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KIRSI RILLA

Hyaluronan Synthase

Intracellular Traffic, Activity at Plasma Membrane, and Impact on Keratinocyte Migration, Proliferation and Formation of Microvilli

Doctoral dissertation

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ABSTRACT

Hyaluronan is a polysaccharide found in the extracellular matrix (ECM) of cells from vertebrates. It is synthesized at the plasma membrane by an enzyme which is attached to the inner surface of the plasma membrane. Hyaluronan synthase (HAS) extrudes the growing hyaluronan chain directly into the extracellular space.

In the course of the presented studies, hyaluronan synthesis rate was experimentally manipulated in keratinocytes to reveal features of the cellular phenotype influenced by hyaluronan. Green fluorescent protein (GFP)-tagged HASs were utilized to study the localization, trafficking and biological effects of HASs by time lapse and 3D confocal imaging.

Epidermal growth factor (EGF) rapidly increased keratinocyte Has2 mRNA levels, which temporally correlated with an increase in hyaluronan secretion. It also increased pericellular and intracellular hyaluronan, and associated with an elongated cell shape and increased motility in scratched monolayer cultures. Inhibition of hyaluronan synthesis by antisense Has2 gene transfection slowed down the migration of keratinocytes. Correspondingly, 4-methylumbelliferone (4-MU) downregulated the basal and EGF-induced hyaluronan synthesis of keratinocytes and accentuated an epithelial morphology with a flat, round cell shape, with depleted lamellipodia, and filopodia and a low migration rate. High concentrations of 4-MU also caused a reversible block in keratinocyte proliferation, and in the organotypic cultures, prevented the hyaluronan accumulation and epidermal hypertrophy induced by EGF. The induction and inhibition of hyaluronan synthesis showed opposite effects on cell morphology and proliferative and migratory activity, indicating that these processes are dependent on active hyaluronan synthesis.

Confocal microscopy of keratinocytes expressing GFP-HAS fusion proteins showed large amounts of intracellular HAS, mainly in the endoplasmic reticulum (ER), Golgi apparatus and endosomes, and a distinct pool on the plasma membrane particularly in microvillous extensions. Inhibition of HAS activity through enzyme mutations or substrate starvation excluded HAS from the plasma membrane, keeping it in the intracellular, latent pool. It was rapidly mobilized from ER and Golgi apparatus after release of a 4-MU and brefeldin A (BFA) block, respectively. The turnover time of the GFP-HAS3 protein was 4-5 h, and the plasma membrane residence approximately 2 h. These results indicate that HAS resides on the plasma membrane only during active hyaluronan synthesis, after which it is endocytosed for degradation or recycling.

Surprisingly, transfection of the GFP-tagged Has3 was not just expressed on membrane protrusions, but induced the formation of elongated microvilli. These microvilli were rapidly destroyed by *Streptomyces* hyaluronidase, while hyaluronan oligosaccharides known to displace hyaluronan from cell surface receptors had no influence. This indicates that overexpression of HAS itself causes the extension of plasma membrane into these hyaluronan-dependent microvilli, described for the first time in this work.

The present studies show that hyaluronan synthesis strongly modulates the morphology of epithelial cells like epidermal keratinocytes, and stimulates their migration and proliferation, resulting in disturbed differentiation of the epidermis. Hyaluronan and HAS are thus potential targets for treatment of epidermal conditions associated with hyperproliferation such as psoriasis and wound healing.

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Medical Subject Headings: cell membrane; cell movement; cell proliferation; endocytosis; epidermal growth factor; glucuronosyltransferase; hyaluronic acid; keratinocytes; microvilli; protein synthesis inhibitors

To Jukka, Sami and Inka

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Kirsi Rilla

ABBREVIATIONS

ABC	Avidin-biotin-complex
BFA	Brefeldin A
BSA	Bovine serum albumin
DAB	3,3'-diaminobenzidine
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EtBr	Ethidium bromide
ER	Endoplasmic reticulum
ERK	extracellular signal regulated kinase
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GlcUA	Glucuronic acid
GlcNac	N-acetylglucosamine
GTP	Guanine triphosphate
ECM	Extracellular matrix
ELSA	Enzyme-linked sorbent assay
HARE	HA receptor for endocytosis
HAS	Hyaluronan synthase
HABC	Hyaluronan binding complex
bHABC	Biotinylated hyaluronan binding complex
HC	hydrocortisone
Hyal	Hyaluronidase (gene)
IαI	Inter-alpha-inhibitor
ICAM-1	Intercellular adhesion molecule-1
IGF	Insulin-like growth factor
IHABP	Intracellular hyaluronan binding protein
IL-1	Interleukin-1
KGF	Keratinocyte growth factor
LYVE-1	Lymph vessel endothelium receptor 1
MD	Membrane domain
4-MU	4-methylumbelliferone
MEM	Minimal essential medium
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
ORF	Open reading frame
PB	0.1 M sodium phosphate buffer
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
REK	Rat epidermal keratinocyte (cell line)

RHAMM	Receptor for hyaluronan mediated motility
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TSG-6	Tumor necrosis factor-stimulated gene 6 protein
UDP	Uridine diphosphate

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV. This thesis contains also unpublished data.

I **Pienimäki J-P, Rilla K, Fülöp C, Sironen RK, Karvinen S, Pasonen S, Lammi MJ, Tammi RH, Hascall VC, Tammi MI:** Epidermal growth factor activates hyaluronan synthase 2 (Has2) in epidermal keratinocytes and increases pericellular and intracellular hyaluronan. J. Biol. Chem. 276:20428-20435, 2001.

II **Rilla K, Pasonen-Seppänen S, Rieppo J, Tammi M, Tammi R:** The hyaluronan synthesis inhibitor 4-methylumbelliferone prevents keratinocyte activation and epidermal hyperproliferation induced by epidermal growth factor. J. Invest. Dermatol. 123:708-714, 2004.

III **Rilla K, Siiskonen H, Spicer AP, Hyttinen JMT, Tammi MI, Tammi RH:** Plasma membrane residence of hyaluronan synthase is coupled to its enzymatic activity. J. Biol. Chem. 280: 31890-31897, 2005.

IV **Kultti A*, Rilla K*, Tiihonen R, Spicer AP, Tammi R, Tammi M:** Hyaluronan synthesis induces microvillous cell surface protrusions. J. Biol. Chem. 2006, in press.
*Equally contributed

CONTENTS

1. INTRODUCTION 15

2. REVIEW OF THE LITERATURE 16

2.1. STRUCTURE AND PROPERTIES OF HYALURONAN 16
2.2. HYALURONAN SYNTHASES 17
2.2.1. Hyaluronan synthase (Has) genes 17
2.2.2. Structure and function of hyaluronan synthases 18
2.2.3. Properties of the HAS isoenzymes 20
2.2.4. Possible regulation points of HAS activity 21
2.2.5. Factors regulating hyaluronan synthesis Has gene expression 23
2.3. HYALURONAN BINDING PROTEINS 26
2.4. HYALURONAN CATABOLISM 27
2.4.1. Degradation mechanisms 27
2.4.2. Hyaluronan degrading enzymes 28
2.5. BIOLOGICAL FUNCTIONS OF HYALURONAN 29
2.5.1. Distribution of hyaluronan in cells and tissues 29
2.5.2. Hyaluronan synthesis and cell proliferation 30
2.5.3. Hyaluronan and cell migration 32
2.5.3.1. Association of hyaluronan content with cell locomotion 32
2.5.3.2. Molecular basis of hyaluronan influence on cell migration 33
2.5.3.2.1. Cell migration 33
2.5.3.2.2. Hyaluronan association with cell protrusions 36
2.5.4. Hyaluronan in cell differentiation and development 38
2.5.4.1. Differentiation 38
2.5.4.2. Development 39
2.5.5. Hyaluronan and wound healing 40
2.5.6. Hyaluronan and cancer 41
2.5.6.1. Hyaluronan correlation with tumor progression 41
2.5.6.2. Hyaluronan and cancer cell properties 41

3. AIMS OF THE STUDY 44

4. MATERIALS AND METHODS 45

4.1. CELL CULTURE (I-IV) 45
4.1.1. Monolayer cultures (I-IV) 45
4.1.2. Organotypic keratinocyte cultures (II) 46
4.1.3. Treatments of cells (I-IV) 46
4.2. TRANSFECTIONS AND GENE CONSTRUCTS (I-IV) 47
4.3. RT-PCR (I) 48
4.4. NORTHERN BLOTTING (I) 48
4.5. QUANTITATION OF HYALURONAN (I-IV) 49
4.5.1. Metabolic labeling (I, II) 49
4.5.2. Hyaluronan ELSA (II-IV) 49
4.5.3. Molecular mass distribution of hyaluronan (II) 50
4.6. MIGRATION ASSAY (I, II) 51
4.7. PROLIFERATION ASSAY (I, II) 51
4.8. MICROSCOPY (I-IV) 51

4.8.1. Hyaluronan staining for light microscopy (I, II) 51
4.8.2. Densitometry of cell-associated hyaluronan (I) 52
4.8.3. Red blood cell exclusion assay (I, IV) 52
4.8.4. Fluorescence stainings (II-IV) 53
4.8.5. Microscopy of organotypic cultures (II) 53
4.8.6. Confocal microscopy (II-IV) 54
4.8.7. Electron microscopy (I, IV) 55

5. RESULTS 56

5.1. REK CELL FEATURES (I, II) 56
5.1.1. REK cell morphology, proliferation and migration rate 56
5.1.2. REK cell hyaluronan metabolism 56
5.2. EFFECT OF EGF ON HYALURONAN SYNTHESIS AND REK PHENOTYPE (I, II) 57
5.2.1. Induction of hyaluronan secretion in REKs by EGF 57
5.2.2. Cell-associated hyaluronan in EGF-treated REKs 58
5.2.3. Effect of EGF treatment on REK migration and proliferation 59
5.2.4. EGF effect on Has mRNA expression 59
5.2.5. Migration inhibition by Has2 antisense transfection 60
5.3. EFFECT OF 4-MU ON REK HYALURONAN SYNTHESIS AND MORPHOLOGY (II) 60
5.3.1. Effect of 4-MU on secretion, molecular mass and localization of hyaluronan .. 60
5.3.2. 4-MU effects on cytoskeleton, migration and proliferation rate 61
5.3.3. 4-MU in organotypic REK cultures 62
5.4. GFP-HAS LOCALIZATION AND TRAFFIC IN REKS (III) 62
5.4.1. Cellular distribution and enzymatic activity of Has-GFP fusion proteins 62
5.4.2. GFP-HAS colocalization with subcellular markers and hyaluronan 63
5.4.3. Effect of BFA and cycloheximide on GFP-HAS3 traffic 64
5.4.4. Effect of hyaluronan synthesis inhibition on GFP-HAS localization 65
5.5. GFP-HAS3 AND PLASMA MEMBRANE PROTRUSIONS (IV) 66
5.5.1. Hyaluronan secretion after GFP-HAS3 transfection 66
5.5.2. Formation of GFP-HAS -positive protrusions rich in hyaluronan 66
5.5.3. The structure of GFP-HAS3–induced protrusions 67
5.5.4. Hyaluronan dependence of cell surface protrusions 68
5.5.5. The protrusions and hyaluronan receptors 68

6. DISCUSSION 69

6.1. STIMULATION OF HYALURONAN SYNTHESIS BY EGF 69
6.2. MECHANISM OF HYALURONAN SYNTHESIS INHIBITION BY 4-MU 70
6.3. HAS TRAFFICKING 71
6.4. HYALURONAN AND PLASMA MEMBRANE PROTRUSIONS 73
6.4.1. Hyaluronan synthesis, microvilli and hyaluronan coat 73
6.4.2. Proteins associated with the microvilli 74
6.5. ROLE OF HYALURONAN AS A REGULATOR OF KERATINOCYTE PROLIFERATION 75
6.6. HYALURONAN SYNTHESIS AND CELL MIGRATION 78
6.6.1. Hyaluronan effect on cell migration 78
6.6.2. Inhibition of migration by 4-MU 78
6.6.3. Mechanisms involved in hyaluronan-induced cell migration 79

7. SUMMARY AND CONCLUSIONS 82

8. REFERENCES 85

1. INTRODUCTION

Hyaluronan, also called hyaluronic acid or hyaluronate, is a high molecular mass polysaccharide composed of N-acetylglucosamine (GlcNac) and glucuronic acid (GlcUA). Hyaluronan is expressed in all tissues of the human body and acts as an important structural organizer of the ECM in connective tissues such as cartilage and skin dermis. A role as a tissue space filler was for long considered to be its only function. However, now the ability of hyaluronan to induce signalling and cell proliferation, and its participation in embryogenesis, cancer metastasis and wound healing are well known examples among the numerous, more specific functions of this molecule.

In the skin epidermis, hyaluronan is actively synthesized and degraded, and present at high concentrations between proliferating and differentiating cell layers. Especially when normal differentiation is disrupted, or tissue injured, hyaluronan accumulates in the epidermis. Hyaluronan synthesis is mainly controlled at transcriptional level in epidermis and other tissues by growth factors and other regulators. The mechanisms involved in the regulation of hyaluronan metabolism in the epidermis and other tissues are complex. While it is known that hyaluronan is produced by HASs at plasma membrane, localization and trafficking of the synthase protein itself during activation has remained largely unknown.

The present results provide new data on HAS properties and function and indicate that enhanced cell locomotion and proliferation of epidermal keratinocytes are associated with hyaluronan synthesis. Hyaluronan is a putative therapeutic target in many clinical conditions, having potential for enhanced wound healing, perhaps with less scar formation. On the other hand, suppression of hyaluronan and its interactions may result in normalization of hyperproliferative epidermis in psoriasis and inflammation, and inhibition of tumor cell growth and invasion.

2. REVIEW OF THE LITERATURE

2.1. Structure and properties of hyaluronan

Hyaluronan is a glycosaminoglycan (GAG) composed of alternating D-glucuronic acid and N-acetyl-D-glucosamine residues **(Fig. 1)**. The molecular mass of intact hyaluronan molecule usually ranges between 10^6 and 10^7 Da, corresponding to 2 500 - 25 000 disaccharides and an extended length of ~2.3 - 23 µm (Toole, 2004).

Hyaluronan has an ability to bind water about thousand times its own weight (Laurent and Fraser, 1992). It is highly soluble in water and exists as an expanded random coil, producing a gel with high viscosity and elasticity (Laurent and Fraser, 1992). The viscosity is related to the size and the concentration of the molecule and is shear-dependent (Laurent and Fraser, 1992). Hyaluronan acts as a diffusion barrier, regulates the transport of other substances, and excludes other macromolecules (Laurent et al., 1996). The water-binding and elastoviscous properties, and immunological non-reactivity make hyaluronan a widely used molecule in various medical applications (Band, 1998; Asari, 2000).

Hyaluronan differs from other GAGs in several ways: It does not contain sulfate groups, it is synthesized on plasma membrane, not in the Golgi apparatus, and it is not covalently linked to any core protein to form proteoglycans (Toole, 2000). Additionally, hyaluronan is the only GAG synthesized both by mammalian cells and some bacteria, like *Streptococcus* and *Pasteurella* (Weigel, 2004), and even *Chlorella* virus contains a functional hyaluronan synthase gene in its genome (DeAngelis et al., 1997).

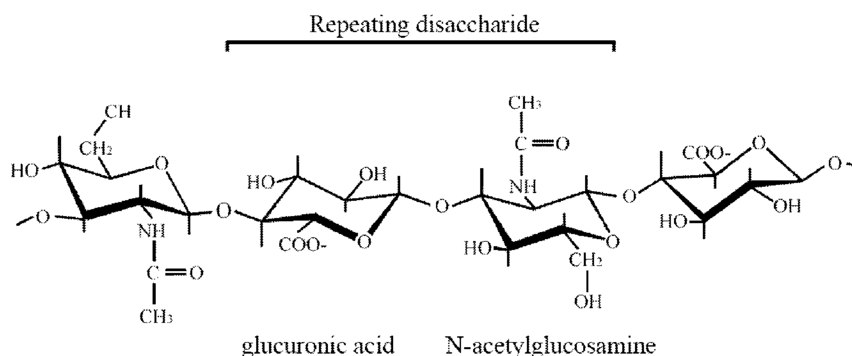

Figure 1. Chemical structure of hyaluronan.

2.2. Hyaluronan synthases

HAS activity was detected already in 1959 (Markovitz et al., 1959). For a long time it was supposed that hyaluronan is produced in the Golgi complex like other GAGs. In 1984, Prehm found that nascent hyaluronan chains were degraded extracellularly by hyaluronidase treatment, and suggested that hyaluronan is synthesized at the inner surface of the plasma membrane (Prehm, 1984).

2.2.1. Hyaluronan synthase (Has) genes

Hyaluronan synthase A (HasA) was first cloned from Group A streptococcus (DeAngelis et al., 1993). After that, members of mammalian Has family were cloned in human (Itano and Kimata, 1996b; Shyjan et al., 1996; Watanabe and Yamaguchi, 1996) and mouse (Itano and Kimata, 1996a; Spicer et al., 1996; Fülop et al., 1997b; Spicer et al., 1997a). In mammalian cells, the HAS family consists of three different isoenzymes, HAS1, HAS2 and HAS3. An additional vertebrate HAS was identified, when the DG42 protein of Xenopus laevis (Rosa et al., 1988) was shown to be a HAS (xHas1) (DeAngelis and Achyuthan, 1996). Other isoenzymes, (xHas2 and xHas3) has also been identified in Xenopus laevis (Spicer and McDonald, 1998).

The three human and mouse Has genes are each localized in different chromosomes (Has1 at 19 and 17, Has2 at 8 and 15, and Has3 at 16 and 8 in human and mouse, respectively) suggesting that the isoenzymes are of ancestral origin and appeared early in the evolution of vertebrates (Spicer et al., 1997b). The amino acid sequences of mammalian isoenzymes are 55-71% identical (Spicer and McDonald, 1998). Additionally, all vertebrate Has genes share at least one exon-intron boundary, suggesting that they evolved from one common ancestral gene (Spicer and McDonald, 1998). At the amino acid and gene structure level, Has2 and Has3 are the most closely related hyaluronan synthases (Spicer and McDonald, 1998).

2.2.2. Structure and function of hyaluronan synthases

HASs are putative transmembrane proteins with predicted molecular mass of ~60 kDa with 6-7 putative membrane domains **(Fig. 2)** (Weigel et al., 1997). Both the NH_2- and COOH -termini and a major part of the large central domain of HAS are located intracellularly (Heldermon et al., 2001). The largest intracellular domain is conserved among HAS proteins (Itano et al., 1999b), and it has been suggested to contain the enzymatically active site of the protein (Weigel et al., 1997). Supporting this idea, several amino acid residues in this domain were shown to be essential for hyaluronan synthesis (Yoshida et al., 2000) **(Fig. 2)** .

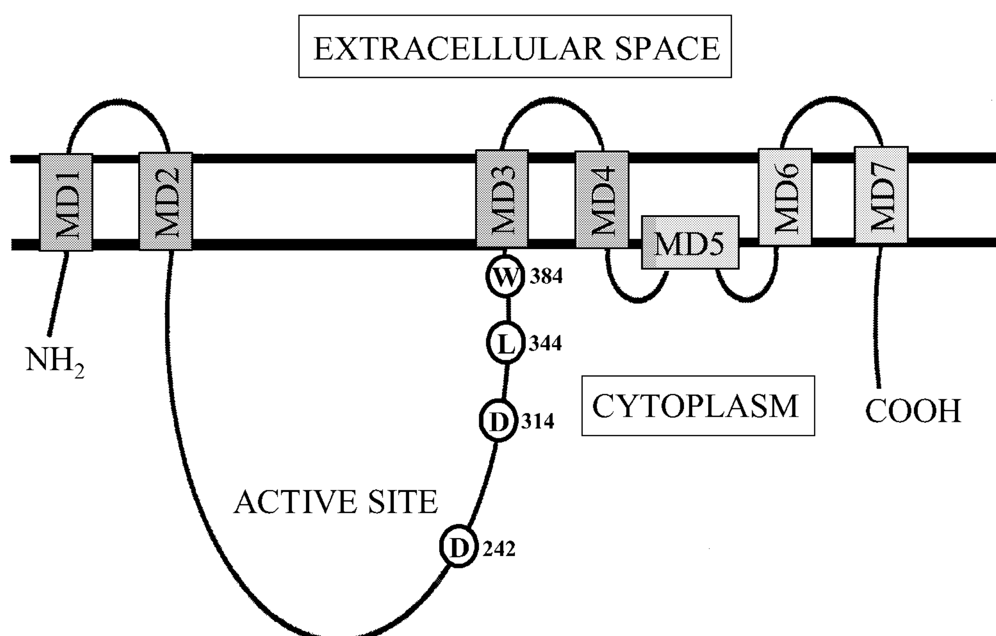

Figure 2. The structure and plasma membrane topology for eukaryotic HAS proteins. The seven putative membrane domains (MDs) are represented. Both amino-terminal and carboxy-terminal regions are located intracellularly. The large intracellular central domain is proposed to contain the enzymatically active site of HAS. Adapted from Weigel et al. (1997).

There is no typical signal peptide in the amino terminus of HAS protein, and the involvement of the normal secretory pathway through ER and Golgi complex remained unresolved until the development of GFP-tagged HAS proteins (Müllegger et al., 2003). The previous publications using BFA and monensin to disrupt the traffic in the ER-Golgi pathways and its effect on hyaluronan synthesis in chondrocytes and in fibrosarcoma cells, had given contradictory results (Goldberg and Toole, 1983, 1984; Calabro and Hascall, 1994). However, Müllegger et al. showed that GFP-XHAS1 accumulates in ER in BFA–treated cells, and is transported to Golgi and subsequently to the plasma membrane after the removal of BFA (Müllegger et al., 2003).

On the plasma membrane, HAS mediates several distinct functions needed for hyaluronan production: HAS is capable of adding both UDP-GlcUA and UDP-GlcNAc to the growing polymer (Prehm, 1983), HAS protein alone is responsible for initiation and elongation of hyaluronan chains (Yoshida et al., 2000), and forms a pore-like structure through which a hyaluronan chain is translocated to extracellular space (Yoshida et al., 2000) **(Fig. 3)**.

The vertebrate enzymes add the alternating monosaccharides to the non-reducing end of the chain, while the streptococcal enzyme extends hyaluronan at the reducing end (Bodevin-Authelet et al., 2005; Tlapak-Simmons et al., 2005). Another difference between eukaryotic and streptococcal enzymes is that cardiolipin seems to activate HAS in these bacteria (Tlapak-Simmons et al., 1998), but not in eukaryotic cells (Yoshida et al., 2000). It was recently suggested, but not unequivocally shown, that an ATP-binding-cassette transporter is required for hyaluronan release by *Streptococcus pyogenes* (Ouskova et al., 2004; Prehm and Schumacher, 2004).

Estimates for the life-time of HAS protein have been obtained by utilizing cycloheximide to block protein synthesis. The turnover time of (4-6 h) seen by some investigators *in vitro* (Bansal and Mason, 1986; Kitchen and Cysyk, 1995), is relatively close to that reported *in vivo* (over 6 hours) (Anggiansah et al., 2003). Because of the relatively short life-time, it is suggested that each HAS makes only one hyaluronan chain before inactivation of the enzyme (Mausolf et al., 1990; Kitchen and Cysyk, 1995). The estimated hyaluronan synthesis rate *in vitro* is 3 monosaccharides/s (Pummill and DeAngelis, 2003), implying that the time required for synthesis of one hyaluronan chain of 2×10^6 Da is about one hour.

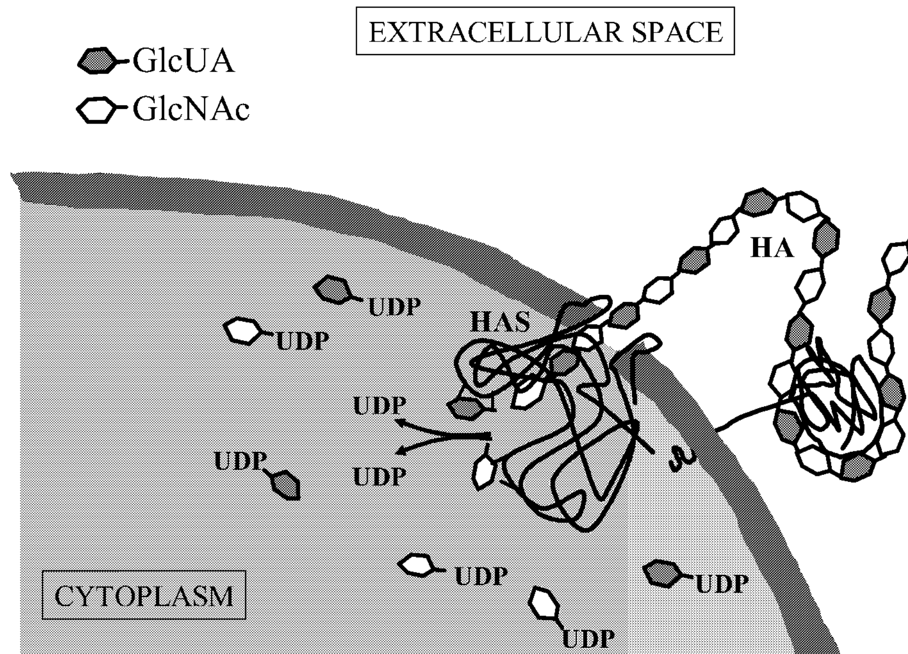

Figure 3. The function of the eukaryotic HAS. The enzyme adds UDP-GlcUA and UDP-GlcNAc alternatively to the non-reducing end of the growing polymer. Simultaneously, the polymer is extruded in to the extracellular space through a pore-like structure formed by synthase itself or with unknown associated proteins. The hyaluronan chain is shed to the ECM or may be bound by receptors.

2.2.3. Properties of the HAS isoenzymes

The members of the HAS protein family have different properties and functional roles. Mouse HAS3 synthesizes shorter hyaluronan chains than HAS2 when expressed in COS-1 cells (Spicer and McDonald, 1998), or in rat fibroblasts (Itano et al., 1999b). Similarly, xHAS1 synthesizes shorter hyaluronan chains than xHAS2 when expressed in COS-1 cells (Köprunner et al., 2000). The studies mentioned above have been done using *in vitro* HAS assays with membrane fractions. One study with CHO cells (Brinck and Heldin, 1999) showed that *in vitro* hyaluronan synthesis by membrane preparations produces hyaluronan chains with different chain lengths depending on HAS isoform, but in living CHO cells the

same differences in chain length are not found. However, in a recent work (Wilkinson et al., 2006), HAS3 synthesized smaller hyaluronan chains than HAS1 and HAS2 even in intact cells. This suggests that for stabilization, HAS may need some accessory factors not present in the membrane preparations used in *in vitro* experiments, or that the activity of hyaluronan degradation is not similar in different situations.

All isoforms of HAS are capable of pericellular hyaluronan coat formation (Brinck and Heldin, 1999), but HAS1 may form a smaller coat as compared to HAS2 and HAS3 (Itano et al., 1999b). HAS isoforms also show different responses to external stimuli (Jacobson et al., 2000) and are differentially expressed after malignant transformation (Itano et al., 2004). Different expression patterns of the HAS isoforms have been reported in different cell lines (Recklies et al., 2001; Yamada et al., 2004). Has2 is the most active (Brinck and Heldin, 1999), and most highly expressed isoform (Nishida et al., 1999), and produces most pronounced effects on hyaluronan production and cell phenotype when transfected to cells (Itano et al., 1999b; Itano et al., 2002).

It has been suggested, that Has1 maintains a low, basal level of hyaluronan resistant to regulation, and is expressed in very low or non-detectable levels in many cell types (Nishida et al., 1999; Yamada et al., 2004). Has2 is required in embryonic morphogenesis and subsequent development because it supports cell migration and invasion (Camenisch et al., 2000). Furthermore, Has isoforms are differentially expressed in the developing embryo and in adult tissues (Spicer and McDonald, 1998; Tien and Spicer, 2005). Because of different expression patterns during early embryogenesis, Has2 knockout is lethal in mice, while Has1 and Has3 knockouts are viable with no obvious phenotypes (Camenisch et al., 2000).

2.2.4. Possible regulation points of HAS activity

HASs are mainly regulated at the transcriptional level, since there are large and rapid changes in Has mRNA that correlate with hyaluronan synthesis (Jacobson et al., 2000; Karvinen et al., 2003b; Yamada et al., 2004) **(Table I)**. The promoter region of Has2 contains several consensus sequences for transcription factor binding sites (Monslow et al., 2004). At present, the response elements for all-trans retinoic acid and EGF were confirmed to be functionally active (Saavalainen et al., 2005).

Differences in the regulatory regions influencing the mRNA turnover among the three Has's (Spicer and McDonald, 1998) suggest that the expression levels may be regulated also by mRNA stability. Glucocorticoids inhibit hyaluronan synthesis by decreasing Has2 transcription, but also by reducing Has2 mRNA stability (Zhang et al., 2000). Since the turnover time of the HAS protein has been reported to differ under different conditions (Bansal and Mason, 1986; Kitchen and Cysyk, 1995), the stability of the HAS protein may also offer a level of regulation.

It has been speculated that interaction of HAS with other membrane components may influence the retention of HAS on the plasma membrane or its activation, thereby influencing polymer size (Toole, 2004). The strength of interaction between HAS and the growing hyaluronan chain, modulated by targeted mutations, has been reported to influence the polymer size (Pummill and DeAngelis, 2003). On the other hand, hyaluronan associated to HAS may inhibit hyaluronan synthesis and its detachment has been suggested to activate the synthase (Lüke and Prehm, 1999).

Activation of an existing HAS enzyme has been suggested to occur via phosphorylation (Klewes and Prehm, 1994), and the intracellular domains of HAS2 have been reported to contain putative phosphorylation sites for PKC and cAMP-dependent kinases (Ohno et al., 2001). PKC stimulates hyaluronan synthesis (Anggiansah et al., 2003), and has been associated with hyaluronan-activated cell locomotion and hyaluronan uptake (Hall et al., 2001). According to a recent finding, HAS is suggested to be regulated by phosphorylation (Goentzel et al., 2006).

The availability of UDP-sugar precursors is a prerequisite for hyaluronan synthesis, and it may limit hyaluronan synthesis rate (Itano et al., 1999b). Treatment of cells with chemicals which interfere with the synthesis of these precursors, like oxidized UDP-sugar precursors (Prehm, 1985), 6-diazo-5-oxo-1-norleucine, preventing UDP-N-acetyl glucosamine synthesis (Chen et al., 1993), and depletion of UDP-glucuronic acid pool through glucuronidation of 4-MU (Kakizaki et al., 2004) all lead to reduced hyaluronan synthesis rate. UDP-glucose dehydrogenase, the key enzyme in the synthesis of UDP-glucuronic acid, is coregulated with HAS (Vigetti et al., 2006). The promoter for this enzyme was recently described to contain a SP-1 binding site (Vatsyayan et al., 2005), a potential regulatory site activated by several signaling pathways.

2.2.5. Factors regulating hyaluronan synthesis Has gene expression

Hyaluronan synthesis is regulated by a number of physiological effectors, like growth factors, cytokines and hormones, several examples of which are shown in **Table I**. While most of them stimulate hyaluronan synthesis, also negative regulators do exist. Some factors like TGF-β exert either stimulatory or inhibitory responses **(Table I)**. This depends on the cell type (mesenchymal versus epithelial), and developmental stage (adult versus fetal) (Ellis et al., 1997). Pharmaceutical agents such as synthetic glucocorticoids, and non-steroidal anti-inflammatory agents also inhibit hyaluronan synthesis (**Table I b**).

Epidermal growth factor (EGF) is a member of epidermal growth factor family. EGF receptors (EGFR, HER-1 ErbB-1) belong to the ErbB family of receptor tyrosine kinases, which regulate growth and development and are overexpressed in many tumor types (Bazley and Gullick, 2005). EGFR activation leads to tyrosine kinase phosphorylation cascades, activating downstream signaling molecules like PI3 kinase, ras, the ERK and also Src kinase (Bazley and Gullick, 2005). EGFR promotes normal keratinocyte growth and survival, suppresses differentiation and stimulates migration and proliferation during wound healing (Hudson and McCawley, 1998). In human keratinocytes, Has2 is one of the direct target genes for EGF signaling (Saavalainen et al., 2005). In oral mucosa, EGF induces the expression of all three Has isoforms (Yamada et al., 2004).

4-methylumbelliferone (4-MU) is a coumarine derivative with spasmolytic activity (Stacchino et al., 1983). Coumarins are natural polyphenols found in medicinal plants with anti-inflammatory, anti-proliferative and anti-tumor actions (Hoult and Paya, 1996). 4-MU has been reported to specifically inhibit hyaluronan synthesis in cultured mammalian cells (Nakamura et al., 1995; Nakamura et al., 1997; Kosaki et al., 1999; Sohara et al., 2001) and in *Streptococcus equi* FM100 cells (Kakizaki et al., 2002). It inhibits melanoma cell adhesion and locomotion (Kudo et al., 2004), and metastasis (Yoshihara et al., 2005). In human breast cancer cells, 4-MU inhibits hyaluronan synthesis, migration and invasion with diminished Has2 and Has3 mRNA levels (Anne Kultti, personal information). 4-MU also reverses the effect of Has2 transfection on hyaluronan synthesis and colony formation of tumor cells (Kosaki et al., 1999). Recently, it was demonstrated that 4-MU inhibition on hyaluronan synthesis is at least partly due to UDP-glucuronic acid depletion (Kakizaki et al., 2004).

Table I. Factors regulating hyaluronan synthesis

a. Hyaluronan synthesis increased

Factor	Has1	Has2	Has3	Cell/Tissue	Reference
Compound K		+		keratinocyte	(Kim et al., 2004)
EGF				fibroblast	(Heldin et al., 1989)
EGF		+	+	keratinocyte	(Pasonen-Seppänen et al., 2003)
EGF	+	+	+	oral mucosal cell	(Yamada et al., 2004)
EGF	+	+	+	fibroblast	(Yamada et al., 2004)
EGF		+		keratinocyte	(Saavalainen et al., 2005).
EGF				neural crest	(Erickson and Turley, 1987)
EGF				mesothelial	(Honda et al., 1991)
EGF				cumulus cell	(Tirone et al., 1997)
estrogen				endometrium	(Tellbach et al., 2002)
estrogen				uterine epithelium	(Mani et al., 1992)
bFGF				fibroblast	(Heldin et al., 1989)
FGF2	+	+		dental pulp	(Shimabukuro et al., 2005a)
FGF2	+	+		periodontal ligament	(Shimabukuro et al., 2005b)
FGF2	+	+	+	fibroblast	(Kuroda et al., 2001)
FSH				cumulus cell	(Tirone et al., 1997)
HGF				epithelial	(Zoltan-Jones et al., 2003)
IFN-γ			+	keratinocyte	(Sayo et al., 2002)
IGF	+	+	+	fibroblast	(Kuroda et al., 2001)
IGF				mesothelial	(Honda et al., 1991)
IL-1β				synoviocyte	(Kawakami et al., 1998)
IL-1β			+	synoviocyte	(Oguchi and Ishiguro, 2004)
IL-1β		+	+	fibroblast	(Yamada et al., 2004)
IL-1β	+	+	+	uterine fibroblast	(Uchiyama et al., 2005)
IL-1β		+	+	fibroblast	(Kaback and Smith, 1999)
IL-6				fibroblast	(Duncan and Berman, 1991)
KGF		+	+	keratinocyte	(Karvinen et al., 2003b)
KGF				keratinocyte	(Jameson et al., 2005)
parathyroid hormone				osteoblast	(Midura et al., 1994)
PDGF		+		mesothelial	(Jacobson et al., 2000)
PDGF				mesothelial	(Heldin et al., 1992)
PDGF				fibroblast	(Heldin et al., 1989)
PDGF		+		vascular endothelial	(Suzuki et al., 2003)
PDGF				trabecular meshwork	(Usui et al., 2003)
PDGF		+		vascular smooth muscle	(Evanko et al., 2001)

progesterone			+	uterine fibroblast	(Uchiyama et al., 2005)
retinoid acid				epidermis	(King, 1981)
retinoic acid				epidermis	(Tammi and Tammi, 1986)
retinoic acid		+		keratinocyte	(Saavalainen et al., 2005).
testosterone				rooster comb	(Jacobson, 1978)
testosterone				prostate	(Li et al., 2001)
TGF-β	+	+		fibroblast	(Sugiyama et al., 1998)
TGF-β				fibroblast	(Heldin et al., 1989)
TGF-β	+			keratinocyte	(Sugiyama et al., 1998)
TGF-β		+		Vascular endothelial	(Suzuki et al., 2003)
TGF-β		+		Trabecular meshwork	(Usui et al., 2003)
TGF-β	+			synoviocyte	(Oguchi and Ishiguro, 2004)
TGF-β	+		-	synoviocyte	(Stuhlmeier and Pollaschek, 2004a)
TNF-α			+	synoviocyte	(Oguchi and Ishiguro, 2004)
tunicamycin				smooth muscle	(Majors et al., 2003)
polyI:C				smooth muscle	(de la Motte et al., 2003)

b. Hyaluronan synthesis decreased

Factor	Has1	Has2	Has3	Cell/Tissue	Reference
glucocorticoid				epidermis	(Ågren et al., 1995)
glucocorticoid		-		fibroblast	(Zhang et al., 2000)
glucocorticoid		-	-	synoviocyte	(Stuhlmeier and Pollaschek, 2004b)
hydrocortisone		-		mesothelial	(Jacobson et al., 2000)
indomethacin				myofibroblast	(August et al., 1994)
4-MU				fibroblast	(Nakamura et al., 1995)
4-MU		-		fibroblast	(Kakizaki et al., 2004)
4-MU				melanoma	(Kudo et al., 2004)
mefenamic acid				myofibroblast	(August et al., 1994)
TGF-α		-	-	synoviocyte	(Kawakami et al., 1998)
TGF-β		-		mesothelial	(Jacobson et al., 2000)
TGF-β		-	-	keratinocyte	(Pasonen-Seppänen et al., 2003)
TGF-β			-	keratinocyte	(Sayo et al., 2002)
vesnanirone				myofibroblast	(Ueki et al., 2000)
Vitamin D				osteoblast	(Takeuchi et al., 1989)

2.3. Hyaluronan binding proteins

Hyaluronan is synthesized as a free polysaccharide chain, but it is capable to interact with other proteins in the cells and ECM (Knudson and Knudson, 1993; Day and Prestwich, 2002). Many of these proteins, like the aggregating proteoglycans (aggrecan, versican, brevican, neurocan), link proteins, TSG-6, CD44, stabilin 2 (HARE) and LYVE1 contain a common structural feature, s.c. link module, a ~100 amino acid hyaluronan binding domain (Day and Prestwich, 2002), while in some hyaladherins like RHAMM basic amino acids mediate the hyaluronan binding (Yang et al., 1994). The heavy chains of Inter-alpha-inhibitor (IαI) are unique in their covalent crosslink to hyaluronan mediated by TSG-6 (Rugg et al., 2005).

Aggregating proteoglycans and link proteins have a structural role in organizing ECM in cartilage (Hardingham and Muir, 1972), other connective tissues (LeBaron et al., 1992) and brain (Delpech, 1982; Ripellino et al., 1985). TSG-6 and IαI are involved in the assembly of hyaluronan-rich matrices in the cumulus oophorus complex and in inflamed tissues (Mukhopadhyay et al., 2004).

There are several cell-associated hyaluronan binding proteins, hyaluronan receptors, both on cell surface and inside the cell (Turley et al., 2002). The most studied receptor for hyaluronan is CD44, a single pass transmembrane protein (Underhill et al., 1987) reviewed by (Lesley et al., 2000; Isacke and Yarwood, 2002; Knudson and Knudson, 2005). The extracellular domain of CD44 contains a link module which binds hyaluronan (Day and Prestwich, 2002) and the intracellular domain interacts with cytoskeletal and signaling proteins (Bourguignon et al., 1998; Turley et al., 2002). CD44 exists as numerous variants because of alternative splicing (Lesley et al., 2000), leading to different properties of the receptor. CD44 mediates three different cellular functions; assembly of pericellular matrices (Knudson et al., 1993), internalization of hyaluronan (Knudson et al., 2002), and signal transduction to cytoskeleton (Turley et al., 2002). CD44 is also binding matrix metalloproteinase-9 at the plasma membrane, thus regulating tissue remodeling (Yu and Stamenkovic, 2000)

The hyaluronan receptor RHAMM (receptor for hyaluronan-mediated motility) (Yang et al., 1994), also known as IHABP (Hofmann et al., 1998), is located on the cell surface, cytoskeleton, mitochondria and nucleus (Turley et al., 2002). RHAMM activates several

signaling pathways including Src, FAK, ERK and PKC (Turley et al., 2002) and regulates actin cytoskeleton via ras (Hall et al., 1996). Hyaluronan-RHAMM interactions are involved with cell migration and tumorigenesis (Hall and Turley, 1995). Thus, CD44 and RHAMM have different structures, are located differentially in the cell, have different mechanisms to bind hyaluronan, and are supposed to regulate signaling by different mechanisms (Turley et al., 2002).

A hyaluronan receptor specific for liver sinusoidal endothelial cells of human spleen and lymph nodes is HARE (hyaluronan receptor for endocytosis) (Zhou et al., 2000), also known as stabilin 2 (Politz et al., 2002). The role of stabilin-1 is still unclear (McCourt et al., 2004) and it does not bind hyaluronan (Prevo et al., 2004).

Layilin is a transmembrane hyaluronan receptor localized in microvilli, filopodia, lamellipodia and membrane ruffles (Bono et al., 2001). The hyaluronan receptor LYVE-1 is expressed in lymphatic endothelial cells and is involved in trafficking of cells within lymphatic vessels and lymph nodes (Jackson, 2004), and in hyaluronan uptake for degradation (Prevo et al., 2001).

2.4. Hyaluronan catabolism

2.4.1. Degradation mechanisms

Hyaluronan is catabolized either locally in tissues (Fraser and Laurent, 1989), or transported from peripheral tissues to lymph and blood to be catabolized in the lymph nodes and liver, respectively (Fraser et al., 1981; Smedsrod et al., 1984). Hyaluronan catabolism involves receptor-mediated uptake, which leads to lysosomal degradation into monosaccharides by hyaluronidase, β-glucuronidase and β-N-acetylglucosaminidase activities (Roden et al., 1989). In liver cirrhosis and liver transplant rejection, serum and tissue levels of hyaluronan are high, indicating that liver malfunction disables the normal catabolism and leads to hyaluronan accumulation (Adams et al., 1989; Engström-Laurent, 1989).

The main receptor responsible for local hyaluronan uptake is CD44, as reviewed by (Knudson et al., 2002). CD44 is involved in the uptake of hyaluronan in keratinocytes

(Tammi et al., 2001), chondrocytes (Hua et al., 1993), macrophages (Culty et al., 1992), dermal fibroblasts (Croce et al., 2003) and transformed and normal fibroblasts (Collis et al., 1998).

The catabolism of hyaluronan is rapid in skin epidermis. The half life of epidermal hyaluronan is approximately 1 day in human skin organ cultures (Tammi et al., 1991) and in organotypic rat keratinocyte cultures (Tammi et al., 2000). For degradation, keratinocytes internalize hyaluronan by a specific endocytotic route (Tammi et al., 2001). Changes in the content and metabolism of hyaluronan occur in many pathological conditions and by pharmacological treatments of skin (Juhlin, 1997). Reactive oxygen species can also degrade epidermal hyaluronan (Ågren et al., 1997b).

2.4.2. Hyaluronan degrading enzymes

Hyaluronidases are a group of enzymes responsible for hyaluronan catabolism (Stern, 2003). They are found in many animal venoms to facilitate the diffusion of other venom constituents. In mammalians they facilitate the entrance of spermatozoa through a layer of hyaluronan-rich matrix produced by cumulus cells surrounding the egg. Hyaluronidases are widely distributed in mammalian tissues, especially abundant in the liver (Kreil, 1995).

Six hyaluronidase-like gene sequences exist in mammals (Stern, 2004). Three of the genes (HYAL-1, HYAL-2 and HYAL-3) are clustered on chromosome 3 (Csoka et al., 1998). Hyal-2 is expressed both as a glycosyl phosphatidyl inositol coupled plasma membrane protein and in a soluble form in lysosomes (Lepperdinger et al., 1998). Hyal-1 is a lysosomal protein also present in plasma and urine (Frost et al., 1997), while the distribution of Hyal-3 is largely unknown. Hyal-2 cleaves high molecular mass hyaluronan into about 50 disaccharide-sized fragments, while Hyal-1 cleaves down to tetrasaccharides (Stern, 2003). Hyal-2 and Hyal-1 are supposed to act together: products of Hyal-2 digestion are further degraded by Hyal-1 (Stern, 2003). Another group of HYAL genes is clustered in chromosome 7 (Csoka et al., 1999). SPAM-1 codes for PH-20 protein (Gmachl et al., 1993), which is expressed in spermatozoa to facilitate sperm penetration through the cumulus mass surrounding ovum (Cherr et al., 1996), and HYALP1 is a pseudogene (Stern, 2003).

2.5. Biological functions of hyaluronan

2.5.1. Distribution of hyaluronan in cells and tissues

Hyaluronan was originally isolated from the vitreous body of the eye (Meyer and Palmer, 1934). Later it has been found to exist in all vertebrates and in all tissues of the human body (Laurent and Fraser, 1992). Although most prokaryotes do not produce it, some bacteria like *Streptococcus* and *Pasteurella* contain hyaluronan in their capsule. In the human body, the highest concentrations of hyaluronan (140-4100 mg/kg) are found in the umbilical cord, synovial fluid, vitreous body and dermis (Laurent and Fraser, 1986). Over half of the total body hyaluronan exists in the skin, as studied in rats (Reed et al., 1988). Although the majority of the body's hyaluronan exists in connective tissues, hyaluronan is also found in neuronal tissues (Ripellino et al., 1985) and in many epithelial tissues (Tammi and Tammi, 2004). In stratified epithelia such as skin epidermis, oral epithelia, esophagus and cervix uteri, hyaluronan is located mainly in the intercellular spaces of basal and spinous layers (Tammi et al., 1994b) while simple epithelia contain little or no hyaluronan (Tammi and Tammi, 2004). Keratinocytes synthesize hyaluronan in cell cultures (Lamberg et al., 1986), organotypic cultures (Tammi et al., 2000) and organ cultures (King, 1981; Tammi and Tammi, 1986; Ågren et al., 1995).

In connective tissues like cartilage hyaluronan has a specific role in the organization and assembly of ECM (Knudson, 2003). Hyaluronan is also associated with the cells, forming a large pericellular coat around many mesenchymal cells, like synovial cells (Clarris and Fraser, 1968), chondrocytes (Knudson, 1996), smooth muscle cells (Evanko et al., 1999), mesothelial cells (Heldin and Pertoft, 1993), and fibrosarcoma cells (Goldberg and Toole, 1984). The hyaluronan-dependent coat is associated with the cell surface via CD44 (Knudson et al., 1993) or HAS (Heldin and Pertoft, 1993), and is suggested to associate with proteoglycans (Evanko et al., 1999; Nishida et al., 1999), IαI (Chen et al., 1994), and TSG-6 (Fülop et al., 1997a).

Hyaluronan is also found in cytoplasmic vesicles (Tammi et al., 1998; Evanko and Wight, 1999) and perhaps even in the nucleus (Evanko and Wight, 1999; Hascall et al., 2004). The amount of the pericellular and cytoplasmic hyaluronan correlates with the proliferative and

migratory activity of the cells (Collis et al., 1998; Evanko and Wight, 1999), and is high for example in colon and breast cancers (Auvinen et al., 1997; Ropponen et al., 1998). Nuclear hyaluronan is sometimes found in the malignant cells of colorectal cancers, breast cancers and basal cell carcinomas (Ropponen et al., 1998; Auvinen et al., 2000; Karvinen et al., 2003a).

Typically, a high level of hyaluronan is associated with embryonic, regenerating, remodeling and healing tissues (Laurent and Fraser, 1992). In the extracellular and pericellular matrices hyaluronan lubricates the tissues, maintains water homeostasis, and filters macromolecules (Laurent et al., 1996). In addition to these physicochemical effects, hyaluronan induces specific signals in cells (Turley et al., 2002). Through these effects hyaluronan is believed to influence cell spreading (Goldberg and Toole, 1984), detachment from the substratum (Abatangelo et al., 1982), proliferation (Brecht et al., 1986; Evanko and Wight, 1999), migration (Evanko et al., 1999) and differentiation of cells (Kujawa and Caplan, 1986; Passi et al., 2004), processes which are crucial during development (Toole, 2001), wound healing (Price et al., 2005) and tumor progression (Toole et al., 2005).

2.5.2. Hyaluronan synthesis and cell proliferation

High hyaluronan content in tissues correlates with rapid proliferation rate, expecially during development (Toole, 1997). Also in vitro, there is often a positive correlation between proliferation and hyaluronan synthesis (Matuoka et al., 1987; Chen et al., 1989). In the epidermis, the proliferation rate and hyaluronan synthesis do not correlate (Tammi and Tammi, 1991). Hyaluronan synthesis increases at the S-phase of cell cycle, and blocking of hyaluronan synthesis arrests the cell cycle before detachment of cells from substratum (Brecht et al., 1986). Hyaluronan synthesis has also been suggested to peak in the G2 phase (Papakonstantinou et al., 1995).

High amounts of hyaluronan exist between the daughter cells during cytokinesis (Tammi and Tammi, 1991), and the formation of hyaluronan-dependent pericellular matrix is associated with mitotic cell detachment and rounding (Evanko et al., 1999). Also intracellular hyaluronan increases in proliferating cells, accumulating at the mitotic spindle and filling the space between separating chromosomes (Evanko and Wight, 1999), which may facilitate nuclear division (Hascall et al., 2004). These results suggest that hyaluronan has a dual role

in mitotic cycle; it helps the cells to detach in early phase of mitosis, and facilitates the segregation of daughter cells in the late phases of mitosis.

Addition of exogenous hyaluronan induces proliferation in many normal cell types, like keratinocytes (Kaya et al., 1997), mesothelial cells (Reijnen et al., 2001) and fibroblasts (Yoneda et al., 1988), as well as in cancer cells, like melanoma (Ahrens et al., 2001), malignant mesothelioma and ovarian carcinoma cells (Bourguignon, 2001). However, it reduces proliferation of synovial, endothelial, and CHO cells (Goldberg and Toole, 1987; West and Kumar, 1989; Dube et al., 2001), and has no effect on kidney epithelial cell or corneal epithelial cell proliferation (Haider et al., 2003; Gomes et al., 2004).

Overexpression of Has3 increases tumor cell proliferation at high cell densities (Liu et al., 2001). Correspondingly, inhibition of proliferation is achieved by Has2 or Has3 antisense inhibition of tumor cells (Simpson et al., 2002; Chao and Spicer, 2005; Nishida et al., 2005; Udabage et al., 2005b; van den Boom et al., 2006). However, overexpression of Has inhibits proliferation of CHO and smooth muscle cells (Dube et al., 2001; Wilkinson et al., 2006)

The effect of hyaluronan on cell proliferation may be mediated via hyaluronan binding to CD44 (Ahrens et al., 2001). Supporting this hypothesis depletion of CD44 inhibits keratinocyte proliferation induced by wounding and growth factors (Kaya et al., 1997), and hyaluronan-induced proliferation of endothelial cells is dependent on CD44 (Singleton and Bourguignon, 2004). Hyaluronan binding to RHAMM may facilitate entry into mitosis (Mohapatra et al., 1996). Intracellular RHAMM associates with actin filaments and microtubules (Entwistle et al., 1996; Assmann et al., 1999) and the MAP kinase cascade (Zhang et al., 1998), all of which are important in cell proliferation. The enhanced expression of both RHAMM and Has2 at the G2 and M phases of the cell cycle suggests a role in the separation of daughter cells after mitosis (Cho et al., 2001). In addition cdc37, a cell cycle regulatory protein, is able to bind hyaluronan (Grammatikakis et al., 1995).

2.5.3. Hyaluronan and cell migration

2.5.3.1. Association of hyaluronan content with cell locomotion

In many developing tissues such as neural crest (Pratt et al., 1975), corneal mesenchyme (Toole and Trelstad, 1971) and cardiac cushion (Markwald et al., 1978; Orkin and Toole, 1978; Camenisch et al., 2000), hyaluronan content is positively correlated with migration (Toole, 1982). Also in cultured embryonic fibroblasts high hyaluronan synthesis correlates with enhanced cell migration (Chen et al., 1989; Ellis et al., 1997). In an *in vitro* scratch wound of a cell lawn of mesothelial cells, hyaluronan synthesis is increased via Has2 expression and hyaluronan was localized in the migrating cells (Yung et al., 2000). Likewise, smooth muscle cells induced to migrate by scratch wound showed rapid but transient accumulation of hyaluronan (Savani et al., 1995). In migrating cells, hyaluronan is found specifically around the trailing end and in areas of plasma membrane ruffling (Evanko et al., 1999).

It has been suggested that addition of exogenous soluble hyaluronan stimulates the migration of especially transformed cells (Hall et al., 2001), like melanoma cells (Yoshinari et al., 1999), colorectal tumor cells (Tan et al., 2001), breast tumor cells (Bourguignon et al., 2000; Tzircotis et al., 2005) and transformed fibroblasts (Sohara et al., 2001). However, it has been shown to induce migration also in normal cells, like hepatic cells (Kikuchi et al., 2005), corneal epithelial cells (Gomes et al., 2004), mesothelial cells (Yung et al., 2000) and proximal tubular cells (Ito et al., 2004). Exogenous hyaluronan does not influence the migration of normal fibroblasts (Hall et al., 2001) and chondrocytes (Maniwa et al., 2001), and in CHO cells (Dube et al., 2001) and osteoclasts (Spessotto et al., 2002) it inhibits migration. The different response depends on the cell type, but the source of the hyaluronan, and possibly contaminating factors may also explain some of the contradictory results.

Besides serving as a general inducer of migration, hyaluronan acts as a chemoattractant for migrating cells (Tsuda et al., 2004). Cell migration is also induced on hyaluronan-coated surface (Thomas et al., 1992; Lewis et al., 2001), and in a three-dimensional matrix (Boudreau et al., 1991).

Furthermore, displacement of the hyaluronan from cell surface by hyaluronan oligosaccharides leads to a flattened and spread cell type, and reduced migration (Banerjee

and Toole, 1992; Evanko et al., 1999). The growth factors that stimulate hyaluronan secretion also stimulate migration (Karvinen et al., 2003b; Pasonen-Seppänen et al., 2003).

In line with these findings increased hyaluronan synthesis resulting from overexpressed Has transgenes leads to increased motility in some cell types like melanoma cells (Ichikawa et al., 1999), and fibroblasts (Li and Heldin, 2001; Itano et al., 2002), while expression of antisense Has constructs have been reported to reduce the migration rate of osteosarcoma cells (Nishida et al., 2005) and zebrafish embryonic cells (Bakkers et al., 2004). However, the excessive hyaluronan synthesis by transfected HAS may also inhibit migration. This was the case in arterial smooth muscle cells (Wilkinson et al., 2006), and in CHO cells (Brinck and Heldin, 1999; Dube et al., 2001). In the CHO cell line, addition of exogenous hyaluronan also reduces migration (Dube et al., 2001).

2.5.3.2. Molecular basis of hyaluronan influence on cell migration

2.5.3.2.1. Cell migration

Cell migration has been divided in to three different steps **(Fig. 4.)**: 1.) The cell is polarized with formation of a broad lamellipodium and repositioning of the microtubule-organizing center towards the side of migration front, 2.) the main cell body including the nucleus is translocated forward, and 3.) the trailing edge is retracted, as reviewed by (Mitchison and Cramer, 1996; Vicente-Manzanares et al., 2005). Protrusion of the lamellipodium requires generation of new actin filaments and reorientation of microtubules (Small et al., 2002). Instead of the polarized migration used by fibroblasts, leukocytes use ameboid movement, and fish keratocytes perform a gliding type of movement (Anderson et al., 1996). The models of the migratory events may have to be re-evaluated since the experimental data has been obtained from experiments using 2D models, while the cellular events in the normal 3D enviroment may be different (Friedl and Wolf, 2003).

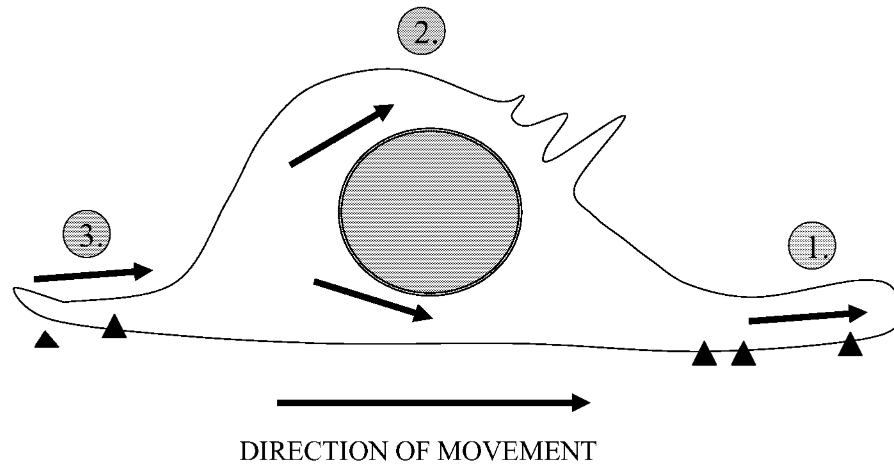

Figure 4. The cell migration in a 2D environment consists of three main steps: Formation of a broad lamellipodium at the leading edge, driven by actin polymerization and formation of new adhesions (triangles) (1), translocation of the main cell body (2), and retraction of the trailing edge with disassembly of focal contacts (3).

Lamellipodium is a thin (~0.2 µm) and broad (several micrometers) leaflet of the cell, and often contains thinner, filopodia-like extensions on its edge (Small and Resch, 2005). The lamellipodium is bound to substratum through attachment sites, which are formed and disassembled continuously, giving traction points for the movement (Small and Resch, 2005). The filopodia are 5 to 70 µm long, very dynamic, extending and retracting at a speed of ~10 µm/min, and probably serve as sensors of the environment (Mejillano et al., 2004). Actin polymerization drives the formation of these two types of protrusions, but with different mechanisms (Mejillano et al., 2004). The protrusion of the lamellipodium is achieved by dendritic actin nucleation of new short actin filaments at 70° angle onto preexcisting filaments through Arp2/3 activation (Mejillano et al., 2004; Small and Resch, 2005).

In the filopodia, actin filaments are organized in a parallel fashion, and elongation proceeds at the tip of the filament. Although the exact mechanisms controlling the growth of lamellipodia and filopodia are still open, it is known that members of the Rho family GTPases, Cdc42 and Rac are involved in these processes. Activation of Cdc42 is thought to stimulate the formation of filopodia, promoting linear actin growth, perhaps by regulating actin filament capping by formins and by bundling proteins (Vicente-Manzanares et al., 2005). Rac activation is associated with dendritic actin growth in lamellipodia by its ability to

control Arp2/3 through activation of WAVE (Jaffe and Hall, 2005; Vicente-Manzanares et al., 2005).

The plus ends of microtubules are selectively oriented towards the leading edge by plus-end binding proteins, and stabilized there, perhaps through the activity of Rac and Cdc42 (Watanabe et al., 2005). The orientation of microtubules enables the position of MTOC and the Golgi complex towards the leading edge and provides the route for polarized secretion of proteins to the lamellipodium (Watanabe et al., 2005).

Traction of the cell body requires generation of contractile forces by antiparallel arrays of actin and myosin II, while the adhesion sites in the lamellipodium provide support for the force (Small and Resch, 2005). The formation of new adhesion sites in the lamellipodium is believed to be controlled by Rac and Cdc42, while RhoA controls their maturation and dissolution at the rear end (Watanabe et al., 2005). RhoA is also supposed to control the acto-myosin contraction, although cdc42 and Rac may also regulate it (Jaffe and Hall, 2005).

Endocytosis and recycling of the membrane material to the leading edge is required for migration. Simultaneus recycling of adhesion molecules, like integrins, may be involved (Mellman, 2000). ECM components are internalized by migrating cells (Mellman, 2000), providing cell entrance through ECM, for example keratinocytes migrating into the wound are phagocytotic (Takashima et al., 1986). In 3D environment, proteolytic remodelling of the ECM by matrix-degrading enzymes, like matrix metalloproteinases (MMPs) is crucial for migration (Friedl and Wolf, 2003). For example, cleavage of dermal collagen by MMP-1 is reguired for keratinocyte migration (Pilcher et al., 1997).

In skin wounds keratinocyte movement has been suggested to occur through 3 different modes: 1) the basal keratinocytes actively migrate into the wound and passively drag the suprabasal layers with them (Usui et al., 2005), 2) the epithelial cells crawl as a sheet and 3) in a leaping fashion, suprabasal cells move on top of each other towards the wound, and after reaching the edge of the keratinocyte layer become attached to the denuded collagen matrix, forming new basal cells which remain static (Garlick and Taichman, 1994; Laplante et al., 2001). Although the mechanism is not settled, it seems that in human skin wounds both leaping and passive dragging of superficial layers are involved (Laplante et al., 2001). During migration to wound area epidermal keratinocytes close to the wound edge retract keratin filaments, dissolve the hemidesmosomal contacts to the basement membrane, and decrease the number of desmosomal contacts and gap junctions (Falabella and Falanga, 2001). They

also loose their apical polarity and acquire a flattened shape with protruding actin-rich lamellipodia (Falabella and Falanga, 2001). The formation of the extending lamellipodia may be induced by EGF (Koivisto et al., 2004). The migration of keratinocytes into the wound *in vivo* requires penetration under the fibrin clot (Kubo et al., 2001).

2.5.3.2.2. Hyaluronan association with cell protrusions

Addition of exogenous hyaluronan to cultured cells has been shown to cause formation of lamellipodia and filopodia (Bernanke and Markwald, 1979; Oliferenko et al., 2000), and a change in cell shape (Bodo et al., 1993). A direct role of endogenous hyaluronan in the lamellipodia formation was revealed in a zebrafish model, where overexpression of HAS2 induced numerous lamellipodia, while antisense inhibition of HAS2 caused a loss of lamellipodia (Bakkers et al., 2004). In the same way, fibroblasts overexpressing HAS2 exhibit prominent lamellipodia (Itano et al., 2002). The exact mechanism for hyaluronan-induced cell shape changes and formation of protrusions is still unknown, but may involve growth factor signaling and CD44 and RHAMM. Hyaluronan-CD44 interaction leads to ErbB2 autophosphorylation, inducing the migration of ovarian carcinoma cells (Bourguignon et al., 1997). Hyaluronan induces ErbB2 phosphorylation via CD44-dependent mechanisms in colon carcinoma and breast cancer cells (Ghatak et al., 2005). Hyaluronan induces a strong ErbB2 activation in endothelial cells, leading to their transition into mesenchymal cells during cardiac valve development (Camenisch et al., 2002). Because CD44 and RHAMM deficient animals do not have any defects in heart development, it is presumable that in this case the ErbB2 signaling induced by hyaluronan is not dependent on CD44 or RHAMM (Camenisch et al., 2002). Hyaluronan may also induce CD44 association with c-Src leading to dephosphorylation of cortactin and diminished association with actin (Bourguignon, 2001). Hyaluronan may induce ERK kinases through PDGF and c-Src via RHAMM (Hall et al. 1996).

Hyaluronan may also induce RhoA, Rac1 and cdc42 via CD44 without growth factor signaling. The intracellular domain of CD44 may bind and activate RhoA in a hyaluronan-dependent manner, leading to CD44 binding to the cytoskeletal protein ankyrin in breast cancer cells (Bourguignon et al., 1999). On the other hand, RhoA may activate migration of endothelial cells via PI3K (Bourguignon et al., 2003). Hyaluronan binding to CD44 activates

also Rac1 (Oliferenko et al., 2000), probably through CD44 binding to the GDP-GTP exchange factors Tiam1 and Vav2, leading to ruffling, formation of lamellipodia and migration (Bourguignon et al., 2000). The role of Rac1 in hyaluronan-dependent lamellipodia formation was confirmed in zebrafish model, where dominant-negative Rac1 inhibited the HAS2 induced lamellipodia formation (Bakkers et al., 2004). Furthermore, hyaluronan induces CD44 interaction with cdc42 and the IQGAP1-protein, known to regulate actin cytoskeleton (Bourguignon et al., 2005). RHAMM has been reported to mediate hyaluronan dependent signals for migration via ras (Turley et al., 2002).

Hyaluronan mediated signals may go through PI3K. Hyaluronan binding to CD44 induces PI3K and Akt activity in breast cancer cells (Bourguignon et al., 2003). Lamellipodia were depleted in Has2 transfected fibroblasts when treated with a PI3K inhibitor, without effect on hyaluronan coat (Itano et al., 2002), and PI3K inhibitors prevented the cell shape change and expression of mesenchymal proteins in epithelial cells overexpressing Has2 (Zoltan-Jones et al., 2003).

CD44 associates with the cytoskeleton via several mechanisms: it interacts with the ERM (ezrin, moesin, radixin) proteins and merlin, all known to bind cytoskeleton (Thorne et al., 2004). Phosphorylation of CD44 is regulated by PKC, and influences on CD44 binding to ERM proteins (Thorne et al., 2004), findings in line with the suggestion that growth factors regulate CD44 binding to ERM proteins (Herrlich et al., 2000). Other ERM binding proteins, like merlin, may act as an antagonist for CD44 (Thorne et al., 2004). CD44 association to actin may also depend on its plasma membrane lipid raft interaction (Oliferenko et al., 2000), which may be regulated (Thorne et al., 2004). The association of CD44 with ankyrin may also have a role in hyaluronan dependent cell migration (Lokeshwar et al., 1996a).

The hyaluronan receptor RHAMM regulates cell migration by inducing the formation and turnover of focal adhesions in a hyaluronan dependent manner (Hall et al., 1994). The intracellular form of RHAMM is known to associate both with microtubules and actin filaments (Assmann et al., 1999), which colocalize with intracellular hyaluronan (Evanko et al., 2004). Although its function there is not completely understood, RHAMM may regulate the stability of the spindle pole (Maxwell et al. 2003). The cytosolic RHAMM associates also directly with ERK and may regulate signaling complexes by binding them to cytoskeleton (Zhang et al., 1998). The hyaluronan receptor layilin also interacts with merlin and radixin, and regulates membrane ruffling (Bono et al., 2005).

2.5.4. Hyaluronan in cell differentiation and development

2.5.4.1. Differentiation

Hyaluronan has been reported to enhance the differentiation of chondrocytes (Kujawa and Caplan, 1986; Suzuki et al., 2005), myofibroblasts (Jenkins et al., 2004) and osteoclasts (Ariyoshi et al., 2005). In contrast, hyaluronan inhibits myoblast differentiation (Kujawa and Caplan, 1986). Keratinocyte hyaluronan synthesis is decreased, when differentiation is induced by calcium (Lamberg et al., 1986).

In normal adult human skin, hyaluronan is located mainly in the basal and spinous layers (Tammi et al., 1994b), while the terminally differentiated cell layers contain little or no hyaluronan. Hyaluronan may assist the keratinocyte movements and shape changes thus facilitating stratification (Tammi et al., 1988). On the other hand, extracellular hyaluronan may inhibit tight cell-cell contacts, required for terminal differentiation (Tammi and Tammi, 1998). Retinoic acid inhibits keratinocyte differentiation, weakens adhesion between keratinocytes (Tammi et al., 1985), and upregulates hyaluronan expression especially in the upper layers of the epidermis, where differentiation normally takes place (Tammi and Tammi, 1986; Tammi et al., 1989). Similarly, in rat organotypic cultures, upregulation of hyaluronan synthesis by EGF and KGF (Karvinen et al., 2003b; Pasonen-Seppänen et al., 2003), correlates negatively with differentiation. Removal of hyaluronan from epidermis enhanced the expression of differentiation related proteins in keratinocytes in organotypic skin cultures (Passi et al., 2004) and in mouse skin (Maytin et al., 2004). Interestingly, the expression of keratins K14 and K5, which are markers for basal, undifferentiated cells, are increased together with Has2 following treatment with a ginsenoside called compound K. The nature of this molecule is not published, but it increases hyaluronan amount and epidermal thickness in aging mice (Kim et al., 2004).

Hyaluronan content in the skin *in vivo* is increased when normal differentiation is disturbed, like in psoriatic skin, where hyaluronan is found in the parakeratotic stratum corneum, and particularly in the perivascular space of dermal papillae (Wells et al., 1991; Tammi et al., 1994a).

2.5.4.2. Development

Hyaluronan facilitates extrusion of the oocyte at ovulation and regulates the penetration of spermatozoa to oocyte (Salustri et al., 1999; Richards, 2005). Formation of three layered embryo during the gastrulation has been suggested to depend on hyaluronan (Müllegger and Lepperdinger, 2002), and Has1 and Has2 are both expressed in the embryonic tissues at this time (Tien and Spicer, 2005). However, the lack of either Has1 or Has2 is not lethal at this developmental stage, suggesting that they can replace each other (Camenisch et al., 2000). Later, hyaluronan is accumulated in the heart tube at the site of future valves due to Has2 upregulation, and the missing hyaluronan is lethal at this stage perhaps because Has2 is the only Has expressed in the heart tube (Tien and Spicer, 2005).

Marked and temporally regulated changes in hyaluronan content are also seen during the development of cornea (Toole and Trelstad, 1971), cartilage (Toole et al., 1972), joint cavity (Pitsillides et al., 1995) and palate (Pratt et al., 1973). In all these locations strong Has2 expression is present (Tien and Spicer, 2005). Because Has3 and Has1 nor Has3/Has1 double knockouts show any skeletal malformations, Has2 is propably responsible for hyaluronan production in these organs during the development. Hyaluronan has been reported to be required for epithelial tube formation in developing prostate (Gakunga et al., 1997), and kidney (Pohl et al., 2000), where hyaluronan resides at the tip of the ureteric bud, promoting its branching (Pohl et al., 2000). In the development of ectodermal appendages, such as hair follicles and teeth, hyaluronan is found both in the epithelial and in the mesenchymal compartments (Ågren et al., 1997a; Felszeghy et al., 2000; Tammi et al., 2005).

Has2 was reported to localize in the dermal compartment and Has3 in the epidermal compartment during development (Tien and Spicer, 2005). However, Has3 knockouts, or Has3/Has1 double knockouts have no defects in the skin during development, suggesting that Has2 can replace Has3 also in the epidermal compartment during the development (Tien and Spicer, 2005). In the interfollicular epidermis, hyaluronan expression is found early on; the beginning of stratification is associated with increased hyaluronan content throughout the epidermis, while later, during the formation of stratum corneum, hyaluronan is excluded from the terminally differentiated cells (Ågren et al., 1997a; Tammi et al., 2005). In mouse epidermis, hyaluronan levels decrease after birth, in parallel with Has2 and Has3 mRNA levels (Tammi et al., 2005).

2.5.5. Hyaluronan and wound healing

Hyaluronan, as an abundant ECM molecule in skin, has an important biological role in the various processes of wound healing (Chen and Abatangelo, 1999). Hyaluronan production by dermal fibroblasts increases transiently in the early wound (Price et al., 2005). Hyaluronan weakens cell attachment to ECM proteins, facilitates tissue swelling, and creates free space between dermal cells (Falabella and Falanga, 2001). Hyaluronan is also abundant in the frontline keratinocytes migrating into the wound (Oksala et al., 1995), and in the adjacent epidermis, showing wound induced hyperplasia (Tammi et al., 2005), normalized by *Streptomyces* hyaluronidase digestion (Maytin et al., 2004). The same growth factors that promote wound healing (Martin, 1997) stimulate also hyaluronan synthesis (Heldin et al., 1989). Furthermore, addition of exogenous hyaluronan (King et al., 1991) enhances the healing of experimental wounds.

CD44 in keratinocytes is increased at the wound area in human (Oksala et al., 1995), and in murine skin (Tammi et al., 2005). Mice with targeted inhibition of the epidermal CD44 expression show delayed wound healing perhaps due to an inhibition of growth factor-mediated signalling (Kaya et al., 1997). CD44 is also increased in hepatocyte wounding reaction (Kikuchi et al., 2005). Also RHAMM influeces hyaluronan-mediated migration after injury (Savani et al., 1995).

Fetal ECM is rich in hyaluronan, which may be a factor contributing to the scarless fetal wound healing (Longaker et al., 1991). Deletion of Hoxb13, a transcription factor with low expression in fetal wounds, results in high levels of hyaluronan, and enhances wound healing with less scarring (Mack et al., 2003). Since Hoxb13 possibly suppresses the expression of Has genes, its absense may create a more fetal-like environment. TGF-β, which may be associated with increased scar formation (Shah et al., 1992), inhibits hyaluronan production in keratinocytes (Pasonen-Seppänen et al., 2003). The lack of scarring in oral mucosa, as compared to skin, may be related to hyaluronan via differential expression of the three Has genes (Yamada et al., 2004).

2.5.6. Hyaluronan and cancer

2.5.6.1. Hyaluronan correlation with tumor progression

Large amounts of hyaluronan exist for example in the stroma of prostate (Lipponen et al., 2001), breast (de la Torre et al., 1993; Auvinen et al., 2000) and ovarian (Anttila et al., 2000; Hiltunen et al., 2002) tumors and in the parenchyme and stroma of colorectal tumors (Ropponen et al., 1998). However, in basal cell carcinoma and late stages of skin squamocellular carcinoma tumors (Karvinen et al., 2003a), oral squamous cell carcinoma (Kosunen et al., 2004) and melanoma (Karjalainen et al., 2000), the amount of hyaluronan is diminished in the tumor cells.

High hyaluronan levels correlate with poor prognosis of patients in colorectal (Ropponen et al., 1998), breast (Auvinen et al., 2000) and ovarian (Anttila et al., 2000) tumors. Elevated concentrations of hyaluronan in serum (Thylen et al., 1999), and in urine (Lokeshwar et al., 1997) are additional indicators of activated hyaluronan production in cancer patients. In tumors derived from squamous epithelia the content of hyaluronan in the malignant cells is negatively correlated with tumor progression (Karjalainen et al., 2000; Karvinen et al., 2003a; Kosunen et al., 2004).

Tumor cells have typically an increased hyaluronan synthesis rate (Kimata et al., 1983). Additionally, tumor cells stimulate stromal cells to produce elevated levels of hyaluronan (Knudson, 1996; Edward et al., 2005), but the factors and mechanisms involved are unknown. Hyaluronan synthesis correlates with the metastatic potential of cancer cells (Kimata et al., 1983; Udabage et al., 2005a).

2.5.6.2. Hyaluronan and cancer cell properties

High levels of Has2 in prostate cancer cells (Tsuchiya et al., 2002) and Has3 in colon carcinoma cells (Bullard et al., 2003) have been measured. Also HAS activity and transcriptional levels are increased after oncogenic malignant transformation of fibroblasts (Itano et al., 2004). In a recent study, both HAS1 and HAS2 proteins were highly expressed in malignant mesotheliomas (Kanomata et al., 2005).

After the first study by Kosaki et al. (1999), several groups have manipulated the hyaluronan synthesis of tumor cells and shown that transfection of tumor cells with expression vectors for Has alters their behavior. Has1 transfection into hyaluronan deficient breast carcinoma cell clones (Itano et al., 1999a) or melanoma cells (Ichikawa et al., 1999) enhanced their metastatic potential. Has3 transfection to prostate cancer cells failed to increase metastasis to lung (Liu et al., 2001), however, it increased the ability to bone marrow metastasis (Simpson et al., 2002). Has2 transfection induces the anchorage-independent growth of fibrosarcoma cells (Kosaki et al., 1999), melanoma cells (Ichikawa et al., 1999), MDCK and MCF-10 cells (Zoltan-Jones et al., 2003) and mesothelioma cells (Li and Heldin, 2001). In contrast, very high levels of Has2 expression can inhibit tumor growth (Itano et al., 2004). Increased growth of the primary tumors were seen in fibrosarcoma cells carrying transfected Has2 and prostate cancer cells carrying transfected Has3 genes (Liu et al., 2001). Elevated hyaluronan production is associated with a switch from epithelial-like to fibroblast-like phenotype (Li and Heldin, 2001), and induces mesenchymal and transformed properties in epithelial cells (Zoltan-Jones et al., 2003).

Antisense inhibition of Has2 and Has3 expression diminishes hyaluronan synthesis and cell surface retention in prostate tumor cells with reduced adhesion to bone marrow (Simpson et al., 2002). Anchorage independent growth, metastasis and invasiveness of cells can be reduced by antisense suppression of Has2 (Itano et al., 2004; Nishida et al., 2005; Udabage et al., 2005b) and Has3 (Bullard et al., 2003).

Increased hyaluronidase expression is reported in colon (Liu et al., 1996), bladder (Lokeshwar et al., 2005b) and prostate (Lokeshwar et al., 1996b) cancers. However, in ovarian tumor progression, there is evidence of diminished hyaluronidase activity (Hiltunen et al., 2002; Tuhkanen et al., 2004). Because HYAL1-3 genes are located in a gene locus often deleted in epithelial tumors, it has been suggested that hyaluronidase is a potential tumor suppressor (Frost et al., 2000). However, recent studies have shown that HYAL1 may act both as a tumor suppressor and promoter, depending on concentration (Lokeshwar et al., 2005a). Additionally, hyaluronidase activity is present only in one ("wild type") of the transcripts produced by alternative splicing (Lokeshwar et al., 2002), making the interpretation of previous mRNA assays more complicated.

Hyaluronan oligosaccharides induce angiogenesis (Deed et al., 1997; Rahmanian and Heldin, 2002) probably via CD44-dependent signaling (Takahashi et al., 2005). However,

oligosaccharides may inhibit tumor growth (Zeng et al., 1998) and predispose to apoptosis by suppressing the P13K/Akt signaling pathway also in a CD44 dependent manner (Ghatak et al., 2002).

Hyaluronan interaction with CD44 (Bourguignon, 2001) and RHAMM (Turley et al., 2002) leads to various signaling cascades involved in cancer cell growth, migration and other tumorigenic properties (Toole, 2004). Another way by which cancer cells benefit from hyaluronan is induction of multidrug resistance, a recent finding by (Misra et al., 2003; Misra et al., 2005).

3. AIMS OF THE STUDY

Hyaluronan is a multifunctional molecule, and its synthesis has been linked to migratory potential of normal and transformed cells. Hyaluronan has a specific role in skin epidermis, and has been suggested to regulate epidermal cell proliferation, migration and differentiation, with a special connection to keratinocyte activation during the wound healing process. After the molecular cloning of hyaluronan synthases, knowledge on the synthesis of hyaluronan has rapidly increased with the possibility of direct experimental manipulation of Has expression. However, the exact role of these enzymes in cellular functions is not precisely known, and the subcellular trafficking, plasma membrane targeting and regulation of their enzymatic activity are poorly understood. The aim of this study was to manipulate hyaluronan synthesis in cells by regulating Has expression and activity, and to study the resulting phenotype of keratinocytes and other epithelial cells.

The most important specific aims of this study were:

1. To investigate the effects of epidermal growth factor (EGF) and the hyaluronan synthesis inhibitor 4-methylumbelliferone on hyaluronan content, localization and size in keratinocyte cultures and their impacts on keratinocyte proliferation, migration and morphology.

2. To image, by utilizing normal and mutated GFP-tagged HASs, the subcellular localization, trafficking and function of HASs.

3. To study the plasma membrane sites of hyaluronan synthesis and the influence of hyaluronan synthesis on cell shape via manipulation of HAS activity by EGF and 4-MU and by transfection of GFP-tagged HASs.

4. MATERIALS AND METHODS

4.1. Cell culture (I-IV)

4.1.1. Monolayer cultures (I-IV)

A spontaneously immortalized epidermal keratinocyte cell line (REK) was originally isolated from neonatal rat epidermis by Baden and Kubilus (Baden and Kubilus, 1983), and further developed by MacCallum and Lillie (MacCallum and Lillie, 1990). This cell line is able to stratify and differentiate at air-liquid interphase (Tammi et al., 2000; Pasonen-Seppänen et al., 2001), but can also be cultured in low to moderate density monolayer cultures. REKs were cultured in minimum essential medium, MEM (Life technologies Ltd, Paisley, Scotland) supplemented with 5% or 10% fetal bovine serum (FBS, HyClone, Logan, UT), 4 mM glutamine (Sigma, St. Louis, MO) and 50 µg/mL streptomycin sulfate and 50 U/mL penicillin (Sigma). They were passaged twice a week at a 1:5 or 1:10 split ratio using 0.05% trypsin (w/v), 0.02% EDTA (w/v) in phosphate-buffered saline (PBS, Reagena Ltd, Kuopio, Finland).

Primary mouse epidermal keratinocytes were isolated from newborn wild type (C57Bl/6J) and CD44 -/- mice (obtained from Dr. Paul Noble and originally developed by Dr. Tak Mak, Toronto, Canada). The Animal Care and Use Committee of the University of Kuopio approved the study protocol. Epidermis and dermis were separated with dispase (type II, Boehringer-Mannheim, Mannheim, Germany), and the cells were released from the tissues with a brief treatment of 0.05% trypsin and 0.02% EDTA (Biochrom, Berlin, Germany). The keratinocytes were cultured in N-MEM. N-MEM contains EMEM without calcium (Biowhittaker, Verviers, Belgium) supplemented with 0.06 mM Ca^{2+}, 8% FBS (HyClone, Logan, UT) treated with Chelex (BioRad, Hercules, CA), 0.4 µg/mL hydrocortisone (hydrocortisone hemisuccinate, Sigma), 0.75 mM aminoguanidine nitrate (Aldrich, Steinheim, Germany), 2 ng/mL EGF (Sigma), and 10^{-10} M cholera toxin (Sigma), and 3T3-fibroblast conditioned medium (1:1).

HaCat human keratinocytes (a gift from Dr. Norbert Fusenig, Heidelberg, Germany) were cultured in DMEM (high glucose, Life Technologies, Paisley, UK) supplemented with 10% serum (FBS, PAA Laboratories). The cells were maintained by plating at 1:10 split ratio

twice a week. For passaging, the cells were treated with EDTA (0.02% in Dulbecco's PBS, Sigma) for 5 min, and then with 0.05% trypsin 0.02% EDTA (Biochrom) for 10 min.

A human breast cancer cell line MCF-7 was cultured in minimum essential medium alpha (MEMα) supplemented with 5% FBS (PAA Laboratories GmbH, Pasching, Austria), 2 mM glutamine (Sigma) and the antibiotics as above. The SKOV-3 human ovarian adenocarcinoma cells (ATCC, Manassas, VA) were cultured in McCoy's 5A medium (Euroclone) with 10% FBS, 2 mM glutamine and antibiotics. The Madine-Darby canine kidney epithelial cells (MDCK, a gift from Dr. MacCallum, Ann Arbor, MI) were cultured in DMEM (Euroclone) supplemented with 10% iron supplemented FBS, 2 mM glutamine and the antibiotics.

4.1.2. Organotypic keratinocyte cultures (II)

For organotypic cultures, keratinocytes were cultured on type I rat collagen, and lifted to the air-liquid interface just after having reached confluence (Tammi et al., 2000). When used, EGF was present in culture medium for the whole culture period (2.5 weeks), while 4-MU was added after 1.5 weeks and kept until the end of the experiment (for 1 week). At the end of the culture, hyaluronan was assayed 16 h after the last change of fresh medium using ELSA, and part of the cultures were fixed for microscopy.

4.1.3. Treatments of cells (I-IV)

The following compounds were added to living cells: Cells were treated with 0.2-200 ng/mL of EGF (Sigma). To inhibit hyaluronan synthesis, 4-MU (0.1-1 mM, Sigma) was used. The synthesis of hyaluronan and the secretory pathways of cells were manipulated by 5 µg/mL BFA (Sigma) to return and keep proteins at the ER, or cycloheximide (50 µg/mL) to block *de novo* protein synthesis in the culture medium for different times. Cells were also treated with high molecular mass hyaluronan (AmVisc™, Anika Therapeutics, Inc., Woburn, MA). To remove pericellular hyaluronan, living cells were treated with *Streptomyces* hyaluronidase (10 TRU/mL, Seikagaku Kogyo Co., Tokyo, Japan). To localize endocytosed material, Alexa Fluor® 594 Hydrazide (0.8 mM, Molecular Probes, Eugene, OR) was added

to the culture medium. To displace hyaluronan from its receptors, 0.2 mg/mL of HA_6, HA_{10}, HA_{12} or HA_{14} oligosaccharides (Seikagaku), Hermes 1 (a partly blocking antibody for CD44, 5 µg/mL, Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City) or R36 antibody (blocking antibody for RHAMM, 20 µg/mL, a generous gift from Dr. Rashmin Savani, University of Pennsylvia School of Medicine, Philadelphia, PA, USA) were added to the cultures. Microtubules were disrupted by a 30 min incubation on ice, followed by nocodazole (63 µM, Sigma) for 30 min at 37°C, and latrunculin B (5 µg/mL, Calbiochem®, La Jolla, CA) was kept in the cultures for 2 h at 37°C to disrupt actin cytoskeleton. Cholesterol was depleted from the cells by treating with 1 % methyl-β-cyclodextrin (Sigma) for 30 min-2 h at 37°C.

4.2. Transfections and gene constructs (I-IV)

The cells were transiently transfected with mammalian expression vectors encoding mouse Has2 or Has3 open reading frames that had been fused in-frame at their N-termini with the enhanced green fluorescent protein (GFP) sequence (Spicer and Nguyen, 1999). FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) was used for all transfections. In some experiments GFP-Has3 containing an inactivating missense mutation (D216A), or C-terminal truncations (R539stop and E510stop, respectively), or human taurine transporter in an EGFP-N1 vector (Molecular Probes, Eugene, OR) were used. The empty EGFP-pCIneo was used as a control for the transfections.

A rat full-length Has2 cDNA in antisense orientation in the PCIneo vector was transfected to REKs to create cell lines with decreased hyaluronan synthesis. To maintain stable transfections, the transfected cells were trypsinized, seeded on a 90 mm dish, and grown with 500 µg/mL G418 (Calbiochem-Nevabiochem Corp., La Jolla, CA). The new selection medium was changed every 3-4 days until separate colonies about 0.5 cm in diameter were found. Individual colonies were trypsinized with sterile cloning cylinders, seeded into 24-well plates, and grown to sufficient numbers for the experiments. The transfected genes were maintained by keeping G418 continuously in the culture medium at 250 µg/mL except during the experiments.

4.3. RT-PCR (I)

Keratinocyte RNA was isolated from the cultures with the TRIzol®-reagent (Life Technologies, Inc.). Equal amounts were aliquoted after spectrophotometric quantitation at 260 nm and treated with DNase (MBI Fermentas, Vilnius, Lithuania). For the RT-PCR reactions with primers specific for Has1, Has2, Has3 and GAPDH, the RNA PCR Core Kit (PerkinElmer Life Sciences, Branchburg, NJ) was used.

For the quantitation of rat Has2 mRNA, a shortened (internal standard) Has2 cDNA, containing identical primer binding sites to those in the wild-type Has2 cDNA was prepared by PCR. RT-PCR was performed with constant amounts of the wild type and different concentrations of the shortened Has2 RNAs. The resulting products were run on an agarose gel, digitized by a BioDocII™ Video Documentation System (Biometra, Göttingen, Germany), and quantitated by EtBr fluorescence by using the NIH Image 1.63 software (Wayne Rashband, NIH, Bethesda).

4.4. Northern blotting (I)

For the direct analysis of Has2 mRNA expression REKs were cultured in ~28 cm^2 dishes until confluency. The total RNA was isolated with the TRIzol®-reagent. RNA was dissolved in distilled H_2O and quantitated with a spectrophotometer. RNA was analyzed by electrophoresis on 1% formaldehyde/agarose gels and transferred onto Hybond-N™ nylon membranes (Amersham Pharmacia Biotech). A Has2-specific probe (1200 base pairs) was obtained from human mRNA by RT-PCR using the primers 5'-GAAACAGCCCCAGCCAAAGAC-3' and 5'-CTCCCCCAACACCTCCAACC-3' and labeled with [α-^{32}P] dCTP by PCR. Hybridization was done according to the ULTRAhyb™ hybridization protocol (Ambion, Austin, TX).

4.5. Quantitation of hyaluronan (I-IV)

4.5.1. Metabolic labeling (I, II)

Metabolic labeling of subconfluent REK cultures with 20 μCi/mL of ^{3}H-glucosamine and 100-200 μCi/mL of ^{35}S-sulphate (Amersham Pharmacia, Little Chalfont, UK) was done as described previously (Tammi et al., 1998). After labeling the medium was collected, and the cell layer was trypsinized, followed by pelleting the cells by centrifugation. The supernatant formed the "trypsinate" sample, and the cell pellet was designated as the "intracellular" sample.

Carrier hyaluronan (6 μg of Healon®, Amersham Pharmacia Biotech, Uppsala, Sweden) was added to all samples precipitated twice with 1.2 mL of ethanol for 2 h at -20°C and suspended in 300 μl of 150 mM sodium acetate, pH 6.5. Proteins were denaturated by boiling the samples for 10 min before papain digestion (Sigma, 200 μg/mL final concentration) at 60°C for 4 h. Papain was inactivated in a boiling water bath for 10 min and the samples were precipitated with 1% cetylpyridinium chloride for 10 min at room temperature. The precipitates were dissolved in 4 M guanidine HCl, and reprecipitated with ethanol at – 20 °C. After centrifugation, the pellets were dissolved in water.

The samples were then mixed with 4.5 μl of 0.5 M sodium acetate, pH 7.0, with 1 mU of *Streptococcus* hyaluronidase, and 25 mU of chondroitinase ABC (both enzymes from Seikagaku, Tokyo, Japan) and incubated for 3 h at 37°C. Aliquots of 40 μl were injected onto a 1 x 30 cm Superdex Peptide column (Pharmacia) eluted at 0.5 mL/min with 0.1 M NH_4HCO_3. The eluent was monitored at 232 nm, and for ^{3}H and ^{35}S activities to quantitate chondroitin sulfate and hyaluronan disaccharides.

4.5.2. Hyaluronan ELSA (II-IV)

After 6 or 24 hour cell culture in fresh medium (5% FBS) the media were harvested for the hyaluronan assay and the number of cells was counted. Maxisorp 96-well Plates (Nunc, Roskilde, Denmark) were precoated with HABC (Tammi et al., 1994b) (hyaluronan binding region of aggrecan and link protein, 1 μg/mL in 50 mM sodium carbonate buffer, pH 9.5) and

after 3 washes with 0.5 % PBS-Tween-20 blocked with 1 % BSA (Sigma) in PBS. Hyaluronan standards (0 - 50 ng/mL, Provisc®, Algon Laboratories, Fortworth, TX) and medium samples diluted 1:5 or 1:10 (100 µl) were pipetted on the plates and incubated for 1 h at 37°C. After washes, 100 µl of biotinylated HABC (bHABC, 1.0 µg/mL) was added, followed by incubation at 37°C for 1 h. After washing, 100 µl horseradish peroxidase streptavidin (1:5000, Vector Laboratories Inc., Burlingame, CA) was added and the plate was incubated at 37°C for 1 h, washed, and incubated with 100 µl of TMB substrate solution (0.5% 3,3',5,5'-tetramethylbenzidine in dimethyl sulfoxide diluted 1:50 with 0.1 M sodium acetate, 1.5 mM citric acid and 0.005% H_2O_2) for 15 min. After addition of 50 µl of 2 M H_2SO_4 the absorbance was measured at 450 nm wavelength.

4.5.3. Molecular mass distribution of hyaluronan (II)

To analyze the molecular mass of secreted hyaluronan REK cultures were incubated for 6 h in the presence of 20 µCi/mL of [^{3}H]-glucosamine. The culture media were collected, precipitated with ethanol and dissolved in 150 mM sodium acetate buffer, pH 6.8 containing 0.1 % CHAPS (3-{(3 cholamidopropyl)dimethyl-aminio}1-propanesulfonate, Sigma). The samples were chromatographed on a 1 x 30-cm column of Sephacryl S-1000 (Pharmacia, Sweden) at 0.4 mL/min with 0.15 M sodium acetate, 0.1% CHAPS, 0.05% Hibitane®, pH 6.8. Two aliquots were taken from each chromatography fraction, one incubated at 60° for 2 h with 0.5 TRU of *Streptomyces* hyaluronidase (Seikagaku Kogyo Co., Tokyo, Japan), and the other in buffer only. All samples were precipitated 1:1 with 2% CPC, and the precipitates recovered with centrifugation at 13.000 g for 15 min. The content of hyaluronan in each fraction was calculated by subtracting the radiolabel in the *Streptomyces* hyaluronidase treated samples (resistant to hyaluronidase) from those incubated in buffer only. The size distribution of hyaluronan in the samples was estimated from the K_{av} values of known hyaluronan standards, provided by the resin manufacturer.

4.6. Migration assay (I, II)

REK cells were grown until confluence and a cell-free area was introduced by scraping the monolayer crosswise with a sterile pipette tip. The cultures were then washed with Hank's balanced salt solution (Euroclone Ltd, Pero, Italy), and fresh medium with 5 % or 10% FBS was added. The effect of exogenous high molecular mass hyaluronan (Healon®, Pharmacia, Uppsala, Sweden or Provisc®, Algon, Fortworth, TX) on migration was studied in medium without FBS. The areas covered by the cells were measured immediately after scraping and 3, 6 or 24 hours later using an Olympus CK 2 inverted phase contrast microscope, a Panasonic Wv-CD 130-L video camera and NIH Image® software. The average distance the outermost cells had migrated was calculated using the formula: (b-a)/2, where a is the area covered by the cells at 0 hours and b is the area covered by the cells after different time points. The results (in pixels) were converted to micrometers.

4.7. Proliferation assay (I, II)

For EGF treatments, 20,000 cells were seeded in 24-well culture plates and grown for 3 days before addition of EGF. For 4-MU treatments, cells were seeded at 40,000 cells/well. Cells from duplicate wells were trypsinized and counted with a hemocytometer after different time points to determine the proliferation rate. The number of detached cells in media was also counted following concentration by low-speed centrifugation.

4.8. Microscopy (I-IV)

4.8.1. Hyaluronan staining for light microscopy (I, II)

Cells were seeded on eight-well chamber slides precoated for 30 min at 37°C with FBS (Nalge Nunc, Naperville, IL) and grown at 37°C for 48 h. The slides were washed with 0.1 M sodium phosphate buffer, pH 7.4 (PB), fixed at room temperature for 30 min with 2% paraformaldehyde (v/v) alone or with 0.5% glutaraldehyde (v/v) and washed 5x2 min with PB. Cells were permeabilized at room temperature with 0.3% Triton X-100 in 3% BSA and

probed with 5 µg/mL of bHABC in 3% BSA overnight at 4°C. After washing with PB, the slides were incubated with avidin-biotin peroxidase (ABC standard kit, Vector Laboratories Inc., Burlingame, CA) for 1 h, and the color was developed with 3,3' diaminobenzidine (DAB) and H_2O_2. The slides were mounted in Supermount (BioGenex, San Ramon, CA). The specificity of the staining for hyaluronan was controlled by removing hyaluronan with *Streptomyces* hyaluronidase (Seikagaku Kogyo Corp., Tokyo, Japan), and the specificity of the bHABC probe was verified by pretreating it with hyaluronan oligosaccharides (average size 20 monosaccharides).

4.8.2. Densitometry of cell-associated hyaluronan (I)

To quantitate the amount of cell associated hyaluronan, the optical density of bHABC – stained cultures was measured. A Leitz BK II microscope with a 16x objective with 0.45 numerical aperture (Leitz, Wetzlar, Germany) was connected to a 12-bit digital camera (Photometrics CH 200, Tucson, AZ) equipped with a KAF 1400 CCD detector (Eastman Kodak Corp., New York, NY). Camera control and image analysis were done with IPLab software (Signal Analytics Vienna, VA). Ten fields (731x841 µm) beginning from a randomly selected corner were systematically sampled along a line across each well, and area-integrated mean optical density (OD) values, including both DAB-positive and background intensities, were calculated for each whole digitized area. In addition, DAB-positive areas were estimated from binary images with a cut-off at an OD value of 0.13. On the basis of the positive area data and the sum of the pixel values that fulfilled the positivity criteria, the mean area-integrated OD values for the DAB-positive material were calculated.

4.8.3. Red blood cell exclusion assay (I, IV)

Fixed horse or sheep red blood cells (Sigma) were suspended in PBS containing 0.1 % BSA, washed three times and suspended with PBS. To visualize the hyaluronan-dependent coat around cells, red blood cells were added to cell cultures, allowed to settle on cells for 15 min at 37 °C, and examined by phase contrast or confocal microscopy.

4.8.4. Fluorescence stainings (II-IV)

For fluorescence stainings, cells were cultured on chamber slides precoated with FBS or on chambered cover glass slides (Nalge Nunc, Naperville, IL) coated with 1 µg/cm^2 of type I collagen (Becton and Bickinson, Bedford, MA). Cultures were fixed with 2% or 4% paraformaldehyde (v/v), permeabilized as described above, and incubated overnight at 4°C with bHABC (1.25-5µg/mL in 1% BSA) to visualize hyaluronan, or with the following primary antibodies in 1% BSA. For the Golgi complex, an anti-58K antibody (mouse ascites fluid, 1:100, Sigma) or trans-Golgi-network marker (diluted ascites, 1:200, Affinity Bioreagents, Golden, Colorado), for endoplasmic reticulum, anti-calnexin antibody (whole rabbit serum, 1:100, StressGen Biotechnologies Corp, Victoria, BC, Canada) and for focal contacts an anti-vinculin mAb (1:1000, Sigma), was used. For CD44 detection, rat anti-CD44, (clone OX50, 0.25 µg/mL, BioSource international, Inc., Camarillo, CA) or human anti-CD44 monoclonal antibody (Hermes 3, 1:100, a gift from Sirpa Jalkanen, Turku), and for microtubules anti-*β*-tubulin (1:500, Roche) was used. After washing, the cells were incubated for 1 h either with Texas Red (TR) or fluorescein isothiocyanate (FITC) -labeled streptavidin (Vector) for bHABC staining and with TR- or FITC-labeled secondary antibodies for immunostainings.

To visualize actin filaments the cells were incubated with Bodipy® FL Phalloidin (4 U/mL, Molecular Probes, Oregon, USA) for 20 min or Alexa Fluor® 594 Phalloidin (4 U/mL, Molecular Probes) for 1 h.

Lipid raft microdomains in plasma membrane were visualized by a Vybrant® Lipid Raft Labeling Kit (Molecular Probes). The cells were fixed with 4 % paraformaldehyde (v/v) on ice for 1 h, and the rafts were stained with an Alexa Fluor ® 594 conjugate of cholera toxin subunit B and a rabbit antibody against the subunit B.

4.8.5. Microscopy of organotypic cultures (II)

A part of the cultures was fixed in Histochoice® (Amresco, Solon, OH, USA), and embedded in paraffin. Hematoxylin/eosin stained sections were morphometrically analysed to measure epidermal thickness with the NIH-Image 1.62/fat software for Macintosh. The cultures were systematically sampled by taking 10 digital images with a CoolSNAP camera

(Photometrics) from each culture at constant distances in each section using a 20 × objective (Nikon Microphot FXA microscope, Japan).

For the double-staining of hyaluronan and CD44, antigen retrieval using TUF™ (Monosan, Uden, The Netherlands) was done. Deparaffinized sections were incubated for 30 min with 50 mM glycine to block autofluorescence, washed with PB, and incubated in 1% BSA in PB for 30 min to block nonspecific binding. The sections were incubated overnight at 4°C with anti-CD44 monoclonal antibody (OX50, 1:20) and bHABC solution (5 µg/mL), washed, and incubated simultaneously with TR-labeled antimouse antibody (Vector, 1:50) and FITC-avidin (Vector, 1:1000) for 1 h at room temperature. The sections were mounted in Vectashield (Vector) mounting medium.

4.8.6. Confocal microscopy (II-IV)

After staining, chamber slides were mounted in Vectashield (Vector) and the cell cultures on chambered coverglasses were left in PB. The micrographs were obtained with an Ultraview confocal scanner (Perkin Elmer Life Sciences, Wallac-LSR, Oxford, UK), on a Nikon Eclipse TE300 microscope, equipped with an Ultrapix CCD camera as a detector (Perkin Elmer) using a 100x or 60x oil immersion objective. Micrographs of living or fixed cells were obtained 24-48 h post-transfection. The images were further modified with Photoshop 6.0 software (Adobe, Mountain View, CA). For the three dimensional imaging, a series of horizontal optical sections were captured through the cell at 200 nm steps. Some of the images were deconvoluted with Microtome deconvolution 7.0. software (Vaytek, Fairfield, Iowa), and the three dimensional images were created and further processed using ImageJ 1.32 (http://rsb.info.nih.gov/ij/) or with Voxblast software (Vaytek). The scale was obtained with FocalCheck™ 6 µm diameter fluorescent microspheres (Molecular Probes, Oregon, USA).

For inspection and imaging of transfected, living cells we utilized Micro-Incubator (CSMI Microincubator (model CSMI, Harvard Apparatus Inc., Holliston, MA) to maintain conditions at 37 °C and 5 % CO_2.

4.8.7. Electron microscopy (I, IV)

For transmission electron microscopy, cells were fixed with 2% paraformaldehyde, 0.5% glutaraldehyde in PB for 20 min at room temperature, and permeabilized with 0.05% saponin in 3% BSA for 10 min on ice. Thereafter the cells were incubated with 10 µg/mL bHABC in 1% BSA, 0.05% saponin overnight at 4°C, followed by washes in 0.05% saponin-PB, and 2 h incubation with streptavidin peroxidase (1:500, Vector). The bound streptavidin was visualized by incubating the cells with 0.05% 3,3'-diaminobenzidine (Sigma) and 0.03% H_2O_2 in PB for 20 min on ice. The cells were postfixed with reduced osmium tetroxide (Stenberg et al., 1984), dehydrated in graded ethanol and embedded in Spurr's resin. Thin sections were mounted on formvar-coated copper grids, stained with uranyl acetate and lead citrate, and observed and imaged using a JEOL 1200 EX microscope (Tokyo, Japan). The level of background was controlled by pretreating the bHABC probe with hyaluronan oligosaccharides (Tammi et al., 1989).

For scanning electron microscopy, cells were grown and transfected on 13 mm coverslips placed in the bottom of 24-well plates. After 48 h, the cultures were washed and fixed with 4% paraformaldehyde for 2 h at room temperature. The coverslips were dehydrated through a graded series of ethanol. After critical point drying, they were shadowed with gold. The cells were photographed on an XL30 TMP environmental scanning electron microscope (FEI Company, the Netherlands) at 15 kV.

5. RESULTS

5.1. REK cell features (I, II)

5.1.1. REK cell morphology, proliferation and migration rate

REKs were flattened, morphologically epithelial in confluent monolayer cultures (I: Fig. 5a). Some elongated cells appeared in subconfluent cultures (II: Fig. 4a), or after artificial wounding, and lamellipodia were abundant in cells near the edge of cell-free areas (unpublished).

The duplication time of REKs was 18-20 h in subconfluent cultures. If the cells were not subcultured immediately after reaching confluency, the proliferation rate rapidly slowed down, stratification was initiated, and granular and cornified cells appeared as signs of differentiation.

A cell boundary introduced by scratching a confluent monolayer culture with a pipette tip, induced cell migration at a typical rate of 7-10 µm/hour (II: Fig. 3a, b). When the cleared area was approximately 1 mm wide, REKs recovered the cleared area in less than 2 days. Low serum concentrations slowed down the migration rate (unpublished data).

When cultured on a collagen gel at the air-liquid interface REKs stratified and differentiated forming a morphologically well-organized epidermis (II: Fig. 4a). After a 2.5 week culture period, vital epidermis consisted of 4-5 vital cell layers and a well-differentiated stratum corneum.

5.1.2. REK cell hyaluronan metabolism

In standard culture conditions, subconfluent REKs secreted approximately 4 ng of hyaluronan to culture medium per 10,000 cells in 24 h (II: Fig. 1c). The secreted hyaluronan was mainly of high molecular mass (II: Fig. 1e). Of the newly synthesized hyaluronan, 59-68% was secreted to culture medium, 16-25% was associated with plasma membrane, and 12-24% was located intracellularly, as measured in 3-h labelling periods at 3-24 hours after medium change (I: Fig. 4). The serum concentration in the culture medium had a small positive correlation with hyaluronan synthesis rate: 3% and 10% serum increased the

hyaluronan secretion by 20% and 30%, respectively, as compared to 0.5% serum (I: Fig. 4). The hyaluronan secretion rate was dependent on cell density and slowed down towards confluency (Rilla, data not shown). The change of fresh culture medium induced a 2-3 fold increase in hyaluronan secretion in 3-6 hours, which gradually slowed down when essential nutrients like glucose were depleted (I: Fig. 4).

In 2-week-old organotypic cultures the concentration of tissue-bound hyaluronan was about 150 ng per mg of epidermal tissue dry weight, and the collagen matrix and medium contained 2800 ng of soluble hyaluronan per mg of epidermal tissue dry weight.

Hyaluronan visualized by bHABC was localized mainly pericellularly in the areas facing cell-cell contacts in confluent cultures (I: Fig. 5a). In subconfluent cultures hyaluronan was localized in the apical surface as dense patches, near the edge of lamellipodia and some also intracellularly (I: Fig. 2). The staining intensity varied between individual cells. In organotypic REK cultures, hyaluronan was located mainly around the basal and spinous cell layers (II: Fig. 4a).

The RT-PCR studies showed that all Has isoforms, (Has1, Has2 and Has3) are expressed by REK cells (I: Fig. 9).

5.2. Effect of EGF on hyaluronan synthesis and REK phenotype (I, II)

5.2.1. Induction of hyaluronan secretion in REKs by EGF

EGF stimulated hyaluronan synthesis more than 2-fold, while the synthesis of other glycosaminoglycans was not significantly altered by EGF treatment. The concentration of EGF resulting in the highest synthesis rate of hyaluronan was 20 ng/mL. Because serum also stimulates hyaluronan synthesis, the effect of serum concentration on normal and EGF-stimulated hyaluronan synthesis was examined. Because EGF increased the rate of hyaluronan synthesis in a similar way in all serum concentrations, and low serum concentrations may affect cell vitality, we decided to use 10% serum in later experiments (I: Fig. 2).

^{3}H-glucosamine incorporation is often used to measure the rate of hyaluronan synthesis. However, the amount of incorporated ^{3}H-glucosamine depends on the specific activity of UDP-GlcNAc pool, which can vary according to the supply of endogenous glucosamine,

competing with the radiolabelled exogenous ^{3}H-glucosamine (Yanagishita et al., 1989). Indeed, it was shown that the specific activity of UDP-GlcNAc showed a tenfold increase during the culture, correlating with the decrease of glucose concentration in the growth medium (I: Fig. 3). Correcting the incorporation rates with the change in specific activity enabled determination of true hyaluronan synthesis rates. While the time after the change of culture medium showed such a dramatic effect, EGF did not change the specific activity of the ^{3}H –hyaluronan (I: Fig. 3)

The hyaluronan synthesis rate in confluent keratinocyte cultures 0-24 h after introduction of EGF was determined using 3-h labeling windows. Treatment with EGF induced a 3–6-fold increase of newly synthesized total hyaluronan, as compared to the control in the 3–9-h labeling windows. In later time points the stimulatory effect of EGF decreased, but the total synthesis at 21–24 h was still more than twice that in the control cultures. In all compartments (medium, pericellular and intracelular) the newly synthesized hyaluronan was increased with similar kinetics, with the highest relative increase (6–10-fold) in the intracellular hyaluronan pool (I: Fig. 4).

To visualize the cell-associated hyaluronan the keratinocyte cultures were stained with the hyaluronan-specific probe (bHABC). The intracellular hyaluronan was specifically indicated by digestion of the cell surface hyaluronan with *Streptomyces* hyaluronidase before staining. The microscopic assays of these cultures confirmed that EGF increased the intracellular and total cell-associated hyaluronan (I: Fig. 5). Interestingly, intracellular hyaluronan increased as early as 0.5 h after introduction of EGF (I: Fig. 6).

In EGF-treated organotypic REK cultures, hyaluronan secretion to culture medium was increased three-fold (II: Fig. 4).

5.2.2. Cell-associated hyaluronan in EGF-treated REKs

After EGF treatment, the normally flattened REKs became rounded or elongated and formed numerous membrane ruffles, filopodia and lamellipodia (I: Fig. 5).

Most of the hyaluronan in control cultures was located on plasma membrane as patches. In EGF-treated cells, the hyaluronan signal intensity was generally increased and hyaluronan covered the membrane ruffles and protrusions. In elongated cells the trailing end showed an intense hyaluronan signal, while the leading lamellipodium showed only localized spots of

hyaluronan. EGF treatment induced the formation of a pericellular hyaluronan coat in the rounded or elongated cells (I: Fig.7). Electron microscopy of vertical sections suggested that in sites where hyaluronan was located under the cell, the plasma membrane was not attached to the substratum (I: Fig. 7).

The intracellular hyaluronan signal of EGF-treated cells was located in small cytoplasmic vesicles and circular structures of various sizes and was most intensive in the rounding cells (I: Fig. 5f).

The staining of EGF-treated organotypic cultures showed that hyaluronan accumulated in the extracellular space of all vital layers, and also intracellularly (II: Fig. 4).

5.2.3. Effect of EGF treatment on REK migration and proliferation

EGF stimulated the speed of cell migration into the cleared area in the scrape wound assay. The migratory activity peaked at the same EGF concentration as for the synthesis of hyaluronan (I: Fig. 8a).

In monolayer cultures, EGF had no significant effect on the proliferation rate of the keratinocytes at EGF concentrations that showed the highest stimulation in hyaluronan synthesis and migration. A small inhibition on proliferation rate occurred at the highest, 200 ng/mL EGF level (I: Fig. 8b). In organotypic cultures, the epidermal thickness was increased after EGF-treatment, indicating that EGF increased the proliferation rate of keratinocytes in stratified cultures (II: Fig. 4).

5.2.4. EGF effect on Has mRNA expression

To find out which of the different Has's is responsible for the enhanced hyaluronan synthesis induced by EGF, we estimated the mRNA levels of Has1, Has2, and Has3 in REK monolayer cultures using RT-PCR. This analysis suggested that Has1 and Has3 mRNA levels were not markedly changed by EGF treatment. In contrast, Has2 level was increased by EGF (I: Fig. 9a). The basal level of Has2 mRNA in the keratinocytes was so low that the level of increase in its two transcripts was difficult to estimate by Northern blot (I: Fig. 9b). Therefore, we used quantitative RT-PCR to determine the increase in Has2 mRNA level in EGF-treated keratinocytes. These analyses for EGF-treated keratinocyte cultures indicated a

low copy average number before the change to fresh medium (6/cell), a detectable increase of Has2 mRNA even after 1 h, a peak at 6 h (54/cell), and a decrease towards the basal level by 24 h. The number of Has2 mRNA copies following change of fresh control medium without EGF followed similar kinetics but was clearly lower compared to EGF-treated cells in all time points (I: Fig. 9d).

5.2.5. Migration inhibition by Has2 antisense transfection

We analysed the migration rate of a REK cell line with reduced synthesis of hyaluronan due to a transfected Has2 antisense gene, to confirm the correlation between migration and Has2 expression. The Has2 antisense cells showed a reduced migration rate compared with its mock-transfected controls (I: Fig. 10), indicating that Has2 has an important role in the migration of REKs.

5.3. Effect of 4-MU on REK hyaluronan synthesis and morphology (II)

5.3.1. Effect of 4-MU on secretion, molecular mass and localization of hyaluronan

When monolayer REK cultures were treated with 4-methylumbelliferone, a dose-dependent decrease in the amount of secreted hyaluronan was detected. When measured with HA-ELSA, the relative inhibition was ~20% at 0.2 mM, while at 1 mM concentration the relative inhibition was ~35% and ~60% after treatments of 6 and 24 h, respectively (II: Fig. 1). A similar dose dependent decrease in the total amount of newly synthesized hyaluronan (medium and cell layer combined) was also found using metabolic labeling with ^{3}H-glucosamine and ^{35}S-sulphate (II: Table 1). 4-MU had less effect on chondroitin sulfate or heparan sulfate synthesis (II: Table 1).

Because EGF strongly induces hyaluronan synthesis in REK cells (I: Fig. 1) we compared the effects of 4-MU on EGF-stimulated and non-stimulated cells. A more prominent, 65 - 85% inhibition in hyaluronan secretion was found in cells treated with EGF as compared to untreated cells.

The molecular mass distribution of the secreted (II: Fig. 1e) or cell layer associated hyaluronan (data not shown) did not change during a 6-h treatment with 4-MU, as analysed with gel filtration (II: Fig. 1e).

In control cultures, hyaluronan visualized with the bHABC probe was typically localized as dense patches mostly on the apical cell surfaces. Instead, hyaluronan in 4-MU-treated REKs had a less intense and more diffusely distributed staining, and most of the remaining hyaluronan was located on the basal side of the cells, often surrounded by a circle of adhesion plaques (II: Fig. 2b and 2f).

5.3.2. 4-MU effects on cytoskeleton, migration and proliferation rate

Phalloidin staining visualized filamentous actin in the cell cortex and in numerous filopodia in REK control cultures. After 4-MU treatment, the lamellipodia and filopodia were severely reduced in size, the cells became flat and spread, and started to show stress fibers, often forming a spindle-like structure in the middle of the cell (II: Fig. 2g, h). Additionally, 4-MU increased the number and size of vinculin-positive adhesion plaques under the cells, as demonstrated by confocal microscopy (II: Fig. 2f). In addition, all EGF-induced features in the REK phenotype including accumulation of cell surface hyaluronan, and the lifted, elongated cell shape, were blocked by 4-MU.

In the presence of 0.1 mM and 0.5 mM 4-MU, a 25% and 60% reduction, respectively, was observed in the migration rate in 24 h. The inhibition was rapid, appearing after a 3 h treatment (II: Fig. 3a-b). This excludes the possible contribution of cell proliferation in filling the cleared area.

Treatment with 4-MU reduced the number of cells dose dependently, and with 0.5 mM concentration, cell proliferation was completely arrested (II: Fig. 3c). The cell counting 4 h after plating resulted in equal numbers for control and 4-MU treated cultures, which indicates that 4-MU does not disturb cell attachment to substratum. No difference in the number of dead, floating cells was detected in control and 4-MU-treated cultures. Addition of high molecular mass hyaluronan (100 μg/mL) did not rescue the proliferation rate of 4-MU-treated cells.

The reversibility of the 4-MU-induced proliferation block was also tested after a 24-h treatment. After transfer into a 4-MU free medium, the cell number was duplicated in 24 h, suggesting that the effect of 4-MU on cell proliferation was fully reversible (II: Fig. 3d).

5.3.3. 4-MU in organotypic REK cultures

The REK cells stratify and form structurally normal, differentiated epidermis when cultured on a collagen gel in the air-liquid interphase (Tammi et al., 2000; Pasonen-Seppänen et al., 2001). In these organotypic cultures, the effect of 4-MU on hyaluronan secreted into culture medium or associated to the tissue was relatively small compared to monolayer cultures. The normal structure or differentiation pattern of the control cultures was not markedly changed by 4-MU. However, 4-MU totally blocked the EGF-induced hyaluronan synthesis and accumulation in the organotypic epidermis, and normalized the basal cell shape, epidermal thickness and the general tissue architecture disturbed by EGF (II: Fig. 4).

5.4. GFP-HAS localization and traffic in REKs (III)

5.4.1. Cellular distribution and enzymatic activity of Has-GFP fusion proteins

To study HAS localization and traffic in rat epidermal keratinocytes, we utilized expression plasmids containing mouse Has2 and Has3 open reading frames (ORFs) with N-terminally coupled GFP. The content of hyaluronan in the medium was clearly higher in the cultures transfected with GFP-Has2 and GFP-Has3 constructs as compared to cells transfected with the vector containing GFP only. Transfection with GFP-Has3 construct with a point mutation in the enzymatically active site (D216A), or with two truncated forms, lacking 16 and 45 amino acids from the C-terminus (R539stop and E510stop, respectively) did not raise the amount of secreted hyaluronan in comparison to control cultures transfected with GFP only (III: Fig. 1).

Both GFP-HAS2 and GFP-HAS3 were abundant inside the cells at vesicular structures, most concentrated around the nucleus, and morphologically resembling the ER-Golgi complex. Both GFP-HAS2 and GFP-HAS3 were found on plasma membranes although GFP-

HAS3 showed more intense and widely spread signal on cell surface. Both GFP-HAS2 and GFP-HAS3 accumulated particularly on cell surface protrusions of varying length and number (III: Fig. 2), which showed upward orientation in z-sections (III: Fig. 2d, f). Sometimes GFP-HAS positive material, not associated with cells, was found extracellularly (unpublished). In addition, there were often GFP-labeled vesicles of variable size in the cytoplasm and some cells also contained large vesicles with a GFP-positive perimeter. Similar intracellular structures resembling endocytic vesicles were also found in EGF-treated keratinocytes (I: Fig. 5f).

In contrast, the truncated and point mutated constructs of HAS3 were not present on plasma membrane at levels detectable by confocal microscopy. Instead, the GFP-HAS3 with longer truncation showed mainly ER-localization and a large part of the GFP-HAS3 with point mutation localized in the Golgi (III: Fig. 4).

5.4.2. GFP-HAS colocalization with subcellular markers and hyaluronan

Different markers were used to confirm the subcellular localization of GFP-HASs. Anti-CD44 immunostaining co-localized with GFP-HAS3 on plasma membrane. The strong perinuclear GFP-HAS3 signal showed partial colocalizations with the 58K Golgi marker, trans-Golgi marker and the ER-marker calnexin (III: Fig. 4). GFP-Has2 transfected cells showed patterns similar to those of GFP-Has3.

Treatment with nocodazole, an agent disrupting the microtubule dependent normal morphology of the Golgi complex, dispersed the perinuclear GFP-HAS3 structures, some of which retained the colocalization of 58K and GFP-HAS3 (III: Fig. 3d). This supports the idea that a significant proportion of GFP-HAS3 resided at the Golgi complex.

To find out if a portion of the intracellular GFP-HAS3 was derived through endocytosis from plasma membrane, a fluid phase marker, Alexa Fluor Hydrazide, was introduced in to the culture medium. The marker was rapidly taken up in a part of the GFP-HAS3 positive, small and the larger vesicles (III: Fig 3 e), which suggests that a portion of the intracellular GFP-HAS3 pool was derived from endocytosed material.

REKs transfected with GFP-Has3 and GFP-Has2 showed elevated levels of cell surface hyaluronan that partially colocalized with GFP-HASs, especially on the microvillus-like projections (III: Fig. 3f). Removal of the pericellular hyaluronan with *Streptomyces*

hyaluronidase digestion helped to confirm that a part of the intracellular hyaluronan colocalized with GFP-HAS3. The origin of the intracellular hyaluronan was studied by digestion of the extracellular hyaluronan with *Streptomyces* hyaluronidase in live cell cultures for up to 4 h before fixation and staining for hyaluronan. This led to almost complete disappearance of the intracellular hyaluronan. The reduced hyaluronan pool on the cell surface presumably diminished the flux into the cell, finally resulting in the disappearance of intracellular hyaluronan.

The R539stop truncated form of GFP-HAS3 (like D510stop) showed a diffuse reticular pattern and colocalized with an ER-marker and to a lesser extent with the Golgi marker (III: Fig 4). Instead, the point mutated D216A was mainly colocalized with the Golgi marker and less with an ER-marker. None of these modified GFP-HAS3 forms was clearly detectable at the plasma membrane, confirmed by the absence of colocalization with CD44. The amount of cell-associated hyaluronan was comparable to untransfected cells and hyaluronan did not show any colocalization with the mutated GFP-HASs. The results indicate that the truncated HASs mainly stayed at the ER, while the point mutated construct reached the Golgi complex, but did not get to the plasma membrane at detectable levels.

5.4.3. Effect of BFA and cycloheximide on GFP-HAS3 traffic

Treatment with BFA reduced the secretion of hyaluronan both in transfected and non-transfected REKs. A 4 h chase after BFA treatment almost restored the amount of hyaluronan released into the culture medium, both in untransfected, GFP-transfected and GFP-Has-transfected cultures, indicating that the effect of BFA was reversible (III, Fig. 5).

Protein synthesis inhibition using a 4 h cycloheximide treatment caused ~50% reduction of hyaluronan secretion into culture medium. When added together with BFA, cycloheximide did not further impair hyaluronan synthesis. To see whether the recovery of hyaluronan synthesis during the chase after BFA was due to newly translated HAS-protein, or redistribution of pre-existing HAS blocked at Golgi and ER, we added cycloheximide into the chase medium of the cultures pretreated with BFA. During a short (4h) chase following BFA block, hyaluronan secretion recovered almost at the same rate as in cultures chased without cycloheximide, suggesting that the recovery was largely due to HAS already existing in the secretory pathway (III: Fig 5).

Confocal analysis showed that BFA treatment resulted in a diffuse pattern of the GFP-HAS3 signal with no signal at plasma membrane. This was similar to the distribution of C-terminally truncated GFP-HAS3 and indicated that GFP-HAS3 had returned from Golgi area to ER.

After a 1-h chase following BFA block, most of the GFP-HAS3 had accumulated to the Golgi complex, and a small amount also to plasma membrane. After a 2-h chase, an intense GFP-HAS3 labeling appeared also on the plasma membrane and in the large endocytic vesicles. The GFP-HAS3 signal at plasma membrane was often more intense in the chased than untreated control cultures, and colocalized strongly with hyaluronan (III: Fig 6f).

After a 2-h cycloheximide treatment the distribution of GFP-HAS3 resembled that in the untreated cells. After 4 h in cycloheximide, the GFP-HAS3 signal was reduced, but still found on the plasma membrane and in small cytoplasmic vesicles. After 5h, the GFP-HAS3 signal was very low, indicating that the turnover time of the GFP-HAS3 protein in REKs is 4-5 h (III: Fig 6g-i).

5.4.4. Effect of hyaluronan synthesis inhibition on GFP-HAS localization

4-methylumbelliferone (4-MU), an inhibitor of hyaluronan synthesis (II), caused a dose-dependent (70%- 90%) reduction in the amount of hyaluronan secretion in cells transfected with the GFP-Has-2 and 3 and the GFP-vector only (III: Fig. 7). Confocal images showed that most of the plasma membrane associated GFP-HAS3 disappeared after 4 h in 4-MU. This location of GFP-HAS3 in 4-MU treated cells resembled that of the point mutated GFP-HAS3 construct, with no colocalization with hyaluronan. The removal of 4-MU from the culture medium rapidly (in 2 h) restored the plasma membrane signal for GFP-HAS3, and the strong colocalization with hyaluronan (III: Fig 6 j-l). The secretion of hyaluronan into culture medium was also rapidly restored after 4-MU removal. The results suggest a connection between hyaluronan synthesis rate and GFP-HAS3 association with the plasma membrane.

5.5. GFP-HAS3 and plasma membrane protrusions (IV)

5.5.1. Hyaluronan secretion after GFP-HAS3 transfection

The MCF-7 human breast cancer cell line secreted about 2 ng of hyaluronan /10 000 cells in 24 hours (IV: Table I). Transient transfection of GFP-HAS3 into these cells induced up to a 100-fold increase in the secretion of hyaluronan into culture medium compared to control cultures transfected with a vector containing a GFP alone (IV: Table I). The proportion of transfected, GFP-positive cells of all cells was 20-50%, which means a marked increase in every cell expressing GFP-HAS3.

The cell associated hyaluronan was also increased in GFP-HAS3 transfected cells (IV: Fig. 1b) as indicated by a particle exclusion assay. No exclusion space existed around cells transfected with a plasmid encoding GFP only (IV: Fig. 1a).

5.5.2. Formation of GFP-HAS -positive protrusions rich in hyaluronan

The confocal microscopy showed that the area of red blood cell exclusion was filled with long GFP-HAS3 positive cell protrusions (IV: Fig. 1b). Similar protrusions were not present in MCF-7 cells transfected with GFP only (IV: Fig. 1a). Staining with the biotinylated HABC (IV: Fig. 1c) showed large amounts of hyaluronan colocalized with GFP-HAS on the protrusions. Very few if any cell surface protrusions positive for hyaluronan were found on cells transfected with GFP only (data not shown).

The ultrastructure of the protrusions, stained for hyaluronan with the biotinylated HABC probe, was observed by transmission electron microscopy. This showed that the protrusions and the plasma membrane of the transfected cells were associated with plenty of hyaluronan (IV: Fig. 1e). During scanning electron microscopy processing, the microvilli were collapsed onto the cells, but cells without GFP-Has3 transfection showed no such microvilli (IV: Fig 1f). The EM analyses showed that the average diameter of the microvilli was 120-130 nm.

There was also an abundant intracellular pool of GFP-HAS3 in MCF-7 cells, representing its traffic along the secretory pathway through the endoplasmic reticulum and Golgi complex, and endocytic vesicles, like in keratinocytes (III).

The growth rate of the microvilli was studied by treatment with BFA, which blocks the traffic of GFP-HAS3 from the Golgi to the plasma membrane. Time lapse imaging of individual cells following removal of BFA indicated that the microvilli started to emerge after 1.5 - 2 h (IV: Fig. 2), corresponding to the time required for GFP-HAS3 transport from the ER to the plasma membrane (III). The microvilli reached their full length during 4 - 5 h and then remained at that state (IV: Fig. 2).

The expression of GFP-HAS3 resulted in a "hedgehog" appearance in all cell types studied so far, including rat (III), human and mouse keratinocytes, SKOV-3 ovarian cancer cells and MDCK kidney epithelial cells (IV: Fig. 3). The length of the microvilli was shorter in other cell lines as compared with the ovarian or breast cancer cell lines.

5.5.3. The structure of GFP-HAS3–induced protrusions

Staining with phalloidin showed that actin filaments were present in the core of the protrusions (IV: Fig. 4), while an antibody against β-tubulin gave no signal in the protrusions (data not shown). Accordingly, degradation of actin filaments with latrunculin B destroyed the protrusions (IV: Fig. 4d), but nocodazole, an agent that disrupts microtubules, had no such effect on cell morphology. These results indicate that the protrusions were based on actin filaments.

Staining for lipid raft microdomains utilizing cholera toxin subunit B showed a partial colocalization between GFP-HAS3 and the lipid rafts (IV: Fig. 5c). Introduction of methyl-β-cyclodextrin (IV: Fig. 5d), an agent that abducts cholesterol from lipid rafts, resulted in disappearance of the protrusions. These data suggest that cholesterol, probably associated with the lipid raft material, is vital for the maintenance of the protrusions.

To control if the protrusions were specific for the GFP-HAS3 protein, we used a GFP-labeled taurin transporter, which is also inserted into plasma membrane. The GFP-taurin transporter localized to plasma membrane, including some cell protrusions (IV: Fig. 6c, d). However, the branched protrusions in taurin transporter transfected cells were all lying on the basolateral aspect of the cell, close to cell substratum (Fig. 6c), while the HAS dependent protrusions mostly projected up from the apical side of the cell (IV: Fig 6b).

5.5.4. Hyaluronan dependence of cell surface protrusions

No protrusions were found on cells transfected with the enzymatically inactive missense mutant (D216A) of GFP-Has3 (IV: Fig. 6 e,f). Likewise, the enzymatically inactive GFP-HAS3 proteins with C-terminal truncations did not induce protrusion formation (data not shown). The dependence of plasma membrane protrusions on hyaluronan was studied by treatment with *Streptomyces* hyaluronidase, an enzyme specific for the degradation of hyaluronan. In live cell cultures, addition of *Streptomyces* hyaluronidase disrupted the protrusions in a few minutes (IV: Fig. 7h, i). Additionally, the protrusions were mostly retracted 3 h after introduction of 4-MU (IV: Fig 7 b), a hyaluronan synthesis inhibitor (II, III), which further indicates that active hyaluronan synthesis is required for their maintenance.

5.5.5. The protrusions and hyaluronan receptors

The GFP-HAS-induced plasma membrane protrusions were strongly positive for the hyaluronan receptor, CD44 (IV: Fig. 7c). To check the effect of signaling through CD44 on the formation of the protrusions, we added soluble hyaluronan in various concentrations up to 1 mg/mL to non-transfected cultures. No induction of protrusions was found with added hyaluronan, as monitored by lipid raft staining. Likewise, cultures incubated with a blocking antibody against CD44 (Hermes 1) did not disrupt the protrusions created by GFP-HAS3. Incubations with high concentrations of HA_6 hyaluronan oligosaccharides, displacing hyaluronan from CD44, were also without effect (IV: Fig. 7g). We also expressed GFP-Has3 in epidermal keratinocytes derived from CD44 knockout mice. Since protrusions were also found on CD44 -/- cells (IV: Fig. 7d, e), we conclude that CD44 is not necessary for their formation.

To probe for the involvement of receptors other than CD44, we incubated the GFP-HAS3 transfected cultures with blocking antibody against RHAMM, resulting in no change in the protrusions and suggesting that RHAMM was not involved, either. The protrusions were also resistant to HA_{10} and HA_{14} oligosaccharides competing hyaluronan off from all currently known hyaluronan binding proteins.

6. DISCUSSION

6.1. Stimulation of hyaluronan synthesis by EGF

The temporal changes in the mean copy number of Has2 mRNA closely correlated with those of hyaluronan synthesis, both in control and EGF-treated cultures, suggesting a transcriptional regulation (I). In preovulatory cumulus oophorus cells, hyaluronan synthesis is also upregulated via Has2, and a raise in hyaluronan secretion shows a temporal correlation with Has2 copy numbers (Dr. Csaba Fülop, Cleveland, personal communication, I).

Has2 was the only isoform responding to EGF in rat (I) as well as in human keratinocyte monolayer cultures (Saavalainen et al., 2005). Of the Has isoforms, Has2 is also the main target for transcriptional regulation mediated by other growth factors like PDGF and TGFβ in vascular endothelial cells and mesothelial cells (Jacobson et al., 2000; Suzuki et al., 2003). However, in oral epithelial monolayer cultures, EGF induced Has1 and Has3 expression (Yamada et al., 2004), and in organotypic keratinocyte cultures, after 10 days treatment, both Has2 and Has3 were upregulated by EGF (Pasonen-Seppänen et al., 2003). This suggests that Has3 expression is dependent on the state of keratinocyte differentiation or duration of the stimulus. The present and previous data suggest that regulation of hyaluronan synthesis by growth factors occurs mainly at transcriptional level.

It was unexpected that EGF stimulated a rapid accumulation of intracellular hyaluronan, located in cytoplasmic vesicles (I). Intracellular hyaluronan accumulation occurs also in serum-stimulated fibroblasts (Evanko et al., 1999) and GFP-HAS transfected cells (III). The disappearance of intracellular hyaluronan after pericellular hyaluronan digestion (III) suggests that the rapidly increased intracellular hyaluronan was endocytosed from cell surface, perhaps by a CD44-dependent mechanism (Tammi et al., 2001).

The dose-dependences of hyaluronan synthesis and cell migration following EGF-treatment were closely correlated (I). The dependence of cell migration on hyaluronan synthesis was also supported by the migration inhibition in the stable cell line expressing Has2 antisense constuct (I). The number of ruffles and microspikes were increased and the cells became elongated shortly after the addition of EGF (I). These functional and morphological features induced by EGF were blocked when hyaluronan synthesis was

inhibited with 4-MU (II), suggesting that they are also associated with enhanced hyaluronan synthesis.

6.2. Mechanism of hyaluronan synthesis inhibition by 4-MU

4-MU has been reported to inhibit hyaluronan production of several cell types including human skin fibroblasts (Nakamura et al., 1995), *Streptococcus equi* FM100 cells (Kakizaki et al., 2002), melanoma cells (Kudo et al., 2004), and pancreatic cancer cells (Nakazawa et al., 2006). 4-MU inhibited also keratinocyte (II, III) and breast cancer cell (IV) hyaluronan secretion dose-dependently. Similar levels of inhibition (50% with 0.5 mM 4-MU) have been reported with human fibroblasts (Nakamura et al., 1995) and melanoma cells (Kudo et al., 2004), while rat 3Y1 fibroblasts seem to be more sensitive, 0.05 mM 4-MU almost totally blocking hyaluronan synthesis.

The fact that 4-MU inhibited hyaluronan synthesis, even in the transfectants in which Has expression is driven by an exogenous promoter, supports the idea of post-transcriptional inhibition of hyaluronan synthesis (III, Kakizaki et al., 2004). The unchanged levels of transfected Has2 mRNA by 4-MU (Kakizaki et al., 2004), and the change of GFP-HAS localization and activity after 4-MU treatment (III, IV) are also in line with a major post-transcriptional effect. Furthermore, the removal of 4-MU rapidly restored the plasma membrane GFP-HAS3 even after long (24 h) treatment (III), indicating that the main target of 4-MU must be at post- transcriptional, probably post-translational level.

Indeed, the inhibitory effect of 4-MU on hyaluronan synthesis is probably due to UDP-GlcUA depletion via glucuronic acid conjugation to 4-MU (Kakizaki et al., 2004), a notion consistent with our own unpublished results. However, at high concentrations, also transcriptional inhibition of endogenous Has2 is involved, suggesting another, still unsolved mechanism (Kakizaki et al., 2004).

In the bacterium *Streptococcus equi* FM100, 4-MU was reported to inhibit HAS activity due to incorrect insertion of the HAS protein in the bacterial membrane, by disrupting the distribution of cardiolipin in the membranes (Kakizaki et al., 2002). This is not inconsistent with our results, but suggests a different mechanism of action on bacterial cells, since cardiolipin is required for HAS activity in these bacteria (Tlapak-Simmons et al., 1998), but

not in eukaryotic cells (Yoshida et al., 2000). Still, association of HAS with lipid rafts (IV) suggests that HAS activity may depend on the lipid environment on plasma membrane.

In keratinocytes, the inhibitory effect of 4-MU on hyaluronan synthesis was more pronounced when hyaluronan synthesis was induced by EGF (II). Furthermore, inhibition of hyaluronan synthesis by 4-MU had little effect on hyaluronan synthesis or morphology of organotypic cultures in normal control cultures, but the EGF-induced hyperproliferation, associated with hyaluronan accumulation, was normalized with 4-MU (II). The stronger inhibition of hyaluronan synthesis in EGF-treated cultures may result from the more severe depletion of the cellular UDP-GlcUA pool (Kakizaki et al., 2004) due to a larger consumption created by the EGF-enhanced hyaluronan synthesis.

6.3. HAS trafficking

The study on GFP-labeled HAS proteins showed their existence throughout the secretory route, including ER, Golgi complex, and plasma membrane **(Fig. 5)**, in line with the results of Müllegger (2003). Immunostainings confirmed the ER-Golgi localization of HAS2 (III). Utilization of mutated and truncated Has constructs, and BFA which disturbs intracellular transport through Golgi, confirmed the trafficking of HAS along the ER-Golgi-pathway (III).

Keratinocyte hyaluronan synthesis can be activated in less than an hour as a response to external stimulus like growth factors (I, II). The relatively large intracellular pool of HAS makes it a potential target for posttranslational regulation; the reservoirs in Golgi could be rapidly mobilized for transport to plasma membrane to hasten the start of hyaluronan synthesis (III). HAS contains putative sites for phosphorylation (Ohno et al., 2001), and its activity may be regulated via phosphorylation (Goentzel et al., 2006).

Blocking GFP-HAS transport to plasma membrane with a 2 h treatment with BFA totally depleted GFP-labeled HAS3 from plasma membrane, suggesting a dwell time of HAS on plasma membrane about 2 h (III). The release of BFA block demonstated that transfer of HAS from ER to Golgi took 1 h and another 1 h was needed to transport from Golgi to the plasma membrane. These results support the experiments with cycloheximide, indicating that the total lifetime of HAS protein is 4-5 h (III). Previous studies utilizing cycloheximide show that HAS turnover time is over 6 h *in vivo* (Anggiansah et al., 2003), but slightly shorter (4-6 h) *in vitro* (Bansal and Mason, 1986; Kitchen and Cysyk, 1995). The fact that the plasma

membrane residence time of HAS protein is approximately the same as the time required for the biosynthesis of one hyaluronan chain (Bansal and Mason, 1986; Kitchen and Cysyk, 1995; Anggiansah et al., 2003; Pummill and DeAngelis, 2003) is in line with our results showing that continuing hyaluronan synthesis keeps HAS at plasma membrane, after which it is internalized for degradation, or even detached into extracellular space together with a hyaluronan chain, as speculated by (Mausolf et al., 1990; Kitchen and Cysyk, 1995).

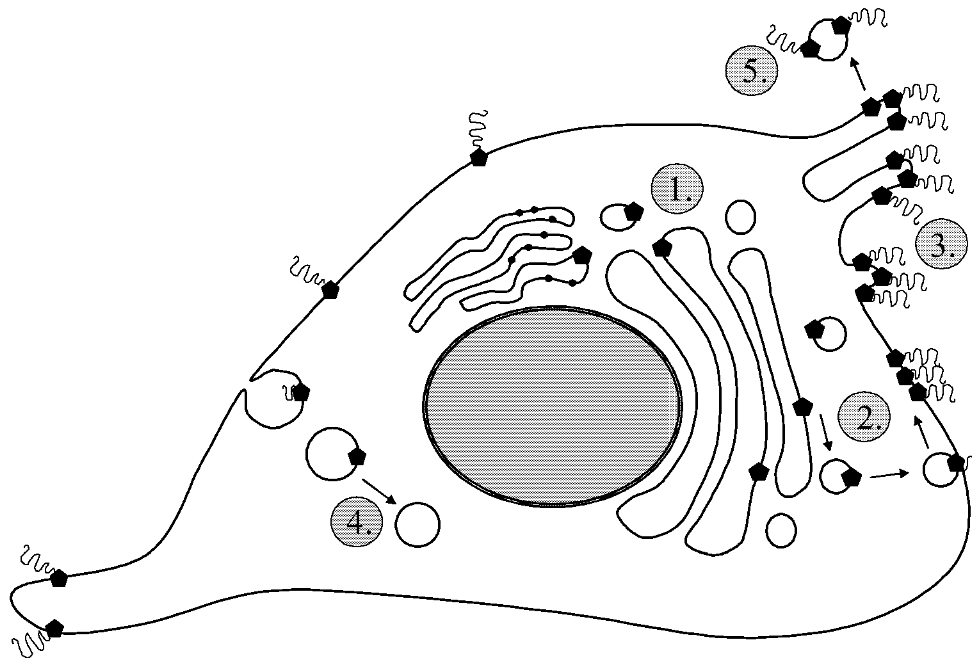

Figure 5. Trafficking of HAS and its association with cellular protrusions. After translation in endoplasmic reticulum (ER) (1), HAS is transported via the secretory pathway through Golgi to plasma membrane (2). It accumulates in plasma membrane protrusions of different size and type (3). It is endocytosed for degradation (4). HAS may also shed into extracellular space together with hyaluronan chain (5).

The result that plasma membrane residence of HAS is coupled to its enzymatic activity (III) is in line with the suggestion that initiation of hyaluronan synthesis leads to rapid entrance of HAS to the cell surface (Kitchen and Cysyk, 1995). Another possible mechanism is that entrance to the plasma membrane activates HAS and continuing hyaluronan synthesis retains it there. Both alternatives are consistent with notion that HAS residence on plasma

membrane is disturbed by inhibition of Golgi vesicle transport to plasma membrane, depletion of sugar precursors, or inactivation of HAS by missense mutation (III).

The GFP-HAS signal on the plasma membrane appeared as discrete spots (III), a finding similar to that of Müllegger et al. (2003) and showed some resemblance to hyaluronan patches on keratinocytes (Tammi et al., 1998). The most intense GFP-HAS signal was found on cell surface protrusions that were also enriched in lipid rafts (IV). However, the determinants in plasma membrane for HAS insertion sites remain unknown.

The activated hyaluronan synthesis by EGF-treatment induced the formation of cytoplasmic circular vesicles with perimeter positive for hyaluronan (I). Overexpression of GFP-HAS induced similar vesicles expressing both hyaluronan and GFP-HAS. The GFP-HAS positive vesicles were derived from plasma membrane through endocytosis, as indicated with a fluid phase marker (III). This suggests that HAS associated with newly synthesized hyaluronan can be endocytosed immediately after or even during the chain elongation (Tammi et al., 2001).

6.4. Hyaluronan and plasma membrane protrusions

6.4.1. Hyaluronan synthesis, microvilli and hyaluronan coat

As discussed above, hyaluronan synthesis rate correlates with certain morphological changes in cultured cells, including the formation of lamellipodia, microspikes and filopodia (I, II, Karvinen et al., 2003b). A definitive proof that hyaluronan synthesis promotes cell surface protrusions was obtained by Has2 antisense inhibition leading to smaller lamellipodia (Rilla et al., 2002) and by 4-MU, which eliminates the protrusions induced by overexpresssion of Has (IV). Furthermore, the microvilli were retracted when HAS-associated hyaluronan on plasma membrane was removed, either by preventing plasma membrane entrance of HAS, or by hyaluronidase digestion of hyaluronan (IV).

Thin cellular processes are hardly detectable by phase contrast microscope, and easily truncated or even disappear after fixation, and their existence may be easily ignored without fluorescent markers. It is thus possible that microvilli extending into hyaluronan-rich coats (IV) exist also naturally in cells with high hyaluronan production (Clarris and Fraser, 1968; Knudson et al., 1996; Cohen et al., 2003), although it has never been substantiated.

In many cells with rapid hyaluronan synthesis, the thickness of the hyaluronan coat (Clarris and Fraser, 1968; McBride and Bard, 1979) is wider than could be expected by a layer of a randomly coiled hyaluronan molecule (Toole, 2001). The present findings of thin microvilli within the hyaluronan coat may explain this observation. The microvilli appear at regular intervals covering the apical surface of the cell, forming a "hedgehog"-like appearance (IV). They probably cooperate by mechanically supporting each other through interactions dependent on hyaluronan chains attached to HAS on adjacent microvilli.

6.4.2. Proteins associated with the microvilli

Although the HAS-induced microvilli were strongly positive for CD44, oligosaccharides displacing hyaluronan from receptors, disruption of the CD44 gene or blocking antibodies against RHAMM or CD44 had no effect on the microvilli, suggesting that they are not dependent on hyaluronan receptors (IV). Hyaluronan synthesis-stimulating growth factors induce the hyaluronan coat formation of mesothelial cells, but exogenous hyaluronan has less effect (Heldin and Pertoft, 1993). In chondrocytes, hyaluronan coat is removed by hyaluronidase digestion but not by oligosaccharides (Sommarin and Heinegard, 1983), suggesting that the coat is not dependent on hyaluronan receptors. However, in other studies the hyaluronan coat on chondrocytes is displaced by hyaluronan hexasaccharides and dependent on CD44 (Knudson, 1993). Instead, the hyaluronan coat induced by HAS transfection requires newly synthesized hyaluronan that is still attached to the membrane-bound HAS (IV). It thus appears that a coat of hyaluronan may rest on either HAS or CD44, but whether CD44 can support the microvilli in other cell types remains to be determined.

The present studies show that the long protrusions triggered by HAS expression are dependent on actin cytoskeleton, but not on microtubules (IV). However, the regulation of actin cytoskeleton in the HAS-induced microvilli remains unknown, since there are no data on direct or indirect interactions between HAS and actin cytoskeleton.

Recent work shows that hyaluronan synthesis enhances multidrug resistance of breast cancer cells (Misra et al., 2005) and multidrug resistance transporter proteins are expressed in microvilli (Rajagopal et al., 2002; Bacso et al., 2004). ErbB receptors, which are activated by hyaluronan (Camenisch et al., 2002) are located in microvilli (Hommelgaard et al., 2004). It

is possible, that hyaluronan synthesis increases multidrug resistance and ErbB-signaling by inducing microvilli.

The role of the microvilli could also be to facilitate cancer cell invasion into surrounding ECM and attachment to other cells. Additionally, the microvilli surrounded by hyaluronan may serve as a protective coat around metastazing tumor cells in the bloodstream and tissues, protecting against immulological attack (McBride and Bard, 1979). They are also structures increasing the plasma membrane surface area, possibly for transport functions, and may help the cells to sense their environment.

6.5. Role of hyaluronan as a regulator of keratinocyte proliferation

High hyaluronan content has been associated with high proliferation rate (Toole, 1997). However, experimental evidence on this issue is contradictory, since adding exogenous hyaluronan or overexpressing or underexpressing HAS enzymes in cell cultures has given contradictory results (references in the literature review).

In epidermis, keratinocyte activation induced by tissue trauma, resulting in epidermal hypertrophy and disturbed keratinocyte differentiation, is associated with increased hyaluronan synthesis (Maytin et al., 2004; Tammi et al., 2005). The same factors that activate keratinocytes (Tomic-Canic et al., 1998), e.g. EGF (I, II) and KGF (Karvinen et al., 2003b), induce hyaluronan production, supporting the hypothesis.

However, the association of hyaluronan with cell proliferation in epidermis is not simple. Hyaluronan is not specifically accumulated between the proliferative basal cells (Tammi and Tammi, 1991), and the removal of hyaluronan by hyaluronidase does not influence the size of the proliferative cell pool (Maytin et al., 2004). Furthermore, EGF (I) and KGF (Karvinen et al., 2003b) when added to keratinocytes in monolayer cultures, do not induce a proliferative response although hyaluronan synthesis is upregulated.

Because the thickness of epidermis is regulated besides through proliferative activity also by the rate of differentiation, it has been suggested that hyaluronan may cause epidermal hyperplasia by delaying differentiation rather than stimulating proliferation (Maytin et al., 2004). Indeed, removal of hyaluronan is probably necessary for the formation of tight cell-cell contacts and a lipid-rich barrier, a prerequisite for terminal differentiation. Accordingly, factors which stimulate hyaluronan synthesis in keratinocytes also disturb differentiation

(Table II), and disturbed differentiation in vivo is associated with hyaluronan accumulation in stratum corneum (Wells et al., 1991; Tammi et al., 1994a).

However, in monolayer cultures inhibition of hyaluronan synthesis was associated with reduced proliferation rate **(Table II)**, and in organotypic cultures the stimulation of hyaluronan synthesis by EGF (Pasonen-Seppänen et al., 2003) and KGF (Karvinen et al., 2003b) were associated with stimulated proliferation rate, suggesting that the association of hyaluronan with proliferation in epidermis is not mediated solely by its influence on differentiation.

While the molecular mechanism controling hyaluronan influence on cell division is unknown at present, the levels of cyclin A and B, key regulators of entry to S-phase and M-phase, are altered in HAS2 overexpressing and underexpressing cells (Li and Heldin, 2001; van den Boom et al., 2006).

The fact that EGF and KGF increased keratinocyte proliferation only in organotypic models but not in monolayer cultures (Karvinen et al., 2003b; Pasonen-Seppänen et al., 2003) shows the importance of the 3D-environment in the influence of hyaluronan on cell division. The role of the environment is also stressed by the findings that HAS2 overexpression leads to increased cell proliferation only in 3D-models (Kosaki et al., 1999) or at high cell densities (Liu et al., 2001). The accumulation of hyaluronan in the cleavage furrow during cytokinesis (Brecht et al., 1986) may also be important in the restricted space in the epidermis in vivo (Tammi and Tammi, 1991). It is possible that hyaluronan may physically contribute to cell detachment and shape changes in tissues, processes perhaps not important in monolayer cultures.

Agents that inhibit hyaluronan synthesis in keratinocytes (Table II) also inhibit proliferation, and this occurs constantly both in 2D and 3D conditions. Furthermore, antisense inhibition of HAS2 in other cell types is associated with reduced cell proliferation in all reported cases cells (Simpson et al., 2002; Chao and Spicer, 2005; Nishida et al., 2005; Udabage et al., 2005b) suggesting that a minimum level of hyaluronan is required for the division of these cells types.

The proliferation rate of rat keratinocytes was almost totally arrested with 0.5 mM 4-MU (II), and a similar finding was done in human breast cancer cells (Anne Kultti, personal communication). However, in human melanoma cells (Kudo et al., 2004) and rat fibrosarcoma cells (Kosaki et al., 1999) no inhibition of proliferation was reported. Earlier,

inhibition of keratinocyte proliferation was correlated to reduced hyaluronan synthesis by TGF-β (Pasonen-Seppänen et al., 2003), cortisol (Ågren et al., 1995) and Has2 antisense transfection (Rilla et al., 2002). The effect of 4-MU confirms the association of keratinocyte proliferation with hyaluronan synthesis, but the mechanism of 4-MU as an antiproliferative factor remains unknown, and may also be partly independent of hyaluronan.

Table II. Correlations of hyaluronan secretion with features typical for keratinocyte activation

	HA secretion	HAS 2/3	Prolife-ration	Migra -tion	Fusiform Shape	Protru-sions	Differen-tiation
EGF[1,5]	+	+[#]	⇔/+[#]	+	+	+	-[#]
KGF[2]	+	+	⇔/+[#]	+	+	+	-[#]
Vitamin A[3]	+	+	+	?	?	?	-[#]
Wounding[8]	+	+	+	+	+*	+*	?
HC[4]	-	- *	- *,[9]	-*	?	?	+[9]
4-MU[5]	-	-	-	-	-	-	?
TGF-β[6]	-	-	-	+**	?	?	+[#]
Has2 AS[7,1]	-	-	-	-	-	-	?

HC, hydrocortisone; AS, antisense;

[#]monolayer/organotypic cultures
? not determined
*unpublished results
**Karvinen, personal communication
[1] **(I)**
[2]Karvinen et al. 2003
[3]Pasonen-Seppänen et al. 2005
[4]Ågren et al. 1998
[5]**(II)**
[6]Pasonen-Seppänen et al. 2003
[7]Rilla et al. 2002
[8]Tammi et al. 2005
[9]Tammi et al. 1981

6.6. Hyaluronan synthesis and cell migration

6.6.1. Hyaluronan effect on cell migration

The migratory rate of keratinocytes was rapid when hyaluronan synthesis was stimulated by EGF (I) and slow when hyaluronan synthesis was inhibited by 4-MU (II). Likewise, the stimulatory effect of EGF on migration was blocked my 4-MU (II). The increase of hyaluronan synthesis by KGF is also associated with enhanced migration of keratinocytes (Karvinen et al., 2003b), while hydrocortisone, an inhibitor of keratinocyte hyaluronan synthesis (Ågren et al., 1995), inhibits migration (Rilla, unpublished). The strongest evidence for the role of hyaluronan comes from the finding that Has2 antisense gene slowed down keratinocyte migration (I).

Increased hyaluronan is associated with migration, whether exogenously added to transformed fibroblasts (Collis et al., 1998; Sohara et al., 2001) or endogenously synthesized by melanoma, fibroblasts or zebrafish embryonic cells (Ichikawa et al., 1999; Itano et al., 2002; Bakkers et al., 2004). However, keratinocytes show only a slight response to exogenous hyaluronan added to growth medium (I, II), but doubles the migration when endogenous hyaluronan synthesis is doubled with KGF (Karvinen et al., 2003b) or EGF (I). A similar effect was found in Has-overexpressing fibroblasts (Itano et al., 2002) and melanoma cells (Ichikawa et al., 1999). These data support the idea that keratinocyte hyaluronan synthesis and migration are tightly connected to each other.

There are also reports suggesting that hyaluronan overexpression inhibits migration. This was the case in arterial smooth muscle cells (Wilkinson et al., 2006) and CHO cells overexpressing different HASs (Brinck and Heldin, 1999). The influence of hyaluronan synthesis on migration rate is likely to depend on the cell type and the level of synthesis increase.

6.6.2. Inhibition of migration by 4-MU

4-MU had a dose-dependent inhibitory effect on keratinocyte migration (II). Inhibition on cell locomotion by 4-MU have been reported also in melanoma cells (Kudo et al., 2004), in transformed fibroblasts (Kosaki et al., 1999) and in breast cancer cells (Kultti, personal

communication). Consistently with the reduced migration, 4-MU had a strong influence on keratinocyte morphology, characterized by a flattened, round cell shape, increased stress fibers and adhesion plaques and reduced size and number of plasma membrane protrusions (II). Similar morphological changes were induced with hyaluronan synthesis inhibition by Has2 antisense transfection into keratinocytes (Rilla et al., 2002) and by blocking protein transport with monensin in fibrosarcoma cells (Goldberg and Toole, 1984). A similar phenotype is also achieved by Rho activation, which is associated with increased stress fibers, focal contacts, cell-cell adhesion and a spread and flattened cell morphology (Hall, 1998). Rac and Cdc42, which have opposite effects on cell phenotype as compared to Rho (Hall, 1998), are connected to hyaluronan–mediated signalling systems through CD44 (Bourguignon et al., 2004; Bourguignon et al., 2005). This suggests that the inhibitory effect of 4-MU on migration may be associated with hyaluronan signaling to cytoskeleton.

The inhibitory effect of 4-MU on cancer cell adhesion and locomotion (Kudo et al., 2004), and metastasis (Yoshihara et al., 2005; Nakazawa et al., 2006), its normalizing effect on hypertrophic epidermis, together with its minor effect on normal epidermal tissue (II) and its safety in clinical use (Takeda and Aburada, 1981) make it a candidate for treatment of cutaneous cancer, and epidermal diseases with excessive keratinocyte proliferation, such as psoriasis.

6.6.3. Mechanisms involved in hyaluronan-induced cell migration

Hyaluronan may facilitate cell migration by several mechanisms **(Fig. 6)**. It has been reported that hyaluronan may facilitate plasma membrane protrusion formation at the leading edge (Evanko et al., 1999), perhaps through receptor-mediated signaling involving CD44 (Bourguignon, 2001), RHAMM (Assmann et al., 1999) and layilin (Bono et al., 2001). CD44 associates with proteins involved in cytoskeletal rearrangements, like ezrin (Legg et al., 2002), cdc42 (Bourguignon et al., 2005) and Rac (Bourguignon et al., 2000), leading to plasma membrane ruffling and formation of cellular projections. Lamellipodial outgrowth mediated via Rac1 is induced by HAS2, suggesting that HAS driven hyaluronan synthesis acts as an autocrine modulator of cell migration (Bakkers et al., 2004), a hypothesis well in line with the current study. The present study indicates that HAS driven hyaluronan synthesis itself modulates plasma membrane dynamics involved in migration (I, II).

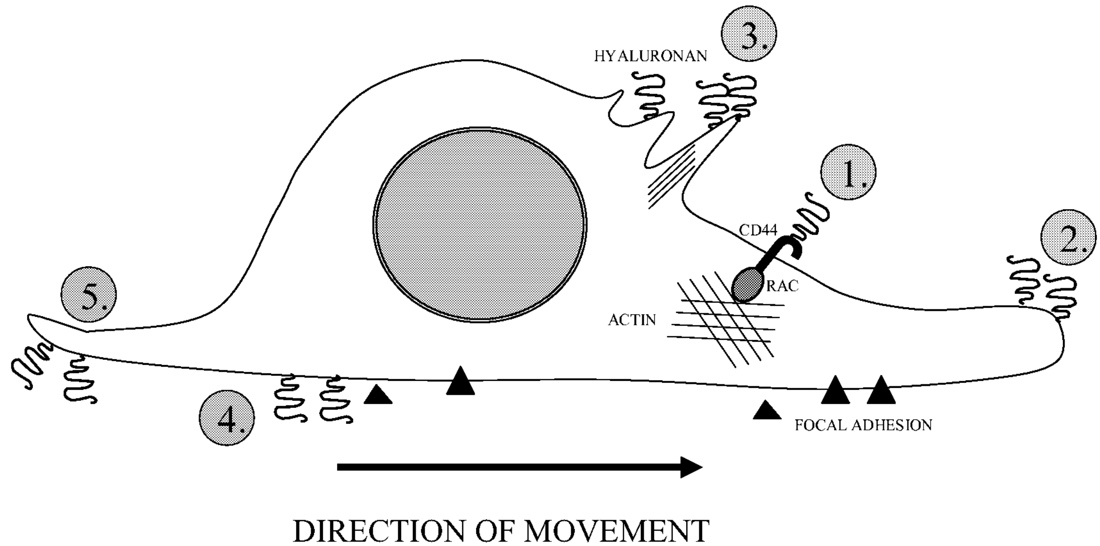

Figure 6. Putative mechanisms of action of hyaluronan synthesis as a stimulator of cell migration. Hyaluronan is supposed to act as a signalling molecule via specific receptors like CD44 or RHAMM to induce cytoskeletal rearrangements via Rac and other mediators (1). By the signals and mechanical forces, hyaluronan induces the formation of lamellipodia (2) and smaller protrusions (3) at the leading edge. Hyaluronan facilitates focal adhesion (triangles) turnover (4) and retraction of the trailing process (5).

Focal adhesion disassembly and retraction of the trailing edge is one of the basic stages of cell migration (Vicente-Manzanares et al., 2005) and suggested to be involved also in hyaluronan stimulated migration (Evanko et al., 1999). Hyaluronan may facilitate focal contact disassembly from substratum or adjacent cells through RHAMM-mediated activation of focal adhesion kinase (Hall et al., 1994). Alternatively hyaluronan may induce CD44 to associate with Rho, which is known to control focal adhesion maturation and dissolution (Watanabe et al., 2005). Alternatively, the newly synthesized hyaluronan itself at the plasma membrane may act as a membrane deforming force acting on focal contacts. In the present work the increased number of focal contacts by 4-MU (II) supports the idea that hyaluronan influences migration by controlling focal adhesion turnover.

The amount of intracellular hyaluronan is high in migratory cells (Evanko and Wight, 1999), for instance in those stimulated by KGF (Karvinen et al., 2003b) and EGF (I). Endocytosis and vesicular traffic to the leading edge is required for migration, providing membrane material for lamellipodia formation (Nabi, 1999) or endocytosis/phagocytosis is used to clear the route for the cells penetrating to the wound (Takashima et al., 1986). The enhanced endocytic activity of migrating cells may be responsible for the increased content of intracellular hyaluronan. The endosomal hyaluronan is destined to degradation, but it may also have an active role through intracellular signaling (Hascall et al., 2004). The rapid increase in intracellular hyaluronan of migratory keratinocytes after introduction of EGF supports the idea of enhanced endocytosis rate in migrating cells (I).

As a water-binding molecule, hyaluronan hydrates, expands and loosens ECM, thus giving space for cells to migrate in. High amounts of hyaluronan also tend to widen the extracellular space between cells, which may as such act as a signal to migration. Such situations exist in wound healing, fetal development and cancer progression. However, hydration is probably not associated with the hyaluronan-induced migration in monolayer cultures (I, II), while it may be a contributing factor in tissues and 3D cultures.

7. SUMMARY AND CONCLUSIONS

This study focused in hyaluronan synthesis and its control in a keratinocyte and a breast cancer cell line. Another central aim was to study the relationship between the rate of hyaluronan synthesis and biological features of the cells like morphology, migration and proliferation. For this aim, EGF was used to induce hyaluronan synthesis in epidermal keratinocytes and 4-MU to decrease hyaluronan synthesis in the same cell line. Utilizing fluorescent tags, the traffic and localization of HAS was studied using 3D confocal microscopy.

The results demonstrate that:

- EGF induces hyaluronan synthesis of keratinocytes by increasing Has2 expression and causes rounding and elongation of cells, formation of protrusions and increased migration in an in vitro wound healing assay. In organotypic cultures EGF-treated cells have a high proliferation rate, leading to epidermal thickening and disturbance of differentiation. The role of hyaluronan in these phenotypic changes is supported by the finding that transfection of Has2 antisense gene reduced the migration rate of the cells, and inhibition of hyaluronan synthesis by 4-MU, prevented both migration and morphological changes associated with high level of hyaluronan synthesis.

- HASs are transported to plasma membrane along the ER-Golgi pathway. When hyaluronan synthesis is inhibited by depletion of sugar precursors or Has mutations, HAS is not present on plasma membrane. Correspondingly, interference of membrane traffic from Golgi to plasma membrane with BFA prevents HAS access to plasma membrane and activity. Therefore, HAS protein resides on plasma membrane only during active hyaluronan synthesis, and is never active when attached to membrane vesicles within the cell. The turnover time of the GFP-HAS3 protein in keratinocytes was 4-5 h, of which ~2 h on plasma membrane, suggesting that one HAS synthesizes one hyaluronan chain.

- HAS is colocalized in intracellular vesicles with hyaluronan and a fluorescent marker from growth medium, indicating that hyaluronan may be endocytosed when still attached to HAS.

Additionally, the stimulation of hyaluronan production by EGF leads to intracellular accumulation of endogenous hyaluronan in perinuclear vesicles, probably due to enhanced endocytosis.

- Experimental overexpression of HAS induces up to 20 μm long microvillus-type cell protrusions in several epithelial cell types. The formation and maintenance of these protrusions is dependent on continuous hyaluronan synthesis and cell surface hyaluronan still bound to HAS, but do not require CD44 or RHAMM or other receptors susceptible to competition with hyaluronan oligomers of the size of dodecasaccharide or smaller. The function of the microvilli is unknown, but they may associate with signalling, membrane channel functions and invasion of the cells.

- Ongoing hyaluronan synthesis is associated with plasma membrane dynamics by enhancing formation of plasma membrane protrusions, like filopodia, lamellipodia, and especially microvilli. The increased lamellipodia and filopodia are typically associated with enhanced cell motility, and may be due to mechanical forces or signaling created by hyaluronan. It can be suggested that hyaluronan acts as an extracellular cytoskeleton, regulating cellular dynamics by mobilizing or stabilizing plasma membrane protrusions.

- This study provides for the first time detailed data on HAS-protein kinetics, and its impact on cell surface protrusions, and suggests that the effects of EGF on keratinocytes are mediated partially by hyaluronan synthesis. The present study shows that hyaluronan regulates normal and disturbed keratinocyte functions, and inhibitors of hyaluronan synthesis, like 4-MU could normalize the hyperproliferative epidermis in inflammation, psoriasis and cutaneous cancers, while stimulation of hyaluronan synthesis may enhance epidermal wound healing.

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APPENDIX: ORIGINAL PUBLICATIONS I-IV

Kuopio University Publications D. Medical Sciences

D 370. Pasonen-Seppänen, Sanna. Regulation of keratinocyte differentiation and hyaluronan metabolism in an organotypic keratinocyte culture.
2005. 109 p. Acad. Diss.

D 371. Laukkanen, Jari. Exercise testing in the prediction of cardiovascular diseases and mortality: a prospective population study in men.
2005. 93 p. Acad. Diss.

D 372. Määttä, Sara. Event-related potenital studies on novelty processing and distractibility.
2005. 54 p. Acad. Diss.

D 373. Juutilainen, Auni. Gender, type 2 diabetes and risk of cardiovascular disease.
2005. 54 p. Acad. Diss.

D 374. Purhonen, Sinikka. Prevention of postoperative nausea and vomiting: with special reference to supplemental oxygen, different antiementics and anesthesia regimens.
2006. 85 p. Acad. Diss.

D 375. Tuomainen, Tomi-Pekka. Body iron, atherosclerosis and coronary heart disease.
2006. 85 p. Acad. Diss.

D 376. Lintula, Hannu. Acute abdominal pain in children with special reference to surgical techniques and effects of opioid analgesia on diagnostic accuracy.
2006. 178 p. Acad. Diss.

D 377. Barengo, Noël C. Physical activity, cardiovascular risk factors and mortality.
2006. 107 p. Acad. Diss.

D 378. Laukkanen, Olli. Genes regulating insulin secretion and insulin signaling as candidate genes for type 2 diabetes in subjects with impaired glucose tolerance.
2006. 101 p. Acad. Diss.

D 379. Ritvanen, Tiina. Seasonal psychophysiological stress of teachers related to age and aerobic fitness.
2006. 88 p. Acad. Diss.

D 380. Pelkonen, Margit. The relationship of smoking habits, physical activity and chronic bronchitis to pulmonary function and of pulmonary function and chronic bronchitis to mortality: a 40-year follow-up in middle-aged men.
2006. 80 p. Acad. Diss.

D 381. Salmenniemi, Urpu. Metabolic and genetic abnormalities clustering with intra-abdonimal obesity.
2006. 95 p. Acad. Diss.

D 382. Pohjola, Leena. TOIMIVA-testit yli 75-vuotiaiden miesten fyysisen toimintakyvyn arvioinnissa.
2006. 173 p. Acad. Diss.

D 383. Kurola, Jouni. Evaluation of pharyngeal devices for prehospital airway management.
2006. 111 p. Acad. Diss.

D 384. Karpansalo, Minna. Predictors of early retirement: a population-based study in men from eastern Finland.
2006. 111 p. Acad. Diss.