if to counteract these effects, betaine activated the prospective tumor suppressor *Nbl1* and repressed the UVB-induced increase in *Akr1b1* transcription (II). *Akr1b1* has previously been shown to be overexpressed in several human cancers, including head and neck carcinomas (Laffin & Petrash, 2012). Betaine could thus represent a practical approach to inhibit *Akr1b1* expression in other contexts as well.

Betaine is also capable of participating in genome-wide epigenetic modifications due to its methylation potential (Stefanska et al., 2012). Hypermethylation in normal cells often works by transcriptional silencing of large genomic regions and specific genes, including growth regulators and proto-oncogenes. In tumor cells, the situation is more complex with global hypomethylation and concomitant inhibition by methylation of critical tumor suppressors. Betaine and other methyl donors participate in maintaining the balance by

regulating the concentration of the methyl donor SAMe and its degradation product SAHe, which also inhibits methylation reactions by DNA methyltransferases. Which of the roles of betaine (osmolyte, antioxidant, metabolic intermediate or regulator of methylation) is most critical in each given setup, must depend on the physiological context.

Overall, the data presented here highlight novel signaling cascades and corroborate existing pathways that could be activated in premalignant states. The results also promote the role of betaine as a modulator of these responses. However, the results necessitate more comprehensive studies on the individual genes and processes that could not yet be characterized in detail. This would be particularly relevant with the potential tumor suppressors shown to be regulated by both UVB and betaine.

7 Summary and Conclusions

This thesis work set out to deepen our understanding of how the epidermis responds to various environmental insults. The focus was on the extracellular matrix molecule hyaluronan and its regulation after physical (UVB) and biochemical (uridine and adenosine nucleotides) stimuli. The nucleotides can be released and elicit auto- and paracrine effects after irritating treatments, including UVB irradiation.

Understanding these basic biochemical, short-term effects may help develop tools by which pathological changes in response to such stress stimuli can be abrogated at the onset. Betaine as an organic osmolyte and metabolic intermediate might prove one useful agent due to its modulating effects on epidermal differentiation and signaling pathways.

The main findings of this thesis can be summarized as follows:

- Keratinocytes respond specifically to different stress mediators by modulating their hyaluronan synthesis and degradation.
- Accumulation of HA occurs fast in response to both UVB and UTP/ATP.
- UVB and extracellular nucleotides induce hyaluronan synthesis by activating distinct intracellular effectors (p38, ERK, CaMKII, PKC, STAT3, CREB), some of which are shared between the treatments (p38, CaMKII).
- Of the purinergic receptors, $P2Y_2$ dominated the nucleotide-induced effects in HaCaT.
- Some of the metabolic changes evoked by UVB and ATP can be counteracted by a pretreatment with betaine.
- The metabolic status of the cells appears to modulate the responses.
- Betaine is a potent regulator of keratinocyte metabolism, particularly differentiation. This is highlighted by increased keratin 2 expression in the epidermal equivalents.

Overall, the results are in line with existing literature. This thesis also strongly encourages future research efforts, including those which develop practical solutions for controlling aberrant HA metabolism and cellular stress.

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Hyaluronan (HA) is the structurally simple, yet functionally complex, glycosaminoglycan of the extracellular matrix. The thesis showed that epidermal HA metabolism is potently regulated by physical (UVB) and biochemical (extracellular nucleotides) stimuli. The organic osmolyte betaine partially modified these effects and keratinocyte differentiation in vitro. The results help to understand keratinocyte responses and aberrant HA metabolism in response to environmentally relevant stress cues.

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