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Hyaluronan and CD44 in Epidermis with Special Reference to Growth Factors and Malignant Transformation

Doctoral dissertation

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ABSTRACT

Hyaluronan is a glycosaminoglycan which forms the main part of the extracellular matrix in the epidermis of skin. Its metabolism in the epidermis is a complex process that can be influenced by several growth factors. Hyaluronan, first thought to be passive space filler, is now known to contribute to several important physiological events such as cell proliferation, migration and differentiation, often mediated through its cell surface receptor CD44. Besides normal tissue homeostasis, hyaluronan and its receptor are involved in the carcinogenesis of several cell types.

The aim of the first part of this thesis was to study the effects of epidermal growth factor (EGF), keratinocyte growth factor (KGF), and transforming growth factor beta (TGF-β) on hyaluronan metabolism in epidermal keratinocytes and in an *in vitro* organotypic culture model of epidermis. The changes in hyaluronan metabolism were correlated with keratinocyte proliferation, migration, differentiation as well as epidermal permeability barrier formation. The second part studied changes in hyaluronan, CD44 and versican during keratinocyte carcinogenesis on histological sections of basal cell carcinoma (BCC), in situ squamous cell carcinoma (ISC), and different grades of squamous cell carcinoma (SCC). Tumor samples were also analyzed for correlations between the expression of hyaluronan/CD44, and MMP-7 and MMP-9, the latter linked to malignant progression due to their ability to degrade matrix components and thus aid in cancer cell invasion.

It was found that mitogenic growth factors (EGF, KGF) increased the synthesis of hyaluronan by epidermal keratinocytes via activation of hyaluronan synthase 2. This increase in epidermal hyaluronan content correlated to increased cell proliferation, migration and epidermal thickness, while keratinocyte differentiation was inhibited. On the other hand, TGF-β decreased the proliferation of keratinocytes causing epidermal atrophy. This was accompanied by decreased Has2 expression and epidermal hyaluronan content, and unchanged keratinocyte differentiation. The study showed a close link between epidermal hyaluronan content, epidermal thickness and keratinocyte differentiation.

In epidermal keratinocyte tumors, hyaluronan and CD44 expressions varied according to the type of the tumor. In BCC, both hyaluronan and CD44 expressions were very low. In ISC, the levels were high with an irregular staining distribution. In squamous cell carcinoma SCC, well-differentiated tumors showed abundant hyaluronan and CD44, while reduced hyaluronan and CD44 signals were found in poorly differentiated, aggressive tumors. The levels of the HA-binding protein versican were increased in the stroma of BCC but not in SCC. Furthermore, expression of MMP-7 was higher in SCC than BCC, and correlated with the loss of CD44 in poorly differentiated SCC. The levels of MMP-9 did not differ between the different tumor types or stages of differentiation.

In conclusion, this study suggested that hyaluronan and CD44 are important mediators of the epidermal homeostasis, regulated by local growth factors. They also correlate with the origin and invasive properties of the epidermal cancers, and the expression of MMP-7.

To Simon and Ella

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Susanna Hartmann-Petersen

ABBREVIATIONS

BCC	basal cell carcinoma
bHABC	biotinylated hyaluronan binding complex
BSA	bovine serum albumin
DAB	3, 3'-diaminobenzidine
DEJ	dermo-epidermal junction
DMEM	Dulbecco's minimal essential medium
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELSA	enzyme-linked sorbent assay
ERK	extracellular signal regulated protein kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FBS	fetal bovine serum
GAPDH	glyceraldehydes-3-phosphate-dehydrogenase
GlcNAc	N-acetyl glucosamine
GlcUA	glucuronic acid
HA	hyaluronan, hyaluronate, hyaluronic acid
HABC	hyaluronan binding complex
HAS	hyaluronan synthase (HAS 1, 2, 3)
HB-EGF	heparin-binding EGF
HBSS	Hank's balanced salt solution
IFN-γ	interferon gamma
ISC	*in situ* squamous cell carcinoma
JNK	c-jun N-terminal kinase
K1	keratin-1
K10	keratin-10
KGF	keratinocyte growth factor
KGFR	keratinocyte growth factor receptor
MAPK	mitogen activated protein kinase
MEM	minimal essential medium
MMP	matrix metalloproteinase (MMP-7, MMP-9)
NMSC	non-melanoma skin cancer
PB	phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI-3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PLC	phospholipase C
RA	retinoic acid
REK	rat epidermal keratinocyte cell line
RHAMM	receptor for hyaluronan mediated motility

RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
UVB	ultraviolet B radiation

LIST OF ORIGINAL PUBLICATIONS

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

I.   **Karvinen S*, Pasonen-Seppänen S*, Hyttinen JM, Pienimäki JP, Törrönen K, Jokela TA, Tammi MI, Tammi R (2003):** Keratinocyte growth factor stimulates migration and hyaluronan synthesis in the epidermis by activation of keratinocyte hyaluronan synthases 2 and 3.J Biol Chem.;278: 49495-49504. * = equal contribution

II.   **Pasonen-Seppänen S, Karvinen S, Törrönen K, Hyttinen JM, Jokela T, Lammi MJ, Tammi MI, Tammi R (2003):** EGF upregulates, whereas TGF-beta downregulates, the hyaluronan synthases Has2 and Has3 in organotypic keratinocyte cultures: correlations with epidermal proliferation and differentiation. J Invest Dermatol.; 120:1038-1044.

III.   **Karvinen S, Kosma VM, Tammi MI, Tammi R (2003):** Hyaluronan, CD44 and versican in epidermal keratinocyte tumours. Br J Dermatol.; 148:86-94.

IV.   **Hartmann-Petersen S, Tammi RH, Tammi MI, Kosma V-M (2009):** Depletion of cell surface CD44 in non-melanoma skin tumors is associated with increased expression of MMP-7 Br J Dermatol, February 2009, Epub ahead of print.

CONTENTS

APPENDIX: original publications I-IV

1   INTRODUCTION

The epidermis of skin consists of tightly packed layers of keratinocytes, which are highly versatile cells capable of rapid proliferation and migration via receptor mediated signals from several growth factors and other mediators - events which are strictly controlled. Disorders in this regulation lead to characteristic disturbances seen in many skin diseases, for example the rapid proliferation and lack of terminal differentiation seen in psoriasis. The balance in regulation of growth is especially disturbed in malignancies arising from the epidermis such as basal cell carcinoma and squamous cell carcinoma, which are the most common cancer types in humans.

Hyaluronan is a glycosaminoglycan, which comprises the extracellular matrix of the vital part of epidermis. Between keratinocytes, it provides an extracellular matrix, which enables transport of nutrients from the dermis, as the epidermis itself lacks blood vessels. Hyaluronan is synthesized by three plasma membrane associated enzymes, named hyaluronan synthases 1, 2 and 3 (Weigel et al, 1997). The hyaluronan content of epidermis is strictly regulated, and the metabolism fast, with a half-life of newly synthesized hyaluronan of less than 24 h (Tammi et al, 1991). Hyaluronan contributes to many of the important cellular functions of the keratinocyte such as adhesion, migration, proliferation and differentiation – all of which are at least in part mediated through its cell surface receptor CD44 (Kaya et al, 1997, Bourguignon et al, 2004). CD44 is expressed by several types of cells, including leukocytes, fibroblasts and epithelial cells. Several isoforms of CD44 are found due to alternative splicing. CD44 binding to its ligand hyaluronan leads to anchoring of hyaluronan to the plasma membrane (Tammi et al, 1998, Knudson, 2003), endocytosis of hyaluronan (Knudson et al, 2002) as well as interactions with growth factor receptors leading to modulation of growth factor effects (Yu et al, 2002). Abnormal expression of hyaluronan has been reported in several cancers and this has been linked to poor prognosis (Setälä et al, 1999, Anttila et al, 2000, Auvinen et al, 2000, Lipponen et al, 2001). Some of these effects in cancer progression have been thought to be mediated through hyaluronan-CD44 interactions with matrix degrading enzymes such as matrix metalloproteinases, and CD44 has been shown to anchor the activated form of MMP-9 to the plasma

membrane of tumor cells, thereby promoting tumor cell invasion (Yu and Stamenkovic, 1999).

In this thesis, the effects of three different growth factors (KGF, EGF and TGF-β) on hyaluronan metabolism were examined in epidermal keratinocytes and in an *in vitro* organotypic culture model. These effects are correlated to such important cellular events as proliferation, migration, differentiation as well as formation of the permeability barrier of the skin. Furthermore, the role of hyaluronan and its receptor CD44 in epidermal keratinocyte tumors (non-melanoma skin cancer, NMSC) were evaluated, and their expression correlated to that of MMP-7 and MMP-9.

2 REVIEW OF THE LITERATURE

2.1. Structure of the skin

The skin is our body's largest organ, accounting for about 15 % of the body mass of an adult. It protects us from outside influences, such as radiation, heat, cold, chemicals, as well as attack from micro-organisms such as bacteria, fungi, parasites and vira. It also acts as a barrier preventing excessive loss of body fluids (Freinkel and Woodley, 2001). Skin is a complex tissue, derived from embryonic mesoderm and ectoderm, arranged in two layers: the dermis and the epidermis. These two layers have their specific characteristics and functions.

2.1.1. Epidermis

The epidermis is the outer layer of the skin comprising of stratified epithelia of tightly packed cells. The main cell type of the epidermis is the keratinocyte, a highly versatile cell, capable of rapid proliferation in response to injury. The average cycle of the epidermal keratinocyte from the basal cell to desquamation as a corneocyte takes around 30 days, but in hyperproliferative skin disorders such as psoriasis, this time can be as short as 3-4 days. Keratins are intermediate filaments that are a hallmark of the keratinocyte and other epithelial cells. They provide structural integrity to the cells. At least 30 subtypes of keratins are known, and different types of keratins are expressed according to type of cell and tissue, differentiation and development as well as disease

state (Smack et al, 1994, Fuchs, 1995). Apart from keratinocytes, the epidermis houses other cell types such as the Langerhans cells, melanocytes and Merkel cells, that all have their specific characteristics and functions (Kanitakis, 2002).
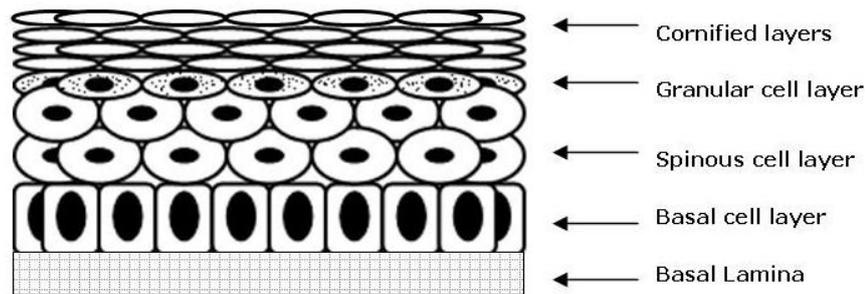
Figure 1. Layers of the epidermis

The epidermis is divided into four different layers according to the differentiation state of the keratinocytes. The basal cell layer is the deepest layer of the epidermis, traditionally thought to consist of stem cell keratinocytes (10%), non-differentiated transit amplifying keratinocytes which are capable of proliferation, as well as a little subpopulation (5-10%) of post-mitotic differentiating cells (Kanitakis, 2002). Recently, this hypothesis has been challenged, and it is thought that the epidermal keratinocyte population is maintained by a population of proliferating cells (reviewed in (Jones et al, 2007)). The basal cells lay adjacent to the basal lamina, to which they anchor via the hemidesmosomes. They are columnar in shape, and have a large nucleus and a basophilic cytoplasm. The basal cells are connected to adjacent cells by intercellular junctions (gap junctions, adherent junctions, and desmosomes). As the basal cells form the proliferative entity of the epidermis, they are the primary targets of several growth factors and cytokines (Kanitakis, 2002). Basal cells contain fine bundles of keratins K5 and K14, which provide the cells with a cytoskeleton flexible enough to allow cell division and migration (Fuchs, 1995). Mutations in the genes encoding these keratins cause a disease called epidermolysis bullosa simplex (EBS), which is a severe blistering disorder causing cytolysis within the basal cell layer (Fuchs, 1995, Bonifas et al, 1991).

Above the basal cell layer lie the spinous cell layers, where the cells migrate from the basal cell layer. The spinous cells are larger in size, and are no longer capable of proliferation. Spinous cells contain large bundles of keratins K1 and K10, which are often referred as differentiation specific keratins. They are abundant in desmosomes, which form the characteristic spines that have given the name of this cell layer (Kanitakis, 2002). The spinous cell layer also contains some growth factor receptors, especially those of KGF (Werner, 1998).

The granular cell layer is named after the characteristic keratohyalin granules in the cytoplasm of the cells. These granules consist primarily of profilaggrin, keratin intermediate filaments as well as loricrin, a protein found in the cornified envelope (Fukuyama et al, 1980). The normal expression and function of profilaggrin gene is essential for normal epidermal structure, as loss of function-mutations in the profilaggrin gene resulting in lack of functional filaggrin protein, have been found as a cause for the scaling skin disorder Ichtyosis Vulgaris, and play an important role in the pathogenesis of atopic eczema (recently reviewed in (McGrath and Uitto, 2008)). Lamellar granules, membrane-bound secretory organelles originating from the Golgi apparatus deliver precursors of stratum corneum lipids into the extracellular space. They contain glycoproteins, glycolipids, phospholipids, free sterols as well as are abundant in lipases, proteases, acid phosphatase and glycosidases. They assemble in the peripheral cytoplasm of the granular cells before being released into the extracellular space. These enzymes convert the pro-barrier lipids into neutral lipid-rich lamellae that coat the surfaces of the corneocytes, thus forming the permeability barrier of the epidermis (Kanitakis, 2002). The granular cells then undergo a keratinisation process leading to dramatic change in cell shape. Protein markers for this keratinisation, which are components of the cornified envelope, include involucrin, loricrin and envoplakin. During the keratinisation process, the cells lose water, and the activity of lipases and proteases dissolves their intracellular organelles, finally leading to the formation of the corneocyte (Candi et al, 2005). This process resembles the apoptotic form for cell death, including fragmentation of DNA and activity of caspases, but differs from apoptosis in the other morphological features. Also tight junctions have been found in the granular

cell layer of the epidermis, suggesting they also have a role in the formation of the permeability barrier of the epidermis (Pummi et al, 2001, Furuse et al, 2002).

The stratum corneum is the outermost part of the epidermis, which provides the major barrier to water loss as well as permeation of chemical and biological substances from the environment. It is comprised of multiple layers of non-viable, terminally differentiated keratinocytes called corneocytes. The thickness of the stratum corneum varies according to the anatomical site, being thickest on the palms and soles. The most important function of the cornified envelope is the barrier function. This is provided by a two-compartment system comprising of the lipid-depleted, protein rich corneocytes, surrounded by a continuous extracellular lipid matrix originating from the lamellar bodies of the granular layer (Kanitakis, 2002). The narrow intercellular space between the corneocytes is filled with mostly non-polar lipids such as ceramides (types 1-9), thus differing from the rest of the epidermis in which polar lipids such as phospholipids dominate (Marks, 2004).

2.1.2. Dermis

The dermis (corium) consists of supportive and elastic connective tissue, as well as contains the vasculature and innervation of the skin. It is divided into two parts, papillary and reticular dermis. Reticular dermis is the deeper layer of dermis, consisting of collagen bundles lying parallel to the skin surface, and a thick elastic network. The papillary dermis forms conical upward projections adjacent to the rete ridges of the epidermis, creating a better surface contact between epidermis and dermis thus allowing better adhesion. The papillary dermis consists of several cell types (fibroblasts, dendrocytes and mast cells), collagen fibrils arranged in loose bundles, and elastic fibres stretching perpendicularly to the dermo-epidermal junction (Kanitakis, 2002).

Fibroblast is the fundamental cell type of the dermis, synthesising all types of fibres and the ground substance. The dermal dendrocytes are mesenchymal dendritic cells present around the capillaries of papillary dermis. They express surface molecules of antigen presenting cells, thus functioning as effectors in immune response, as well as being capable of phagocytosis (Kanitakis, 2002). Mast cells are cells of bone marrow origin, and are generally distributed to connective tissue throughout the body. The

activation of mast cells in dermis is associated with the pathogenesis of various immunological skin diseases such as atopic dermatitis and contact dermatitis (Navi et al, 2007).

Collagens form the main part of dermal fibres, accounting for approximately 75% of skin dry weight. They give the skin mechanical resistance, as well as elasticity. More than 20 different collagens have been identified. In the dermis, collagen types I, III and V account for the main part of the collagens. Type I and III collagens form the bundles of fibrils in dermis, while type V collagen assists in regulating the diameter of the fibrils. Collagen type IV is found within the dermo-epidermal junction (DEJ) and the basement membranes of the DEJ, dermal vessels and nerves. Type VI collagen is also abundant in dermis, organising the matrix during development, and filling the interfibrillar spaces as fine, beaded filaments. Finally, type VII collagen forms anchoring fibrils at the DEJ. Elastic fibres of the dermis form a continuous network throughout the dermis, and return the skin to its normal form after it has been stretched or deformed. The turnover of elastic fibres is normally very slow, but chronic exposure to UVB of the sun can induce breakdown of these fibres by matrix metalloproteinases, giving rise to a condition known as solar elastosis (Saarialho-Kere et al, 1999).

The so called ground substance between the fibrillar components of dermis consists of glycoproteins and proteoglycans, including hyaluronan, fibronectin, chondroitin/dermatan sulphate proteoglycans (biglycan, decorin, versican) and heparan sulphate proteoglycans. These components, produced by the dermal fibroblasts, interact with the fibres and cells of the dermis, linking them to each other. They are also capable of binding growth factors and cytokines, thus being capable of inducing proliferation, differentiation and tissue repair. The dermatan sulphate molecule, decorin, can also act as a ligand to the epidermal growth factor receptor (Iozzo et al, 1999).

The dermal-epidermal junction (DEJ) is a complex basement membrane, synthesised by epidermal keratinocytes and dermal fibroblasts. Its fundamental role is providing mechanical support, and mediating the adhesion of epidermis to dermis, as well as regulating the exchange of metabolic products. In wound healing, the DEJ serves as a support for keratinocyte migration (Kanitakis, 2002). The DEJ has four distinct layers, visible in light microscopy. The deepest layer is the sub-basal lamina of

the papillary dermis, comprising of the anchoring fibrils made of type VII collagen. Above it is the lamina densa, the basal lamina proper, consisting primarily of type IV collagen as well as basement membrane specific proteoglycans like perlecan and heparan sulphate. The lamina lucida is a ca 40 nm wide space above the lamina densa, consisting of anchoring filaments which traverse the narrow space. The main component of the filaments is laminin-5, which is a major attachment factor for keratinocytes (McMillan et al, 2003, Schneider et al, 2007). Normal production and function of laminin-5 is extremely important, as it serves as a base for keratinocyte migration, initiates the formation of hemidesmosomes after injury and accelerates basement membrane restoration (Schneider et al, 2007). The lack of functional laminin-5 due to a genetic defect gives rise to Herlitz form for junctional epidermolysis bullosa presenting as severe blistering at birth and is lethal (Schneider et al, 2007). The outmost layer of the DEJ consists of the plasma membrane of the keratinocytes of the basal cell layer of epidermis, and their hemidesmosomes. The hemidesmosomes are thickened areas in the basal membrane consisting of integrins and the bullous pemphigoid antigens, linking the basal cells of the epidermis to the anchoring filaments of the lamina lucida (Nievers et al, 1999).
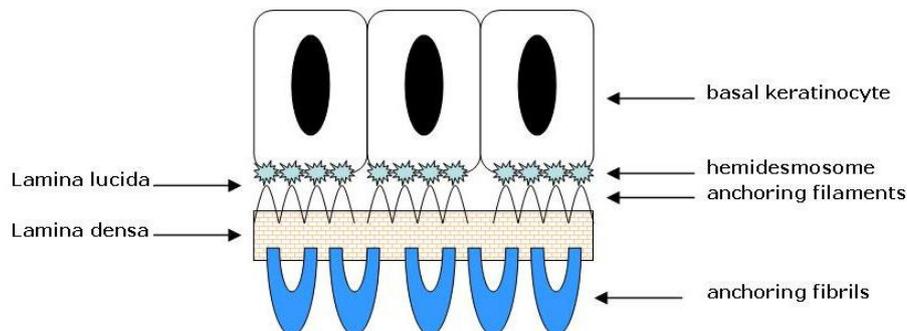
Figure 2. Dermal-epidermal junction

2.2. Growth factors and their receptors

Growth factors are a heterogeneous group of molecules with effect on cell growth. Some of the main groups of growth factors include the epidermal growth factor

group (EGF, HB-EGF, TNF-alpha), fibroblast growth factors, and transforming growth factors. Some growth factors induce their effect in several cell types, while others are more cell type specific.

2.2.1. Epidermal growth factor

EGF was one of the first known growth factors, described by Stanley Cohen as a polypeptide purified from mouse submaxillary glands, which affects epidermal growth and differentiation in mice (Cohen, 1965, Cohen, 1983). The EGF receptor (EGFR, also known as HER-1 and ErbB1) was characterized shortly after its ligand. It is a 170-kD glycoprotein consisting of an extracellular, glycosylated, EGF binding domain, a small transmembrane region, and an intracellular tyrosine kinase domain with multiple phosphorylation sites (King et al, 1990). In the epidermis, EGFR is primarily found in the basal cell layer and to some degree also in the suprabasal layers (Nanney et al, 1990). In the past decades, several EGF-like growth factors as well as four EGFR-like receptors have been identified (Jost et al, 2000). Apart from EGF itself, keratinocytes are able to produce and secrete four other EGF family members; TGF-α, HB-EGF (heparin-binding EGF), amphiregulin and epiregulin, which are also important in normal keratinocyte proliferation and differentiation. They all bind to EGFR (ErbB1), and act as autocrine regulators in the epidermis (Hashimoto, 2000). Keratinocytes also express two other EGF receptors, ErbB2 and ErbB3, which bind other EGF family members (betacellulin, neuregulins). This thesis, however, will primarily concentrate on EGF and its receptor.

The binding of EGF to its receptor leads to dimerisation of receptor molecules and phosphorylation of tyrosine residues in the intracellular domains of the receptor. This in turn leads to activation of several kinase pathways leading to the cellular effects of EGF. One of the most well known pathways activated by EGFR phosphorylation is the Ras/Raf/MEK/Erk kinase pathway. This is the main pathway responsible for EGF mediated increase in cell proliferation, and the inhibition of the pathway by specific inhibitors leads to a stop in the cell cycle in the G1 phase (Liu et al, 2004). EGFR phosphorylation also activates the PLC-gamma/PKC and PI3K/AKT, and leads to the NFkappaB activation, thus increasing cell survival.

EGF is known to be a potent growth factor that controls such important cellular events as cell proliferation, migration and differentiation as well as cell survival (Jost et al, 2000). Abnormal expression and activity of EGF and its receptor have also been linked to several malignancies such as breast cancer (Cohen et al, 1998, Navolanic et al, 2003) and tumors of epidermal origin (Salomon et al, 1995). In skin, EGFR has been found to be vital to the proliferation of epidermal keratinocytes, which depend on EGF stimuli.

2.2.2. Keratinocyte growth factor

Keratinocyte growth factor (KGF, FGF-7) is one of the more recently identified growth factors. It is a member of the fibroblast growth factor family, which was originally isolated from the supernatant of human embryonic fibroblasts. It is a monomeric polypeptide with a molecular weight of 26-28kD (Rubin et al, 1989). KGF is specifically targeted to epithelial cells, especially keratinocytes, which themselves are unable to produce it (Rubin et al, 1989, Finch et al, 1989, Rubin et al, 1995). In the skin, it is produced by dermal fibroblasts, and affects keratinocytes as a paracrine mediator. KGF binds to, and activates a specific tyrosine kinase receptor, KGFR, which is a splice variant of the FGF2 receptor (Miki et al, 1992). In epidermis, KGFR is expressed mainly in the spinous cell layer, a layer that determines the differentiation rate of keratinocytes. Some KGFR expression is also seen in the basal cell layer, but not in the granular cells. KGF enhances keratinocyte proliferation and migration, but delays their differentiation. It has also been found to be an important factor in the normal development, growth and differentiation of the hair follicle (Werner, 1998, Danilenko et al, 1995). Many authors have found KGF to be an important mediator of wound healing in vitro as well as in vivo (Pierce et al, 1994, Kopp et al, 2004). However, some reports of knockout experiments suggest that KGF has no effect on wound healing, but is important for hair follicle development (Guo et al, 1996).

2.2.3. Transforming growth factor beta

The TGF-β superfamily is a large family of mediators including the TGF-β isoforms (TGF-β1-5), activins, inhibins, bone morphogenetic protein (BMP) as well as Mullerian inhibiting substance (Hashimoto, 2000). They are multifunctional cytokines that regulate cell growth and differentiation, tissue remodeling, immune response and angiogenesis (Bottinger et al, 1997, Letterio and Roberts, 1997). All TGF-β isoforms are secreted as biologically inactive precursors that must be activated before exerting their effects on target cells. TGF-β signals through binding to membrane receptors with serine-threonine kinase activity. The TGF-β receptors consist of type 1 and type 2 receptors. TGF-β binds to a type 2 receptor and type 1 receptor is recruited to the complex. The type 2 receptor phosphorylates the type 1 receptor in order to activate it. The activated receptor complex phosphorylates a group of SMAD proteins as cytoplasmic mediators that transmit the signal to the nucleus (Hashimoto, 2000, Massague, 1998). In keratinocytes, TGF-β is known to exhibit a range of biological effects including inhibition of growth and extracellular matrix production (Shipley et al, 1986, Hashiro et al, 1991). TGF-β also plays an important role in fibrosis, a pathological event seen is skin diseases such as keloid (Uitto and Kouba, 2000) and systemic scleroderma (Ludwicka et al, 1995), as well as skin damage due to radiotherapy (Martin et al, 2000).

2.2.4. Growth factors and growth factor receptors in cancer

Overexpression of the epidermal growth factor receptor has previously been linked to several malignancies, including breast (Lichtner et al, 1995), ovary, colon and lung cancer as well as head and neck squamous cell carcinoma (reviewed in (Salomon et al, 1995)). Head and neck SCC tumors tend to express high levels of EGFR, and the degree of expression correlates with poor clinical outcome (Pomerantz and Grandis, 2003, Pomerantz and Grandis, 2004). A monoclonal antibody targeting the EGFR (cetuximab) is already in use for treatment of head and neck SCC, and is showing

promising results (Burtness, 2005, Blick and Scott, 2007). There is no data yet on the use of cetuximab on SCC of the skin.

TGF-β has been reported to have a dual role in carcinogenesis. The presence of TGF-β has been thought to act as a tumor suppressor in early carcinogenesis due to its anti-proliferative activity. However, there have been reports suggesting a tumor promoter role in end stage carcinogenesis. In skin chemical carcinogenesis experiments, various carcinogens induced TGF-β expression (Jost et al, 2000, Wang, 2001). Many reports have also stated high levels of TGF-β in malignant skin tumors in both naturally occurring clinical specimens as well as experimentally induced tumours in animal models (Cui et al, 1996). Elevated levels of TGF-β and its receptors have been found in the stroma of BCC (Furue et al, 1997). TGF-β has been noted to increase the activity of gelatinases A and B (MMP-2 and MMP-9, respectively), molecules known to promote tumor invasion, in tumor cells (prostate) (Sehgal et al, 1996) as well as in tumor stroma (Wright et al, 1993). The role of KGF and its receptor FGFR2b in tumorigenesis has been studied extensively (Finch and Rubin, 2006). Even though KGF and its receptor are expressed in many epithelial cancer cell lines, there is little evidence suggesting that they play a role in tumorigenesis (Finch and Rubin, 2006).

2.3. Keratinocyte migration and wound healing

The migration of keratinocytes is a specific function seen in healing of skin wounds. The ability of epidermal keratinocytes to rapidly cover an open wound is a biologically crucial motility response specific to these cells. After wounding of the skin, basal epidermal keratinocytes undergo structural changes in their transformation from a stationary to a migratory cell. These changes include flattening of the cell shape and dramatic reorganization of the cytoskeleton, leading to the formation of long projections called lamellipodia, as well as membrane ruffles at the cell front (Kirfel and Herzog, 2004). In the normal, unwounded epidermis, the keratinocytes are attached to each other by desmosomes and to the basement membrane by hemidesmosomes. When the cells become migratory, an internalization of these structures is seen, enabling the cells to detach from the surrounding cells (Kirfel and Herzog, 2004).

Upon wounding, the keratinocytes on the wound edge come to contact with dermal collagens I and III as well as the constituents of the fibrin clot (fibronectin, fibrin, vitronectin). During their migration over the dermal ECM, the keratinocytes synthesize and deposit various ECM components such as laminin 5, fibronectin and collagen IV, thereby forming a provisional basement membrane, which however is lacking under the migrating keratinocytes themselves (Larjava et al, 1993). The role of integrins is also vital, as they are molecules that are able to bind dermal matrix components during wound repair. During cell migration, new integrin-substrate adhesions are formed at the tips of the lamellipodia, whereas adhesion sites at the rear end of the migrating cell must be disrupted in order for the cell to move (Kirfel and Herzog, 2004). Detachment of the cell from its substratum may involve pericellular proteolysis in which matrix metalloproteinases (MMPs) play a central role (Gill and Parks, 2008, Toriseva and Kähäri, 2008). Excessive activation of some MMPs, such as MMP-9, can on the other hand impair cell migration and lead to breakdown of some necessary matrix proteins and growth factors. This is seen in diabetic peripheral arterial disease, which commonly presents a problem with chronic, non-healing wounds (Signorelli et al, 2005).

The role of several growth factors has long been recognized to be vital in wound healing. Growth factors on the wound region are derived from different sources, including blood platelets of the fibrin clot, dermal fibroblasts and inflammatory cells. Migrating keratinocytes themselves produce a variety of growth factors relevant for wound healing. The epidermal growth factor family has been considered to be one of the key regulatory factors for keratinocytes at the wound edge (Koivisto et al, 2006). The EGF-family of growth factors promotes both keratinocyte migration and proliferation (Jost et al, 2000). KGF, produced by dermal fibroblasts, is upregulated more than 100-fold within 24 h of wounding, and is identified as a regulator of keratinocyte migration and proliferation (Werner, 1998). Furthermore, TGF-β1, while inhibiting keratinocyte proliferation, has been found to stimulate their migration at the wound edge (Schmid et al, 1993). TGF-β1 also increases the synthesis of laminin-5 in migrating cells, thereby influencing cell-matrix interactions (Kainulainen et al, 1998).

2.4. Hyaluronan

2.4.1. Structure and physicochemical properties of hyaluronan

Hyaluronan is a linear high molecular mass polysaccharide built from repeating disaccharide units consisting of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc). The molecule consists of about 2000-25000 of these units, reaching a molecular mass of 10^6 to 10^7 Da, and extended length of 2-25 μm. It belongs to a group of molecules called glycosaminoglycans, along with molecules such as heparan sulfate and chondroitin sulfate, hyaluronan being the only non-sulfated glycosaminoglycan. Because of its special molecular characteristics and considerable size, hyaluronan forms a gel-like matrix capable of binding great amounts of water (Laurent and Fraser, 1992). In tissues, hyaluronan is commonly found in ECM of connective tissues like cartilage, vitreous body of the eye, and in the skin, particularly dermis. The molecular properties of hyaluronan give it distinct functions. First, it contributes directly to tissue homeostasis due to its biophysical properties, forming a stiffened and expanded random coil with strong hydrogen bonds between adjacent sugar units. Second, interactions with link-proteins and proteoglycans (hyaladhedrins) add to its importance to the structural integrity of extra- and pericellular matrices. Third, by interacting with specific cell surface receptors, HA influences important cellular functions such as morphogenesis and tissue remodeling, cell migration, proliferation, differentiation as well as inflammation and tumor development (Toole, 2001). Under the following subtitles, an overview is given on the properties and functions of hyaluronan.
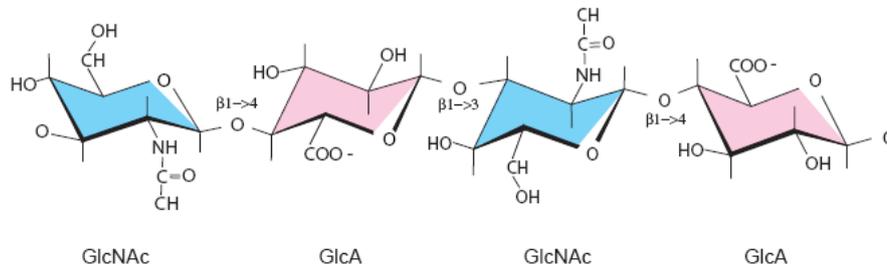
Figure 3. Structure of hyaluronan (two repeating disaccharides shown)

2.4.2. Hyaluronan synthases

Hyaluronan is synthesized on the inner leaflet of the plasma membrane by specific enzymes named hyaluronan synthases. It protrudes out of the cell as a growing chain, by alternative addition of glucuronic acid and N-acetylglucosamine from their UDP-sugars to the cytosolic end of the chain (Weigel et al, 1997). The synthesis of hyaluronan is mainly regulated through the activity of three genes in mammals, designated Has1, Has2 and Has3, located in human chromosomes 19, 8 and 16, respectively (Spicer et al, 1997). The biosynthesis of hyaluronan can be achieved by the expression of any one Has gene, although several of the genes can be activated simultaneously in a tissue. The gene products, the hyaluronan synthases (HAS), are integral membrane proteins with an active site located in the cytoplasmic side of the plasma membrane (Weigel et al, 1997). The size of the synthesized hyaluronan, a property known to greatly influence the biochemical and biological activity of the molecule, varies according to the different hyaluronan synthases. Hyaluronan synthesized *in vitro* by HAS3 has a significantly lower molecular mass, than the product of HAS1 or HAS2. Furthermore, HAS1 and HAS3 produce hyaluronan of variable lengths, whereas the hyaluronan produced by the HAS2 gene is very large in size with less size variation (Itano et al, 1999). In arterial smooth muscle cells, the hyaluronan produced by Has1 and Has2 was also found to be of higher molecular mass than that

produced by Has3 (Wilkinson et al, 2006). However, studies in intact cells have shown that all HAS isoforms can produce hyaluronan of large molecular size (Brinck and Heldin, 1999).

The hyaluronan synthase activity is known to fluctuate with the cell cycle, and peak at mitosis, creating a hydrated microenvironment to the cell and promote partial detachment and rounding of the dividing cell. This is supported by the finding that inhibition of hyaluronan synthesis leads to cell cycle arrest at mitosis, just before cell rounding and detachment (Brecht et al, 1986). At mitosis, hyaluronan is enriched in intracellular compartments, especially around the nucleus (Evanko and Wight, 1999), as well as in the pericellular matrix (Tammi and Tammi, 1991, Evanko et al, 1999), where it most likely provides both signaling and structural functions.

Recent studies have shown that the HAS molecules travel along the endoplasmatic reticulum and Golgi networks before reaching their active site at the plasma membrane. Though they are not believed to be active in hyaluronan synthesis in the intracellular locations, they can form a kind of reserve of molecules, that can be transported to the plasma membrane when increased hyaluronan synthesis is required (Rilla et al, 2005). It is not known, which factors regulate this transport, but recent results indicate that phosphorylation sites are present in HAS molecules, and phosphorylated forms of HAS3 have been found. This phosphorylation can be enhanced by effectors such as membrane-permeable cAMP (Goentzel et al, 2006).

2.4.3. Regulation of hyaluronan synthesis

Several factors, including growth factors and hormones, are known to have a role in the regulation of hyaluronan synthesis in both physiological and pathological conditions, but the exact mechanism for this regulation is yet to be fully unraveled. Hyaluronan synthesis can be regulated at different levels: at Has mRNA transcription, mRNA stabilization, enzymatic activity and stability of the three HAS isoenzymes, as well as availability of the precursor sugars used in hyaluronan production. Growth factors are known to influence hyaluronan synthesis at the level of Has mRNA transcription, including platelet derived growth factor (PDGF), EGF, FGF and TGF-β, but their effects vary significantly in different cell types (Heldin, 2003). For example,

EGF is known to increase hyaluronan synthesis via Has2 in keratinocytes (Pienimäki et al, 2001), and increase pericellular hyaluronan matrix formation in mesothelial cells (Heldin and Pertoft, 1993), but not in chick embryo mesodermal cells (Munaim et al, 1991). In mouse keratinocytes, Has3 mRNA is upregulated by IFN-γ, but downregulated by TGF-β (Sayo et al, 2002). On the other hand, TGF-β has been found to stimulate hyaluronan production via increased Has1 and Has2 expression in fibroblasts (Sugiyama et al, 1998). In mesothelial cells, TGF-β has been shown to reduce Has2 mRNA, but not significantly affect the expression of Has1 or Has3, whereas PDGF increases the expression of Has2 in these cells, with only a slight induction of Has1 and Has3 expression (Jacobson et al, 2000). In dental pulp cells, FGF-2 has been found to increase hyaluronan synthesis via increased expression of Has1 and Has2 (Shimabukuro et al, 2005b). Glucocorticoids, hormones known to decrease hyaluronan synthesis in different cell types (Jacobson et al, 2000, Ågren et al, 1995), have been found to decrease Has2 gene transcription as well as message stability in dermal fibroblasts and in osteoblasts (Zhang et al, 2000). Recently, a natural antisense Has2 (HASNT) has been identified. It has been hypothesized to represent a natural post-transcriptional mechanism to regulate Has2 mRNA, *de novo* hyaluronan biosynthesis, as well as hyaluronan-related cell functions (Chao and Spicer, 2005). No similar antisense mechanisms have been found for Has1 or 3.

Intracellular signaling pathways involved in the regulation of Has expression and hyaluronan mediated signaling are currently under extensive research to further understand the mechanisms in the regulation of hyaluronan synthesis. Since nearly all cell surface receptors utilize mitogen activated protein kinases (MAPK) in their intracellular signaling, a significant amount of research has been directed towards these pathways. In fibroblast like synoviocytes, TGF-β stimulates hyaluronan production via increased Has1 expression, and this effect is mediated via the p38/MAPK pathway (Stuhlmeier and Pollaschek, 2004). In a study by Bastow and co-workers concerning hyaluronan-rich matrix assembly during formation of joint cavities, mechanical stimulus provided the activation of the MEK/ERK pathway, which in turn led to an increase in a hyaluronan-rich pericellular matrix. When MEK signaling was blocked with specific inhibitors, a reduction of the levels of newly secreted hyaluronan was

observed, suggesting a role of increased synthesis in the process (Bastow et al, 2005). Increased phosphorylation and kinase activity of ERK2 has also been reported due to hyaluronan-CD44 interaction in ovarian cancer cells, leading to increased migration and cancer progression (Bourguignon et al, 2005).

Also the activity of the phosphatidylinositol 3-kinase (PI3K) has been related to controlling HA synthesis. PI3K is known to function in intracellular signaling involved in such important cellular responses as cell growth, inhibition of apoptosis, actin cytoskeleton reorganization and cellular motility. Also, Kamikura and co-workers have reported, that membrane PI3K activity is needed for hyaluronan synthesis in several tumor cell lines (Kamikura et al, 2000).

NF-κB is a transcription factor involved in the transportation of signals from cell surface receptors to the nucleus, leading to activation of target genes. It has been shown, that hyaluronan-fragments are able to activate NF- κB in several cell types via the hyaluronan-receptor CD44. This activation was found to be dependent of hyaluronan size, high molecular weight hyaluronan or hyaluronan disaccharides were unable to activate the signaling cascade (Fitzgerald et al, 2000). Recently, a NF-κB binding site was found in the promoter region of human Has2 gene, suggesting evolutionary conservation of Has2 transcriptional regulation (Monslow et al, 2004). Protein kinase C (PKC) is another intracellular signaling molecule linked both to ERK/MAPK cascades as well as NF-κB. PKC has been found to increase hyaluronan synthesis in rabbit joint synoviocytes by increasing *de novo* protein synthesis (Anggiansah et al, 2003, Saavalainen et al, 2007).

2.4.4. CD44 and other hyaluronan receptors

Hyaluronan mediates its cellular effects through specific cell surface receptors. Several hyaluronan-binding receptors have been identified (CD44, RHAMM, LYVE-1, HARE) and are expressed in various cell types. The keratinocytes of the skin are known to express CD44 (Tuhkanen et al, 1997) and RHAMM (Turley and Harrison, 1999), thus these two receptors will be discussed in detail.
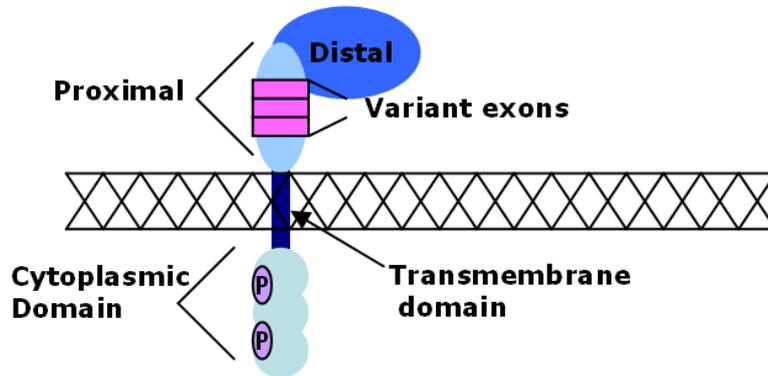
Figure 4. Structure of CD44

The main hyaluronan receptor, CD44, is a cell surface protein expressed widely in various cell types such as leukocytes, fibroblasts, epithelial cells and keratinocytes. Several isoforms of this receptor are found, due to extensive alternative splicing. The molecule of CD44 consists of an extracellular domain of 248 amino acids, a transmembrane domain of 21 amino acids, and a 72 amino acid cytoplasmic domain. The variant forms of CD44 primarily differ from each other in the amino acid composition of their extracellular domain, whereas the transmembrane and cytoplasmic domains are highly conserved (Isacke and Yarwood, 2002). However, some variation also occurs in the intracellular domain. Expression of the so-called "tailless" isoform of CD44, in which the cytosolic domain only consists of three amino acids, affects hyaluronan binding and internalization. The inhibition of this isoform in human articular chondrocytes results in enhanced hyaluronan internalization, whereas its expression even in small amounts negatively modulates the CD44-mediated uptake of HA (Knudson et al, 2002). The alternative splicing of CD44 in normal tissues is regulated, and occurs only in particular cell types and activation states. Abnormal isoform expression patterns are found in malignancies and other pathological conditions indicating a breakdown in this regulation. The hyaluronan binding ability of CD44 is also tightly regulated, and inactive forms without ligand binding capacity do exist. CD44 functions in anchoring hyaluronan on the plasma membrane (Tammi et al, 1998, Knudson, 2003). This interaction leads to cell rolling (DeGrendele et al, 1996),

migration (Bourguignon et al, 2005, Bourguignon et al, 2001), proliferation, differentiation (Bourguignon et al, 2006), and chemotaxis (McKee et al, 1996), according to cell type. CD44/hyaluronan interactions also lead to internalization of hyaluronan (Knudson et al, 2002), which plays a key role in some of the above mentioned cellular events. The most studied of the functions of CD44 is its association in cell migration. CD44 mediates the migration of several cell types, including endothelial cells and melanoma cells (Ichikawa et al, 1999, Singleton and Bourguignon, 2002). The phosphorylation of CD44 following HA-binding activates intracellular tyrosine kinases and signaling molecules including c-Src and Tiam1, leading to regulation of certain cytoskeletal components (ankyrin, ERM-proteins), and changes in cell locomotion (Turley et al, 2002). Cell migration requires active rearrangements of the cytoskeleton. CD44 is localized in the microvilli and regions of actin polymerization in cultured cells, which suggests that it associates with the actin cytoskeleton, even though the molecule in itself has no actin-binding sites. This indirect interaction has been suggested to be mediated by the ERM-proteins (Thorne et al, 2004).

CD44 has been found to be associated with the specific cholesterol enriched domains on the plasma membrane called lipid rafts. These domains have been found to serve as platforms for several transmembrane receptors, thus being important in receptor-mediated signalling events. Depletion of cholesterol from lipid rafts is known to activate such important receptors as the death-receptor Fas (Gniadecki, 2004) and EGFR (Lambert et al, 2006). CD44 has been found to be linked to the actin cytoskeleton via lipid rafts, and this association has been suggested to stabilize the interaction of CD44 with the actin cytoskeleton (Oliferenko et al, 1999). Furthermore, CD44 localisation to lipid rafts is crucial for hyaluronan internalisation, as prevention of CD44 association with lipid rafts by sequestration of cholesterol inhibits hyaluronan internalisation as well as receptor turnover. It does, however, not affect the ability of CD44 to bind hyaluronan (Thankamony and Knudson, 2006).

CD44 has also been found to function as a co-receptor, being able to form complexes with growth factors and their receptors, as well as members of the matrix metalloproteinase family. CD44 can act as a co-receptor for the ErbB family of receptors and in this way modulates their tyrosine kinase activity. For example, heparin-

binding EGF (HB-EGF), bound to the heparan sulphate on CD44 can be cleaved by MMP-7 (matrilysin) and thereby activate ErbB4 (Yu et al, 2002). It is not known which domains of CD44 play a role in this process, but it has been suggested that CD44 association to lipid rafts and association of its cytoplasmic domain to actin cytoskeleton may be important in promoting such heterogeneous receptor interactions (Thorne et al, 2004). CD44 is also able to anchor the active form of MMP-9 on the surface of tumor cell lines, mediate collagen IV degradation, and thus promote tumor cell invasion (Yu and Stamenkovic, 1999).

The receptor for hyaluronan-mediated motility (RHAMM) is another hyaluronan binding membrane receptor that has been linked to migration of cells. It has been reported to occur on the cell surface, as well as in the cytoplasm and the nucleus (Entwistle et al, 1996, Pilarski et al, 1999). It is expressed as several variants, formed by alternative splicing. Different forms of RHAMM are expressed by several mammalian cell types, including fibroblasts, endothelial cells, keratinocytes and several malignant cell types, and its expression is increased in tissue injury as well as tumors. Interactions between RHAMM and hyaluronan on the cell surface have been shown to activate signalling cascades related to cell migration and proliferation (Turley and Harrison, 1999, Savani et al, 2001). Intracellular RHAMM has been demonstrated to associate with cytosolic hyaluronan and mitotic spindle microtubules in arterial smooth muscle cells, thus possibly controlling microtubule assembly during mitosis (Evanko et al, 2004).

2.4.5. Hyaluronan binding matrix molecules

In addition to its cell surface receptors, hyaluronan is bound by a group of proteins, generally called hyaladherins. The hyaladherins are a heterogeneous group of molecules. Many of them contain a common structural domain of about 100 amino acids, termed the link module, which is involved in binding of hyaluronan (Day, 1999). However, a growing number of the newly isolated binging proteins lack this domain, and are unrelated to each other on the primary sequence level.

The link module family of hyaladherins includes some of the most well known hyaluronan binding proteoglycans such as aggrecan, versican, neurocan and brevican.

These proteoglycans form large link protein stabilized complexes with hyaluronan that provide the load bearing function in articular cartilage, give elasticity to blood vessels, and contribute to the structural integrity of tissues such as the skin and brain (Day and Prestwich, 2002). The hyaluronan receptor CD44 contains one link module, whereas HA-binding proteoglycans and the link protein contain two.

Versican belongs to a family of large aggregating chondroitin sulphate proteoglycans found in the ECM. Unlike aggrecan, which is almost exclusively expressed in the cartilage and brain, versican is widely expressed in the ECM of various tissues and organs during development (Wight, 2002). In the epidermis, versican is normally expressed in the basal cell layer, but not in the differentiating keratinocytes, suggesting a role in keratinocyte proliferation (Zimmermann et al, 1994). It is also expressed in the dermis in areas of solar elastosis due to chronic UV-exposure (Saarialho-Kere et al, 1999). In vitro studies have shown that MMP-12, which also is present in solar elastosis (Saarialho-Kere et al, 1999), cleaves versican in the hyaluronan-binding sites indicating that versican located in areas of solar elastosis is unable to bind hyaluronan (Hasegawa et al, 2007). Versican binding to hyaluronan is well known, and is stabilized by link protein (Wight, 2002, Wu et al, 2005). Some studies have suggested that versican may influence tumor behavior via its interaction with hyaluronan and subsequent activation of CD44 (Serra et al, 2005), while other studies suggest that versican can bind to and activate CD44 directly, independent of the association with hyaluronan (Kawashima et al, 2000). The expression of versican is also increased in early atherosclerosis and restenosis, in association with an increase in hyaluronan (Wight and Merrilees, 2004).

2.4.6. Breakdown of hyaluronan by hyaluronidases

Hyaluronan is catabolized in the tissues by to the activity of a group of hyaluronan-degrading enzymes called hyaluronidases. Five different hyaluronidases have been isolated, termed Hyal1, 2, 3, 4 and PH-20 (Stern, 2004). Hyal1 and Hyal2 are found in most tissues and body fluids, and are the main hyaluronan degrading enzymes. Hyal3 is found in testis and bone marrow, but little is known about its specific function. PH-20 is a hyaluronidase found in testis, which is sperm-associated and has a role in

fertilization (Heldin, 2003, Kreil, 1995). Hyal1, 2 and 3 are active in acidic conditions and are believed to have a mainly lysosomal location. PH-20 on the other hand, is active in neutral conditions. As hyaluronidases are relatively newly characterized enzymes, quite little is yet known about their role in physiological and pathological processes.

2.4.7. Biological functions of hyaluronan

Hyaluronan has been found to be an important mediator in many biological events under embryonic development as well as several physiological and pathological processes.

In embryonic development, hyaluronan is especially prominent in sites of cell migration, such as pathways of neural crest cell migration and in the developing cardiovascular system. Especially, hyaluronan biosynthesis via Has2 has been found to be crucial in the development of the cardiovascular system. Developing mouse embryos deficient of Has2 activity die during mid-gestation due to severe cardiac and vascular abnormalities, whereas those lacking Has1 or Has3 were viable and fertile without major defects (Camenisch et al, 2000). In a study performed on zebrafish embryos, the important role of Has2-mediated hyaluronan production was further linked to embryonic cell migration. Has2 mediated hyaluronan production induces the autocrine downstream activation of Rac1, a factor known to be important for lamellipodia formation and cell migration. In zebrafish embryos, this unique event was crucial to the migration of converging lateral cells during gastrulation. Interestingly, this autocrine stimulation of migration in embryonic development shares morphological similarities with metastatic tumor cells (Bakkers et al, 2004).

In the physiological function of the ovaries during ovulation, increased hyaluronan synthesis is seen in the preovulatory cumulus cell-oocyte complex (COC) due to the activity of Has2 (Fulop et al, 1997, Salustri et al, 1999). This accumulation creates a spongy, elastic matrix that facilitates the extrusion of the oocyte at ovulation. This matrix may also present a physiological barrier for penetration of the oocyte by deficient spermatozoa (Salustri et al, 1999).

The cartilage is one of the tissues with greatest hyaluronan concentration, and HA is known to play an important role in the maintenance of the cartilage matrix

(Nishida et al, 1999). The primary function of the chondrocytes is to maintain the complex ECM of the cartilage, which primarily consists of proteoglycan aggregates (mainly aggrecan) in a collagen network. In this matrix, hyaluronan forms the central filament of the proteoglycan aggregate, which has potent viscoelastic properties and is responsible for controlling the osmotic pressure of the matrix. The synthesis of hyaluronan in cartilage is predominantly due to Has2 expression (Nishida et al, 1999). In aging and osteoarthritis, hyaluronan content and synthesis is increased in articular cartilage, but hyaluronan is mainly present as small oligosaccharides, either due to enzymatic degradation or the activity of free radicals of nitric oxide (NO). When high molecular mass hyaluronan interacts with its receptor CD44 it promotes cell survival. These effects are however counteracted by hyaluronan oligosaccharides leading to increased apoptosis (Knudson and Knudson, 2004).

In inflammation, hyaluronan plays an important role in several ways. Extravasation of leukocytes from the blood into the vascular wall involves hyaluronan anchored to endothelial cells by its cell-surface receptors CD44 and RHAMM, and is also mediated by CD44 found on the surface of leukocytes (DeGrendele et al, 1996, Siegelman et al, 1999). In several disease states involving inflammation such as ulcerative colitis (Rosenberg et al, 1995, Bandyopadhyay et al, 2008)and asthma (Rothenberg, 2003, Lauer et al, 2008), there is an abnormal accumulation of hyaluronan and CD44 in the extracellular matrix. Monocytes, that have an even distribution of the hyaluronan receptor CD44 on their surface, adhere to hyaluronan-cables produced by cells in response to endoplasmatic reticulum stress (Lauer et al, 2008). In this way, hyaluronan can function as a signal of cellular stress, and help to engage the recruited leukocytes on the site of inflammation (Hascall et al, 2004). Hyaluronan even plays an important role in the immune response in the skin, including trafficking of leucocytes in and out of the inflamed skin, maturation and migration of epidermal Langerhans cells, as well as dendritic cell induced T-cell activation in antigen presentation (Mummert, 2005).

Marked changes in the tissue content and distribution of hyaluronan are characteristic for atherosclerosis, as well as restenosis after surgical interventions such as balloon angioplasty (Toole et al, 2002). Hyaluronan is present in both early and late

atherosclerotic lesions and this is accompanied by an increase in hyaluronan-associated molecules such as CD44 and versican. Injury to blood vessels, as often seen due to balloon catheter angioplasty induces a dramatic increase in hyaluronan content. As hyaluronan is known for its ability to bind large amounts of water, the rapid expansion of restenotic lesions could be due to edematous changes created by hyaluronan and its associated molecules. This is supported by findings in myocardial infarcts, in which hyaluronan also is also accumulated in the edematous lesions, and where removal of hyaluronan by using hyaluronidases reduces tissue damage (Sunnergren and Rovetto, 1985, Waldenstrom et al, 1991). Another mode of hyaluronan influence on atherosclerosis is the known association of hyaluronan with inflammation, which is a phenomenon seen in early stages of atherosclerosis (Toole et al, 2002).

2.4.8. Hyaluronan and cancer

The development and progression of malignant tumours is a multi-stage process accompanied by various cellular, biochemical and genetic alterations. These events include tumor cell interaction with ECM components, including hyaluronan. The involvement of hyaluronan in malignant transformation, tumor progression and metastasis is well established. The physicochemical properties of hyaluronan help in the formation of open space, which aids in the in growth of new blood vessels (Rooney et al, 1995). The space HA provides also aids tumor cells to migrate, and HA actively supports tumor cell invasion by interacting with its cell surface receptors (Tammi et al, 2002). Elevated levels of hyaluronan have been found in many solid malignancies such as carcinoma of the breast (Auvinen et al, 2000), colon (Wang et al, 1996), thyroid (Böhm et al, 2002), and lung (Pirinen et al, 2001), and linked to poor prognosis in tumors of the stomach (Setälä et al, 1999), ovaries (Anttila et al, 2000), and prostate (Lipponen et al, 2001). On the contrary, in squamous cell tumors, the loss of hyaluronan has been found to be accompanied by the loss of its cell surface receptor CD44 (Hirvikoski et al, 1999, Pirinen et al, 2000) In some malignancies, increased hyaluronan content is found in tumor cells themselves (stomach (Setälä et al, 1999)), whereas others show an increase in the stroma directly adjacent to the tumor (thyroid, prostate, lung,

breast (Auvinen et al, 2000, Lipponen et al, 2001, Böhm et al, 2002, Pirinen et al, 2001).

A study screening several different types of solid tumors showed that hyaluronan content is considerably up-regulated in well-differentiated tumors and associated stroma irrespective of tumor origin, while it is down-regulated in cells of poorly differentiated tumors where stromal expression is retained (Boregowda et al, 2006). This is shown previously in tumors of lung, colon, breast, ovary as well as prostate, which all arise from single layer epithelium, where the expression of hyaluronan is normally lacking. This increase has been found to correlate to aggressive behaviour and high metastasis ratio, and an increased hyaluronan expression is an independent factor for unfavourable prognosis (Setälä et al, 1999, Anttila et al, 2000, Ropponen et al, 1998). Recent research has indicated that tumor cells are able to stimulate fibroblast hyaluronan expression, thus facilitating tumor growth and invasion (Edward et al, 2005).

In an *in vitro* assay, increased hyaluronan synthesis due to overexpression of the hyaluronan synthase Has2, as well as overexpression of CD44 has been correlated to increased invasiveness of breast cancer cells (Udabage et al, 2005). On the other hand, inhibition of cell surface hyaluronan expression by a specific inhibitor, 4-methylumbelliferone (4-MU), has been shown to inhibit metastasis of melanoma cell *in vivo* (Yoshihara et al, 2005). This further underlines the connection between changes in hyaluronan metabolism and aggressiveness of several cancers.

2.4.9. Hyaluronan and CD44 in the skin

In 1947, Meyer et al showed that the skin is one of the richest sources of hyaluronan in the human body. In rats, about half of the total body hyaluronan resides in the skin (Reed et al, 1988). Hyaluronan is found in great amounts in both dermis and epidermis, and to some extent in skin appendages (Wang et al, 1992). In epidermis, hyaluronan is the main component of the ECM, and is found in all vital cell layers, but not in the terminally differentiated keratinocytes in the stratum corneum. When taking into consideration the narrow spaces between the keratinocytes, the hyaluronan content in the epidermis is very high, 90 µg/g wet weight (Tammi et al, 1994). In the dermis, highest hyaluronan concentration lies in the papillary dermis, right below the basement

membrane, decreasing towards the reticular dermis (Wang et al, 1992). The hyaluronan content of dermis is 440-520 µg/g wet weight (Tammi et al, 1994). Dermal hyaluronan is synthesized as a molecule with high molecular mass, but rapidly undergoes degradation. This fragmentation is suggested to facilitate the diffusion of the molecules away from the tissue (Tammi et al, 1991). The hyaluronan content of the skin is higher during embryogenesis than in the adult skin (Ågren et al, 1997). In the epidermis, the half-life of HA is exceptionally short**,** less than 24 h, indicating rapid catabolism by keratinocytes (Tammi et al, 1991).

Epidermal keratinocytes are known to have a high expression level of CD44, the expression pattern of which resembles hyaluronan (Wang et al, 1992). This hyaluronan-CD44 co-localization is thought to be responsible for hyaluronan uptake in several cells. It has also been shown, that mice with blocked CD44 expression accumulate hyaluronan in the epidermis indicating impaired hyaluronan uptake to the cells (Kaya et al, 1997). Several studies have shown that keratinocytes have a pool of hyaluronan resistant to trypsin digestion, suggesting an intracellular localization. This pool was shown to originate from the extracellular pool of hyaluronan, indicating an internalization process after the synthesis and extrusion of hyaluronan out of the cell.

Interestingly, intracellular hyaluronan has a lower molecular mass in comparison to extra-and pericellular hyaluronan, indicating either the preference of lower molecular mass in the internalization process, or the presence of enzymatic degradation by hyaluronidases. The biological significance of the hyaluronan synthesis and endocytosis is not known at present (Tammi et al, 2001).

2.4.10. Regulation of hyaluronan synthesis in keratinocytes

Previous results from our laboratory (Pienimäki et al, 2001), as well as others, have shown, that all the three known hyaluronan synthases are expressed in epidermal keratinocytes (Sugiyama et al, 1998). Human keratinocytes, however, have by some authors been reported only to express Has1 and Has3 (Sayo et al, 2002), whereas others report low, but detectable levels of Has2 mRNA in HaCaT cells with an increase mRNA after ginsenoside (active ingredient of natural ginseng products) treatment with subsequent increase in hyaluronan expression (Kim et al, 2004).

Treatment of keratinocytes with EGF has been reported to increase hyaluronan biosynthesis by up-regulating Has2 mRNA transcription (Pienimäki et al, 2001). This is supported by the finding, that Has2 is a primary EGF and retinoic acid responding gene in human keratinocytes (Saavalainen et al, 2005). Retinoic acid, known to retard terminal differentiation of keratinocytes, has been found to stimulate hyaluronan synthesis rate in human skin organ cultures, leading to accumulation of HA in the superficial layers of epidermis (Tammi and Tammi, 1986, Tammi et al, 1989). On the other hand, factors known to suppress keratinocyte proliferation and epidermal thickening, such as hydrocortisone and the coumarine derivative 4-methylumbelliferone (4-MU), are also known to suppress hyaluronan synthesis in the epidermis (Ågren et al, 1995, Rilla et al, 2004).

Factors like skin injury have a direct influence on keratinocyte hyaluronan synthesis via increased expression of Has2. This in turn leads to increased cell migration and proliferation (Tammi et al, 2005). In skin wound healing, hyaluronan is abundant in the front line keratinocytes migrating into the wound (Oksala et al, 1995). Hyaluronan may contribute to cell migration as a structural component of the extracellular space, creating a highly hydrated, elastic matrix that may help cell movement by facilitating cell detachment as well as providing the space needed for migration (Tammi et al, 2002). Also, the expression of the hyaluronan synthase Has2 has been found to be crucial to keratinocyte migration as shown in studies with antisense Has2 transfection. The lack of Has2 in the antisense Has2 transfected cells reduce the migration of the cells, indicating that increased hyaluronan biosynthesis via Has2 is needed for keratinocyte migration (Jost et al, 2000, Rilla et al, 2002).

2.5. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are proteolytic enzymes capable of degrading all components of the ECM. In humans, 24 different metalloproteinases have been identified, and they are divided into several subgroups according to the targets of their proteolytic activity (Visse and Nagase, 2003). These subgroups include collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs (Kerkelä and Saarialho-Kere, 2003). The activity of metalloproteinases is

controlled on many levels including their DNA transcription, mRNA activity and synthesis rate, as well as by inhibition of their proteolytic activity (reviewed in (Yan and Boyd, 2007)). Tissue inhibitors of metalloproteinases (TIMP) are a group of specific molecules controlling the activity of the enzymes (reviewed in (Clark et al, 2008)). Matrix metalloproteinases are expressed in normal tissues in various physiological processes such as wound healing and embryogenesis. However, increased MMP expression has been linked to development of many cancers, including BCC and SCC of the skin (Kerkelä and Saarialho-Kere, 2003).
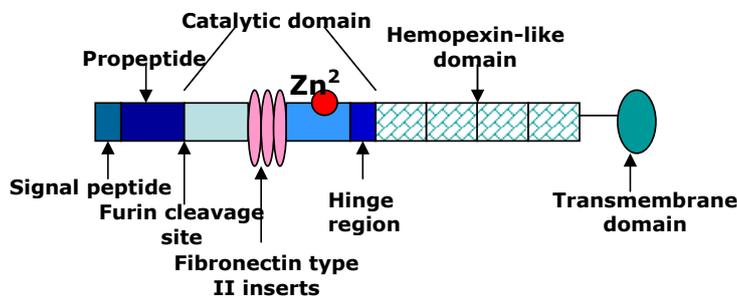
Figure 5. Structure of matrix metalloproteinase. (Modified from Kerkelä and Saarialho-Kere, 2003).

2.5.1. MMP-7

MMP-7 belongs to the MMP subgroup of matrilysins. It was originally identified as the small putative uterine metalloproteinase (PUMP) (Muller et al, 1988). Its known substrates include type IV collagen, elastin, fibronectin, versican, aggrecan and decorin (reviewed in (Kerkelä and Saarialho-Kere, 2003)). Furthermore, it is known to degrade cell surface proteins, such as the adhesion molecule E-cadherin (McGuire et al, 2003), pro-HB-EGF, and syndecan (Ding et al, 2005). MMP-7 has also been reported to cleave cell-associated FasL to its soluble form, thus inducing apoptosis (Powell et al, 1999). MMP-7 is expressed by exocrine and mucosal epithelium in many tissues and is suggested to play a role in the early stages of tumorigenesis and cancer progression (reviewed in (Kerkelä and Saarialho-Kere, 2003, Ii et al, 2006)). Mechanisms involved in MMP-7 induced cancer invasion are modulation of the ECM

and induction of angiogenesis (reviewed in (Ii et al, 2006)) as well as activation of other MMPs, such as MMP-9 (Wang et al, 2005).

In skin, MMP-7 expression has been reported in areas of solar elastosis due to photodamage to the skin. In these areas, it co-localises with versican, its known substrate (Saarialho-Kere et al, 1999). High MMP-7 expression has been reported in several tumor types, including SCCs of the skin (Kerkelä and Saarialho-Kere, 2003, Kivisaari et al, 2008), oesophageal cancer (Tanioka et al, 2003), and head and neck SCCs (Weber et al, 2007). In BCC, MMP-7 has been localized to the tumor-stroma interface in the aggressive forms, but not in other BCC subtypes (Karelina et al, 1994). In SCC, MMP-7 protein is expressed in SCC cells at the stromal interface surrounding tumor nests (Karelina et al, 1994, Lengyel et al, 1995). Interestingly, SCC rising in patients with recessive dystrophic epidermolysis bullosa, which are known to be especially aggressive with higher metastasis ratio, have increased MMP-7 staining in comparison to sporadic SCC (Kivisaari et al, 2008).

2.5.2. MMP-9

MMP-9 belongs to the group of gelatinases, and is produced by keratinocytes, monocytes, macrophages, and many malignant cells. Gelatinases are able to degrade type IV, V, VII, X, XI and XIV collagens as well as gelatin, elastin, aggrecan and fibronectin, and are believed to play an important role in cancer cell invasion (reviewed in (Kerkelä and Saarialho-Kere, 2003)). MMP-9 is also linked to the angiogenic switch in carcinogenesis, and inhibition of MMP-9 has been found to reduce tumor number and growth in mouse-studies (Bergers et al, 2000). MMP-9 can be proteolytically activated by several other MMPs including MMP-7 (Wang et al, 2005) and MMP-3 (Ogata et al, 1992).

In skin cancer, MMP-9 has been detected in the malignant keratinocytes in SCC, but not in normal keratinocytes or premalignant lesions such as actinic keratoses (Kerkelä and Saarialho-Kere, 2003, Karelina et al, 1994, Lengyel et al, 1995). In SCC, MMP-9 protein is expressed in SCC cells at the stromal interface surrounding tumor nests (Karelina et al, 1994, Lengyel et al, 1995). The level of MMP-9 protein is elevated

in cutaneous SCC in comparison to BCC (Dumas et al, 1999). Furthermore, expression of MMP-9 by inflammatory cells is thought to be functionally involved in epithelial carcinogenesis (Coussens et al, 2000, Sillanpää et al, 2007, Boyd et al, 2008).

2.5.3. Hyaluronan and CD44 interaction with MMPs

There is a known interaction between hyaluronan, CD44 and matrix metalloproteinases in cancer development. In mammary cancer and melanoma cells, clustering of CD44 results in the docking of MMP-9 on the surface of the cells, leading to increased tumor invasiveness (Yu and Stamenkovic, 1999, Yu and Stamenkovic, 2000). In oral SCC, strong stromal staining correlates to irregularity in CD44 staining, a factor which predicted a more advanced disease and shorter survival (Kosunen et al, 2007). Proteolytically active MMP-7 forms a complex with CD44 and ErbB4 in the uterine epithelium leading to basement membrane destruction and tumor progression (Yu et al, 2002).

2.6. Epidermal keratinocyte tumors (non-melanoma skin cancer)

Tumors of keratinocyte origin are the most commonly occurring cancers in humans. The two most common forms of these tumors are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), which sometimes arises from a premalignant lesion called actinic keratosis. They are also often called non-melanoma skin cancers (NMSC) to distinguish them from the more malignant skin cancer, melanoma, arising from epidermal melanocytes. Morbus Bowen is a form of *in situ* SCC. Benign keratinocyte tumors, such as keratoacanthomas can clinically resemble SCC (LeBoit et al, 2006).

2.6.1. Basal cell carcinoma

Basal cell carcinoma (BCC) is the most common tumor in humans, and the incidence of this type of skin cancer is rising rapidly. BCCs develop predominantly in sun-damaged skin in fair-skinned individuals that are prone to sunburn (Green and Battistutta, 1990, Lear et al, 1997). The main etiological factors for BCC are sunburns

in childhood as well as intense intermittent exposure to UV-light (Armstrong and Kricker, 2001). Immunodeficiency due to transplantation or AIDS, increases the risk for BCC as well as SCC (Bagheri and Safai, 2001). Other risk factors include exposure to arsenic (Guo et al, 2001) or ionizing radiation (Lichter et al, 2000, Yoshinaga et al, 2005). Also the use of sun beds has been correlated with an increased risk of developing BCC (Faurschou and Wulf, 2007).

Clinically, BCC typically presents with a pearly appearance with teleangiectasia as a papule or nodule that may be ulcerated (LeBoit et al, 2006). Superficial forms of BCC can be mistaken for dermatitis appearing as erythematous patches. BCC can also present a pale, slowly growing scar like lesion or be pigmented and thus be mistaken for malignant melanoma. Erosive lesions, especially in the lower limbs, can be mistaken as traumatic wounds (LeBoit et al, 2006). The most common location for BCC is the head and neck area, due to the common sun exposure especially to these areas of the body.

Deficiency of DNA-repair due to inherited disease is also an important etiological factor for BCC, and this explains its increased incidence in diseases such as xeroderma pigmentosum and Gorlins syndrome, also known as basal cell nevus syndrome. In both of these syndromes, as well as sporadic BCC, mutations in the "patched" (PTCH1, chromosome 9q22.3) gene are found (Aszterbaum et al, 1998). This gene is a tumor suppressor and a key regulator of the Hedgehog growth stimulatory pathway (Bodak et al, 1999). Mutations in the tumor suppressor gene p53 are seen in over 50% of BCCs, more often in aggressive than non-aggressive forms (Ziegler et al, 1994, Bolshakov et al, 2003).

According to the World Health Organisation guidelines, BCCs are divided into several main histological subtypes; nodular, superficial, micronodular, infiltrating, fibroepithelial, basosquamous, keratotic as well as those with adnexal differentiation. Other, less common subtypes include the pigmented and morpheiform BCCs (LeBoit et al, 2006). Histologically, nodular BCC is seen as large nodular masses of basaloid cells extending from the epidermis to the dermis, whereas in the micronodular subtype, the noduli are smaller. Sometimes, cystic spaces are formed inside the tumour due to necrosis. In superficial BCC, the lobules of basaloid cells are confined to the papillary dermis. In infiltrating BCC, the tumor appears as strands of basaloid cells. In BCC with

adnexal differentiation, presence of ductal, sebaceous and trichelimmal elements can be seen. BCC's are considered locally invasive tumors and metastases are very rare (Lo et al, 1991).

2.6.2. Squamous cell carcinoma

Squamous cell carcinoma is the second most common skin cancer, which rises from keratinocytes of the squamous cell layer of the epidermis (LeBoit et al, 2006). The most important risk factor for SCC is chronic cumulative exposure to ultraviolet radiation, and most SCCs arise on the sun-exposed skin of elderly people (Armstrong and Kricker, 2001). Therapeutic measures using psoralen together with UVA (PUVA) have been linked to increased SCC ratio (Lindelof et al, 1999). Immunosuppression greatly increases the incidence of SCC, increases of up to 65-250 times have been reported in transplant patients (Ulrich et al, 2008). Infection with human papillomavirus (HPV) has also been linked to development of SCC, especially in organ transplant recipients (Nindl and Rosl, 2008). Studies have shown mutations of the p53 tumor suppressor gene in SCC due to UV radiation (Ziegler et al, 1994, Bolshakov et al, 2003). Many SCC arise from premalignant lesions such as solar (actinic) keratosis (AK). The transformation rate from AK to invasive SCC varies in different studies and is reported between 0.025% and 16% (Glogau, 2000). An *in situ* form of SCC exists and is also called Morbus Bowen. Histologically, it presents as hyperkeratosis, parakeratosis and increased cellularity in the epidermis with an intact basement membrane, and chronic inflammatory infiltrate in the upper dermis. Besides the skin, Morbus Bowen often resides on mucosa such as genitalia (LeBoit et al, 2006). Clinically, SCC presents as a shallow ulcer, often with a keratinous crust.

Histologically, SCC presents as masses of epidermal cells proliferating into the dermis. The typical squamous cell appearance is seen, with abundant eosinophilic cytoplasm and a large nucleus. Horn pearls are seen as a result of the keratinisation process in the cells. The degree of anaplasia in the tumor nests is used to grade the tumors into "well", "moderately" and "poorly" differentiated. In the dermis, a marked inflammatory reaction is often present, with an abundance of lymphocytes (LeBoit et al, 2006).

The majority of SCCs are locally aggressive and can be cured (Motley et al, 2002). The SCCs that develop in immunocompromised patients such as recipients of solid organ transplantations or those infected with human immunodeficiency virus, have a tendency of being more aggressive (LeBoit et al, 2006).

2.6.3. Hyaluronan, CD44 and versican in non-melanoma skin cancer

In a number of different tumor types, there is a close correlation between tumor progression and hyaluronan production, either by tumor cells or stromal cells. In BCC, increased expression of hyaluronan in the tumor stroma has been linked to increased proliferative capacity of the tumors (Bertheim et al, 2004). No previous publications exist on the expression of hyaluronan in SCC of the skin.

The expression of the hyaluronan receptor CD44 has also been linked to several malignancies. There is considerable evidence confirming that the interaction of CD44 with hyaluronan promotes tumor cell growth, migration and metastasis in several tumor types (Bartolazzi et al, 1994, Peterson et al, 2000). In non-melanoma skin cancer, CD44 is expressed on the cells of *in situ* and invasive SCC, but is absent or only focally expressed in BCC (Yasaka et al, 1995, Prieto et al, 1995, Seelentag et al, 1996, Simon et al, 1996). The importance of this difference, however, is not clear. While some authors have linked the difference between SCC and BCC in CD44 expression to the ability of the tumors to metastasize (Prieto et al, 1995), others state, that the expression of different splice variants of CD44 in SCC and BCC has not been correlated with the invasive or metastatic potential of the tumors, but is related to the degree of tumor differentiation (Seelentag et al, 1996). Studies with melanoma cells *in vitro* have demonstrated a role for hyaluronan and CD44 in facilitating growth and migration. However, in primary melanomas reduced hyaluronan and CD44 has been correlated with an unfavourable prognosis (Karjalainen et al, 2000).

Versican is a large proteoglycan, which associates to hyaluronan in tissue matrices. This association has been suggested to promote tumor growth in breast (Nara et al, 1997) and prostate (Ricciardelli et al, 1998) cancers, but has not been previously studied in NMSC.

3   AIMS OF THE STUDY

Hyaluronan is an ECM molecule that is abundant in the epidermis of the skin, where other ECM components such as fibrillar collagens are absent. We hypothesize, that hyaluronan not only functions in filling the narrow spaces between the tightly packed cells, but actively participates in important cell functions, such as cell proliferation, migration and differentiation, in response to growth factor stimuli.

The aims of this study were as follows:

1. To examine the effects of three different growth factors (EGF, KGF, TGF-β) on the important functions of the keratinocyte: proliferation, migration and differentiation

2. To correlate these growth factor effects in the changes they induce in hyaluronan metabolism

3. To analyse the expression pattern of hyaluronan, its surface receptor CD44 and MMP-7 and MMP-9 in epidermal keratinocyte tumors and to correlate hyaluronan/CD44 expression to that of the ECM degrading MMP-7 and MMP-9, as well as to the invasiveness of the tumors

4 MATERIALS

4.1. Monolayer cell cultures (I)

A spontaneously immortalised newborn rat epidermal keratinocyte (REK) cell line, originally isolated by Baden and Kubilus (Baden and Kubilus, 1983) was used in all experiments. Cultures of REK**s** were grown in Minimum essential medium (Life Technologies, Paisley, UK) with added 5 (I) or 10 (II) % fetal bovine serum (FBS, HyClone, Logan, UT), 4 mM L-glutamine (Sigma, St Louis, MO), and penicillin-streptomycin (50 units/mL and 50 µg/mL, Sigma). REKs were passaged twice a week at a 1:5 split ratio using 0.05% trypsin, 0.02% EDTA in phosphate buffered saline. After 60-70 passages, new cells were taken from the liquid nitrogen, because in the course of time, the cells lose the differentiation potential in cell culture.

4.2. Organotypic cell cultures (I, II)
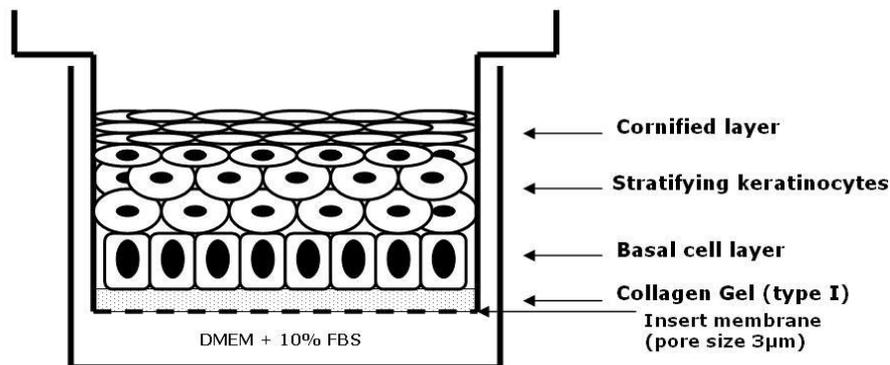
Figure 6. The organotypic culture model

The REK organotypic culture model was modified from that used by Lillie (Lillie et al, 1988) and Tammi (Tammi et al, 2000). REKs were cultured at air-liquid interface on type I collagen support (from rat tail; Becton Dickinson Labware, Bedford, MA). To prepare collagen substrates, 8 volumes of type I collagen (~3.2mg/ml) was

dissolved on ice in a solution consisting of 1 volume 10x EBSS (Life Technologies LTD), 0.3 volumes 7.5% sodium bicarbonate (Life Technologies LTD) and 0.2 volumes 1 M NaOH. 800µl of dissolved collagen was added to individual 24 mm diameter tissue culture inserts (3.0 µm pore size) (Costar Transwell®, Cambridge, MA), which were then incubated overnight at 37˚C in a humified atmosphere to polymerize the collagen.

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

4.3. Growth factors (I, II)

EGF (Sigma) was dissolved in sterile water and added to culture medium in the final concentrations of 2-20 ng/ml. KGF (Sigma) was dissolved in 0.1% BSA in PBS and was used in the final concentration of 0.1-100 ng/ml. TGF-β (Life Technologies LTD) was dissolved in sterile water and used in the final concentrations of 1-4 ng/ml.

4.4. Paraffin embedded skin cancer samples (III, IV)

The tissue material consisted of 180 skin samples removed in Kuopio University Hospital between 1996 and 1999. Paraffin-embedded samples were collected from the archives of the Department of Pathology and sectioned at 5µm. Hematoxylin and eosin-stained sections were used to type and re-grade the lesions histologically. The samples consisted of 114 BCC, 31 ISC and 35 SCC, grades I-III. Permission was obtained from the Kuopio University Ethics board.

5   METHODS

5.1. Proliferation assays (I, II)

In monolayer cultures, the REKs were seeded into 24-well plates at 60.000 cells/well, KGF (1-100 ng/ml) was added the next day, and the number of cells was counted by haemocytometer 24, 48, and 72 h later.

To examine cell proliferation in organotypic cultures, two-week old REK cultures grown in the presence of either EGF (0, 2, 20 ng/ml) or TGF-β (0, 1, 4 ng/ml) were incubated with 5-bromo-2'deoxyuridine (BrdU) for 1 h, washed with PBS, and fixed overnight in Histochoice®. Deparaffinized, TUF-treated sections were incubated at 95ºC, immunostained with the anti-BrdU antibody, and counterstained with propidium iodide (0.01 μg/ml; Sigma) according to the manufacturers instructions (5-bromo-2'-dioxyuridine Labeling and Detection Kit I, Roche Diagnostics Corporation, IN). Approximately 10 fields per section were counted for the labeled cells in six separate experiments with a Nikon Microphot FXA microscope using a 10 x objective.

5.2. Migration assay (I)

Keratinocyte migration was studied *in vitro* with a scratch wound assay. REKs were seeded at 600 000 cells/well on 6-well plates, and grown for 24h. A standardized area was scraped clean with a sterile 1000μl pipette tip, and the medium containing KGF in concentrations from 1 to100 ng/ml was added. The area covered by the cells in eight (8) crossing areas in duplicate wells of each KGF concentration was measured immediately after scraping and after 24 h incubation using Olympus CK2 inverted phase contrast microscope, a Panasonic Wv CD 130-L video camera, and NIH Image Software.

5.3. Hyaluronan assays

5.3.1. Metabolic labeling assay (I)

REKs were seeded on 6-well plates at 200 000 cells/well, and grown until subconfluent. Fresh medium containing [^{3}H] glucosamine (20μCi/ml) and [^{35}S]SO$_4$

(10μCi/ml) (Amersham Biosciences, Little Chalfont, UK), and the appropriate amounts of KGF (0, 1, 10, 100 ng/ml) were added to the cells and incubated for 6 or 18h. The medium and two 0.3ml HBSS (EuroClone) washes of the cell layer were combined and designated "medium". Cell surface-associated hyaluronan was detached with 0.5ml of 0.05% trypsin (w/v), 0.02%EDTA (w/v) for 10 min in 37˚C, and the cells were pelleted and washed with 250 µl HBSS. The trypsin solution and HBSS wash were combined and called "pericellular", whereas the pellet was designated as the "intracellular" hyaluronan pool. Hyaluronan and other glycosaminoglycans were purified and quantitated from the different cellular compartments after determination of the specific activity of the hexosamines as described in detail previously (Tammi et al, 1998)**.**

5.3.2. Hyaluronan synthase activity *in vitro* (I)

The assay was done essentially as described before by Spicer (Spicer, 2001). Subconfluent REKs were incubated for 14 h with 0-100ng/ml KGF, membrane fractions isolated and incubated with 0.5mM UDP-GlcNAc and 0.05 mM UDP-GlcA (both from Sigma), the latter containing 2.5 μCi of UDP-[^{14}C]GlcA (PerkinElmer Life Sciences), for 2 h at 37˚C. The samples were boiled with 1% SDS, incorporated activity separated by paper chromatography, quantified by liquid scintillation counting, and expressed as picomoles of GlcA incorporated/mg of protein in the membrane fraction (Spicer, 2001).

5.3.3. Hyaluronan disaccharide analysis with electrophoresis (I)

Medium samples (400µl) were boiled for 10 min and digested with 40µl of proteinase K (Sigma, 600µg/ml in ammonium acetate, pH 6.5) for 1.5 h at 60˚C. After proteinase K inactivation by boiling for 10 min, 50µl of 50% trichloroacetic acid was added to precipitate proteins by centrifugation (15 min, 13 000 xg). Dialyzed supernatants were evaporated, dissolved in 100 mM ammonium acetate, pH 6.5, and digested for 3 h at 37˚C with 2 milliunits of *Streptococcal* hyaluronidase (Seikagaku), dried, and derivatized overnight at 37˚C in 5 µl of 0.1 M 2-aminoacridone (Lambda Fluoreszenztechnologie GmbH, Graz, Austria) in 3:17 (v/v) acetic acid:dimethyl sulfoxide, and 5 µl of 1 M NaBH$_3$CN. The 2-aminoacridone-derivatized disaccharides

were stored at -20˚C until electrophoresis as described before (Rilla et al, 2002), with the following modification; 30% polyacrylamide gels were cast in the laboratory in 100 mM Tris borate buffer, pH 8.9, and the same buffer was used as the running buffer. The intensities of the hyaluronan disaccharide bands derived from the samples and hyaluronan standards (Healon®, Amersham Biosciences) were digitized on a UV-light box using a CCD camera. Quantitative image processing was done with NIH-Image.

5.3.4. Hyaluronan ELSA (I)

Organotypic cultures were changed into 1.5 ml of serum-free medium with KGF (0, 2 and 20 ng/ml) and continued for 24 h. The medium, epidermis, and collagen support were each analyzed separately, the latter two after extraction with 2 x 2 ml of acetone at 4 ℃ over 2 days, and digestion of the residue overnight at 60 ℃ with 250 µg/ml papain (Sigma) in 5 mM cysteine and 5 mM EDTA. After incubation, the samples were boiled for 10 min to inactivate the enzyme, centrifuged at 13 000 x g, and the pellet discarded. Maxisorp Plates (Nunc, Roskilde, Denmark) were coated overnight at 4 ℃ with 1 µg/ml hyaluronan-binding complex (140), washed with PBS containing 0.5% Tween 20 (Tween-PBS), and blocked with 1% BSA for 1 h at 37 ℃. Standard hyaluronan (Provisc, Algon Laboratories Inc., Fort Worth, TX) at 150ng/ml concentrations and samples diluted into 1% BSA in PBS (100µl) were added to the wells for 1 h at 37 ℃, the plates were washed with Tween-PBS, and incubated with 1 µg/ml bHABC for 1 h at 37 ℃, washed with Tween-PBS, and 1 mg/ml O-phenylenediamine dihydrochloride (Sigma) and 0.03% H_2O_2 in 0.1 M phosphate citrate buffer, pH 5, were added at 37 ℃. The reaction was stopped after 15 min with 50 µl of 4 M H_2SO_4 and the absorbance read at 490 nm. Each sample and standard was done in triplicate.

5.3.5. Molecular mass of hyaluronan (I)

Aliquots (0.5 ml) of radiolabeled culture medium, trypsin supernatant, and cell extract were subjected to gel filtration on a 1 x 30 cm column of Sephacryl S-1000 (Amersham Biosciences), equilibrated, and eluted at 0.4 ml/min with 0.15 M sodium

acetate, 0.1% CHAPS (Sigma), 0.05% Hibitane®, pH 6.8. From each fraction, one aliquot was incubated overnight 37˚C with 12.5 milliunits of *Streptomyces* hyaluronidase (Seikagaku), whereas another received buffer only. Both aliquots were precipitated in 1% cetylpyrinidium chloride (Sigma) with 5µg of carrier hyaluronan. The increase of [^{3}H] glucosamine in the supernatant of the hyaluronidase-treated aliquot was a specific measure of hyaluronan. The void volume of Sephacryl S-1000 column (V_0) was considered to be in the first hyaluronan-positive fractions emerging from the chromatogram of Healon GV with a mean molecular mass of 6 x 10^6 Da (Amersham Biosciences), and the total volume V_t in the elution position of glucuronic acid. The size distribution of hyaluronan in the samples was estimated from the K_{av} values of known hyaluronan standards, provided by the resin manufacturer.

5.3.6. Hyaluronan staining of monolayer cultures (I)

To localize hyaluronan, a specific probe, bHABC, was used as described by Tammi in 1994 (Tammi et al, 1994). In monolayer cultures, the cells were grown on 8-well chamber slides, fixed with 2% paraformaldehyde with 0,5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PB), for 20 min, washed with PB, and permeabilized with 0.3% Triton X-100 in 1% BSA-PB for 30 min. The samples were then incubated overnight with bHABC (5µg/ml in 1% BSA) in 4˚C. After washes with PB, avidin-biotin peroxides complex (1:200, Vector Laboratories Inc., Burlingame, CA) was added for 1h. Hyaluronan was visualized with 0.05% 3,3´–diaminobenzidine (DAB, Sigma, St. Louis, MO) and 0.03% H_2O_2 for 5 min. To examine intracellular hyaluronan, *Streptomyces* hyaluronidase (10 turbidity reducing units/ml, 10 min at 37˚C, Seikagaku, Tokyo, Japan) digestion was done prior to the permeabilization to remove extracellular and pericellular hyaluronan.

5.3.7. Hyaluronan staining of paraffin embedded tissues (I, II)

To stain for hyaluronan in paraffin embedded tissues (organotypic cultures, paraffin embedded skin cancer samples), the paraffin sections were rehydrated in graded ethanol. The staining procedure was performed as described before (Tammi et al, 2000).

The sections were deparaffinised in xylene and rehydrated in graded alcohols, followed by washings in PB. After blocking the endogenous peroxidase by 1% hydrogen peroxide for 5 min, the sections were preincubated in 1% BSA for 30 min to block non-specific binding. The sections were incubated overnight with bHABC (protein concentration $3\mu g/mL^{-1}$, diluted in 1% BSA in PB at 4°C), washed thoroughly with PB and treated with avidin-biotin peroxidase complex (ABC; Vector, Burlingame, CA, dilution 1:200) for 1 h in room temperature. Following washes with PB, the slides were incubated in 0.05% 3,3'–diaminobenzidine (DAB, Sigma, St. Louis, MO) and 0.03% H_2O_2 for 5 min. The slides were dehydrated in graded alcohols and mounted in DPX (BDH Laboratory Supplies, Poole, Dorset, UK) without counterstaining. The specificity of the staining was tested by digesting the sections with *Streptomyces* hyaluronidase (100 turbidity reducing units/ml in 50mM sodium acetate buffer, pH 5.0, for 3h: Seikagaku, Tokyo, Japan) in the presence of protease inhibitors, or preincubating the bHABC probe with hyaluronan oligosaccharides prior to staining. To view overall morphology, the sections were stained with haematoxylin/eosin.

5.3.8. Optical density measurements (I)

Optical densities of the cells stained for hyaluronan using DAB were analyzed by a Leitz BK II microscope with 16x/0.45 numeric aperture objective (Leitz, Wetzlar, Germany) and digital camera (Photometrics CH 200, Roper Scientific Inc. Trenton, NJ) as described previously (Tammi et al, 1998). Area-integrated mean optical density values for the DAB chromogen were calculated for each whole digitized area, excluding possible artefact areas. In the densitometric assays, the hyaluronan remaining after *Streptomyces* hyaluronidase treatment of non-permeabilized cells was designated as "intracellular" whereas in the chemical assays the term intracellular represents hyaluronan resistant to peeling off the pericellular hyaluronan with trypsin-EDTA. These two techniques give parallel results for the intracellular pool, but the values of the trypsin-EDTA method are somewhat higher (Tammi et al, 2001).

5.4. RNA isolation and semi-quantitative RT-PCR (I, II)

Has1	5′-GCT CTA TGG GGC GTT CCT C-3′ (left) 5′-CAC ACA TAA GTG GCA GGG TCC-3′ (right)	57°C	35–37 ×
Has2	5′-TCG GAA CCA CAC TGT TTG GAG TG-3′ 5′-CCA GAT GTA AGT GAC TGA TTT GTC CC-3′	62°C	33–35 ×
Has3	5′-ACT CTG CAT CGC TGC CTA CC-3′ 5′-ACA TGA CTT CAC GCT TGC CC-3′	66°C	33 ×
CD44	5′-TTG GGG ACT ACT TTG CCT CTT A-3′ 5′-CCA CTG CTG ACA TCC TCA TCT A-3′	55°C	33 ×
Profilaggrin	5′-CTC AGG GCA TCG CTC GTC A-3′ 5′-GCT GGT GGC GGT CTT CGT G-3′	64°C	34 ×
GAPDH	5′-TGA TGC TGG TGC TGA GTA TG-3′ 5′-GGT GGA AGA ATG GGA GTT GC-3′	60°C	32 ×

Table 1. Primers, annealing temperatures and number of PCR cycles used in RT-PCR (from I)

Total RNA was isolated using TRIzol® reagent (Life Technologies LTD). Organotypic REK cultures were put in TRIzol® and homogenized with a needle and a syringe. RNA was isolated according to the manufacturer and dissolved in 20µl of sterile water. To remove genomic DNA contamination, the samples were treated with 10 U of RNase-free DNase I (Roche Diagnostics GmbH, Penzberg, Germany). After DNase treatment, the RNA concentration was measured with a spectrophotometer at 260 nm and the samples were diluted to equal concentration. 0.1 µg of RNA was taken for the RT-PCR, done with the GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems, Foster City, CA). The primers used for the RT-PCR of rat Has1, Has2, Has3, CD44, profilaggrin and GAPDH as well as the annealing temperatures and the number of PCR cycles are shown in Table 1. The RT-PCR products electrophoresed in a 1.5% agarose gel were digitalized by BioDocII Video Documentation system (Biometra, Göttingen, Germany), and the band densities of ethidium bromide fluorescence were measured with the NIH-Image 1.62/fat software.

5.5. Immunoblotting (I, II)

The organotypic REK cultures (without collagen matrix) were homogenized in 8 M urea, 50 mM Tris, pH 7.6, 100 mM dithiothreitoil, 0.13 M 2-mercaptoethanol,

100μg/ml phenylmethylsulfonyl fluoride (PMSF), 20μg/ml sodium orthovanadate and 100μg/ml aprotinin (modified from (Haydock et al, 1993)) with Ultra Turrax (Ystral, Germany), and centrifuged 13, 000 x g for 15 min. The supernatant was used for the determination of protein concentration (Bradford, 1976) and Western blotting.

15-20μg of protein was resolved in a 10% SDS-PAGE, and transferred onto Immobilon™-NC membranes (Millipore, Bedford, MA) by a 35mA per cm^2 constant current with a Sammy™ semidry blotter (Schleicher and Schuell, Dassel, Germany). The blots were blocked in 10 mM Tris, 150mM NaCl, pH 7.4 (Tris-saline blocking buffer) containing 1-5% non-fat milk powder and 0.1-0.3% Tween-20 overnight at 4˚C, and incubated with the primary antibody for 2h, using the following dilutions: polyclonal anti-filaggrin 1:9000 and monoclonal anti-keratin 10 1:100, diluted in blocking buffer. After washes with the blocking buffer, the membranes were incubated for 1h with a horseradish peroxidase-conjugated anti-rabbit IgG (Zymed Laboratories Inc, San Francisco, CA), dilution 1:20 000 for filaggrin, and with anti-mouse IgG (Santa Cruz Biotechnologies, Santa Cruz, CA), dilution 1: 20 000 for keratin 10. The immune complexes were visualized using the NEN™ chemiluminescence detection kit according to instructions from the manufacturer (PerkinElmer Life Sciences).

5.6. Immunohistochemical stainings (I-IV)

The Histochoice-fixed, deparaffinised sections of organotypic cultures (I, II) were first incubated in target unmasking fluid (TUF™, Monosan, Uden, Netherlands) at 95ºC, then for 5 min with 1% hydrogen peroxide to block endogenous peroxidases, washed with PB, and incubated in 1% BSA in PB to block non-specific binding. The sections were incubated overnight at 4 ºC with polyclonal anti-filaggrin antibody (1: 5000, diluted in 1% BSA in PB, generous gift from Dr. Beverly Dale-Crunk, University of Washington, Seattle, WA), anti-keratin 10 antibody (Monosan, 1:10 dilution in 1% BSA) or with anti CD44 (OX50 1:50, Biosource, Camarillo, CA), followed by an 1 h incubation with biotinylated anti-mouse IgG antibody (1:50, Vector) or biotinylated anti-rabbit antibody (1:70, Vector). The bound antibodies were visualized with avidin-biotin peroxidase complex (ABC, Vector, dilution 1:200) and 0.05% 3,3'–diaminobenzidine (DAB, Sigma, St. Louis, MO) and 0.03% H_2O_2 for 5 min. The slides

were counterstained with haematoxylin, dehydrated in graded alcohols and mounted in DPX (BDH Laboratory Supplies, Poole, Dorset, UK). The controls included samples treated the same way, but without the primary antibody.

To stain the NMSC samples for CD44 (III), the sections were blocked for endogenous peroxidase activity and non-specific binding as described above, and incubated overnight at 4˚C with the primary antibody Hermes 3 (a generous gift from Dr Sirpa Jalkanen University of Turku, Finland) diluted 1:100 in BSA/PB. The sections were sequentially incubated with a biotinylated anti-mouse antibody (Vector, 1:100), and avidin-biotin peroxidase complex (Vector) for 1h in room temperature. The colour was developed with DAB and H_2O_2 as described above with hyaluronan staining. After counterstaining with Mayers haematoxylin, the slides were dehydrated, cleared, and mounted in DPX. Hermes 3 detects an epitope in the standard backbone of CD44.

The staining protocol for versican (III) differed from that of CD44 at two points. After deparaffinization the sections were treated with an antigen unmasking solution for 10 min at 95˚C. Secondly, the primary antibody used was anti-large proteoglycan (Seikagaku: dilution 1:1000).

To stain for MMP-7 and MMP-9 (IV), the sections were deparaffinised in xylene and rehydrated in graded alcohols, followed by washings in 0.1 mol PB, pH 7.4. The sections were heated in a microwave oven in 0.01M citrate buffer (pH 6.0) for 3 x 5 min, incubated in the citrate buffer for 18 min and washed for 2 x 5 min in PBS at room temperature. Endogenous peroxidase activity was blocked by 5 % hydrogen peroxide for 5 min, followed by a wash with distilled water for 2 x 5 min and with PBS for 2 x 5 min. Non-specific binding was blocked with 1.5% normal serum (Vectastain ABC Elite Kit, Vector, Burlingame, CA) in PBS, for 35 min at room temperature. The sections were incubated overnight at 4ºC with the mouse anti-MMP-9 primary antibody (1:3000, Gelatinase B 92 kDa type, clone 56-2A4, Chemicon, Temecula, CA) or mouse monoclonal anti-human-MMP-7 primary antibody (1:800, clone 141-7B2, Chemicon), diluted in 1.5% Normal Serum. Next, the slides were washed with PBS 2 x 5 min and incubated with the biotinylated secondary antibody (1:20 000, Vectastain ABC Elite Kit, Vector) for 45 min at room temperature and washed with PBS 2 x 5 min. The slides were then incubated with preformed avidin-biotin peroxidase complex (Vector) for 50

min at room temperature and washed twice for 5 min with PBS, developed with DAB 0.05% (Sigma) containing 0.03% H^2O^2 for 5 min, counterstained with Mayers haematoxylin, dehydrated in graded alcohols and mounted in Depex mounting medium. Placental and ovary tissues were used as positive controls for MMP-9 and 7, respectively. The negative control was skin tissue, but instead of incubation with the primary antibody the slide was incubated with the blocking solution overnight.

Marker	Antibody	Manufacturer	Type	Dilution
keratin 10 (I,II)	anti-keratin 10	Monosan	murine monoclonal	1:10
filaggrin (I,II)	anti-filaggrin	gift from Dr Beverly Dale-Crunk	rabbit polyclonal	1:5000
CD44 (I,II)	OX50	Biosource	mouse monoclonal (anti-rat)	1:50
CD44 (III)	Hermes 3	gift from Dr Sirpa Jalkanen	mouse monoclonal (anti-human)	1:100
Versican (III)	anti-large proteoglycan	Seikagaku	mouse monoclonal	1:1000
MMP-9 (IV)	clone 56-2A4	Chemicon	mouse monoclonal	1:3000
MMP-7 (IV)	clone 141-7B2	Chemicon	mouse monoclonal	1:800

Table 2. Summary of the antibodies used in this thesis

5.7. Permeability studies (I, II)

The permeability of epidermis was tested by clamping the organotypic cultures between two chambers filled with PBS and equilibrated at 37 ºC, as described previously (Pasonen-Seppänen et al, 2001). Tritiated corticosterone was added to the apical side of the epidermis, and aliquots were withdrawn repeatedly from the basal side for liquid scintillation counting. The permeability coefficient (P, cm/s) was calculated under sink conditions by dividing the steady state flux (dpm/s x cm^2) through the epidermis by the concentration of corticosterone (dpm/cm^3) in the donor chamber.

5.8. Microscopical methods

5.8.1. Electron microscopy (I)

Paraformaldehyde (2%) and glutaraldehyde (0.5%) fixed REKs were permeabilized with 0.05% saponin in 3% BSA-PB for 10 min on an ice bath.

Hyaluronan staining was performed as described above, except that all solutions and buffers contained 0.05% saponin, and the incubations were done at 4ºC. Hyaluronan staining was followed with a 15 min postfixation in 1% reduced osmium tetroxide. After dehydration in graded ethanol, the samples were embedded in Spurr's resin. Thin sections were cut out on Formwar-coated copper grids, stained with uranyl acetate and lead citrate, and viewed in a type 1200 EX microscope from JEOL (Tokyo, Japan).

5.8.2. Confocal microscopy (I)

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

5.8.3. Morphometric analysis of epidermal thickness (I, II)

Hematoxylin-eosin-stained sections were systematically sampled by taking six digital images with a CoolSNAP camera (Roper Scientific) from each culture at constant intervals using a 20 x objective and a 1.25 x intermediate lens (Nikon Microphot FXA microscope). The heights of the basal cells, vital epidermis, and stratum corneum were each measured using the NIH Image 1.62/fat software for Macintosh (Wayne Rashband, National Institute of Health, Bethesda, MD). Thresholding of areas exhibiting background intensity was used to exclude the areas between separated corneocytes in the stratum corneum measurements. Data from six cultures was analyzed for each group.

5.9. Evaluation of skin cancer samples (III, IV)

The stainings for hyaluronan, CD44, versican and MMP-7 and MMP-9 were evaluated by two observers (SH-P, V-MK), unaware of the clinical data. In the BCC and SCC, the staining was classified as homogenous when all parts of the tumor epithelium showed the same staining intensity, and as irregular when signal intensity varied in the tumor area or was negative in parts of the tumor. In ISC samples, the staining was homogenous if it extended evenly throughout the epithelium, and irregular if some layers were more intensely stained. The staining intensity was compared to the normal epidermis in hyaluronan and CD44 samples, and to the skin appendages in MMP samples. Staining intensity less than that of the control tissue was considered weak and equal to the control as moderate. In the case of hyaluronan and CD44, a staining intensity stronger than that of the normal epidermis was considered as intense. Peritumoral versican staining was classified as negative or positive if the intensity equalled or exceeded that around normal hair follicles.

5.10. Statistical analysis

5.10.1. *In vitro* studies (I, II)

The statistical significances of the differences between control and growth factor treated groups in the migration; RT-PCR assays and hyaluronan-ELSA measurements were tested using paired-samples t-test. The data from the proliferation and permeability assays were analyzed with the non-parametric Mann-Whitney U-test. A difference was considered statistically significant when the associated p-value was less than 0.05.

5.10.2. Epidermal tumor staining (III, IV)

The statistical calculations were performed using the SPSS for Macintosh program (v. 10.0 SPSS Inc., Chicago, IL). The χ^2-test was used to compare staining intensity and regularity. The correlations of staining intensities were analysed between different tumor types, and also between different grades of SCC. Because of the low

number of grade 2 and grade 3 SCC samples, their results were combined for statistical analysis. Probability values less than 0.05 were regarded as significant.

6   RESULTS

6.1. Hyaluronan metabolism in organotypic and monolayer keratinocyte cultures (I, II)

In this study, we examined the influence of growth factors on the synthesis of hyaluronan, and the hyaluronan content in the extracellular, pericellular and intracellular compartments in the REK keratinocytes in organotypic and monolayer cultures.

In stainings performed on organotypic cultures, EGF caused a dose-dependent increase of hyaluronan in the epidermis. The increase was most significant in the spinous cell layer (II, Fig. 3). This increase in hyaluronan in the epidermis, as well as in growth medium and collagen matrix, was confirmed by the ELSA assay (II, Fig. 2A). The treatment of organotypic cultures with EGF increased the expression of Has2 and Has3 mRNA (II, Fig. 2B). Has1 mRNA was also present in the cultures in very small amounts, but it was not affected by EGF.

In organotypic cultures, TGF-β did not influence hyaluronan content in the epidermis, in comparison to control cultures (II, Fig. 3). However, the hyaluronan content in the matrix compartment (growth medium) was significantly lower than that of the control cultures, indicating a lower synthesis rate of hyaluronan (II, Fig. 2A). This was supported by the reduction of the Has 2 and Has3 mRNA expression in response to TGF-β as measured by the RT-PCR assay (II, Fig. 2B).

Like EGF, KGF stimulated the accumulation of hyaluronan in the epidermis in organotypic cultures. This increase was most prominent in the spinous cell layer, but some increase was also found in the basal cell layer (I, Fig. 4. b-c). KGF increased the amount of hyaluronan in the epidermis, and also in the medium and collagen matrix, as measured by the ELSA assay (I, Fig. 4g). RT-PCR performed on the organotypic cultures, showed an increase in Has 2 and Has 3 mRNA in response to KGF (I, Fig. 4. h-i).

In organotypic cultures, KGF also increased the levels of the main HA-receptor CD44 in the epidermis, mainly in the spinous cell layer but also in the basal cell layer (I, Fig 4. c-d). RT-PCR showed an increase in the mRNA for CD44 after KGF treatment (I, Fig 4. h)

In monolayer cultures, KGF induced a dose-dependent increase in newly synthesized hyaluronan in the growth medium, representing the extracellular compartment, as measured by electrophoresis (I, Table II) and a metabolic labeling assay (I, Fig 2. d). The accumulation of HA in the culture medium was associated with a dose-dependent increase of HAS activity as measured by the hyaluronan synthase assay (I, fig. 2a). The RT-PCR performed for the three Has isoforms indicated that they were all expressed in the keratinocyte monolayer cultures. The levels of Has1 and Has3 mRNA were not markedly affected by the addition of KGF to the growth medium, whereas the Has2 mRNA levels were elevated in response to KGF. This up-regulation of Has2 mRNA expression started about 3 h after KGF addition and lasted up to 24 h (I, Fig. 2b, c). This suggested that up-regulation of Has2 was mainly responsible for the induction of hyaluronan synthase activity and increased hyaluronan content in response to KGF. When localisation of newly synthesized HA was studied by a 24 h metabolic labeling experiment, we found that KGF did not significantly affect the amount of pericellular hyaluronan, but a marked increase in the intracellular and extracellular hyaluronan content was seen (I, Fig. 2e). Histological stainings confirmed the accumulation of HA in the cells and especially in the intracellular compartment in response to KGF (I, Fig. 1a-d). The localisation of HA in the intracellular compartment was confirmed by electron microscopy (I, Fig. 1e) Transmission electron microscopy showed, that the intracellular HA was located in membrane coated vesicles (I, Fig. 1f). The accumulation of hyaluronan in the intracellular vesicles rather than the pericellular compartment due to KGF suggests that KGF not only stimulates the synthesis of hyaluronan, but also its uptake to the cells from the cell surface. Using densitometry of the histochemical stainings, we found that while the increase in total cell associated HA was first evident after 24 h treatment (I, Fig. 1i), the content of the intracellular hyaluronan was almost doubled after 4h (I, Fig. 1j), and at an elevated level after just 10 min (I, Fig. 1k). As this rapid increase of HA in the intracellular compartment is unlikely due to translational of transcriptional regulation of the synthases, it supports the theory of endocytosis from the cell surface via the activation of receptors. Furthermore, the addition of hyaluronidase to the culture medium inhibited this increase in

intracellular hyaluronan (I, Fig. 1k), indicating that the intracellular hyaluronan has been exposed to the extracellular environment before entering the vesicles.

As the molecular mass of the newly synthesized hyaluronan greatly influences its physiological effects, we examined the size of the hyaluronan synthesized following KGF stimuli, using gel filtration of radiolabeled culture medium, trypsin supernatant, and cell extract. The molecular mass of the newly synthesized hyaluronan in response to KGF varied in the three compartments. In the extracellular compartment (growth medium) KGF increased hyaluronan chains in the intermediate (0.4-2×10^6) size range, whereas the newly produced intracellular hyaluronan was in small fragments of below 90 kDa in size. KGF had no marked influence on the size of the cell surface hyaluronan (I, Fig.3).

6.2. Proliferation and differentiation in growth factor treated cultures (I, II)

In organotypic cultures, KGF did not significantly change the bromodeoxyurine (BrdU) labelling, indicating that there was only a minor stimulation of cell proliferation in the organotypic cultures (I, Table III). This finding was in line with the proliferation assay from monolayer cultures (I, Table II). Morphologically, the height of the basal cells was increased, and the whole vital layer was somewhat thickened as compared to the control cultures, although not on a statistically significant level (I, Table III). KGF induced a marked reduction in the expression of early epidermal differentiation marker keratin 10, as indicated by immunostaining of organotypic cultures (I, Fig. 5a-b) as well as western blot assay (I, Fig. 5e). There was also a minor reduction in the late differentiation marker filaggrin (I, Fig. 5c-e) indicating, that although KGF retarded terminal differentiation, it didn't cause total inhibition. This was also supported my permeability studies, where a minor,but not statistically significant increase in permeability was observed (I, Fig 5f).

EGF induced a dose-dependent stimulation of proliferation in the organotypic cultures as shown by the BrdU labelling (II, Fig. I). A significant increase of the epidermal thickness was seen, mainly due to hypertrophy of the whole vital part of the epidermis (II, Fig 1A, C). In immunohistochemical stainings, EGF markedly reduced the expression of the differentiation markers keratin 10 and filaggrin. Western blot

assay confirmed the results of the immunohistochemical stainings, showing that the bands for filaggrin and keratin 10 were almost totally absent after treatment with EGF (II, Fig. 5A). However, EGF had no effect on profilaggrin as shown by western blot as well as RT-PCR for profilaggrin mRNA (II, Fig. 5A, B), indicating that EGF acts by blocking the processing of profilaggrin to its active form. As transepidermal permeability is a functional indicator of epidermal differentiation, we examined the effects of EGF on epidermal permeability in organotypic cultures by calculating the steady state flux of tritiated corticosterone through the organotypic cultures. EGF significantly increased the tracer diffusion through epidermis (II, Fig. 5C), indicating that EGF retarded the terminal differentiation of the keratinocytes.

TGF-β decreased BrdU labelling in the cultures (II, Fig. 1B), and epidermal thickness (II, Fig. 1A,C), indicating a reduction in the cell proliferation as previously reported (Nickoloff et al, 1988, Alexandrow and Moses, 1995). TGF-β had only minor effects in the expression of the two differentiation markers in immunohistochemical stainings (II, Fig. 4E-F), and no difference to control was seen in western blot assay (II, Fig. 5A). The permeability studies showed no difference in epidermal permeability after TGF-β treatment (II, Fig. 5C), confirming that TGF-β did not influence epidermal differentiation.

6.3. Migration in response to KGF (I)

KGF is a growth factor known to play an important role in wound healing. KGF changed the morphology of the monolayer REK cultures, where the cells obtained an elongated shape as well as a rounded (lift up) appearance (I, Fig. 1 a and b), typically seen in migrating cells. Electron microscopy of such cells revealed numerous microvilli on the upper cell surface (I, Fig. 1g). The migration assay performed by scraping wounds into nearly confluent cultures and following the migration of the cells to the wounded area showed a dose-dependent increase in REK migration after KGF treatment (I, Table II). Surprisingly, TGF-β also increased the migration of REK cells (Karvinen et al., unpublished results).

6.4. Hyaluronan, CD44 and versican expression in epidermal keratinocyte tumours (III)

In the normal epidermis adjacent to the tumors, the intercellular spaces of the basal, spinous, and granular cell layers are hyaluronan positive, but no staining is present in the upper granular layers or in the stratum corneum (see adjacent areas to tumors, III, Figs. 1e and 2a). In the normal dermis, a diffuse hyaluronan staining is generally found throughout the tissue, though most intensely right below the basement membrane and around the skin appendages and dermal blood vessels.

In SCC *in situ* lesions, hyaluronan formed a net-like pattern in the epidermis, localizing on the plasma membranes of the cells. However, in many of the tumors the staining was not homogenous throughout the epithelium, but instead hyaluronan negative areas were seen inside the afflicted area (III, Fig. 1a, Table 1). The staining intensity of these tumors was the highest noted among the keratinocyte tumor groups examined (III, Table 1). In the invasive SCCs, the net-like localization of hyaluronan on the plasma membranes of the cells was seen (III, Fig 1c, e). The staining intensity was mostly moderate in both the well differentiated (grade I) and less differentiated (grades II-III) tumours. However, while a majority of the well differentiated tumors showed a homogenous staining for hyaluronan, the irregularity of the staining pattern was seen in many of the less differentiated tumours, which also showed low staining intensity more often than in the well differentiated tumors (III, Table 1). No staining of the cell nuclei was found in any of the SCC samples (III, Table 1).

In BCCs, the hyaluronan localisation was strikingly different from that of the normal epidermis or SCC (III, Fig. 2 a, c, e). In almost half of the tumors, the typical plasma membrane staining for hyaluronan was almost totally absent - and instead staining of the nuclei of the cancer cells was seen (III, Fig 2 c,e, Table 2). When hyaluronan was digested from the cells using *Streptomyces* hyaluronidase, a known treatment to examine the genuinity of hyaluronan staining, the nuclear staining was totally abolished (III, Fig 2f). This was also the case when the probe was pre-treated with oligosaccharides, confirming the specificity of the staining (data not shown). In the BCCs, the majority of the tumors showed an irregular staining pattern for hyaluronan (III, Table 1).

The staining for hyaluronan in the stromal tissue adjacent to the tumors showed a staining comparable to that of the normal tissue in most of the SCCs. The staining intensity was moderate in most of the cases, but a higher staining intensity was found in 4 of the cases examined. In the BCCs the stromal staining was found to be low in 20%, moderate in 75% and high in 5% of the cases, showing a statistically significant difference to the staining pattern of the SCCs.

In the normal epidermis, CD44 staining resides on the plasma membranes of the basal and spinous cell layers, but not in the granular of cornified cell layers. In ISCs, the localisation and staining intensity for CD44 resembled that of the normal epidermis in most cases (III, Fig. 1b). However, the staining was often irregular, leaving CD44-negative areas, thus resembling the staining pattern for hyaluronan in these tumours (III, Table 2). In the well differentiated SCCs, the staining pattern for CD44 was mostly homogenous and of moderate intensity (III, Fig d; Table 2). This was comparable to the CD44 pattern found in normal epidermis. However, in the poorly differentiated tumors, CD44 expression was often reduced in large areas of the tumors, and showed a low staining intensity (III, Fig. 1f; Table 2). In the BCCs, CD44 signal was found to be weak, and only present in small areas of the tumor, being irregular in 96% of the tumors (III, Fig. 2b,d ; Table 2).

The staining of the tumors for the hyaluronan-binding proteoglycan versican was examined in a randomly selected fraction of the tumor samples. In normal dermis, some positive staining for versican was found around the hair follicles and dermal blood vessels as well as in the loose connective tissue. In seven of the 25 BCCs, the peritumoral stroma was positive for versican, while none of the ISC or SCC samples showed tumor-related versican staining (III, Fig. 2i).

6.5. The expression of MMP -7 and MMP-9 in epidermal keratinocyte tumors (IV)

The normal epidermis around the tumors showed a diffuse, weak staining for both MMP-7 and MMP-9 in the vital cell layers, but no staining was found in the stratum corneum. The staining was seen as granules in the cells and not as the pericellular net-like staining found in HA and CD44 stainings. In the dermis, the dermal fibroblasts were positive for MMP-9, but no staining of the loose connective tissue was

seen. Some of the skin appendages as well as dermal blood vessels, however, showed a strong and homogenous staining for both of the MMPs examined.

BCCs showed a weak but specific staining for MMP-7, homogenously spread throughout the tumor (IV: Table 1, Fig. 2). The stroma adjacent to the tumors was negative, unless infiltrated with inflammatory cells, which were strongly positive for MMP-7. The in situ and invasive SCCs also showed a weak staining intensity for MMP-7, although more often than in BCC the staining was of moderate intensity (IV, Table 1). Many SCCs, as well as BCCs showed infiltration of MMP-7 positive inflammatory cells in the stroma adjacent to the tumors. Interestingly, solar elastosis due to intensive UV damage was seen in a majority of the samples, and was strongly positive for MMP-7.

Most of the BCCs investigated showed a moderate staining intensity for MMP-9 (IV, Table 2). The staining was in most cases present throughout the tumor and classified homogenous. In almost all of the BCCs, stromal staining for MMP-9 was seen, mostly due to the accumulation of inflammatory cells in the stroma adjacent to the tumors. In the *in situ-* and invasive SCCs, the staining was also homogenous, and of moderate staining intensity (IV, Table 2).

6.6. Correlations between hyaluronan, CD44 and matrix metalloproteinases in epidermal keratinocyte tumors (IV)

Statistical analysis of the MMP-7, MMP-9, hyaluronan and CD44 stainings was performed to find out any possible correlations between the expressions of these molecules. Analysis performed by the χ^2-test showed an inverse correlation between MMP-7 and CD44 intensities in both BCC (p=0.022) and SCC (p=0.002). Furthermore, in SCC, increased expression of MMP-7 correlated with irregular and diminished CD44 expression. The expression difference of MMP-7 in the two tumor types (BCC versus SCC) was also significant (p=0.025). No correlation was however found between MMP-7 and hyaluronan, or MMP-9 and CD44 or hyaluronan.

7 DISCUSSION

Epidermis of the skin is a versatile, rapidly renewing tissue that is under strict growth regulation via activity of several growth factors and other mediators. The epidermis is divided into four different layers that all have distinct characteristics. The basal cell layer consists of the proliferative pool of keratinocytes. From the basal cell layer the keratinocytes migrate to the spinous cell layer and begin to express differentiation related molecules, like keratins (1 and 10), involucrin, filaggrin and loricrin (Freinkel and Woodley, 2001). These molecules are expressed by the cells in a distinct order and they control the process of differentiation. The expression of differentiation markers is controlled by receptor mediated signals from growth factors and thereby tightly regulated. Above the spinous cell layer lie the granular cell layers, where the cells undergo the process of keratinisation, losing water and dissolving their intracellular organelles due to activity of several enzymes. The end product is a non-viable cell called corneocyte, which forms the stratum corneum – the outermost part of epidermis that due to its lamellar structure and content of non-polar lipids provides the major barrier of permeation of chemical and biological substances from the environment, as well as water loss from the body. The average life-cycle of a keratinocyte is about 30 days, but when the regulatory mechanisms fail, as in hyperproliferative skin diseases like psoriasis, it can be as short as 3 days. Another typical example of failure in mechanisms that control cell proliferation and behaviour is cancer. As the skin is constantly under attack from external carcinogenic influences such as solar ultraviolet radiation, it is no wonder that skin cancer is the most common cancer in humans. As we more and more value a tanned appearance, the incidence of skin cancer is rapidly escalating.

Hyaluronan is a glycosaminoglycan that forms the main part of the ECM of the epidermis. Hyaluronan is present in all the vital cell layers of the epidermis, but not in the stratum corneum. Interestingly, during embryogenesis, the hyaluronan content in skin is higher than that of adult skin (Ågren et al, 1997), suggesting that hyaluronan plays a role in conditions, where rapid proliferation and migration of cells is required. Hyaluronan plays a key role in wound healing, as well as the normal growth and

differentiation of the epidermis (Tammi and Tammi, 1991, Tammi et al, 2005, Oksala et al, 1995), events which are influenced by the activity of growth factors and other mediators (Pienimäki et al, 2001, Tammi et al, 1985). Also UVB-radiation, a key etiological factor of epidermal keratinocyte cancers, has been shown to increase hyaluronan synthesis in the epidermis (Averbeck et al, 2007). An increase in the amount of hyaluronan has been shown in several cancers, and linked to poor disease outcome (Setälä et al, 1999, Anttila et al, 2000, Auvinen et al, 2000, Ropponen et al, 1998).

The aim of this thesis was to examine the correlation of hyaluronan synthesis with growth and differentiation of keratinocytes in normal cells, as well as in cancers of epidermal keratinocyte origin. To examine the behaviour of normal rat keratinocytes, both a monolayer culture consisting of proliferating keratinocytes, as well as an organotypic skin culture model was used. Using an organotypic model provides the advantage to observe not only the proliferation of keratinocytes, but also their differentiation and the functionally important permeability barrier formation. This enables examination of how mediators, such as growth factors, influence all these physiological processes and how they correlate to hyaluronan biosynthesis, using several biochemical methods. To examine the synthesis of hyaluronan in response to growth factors, metabolic labeling, HA-disaccharide electrophoresis and HA-ELSA were used and all have given similar results. Metabolic labeling facilitates assays of short time periods and determination of the content of newly synthesized hyaluronan not only in extracellular, but also in pericellular and intracellular compartments - something which the sensitivity of the other used methods do not allow. Also the measurement of the amount of hyaluronan by metabolic labeling is based on the assumption that hyaluronan and chondroitin sulphates use the same UDP-hexosamine pool, as the amount of hyaluronan is calculated by correlating the amounts of incorporated glucosamine and sulphate in chondroitin sulphate. The assumption was correct, since HA-electrophoresis provided similar results, indicating that the dual labeling method is trustworthy. In addition to biochemical methods, hyaluronan content in the cells was examined by staining with a biotinylated hyaluronan binding probe. The results from the histochemical stainings supported those of the biochemical methods, showing that the stainings also are able to give good qualitative and at least semi-

quantitative data of the amount of hyaluronan in skin, in addition to providing information on localisation. This method was then also used to evaluate the paraffin embedded human tumor samples, for which the other assays are not applicable. However, research done on hyaluronan in ovarian cancer samples using both staining and a biochemical methods gave similar results from both methods (Hiltunen et al, 2002), a further support to the idea, that staining is a valid method in studying hyaluronan in cancer samples.

7.1. Growth factor effects on the regulation of hyaluronan synthesis in monolayer and organotypic cultures

One of the main aims of this study was to examine the effects of EGF, KGF and TGF-β on the synthesis of hyaluronan in organotypic and monolayer rat keratinocyte cultures.

The results showed that KGF greatly enhances hyaluronan synthesis in both monolayer and organotypic cultures (I). This increased synthesis is associated with an increased expression of Has2 and Has3 in the organotypic cultures, while in monolayer cultures only Has2 expression was increased in comparison to control cultures. EGF greatly enhances the biosynthesis of hyaluronan in monolayer keratinocyte cultures, primarily via enhanced expression of Has2 (Pienimäki et al, 2001). The results from organotypic cultures show a similar increase in hyaluronan synthesis in response to EGF treatment, due to upregulation of Has2 and Has3 (II). Furthermore, TGF-β inhibited the synthesis of hyaluronan, simultaneously with a downregulation of Has2 and Has3 expression (II). This correlates with previous findings from human keratinocytes, where TGF-β was found to suppress Has3 expression (Sayo et al, 2002). The results indicate that the Has2 gene is an important target of growth factor regulation irrespective of the differentiation state of the keratinocytes, while the regulation of the Has3 gene is modified by the cellular interactions or the state of cell differentiation. The results indicate that the different growth factors induce their effects on hyaluronan through transcriptional regulation, as significant changes are seen in the mRNA of hyaluronan synthases following growth factor treatment. The rat keratinocytes are known to express all three Has isoforms, although the level for Has1 is much lower than

that of Has2 and Has3 (Pienimäki et al, 2001). Previously, contradictory results were obtained from keratinocytes of different species, as human keratinocytes were reported only to express Has1 and Has3 (Sayo et al, 2002) and mouse keratinocytes Has1 and Has2 (Sugiyama et al, 1998). More recently, however, using quantitative PCR, Saavalainen et al showed that human keratinocytes do express all three isoforms, and that the level of Has1 is lower than Has2 and 3, as in rat keratinocytes (Saavalainen et al, 2005).

The fact that KGF increases hyaluronan synthesis independently of the differentiation state of the keratinocytes or the cellular environment, suggests that hyaluronan synthesis is an important target of KGF. This hypothesis is also supported by the way KGF rapidly up-regulates Has2 mRNA and this elevated level is maintained following KGF administration (I). Not much other data is published on KGF effects on hyaluronan synthesis, but recently Jameson et al reported, that skin γδ-T-cells produce KGF, which induces keratinocyte hyaluronan production during wound repair (Jameson et al, 2005). Other fibroblast growth factor family members, however, have been reported to influence hyaluronan synthesis. Basic fibroblast growth factor (FGF-2) has been found to stimulate hyaluronan synthesis and pericellular hyaluronan coat formation in mesodermal chick embryo limb cells (Munaim et al, 1991), as well as dose-dependently stimulate hyaluronan synthesis and the expression of Has1 and Has2 in human periodontal ligament cells (Shimabukuro et al, 2005a). Similar rapid up-regulation of Has2 has been reported with EGF on both REK and human keratinocytes (Pienimäki et al, 2001, Saavalainen et al, 2005). These findings suggest that hyaluronan synthesis is generally regulated in the transcriptional level.

Several previous studies have reported that increased pericellular and intracellular hyaluronan is seen in proliferating cells (Brecht et al, 1986, Evanko and Wight, 1999, Tammi and Tammi, 1991). Interestingly, a significant increase of hyaluronan following KGF treatment of monolayer cultures occurred in the intracellular, rather than pericellular compartment (I). The pericellular pool of hyaluronan is assumed to contain the molecules that are under synthesis, as well as those associated to cell-surface receptors (Tammi et al, 1998). A plausible explanation

to the relatively low pericellular hyaluronan content is reduction of the receptor-bound pool, possibly due to a more rapid uptake of hyaluronan into the cells by endocytosis.

7.2. Growth factor effects on CD44 expression

In organotypic cultures, an increase in extracellular hyaluronan correlated with elevated intracellular hyaluronan, as well as increased expression of CD44. In fact, in KGF (I) and EGF (II) treated cultures, the CD44 mRNA was upregulated in a pattern similar to that of the hyaluronan synthases Has2 and Has3. In TGF-β treated cultures a down-regulation of mRNA for both CD44 and the hyaluronan synthases was seen (II). Previously, EGF has been shown to increase CD44 expression and enhance invasion of astrocytoma cells *in vitro* (Monaghan et al, 2000).

The correlation between increased CD44 expression and elevated intracellular hyaluronan suggests, that hyaluronan was internalized via CD44, probably for degradation, a pathway suggested by Tammi et al. (2001). Interestingly, endocytosis also generally enhances cell migration, and in the experiments with KGF (I), increased intracellular hyaluronan was associated with increased migration. In monolayer keratinocyte cultures, EGF induces a similar increase in intracellular hyaluronan (Pienimäki et al, 2001), a finding reproduced with the present organotypic cultures (II). Both KGF and EGF promote endocytosis (Pol et al, 2000, Cardinali et al, 2005), supporting the idea that the rapid increase in intracellular hyaluronan following KGF and EGF administration occurs via endocytosis.

7.3. Hyaluronan synthesis in correlation to keratinocyte proliferation and epidermal thickness

This study shows that enhanced hyaluronan synthesis due to increased expression and activity of Has2 is associated with an increase in epidermal thickness, and increased proliferation rate of the cells (I, II). Furthermore, a decline in hyaluronan synthesis, as seen in response to TGF-β, is seen simultaneously with decreased proliferation and epidermal atrophy (II). These findings correlate with earlier findings with other mediators like hydrocortisone, which inhibits hyaluronan synthesis, simultaneously decreasing keratinocyte proliferation and epidermal thickness (Ågren et

al, 1995). It is also in line with the fact that glucocorticoids are powerful down-regulators of Has2 mRNA (Jacobson et al, 2000, Zhang et al, 2000). When hyaluronan synthesis is inhibited by the specific inhibitor 4-methylumbelliferone, a decline in keratinocyte proliferation is seen (Rilla et al, 2004). Furthermore, reduced proliferation and hyaluronan synthesis is seen when Has2, which we found to be the primary growth factor responding hyaluronan synthase, is inhibited by antisense mRNA (Rilla et al, 2002). On the other hand, retinoic acid, which stimulates hyaluronan synthesis, also increases epidermal thickness by increasing the number of spinous cell layers (Tammi et al, 1985). Thus hyaluronan synthesis rate correlates directly with keratinocyte proliferation and the thickness of the vital layers of the epidermis. This is further supported by *in vitro* and clinical studies, where treatment with intermediate size hyaluronan fragments (HAFi: 50-400kDa), induced increased keratinocyte proliferation and significant thickening of epidermis in mice, as well as patients with skin atrophy (Kaya et al, 2006). It has been suggested that stimulation of cell proliferation by hyaluronan occurs through changes in signalling via its cell-surface receptor CD44 (Turley et al, 2002). This is supported by studies with CD44 knockout mice, showing that CD44 deficiency associates with reduced hyaluronan staining in their epidermis, which also is thinner than that of wild-type mice, and has a defective permeability barrier (Bourguignon et al, 2006). Also, the positive effect of intermediate size hyaluronan fragments on skin atrophy was not seen in CD44 knockout mice, indicating that hyaluronan interaction with its receptor is required (Kaya et al, 2006).

7.4. Hyaluronan and keratinocyte migration

The results indicate that KGF, a growth factor known to be important for healing of epidermal wounds, significantly increases keratinocyte migration, simultaneously with increasing the synthesis of hyaluronan via Has2 (I). This suggests that increased hyaluronan synthesis plays a central role in KGF-mediated migration. Similar correlation has previously been found following EGF treatment of keratinocytes (Pienimäki et al, 2001). There is also strong evidence that cell motility is controlled by the Has genes. Studies with antisense Has2 transfection have shown, that the lack of this synthase activity cripples the capability of the keratinocytes to migrate, indicating that

increased hyaluronan biosynthesis via Has2, rather than abundance of pericellular HA, is needed for keratinocyte migration (Rilla et al, 2002). Interestingly, endocytosis also generally enhances cell migration (Nabi, 1999), and in the experiments with KGF (I), increased intracellular hyaluronan was associated with increased migration.

A connection between hyaluronan and cell migration has been observed during healing of epidermal wounds (Tammi et al, 2005, Mack et al, 2003). In wounded mouse epidermis, the amount of hyaluronan is elevated due to upregulation of Has2 and Has3 mRNA (Tammi et al, 2005). Also, HOXB13 knockout mice, which show enhanced wound healing, have elevated epidermal and dermal hyaluronan content (Mack et al, 2003). The connection between increased epidermal hyaluronan and enhanced wound healing could be explained by the fact that hyaluronan provides a loose extracellular matrix that allows cells to detach and migrate. On the other hand, as shown with breast tumor cells, hyaluronan can have a more direct role in stimulating migration via signalling through its cell surface receptor CD44 (Bourguignon et al, 2000).

7.5. Growth factor effects on keratinocyte differentiation

The majority of KGF receptors lie in the spinous cell layer of epidermis. This explains why the most sensitive cellular response for KGF in the organotypic cultures was seen in the spinous cell layer, which showed an intense signal for hyaluronan. KGF-treated cultures also showed a lower expression of the early differentiation marker keratin 10, whereas the expression of the late differentiation marker filaggrin was less affected. Furthermore, KGF had no significant effect on the diffusion barrier. These results closely correspond to those made by Andreadis et al (Andreadis et al, 2001) with a human keratinocyte organotypic culture, thus confirming the general validity of the culture model. Some authors, however, have reported that KGF promotes the expression of keratin 10 and filaggrin in keratinocytes, when differentiation is induced by high Ca^{2+} concentrations (Marchese et al, 1990, Marchese et al, 2001). Other authors have reported, that hyaluronan synthesis is decreased, when differentiation is induced by high Ca^{2+} concentrations (Lamberg et al, 1986). Thus the effects of KGF on differentiation can vary due to external environment, but in general there seems to be an inverse

correlation between hyaluronan synthesis and induction of keratinocyte terminal differentiation.

The treatment of organotypic cultures with EGF induces a similar inverse correlation between hyaluronan content in the spinous cell layer and the indicators of epidermal differentiation as was seen with KGF (II). However, unlike KGF, EGF also inhibited the maturation of the late differentiation marker filaggrin, and compromised the function of the permeability barrier (II). This inhibition of differentiation markers is in agreement with previous studies (Marchese et al, 2001). The correlation with stimulation of hyaluronan biosynthesis and inhibition of epidermal differentiation, has been reported previously with vitamin A (Tammi et al, 1989). Conversely, it has been reported that pharmacological concentrations of hydrocortisone enhance differentiation while inhibiting hyaluronan synthesis (Ågren et al, 1995). The tight correlation between the status of epidermal differentiation and the synthesis of hyaluronan in the spinous cells may indicate that hyaluronan has a direct, inhibitory impact on keratinocyte terminal differentiation. This is further supported by the findings by Passi et al, who treated organotypic keratinocyte cultures with *Streptomyces* hyaluronidase to remove intercellular hyaluronan. These cultures showed significantly increased expression of both early and late differentiation markers in comparison to control cultures (Passi et al, 2004). Also the interaction of hyaluronan with its receptor CD44 has been suggested to be important in the regulation of epidermal differentiation. In CD44 knockout mice, the epidermis is thinner and the hyaluronan staining reduced, simultaneously with reduced expression of differentiation markers and reduced barrier formation (Bourguignon et al, 2006).

TGF-β, which decreased epidermal thickness without affecting its hyaluronan content, had no effect on the expression of the differentiation markers or epidermal permeability. This is in agreement with previous results with normal human keratinocytes, where TGF-β induces reversible growth arrest in the cells but is unable to induce squamous cell differentiation (George et al, 1990, Saunders and Jetten, 1994).

7.6. Hyaluronan and CD44 expression in non-melanoma skin cancer

This study is to our knowledge the first one describing the exact localisation, staining intensity and distribution of hyaluronan and its receptor CD44 in NMSC. It shows that although both BCC and SCC originate from epidermal keratinocytes, their staining for hyaluronan and CD44 is quite different.

During epidermal development, hyaluronan content is lower in the proliferating basal cell layer compared to the spinous cell layers housing the differentiating keratinocytes (Ågren et al, 1997). This is congruent with the findings in epidermal malignancies, where a lower general hyaluronan staining was found in the BCCs presumably arising from the proliferating basal cells, than in well-differentiated SCCs. Basal cell carcinomas showed an irregular staining pattern for hyaluronan, associated with the loss of CD44 – a situation very rare in stratified epithelia and their carcinomas (III). A similar pattern, however, is seen in the embryonic hair follicle bulge, an area where the follicular stem cells are located (Ågren et al, 1997, Tuhkanen et al, 1999). Interestingly, some authors have suggested, that BCC arises from the undifferentiated cells of the hair follicle bulge (Weinstock, 1994, Kruger et al, 1999). However, the other possible origin of this tumor – the basal cell layer of epidermis – also shows a reduced CD44 expression compared to that of the spinous cell layers (Tuhkanen et al, 1998). Thus the hyaluronan and CD44 expression patterns of the BCCs may reflect the tumors origin in the population of the undifferentiated cells.

The other distinct feature found was the nuclear HA staining pattern, found in about half of the BCCs examined (III). This finding is exceptional, though similar staining has been occasionally seen in breast (Auvinen et al, 1997) and colon (Ropponen et al, 1998) cancers. The importance of nuclear hyaluronan staining is not clear, but it has been suggested that intracellular hyaluronan may participate in cell signaling (Evanko and Wight, 1999). Further studies are needed to examine whether nuclear staining in BCC is of clinical significance, as we did not see possible correlations to different forms of BCC.

Increased stromal expression of the hyaluronan-binding proteoglycan versican in a subset of the tumors was found in BCC, but not SCC (III). Also the expression of small, collagen-binding proteoglycans decorin and biglycan has been found to be

increased in BCC (Hunzelmann et al, 1995). The increase of peritumoral versican may reflect the general activation of connective tissue seen in BCC (Miller, 1991), and may be an important factor in the growth of BCC.

The hyaluronan staining intensity was high in *in situ* and well differentiated SCCs, but decreased again in the poorly differentiated SCC (III). The low expression of the hyaluronan receptor CD44 correlated to that of hyaluronan in BCC and well-differentiated SCC, but was almost lacking from the poorly differentiated tumors (III). The initial phase of transformation to SCC (ISC and well-differentiated SCC) seems to involve a high expression of cell-surface hyaluronan (III). This is consistent with the findings in *in situ* oesophageal carcinoma (Wang et al, 1996), well differentiated SCCs of the larynx (Hirvikoski et al, 1999) and lung (Pirinen et al, 1998). The high hyaluronan content may give the transformed cells a growth advantage, as hyaluronan has been found to support mitosis (Brecht et al, 1986), and cell migration (Tammi et al, 2002) and protect from apoptosis (Yasuda et al, 2001). These events can be mediated by signaling through CD44 (Bourguignon et al, 2001, Yasuda et al, 2001, Bourguignon et al, 2007). Also the depletion of hyaluronan noted in poorly differentiated tumors, is consistent with the findings in other tumors of squamous cell origin such as those of the oesophagus (Wang et al, 1996), lung (Pirinen et al, 1998) and larynx (Hirvikoski et al, 1999). Furthermore, a study on several solid malignancies showed, that hyaluronan is considerably up-regulated in well-differentiated tumors irrespective of tumor origin, while it is down-regulated in cells of poorly differentiated tumors (Boregowda et al, 2006).

In SCCs, the loss of hyaluronan has been found to be accompanied by the loss of its cell surface receptor CD44 (Hirvikoski et al, 1999, Pirinen et al, 2000) which is consistent with the findings in this thesis (III). The expression of CD44v6 has been shown to correlate with depth of invasion both in BCC and cutaneous SCC (Son et al, 2008). In this thesis, we did not specifically examine the expression of variant forms of CD44.

It is not known at present, whether the changes in hyaluronan content in the well- and poorly differentiated tumors can be due to changes in the synthesis of hyaluronan or its metabolism. In *in vitro* experiments with epithelial cells and

fibroblasts, oncogenic Ras-transformation increased hyaluronan synthesis due to increased Has2 expression (Itano et al, 2004). The increase of hyaluronan in the well-differentiated tumors could be due to Has activation induced by for example growth factor signaling, as EGFR has been found to be overexpressed in SCC of the skin, and correlates to enhanced migration (McCawley et al, 1997, Shimizu et al, 2001). On the other hand, in some cancers such as those arising from the bladder (Pham et al, 1997) and prostate (Lokeshwar et al, 2001), a high expression of the hyaluronan degrading enzyme Hyal1 is found. Besides high Hyal1 expression, these tumors also have high levels of low molecular weight hyaluronan, which is thought to induce angiogenesis and malignant behaviour, and the levels are higher in more aggressive tumors (Pham et al, 1997, Lokeshwar et al, 1997).

As HA and CD44 may also support cell adhesion and aggregation (Milstone et al, 1994), their loss in the poorly differentiated squamocellular carcinomas may be a part of a general dysadhesive process in the cancers. Possible mechanisms involved in the reduction of HA and CD44 include reduced expression or increased degradation. Enhanced shedding of CD44 due to the activation of MMPs, capable of degrading CD44 has been shown to play a role in tumor cell migration (Okamoto et al, 1999).Especially interaction between MT1-MMP and CD44 in tumor cells has been shown to promote cell invasion (Marrero-Diaz et al, 2009).This is one possible mechanism in the reduction in HA and CD44 levels, found in the poorly differentiated SCCs, and therefore the expression of MMP-7 and MMP-9 was examined in the current study to reveal possible correlations.

7.7. Correlation of hyaluronan and CD44 expression to MMP-7 and MMP-9

Positive staining for MMP-7 was found in BCC, ISC and invasive SCC (IV). Although mostly weak, there was a generally higher staining intensity in SCC than BCC, in line with previous results (Kerkelä and Saarialho-Kere, 2003, Karelina et al, 1994). An inverse correlation was found between the staining intensities of CD44 and MMP-7 and higher MMP-7 levels were correlated to irregular CD44 staining pattern (IV). MMP-7 is a broad spectrum metalloproteinase which can degrade various ECM components, but also cell surface proteins like the adhesion molecule E-cadhedrin

(McGuire et al, 2003). While there is no information in the literature whether MMP-7 also can degrade CD44, the inverse correlation in our results would suggest that. Previous research with uterine epithelium has shown that CD44 recruits the proteolytically active MMP-7 via binding to the heparan sulphate side chains of CD44 and leads to destruction of the basement membrane (Yu et al, 2002). As the major epidermal form for CD44 is a proteoglycan substituted with heparan sulphate side chains (Tuhkanen et al, 1997), the epidermal cells are probably capable of binding the activated MMP-7. Interestingly, UVB, which is the major risk factor for skin cancers, is shown to induce MMP-7 expression in keratinocytes (Skiba et al, 2005), as well as cause rapid down-modulation of CD44 immunostaining (Calikoglu et al, 2006). Increased levels of MMP-7 have been found in oesophageal cancers (Tanioka et al, 2003), head and neck SCC (Weber et al, 2007) as well as SCC of the skin (Kerkelä and Saarialho-Kere, 2003, Kivisaari et al, 2008), supporting the findings of this thesis where we found a tendency towards higher staining intensity in SCC in relation to BCC. In keratoacanthomas, which are benign skin tumors that histologically can resemble well differentiated SCC, the staining for MMP-7 is low or absent (Kuivanen et al, 2006). In chronic wounds, MMP-7 is absent from the wound epithelium, but induced if malignant transformation to SCC occurs in the wound (Impola et al, 2005). These clear indications of lacking or low expression in benign cutaneous changes and increase in malignant lesions underline the role of MMP-7 in malignancy and even suggest possible use of MMP-7 as a marker of malignant transformation. In this study as well as earlier reports (Tanioka et al, 2003, Tanioka et al, 2003, Leinonen et al, 2006), MMP-7 stainings were performed using an antibody against the pro-MMP7 protein. This does not represent the total MMP-7 activity and may affect the results.

Our data indicate that MMP-9 expression is higher in SCC than in BCC (IV), and is in line with previous results (Kerkelä and Saarialho-Kere, 2003). We could, however, not show any association between CD44 and MMP-9 expressions, which has previously been found in breast cancer and malignant melanoma (Yu and Stamenkovic, 1999). As the antibody used in this study detects both active and pro-MMP-9, it may influence the result, although tissue and cell type differences also may exist. However, stromal, rather than tumor cell-associated MMP-9 may relate to the level of CD44 in

cancer (Kosunen et al, 2007), and expression of MMP-9 by inflammatory cells and mast cells but not the actual tumor cells has previously been linked to epidermal carcinogenesis (Coussens et al, 2000).

The loss of hyaluronan in advanced SCC did not correlate to MMP-7 or MMP-9 expression, although it closely correlates to CD44 (III, IV). This suggests that hyaluronan and MMPs are not directly related. The loss of hyaluronan in SCC may be caused by the reduced CD44 expression, but also other factors such as reduced expression of the hyaluronan synthases or increased expression of hyaluronidases, and further studies are needed to shed light on this subject.

8   SUMMARY AND FUTURE DIRECTIONS

The main findings of this thesis are:

- Mitogenic growth factors EGF and KGF increase hyaluronan synthesis by increasing the expression of Has2 and Has3. This increased synthesis correlates with increased keratinocyte proliferation and migration, a decrease in keratinocyte differentiation, and an increase in epidermal thickness.

- The anti-proliferative growth factor TGF-β1 decreases epidermal proliferation in organotypic cultures, inducing epidermal atrophy. Simultaneously, a decrease in hyaluronan synthesis, Has2 and Has3 mRNA expression is seen, further underlining the link between hyaluronan synthesis and cell proliferation.

- The content of hyaluronan and its receptor CD44 in epidermal keratinocyte tumors varies between BCC and SCC, indicating the different origin of the tumors from non-differentiated basal cells and differentiating squamous cells, respectively.

- The expression of MMP-7 is stronger in SCC than BCC, and increased expression of MMP7 in both types of tumors correlates with a decrease in CD44 expression

To summarize, the results support the existing hypothesis that hyaluronan synthesis by epidermal keratinocytes is directly correlated to keratinocyte migratory phenotype and increased epidermal thickness, and inversely with keratinocyte differentiation. Thus, hyaluronan is important for keratinocyte proliferation and migration, especially in situations like wound repair where rapid keratinocyte response is needed to cover the wound space. In keratinocyte tumors, hyaluronan staining intensity correlates to tumor origin and in SCC with tumor aggressiveness, and is also closely linked to the staining intensity of its receptor CD44. Furthermore, CD44 expression is inversely correlated to that of MMP-7, a metalloproteinase which is associated with malignant transformation of keratinocytes. As future perspectives, further research is needed to investigate, why hyaluronan and CD44 become absent in aggressive forms of SCC. Is this due to direct interactions with MMPs, such as MMP-7, or due to other mechanisms such as increased expression of hyaluronidases.

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APPENDIX: ORIGINAL PUBLICATIONS I-IV

Kuopio University Publications D. Medical Sciences

D 427. Saarelainen, Soili. Immune Response to Lipocalin Allergens: IgE and T-cell Cross-Reactivity.
2008. 127 p. Acad. Diss.

D 428. Mager, Ursula. The role of ghrelin in obesity and insulin resistance.
2008. 123 p. Acad. Diss.

D 429. Loisa, Pekka. Anti-inflammatory response in severe sepsis and septic shock.
2008. 108 p. Acad. Diss.

D 430. Joukainen, Antti. New bioabsorbable implants for the fixation of metaphyseal bone : an experimental and clinical study.
2008. 98 p. Acad. Diss.

D 431. Nykänen, Irma. Sepelvaltimotaudin prevention kehitys Suomessa vuosina 1996-2005.
2008. 158 p. Acad. Diss.

D 432. Savonen, Kai. Heart rate response to exercise in the prediction of mortality and myocardial infatction: a prospective population study in men.
2008. 165 p. Acad. Diss.

D 433. Komulainen, Pirjo. The association of vascular and neuroprotective status indicators with cognitive functioning: population-based studies.
2008. Acad. Diss.

D 434. Hassinen, Maija. Predictors and consequences of the metabolic syndrome: population-based studies in aging men and women.
2008. Acad. Diss.

D 435. Saltevo, Juha. Low-grade inflammation and adiponectin in the metabolic syndrome.
2008. 109 p. Acad. Diss.

D 436. Ervasti, Mari. Evaluation of Iron Status Using Methods Based on the Features of Red Blood Cells and Reticulocytes.
2008. 104 p. Acad. Diss.

D 437. Muukka, Eija. Luomun tie päiväkotiin: luomuruokailun toteutettavuus ja ravitsemuksellinen merkitys päiväkotilapsille.
2008. 168 p. Acad. Diss.

D 438. Sörensen, Lars. Work ability and health-related quality of life in middle-aged men: the role of physical activity and fitness.
2008. 83 p. Acad. Diss.

D 439. Maaranen, Päivi. Dissociation in the finnish general population.
2008. 97 p. Acad. Diss.

D 440. Hyvönen, Juha. Suomen psykiatrinen hoitojärjestelmä 1990-luvulla historian jatkumon näkökulmasta. 2008. 279 p. Acad. Diss.

D 441. Mäkinen, Heidi. Disease activity and remission in rheumatoid arthritis: comparison of available disease activity measures and development of a novel disease sctivity indes: the mean overall index for rheumatoid arthritis (MOI-RA).
2008. 129 p. Acad. Diss.

D 442. Kousa, Anne. The regional association of the hardness in well waters and the incidence of acute myocardial infarction in rural Finland.
2008. 92 p. Acad. Diss.