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Regulation of Keratinocyte Differentiation and Hyaluronan Metabolism in an Organotypic Keratinocyte Culture

Doctoral dissertation

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ABSTRACT

Organotypic keratinocyte cultures provide an alternative to animal tests, and are more useful than submerged monolayer keratinocyte cultures for selected aspects of epidermal biology. Keratinocytes cultured at the air-liquid interface can stratify and form a structure resembling normal epidermis in many ways. However, a permeability barrier comparable with that of intact skin has not been achieved in organotypic cultures. The primary focus of this thesis work was to develop and characterize an organotypic keratinocyte culture model, which can be used in studies of drug penetration, keratinocyte differentiation and hyaluronan metabolism.

A continuous rat epidermal cell line (rat epidermal keratinocyte; REK) formed a morphologically well-organized in vitro epidermis in the absence of feeder cells when grown for 2 weeks on type I collagen gel at air-liquid interface. The present results show that vitamin C supplementation increased the normal wavy pattern of stratum corneum (SC), the number of keratohyalin granules, and the quantity and organization of intercellular lipid lamellae in the interstices of SC of the in vitro epidermis, suggesting improved keratinocyte differentiation. These morphological improvements in vitamin C supplemented cultures were accompanied by an enhanced barrier function, as indicated by reduced permeation rates of tritiated corticosterone and mannitol, and transepidermal water loss (TEWL).

This organotypic culture model offered a good model to study the regulation of epidermal hyaluronan (HA) metabolism, without interference by dermal or feeder cells. HA, synthesized by three different hyaluronan synthase enzymes (HAS1, 2, 3) in the plasma membrane, is the main extracellular matrix molecule in the vital cell layers of skin epidermis, and contributes to many cellular functions.

The effects of mitogenic growth factors (EGF, KGF, all-*trans* retinoic acid) and an anti-proliferative growth factor (TGF-β) on epidermal morphology and HA synthesis were investigated. EGF and KGF increased epidermal thickness and stimulated HA synthesis by increasing the mRNA of *Has2* and *Has3*, but decreased keratinocyte differentiation. Conversely, TGF-β inhibited HA synthesis and *Has2* and *Has3* mRNA expression and induced epidermal atrophy. The mechanism of all-*trans* retinoic acid (RA) action on HA synthesis was complex, since it increased EGF-receptor (EGFR) and Erk1/2 activity, and inhibitors of EGFR (AG1478), MEK (UO126) and MMPs (GM6001) blocked all-*trans* RA –induced HA synthesis, *Has* activation and epidermal hyperplasia.

This work provides new insights into the regulation of keratinocyte differentiation and hyaluronan metabolism. The results indicate that vitamin C is an important regulator of epidermal differentiation, and thus permeability barrier formation. And the data suggest that HA synthesis is associated with keratinocyte proliferation and migration but compromised terminal differentiation.

To Jarmo and Roope

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Sanna Pasonen-Seppänen

ABBREVIATIONS

ADAM	a disintegrin and metalloprotease
AG1478	an inhibitor of EGF-receptor
bHA	biotinylated hyaluronan
bHABC	biotinylated hyaluronan binding complex
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's minimal essential medium
EBSS	Earle's balanced salt solution
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELSA	enzyme-linked sorbent assay
ERK	extracellular signal regulated protein kinase
FGF	fibroblast growth factor
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
GM6001	an inhibitor of matrix metalloproteinase (MMP)
GPI	glycosylphosphatidylinositol
HA	hyaluronan
HABC	hyaluronan binding complex
HAS	hyaluronan synthase (HAS1, 2, 3)
HB-EGF	heparin binding EGF
HBSS	Hank's balanced salt solution
JNK	c-Jun N-terminal kinase
KGF	keratinocyte growth factor
MAPK	mitogen activated protein kinase
MEM	minimal essential medium
MEK	MAP/ERK kinase
P	permeability coefficient (cm s^{-1})
PB	phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI-3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PLC	phospholipase C
RA	retinoic acid
REK	rat epidermal keratinocyte
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
SC	stratum corneum

SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electroforesis
STAT	signal transducer and activator of transcription
TEWL	transepidermal water loss (g/ m^2 per h)
TGF-β	transforming growth factor beta
TGase	transglutaminase
TPA	12-O-tetradecanoylphorbol 13-acetate
UO126	an inhibitor of MEK

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals (I-V). Some unpublished data are also presented.

I.   **Pasonen-Seppänen S, Suhonen M, Kirjavainen M, Urtti A, Miettinen M, Tammi M, Tammi R:** Formation of permeability barrier in epidermal organotypic culture for studies on drug transport. Journal of Investigative Dermatology 117:1322-1323, 2001

II.   **Pasonen-Seppänen S, Suhonen M, Kirjavainen M, Suihko E, Urtti A, Miettinen M, Hyttinen M, Tammi M, Tammi R:** Vitamin C enhances differentiation of a continuous keratinocyte cell line (REK) into epidermis with normal stratum corneum ultrastructure and functional permeability barrier. Histochemistry and Cell Biology 116: 287-297, 2001

III. **Pasonen-Seppänen S, Karvinen S, Törrönen K, Hyttinen JMT, Jokela T, Lammi M, Tammi M, Tammi R:** EGF upregulates, whereas TGF-β downregulates, the hyaluronan synthases *Has2* and *Has3* in organotypic keratinocyte cultures: correlations with epidermal proliferation and differentiation. Journal of Investigative Dermatology 120:1038-1044, 2003

IV.   ***Karvinen S, *Pasonen-Seppänen S, Hyttinen JMT, Pienimäki JP, Törrönen K, Jokela TA, Tammi MI, Tammi R:** Keratinocyte growth factor stimulates migration and hyaluronan synthesis in the epidermis by activation of keratinocyte hyaluronan synthases 2 and 3. Journal of Biological Chemistry 278:49495-49504, 2003
      *These authors contributed equally to this work

V.   **Pasonen-Seppänen S, Maytin E, Törrönen K, Hyttinen JMT, Hascall V, MacCallum D, Kultti A, Tammi M, Tammi R:** All-*trans* retinoic acid induces hyaluronan production in epidermal keratinocytes by increased *Has2* and *Has3* expression partly mediated by EGFR-Erk signaling (Manuscript)

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1. INTRODUCTION

The outer part of the skin, the epidermis, is composed of keratinocyte layers characterized by various differentiation stages. The final stage in keratinocyte differentiation is to produce a keratinized cell layer of dead corneocytes, which, together with their lipid-rich interstices and with granular cell layer tight junctions form the permeability barrier of the skin (Elias, 1983; Furuse et al., 2002). This differentiation process is tightly regulated by autocrine, juxtacrine and paracrine signaling molecules, and hormones, and is disturbed in pathologic situations such as wounding and psoriasis. It is important to know the signaling molecules, and the exact regulatory mechanisms involved in keratinocyte differentiation since they are current and future targets for successful therapeutic interventions in the skin. Epidermal growth factor (EGF), keratinocyte growth factor (KGF), all-*trans* retinoic acid (RA), transforming growth factor beta (TGF-β) and extracellular calcium are major factors known to control keratinocyte differentiation (Haake et al., 2001). In addition, the extracellular matrix (ECM) components have active and vital role in defining the functions of keratinocytes (Falabella and Falanga, 2001).

Hyaluronan is the main extracellular matrix molecule in the vital cell layers of the epidermis. This large unsulfated glycosaminoglycan is synthesized by three different plasma membrane associated enzymes (HAS1, 2, 3) (Weigel et al., 1997). In the epidermis, hyaluronan contributes to cellular functions such as adhesion, migration, proliferation and differentiation, at least in part by signaling through its cell surface receptor, CD44 (Kaya et al., 1997; Bourguignon et al., 2004). In epidermal activation such as occurs in injured skin, psoriasis and well differentiated squamous cell cancers, the amount of hyaluronan is elevated (Tammi et al., 1994b; Karvinen et al., 2003; Tammi et al., 2005), as well as in variety of other disease processes (Toole et al., 2002). Thus, understanding of the regulatory mechanisms controlling its synthesis is important. Unlike many other ECM components, the turnover rate of hyaluronan is rapid in the epidermis, the half-life of hyaluronan being less than one day (Tammi et al., 1991). This

allows hyaluronan to quickly respond to environmental changes like wounding, and signal to the surrounding cells.

In the present thesis, a novel organotypic keratinocyte culture model was established using immortalized rat keratinocytes, and its morphology and barrier properties were characterized. Utilizing this *in vitro* model, the effects of some potent modulators of keratinocyte growth on keratinocyte differentiation and hyaluronan metabolism were investigated.

2. REVIEW OF THE LITERATURE

2.1. Structure of the skin

Histologically, skin is divided into three different layers; epidermis, dermis and hypodermis, each with particular properties (**Fig 1**). The uppermost (epidermis) is a stratifying layer of epithelial cells, keratinocytes, whose main function is to form the permeability barrier of the skin. This barrier protects the underlying tissue from the entrance of infectious agents and other potentially harmful substances, and prevents the dehydration of the organism. Furthermore, epidermis synthesizes vitamin D. Dermis is comprised mainly of connective tissue, collagen bundles and elastic fibers that are responsible for the structural strength of the skin. Blood capillaries, nerves, sweat and sebaceous glands, and hair follicles are also located in the dermis. The undermost layer of the skin is hypodermis, or subcutaneous tissue, which is composed of fat and connective tissue. One of the important functions of the hypodermis is the insulation of the body.
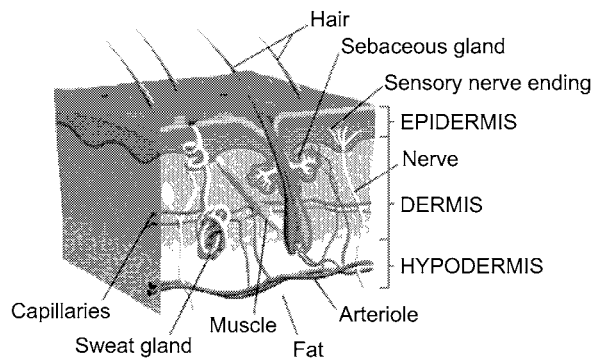
Figure 1. Structure of the skin.

2.1.1. The epidermis

The avascular epidermis consists of four morphologically and biochemically different cell layers, each with a distinct maturation state of keratinocyte, the major cell type in the epidermis (90-95%). Keratinocytes are attached to each other by desmosomes, adherens junctions, gap junctions and tight junctions. Other cells in epidermis include the melanocytes, which are the melanin pigment producing cells, Langerhans cells,

which are part of the immune system, and Merkel cells, which are mechanoreceptors. The epidermal structure is excellently reviewed in (Haake et al., 2001). The amount of the extracellular matrix (ECM) in the epidermis is low, like in other epithelia. The main ECM molecule in the human epidermis is hyaluronan (Tammi et al., 1994a).

Dermal-epidermal junction

Epidermis rests on the basal lamina, which binds the epidermis to the underlying dermis, and provides regulatory signals controlling keratinocyte proliferation, migration and differentiation. The basal lamina is a complex multi-molecular ECM structure composed of three layers: the lamina lucida, the lamina densa and the sublamina densa. Keratinocytes in the basal layer are connected to the basal lamina via hemidesmosomes (**Fig 2, 4**). In hemidesmosomes, the keratin filaments are attached to the hemidesmosomal inner plaque, which contains BP230 and plectin proteins. These proteins interact with those of the outer (intracellular) plaque, the transmembrane BP180 and α6β4 integrin proteins. Integrins are group of cell surface ECM receptors, which are also implicated in transducing signals from ECM to the cell inferior (Heino, 2000). In the dermal-epidermal junction, α6β4 integrins and BP180 anchor basal cells to the underlying basal lamina via laminin 5 protein (**Fig 2**; Borradori and Sonnenberg, 1999). In addition to laminins, collagen type IV, type VII and type I, heparan sulfate proteoglycans and entactin are important components of this structure. Both epidermal keratinocytes and dermal fibroblasts participate in the molecular organization of the dermal-epidermal junction and the production of the basal lamina components (Sorrell and Caplan, 2004).
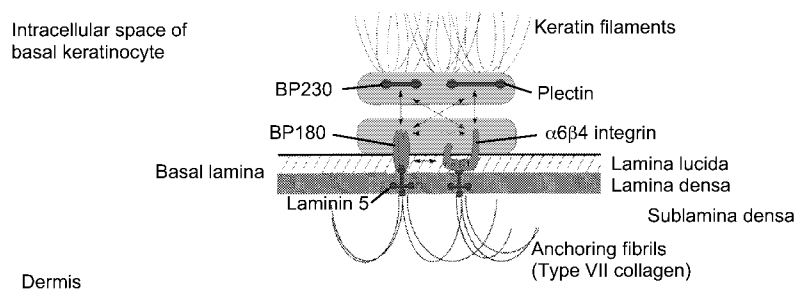
Figure 2. Schematic presentation of the dermal-epidermal junction.

Basal and spinous cell layer

In the continually renewing epidermis, proliferation in the basal cell layer is required to compensate the loss that occurs through desquamation from the surface of the stratum corneum (SC). Basal cell proliferation is highly regulated by a variety of intrinsic and extrinsic factors like growth factors, hormones, vitamins and ECM components (Hashimoto, 2000; Freedberg et al., 2001). There are three distinct types or cells in the basal layer: mitotically active stem cells, transit-amplifying cells, and some (5-10%) post-mitotic differentiating cells (**Fig 3**) (Haake et al., 2001). Stem cells are undifferentiated cells, which are clustered in specific locations in the interfollicular epidermis, the germinal matrix of the hair follicles, and the bulge region of the hair follicle (Potten et al., 1997). These cells have long-term proliferative capability but the duration of their cell cycle is long, approximately 2 times longer than in transiently amplifying cells (Potten and Loeffler, 1987). Stem cells express high levels of integrin β1, which may make them less motile or more adhesive to the basal lamina than transit-amplifying cells or post-mitotic cells (Watt 2002). Stem cells produce new stem cells and transit-amplifying cells, which lose the potential for proliferation when they detach from the basal lamina, and start the differentiation program while migrating from the basal to the spinous cell layer. While the molecular mechanisms involved in regulating the fate of stem cells are still unclear, Rac1 was recently shown to be an important regulator (Benitah et al., 2005). Rac1 exerts it effects in epidermis by suppressing c-Myc, which promotes epidermal differentiation by disrupting stem cell adhesion and by affecting cytoskeleton (Benitah et al., 2005).
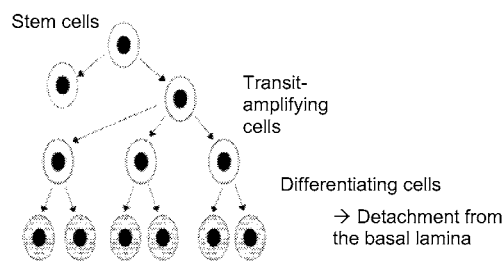
Figure 3. The different cell populations in the basal cell layer.

During this differentiation, when proliferating basal cells eventually mature to lifeless flattened squames of the stratum corneum, certain genes are activated while others are down-regulated. This differentiation process takes approximately 14 days in humans (Haake et al., 2001).

Basal cells are often columnar in shape and express keratins 5 and 14 (Fuchs, 1990). In the spinous cell layer, keratinocytes synthesize a different set of macromolecules, such as keratins 1, 2 and 10 (Fuchs, 1990). Keratins are structural proteins that provide epidermal integrity. These intermediate filaments are especially abundant in epidermal keratinocytes, accounting for up to 85 % of the total protein of fully differentiated keratinocytes. Keratin bundles are localized in the cytoplasm between the nuclear envelope and the desmosomes and hemidesmosomes in the plasma membrane (**Fig 2, 4**). Different keratin filaments are expressed in the different states of keratinocyte differentiation, and their abnormal expression is the molecular basis for many severe skin diseases (e.g. mutations in keratin 5 and 14 cause epidermolysis bullosa simplex (Fuchs and Cleveland, 1998)). The expression of the cornified envelope protein involucrin appears also in the spinous cell layer (Eckert et al., 2005). Adjacent spinous cells are attached to each other by numerous desmosomes, which mediate strong adhesion between epidermal keratinocytes. In desmosomes, the keratin cytoskeleton is connected to the neighboring cells via cytoplasmic plaque proteins (plakoglobin, desmoplakin, plakophilin) and transmembrane proteins, which belong to the cadherin family (desmogleins, desmocollins) (**Fig 4**). Ca^{2+} is required for the formation of these cell-cell contacts (Kitajima 2002).

Granular cells

Major changes in cellular architecture occur is the granular layer. Granular cells are wide and flattened, and several keratohyalin granules are present in these cells (**Fig 4**). These granules contain the cornified cell envelope protein, loricrin, and the high molecular weight, histidine-rich profilaggrin, both important in keratinocyte differentiation (Haydock et al., 1993). To yield active, mature filaggrin, profilaggrin is dephosphorylated and the linker peptides are removed by site-specific proteolysis

(Resing et al., 1984). This maturation of the profilaggrin precursors to filaggrin monomers occurs during the transition of a granular cell to a cornified cell. Filaggrin has at least two functions: 1) in stratum corneum, it aggregates keratin filaments into tightly aligned bundles (Fuchs, 1990), and 2) in the upper stratum corneum it is proteolyzed into free amino acids, which are important in water retention (Scott et al., 1982). Numerous lamellar granules (bodies) are also present in the granular cell layer (**Fig 4**). These organelles originate in the Golgi apparatus (Madison and Howard, 1996), and contain stacks of lipid lamellae surrounded by a membrane.

Cornified envelope

In the upper granular cell layer, so-called transition zone below the stratum corneum, extensive cellular remodeling takes place. Proteases and nucleases destroy cell organelles, and plasma membrane is replaced with the cornified envelope. Transglutaminases (TGases) comprise a family of calcium-dependent enzymes that are responsible for envelope formation beneath the plasma membrane (Roop, 1995; Eckert et al., 2005). TGases 1, 3 and 5 are active in skin epidermis and participate to varying degrees in interprotein cross-link formation in cornified envelope assembly (Eckert et al., 2005). In the cornified envelope formation, involucrin is cross-linked to the membrane-bound proteins and forms an initial scaffold for incorporation of other precursors (e.g. small proline-rich proteins (SPR), cystatin A, loricrin, periplakin and envoplakin) that strengthen this envelope. Loricrin is the major component of the envelope (Eckert et al., 2005). From the inner surface of this scaffolding, cytoskeletal keratin filament bundles are attached to it and finally, in the stratum corneum, the intercellular ceramide lipids are covalently bound to the outer surface of this protein envelope (Behne et al., 2000; Steinert, 2000).

During the process of epidermal terminal differentiation (keratinization), proliferative keratinocytes progress through intermediate stages and ultimately evolve into stacks of non-dividing, dead corneocytes, which, together with their lipid-rich interstices, form the normal skin permeability barrier (Elias, 1983). Stratum corneum consists of approximately 15-100 cell layers depending on the anatomical site, and is shed usually

at a rate equal to the rate of epidermal cell regeneration, thereby maintaining a SC with a virtually constant thickness (Roop, 1995). In corneocyte desquamation, the hydrolysis of cholesterol sulfate, and the breakdown of desmosomes by tryptic and chymotryptic enzymes are implicated (Hansson et al., 1994).

Cornified cell envelope
Desquamation
Stratum corneum
Tight junction
Transition zone
Keratohyalin granule
Granular layer
TGases 1, 3, 5
Involucrin
Cornifin
K1, K10
Loricrin
Filaggrin
Lamellar granule
Spinous layer
Desmosome
Keratin filament
Nucleus
K5, K14
Basal layer
Hemidesmosome
Basal lamina
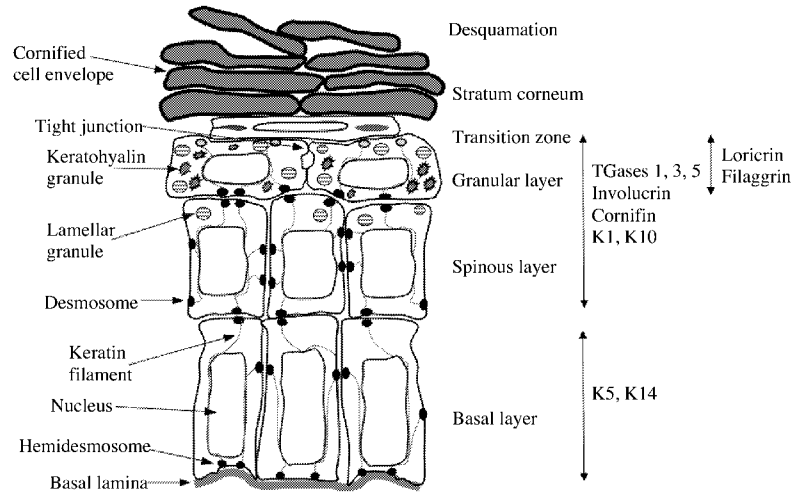
Figure 4. Schematic diagram of the epidermis.

2.1.2. The permeability barrier of the skin

The principal site for epidermal barrier is in SC, since the removal of the corneocyte cell layers by tape-stripping remarkably increases epidermal permeability (Scheuplein, 1976). The barrier function is provided by cornified cell envelope structure and patterned lipid lamellae in the extracellular space between flat, polygonal cornified cells (Steinert, 2000). In routine histology, it is difficult to imagine that SC forms an effective barrier, since in paraffin embedding, the missing lipids cause the typical, artifactual "basket-weave" structure. Frozen sections reveal that corneocytes are tightly attached to each other, and electron microscopy with ruthenium tetroxide fixation demonstrates the stacked and patterned lipid sheets in the intercellular space of SC (Madison et al., 1987).

The barrier function depends upon the presence of a unique mixture of lipids in the SC and their organization. The importance of SC lipids has been shown in chloroform: methanol extraction, which dramatically increases water permeability (Scheuplein and Blank, 1971). Intracellular lamellar granules contain most of the precursors of SC intercellular lipids, and the enzymes responsible for their modifications (Menon et al., 1992; Madison et al., 1998). The contents of lamellar granules are secreted in granular cell layer where the transformation of lamellar lipids occurs. Phospholipids are broken down by phospholipases to free fatty acids (Menon et al., 1986), and glucosylceramides are converted to ceramides by β-glucocerebrosidase (Holleran et al., 1994). The lipid composition of the SC is highly unique. Unlike other biological membranes, it is almost devoid of phospholipids, and is comprised mainly of ceramides (18-41%), cholesterol (14-33%), cholesteryl esters (5-10%), and free fatty acids (9-20%) with a small fraction of cholesterol sulfate (2%) (Wertz and Downing, 1989). Sphingolipids, i.e. ceramides have been shown to be of major importance for the epidermal barrier (Wertz et al., 1983; Bouwstra et al., 1996).

The low permeability of the SC is not only due to the unique lipid composition, but also to the unique structural organization of the intercellular lipids (Friberg et al., 1990). During the secretion of granular lipids, glucose and linoleate residues are removed from acylglucosylceramides by enzymes producing ω-hydroxyceramides which are ester-linked to the cornified cell envelope proteins, involucrin and loricrin (Swartzendruber et al., 1987; Wertz and Downing, 1987; Marekov and Steinert, 1998) to form the corneocyte lipid envelope (Behne et al., 2000). Although the corneocyte lipid envelope does not display intrinsic barrier properties, it has been shown to be crucial for the proper orientation of SC lipid lamellae (Downing, 1992). Because of the amphiphilic structure of the ceramides, they are arranged in multiple lamellar or bilayer structures - lipid stacks, which fuse in an edge-to-edge fashion in the SG/SC interface (Landmann, 1986). In ruthenium tetroxide fixed skin, these lipid lamellae show a repeating pattern with alternating major and minor electron-dense bands, which are sometimes called as Landmann units (Landmann, 1986; Swartzendruber et al., 1989). This highly organized

lipid matrix in the SC is the main barrier for the transepidermal diffusion. The thickness of SC contributes little to the barrier function (Elias et al., 1981a).

So far, the transepidermal permeation pathways for polar and nonpolar molecules across the SC have remained unresolved, but it is evident that the transport occurs through passive diffusion (Scheuplein and Blank, 1971). Highly lipophilic compounds with low molecular weigh demonstrate the greatest permeability through the SC. While SC is the main barrier, in some circumstances (i.e. for lipophilic molecules) the water-rich viable epidermis and dermis can be rate limiting. There are three different permeation routes through the skin: 1) transepidermal pathway across the keratinized horny layer intercellularly, 2) intracellularly and 3) via the hair follicles and sweat glands (Fig 5). The predominant route is most likely via the intercellular lipid pathway, since this is the only continuous phase in the SC (Williams and Elias, 1987). Due to the intercellular lipid bilayers with alternating hydrophilic and lipophilic regions (Fig 5), both polar and nonpolar molecules are able to diffuse across the SC. However, the penetration through the skin is low, and limits the use of transdermal route in drug delivery. Using chemical and physical enhancers, transdermal drug delivery has been made possible, but is still generally limited to compounds of molecular weight less than 500 Da.
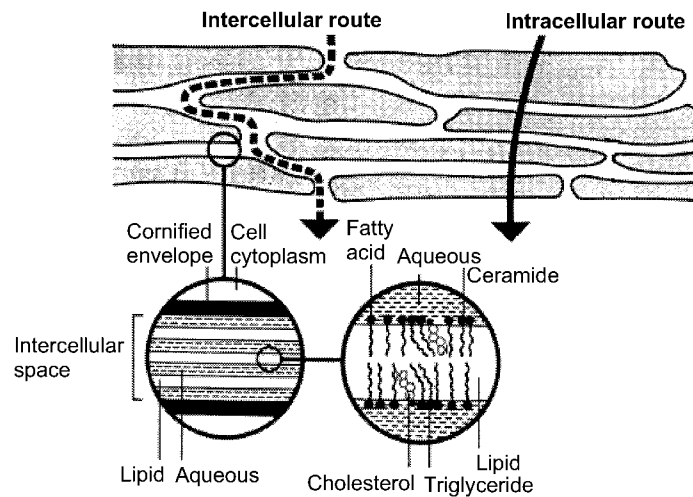
Figure 5. A scheme of the intercellular and the intracellular permeation routes in the SC (Modified from Suhonen et al., 1999).

The role of tight junctions in permeability barrier

Traditionally, it has been thought that SC intercellular lipid lamellae and cornified cell envelopes are crucial for the epidermal barrier (Steinert, 2000; Madison, 2003). However, recent studies have suggested that tight junctions in the granular cell layer are also important for the epidermal barrier (Pummi et al., 2001; Furuse et al., 2002; Turksen and Troy, 2002). In tight junctions, the opposing plasma membranes of adjacent cells are bound tightly together with claudin proteins. This structure functions as a barrier for paracellular pathway in epithelium (Turksen and Troy, 2004). Furuse and coworkers (2002) showed that the deficiency of the tight junction structural protein, claudin-1 (Cln-1), leads to dehydration and increased TEWL in mice. These Cln-1$^{-/-}$ mice died within 1 d of birth. The barrier function was also disturbed in transgenic mice overexpressing claudin 6, and the expression of keratinocyte differentiation markers was aberrant (Turksen and Troy, 2002). Furthermore, in the epidermal differentiation defect associated with psoriasis and lichen planus, the localizations of the tight junction proteins ZO-1 and occludin are disturbed, stressing the importance of tight junction proteins in keratinocyte differentiation (Pummi et al., 2001). The results demonstrate that tight junctions function as a barrier at least for small molecules (~ 600 Da) (Furuse et al., 2002; Turksen and Troy, 2002).

2.2. The regulation of epidermal keratinocyte growth

The constantly renewing epidermal tissue must maintain a fine equilibrium between proliferation and differentiation under various environmental conditions and challenges. These processes are highly regulated by autocrine, juxtacrine and paracrine signals, but disturbed in pathologic situations like wounding (Stoscheck et al., 1992) and psoriasis (King et al., 1990). Both epidermal keratinocytes and dermal fibroblasts produce these signaling molecules, indicating that dermal-epidermal interactions are essential in regulating epidermal tissue homeostasis. Various growth factors, vitamins and cytokines, such as epidermal growth factor (EGF) (Nickoloff et al., 1988), keratinocyte growth factor (KGF) (Marchese et al., 1990), interleukin-1 (Angel and Szabowski, 2002), insulin-like growth factor (IGF) (Kratz et al., 1992), and vitamin A (all-*trans* retinoid acid) (Fisher and Voorhees, 1996) are crucial for keratinocyte proliferation, but

simultaneously most of them suppress differentiation. Transforming growth factor-beta (TGF-β) restricts keratinocyte growth (Hashimoto, 2000), and vitamin D (Bikle et al., 2003) and calcium (Eckert, 1989) stimulate keratinocyte differentiation. Furthermore, the extracellular matrix components influence the behavior of epidermal keratinocytes (Falabella and Falanga, 2001). Especially during re-epithelialization of skin wounds, the extracellular matrix has an important role in the regulation of keratinocyte motility (Li et al., 2002; Li et al., 2004). For example, fibronectin and type IV collagen stimulate, and laminin inhibits keratinocyte migration (Falabella and Falanga, 2001).

2.2.1. Vitamin A

Vitamin A and its derivatives (retinoids) are important regulators of keratinocyte proliferation and differentiation. Intracellularly, vitamin A is converted to all-*trans* retinoic acid (RA), which exerts its molecular actions on cell behavior through two groups of nuclear receptors, the RA receptor (RARα, β and γ) and the retinoic X receptor (RXRα, β and γ) (Fisher and Voorhees, 1996). RXRα/RARγ heterodimers are the main functional units mediating RA-induced effects in epidermal keratinocytes (Fisher and Voorhees, 1996). The receptor-ligand complex stimulates or antagonizes gene transcription by interacting with RA responsive elements (RAREs) in the promoters of RA-inducible genes, or by interacting with other transcription factors such as AP1 (Presland et al., 2001; Bastien and Rochette-Egly, 2004). The effects of retinoids vary between different cells, depending on the physiologic condition of the tissue at the time of treatment (Amos and Lotan, 1990). The inhibitory effects of all-*trans* RA on keratinocyte differentiation are well documented. All-*trans* RA suppresses or retards the expression of keratins 1 and 10 (Fuchs and Green, 1981) and filaggrin (Fleckman et al., 1985), and the production of cornified envelopes (Yuspa and Harris, 1974). It also disturbs the morphology of the stratum corneum and the formation of permeability barrier (Elias et al., 1981b).

In normal human skin, all-*trans* RA (10^{-7} M) induces keratinocyte proliferation, leading to thickening of the epidermis (Fisher and Voorhees, 1996), but in fast growing transformed cells, or in abnormal epidermis such as psoriatic skin, retinoids inhibit

proliferation (Chandraratna, 1996). However, their mechanism of action has not been fully elucidated. Recent studies have suggested that retinoid stimulation in epidermis is associated with increased heparin-binding epidermal-growth-factor-like growth factor (HB-EGF) expression in the spinous cell layer, which mediates the mitogenic signals of all-*trans* RA to epidermal keratinocytes in the basal cell layer (Xiao et al., 1999; Varani et al., 2001).

2.2.2. Epidermal growth factor

Epidermal growth factor (EGF) plays a central role in the regulation of epidermal keratinocyte function. It belongs to the EGF-family, which comprises several mitogenic members. Human keratinocytes synthesize four EGF family growth factors, TGF-α, amphiregulin, HB-EGF and epiregulin, which convey their proliferative signals via autocrine, juxtacrine (TGF-α, HB-EGF) or paracrine manner. Almost all EGF family members are synthesized as membrane-anchored forms, which are proteolytically cleaved from the plasma membrane by metalloproteases (MMPs, ADAMs) (Nanba and Higashiyama, 2004; Blobel, 2005). These growth factors exert their functions by binding to EGF-receptors (EGFR). This receptor tyrosine kinase family include four isoforms, EGFR (also referred to as ErbB1 or Her1); ErbB2 (Neu/Her2); ErbB3 (Her3) and ErbB4 (Her4). The first three of them are expressed in human keratinocytes (Hashimoto, 2000). EGFR is primarily expressed in the basal cell layer and, to a lesser degree, the first suprabasal layers (Nanney et al., 1990). Ligand binding to EGFR causes receptor dimerization and tyrosine kinase activation, which usually leads to the conventional mitogen activated protein kinase (MAPK) signal transduction pathways but also other signaling cascades (Jorissen et al., 2003; Holbro and Hynes, 2004). Signaling through EGFR regulates several cellular processes, like proliferation, cell adhesion, expression of matrix-degrading proteinases, and cell migration. All of these are important in re-epithelialization during wound healing. Thus the expression of EGF, TGF-α, HB-EGF and EGFR are increased at the wound site (Werner and Grose, 2003).

2.2.3. Keratinocyte growth factor

Keratinocyte growth factor (KGF), synthesized and secreted by stromal fibroblasts, is a member of the fibroblast growth factor superfamily (FGF-7). Although its mitogenic activity is restricted to epithelial cells, KGF is not expressed in epidermal keratinocytes (Rubin et al., 1995). Keratinocytes synthesize and secrete interleukin-1 (IL-1), which induces KGF expression in dermal fibroblasts (Angel and Szabowski, 2002). This double paracrine pathway involving IL-1 and KGF plays an important role in controlling epidermal homeostasis. A characteristic feature of KGF, and other FGFs is their interaction with heparin and heparan sulfate proteoglycans. This interaction is essential for the activation of the signaling receptors (Ornitz, 2000). KGF binds to a specific tyrosine kinase receptor, KGFR (FGFR2IIIb), a splice variant of fibroblast growth factor receptor 2, only expressed in epithelial cells (Miki et al., 1992). KGFR is located mostly to the spinous cell layer, but some KGFR expression occurs also in the basal cell layer (LaRochelle et al., 1995). KGF has been shown to enhance keratinocyte proliferation (Marchese et al., 1990), and migration (Tsuboi et al., 1993), but delay differentiation (Hines and Allen-Hoffmann, 1996; Andreadis et al., 2001). Its expression is highly upregulated during wound healing. Marchese et al. (1995) showed that 24 h after wounding KGF mRNA expression increased 100-fold, suggesting its importance in the wound healing process. Surprisingly, mice lacking KGF-gene did not show any obvious delay in wound healing (Guo et al., 1996), indicating that it may be compensated by other growth factors. However, the lack of KGFR leads to a severe delay in wound re-epithelialization (Werner et al., 1994).

2.2.4. Vitamin D

One of the most potent regulators of keratinocyte growth is vitamin D. The biologically active form of vitamin D is 1,25-dihydroxyvitamin D_3 (Garach-Jehoshua et al., 1999), which inhibits keratinocyte proliferation and induces differentiation (Gurlek et al., 2002). The lipophilic vitamin D_3 binds intracellularly to its receptor, VDR that forms heterodimers with RXR and thus activates transcription by binding to vitamin D response elements (VDREs) within the promoter of vitamin D responsive genes. How vitamin D conveys the growth inhibitory effects on keratinocytes is not completely

clear. There is evidence that vitamin D inhibits human keratinocyte growth by increasing the release of TGF-β2 (Haugen et al., 1996) and reducing c-myc and the EGFR mRNA levels (Matsumoto et al., 1990). On the other hand, there are some contradictory studies where vitamin D promotes keratinocyte proliferation (Gniadecki, 1996; Garach-Jehoshua et al., 1999). However, generally speaking the most important role of vitamin D in keratinocytes is to promote differentiation. It increases involucrin, loricrin and transglutaminase mRNA, and thus stimulates cornified encelope formation (Bikle et al., 2001). The pro-differentiative effects of vitamin D are connected to the intracellular calcium concentration (Bikle et al., 2003). Calcium concentration has been shown to be essential for keratinocyte differentiation in several *in vitro* and *in vivo* experiments (Bikle et al., 2001). Vitamin D increases the expression of calcium sensing receptor (CaR) and the PLC family members, which leads to the intracellular rise of free calcium (Bikle et al., 2004). Calcium is required for example for desmosome formation and transglutaminase cross-linking. In cell cultures, a calcium concentration above 0.1 mM promotes keratinocyte differentiation. In epidermis, there is a steep gradient of calcium, increasing from proliferative basal cell layer to the granular layer (Menon et al., 1985). This calcium gradient is required for normal epidermal differentiation and function. It is suggested that vitamin D is required for maintenance of this calcium gradient (Bikle et al., 2004).

2.2.5. Transforming growth factor-beta

TGF-β is one of the most extensively studied negative regulators of keratinocyte growth. It belongs to the TGF-β growth factor family, whose members inhibit DNA synthesis and cell division in various cell types. TGF-β signaling controls cell cycle by regulating cyclin-dependent kinases (cdks), which are responsible for cell cycle progression with cyclin proteins (Donovan and Slingerland, 2000). TGF-β transduces its signals through binding to the cell surface receptors with serine/threonine kinase activity. Ligand binding allows type II receptor to phosphorylate type I receptor, which conveys the signal to the SMAD-proteins (Shi and Massague, 2003). These proteins translocate to the nucleus where they activate transcription. In skin, the biological effects of TGF-βs are broad. It suppresses keratinocyte proliferation, whereas it induces

synthesis of dermal ECM proteins and basement membrane components, extracellular proteases and their inhibitors, as well as cell surface proteins including integrins α5, αv, β1, β4 and β5 (Freedberg et al., 2001). At the wound site, the expression of TGF-β is upregulated (Werner and Grose, 2003). It stimulates angiogenesis, fibroblast proliferation and matrix deposition. Furthermore, it stimulates keratinocyte migration and increases keratinocyte fibronectin production (Falabella and Falanga, 2001). Once the injured tissue is re-epithelialized, TGF-β inhibits keratinocyte hyperproliferation, reverts the active phenotype of keratinocytes to a regular one, and promotes their differentiation (Freedberg et al., 2001).

2.3. Hyaluronan

Hyaluronan is a large unsulfated glycosaminoglycan composed of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) disaccharide repeats (**Fig 6**), with a molecular mass up to 10^7 Da. It can have an extended length of up to 25 μm, depending on tissue source and physiologic conditions. Hyaluronan was purified from bovine vitreous already in 1934 (Meyer and Palmer, 1934), and its chemical structure was solved in 1954 (Weissmann and Meyer, 1954). However, its true biological functions were unclear for many decades. The last decade has provided much information about hyaluronan and its biological effects. Earlier it was thought that this simple carbohydrate is just a passive space-filling component in the extracellular matrix (ECM). Hyaluronan has an important role in ECM organization and modification through its interactions with other ECM molecules such as aggrecan and versican. It is also implicated in complex biological processes such as morphogenesis, cell adhesion, migration and proliferation through interactions with its specific cell surface receptors such as CD44 and RHAMM (Knudson and Knudson, 1993; Hall et al., 1994; Sherman et al., 1994; Evanko et al., 1999). Hyaluronan is found in all vertebrate tissues and on the surface of certain *Streptococcus* and *Pasteurella* bacterial pathogens (Mortimer and Vastine, 1967; Rosner et al., 1992). Exceptionally high levels of hyaluronan are found in connective tissues such as synovial fluid, cartilage and skin dermis (Laurent and Fraser, 1992). It is also highly expressed in the multilayered epithelia like in skin epidermis (Tammi et al., 1988). In addition, there are a number of pathophysiological

processes where high hyaluronan levels are involved, like rheumatoid arthritis, inflammatory and vascular diseases, as well as cancer (Laurent and Fraser, 1992; Hall and Turley, 1995; Toole et al., 2002).
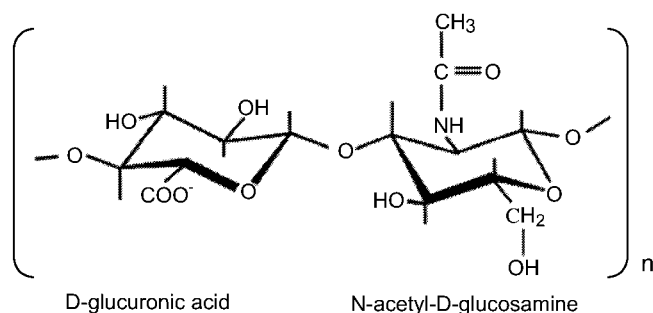
Figure 6. The structure of hyaluronan.

2.3.1. Hyaluronan synthases

In contrast to other glycosaminoglycans, synthesized in the Golgi apparatus and dependent on a core protein synthesis, hyaluronan is produced by hyaluronan synthases (HAS) at the inner surface of the plasma membrane and extruded through the membrane into the extracellular space (Weigel et al., 1997). N-acetyl-D-glucosamine and D-glucuronic acid residues are added to the growing chain by HASs from the corresponding UDP-sugar donors (Prehm, 1983). The knowledge related to hyaluronan synthetic system is somewhat incomplete. *Streptococcal* HAS has been shown to catalyze the addition of sugar units at the reducing end (Bodevin-Authelet et al., 2005; Tlapak-Simmons et al., 2005) originally suggested from experiments on eukaryotic cells (Prehm, 1983). However, recent studies with *Xenopus laevis* HAS1 and recombinant human HAS2 have convincingly demonstrated that vertebrate hyaluronan polymerization occurs at the non-reducing terminus (Hoshi et al., 2004; Bodevin-Authelet et al., 2005). The HAS-enzymes are transmembrane proteins, with the active site located in the cytoplasm (DeAngelis et al., 1993; DeAngelis and Weigel, 1994). These enzymes have unusual characteristics since they have both β1-4GlcNAc- and β1-3GlcA-transferase activity, and are able to translocate hyaluronan chains through the membrane (Prehm, 1983; DeAngelis and Weigel, 1994; Weigel et al., 1997). Once out

of the cell, hyaluronan forms complexes with hyaluronan binding proteins and receptors to form pericellular coats and extracellular matrices.

Hyaluronan synthase activity and its membrane localization in *Streptococcus pyogenes* were discovered already in 1959 by Markovitz and co-workers (Markovitz et al., 1959). It took over three decades to clone the first gene encoding hyaluronan synthase (HAS A) from *Streptococcus pyogenes* (DeAngelis et al., 1993). In the same decade, several laboratories identified the mammalian *Has* genes (*Has1, 2, 3*) (Itano and Kimata, 1996a, b; Shyjan et al., 1996; Spicer et al., 1996; Watanabe and Yamaguchi, 1996; Fulop et al., 1997; Spicer et al., 1997a). The expression of any *Has* gene in transfected mammalian cells leads to hyaluronan biosynthesis. The three genes encoding hyaluronan synthases are located in separate chromosomes – human *Has1* gene locates in chromosome 19q13.3-13.4, *Has2* in 8q24.12 and *Has3* in 16q22.1 (Spicer et al., 1997b). The structures of the genes imply that they have arisen from a common ancestral gene through a process of sequential gene duplication (Spicer and McDonald, 1998). The gene products (~65 kDa) of the three *Has*-isoforms are very similar in amino acid sequence (55-71%) and molecular structural characteristics (Spicer and McDonald, 1998), while the synthase activity, stability, product length and product's ability to form pericellular matrices may differ to some extent (Itano et al., 1999). The catalytic activity of HAS3 is higher than that of HAS2, which is in turn more active than HAS1, whereas HAS3 produces lower molecular mass hyaluronan ($2\text{-}3\times10^5$ Da) than HAS2 and HAS1 (2×10^6 Da) in *in vitro* incubations of cell homogenates (Spicer et al., 1997a; Brinck and Heldin, 1999; Itano et al., 1999). Hyaluronan chains of different lengths have different effects on cell behavior, suggesting that regulation between HAS isoenzymes may have important consequences on cellular function. So far, a specific cellular role has been documented only for *Has2*. *Has2* deficient mice die during midgestation (E9.5-10) because of a failure in the development of the heart (Camenisch et al., 2000), indicating that hyaluronan synthesis by HAS2 cannot be compensated with HAS1 or HAS3 during this part of embryogenesis. *Has1* and *Has3* null mice are viable and fertile with only minor defects in skin phenotype (Spicer et al., 2002).

2.3.2. Regulation of hyaluronan synthases

The regulation of HAS activity is under extensive research. Many growth factors and cytokines stimulate hyaluronan production, but the exact regulation mechanisms have not been unraveled. In hyaluronan synthesis, there are different levels that may be under regulation - *Has* mRNA transcription, mRNA stabilization, HAS enzyme activity, HAS enzyme stability, and the availability of precursor sugars like UDP-GlcUA, controlled by the activity of UDP-Glc dehydrogenase. In addition, there is also some evidence for a natural antisense *Has2* mRNA (HASNT), which participates in the regulation of hyaluronan synthesis (Chao and Spicer, 2005). In most studies with different growth factors, the production of hyaluronan is controlled at the level of *Has* gene transcription, reflecting the fact that hyaluronan synthesis is dependent on continuous *de novo* protein synthesis (Jacobson et al., 2000; Pienimäki et al., 2001; Sayo et al., 2002; Yamada et al., 2004). However, there is also some evidence that the TPA-stimulated hyaluronan synthesis in foreskin fibroblast cultures is partly independent of *de novo* protein synthesis (Suzuki et al., 1995), a situation similar to the PKC stimulated hyaluronan synthesis in rabbit joint synoviocytes (Anggiansah et al., 2003), suggesting that PKC is able to activate HASs directly (Heldin, 1998). Growth factors and cytokines, like EGF, PDGF, TGF-β, IL-1β and IFN-γ can stimulate hyaluronan production by upregulating *Has* message levels. This inducibility seems to be widespread, but shows great variation between cell types and species. Furthermore, the cell density in cell cultures (Jacobson et al., 2000), and the differentiation state greatly affect hyaluronan synthesis. In mouse keratinocytes, *Has3* mRNA is upregulated by IFN-γ, and downregulated by TGF-β (Sayo et al., 2002). On the other hand, in mouse fibroblasts, TGF-β stimulates hyaluronan production by increasing the levels of *Has1* and *Has2* message (Sugiyama et al., 1998). Quantitative RT-PCR revealed that EGF stimulates the expression of all *Has* genes in cultured human oral mucosal epithelial cells (COME) (Yamada et al., 2004), but only *Has2* expression in monolayer cultures of rat epidermal keratinocytes (Pienimäki et al., 2001). These changes in the message level were correlated with actual changes in hyaluronan synthesis. Glucocorticoids are well known inhibitors for hyaluronan synthesis in different cell types (Ågren et al., 1995; Jacobson et al., 2000). These widely used therapeutic agents decrease both *Has2* gene transcription and

message stability in dermal fibroblasts and in osteoblasts (Zhang et al., 2000). Glucocorticoids inhibit all *Has* genes in synoviocytes and leucocytes isolated from the synovial fluid of rheumatoid arthritis patients, and *Has1* activation is inhibited by blocking the p38 mitogen-activated protein kinase signaling pathway (Stuhlmeier and Pollaschek, 2004).

Recently, some of response elements in the promoter region of the human *Has2* gene have been identified. The proximal *Has2* promoter contains putative binding sites of the transcription factors Sp1, NF-Y and NF-kB (Monslow et al., 2004). These binding sites are common to all *Has2* orthologues, suggesting evolutionary conservation of *Has2* transcriptional regulation by the respective effectors. Saavalainen and co-workers (2005) found that human *Has2* promoter has several binding sites for STATs, the main transcription factors for the EGF signaling pathway, and a functional RARE cluster approximately 1 Kb upstream of the transcription initiation site. In addition, the promoter sequence for murine *Has1* contains several potential binding motifs for transcription factors (Yamada et al., 1998). The proximal promoter region of all three *Has* genes have constitutive activity, of which *Has2* has the lowest basal level (Monslow et al., 2004). Thus the *Has2* gene may be the main candidate for modulating hyaluronan synthesis rate.

2.3.3. CD44 and other hyaluronan binding proteins, the hyaladherins

Hyaluronan is involved in many biological functions through its interaction with hyaluronan binding proteins, hyaladherins. Many of them contain a common structural domain of ~100 amino acids in length, termed a Link module that is involved in hyaluronan binding. However, several hyaladherins lack this domain (Day and Prestwich, 2002). Based on their localization, hyaladherins are divided into two groups: 1) ECM-proteins like aggrecan, versican and inter-α-inhibitor (IαI), and 2) pericellular proteins such as CD44, RHAMM, layilin and LYVE-1. Furthermore, these hyaladherins may appear as soluble forms (CD44, RHAMM) or be located in cytoplasmic compartments (RHAMM) (Day and Prestwich, 2002; Tammi et al., 2002). The main hyaluronan binding receptor in epidermal keratinocytes is CD44 (Tuhkanen et al.,

1997), while RHAMM (Turley and Harrison, 1999) and versican (Zimmermann et al., 1994) are also expressed in these cells.

CD44

CD44 is a widely expressed cell surface hyaluronan binding receptor. The molecular weight of this single-pass transmembrane glycoprotein varies between 80 to 250 kDa due to extensive alternative mRNA splicing and post-translational modifications (Knudson and Knudson, 1999). Post-translational modifications such as N- and O-glycosylation and sulfation, phosphorylation, as well as clustering of CD44 molecules in the plasma membrane, regulate its ability to bind hyaluronan (Isacke and Yarwood, 2002). The interaction between CD44 and hyaluronan lead to pericellular coat formation (Knudson et al., 1996), and intracellular signaling cascades that regulate cell migration and proliferation (Kaya et al., 2000; Bourguignon et al., 2003; Singleton and Bourguignon, 2004), and hyaluronan internalization (Knudson et al., 2002). This interaction is important in a variety of physiological and pathophysiological processes, including tumor metastasis, wound healing, and leukocyte extravasation at sites of inflammation (Bajorath, 2000).

Of the many biological properties of CD44, its role in cell migration is probably most extensively studied. It has been demonstrated to mediate migration in several cell types such as melanoma cells, fibroblasts and endothelial cells (Ichikawa et al., 1999; Singleton and Bourguignon, 2002), but the specific signaling cascades initiated from hyaluronan-CD44 interaction are still partly unclear and contradictory. Hyaluronan binding to CD44 can cause clustering of CD44 molecules in the plasma membrane (Liu et al., 1998), which is associated with the phosphorylation of CD44, and interactions with p185[HER2] tyrosine kinase and the intracellular signaling molecules like c-Src and Tiam1, a guanidine nucleotide exchange factor (reviewed in Turley et al., 2002). This hyaluronan stimulated CD44 signaling is involved in the regulation of cytoskeletal components such as ankyrin and ERM-proteins, and changing cell locomotion (reviewed in Turley et al., 2002). In tumor cell lines, hyaluronan-CD44 interaction can also induce migration by modulating the secretion and activation of MMP-2, or by

anchoring the active form of MMP-9 on the cell surface of tumor cells (Isacke and Yarwood, 2002). This interaction results in the promotion of tumor cell invasiveness and angiogenesis, both of which are important events in tumor progression (Toole et al., 2002). Hyaluronan-CD44-MMP-9 interaction facilitates the cleavage of latent TGF-β, which promotes tumor growth (Yu and Stamenkovic, 2000). Furthermore, soluble CD44 has been shown to induce cell motility, but these results are conflicting with the results suggesting that soluble CD44 inhibits migration by disrupting endogenous CD44-hyaluronan interaction (reviewed in Cichy and Pure, 2003).

In addition to enhanced cell migration, hyaluronan-CD44 interaction causes increased cell proliferation. In the same way as in cell migration, the regulatory mechanisms are partly unclear. Ahrens and co-workers (2001) demonstrated that hyaluronan-CD44 interaction in melanoma cells increases cell proliferation probably due to enhanced TGF-β1 and bFGF production after interaction. In endothelial cells, proliferation induced by the hyaluronan-CD44 interaction is suggested to be a consequence of ankyrin-IP$_3$-Ca^{2+} signaling cascade (Singleton and Bourguignon, 2004). Kaya et al. (1997) demonstrated that CD44 deficiency results in defective keratinocyte proliferation in response to epidermal injury and growth factor stimulation, indicating the importance of CD44 in cell proliferation. It has also been suggested that the deficiency of epidermal CD44 may play a pathogenetic role in lichen sclerosus et atrophicus, where hyaluronan accumulates in the superficial dermis (Kaya et al., 2000). However, the deletion of CD44 causes no detectable developmental defects and no apparent structural epidermal abnormalities (Protin et al., 1999).

RHAMM

RHAMM, the receptor for hyaluronan-mediated motility, is alternatively spliced, like CD44. RHAMM is expressed in most normal mammalian cell types, and its expression is increased in tissue injury and tumors (Turley and Harrison, 1999). This hyaluronan receptor has been reported to occur at cell surface, in cytoplasmic compartments and in the nucleus. Furthermore, it may also be shed or secreted. On cell surface, RHAMM may be linked via a GPI-linkage or through associations with integral membrane

proteins, because it does not have a transmembrane domain. RHAMM-hyaluronan interactions in the cell surface induces signaling cascades that contribute to cell migration and proliferation (Turley and Harrison, 1999; Savani et al., 2001). Cell surface RHAMM-hyaluronan interaction has been shown to activate protein tyrosine kinases, such as Src and focal adhesion kinase, FAK, as well as ERK kinases and protein kinase C (Hall et al., 2001; Turley et al., 2002). This interaction can also modify growth factor signaling (Zhang et al., 1998). The major role of intracellular RHAMM proteins, also called IHABPs, is cytoskeletal assembly (Turley et al., 2002). These proteins associate directly with ERK and Src, and connect these signaling complexes to the cytoskeleton (Turley et al., 2002). This way RHAMM is able to rearrange cytoskeleton for cell division or locomotion. Recently, intracellular RHAMM was demonstrated to associate with intracellular hyaluronan and mitotic spindle microtubules in arterial smooth muscle cells (Evanko et al., 2004). Thus, RHAMM with hyaluronan may have a role in controlling microtubule assembly during mitosis. Furthermore, it is speculated that intracellular RHAMMs are involved in "inside-out" signaling like integrins. Both cell surface and intracellular RHAMMs regulate Ras, which controls multiple downstream signaling pathways like the MAPK pathway. Deletion of RHAMM does not result in embryonic lethality (Tammi et al., 2002), but may delay wound repair due to defects in wound contraction and cell migration (Tolg and Turley, 2003).

Versican

Versican is a chondroitin sulfate proteoglycan with a hyaluronan binding domain at the NH_2-terminal end, and a C-type lectin domain in the C-terminus (Day and Prestwich, 2002). Versican, like other lecticans (aggrecan, neurocan and brevican) interacts with hyaluronan and link proteins in the extracellular matrix. Hyaluronan- and versican-rich pericellular matrix is required for vascular smooth muscle cell migration and proliferation (Evanko et al., 1999). It is suggested that hyaluronan-dependent pericellular matrix increases cell detachment, and thus favors cell migration and division (Evanko et al., 1999). In skin epidermis, versican is expressed in the dividing

basal cells, but not in differentiated keratinocytes, suggesting a role in cell proliferation (Zimmermann et al., 1994).

2.3.4. Hyaluronan in skin and its diseases

Already in 1947, it was shown that skin is one of the richest sources of hyaluronan in the body (Meyer, 1947). Reed et al. (1988) reported that about half of the total body hyaluronan in the rat is located in the skin. Hyaluronan localization with specific staining methods was impossible until the discovery of proteins that specifically bind hyaluronan. Biotinylated hyaluronan binding region (bHABR) prepared from bovine aggregan stains human skin intensely (Tammi et al., 1988). Hyaluronan is found both in dermis and epidermis. Skin appendages are also hyaluronan positive, but the staining intensity varies greatly (Wang et al., 1992). In dermis, highest hyaluronan staining intensity is found just below the basement membrane, and the intensity of bHABC staining decreases from papillary dermis to reticular dermis (Wang et al., 1992). In human epidermis, hyaluronan is found in all vital cell layers, but not in terminally differentiated SC (Tammi et al., 1988). CD44, the main hyaluronan binding receptor in the keratinocytes, is localized as hyaluronan in the epidermis - in the basal and spinous cell layer (Wang et al., 1992). Considering the small space between the neighboring keratinocytes in the epidermis, the hyaluronan concentration is high. In human epidermis, it is 90 μg/g wet weight, and in dermis 440-520 μg/g wet weight (Tammi et al., 1994a). During embryogenesis, hyaluronan concentration is higher than in adult skin (Ågren et al., 1997a; Tammi et al., 2005).

Hyaluronan functions in epidermis

In skin epidermis, hyaluronan forms a loose extracellular matrix between keratinocytes. This matrix facilitates the exchange of metabolites between dermal circulation and epidermal keratinocytes, and allows keratinocyte movements and changes of cell shape during differentiation (Tuhkanen et al., 1998). Hyaluronan seems to be important for cell stratification, since other stratified epithelia like that in the mouth (Tammi et al., 1990) and esophagus (Wang et al., 1996) show a hyaluronan distribution similar to that in skin epidermis, whereas hyaluronan is not detected in most simple epithelia (Wang et

al., 1996). There is a lot of evidence showing the tight connection between hyaluronan synthesis and keratinocyte proliferation, but the exact mechanisms are still unclear (Rilla et al., 2002; Rilla et al., 2004). In hyperplastic epidermis the amount of hyaluronan is elevated (Tammi and Tammi, 1986), and in atrophic tissue the amount is decreased (Ågren et al., 1995). On the other hand, in human skin organ culture, rapid elevation in keratinocyte proliferation is not followed with increased hyaluronan production (Tammi and Tammi, 1991). During late mitosis, in cytokinesis, hyaluronan is intensively stained between the daughter cells suggesting that hyaluronan facilitates the separation of keratinocytes following mitosis (Tammi and Tammi, 1991). Furthermore, in the developing and adult epidermis the correlation between keratinocyte proliferation and hyaluronan synthesis is not obvious, since the content of hyaluronan in the proliferating basal cell layer is low compared to upper vital cell layers (Ågren et al., 1997a).

There are several possibilities how hyaluronan may favor cell (keratinocyte) migration: 1) It may alter or weaken cell-matrix or cell-cell adhesion, 2) Being a highly hydrated molecule, it may cause tissue swelling that creates space between ECM and cells, and 3) By interacting with its cell surface receptors, it may induce signaling cascades that induce cell migration (Bourguignon et al., 2000, 2001), as described earlier. During wound healing, hyaluronan and CD44 expressions are increased (Oksala et al., 1995; Tammi et al., 2005). By facilitating cell detachment and migration it fastens wound closure (Mack et al., 2003). In keratinocyte cultures, where hyaluronan production is suppressed, the migration rate is reduced (Rilla et al., 2002; Rilla et al., 2004).

In psoriatic skin, epidermal homeostasis is disturbed. Keratinocyte proliferation is enhanced, differentiation is aberrant and inflammation is present in various forms. Surprisingly, neither the localization nor the concentration of epidermal hyaluronan is changed in psoriasis (Tammi et al., 1994b). However, hyaluronan is accumulated in the psoriatic dermal papillae possibly due to elevated hyaluronan synthesis in the epidermis, and increased diffusion through an abnormally permeable basal lamina (Tammi et al., 1994b).

Since hyaluronan is involved in cell proliferation and migration, it is not surprising that several tumors are associated with elevated hyaluronan levels. In most tumors, like in breast (Auvinen et al., 2000), prostate (Lipponen et al., 2001) and ovary cancers (Anttila et al., 2000), increased hyaluronan concentration is an unfavorable prognostic factor (Toole et al., 2002). In skin tumors of keratinocyte origin, like squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), the expression of hyaluronan varies. In well differentiated SCC cells, hyaluronan staining intensity is enhanced unlike in BCC and less differentiated SCC with a reduced HA signal (Karvinen et al., 2003). In skin melanomas, reduced hyaluronan level correlates with poor prognosis (Karjalainen et al., 2000).

2.3.5. Hyaluronan metabolism in skin

Both dermal fibroblasts and epidermal keratinocytes synthesize high molecular mass hyaluronan. In human skin organ cultures, the average size of newly synthesized epidermal hyaluronan is $\sim5 \times 10^6$ Da, and that of dermal hyaluronan is $\sim2\text{-}3 \times 10^6$ Da (Tammi et al., 1991). Compared to other ECM components, the turnover rate of hyaluronan is exceptionally fast, its half-life being approximately 1 day in the epidermis (Tammi et al., 1991), while it is 27-29 days in articular cartilage (Morales and Hascall, 1988). Hyaluronan degradation occurs locally, or in the lymph nodes and liver. Since in normal skin, basal lamina restricts effectively the diffusion of hyaluronan to the underlying dermis (Tammi et al., 2000), and the molecular mass of newly synthesized epidermal hyaluronan decreases from several million to 0.5×10^6 Da during a 24 h chase in epidermis (Tammi et al., 1991), epidermal hyaluronan must be degraded locally.

Reactive oxygen species (Ågren et al., 1997b) and hyaluronidases (Frost and Stern, 1997) contribute to epidermal hyaluronan degradation. Fragmented hyaluronan, and also newly synthesized hyaluronan, bind to cell surface CD44, which mediate both hyaluronan immobilization, and uptake and degradation (Knudson et al., 2002). According to Kaya et al. (2000) in the absence of epidermal CD44, hyaluronan accumulates in the dermis, probably because keratinocytes are unable to bind and degrade hyaluronan. Epidermal hyaluronan endocytosis occurs via a novel endocytic

route, where clathrin coated pits or caveolae are not involved (Tammi et al., 2001). Internalized hyaluronan is probably fragmented by hyaluronidases and later completely degraded in the lysosomes by exoglycosidases.

Hyaluronidases are a group of enzymes involved in hyaluronan degradation. So far, six hyaluronidase-like genes have been identified in human genome (Csoka et al., 2001). Hyal-1 and Hyal-2 are the major hyaluronidases in somatic tissues (Stern, 2003). Both have acidic pH-optimum, suggesting lysosomal localization. Furthermore, Hyal-2 can also be anchored via a GPI-link to the cell surface, where it participates in hyaluronan binding with hyaluronan receptors (Stern, 2004). These enzymes may also be secreted, and located extracellularly, since high hyaluronidase activities are found in cell culture media (Heldin, 2003; Stern, 2003). Hyal-2 has been suggested to be less active than Hyal-1, which may explain why Hyal-2 only degrades high molecular weight hyaluronan to ~20 kDa fragments (Lepperdinger et al., 1998; Stern, 2003). Lysosomal Hyal-1 cleaves hyaluronan down to a size of tetra-hexasaccharides (Stern, 2004). Due to their role in hyaluronan catabolism, hyaluronidases may have important roles in angiogenesis, migration and differentiation, since hyaluronan chains of different size have different biological activities in these processes. For instance, intermediate sized hyaluronan has been shown to be highly angiogenic (Stern, 2003), and very small hyaluronan oligomers promote endothelial cell differentiation (Stern, 2004) and suppress synovial cell death (Xu et al., 2002). Furthermore, changes in polymer size elicite different signaling pathways and affect HaCat keratinocyte migration (K. Törrönen, personal communication). The exact mechanisms involved in the local degradation of hyaluronan in epidermis are not clear, but keratinocytes have the necessary enzymatic machinery for that. Human foreskin primary keratinocytes have hyaluronidase activity (Frost and Stern, 1997), and rat epidermal keratinocytes express *Hyal-2* mRNA (R. Tammi, personal communication). *Hyal-1* has been found in human skin fibroblasts (Stair-Nawy et al., 1999).

2.3.6. Regulation of hyaluronan metabolism

Growth factors, hormones and vitamins that have a key role in the regulation of epidermal homeostasis, such as EGF, TGF-β and vitamin A, also influence hyaluronan metabolism. Basically, hyaluronan synthesis is mostly regulated at *Has* mRNA level in keratinocytes as well as in other cell types, as described earlier. In epidermal keratinocytes, EGF (Pienimäki et al., 2001) and interferon gamma (Sayo et al., 2002) have been shown to upregulate hyaluronan production. During wound healing dermal fibroblasts produce these growth factors and cytokines, which may stimulate keratinocyte migration and proliferation at least partly by upregulating hyaluronan synthesis (Pienimäki et al., 2001). All-*trans* retinoic acid, an inhibitor of keratinocyte differentiation, stimulates epidermal hyaluronan production, and leads to the accumulation of hyaluronan in the upper spinous cells and the stratum corneum (Tammi et al., 1989). In the upper epidermal cell layers, increased hyaluronan level may disturb keratinocyte differentiation. Factors that suppress keratinocyte proliferation and epidermal thickening suppress epidermal hyaluronan production (Ågren et al., 1995; Sayo et al., 2002; Rilla et al., 2004). Based on these studies, it seems that epidermal hyaluronan synthesis is connected with keratinocyte proliferation and epidermal thickness.

There are relatively little data concerning the regulation of hyaluronan catabolism, and actually no data at all concerning the regulation of hyaluronan catabolism in the epidermis. Lactate and staurosporine upregulate *Hyal-1* and *Hyal-2* mRNA levels and hyaluronidase activity at pH 3.7 in human dermal fibroblasts (Nicoll et al., 2002), while TGF-β inhibits hyaluronidase activity at the same pH in rat lung fibroblasts (Li et al., 2000). In bovine chondrocytes, IL-1 and TNF-α upregulate *Hyal-2* and *Hyal-3* mRNA levels, but have no effect on hyaluronidase activity (Flannery et al., 1998). It is not established that elevated hyaluronan production, for example in cancer cells, correlates with increased hyaluronidase activity (Hiltunen et al., 2002).

2.4. Organotypic keratinocyte cultures

Organotypic keratinocyte cultures have been widely used to examine keratinocyte differentiation, epidermal-dermal interactions, and wound healing (Hinterhuber et al., 2002; Steude et al., 2002; Passi et al., 2004). Furthermore, these artificial skin models offer an alternative to cadaver and animal skin in drug transport and metabolism, and in skin irritation studies (de Brugerolle de et al., 1999; Asbill et al., 2000; Schmook et al., 2001; Pappinen et al., 2005). The preliminary development work in this field derives from the work of Rheinwald and Green (1975) when they noted that single keratinocytes are able to form stratified and keratinized colonies. These conventional, submerged keratinocyte cultures express many epidermal differentiation markers (Jepsen et al., 1980; Haydock et al., 1993). However, an epithelium with stratum corneum was produced not until these cells were grown at the air-liquid interface (Lillie et al., 1988). In organotypic keratinocyte cultures, a kind of reconstructed epidermis, keratinocytes are grown on a matrix resembling dermis, and kept at air-liquid interface. In air-liquid interface, cells are fed from below and exposed to air above. Culturing keratinocytes at the air-liquid interface greatly facilitates formation of a tissue resembling normal epidermis.

In organotypic keratinocyte cultures, there are many variations as to the supporting matrices employed, and the origin of keratinocytes. One common model is a dermal equivalent made of collagen type I gel seeded with keratinocytes (Cumpstone et al., 1989; Schoop et al., 1999; Suhonen et al., 2003). In these stratified keratinocyte cultures, the general histology and the expression of differentiation markers are closely similar to that in human skin, but the 'dermal'-epidermal zone is different. Keratinocytes are able to produce some basement membrane components, but not all (El-Ghalbzouri et al., 2005). In some systems collagen gel is populated with fibroblasts, which promote keratinocyte growth (Schoop et al., 1999; Kim et al., 2002; El-Ghalbzouri et al., 2002). Such feeder cells may be human dermal fibroblasts (Schoop et al., 1999) or irradiated mouse 3T3 fibroblasts (Mak et al., 1991). Besides producing keratinocyte growth promoting soluble factors, fibroblasts facilitate the formation of a

structure resembling native basement membrane (Boyce et al., 2002; El-Ghalbzouri et al., 2002; El-Ghalbzouri et al., 2005). In keratinocyte-fibroblast cocultures, the expression and organization of basement membrane proteins are improved in the presence of vitamin C (Boyce et al., 2002). In one dermal model the collagen gel is covered with a basement membrane produced by MDCK cells (Tammi et al., 2000; Passi et al., 2004). A closer approximation to the *in vivo* situation is achieved when keratinocytes are grown on a de-epidermized and devitalized cadaver dermis (Regnier et al., 1993; Gibbs et al., 1997). In this model, the original basement membrane molecules remaining on the dermis appear to enhance epidermal differentiation (Ralston et al., 1999). However, the presence of dermal support is not necessary for epidermal growth, if the composition of culture medium is correct (Poumay et al., 2004). In such simple reconstructed epidermis, human keratinocytes are seeded on inert polycarbonate culture inserts and grown at the air-liquid interface (Poumay et al., 2004). The commercially available SkinEthic™ reconstructed epidermis represents this model.

Depending on the research interest, keratinocytes originated from different species are used in organotypic cultures. Typically, these *in vitro* skin models have been constructed with primary or early passaged human keratinocytes and fibroblasts obtained from plastic surgery (Gibbs et al., 1997) or from circumcision (Godwin et al., 1997). Primary rat and mouse keratinocytes have also been used in air-liquid interface keratinocyte cultures (Cumpstone et al., 1989; Pu et al., 1995). Because of the limited availability of skin material, and its variable quality, continuous cell lines offer a good alternative to primary keratinocytes. The immortalized human keratinocyte line HaCat is widely used, and it has been shown to stratify and reconstitute a rather normally structured epidermis in organotypic culture conditions (Schoop et al., 1999). However, the ultimate steps of terminal differentiation, in other words, the formation of stratum corneum, do not occur irrespective of the type of dermal substratum or culture conditions in HaCat cell line (Boelsma et al., 1999). The rat epidermal keratinocyte line, REK, has been demonstrated to exhibit a high differentiation potential under *in vitro* conditions (Tammi et al., 2000; Passi et al., 2004).

3. AIMS OF THE STUDY

Organotypic keratinocyte cultures provide a good model to study different aspects of keratinocyte biology in well-standardized cell culture conditions. The primary focus of the present study was to develop and characterize a keratinocyte culture model with epidermal morphology and barrier properties resembling those in intact skin. This culture model offered a tool to study hyaluronan metabolism in epidermal keratinocytes. The specific aims of this research were the following:

1. To develop an organotypic keratinocyte culture model, which can be used in studies on drug penetration, keratinocyte differentiation and epidermal hyaluronan metabolism.

2. To investigate the role of vitamin C on epidermal permeability barrier formation and stratum corneum ultrastructure in organotypic keratinocyte culture.

3. To examine and compare the effects of mitogenic growth factors (EGF, KGF, all-*trans* retinoic acid) and an anti-proliferative growth factor (TGF-β) on epidermal morphology and hyaluronan synthesis in organotypic keratinocyte culture.

4. To correlate hyaluronan metabolism with keratinocyte differentiation.

5. To study the signaling pathways involved in the induction of hyaluronan synthesis by all-*trans* retinoic acid.

4. MATERIALS

4.1. Monolayer cell cultures (I-V)

A spontaneously immortalized newborn rat keratinocyte cell line (REK) originally isolated by Baden and Kubilus (Baden and Kubilus, 1983) was used in these studies. Stock cultures of REKs were grown in Minimum essential medium (Life Technologies, Paisley, U.K.) with 10% fetal bovine serum (FBS, HyClone, Logan, UT). REKs were passaged twice a week at a 1:5 split ratio. After 60-70 passages, new cells were taken from the liquid nitrogen, because in the course of time REKs lose the differentiation potential in cell culture.

4.2. Organotypic cell cultures (I-V)

The REK organotypic culture model is modified from (Lillie et al., 1988) and (Tammi et al., 2000) (**Fig. 7**). For organotypic cultures, REKs were cultured at the air-liquid interface on type I collagen support (from rat tail; Becton Dickinson Labware, Bedford, Mass., USA). To prepare collagen substrates, 8 vol of type I collagen (~3.2 mg/ml) was dissolved on ice in a solution consisting of 1 vol 10X EBSS (Life Technologies LTD), 0.3 vol 7.5% sodium bicarbonate (Life Technologies LTD) and 0.2 vol 1 M NaOH. 800 μl of dissolved collagen was added to individual 24 mm diameter tissue culture inserts (3.0 μm pore size) (Costar Transwell®, Cambridge, MA), which were then incubated overnight at 37°C in a humidified atmosphere to polymerize the collagen.

REKs were seeded on the collagen mats, which were washed with Dulbecco's Modified Minimal Essential Medium (DMEM) before use (4 g glucose/liter, ≈22 mmol/l) (Life Technologies LTD). The subcultivated REKs were grown for 3 days in DMEM culture medium, with 10% FBS, 4 mM L-glutamine (Sigma) and 50 μg/ml streptomycin sulfate and 50 U/ml penicillin (Sigma), present both beneath the insert and on the surface of the cells before lifting the confluent cultures to the air-liquid interface. To examine the effects of vitamins and growth factors, the culture medium was supplemented with the test substances from the day after the culture was lifted to the air-liquid interface.

Cultures were harvested at various time points for histology, RT-PCR, hyaluronan-ELSA, western blotting and permeability tests.
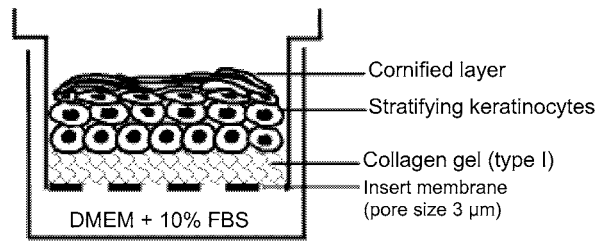
Figure 7. Schematic diagram of the REK organotypic culture model.

4.3. Mouse tail skin (V)

Mice (C57Bl/6J) used in this study were supplied by the National Laboratory Animal Centre (Kuopio, Finland). The mouse studies were approved by the Animal Care and Use Committee of Kuopio University and followed the National Institutes of Health guidelines for animal care. All-*trans* retinoic acid containing cream (Avitcid 0.05%, Tretinoin, Orion Pharma, Finland) was applied once a day to mouse tail and ear skin for 4-14 days. 24 h after the last application of the retinoic acid cream, the animals were sacrificed by cervical dislocation. Skin samples for histology, hyaluronan assay and RT-PCR were collected. Epidermis and dermis were separated by a 15 min treatment with 0.4% EDTA at 60°C.

4.4. Cadaver skin (I-II)

For the permeability experiments, cadaver abdominal skin was obtained from the Kuopio University Hospital, Kuopio, Finland, with the permission from The National Board of Medicolegal Affairs. Epidermis was separated from the underlying dermis by heating in distilled water at 60°C.

4.5. Growth factors and vitamins (I-V)

Epidermal growth factor, EGF (Sigma, St. Louis, MO) was dissolved in sterile water and added to culture medium in the final concentrations of 2-20 ng/ml (III). Keratinocyte growth factor, KGF (Sigma) was dissolved in 0.1% BSA in phosphate

buffered saline (PBS) and was used at 0.1-100 ng/ml final concentrations in complete medium (IV). Transforming growth factor-beta, TGF-β (Life Technologies) was dissolved in sterile water and used at 1-4 ng/ml final concentrations in the culture medium (III). L-ascorbic acid, vitamin C (Sigma) was diluted in culture medium, sterile filtered and added to culture medium in the final concentration of 40 μg/ml (II). All-*trans* RA (Sigma), EGF-receptor inhibitor AG1478 (LC Laboratories®, Woburn MA, USA), the matrix metalloproteinase inhibitor GM6001 (Calbiochem, La Jolla, CA, USA) and the MEK-kinase inhibitor UO126 (Calbiochem), were dissolved in sterile filtered DMSO (V). All-*trans* RA was used at 0.1 - 3 μM, AG1478 at 100 nM, GM6001 at 1-10 μM and UO126 at 1-10 μM final concentrations in complete medium.

5. METHODS

5.1. Proliferation assay (IV)

The REKs were seeded into 24-well plates at 60,000 cells/well, KGF (1-100 ng/ml) added on the next day, and cells were counted 24, 48 and 72 h later.

5.2. Migration assay (IV)

Keratinocyte migration was studied *in vitro* with a scratch wound assay. 600,000 cells/well were seeded on 6-well plates, and grown for 24 h. A standardized area of cells was scraped clean with a sterile 1000-μl pipette tip, and the medium containing the appropriate concentration of KGF was added. The area covered by the cells in eight crossing areas in duplicate wells of each growth factor concentration was measured immediately after scraping, and 24 h later using an Olympus CK2 inverted phase contrast microscope (Olympus Optical Co. Ltd. Tokyo, Japan), a Panasonic Wv CD 130-L video camera (Matsushita Electric Works, Tokyo, Japan), and NIH Image software.

5.3. Metabolic labeling and analysis of glycosaminoglycan synthesis (IV, V)

5.3.1 Monolayer cultures (IV)

Subconfluent REKs with appropriate amounts of KGF (0, 1, 10, 100 ng/ml) in the culture medium were labeled for 6 or 18 h with 20 μCi/ml of [^{3}H]-glucosamine (Perkin Elmer, Life Sciences Inc.) and 10 μCi/ml [^{35}S]-sulfate (Amersham Pharmacia Biotech). Labeled glycosaminoglycans were purified and quantitated from the culture medium, cell trypsinization solution (pericellular compartment) and cell pellet (intracellular compartment) as described (Tammi et al., 1998).

5.3.2 Organotypic cultures (V)

Ten days after raising to the air-liquid interface, the cultures were metabolically labeled for 24h with 20 μCi/ml of [^{3}H]glucosamine and 100 μCi/ml of [^{35}S]SO$_4$ in culture medium. The three "compartments" of the cultures (medium, collagen gel, epidermis) were analyzed separately. At the end of the labeling, the medium (1.5 ml) was collected and the culture inserts were washed once with 0.5 ml PBS, which was added to the medium compartment. Thereafter the collagen and the epidermis were separated with fine tweezers.

5.3.3. Purification of radiolabeled hyaluronan and other glycosaminoglycans (IV, V)

Carrier hyaluronan (6 μg in 40 μl, Healon; Pharmacia, Uppsala, Sweden) was added to each medium, collagen and epidermal sample to evaluate the recovery of the samples. Papain (Sigma) digestion was performed at 60°C overnight in 150 mM sodium acetate, pH 5.8 containing 5 mM cysteine-HCl and 5 mM sodium-EDTA. The samples were heated at 100°C for 10 min, centrifuged, and supernatants containing hyaluronan and other glycosaminoglycans recovered. 1% cetylpyridinium chloride (CPC) in 20 mM NaCl was added to each supernatant, followed by incubation for 1 h at room temperature. The samples were centrifuged at 13 000 g for 15 min at room temperature and each supernatant was carefully removed by aspiration. The CPC-precipitates were washed with H$_2$O and dissolved in 50 μl 4 M guanidine-HCl and reprecipitated with 900 μl of ethanol at -20°C for 1 h. The samples were centrifuged and dissolved in 50 μl 0.5

M ammonium acetate, pH 7.0, and digested for 3 h at 37° with 25 mU chondroitinase ABC and 1 mU of *Streptococcal* hyaluronidase (both from Seikagaku Kogyo, Tokyo, Japan), and 39 μl injected onto a 1x30 cm Superdex Peptide column (Pharmasia), eluted at 0.5 ml/min with 12 mM NH_4HCO_3. The eluent was monitored at 232 nm, and aliquots of the 350 μl fractions were counted for ^3H and ^{35}S. The carrier hyaluronan produced a disaccharide peak at 232 nm, which was used to correct for any losses in the purification (recovery 60-70%). The chemical quantitation of hyaluronan and other glycosaminoglycans was done from different compartments as described (Tammi et al., 2000).

5.4. Molecular mass distribution of hyaluronan (IV, V)

To analyze the size of secreted (V), cell associated and intracellular hyaluronan (IV), monolayer REK cultures were incubated for 24 h with or without KGF (100 ng/ml), and organotypic REK cultures were incubated with or without all-*trans* RA or EGF-receptor inhibitor AG1478, and 20 μCi/ml of [^3H]-glucosamine (Amersham). Radiolabeled samples (0.5 ml) were chromatographed on an 1x30 cm column of Sephacryl S-1000 (Amersham Biosciences), equilibrated and eluted at 0.4 ml/min with 0.15 M sodium acetate, 0.1% CHAPS (Sigma), 0.05% Hibitane®, pH 6.8. Two aliquots were taken from each fraction, one was incubated with 12.5 milliunits of *Streptomyces* hyaluronidase overnight at 37°C, the other in buffer only. Samples were precipitated in 1% cetyl pyridium chloride (Sigma) with 5 μg of carrier hyaluronan. The size distribution of hyaluronan in the samples was estimated as described (Tammi et al., 1991).

5.4. *In vitro* hyaluronan synthase assay (IV)

The assay was performed as described by Spicer (2001). Subconfluent REKs were treated with 0-100 ng/ml KGF for 14 h, and membrane fractions were extracted from the cultures. Protein concentration was measured with the Bradford method (Bradford, 1976) from membrane fractions, and an equal amount of protein from each sample was taken to the assay. The *in vitro* synthase assay was carried out with UDP-GlcNAc (0.5 mM), UDP-GlcA (0.05 mM) (both from Sigma), the latter containing 2.5 μCi of UDP-[^{14}C]GlcA (Perkin Elmer Life Sciences), resulting in ^{14}C-labeled hyaluronan. The

samples were boiled with 1% SDS to stop the reaction, and the incorporated activity separated from precursor by chromatography on Whatman 3MM paper, and incorporated activity quantified by liquid scintillation counter. The results were expressed as picomoles of GlcA incorporated/mg of protein in the membrane fraction.

5.5. Hyaluronan disaccharide analysis with electrophoresis (IV)

To analyze secreted hyaluronan levels, medium samples (400 μl) were subjected to proteinase K (Sigma, 600 μg/ml) digestion and trichloroacetic acid (50%) precipitation as described (Rilla et al., 2002). Dialyzed supernatants were evaporated, dissolved in 100 mM ammonium acetate, pH 6.5, and digested for 3 h at 37°C with 2 mU of *Streptococcus* hyaluronidase (Seikagaku), dried and derivatized overnight at 37°C in 5 μl of 0.1 M 2-aminoacridone (AMAC, Lambda Fluoreszenztechnologie GmbH, Graz, Austria) in 3:17 (v/v) acetic acid:dimethylsulfoxide, and 5 μl of 1 M NaBH$_3$CN. The AMAC-derivatized disaccharides were subjected to 30% PAGE as previously described (Rilla et al., 2002). The intensities of the hyaluronan disaccharide bands derived from the samples and hyaluronan standards (Healon®, Amersham Pharmacia Biotech, Uppsala, Sweden) were digitized on a UV-light box using a CCD camera. Quantitative image processing was done with NIH-Image.

5.6. Hyaluronan assay (III-V)

Hyaluronan content was measured from all-*trans* retinoic acid and inhibitor treated monolayer cultures (medium, 6-well plates), from mouse skin epidermis and dermis, and from three different compartments of the organotypic keratinocyte cultures (medium, collagen gel and epidermis) with an enzyme linked sorbent assay (ELSA). Before the assay, the tissue samples were extracted with acetone, weighed, digested with papain and diluted with 1% BSA in PBS.

The ELSA assay was performed as described previously (Hiltunen et al., 2002). Briefly, 96-well Maxisorp Plates (Nunc, Roskilde, Denmark) were precoated with the hyaluronan binding complex (HABC, 1 μg/ml) overnight at 4°C, washed with 0.5% Tween-PBS and blocked with 1% BSA for 1 h at 37°C. The dilutions of standard

hyaluronan (Provisc, Algon Laboratories, Fort Worth, TX) and samples were aliquoted to the wells. The bound hyaluronan was visualized with bHABC using a horseradish peroxidase streptavidin complex (1:20 000, Vector Laboratories Inc., Burlingame, CA) and TMB-substrate solution (3,3',5,5'-tetrametylbenzidine in dimethyl sulfoxide, Sigma) in 0.1 M sodium acetate buffer containing 1.5 mM citric acid and 0.005% H_2O_2. The absorbances were read at 450. Each sample and standard were done in triplicate.

5.7. Histology and immunocytochemistry (I-V)

5.7.1. Hyaluronan staining in monolayer cultures (IV, V)

To localize hyaluronan, a specific probe, biotinylated hyaluronan binding complex (bHABC) was used (Tammi et al., 1994a). REKs grown in 8-well chamber slides were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PB), for 20 min, washed with PB, and permeabilized with 0.3% Triton X-100 in 1% BSA-PB for 30 min. Thereafter the cells were incubated with bHABC (5μg/ml in 1% BSA) overnight at 4°C. After washing, avidin-biotin peroxidase complex (Vector Laboratories Inc., Burlingame, CA) was added for 1 h. Hyaluronan was visualized with 0.05% 3,3'-diaminobenzidine (DAB, Sigma) and 0.03% H_2O_2 for 5 min. *Streptomyces* hyaluronidase (10 turbidity reducing units/ml, 10 min at 37°C, Seikagaku, Tokyo, Japan) digestion was done prior to the permeabilization to some of the cultures to remove the extracellular hyaluronan.

5.7.2. Tissue fixation and processing (I-V)

For immunohistochemistry, tissue samples were fixed with Histochoice® (Amresco, Solon, OH), and for hyaluronan stainings with 2% paraformaldehyde containing 0.5% glutaraldehyde overnight, washed with sodium phosphate buffer (PB), pH 7.4, dehydrated in graded ethanol, embedded in paraffin, and cut into 3 μm thick vertical sections. To view overall morphology and to perform morphometry, the sections were stained with hematoxylin/eosin.

5.7.3. Histological demonstration of hyaluronan in organotypic cultures (III-V)

Sections were rehydrated, treated with 1% H_2O_2 for 5 min to block endogenous peroxidases, blocked with 1% BSA for 30 min and stained for hyaluronan using bHABC (5 μg/ml), avidin-biotin peroxidase (1:200 dilution, Vectastain Kit, Vector Laboratories), and DAB (Sigma) as described (Tammi et al., 2000). Sections counterstained with Mayer's hematoxylin were washed, dehydrated, and mounted in DPX (Gurr®, BDH Laboratory Supplies, Poole, UK). To ascertain the specificity of the staining, the sections were pretreated with *Streptomyces* hyaluronidase to remove hyaluronan from the tissue.

5.7.4. Keratin 10, involucrin, filaggrin and CD44 stainings (II-V)

Histochoice® fixed cultures were deparaffinized and treated with target unmasking fluid (TUF, Monosan, Uden, The Netherlands) according to manufacture's instructions. After blocking with H_2O_2 and 1% BSA, sections were incubated with the primary antibodies overnight at 4°C and thereafter with the secondary antibodies for 1 h at room temperature using the following dilutions: monoclonal anti-keratin 10, 1:10 – 1:50 (Monosan); monoclonal anti-involucrin, 1:50 (Novocastra Laboratories, Newcastle upon Tyne, UK); polyclonal anti-filaggrin, 1:5000 – 1: 9000 (a gift from Dr. Beverly Dale-Crunk); monoclonal CD44 antibody, 1:50 (OX50; Biosource, CA, USA); biotinylated anti-mouse antibody, 1:50 (Vector Laboratories, CA, USA), and biotinylated anti-rabbit antibody, 1:70 (Vector Laboratories). The bound antibodies were visualized with the avidin-biotin-peroxidase method with DAB chromogen. Sections counterstained with hematoxylin were dehydrated and mounted in DPX. Sections treated in the same way but without the primary antibody served as negative controls.

5.8. Microscopic methods (I-V)

5.8.1. Morphometry

Epidermal thickness (II, III, IV)

The thickness of SC was measured from cryosections, where the compact SC structure is retained. The paraformaldehyde/glutaraldehyde fixed cultures were embedded in

OTC (Tissue-Tek, Sakura, The Netherlands), and snap frozen in liquid nitrogen (II). Five-μm-thick sections were cut with a cryomicrotome, postfixed with acetone, air dried, and stained with hematoxylin and eosin. Images were systematically taken with a Polaroid digital camera (Polaroid, Cambridge, Mass., USA), and the thickness of SC was measured with the NIH-Image 1.62/fat software for Macintosh.

In III and IV, the thickness of the SC and the vital epidermis and the height of the basal cells were recorded from 3-μm-thick paraffin sections with the NIH-Image software. The cultures were systematically sampled by taking 6-10 digital images with a CoolSNAP camera (Photometrics) from each culture at constant intervals using a 20x objective and a 1.25x intermediate lens (Nikon Microphot FXA microscope, Japan). Thresholding of areas exhibiting background intensity was used to exclude the areas between separated corneocytes in the SC measurements.

Keratohyalin granules (II)

Hematoxylin and eosin stained histological sections were examined with a Leitz Ortholux BK-microscope (Leitz, Wezlar, Germany) and a Photometrics CH 200 camera (Photometrics, Tucson, AZ, USA) using a constant exposure time and 8-bit dynamic range. Digitized images were subtracted from the optical background at 658 x 517 pixel resolution with IPLab software (Scanalytics Inc., Fairfax, VA, USA). A single pixel side corresponded to 1.23 μm. The grey distribution for each image was normalized with the batch processing mode of Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA). Finally, the images were processed with the "unsharp mask" feature (radius 4, amount 220, threshold 18). Manually delineated total epidermal area was measured with IPLab software. After that, a zone containing keratohyalin granules was traced and segregated using a predetermined threshold value. In the beginning, the threshold was empirically determined by superimposing binarized granules on the original gray level image to obtain the best agreement between these two images. A region containing keratohyalin granules was delineated, and in this region binarized structures with areas from 1 to 80 pixels (0.052 to 0.22 μm^2, respectively) were regarded as keratohyalin granules, and their cumulative number and area were

determined from each section. Three sections of each sample from eight sample pairs (with and without vitamin C) were measured, and from each section 10 to 20 fields were digitized. The number of granules was calculated per measured epidermal area.

5.8.2. Image analysis of cell-associated hyaluronan (IV)

REKs grown on 8-well chambers were stained for hyaluronan with DAB as a chromogen. Optical densities were analyzed by a Leitz BK II microscope with 16x/0.45 numeric aperture objective (Leitz, Wetlar, Germany) and a digital camera (Photometrics CH 200). The optimum wavelength for DAB was found at 543 nm using an interference filter (Schott, Wiesbaden, Germany). IPLab software was used for camera control and image analysis. Multiple fields were systematically sampled from each well. Area-integrated mean optical density values for the DAB chromogen were calculated for each whole digitized area, excluding possible artifact areas. In the densitometric assays, the hyaluronan remaining after *Streptomyces* hyaluronidase treatment of non-permeabilized cells was designated as "intracellular".

5.8.3. Electron microscopy for monolayer cultures (IV)

Paraformaldehyde (2%) and glutaraldehyde (0.5%) fixed REKs were permeabilized with 0.05% saponin in 3% BSA-PB for 10 min on an ice bath. Hyaluronan staining was done as described above, except that all solutions and buffers contained 0.05% saponin, and the incubations were done at 4°C. Hyaluronan staining was followed with a 15 min-postfixation in 1% reduced osmium tetroxide. After dehydration in graded ethanol, the samples were embedded in Spurr's resin. Thin sections were cut on Formvar-coated copper grids, stained with uranyl acetate and lead citrate, and viewed in a type 1200 EX microscope from JEOL (Tokyo, Japan).

5.8.4. Electron microscopy for organotypic cultures (I-II)

Cultures were first fixed overnight at 4°C in 0.1 M cacodylate-buffered 2.5% glutaraldehyde pH 7.4, followed by a 2 h postfixation in 1% osmium tetroxide or in 0.4% ruthenium tetroxide (Electron Microscopy Sciences, Ft. Washington, PA, USA) with 0.2% aqueous potassium ferrocyanide to fix stratum corneum lipids (modified

from Fartasch and Ponec, 1994). After dehydration in graded ethanol and propylene oxide the specimens were embedded in Epon. Thin sections were cut, stained with uranyl acetate and lead citrate, and viewed in a JEOL-1200 EX electron microscope.

5.8.5. Confocal microscopy (III, IV)

For confocal fluorescence analysis of hyaluronan and CD44 (III), the culture sections were stained with bHABC and OX50 antibody, respectively. Briefly, the deparaffinized, TUF-treated sections were incubated in 50 mM glycine for 30 min and blocked with 1% BSA. For double staining, the sections were incubated with OX50 (1:20) and bHABC (5 μg/ml) in 1% BSA overnight at 4°C, washed and incubated simultaneously with Texas-Red-labeled anti-mouse antibody (1:50) and fluorescein isothiocyanate-avidin (1:1000) for 1 h at room temperature. The sections were coverslipped with Vectashield mounting medium (Vector) and viewed with an Ultraview ® confocal scanner (Perkin Elmer Life Sciences, Wallac-LSR, Oxford, U.K.) on a Nikon Eclipse TE300 microscope using a 100 x oil immersion objective.

To examine cell proliferation, the cultures were labeled with 5-bromo-2'-deoxyuridine (BrdU) for 1 h (III, IV). Samples were processed for histology as described above. The TUF-treated sections were stained according to the manufacturer's instructions (5-Bromo-2'-deoxyuridine Labeling and Detection Kit I, Roche Diagnostics Corporation, IN), and viewed with Nikon Eclipse TE300 microscope using a 10 x objective.

5.9. RNA isolation and semi-quantitative RT-PCR (II-V)

Total RNA was isolated using TRIzol® reagent (Life Technologies) or Eurozol® reagent (Euroclone Ltd, UK). Mouse tail epidermis or organotypic REK cultures were put in TRIzol® or Eurozol® and homogenized with a needle and a syringe. RNA was isolated according to the protocol of the manufacturer and dissolved in 20 μl of sterile water. To remove genomic DNA contamination, the samples were treated with 10 U of RNase-free DNase I (Roche Diagnostics GmbH, Penzberg, Germany). After DNase treatment, the RNA concentration was measured with a spectrophotometer at 260 nm and the samples were diluted to equal concentrations.

0.1 μg of RNA was taken for the RT-PCR, done with the GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems, Foster City, CA). The primers used for the RT-PCR of rat and mouse *Has1*, *Has2*, *Has3*, *HB-EGF*, *CD44*, *profilaggrin* and *GAPDH*, the annealing temperatures, and the number of PCR cycles are shown in **Table I**. The RT-PCR products electrophoresed in a 1.5% agarose gel were digitized by BioDocII Video Documentation system (Biometra, Göttingen, Germany), and the band densities of ethidium bromide fluorescence were measured with the NIH-Image 1.62/fat software.

Table I. Primers and conditions in the RT-PCR reactions (r, rat; m, mouse)

Gene	Primers	Annealing	Cycles
r*Has1*	5'-GCT CTA TGG GGC GTT CCT C-3' (left)	57°C	35-37x
	5'-CAC ACA TAA GTG GCA GGG TCC-3' (right)		
r*Has2*	5'-TCG GAA CCA CAC TGT TTG GAG TG-3'	62°C	33-35x
	5'-CCA GAT GTA AGT GAC TGA TTT GTC CC-3'		
r*Has3*	5'-ACT CTG CAT CGC TGC CTA CC-3'	66°C	33-35x
	5'- ACA TGA CTT CAC GCT TGC CC-3'		
r*HB-EGF*	5'-AGG AAA GGC AAG GGG TTA GGA-3'	58°C	33x
	5'-CCT CCT CGC CTA TGG TAC CTG-3'		
r*CD44*	5'-TTG GGG ACT ACT TTG CCT CTT A-3'	55°C	33x
	5'-CCA CTG CTG ACA TCC TCA TCT A-3'		
r*Profilaggrin*	5'-CTC AGG GCA TCG CTC GTC A-3'	64°C	34x
	5'-GCT GGT GGC GGT CTT CGT G-3'		
r*GAPDH*	5'-TGA TGC TGG TGC TGA GTA TG-3'	60°C	30-33x
	5'-GGT GGA AGA ATG GGA GTT GC-3'		
m*Has2*	5'-ACA GGC ACC TTA CCA ACA GGG TGT-3'	66°C	35x
	5'-GCA TGC ATA GAT CAA AGT TCC CAC G-3'		
m*Has3*	5'-ACT GCC TTC AAG GCC CTT GG-3'	60°C	35x
	5'-AAT GTT CCA GAT GCG GCC AC-3'		
m*CD44*	5'-CCC ACC ATG GAC CAA ATG A-3'	58°C	31x
	5'-GGT GCT CCG GAT AAA GAA GGA-3'		
m*HB-EGF*	5'- TCC AGG ACT TGG AAG GGA CAG -3'	59°C	32x
	5'- CAG CCA CCA CAG CCA AGA CT -3'		
m*GAPDH*	5'-TGC AGT GGC AAA GTG GAG AT-3'	62°C	31x
	5'-AGT GAT GGC ATG GAC TGT GG-3'		

5.10. Immunoblotting (II-V)

The organotypic REK cultures (epidermis without collagen matrix) were homogenized in 8 M urea, 50 mM Tris, pH 7.6, 100 mM dithiothreitol, 0.13 M 2-mercaptoethanol, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml sodium orthovanadate and 100 μg/ml aprotinin (modified from (Haydock et al., 1993)) with Ultra Turrax (Ystral, Germany), and centrifuged at 13 000 g for 15 min. The supernatant was used for the determination of protein concentration (Bradford, 1976) and Western blotting.

15-20 μg of protein was resolved in a 10% sodium dodecyl sulfate-polyacrylamide gel elctrophoresis (SDS-PAGE), and transferred onto Immobilon™-NC membranes (Millipore, Bedford, MA) by 35 mA per cm^2 costant current with a Sammy™ semidry blotter (Schleicher and Schuell, Dassel, Germany). The blots were blocked in 10 mM Tris, 150 mM NaCl, pH 7.4 (Tris-saline blocking buffer) containing 1 - 5% fat-free milk powder and 0.1 - 0.3% Tween-20 overnight at 4°C, and incubated with the primary antibody for 2 h, using the following dilutions: polyclonal anti-filaggrin 1:9000; monoclonal anti-keratin 10 1:100; polyclonal anti-pEGFR 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-pERK1/2 1:500 (Santa Cruz); monoclonal anti-pPI3K 1:250 (Santa Cruz); monoclonal anti-pJNK 1:200 (Santa Cruz); monoclonal anti-pELK 1:500 (Santa Cruz), monoclonal pSTAT3 1:250 (Santa Cruz) and monoclonal anti-p cJUN 1:250 (Santa Cruz). Thereafter the blots were incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h, using the following dilutions: anti-rabbit IgG 1:20 000 (Zymed Laboratories, CA, USA) and anti-mouse IgG 1:5000 – 1:20 000 (Santa Cruz). After antibody incubation the blots were washed four times with 0.1 - 0.5% Tween-20 in Tris-saline buffer. The protein bands were visualized using NEN™ chemiluminescent detection system (Life Science Products, Boston, MA, USA) and Kodak Biomax Light Film (Eastman Kodak Company, NY, USA).

5.11. Permeability studies (I-IV)

The permeability of epidermis was measured using two-chamber diffusion cells (Side-Bi-Side; Crown Glass, Somerville, NJ, USA) with an average effective diffusional area

of 0.64 cm^2. Cadaver skin (I, II) or three-week-old organotypic culture (I-IV) was clamped between the cell halves. 3 ml of PBS, pH 7.4, was pipetted into both chambers and equilibrated at 37°C. The donor chamber was spiked with a labeled test substance (tritiated coriticosterone or mannitol; NEN Life Science Products; 20 000 – 40 000 dpm/10 μl), and aliquots were taken from the receiving chamber for scintillation counting. Permeability experiments were carried out for 4 days, which was long enough to make sure that the steady-state phase was reached. The permeability coefficients (P, cm/s) for the test substances were calculated at steady state under sink conditions according to **Eq (1)**:

$$P = \frac{1}{AC_d} \frac{dQ}{dt} \qquad\qquad \textbf{Eq (1)}$$

Where A is the diffusional area of the diffusion cell (cm^2), C$_d$ is the concentration in the donor chamber (dpm/ml), and dQ/dt is the steady state flux (dpm/s/cm) through the skin (dQ is the amount of the permeant in the receiver chamber, dpm; dt is time, s).

5.12. Transepidermal water loss (TEWL) (I)

The TEWL (g/m^2/h) was measured by using a Delfin-SWL system (Delfin Technologies, Kuopio, Finland). In this system, a probehead is kept in contact with the culture surface or skin for 1 min and the sensors inside the evaporimeter probehead measure the humidity of the air. Cultures were taken out from the incubator 1 h before the measurement to equilibrate the humidity in the cultures. The measurement was carried out at room temperature (21-23°C). TEWL control values were obtained from the forearm skin of healthy persons.

5.13. Statistical analysis (II-V)

In morphometric measurements the significance of the differences between groups were tested using repeated measures ANOVA (II, V) or paired samples t-test (III-IV). t-test was also used to analyze hyaluronan ELSA results (IV). The data from the proliferation assay and the permeability and TEWL measurements were analyzed by the non-

parametric Mann-Whitney U-test. A difference was considered statistically significant when the p value was less than 0.05.

6. RESULTS

6.1. CHARACTERIZATION OF THE ORGANOTYPIC KERATINOCYTE CULTURE MODEL

6.1.1. Morphology and differentiation markers (I, II)

A continuous rat epidermal cell line (rat epidermal keratinocyte; REK) formed a morphologically well-organized epidermis in the absence of feeder cells when grown for 2 weeks on a collagen gel in culture inserts at an air-liquid interface (I, Fig. 1a; II, Fig. 2). Vital epidermis (basal-granular cell layers) consisted approximately 5 cell layers and was 21 ± 2 μm thick (II, Fig. 1). Two-week-old cultures expressed the suprabasal differentiation markers keratin 10, involucrin and filaggrin, indicating that keratinocytes differentiate properly (II, Fig. 3). Granular cells contained keratohyalin granules and lamellar bodies, and cornified envelopes and tightly packed keratin filaments were present in the corneocytes (II, Fig. 5). Morphologically, vitamin C supplementation of the culture medium further improved epidermal morphology by enhancing the number and the size of keratohyalin granules (II, Table 1), and the expression of profilaggrin and filaggrin, both at mRNA and protein levels (II, Fig. 4).

6.1.2. Stratum corneum ultrastructure (I, II) and lipid profile (unpublished)

To study the ultrastructure with electron microscope, the organotypic cultures were postfixed with 0.5% ruthenium tetroxide to stabilize the stratum corneum lipids, the main determinants of the epidermal permeability barrier. Suprabasal cells, and especially the granular cells in control and vitamin C treated cultures, contained lamellar bodies (I, Fig 1b; II, Fig 5c), which were extruded in the stratum granulosum / stratum corneum interface (I, Fig. 1c; II, 6a). However, vitamin C had a strong influence

on SC structure, it enhanced the normal wavy pattern of the stratum corneum in paraffin sections (I, Fig. 2d, f), and the quantity and organization of the intercellular lamellar lipid layers in the interstices of the stratum corneum (II, Fig. 6d). Most of the intercellular lipids showed a typical repeating pattern of Landmann units like in normal skin. However, there were some abnormal lipid droplets as well, a common finding in air-exposed keratinocyte cultures (II, Fig. 6c, e) (Fartasch and Ponec, 1994; Stark et al., 1999).

Since disturbed lipogenesis is common in reconstructed skin models (Fartasch and Ponec, 1994; Vicanova et al., 1998), the lipid composition of REK culture (supplemented with vitamin C) stratum corneum was analysed with thin-layer chromatography by Dr. Philip Wertz, University of Iowa (unpublished data). The analysis revealed that the content of SC phospholipids was markedly higher and triglyceride content lower in organotypic REK culture than in normal human skin. However, the amount of ceramides and glycosylceramides, most important lipids for barrier function, corresponded to that in human skin (**Table II**).

Table II. Composition of stratum corneum lipids (weight %)

Lipid type	Organotypic REK culture	Human skin
Phospholipids	16	4.9
Cholesterol sulfate	n.d.	1.5
Glucosylceramides	0.3	trace
Ceramides	21.7	18.1
Sterols	21.1	14
Free fatty acids	20.1	19.3
Triglyceride	2.1	25.2
Sterol esters	10.3	5.4
Monoacylglycerol	8.4	n.d.
Squalene	n.d.	4.8
n-Alkanes	n.d.	6.1

Human skin data is adapted from Lampe MA, Williams ML, Elias PM: Human epidermal lipids: Characterization and modulations during differentiation. *Journal of Lipid Research* 24:131-140, 1983. n.d., not determined.

6.1.3. Barrier function (I, II)

To characterize the barrier properties of organotypic REK culture, the permeability of frequently used indicator substances, mannitol and corticosterone, and the transepidermal water loss (TEWL) were examined. The relatively normal morphology of REK organotypic cultures was reflected in the permeability barrier properties, which were close to those of human skin (I, Table I). The permeability for corticosterone and TEWL were relatively high in 1-week old cultures, but significantly lower at 2 weeks (II, Fig. 7). The epidermal barrier matured by three weeks in REK culture, since the permeability for corticosterone remained relatively constant after that (II, Fig. 7a). The morphological improvements observed with vitamin C correlated with enhanced epidermal barrier function, as indicated by a marked reduction of the permeation rates of tritiated corticosterone and mannitol, and transepidermal water loss (TEWL) (II, Fig. 7). In vitamin C treated cultures, transdermal permeabilities of corticosterone and mannitol were 0.22×10^{-6} and 0.59×10^{-6} cm/s, and in human cadaver skin these permeabilities were 0.18×10^{-6} and 0.17×10^{-6} cm/s, respectively. TEWL was 19.4 g m^2/h in three-week-old vitamin C treated cultures, and in human skin it was 10.1 g m^2/h (I, Table I).

6.2. HYALURONAN METABOLISM IN ORGANOTYPIC AND MONOLAYER KERATINOCYTE CULTURES

6.2.1. Proliferation, migration and hyaluronan synthesis in EGF- and KGF-treated cultures (III, IV)

EGF (20 ng/ml) induced proliferation in organotypic cultures as indicated by increased BrdU-labeling (III, Fig. 1b). Accordingly, basal cells were taller and the whole vital epidermis showed hypertrophy in EGF-treated cultures (III, Fig. 1a, c). KGF had a similar but smaller effect on keratinocyte proliferation in organotypic cultures (IV, Table III), while in the monolayer cultures it did not affect cell proliferation (IV, Table II).

In monolayer cultures, KGF changed the cells to a more elongated shape (IV, Fig. 1b), with numerous microvilli seen in EM on the upper cell surface (IV, Fig. 1g). This phenotype is typical for migrating cells. Therefore, keratinocyte migration was studied. In an *in vitro* wounding assay, KGF showed a dose-dependent stimulation of REK migration from wound edges to the cleared area (IV, Table II).

KGF increased hyaluronan content in monolayer REK cultures as determined by electrophoresis of hyaluronan derived fluorotagged disaccharides (IV, Table II). Accordingly, HAS activity was increased 2-fold in KGF-treated (10 ng/ml) cultures (IV, Fig. 2a). Enhanced hyaluronan production was confirmed using metabolic double labeling with [^{3}H]glucosamine and [^{35}S]sulfate and histochemical stainings. Both total hyaluronan and the intracellular hyaluronan were increased after 24 h KGF (100 ng/ml) treatment (IV, Fig. 2e). Intracellular hyaluronan showed a 8.5-fold increase in the cultures treated with 100 ng/ml KGF, and was doubled already with 1 ng/ml. The location of hyaluronan in the intracellular compartment was confirmed with confocal microscopy (IV, Fig. 1e). This intracellular hyaluronan was localized in membrane-coated vesicles seen in EM (IV, Fig. 1h). Quantitative denstitometry of the histological stainings showed that intracellular hyaluronan was elevated already 10 min after KGF addition (IV, Fig. 1k). Together, these results suggest that KGF stimulates hyaluronan synthesis and strongly promotes hyaluronan endocytosis.

The molecular mass of radiolabeled hyaluronan was estimated with gel filtration. In monolayer REK cultures, secreted and pericellular hyaluronan were of high molecular mass, approximately 6×10^6 Da. Most of the intracellular hyaluronan was small fragments, below 90 kDa in control cultures. KGF changed the molecular mass distribution of the secreted and intracellular hyaluronan. It strongly increased hyaluronan chains in the intermediate size range, $0.4-2 \times 10^6$ Da especially in the intracellular compartment (IV, Fig. 3).

In organotypic cultures, EGF and KGF increased epidermal hyaluronan synthesis as studied biochemically with hyaluronan ELSA. In 2-week-old control cultures, the

concentration of epidermal hyaluronan was ~150 ng/mg of tissue dry weight. The collagen matrix and the medium, into which a part of the hyaluronan escapes, contained ~2800 ng/mg of tissue dry weight. EGF and KGF caused a dose-dependent increase of hyaluronan in the epidermis, collagen and medium (III, Fig. 2a; IV, Fig. 4g). In EGF (20 ng/ml) and KGF (20 ng/ml) treated cultures, the amount of epidermal hyaluronan was increased 3.6-fold and 4-fold, respectively. In the matrix and medium compartments, a 4.9-fold and 3-fold increase of hyaluronan concentration was found in the EGF- and KGF -treated cultures, respectively (III, Fig. 2a; IV, Fig. 4g).

In control cultures, hyaluronan was found in all vital cell layers, with the highest staining intensity in the spinous cell layer (III, Fig. 3a; IV, Fig. 4c). EGF and KGF increased hyaluronan staining intensity especially in the spinous cell layer (III, Fig. 3c; IV, Fig. 4d). Furthermore, hyaluronan was found also in the stratum corneum in growth factor treated cultures (III, Fig. 3c; IV, Fig. 4d). In addition to the pericellular hyaluronan, EGF-treated cultures contained dramatically increased amounts of intracellular hyaluronan, which was confirmed by confocal microscopy (III, Fig. 3h). The expression of the hyaluronan receptor CD44 was relatively low in control cultures, but increased after EGF and KGF stimulation (III, Fig. 3d; IV, Fig. 4f). In the EGF-treated cultures, the CD44 signal was mainly detected in the basal cell layer, whereas in the KGF-treated cultures the increased expression was localized to the spinous cell layer.

To reveal the hyaluronan synthase genes responsible for the increased hyaluronan synthesis in EGF- and KGF-treated cultures, semi-quantitative RT-PCR was performed. All *Has* isoenzymes are expressed in monolayer REK cultures (Pienimäki et al., 2001). In the organotypic cultures, *Has1* expression was very low, and showed no change with either EGF or KGF, while cultures treated with EGF or KGF (20 ng/ml) showed an elevated expression of *Has2*, *Has3* and *CD44* (III, Fig. 2b; IV, Fig. 4h).

6.2.2. Differentiation in EGF- and KGF-treated cultures (III, IV)

In EGF- and KGF-treated cultures, the differentiation of keratinocytes was deficient. The expression of keratin 10 and filaggrin was decreased as studied using immunohistochemistry and Western blotting (III, Fig. 4, 5; IV, Fig. 5). EGF had no effect on the high molecular mass profilaggrin, but it blocked the processing of profilaggrin into its mature, low molecular mass form (III, Fig. 5). KGF caused a marked reduction in the expression of the early epidermal differentiation marker, keratin 10, but it had only a minor effect on filaggrin maturation (IV, Fig. 5e). These alterations in the differentiation program were accompanied with defective epidermal barrier function in EGF- and KGF- treated cultures, as indicated with increased coritcosterone permeability (13.7×10^{-6} cm/s control; 21.1×10^{-6} cm/s EGF, $p<0.01$; 17.3×10^{-6} cm/s KGF) (III, Fig. 5c; IV, Fig 5f).

6.2.3. Hyaluronan synthesis and *Has* and *CD44* mRNA expression in TGF-β –treated cultures (III)

The epidermal morphology correlated with the hyaluronan content in that a low hyaluronan synthesis rate was observed in the TGF-β–treated cultures with slightly atrophic epidermis, low proliferation rate and few suprabasal cell layers (III, Fig. 1). Cultures containing 4 ng/ml TGF-β showed a 54% decrease in keratinocyte proliferation as indicated by BrdU-labeling. Compared to control cultures, hyaluronan content was not changed in the epidermal compartment after TGF-β treatment (III, Fig. 2a). However, in the medium and matrix compartments the concentration of hyaluronan was significantly decreased (III, Fig. 2a). In contrast to EGF and KGF, TGF-β reduced *Has2*, *Has3* and *CD44* mRNA expression (III, Fig. 2b). TGF-β had no effect on keratinocyte differentiation markers (III, Fig. 5).

6.2.4. The effects of all-*trans* retinoid acid and EGFR-, MEK- and MMP-inhibitors on keratinocyte growth and hyaluronan metabolism (V)

Organotypic REK cultures were treated with 0.1 μM all-*trans* retinoic acid (RA) for 10 days to investigate the effects of RA on epidermal morphology and hyaluronan synthesis. All-*trans* RA caused epidermal hyperplasia (V, Fig. 2c, d) as shown in

previous reports (Fisher and Voorhees, 1996; Chapellier et al., 2002). The number of vital cell layers was increased and the epidermal morphology was partly disorganized in all-*trans* retinoic acid treated cultures. The RA -induced hyperplasia was reversed with the EGFR-inhibitor, AG1478 (V, Fig. 2e, f), suggesting that the mitogenic effects of all-*trans* retinoic acid are mediated through EGFR. An inhibitor of MEK, UO126 (V, Fig. g, h), had somewhat similar effects on all-*trans* RA –induced epidermal hyperplasia as the EGFR-inhibitor, while a broad spectrum inhibitor of matrix metalloproteinases (MMPs) totally blocked all-*trans* RA –induced epidermal hyperplasia (V, Fig. 2i, j).

Hyaluronan synthesis was studied histologically (V, Fig. 2), and biochemically with an ELSA-assay (V, Fig. 3a) and metabolic labeling (V, Fig. 3b). Hyaluronan production was elevated in all-*trans* RA –treated cultures, as seen in previous reports (King, 1984; Tammi and Tammi, 1986). All-*trans* RA caused hyaluronan accumulation especially in the suprabasal cells, where hyaluronan located both pericellularly and intracellularly. All of the studied inhibitors (AG1478, GM6001 and UO123) reduced all-*trans* RA –induced hyaluronan staining intensity. Furthermore, all-*trans* RA had an obvious effect on CD44 expression (V, Fig. 2d). In control cultures, CD44 located mostly in the basal cell layer. All-*trans* RA greatly increased CD44 staining intensity, and it was also found in the spinous cell layer and in the collagen matrix in these cultures. All inhibitors also reversed the effect of all-*trans* RA on CD44 expression.

Biochemical methods supported the histological findings. The total hyaluronan concentration of REK organotypic cultures (including hyaluronan in epidermis, collagen matrix and medium) was measured with the ELSA assay. The EGFR receptor inhibitor AG1478 (0.1 μM) caused a 30 % decrease in hyaluronan concentration, suggesting that either endogenous growth factors or those in the medium with serum stimulated hyaluronan production under basic culture conditions. All-*trans* RA raised the total hyaluronan concentration by 49%. This RA –induced increase in hyaluronan concentration was partially reversed by the EGFR inhibitor (AG1478), while the MEK-kinase inhibitor (UO126) and the MMP-inhibitor (GM6001) totally blocked the stimulatory effect of all-*trans* RA (V, Fig. 3a).

Glycosaminoglycan synthesis rate was studied using metabolic labeling (V, Fig. 3b-d). After 24 h, total hyaluronan concentration was approximately 400 ng/mg (dry weight) in control cultures, and this was more than doubled with all-*trans* RA (0.1 μM) treatment (2.5x). All-*trans* RA stimulated also the synthesis of sulfated glycosaminoglycans in REK cultures. All-*trans* RA induced glycosaminoglycan synthesis was suppressed with the inhibitors of EGFR signaling, MEK signaling, and MMP activity. Gel filtration analysis revealed that neither all-*trans* RA nor AG1478 changed the molecular mass distribution of the secreted hyaluronan (in medium and collagen) (V, Fig. 3e). Most of the newly synthesized hyaluronan was of high molecular mass in the organotypic REK cultures.

In monolayer REK cultures, all-*trans* RA increased hyaluronan synthesis 2-fold compared to the control cultures as studied biochemically with hyaluronan ELSA (V, Fig. 4a). Like in the organotypic cultures, the RA –induced hyaluronan production was reversed with the EGFR, MEK and MMP inhibitors in the monolayer REK cultures.

In vivo results correlated with those in the organotypic cultures. All-*trans* RA induced epidermal hyperplasia and hyaluronan accumulation in mouse tail and ear skin treated with RA for 4-14 days (V, Fig. 1a-d). Histologically, an intense hyaluronan signal was present in all-*trans* RA –treated ear epidermis (V, Fig. 1c). In tail epidermis, hyaluronan accumulation was obvious in orthokeratotic skin regions (V, Fig. 1d), while its expression was not changed in the parakeratotic scale regions. Thus, the total content of tail hyaluronan was increased only slightly (from 30±9 ng/mg to 50 ng/mg) after 4 days RA treatment (V, Fig. 1e) Furthermore, the expression of CD44 was increased in mouse skin treated with RA as suggested by immunohistochemical stainings (V, Fig. 1d).

6.2.5. *Has2*, *Has3*, *CD44* and *HB-EGF* mRNA expression after all-*trans* RA stimulation

Organotypic cultures were treated with 0.1 μM RA with or without the EGFR inhibitor (AG1478) for 1 to 12 h, and analyzed for several mRNA levels with semi-quantitative

RT-PCR. *Has2*, *Has3*, *CD44* and *HB-EGF* mRNA were rapidly upregulated after all-*trans* RA exposure. The EGFR-inhibitor AG1478 blocked the all-*trans* RA –induced *Has2*, *Has3* and *HB-EGF* expression, and suppressed their expression also in the control cultures. This suggests that EGFR ligands such as HB-EGF and TGF-α are endogenously expressed in organotypic REK cultures and their effects are inhibited by AG1478. Increased *HB-EGF* expression in all-*trans* RA treated cultures and mouse tail skin supports the idea that the mitogenic effects of all-*trans* RA are partly mediated through EGFR signaling pathway.

6.2.6. Alterations in protein phosphorylation after all-*trans* retinoic acid treatment

Protein phosphorylation was studied 1-8 h after RA-treatment in Western blotting. After 1 h, there were no differences between control and all-*trans* RA –treated cultures. However, the inhibitory effect of AG1478 on Erk1/2 phosphorylation was seen already after 1 h. After 2-h RA (0.1 μM) stimulation, EGFR and Erk1/2 phosphorylations were increased compared to control, and after 4-h RA exposure Erk1/2 phosphorylation was prominently elevated. All-*trans* RA –induced Erk1/2 stimulation was completely inhibited with AG1478 at 2 and 4 hours. The phosphorylation of Stat3 transcription factor, JNK and c-JUN were also elevated in 4-h RA treated cultures. Neither all-*trans* RA nor AG1478 influenced the phosphorylation level of PI3K and the transcription factor Elk.

6.2.7. Keratinocyte differentiation in all-*trans* retinoic acid treated cultures

Keratinocyte differentiation was compromised in all-*trans* RA treated cultures as indicated by K10 and filaggrin expression in Western blotting and immunohistochemistry. After a 10-day RA treatment, K10 expression was obviously reduced, and was shifted from the spinous and granular cell layers to SC. Furthermore, profilaggrin maturation was deficient in these cultures. However, in contrast to the RA –induced hyaluronan synthesis and tissue hyperplasia, the retarded differentiation caused by all-*trans* RA was not reversed with EGFR-, MEK- and MMP-inhibitors. This implies that the effects of all-*trans* RA on differentiation and hyaluronan synthesis are mediated through different signaling pathways.
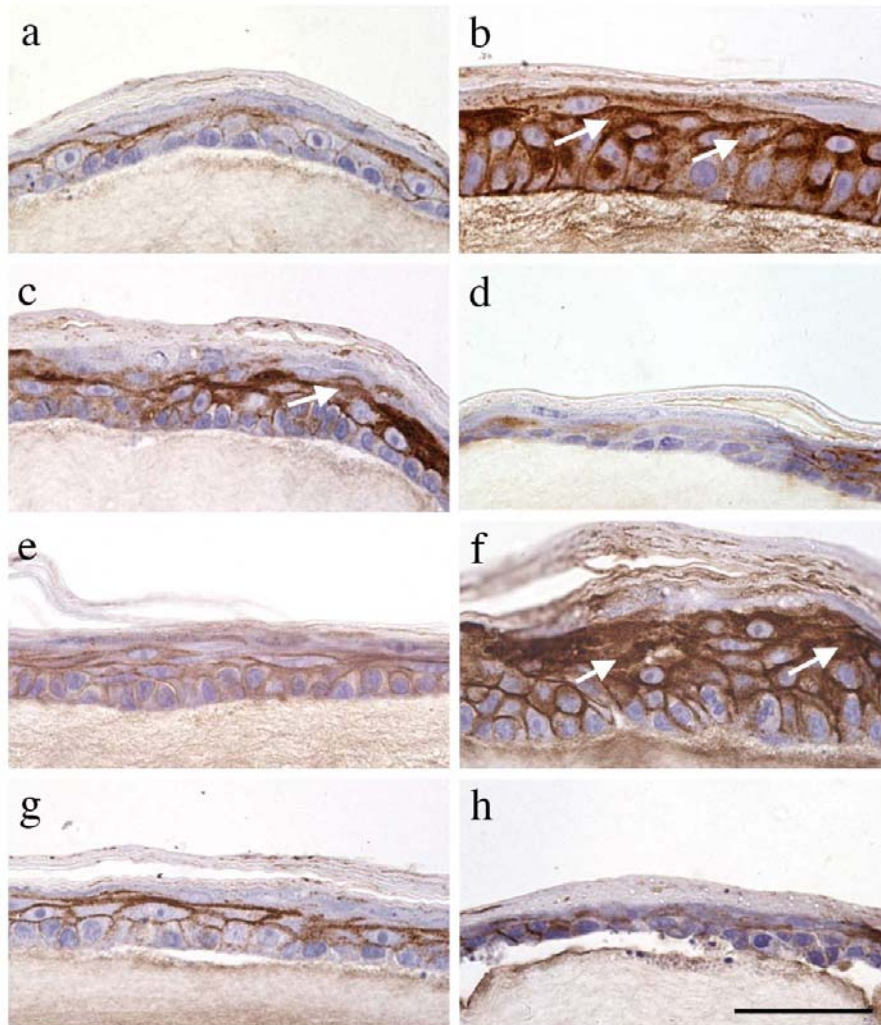
Figure 8. Epidermal morphology and hyaluronan localization in control (*a*), EGF (20 ng/mg, *b*), KGF (20 ng/mg, *c*), TGF-beta (4 ng/mg, *d*), vitamin C (40 μg/ml, *e*), all-*trans* RA (0.1 μM, *f*), all-*trans* RA + AG1478 (0.1 μM, *g*) and all-*trans* RA + GM6001 (5 μM, *h*) –treated organotypic REK cultures. In the mitogenic growth factor –treated cultures, hyaluronan was localized also intracellularly (*arrows* in *b*, *c*, *f*). *Scale bar* 50 μm.

7. DISCUSSION

7.1. Organotypic keratinocyte culture

In reconstructed skin models, keratinocytes are cultivated at the air-liquid interface on various substrates serving as a dermal equivalent. Such skin models are used to study keratinocyte gene expression (Cerezo et al., 2003), wound healing (Steude et al., 2002), skin irritation (de Brugerolle de et al., 1999; Pappinen et al., 2005), drug permeability (Asbill et al., 2000; Suhonen et al., 2003) and epidermal-dermal interactions (Hinterhuber et al., 2002). In organotypic cultures, keratinocytes stratify, express several differentiation markers, and form an apparently normal stratum corneum. Hence, they offer a more reliable tool to study keratinocyte biology than classical submerged monolayer keratinocyte cultures. The establishment and development of such three-dimensional keratinocyte cultures are often a time consuming and difficult process, because the complex epidermal structure is properly assembled only during undisturbed keratinocyte differentiation. Therefore, in several reconstructed epidermal cultures, the stratum corneum ultrastructure is abnormal, and permeability higher than in intact human skin (Nolte et al., 1993; Schmook et al., 2001), because of deficient keratinocyte differentiation (Schoop et al., 1999). In recent decades, a number of different kinds of skin equivalents have been developed for clinical and research purposes, but a simple model replicating the epidermal differentiation program has been missing. In this thesis work, an immortalized REK line, originally isolated by Baden and Kubilus (1983), was utilized for developing an organotypic keratinocyte culture. REKs were grown on type I collagen gel without feeder cells, unlike nearly all organotypic models in current use (Parenteau et al., 1992; Schoop et al., 1999; Asbill et al., 2000; Kim et al., 2002). This made it possible to study keratinocyte differentiation and epidermal hyaluronan metabolism without the influence of dermal cells, which participate in hyaluronan synthesis and catabolism, and which secrete various soluble growth factors.

7.2. The effects of vitamin C on SC ultrastructure and epidermal barrier function

In papers I-II, it was shown that REKs are able to produce a skin equivalent in which morphology and barrier properties resembles those of intact human skin. The expression patterns of the differentiation markers keratin 10, involucrin and filaggrin, corresponded to that of epidermis *in vivo*. However, like other organotypic cultures (Nolte et al., 1993; Pouliot et al., 1999), the basic REK culture model showed some imperfections in the stratum corneum ultrastructure, such as disorganized intercellular lipid lamellae and intercellular lipid accumulations. These defects in the SC structure are suggested to be due to a hyperproliferative state of the air-exposed cultures (Fartasch and Ponec, 1994).

Ponec and coworkers (1997) showed that vitamin C supplementation of the human keratinocyte culture medium improves these deficiencies by normalizing epidermal morphology, and by regulating the synthesis of competent barrier lipids. It stimulates keratinocyte glucosylceramide and ceramide production (Ponec et al., 1997; Uchida et al., 2001). Similarly as vitamin C functions as a cofactor in prolyl hydroxylation in collagen synthesis (Kivirikko et al., 1989) and in HIF (hypoxia-inducible factor) transcriptional cascade (Knowles et al., 2003), it may also promote the hydroxylation of fatty acids and sphingoid bases (Ponec et al., 1997). Supplementation of the REK culture medium with vitamin C confirmed the previous results – it improved the general epidermal morphology and the SC ultrastructure. In vitamin C treated cultures, the amount of intercellular lipid lamellae between corneocytes was higher than in control cultures, and they showed the proper arrangement, the Landmann unit pattern (Swartzendruber et al., 1989), which is critical for epidermal barrier function (Elias and Menon, 1991; Bouwstra et al., 1996).

Barrier formation was studied in side-by-side diffusion chambers, and the permeabilities were compared to human cadaver skin. Because hydration of SC influences the permeation rate (Cumpstone et al., 1989), the cadaver skin was prehydrated prior to the study to ensure that water content was comparable in organotypic REK cultures and cadaver skin. In organotypic REK cultures, the morphological improvements obtained

with vitamin C correlated with enhanced barrier function as indicated by reduced TEWL and the permeability of corticosterone and mannitol. The permeability results are in line with the results from Dr. Steven Boyce's group. They also demonstrate that incubation of cultured skin substitutes with vitamin C results in better epidermal barrier (Boyce et al., 2002). The barrier for corticosterone and mannitol developed in three weeks in REK cultures. Thereafter the permeation rate did not change, even though the number of the cornified cell layers increased in extended cultures because of the lack of desquamation in organotypic cultures. This is consistent with the *in vivo* finding that epidermal barrier function does not correlate with the number of cell layers in the stratum corneum (Elias et al., 1981a). In addition to corticosterone and mannitol, the permeability of 16 other compounds with different charge, molecular mass and octanol-water partition coefficient have been characterized in organotypic REK cultures (supplemented with vitamin C) (Suhonen et al., 2003). These permeability studies demonstrate that organotypic REK culture provides a close estimate of human epidermal permeabilities over the whole range of the solutes used, with about 2-fold higher permeability (range 0.3–5.2) than those obtained from human cadaver epidermis (Suhonen et al., 2003). The permeability in REK cultures corresponds to that of a human skin equivalent model (Asbill et al., 2000).

7.3. Stratum corneum lipids in the organotypic REK culture

In spite of continuous efforts, the permeability has remained higher in reconstructed skin models compared to human skin (Nolte et al., 1993; Asbill et al., 2000). The higher permeability in organotypic cultures probably reflects abnormalities in the barrier lipid profile (Vicanova et al., 1998), as well as in the intercellular organization of the lamellar body contents (Fartasch and Ponec, 1994). In the vitamin C exposed organotypic REK culture, SC lipids generally corresponded to those of human, but the contents of phospholipids were higher, as analyzed with thin-layer chromatography (Dr. Philip Wertz, unpublished data). During keratinocyte differentiation the changes in their lipid composition include replacement of polar lipids by neutral lipids, and the marked decline of phospholipids which means that ceramides, free fatty acids and cholesterol

are the main SC lipid species (Wertz and Downing, 1989). Higher content of phospholipids in the organotypic REK culture is in line with the somewhat higher permeability than that in cadaver skin. Furthermore, rodent skin has naturally a less effective barrier function than human skin (Schmook et al., 2001), which may also partly explain the differences in the permeation rate of REK culture model and cadaver skin. However, the amount of ceramides, important in barrier function (Wertz et al., 1983; Bouwstra et al., 1996), was relatively similar in organotypic REK cultures and human skin (**Table II**). In spite of these differences in the barrier lipid profile and function compared to human skin, the REK organotypic cultures offer an excellent model for permeability tests of topical drugs, combining the easily maintained and reproducible cultures from a continuous cell line with the reasonably good permeability characteristics.

7.4. The influences of vitamin C on keratinocyte gene expression

Our findings revealed that barrier lipids are not the only targets of vitamin C on epidermal differentiation, since it also affected keratincyte gene expression. It increased profilaggrin mRNA expression, as well as the size and the number of keratohyalin granules. Furthermore, the overall morphology was improved after vitamin C supplementation. It is known that vitamin C modulates chondrocyte growth, protein synthesis and maturation (Farquharson et al., 1998; Freyria et al., 1999), and promotes fibroblast proliferation and collagen synthesis (Phillips et al., 1994). With the exception of collagen synthesis, the molecular mechanisms of vitamin C action are open. Our results were the first to suggest that vitamin C has a direct effect on keratinocyte gene expression, but thereafter it has been shown to upregulate several other genes in human keratinocytes (Catani et al., 2002; Savini et al., 2002). As well as acting as an antioxidant, free radical scavenger, it seems to be a pro-differentiating agent for keratinocytes. In addition to profilaggrin, it stimulates cornified envelope formation (Savini et al., 2002), a structure contributing to the epidermal barrier function. Vitamin C induced differentiation has been demonstrated to proceed through a PKC-dependent activation of the activating protein 1 (AP-1) transcription factor (Savini et al., 2002).

The results from Kim and co-workers (Kim et al., 2002) also support the idea that vitamin C regulates keratinocyte differentiation, but it remained unclear whether vitamin C affects keratinocytes directly or indirectly through effects on fibroblasts. In addition to its effects on keratinocyte gene expression, vitamin C may improve keratinocyte differentiation pattern through basement membrane. In cultured human skin substitutes, vitamin C stimulates the expression and organization of the basement membrane antigens (Boyce et al., 2002), which have been suggested to promote keratinocyte differentiation (Ralston et al., 1999). Whether the basement membrane proteins are also expressed in vitamin C treated organotypic REK cultures, remains to be elucidated.

7.5. Hyaluronan and keratinocyte differentiation

In general, undifferentiated cells seem to produce more hyaluronan than differentiated cells (Ågren et al., 1997a; Suzuki et al., 2005), and hyaluronan production associates with cell proliferation and migration, e.g. during wound healing (Mack et al., 2003; Tammi et al., 2005). However, its role in differentiation is less investigated, but it is hypothesized to suppress keratinocyte differentiation (Ågren et al., 1995). The results obtained in studies III-V support this idea. EGF (III), KGF (IV) and all-*trans* retinoic acid (V) stimulated epidermal hyaluronan synthesis, which associated with disturbed epidermal differentiation characterized by diminished keratin 10 expression, defective profilaggrin maturation and barrier formation. A conspicuous finding was that in cultures with enhanced hyaluronan production, hyaluronan was accumulated especially in the upper spinous and granular cell layer, the site of initiation of terminal differentiation and almost devoid of hyaluronan in normal human skin (Tammi et al., 1988). A similar accumulation of hyaluronan in the upper spinous cell layer and delayed differentiation has been demonstrated in human skin organ cultures treated with all-*trans* retinoic acid (Tammi et al., 1985; Tammi et al., 1989).

It is difficult to evaluate whether hyaluronan has some intrinsic role in differentiation or, whether its concentration changes as a result of altered differentiation. Passi and co-

workers (2004) demonstrated the role of hyaluronan in epidermal differentiation by removing it with *Streptomyces* hyaluronidase -treatment. The loss of epidermal hyaluronan in the upper spinous and granular cell layers induces terminal differentiation in organotypic keratinocyte cultures (Passi et al., 2004). Also, in mouse skin with disrupted barrier, the removal of hyaluronan in the upper epidermal cell layers accelerates keratinocyte differentiation (Maytin et al., 2004). Furthermore, hydrocortisone, which promotes keratinocyte differentiation, reduces epidermal hyaluronan production (Ågren et al., 1995). Our unpublished data with vitamin C in organotypic REK cultures are parallel with these studies. During differentiation induced by vitamin C, hyaluronan production was greatly diminished as compared to control cultures as studied biochemically with hyaluronan ELSA. Based on these results, it appears that hyaluronan plays a regulatory role in keratinocyte differentiation, and the concentration of hyaluronan must be reduced to initiate keratinocyte differentiation. Accordingly, hyaluronan synthesis was decreased (Lamberg et al., 1986; Akiyama et al., 1994) and hyaluronidase activity increased (Frost and Stern, 1997) after keratinocytes were induced to differentiate with elevated extracellular Ca^{2+}. Hyaluronan seems to influence the signaling pathways regulating differentiation, but as yet almost no data are available on this topic. In barrier formation, this hydrophilic molecule, if not removed in time, may interfere the exocytosis of lamellar body contents or disturb the organization of the intercellular lipids that are important for the epidermal barrier. Due to the hydrophilicity, it is able to increase water content in the upper epidermal cell layers and cause increased permeability, a situation found in psoriatic skin (Micali et al., 2001). Interestingly, in addition to pericellular hyaluronan, hyaluronan was also localized intracellularly in cultures with compromised differentiation. It is possible that especially this intracellular hyaluronan interferes differentiation, perhaps by lowering intracellular calcium concentration, or disturbing it in some other way.

7.6. Hyaluronan and proliferation

Studies III-V show that enhanced hyaluronan synthesis is associated with elevated proliferation rate and increased epidermal thickness, and a decline in hyaluronan

synthesis is associated with diminished proliferation and epidermal atrophy, respectively. The idea of hyaluronan contributing to the regulation of epidermal thickness and keratinocyte proliferation rate receives support from earlier studies of human skin organ cultures, indicating that hydrocortisone inhibits hyaluronan synthesis and simultaneously reduces keratinocyte proliferation and epidermal thickness (Ågren et al., 1995). A similar suppressing effect on keratinocyte proliferation is observed with 4-methylumbelliferone, an inhibitor of hyaluronan synthesis (Rilla et al., 2004). Furthermore, cells expressing antisense *Has2* show reduced hyaluronan synthesis and retarded proliferation (Rilla et al., 2002). In contrast, all-*trans* retinoic acid causes elevated hyaluronan synthesis and epidermal hyperplasia in human skin organ cultures (Tammi et al., 1985). Thus, hyaluronan synthesis correlates directly with keratinocyte proliferation, and the thickness of vital epidermis. Hyaluronan may support epidermal hyperplasia by providing a loose matrix for the changes in cell shape that occur during mitosis, promoting stratification of keratinocytes, and facilitating diffusion of nutrients. However, it is not clear at present whether it stimulates cell proliferation directly by inducing signaling e.g. via CD44 (Turley et al., 2002), or facilitates proliferation physically.

There is a large amount of evidence showing that pericellular, and also intracellular hyaluronan content is increased in proliferating cells (Brecht et al., 1986; Tammi and Tammi, 1991; Evanko et al., 1999). In mitotic cells, hyaluronan localized between the dividing cells facilitates cell detachment during cytokinesis (Tammi and Tammi, 1991; Evanko and Wight, 1999). Recently, intracellular hyaluronan has been shown to associate with RHAMM and mitotic spindle microtubules suggesting that it may also have a role in chromosome rearrangements during mitosis (Evanko et al., 2004). On the other hand, there are some data that challenge the importance of hyaluronan in cell proliferation. During epidermal development, hyaluronan content is lower in the proliferating basal cell layer, compared to the spinous cell layer (Ågren et al., 1997a). This is in accordance with our findings in hyperplastic REK cultures, in which proliferating cells mostly reside in the basal cell layer while hyaluronan staining was most intense in the spinous and granular cell layers. Furthermore, after epidermal barrier

disruption, which leads to hyperproliferation, keratinocyte proliferation is not altered if hyaluronan is removed with *Streptomyces* hyaluronidase treatment (Maytin et al., 2004). It appears that the presence of hyaluronan promotes mitosis but its amount does not necessarily correlate with cell proliferation.

7.7. Hyaluronan and migration

The results obtained from study IV indicate that KGF has a strong effect on keratinocyte migration, which correlates with increased hyaluronan synthesis, suggesting that hyaluronan plays an essential role in the KGF-stimulated keratinocyte motility. Similar connection between enhanced migration and increased hyaluronan synthesis is found in keratinocytes stimulated with EGF (Pienimäki et al., 2001), human melanoma cells (Ichikawa et al., 1999), vascular smooth muscle cells (Evanko et al., 1999) and fibroblasts (Hall et al., 2001). Suppressed hyaluronan synthesis, on the contrary, has been shown to associate with reduced keratinocyte motility (Rilla et al., 2004). There is strong evidence that the expression of *Has* genes control cell motility. It was recently demonstrated that transfection of the *Has* genes into fibroblasts increased cell migration (Itano et al., 2002), and transfection of the antisense *Has2* suppressed REK migration (Rilla et al., 2002). However, there are also contradictory results, indicating that the influence of hyaluronan synthesis on motility depends on the cell type and the expression level (Brinck and Heldin, 1999; Dube et al., 2001).

The connection between hyaluronan and cell migration is obvious during wound healing (Mack et al., 2003; Tammi et al., 2005). At the wound edge, hyaluronan is abundant in migrating keratinocytes (Oksala et al., 1995). Furthermore, in wounded mouse epidermis *Has2* and *Has3* are strongly upregulated and hyaluronan content elevated (Tammi et al., 2005). During wound healing, local growth factors and cytokines, like KGF, EGF, IL-1β and IFN-γ, stimulate hyaluronan synthesis, which favours wound closure. Recently, it was indicated that HOXB13 knockout mice, which show enhanced wound healing, exhibit elevated epidermal and dermal hyaluronan content (Mack et al., 2003). During re-epithelialization, hyaluronan provides a loose matrix favourable for

keratinocyte migration. Furthermore, it probably has also a more direct role acting as a signaling molecule that influences the intracellular locomotory system through cell surface receptors (Bourguignon et al., 2000, 2001). However, it is interesting that exogenous hyaluronan does not have a similar stimulatory effect on keratinocyte migration as endogenous hyaluronan, suggesting that hyaluronan synthesis i.e. *Has* activation is vital for migration (Rilla et al., 2002).

Overall, the data are consistent with the hypothesis that hyaluronan synthesis supports keratinocyte proliferation and migration, and suppresses differentiation.

7.8. The expression of *Has* and *CD44*

Although hyaluronan synthases have been discovered several years ago (DeAngelis et al., 1993; Itano and Kimata, 1996a, b; Shyjan et al., 1996; Spicer et al., 1996; Watanabe and Yamaguchi, 1996; Fulop et al., 1997; Spicer et al., 1997a), the precise regulatory mechanisms of each *Has* gene and HAS enzyme are still largely unknown. The present results demonstrated that the levels of *Has2* and *Has3* mRNA regulated by EGF, TGF-β (III), KGF (IV) and all-*trans* retinoic acid (V) corresponded to the synthesis of hyaluronan in organotypic REK cultures. These findings are parallel with previous results showing that hyaluronan synthesis is regulated mainly at the mRNA level (Jacobson et al., 2000; Pienimäki et al., 2001; Yamada et al., 2004). In line with the present findings, all-*trans* retinoic acid upregulates *Has2* in human keratinocyte cell line (HaCat) (Saavalainen et al., 2005) and TGF-β suppresses *Has3* expression in human keratinocytes (Sayo et al., 2002). Furthermore, similar correlations between *Has2* mRNA and hyaluronan synthesis were observed after KGF (IV), EGF (Pienimäki et al., 2001) and all-*trans* RA (V) (Saavalainen et al., 2005) administration to monolayer REK cultures, suggesting that hyaluronan synthesis, i.e. *Has* mRNA expression, is an important target of these growth factors. These growth factors may contribute to epidermal homeostasis in part by modulating *Has* gene expression and epidermal hyaluronan concentration.

The mRNA levels of CD44, a major hyaluronan binding receptor in epidermal keratinocytes (Tammi et al., 1998) was modulated in the pattern somewhat similar to *Has2* and *Has3*, i.e. with an upregulation by EGF, KGF and all-*trans* retinoic acid, and a tendency to downregulation by TGF-β. Although the changes in CD44 mRNA level were modest as compared to those of *Has2* and *Has3*, CD44 protein appeared markedly upregulated in EGF-, KGF- and all-*trans* retinoic acid -treated cultures. As mentioned above, CD44 has been suggested to mediate the effects of hyaluronan on cell migration and proliferation (Kaya et al., 1997; Bourguignon et al., 2001). Furthermore, it is involved in the organization of the pericellular hyaluronan matrix and internalization of hyaluronan (Tammi et al., 2001; Knudson et al., 2002). In the present studies, enhanced extracellular hyaluronan in organotypic REK cultures correlated with increased CD44 expression and elevated intracellular hyaluronan, suggesting that hyaluronan was internalized via CD44 probably for degradation. It has been indicated that in hyaluronan endocytosis in REKs, hyaluronan binds to clustered CD44 before internalization (Tammi et al., 2001). Apparently, CD44 plays a regulatory role in hyaluronan metabolism (Kaya et al., 2000), and thus its expression is regulated by the same set of growth factors that regulate *Has* genes. It can be anticipated that elevated synthesis is coupled to increased catabolism (Tammi et al., 2000), and decreased synthesis to diminished catabolism (Ågren et al., 1995) to maintain a balance in epidermal hyaluronan concentration. Hyaluronan catabolism cannot be studied reliably in this organotypic culture model, because of the lack of the basement membrane. Basement membrane has been shown to restrict effectively the diffusion of hyaluronan out of the epidermis, resulting in increased catabolism within the epidermis (Tammi et al., 2000).

7.9. All-*trans* retinoic acid induced signaling involved in hyaluronan synthesis

Although retinoids have been used in several skin diseases such as psoriasis and acne, their exact regulatory mechanisms on keratinocyte growth are largely unknown. It has been established that retinoids modulate cell growth, differentiation and apoptosis by regulating gene expression primarily via association with their nuclear receptors. Retinoid receptors (RARs and RXRs) are ligand-activated transcription factors, which

can either enhance or suppress the expression of target genes by interacting with RARE in the promoters (Bastien and Rochette-Egly, 2004). However, only few primary RA inducible genes with functional RAREs have been found so far in keratinocytes (Xiao et al., 1999). Recently, human *Has2* gene was found to be a potential all-*trans* RA responding gene in keratinocytes (Saavalainen et al., 2005). In organotypic REK cultures, *Has2* and *Has3* were upregulated, and were responsible for the elevated hyaluronan synthesis after all-*trans* RA exposure. However, *Has2* and *Has3* were most upregulated at 4h and 8h after RA stimulation, suggesting that in this time scale, other signaling mechanisms than nuclear receptors may also be involved in *Has* activation.

It has been reported that in epidermis, the hyperplastic response of all-*trans* RA is connected to HB-EGF expression (Xiao et al., 1999; Varani et al., 2001). Our results also demonstrated that all-*trans* RA enhances *HB-EGF* mRNA expression in mouse skin epidermis and in organotypic REK cultures. Using a broad-spectrum MMP inhibitor GM6001, it was demonstrated that all-*trans* RA –induced hyperplasia and hyaluronan synthesis were inhibited, suggesting that the MMPs inhibited by GM6001 (i.e. MMP-1, -2, -3, -9 (Santiskulvong and Rozengurt, 2003)) are responsible for HB-EGF release. The present data support the hypothesis that HB-EGF is the paracrine factor that contributes to all-*trans* RA-induced epidermal hyperplasia and hyaluronan synthesis. The use of the EGFR inhibitor AG1478 and the MEK inhibitor UO126 supported the idea that EGFR signaling is stimulated by all-*trans* RA-treatment.

Western blotting revealed that all-*trans* RA phophorylates and activates EGFR, leading to Erk1/2 activation. Recently, it has been reported that low concentrations of all-*trans* RA increase EGF production, EGFR expression and Erk1 activity in a SCC line (Crowe et al., 2003). It is noteworthy that in organotypic REK cultures, EGFR and Erk were activated already after a 2-hour-RA-stimulation, raising the question whether all-*trans* retinoic acid is also able to trigger the EGFR signaling cascade directly, without *de novo* protein synthesis. However, the precise signaling events between all-*trans* RA and EGFR are still unknown. Estradiol, which also influences the expression of target genes mainly through its nuclear receptors, has been demonstrated to stimulate membrane-

bound G-proteins, leading to the activation of MMPs and liberation of HB-EGF and EGFR transactivation (Razandi et al., 2003). In neuronal cells, all-*trans* RA induces rapid Erk activation, proposed to be mediated through RA's binding to cytoplasmic RARs, which activates Erk by direct interaction with upstream components of the signaling pathway (Canon *et al*, 2004). Further investigation is needed to define if all-*trans* retinoic acid could trigger similar kind of signaling cascades leading to EGFR transactivation also in epidermal keratinocytes.

In summary, all-*trans* retinoic acid caused epidermal hyperplasia and enhanced hyaluronan synthesis by 1) upregulating the expression of *HB-EGF* and *Has2* and *Has3* possibly via its nuclear receptors and 2) activating EGFR signaling, which leads to Erk1/2 activation (**Fig 9**).
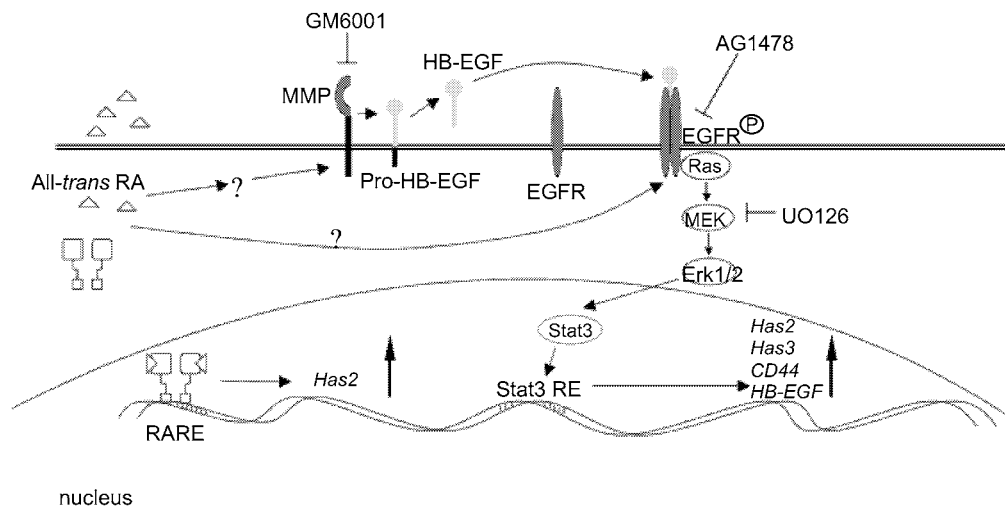

Figure 9. Schematic presentation of the alternatives for all-*trans* RA –induced signaling leading to epidermal hyperplasia and enhanced hyaluronan production in organotypic REK cultures.

8. SUMMARY OF RESULTS

The main scientific findings of the current thesis are:

- Rat epidermal keratinocytes grown on type I collagen at the air-liquid interface formed a structure resembling normal epidermis, with barrier properties close to those in human skin.

- Vitamin C affects keratinocyte gene expression without the influence of dermal cells. It stimulated profilaggrin mRNA expression and facilitated epidermal barrier formation.

- Mitogenic growth factors, EGF and KGF, elevated keratinocyte hyaluronan synthesis by upregulating *Has2* and *Has3* mRNA. These growth factors also stimulated CD44 expression and hyaluronan uptake.

- TGF-β inhibited *Has2* and *Has3* expression and hence decreased epidermal hyaluronan production.

- All-*trans* retinoic acid upregulated the expression of *Has2, Has3, CD44* and *HB-EGF* in keratinocytes.

- All-*trans* retinoic acid –induced hyaluronan production and epidermal hyperplasia were inhibited by EGFR-, MEK- and MMP-inhibitors, suggesting that EGFR signaling pathway contributed to these effects.

To summarize, an *in vitro* epidermal model was established in the present work. This cell culture model, currently used in drug transport, toxicity and basic skin biology research in many laboratories, can reduce the need for animal tests. Furthermore, the results support the hypothesis that hyaluronan synthesis correlates directly with epidermal thickness and keratinocyte migratory phenotype, and inversely with keratinocyte differentiation. Alterations in hyaluronan synthesis and concentration regulate epidermal keratinocyte behavior – hyaluronan appears to keep epidermal keratinocytes in an undifferentiated state able to proliferate and migrate. Thus, its expression is essential during wound healing. Moreover, for the initiation of proper keratinocyte terminal differentiation, the amount of this molecule must be decreased in the upper spinous and granular cells.

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APPENDIX: ORIGINAL PUBLICATIONS I-V

Kuopio University Publications D. Medical Sciences

D 353. Väisänen, Jussi. Non-competitive NMDA receptor antagonists in rodent modelling of schizophrenia.
2005. 90 p. Acad. Diss.

D 354. Soininvaara, Tarja. Bone mineral density changes after total knee arthroplasty.
2005. 83 p. Acad. Diss.

D 355. Kukkonen, Jarmo. Terveydenhuollon vaikuttavuuden arviointi rutiinisti kerätyn tiedon pohjalta.
2005. 252 p. Acad. Diss.

D 356. Papp, Anthony. Experimental thermal injury: new methods in assessing tissue damage.
2005. 78 p. Acad. Diss.

D 357. Berg, Marja. CT angiography in the assessment of atherosclerotic carotid and renal arteries.
2005. 143 p. Acad. Diss.

D 358. Miettinen, Timo. Whiplash injuries in Finland: incidence, prognosis and predictive factors for the long-term outcome.
2005. 86 p. Acad. Diss.

D 359. Kotaniemi-Syrjänen, Anne. Wheezing requiring hospitalisation in infancy - outcome at early school age: viral aetiology of wheeze and predictive factors for outcome.
2005. 97 p. Acad. Diss.

D 360. Huopio, Jukka. Predicting fractures in middle-aged women.
2005. 86 p. Acad. Diss.

D 361. Gül, Mustafa. Cytotoxic and antifungal acetophenone-derived Mannich bases: effects on redox thiols and heat shock proteins.
2005. 68 p. Acad. Diss.

D 362. Virtanen, Jyrki. Homocysteine, folate and cardiovascular diseases.
2005. 65 p. Acad. Diss.

D 363. Tuomainen, Petri. Physical exercise in clinically healthy men and in patients with angiographically documented coronary artery disease with special referense to cardiac autonomic control and warm-up phenomenon.
2005. 125 p. Acad. Diss.

D 364. Lindgren, Annamarja. Cancer incidence in hypertensive patients.
2005. 94 p. Acad. Diss.

D 365. Töyry, Saara. Burnout and self-reported health among Finnish physicians.
2005. 102 p. Acad. Diss.

D 366. Haapalahti, Mila. Nutrition, gastrointestinal food hypersensitivity and functional gastrointestinal disorders in schoolchildren and adolescents.
2005. 612 p. Acad. Diss.

D 367. Lindi, Virpi. Role of the Human PPAR-γ2 Gene on Obesity, Insulin Resistance and Type 2 Diabetes.
2005. 102 p. Acad. Diss.