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Hanna Siiskonen

Hyaluronan and hyaluronan synthases

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HANNA SIISKONEN

Hyaluronan and hyaluronan synthases

Studies on their subcellular localization and processing in cell culture models and on hyaluronan metabolism in UV-induced cutaneous tumors

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ABSTRACT

Hyaluronan is a large, hydrophilic glycosaminoglycan consisting of repeating units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA). It is synthesized by hyaluronan synthases (HAS1-3), plasma membrane-inserted enzymes which extrude the growing hyaluronan chain into the extracellular matrix. Hyaluronan is abundant in vertebrates, and its content is highest in the skin, joints and vitreous of the eye. Besides its essential role in normal tissue homeostasis, hyaluronan and its receptors are involved in the tumorigenesis of several cell types.

The aim of this thesis was to study the intracellular traffic and function of the hyaluronan synthases and to investigate the possible intracellular roles of hyaluronan utilizing transient transfection of HAS fusion proteins with fluorescent tags and microinjections. In addition, the changes in hyaluronan, its plasma membrane receptor CD44, and enzymes involved in its metabolism were analyzed during carcinogenesis of cutaneous squamous cell carcinoma (SCC) and melanoma using histochemical stainings of mouse and human tissue specimens.

The results showed that the HAS protein is transported from the endoplasmic reticulum and the Golgi apparatus to the plasma membrane and it resides at the cell surface only when active hyaluronan synthesis is possible. The studies also found that HAS1 requires cytokines or a high concentration of substrates to form a pericellular hyaluronan coat, while HAS2 and HAS3 produce high levels of hyaluronan and form coats without stimulation. Microinjection of fluorescent hyaluronan binding complex (fHABC) into living cells did not reveal any specific hyaluronan-dependent binding sites in the cytosol. Microinjected hyaluronan fragments (4-120 monosaccharide units) were not enriched in any specific sites in the cytosol. Cytosolic injection of hyaluronidase did not affect hyaluronan synthesis, suggesting that the growing hyaluronan chain is translocated by the hyaluronan synthase itself and is not accessible to enzymatic degradation during the process.

In the ultraviolet light-induced tumors, squamous cell carcinoma and melanoma, the content of hyaluronan and its CD44 receptor were increased during the early phases of tumorigenesis, along with increased expression of all HASs. Instead, in advanced stage tumors the amounts of hyaluronan and CD44 are decreased with reduced levels of HASs and increased expression of the hyaluronidase HYAL2.

In conclusion, this thesis provides new information about the traffic and activity of hyaluronan synthases and intracellular hyaluronan. In addition, the results suggest that hyaluronan and CD44 are important during the onset of malignant transformation in epidermal cells.

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TIIVISTELMÄ

Hyaluronaani on suuri, vettä sitova glykosaminoglykaani, joka koostuu vuorottelevista N-asetyyliglukosamiinin (GlcNAc) ja glukuronihapon (GlcUA) muodostamista sokeriyksiköistä. Hyaluronaania tuottavat solukalvolle asettuneet hyaluronaanisyntaasit (HAS1-3), jotka työntävät kasvavan hyaluronaanimolekyylin solunulkoiseen tilaan. Hyaluronaania on löydetty monista selkärankaisista ja sen pitoisuudet ovat korkeimmat ihossa, nivelissä ja silmän lasiaisessa. Hyaluronaani on keskeinen molekyyli elimistön sisäisen tasapainon ylläpidossa, mutta se osallistuu myös monien syöpien kehittymiseen.

Tämän väitöskirjan tarkoituksena oli tutkia hyaluronaanisyntaasien solunsisäistä kuljetusta ja niiden toimintaa, sekä selvittää solunsisäisen hyaluronaanin mahdollisia tehtäviä käyttäen menetelminä fluoresoivien HAS-proteiinien yli-ilmentämistä soluissa sekä mikroinjektioita. Lisäksi tutkittiin hyaluronaanin, sen CD44-reseptorin ja hyaluronaanisyntaasien sekä hajottamiseen osallistuvien hyaluronidaasien (HYAL1-2) muutoksia ihon levyepiteelikarsinooman ja melanooman kehittymisessä analysoimalla hiiren ja ihmisen kudosnäytteiden vasta-ainevärjäyksiä.

Tulokset osoittivat, että hyaluronaanisyntaasit kuljetetaan solulimakalvostosta ja Golgin laitteesta solukalvolle ja syntaasi pysyy solukalvolla vain voidessaan tuottaa hyaluronaania. Tuloksista selvisi myös, että HAS1 tuottaa solun pinnalle hyaluronaanivaipan suuremman substraattipitoisuuden tai sytokiinien läsnä ollessa, toisin kuin HAS2 tai HAS3, jotka eivät tarvitse stimulaatiota. Solujen sisältä ei löytynyt erityisiä hyaluronaania sitovia kohteita, kun soluihin injektoitiin fluoresoivaa hyaluronaania sitovaa koetinta. Hyaluronaanifragmentit (4-120 sokeriyksikköä) eivät kertyneet mihinkään spesifiseen paikkaan soluissa. Solulimaan injektoitu hyaluronidaasi ei vaikuttanut hyaluronaanin synteesiin. Tämä osoittaa, ettei tuotettava hyaluronaanimolekyyli ole sytosolissa hajottavan entsyymin saatavilla, vaan syntaasientsyymi itse siirtää hyaluronaanin solukalvon läpi synteesin aikana.

Tutkimuksessa selvisi myös, että hyaluronaani on lisääntynyt ihon levyepteelikarsinooman ja melanooman varhaisvaiheissa ja samalla hyaluronaani-syntaasien määrä ihosoluissa on kohonnut. Sen sijaan pitkälle edenneissä kasvaimissa hyaluronaani on vähentynyt, mikä selittyy vähentyneellä hyaluronaanisyntaasien määrällä ja lisääntyneellä hyaluronidaasien määrällä.

Tämä väitöskirja antaa uutta tietoa hyaluronaanisyntaasien solunsisäisestä kuljetuksesta ja toiminnasta sekä solunsisäisestä hyaluronaanista. Tulokset osoittavat lisäksi, että hyaluronaani ja sen CD44-reseptori ovat tärkeitä ihosyövän kehittymisen alkuvaiheissa.

Yleinen Suomalainen asiasanasto: hyaluronaani; ihosyöpä; solut – aineenvaihdunta

A smooth sea never made a skillful sailor.
(English proverb)

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Kuopio, January 2013

Hanna Siiskonen

List of the original publications

This dissertation is based on the following original publications:

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Plasma membrane residence of hyaluronan synthase is coupled to its enzymatic activity.
J Biol Chem, 280(36): 31890-31897, 2005.

II Siiskonen H, Kärnä R, Hyttinen J, Tammi RH, Tammi MI, Rilla K.
HAS1 is a substrate-activated hyaluronan synthase with low basal activity.
Manuscript, 2013.

III Siiskonen H, Rilla K, Kärnä R, Bart G, Jing W, Haller MF, DeAngelis PL, Tammi RH, Tammi MI.
Hyaluronan in cytosol – microinjection-based probing of its existence and suggested functions.
Glycobiology 23(2): 222-231, 2013.

IV Siiskonen H*, Törrönen K*, Kumlin T, Rilla K, Tammi MI, Tammi RH.
Chronic UVR causes increased immunostaining of CD44 and accumulation of hyaluronan in mouse epidermis.
J Histochem Cytochem, 59(10): 908-917, 2011.

V Siiskonen H, Poukka M, Tyynelä-Korhonen K, Sironen R, Pasonen-Seppänen S.
Inverse expression of hyaluronidase 2 and hyaluronan synthases 1-3 is associated with reduced hyaluronan content in malignant cutaneous melanoma.
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* Equal contribution.
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This thesis also contains unpublished data.

Contents

Abbreviations

ABC	adenosine triphosphate-binding cassette
Akt	protein kinase B (PKB)
AP	activating protein
BFA	Brefeldin A
bFGF	basic fibroblast growth factor
bHABC	biotinylated HABC
fHABC	fluorescent HABC
cAMP	cyclic adenosine mono-phosphate
CD38	cluster of differentiation 38
CD44	cluster of differentiation 44/ hyaluronan receptor
CD147	cluster of differentiation 147/ emmprin
CD168	cluster of differentiation 168/ RHAMM
CDC37	cell division cycle 37
CHO	Chinese hamster ovary cell line
CRSBP-1	cell surface retention sequence binding protein-1
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
emmprin	extracellular matrix metalloproteinase inducer
EMT	epithelial to mesenchymal transition
ERK	extracellular signal-regulated kinase
GAG	glycosaminoglycan
GFP	green fluorescent protein
GlcNAc	N-acetylglucosamine
GlcUA	glucuronic acid
HA	hyaluronan
HABC	hyaluronan binding complex of the cartilage aggrecan G1 domain and link protein
HABP	hyaluronan binding protein
HaCat	a human keratinocyte cell line
HARE	hyaluronan receptor for endocytosis
HAS	hyaluronan synthase protein
HAS/*Has*	hyaluronan synthase gene, human/animal
HC	heavy chain
HGF	hepatocyte growth factor
HYAL	hyaluronidase
IαI	inter-alpha-inhibitor

IFN	interferon
IGF	insulin-like growth factor
IHABP	intracellular HABP
IL-1β	interleukin 1 beta
KGF	keratinocyte growth factor
LYVE-1	lymph vessel endothelium receptor 1
MAPK	mitogen-activated protein kinase
MβCD	methyl-beta-cyclodextrin
MCF-7	a human breast adeno-carcinoma cell line
MD	membrane domain
MDR1	multridrug resistance 1, P-glycoprotein
MMP	matrix metalloproteinase
4-MU	4-methylumbelliferone
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
O-GlcNAc	O-linked-N-acetylglucosamine
PDGF	platelet derived growth factor
PDGF-BB	platelet derived growth factor, dimer of two beta polypeptides
PHYAL	hyaluronidase pseudogene
PI3K	phosphoinositide-3-kinase
PMA	phorbol-12-myristate-13 acetate
Poly I:C	polyinosinic:polycytidylic acid
PTH	parathyroid hormone
RA	retinoic acid
Ras	a small guanosine triphosphatase
RHAMM	receptor for hyaluronan-mediated motility
ROS	reactive oxygen species
SCC	squamous cell carcinoma
SP	specificity protein
SPAM1	sperm adhesion molecule-1
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor beta
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
TSG-6	tumor necrosis stimulated gene 6
UDP	uridine diphosphate
YY1	ying-yang 1

1 Introduction

Hyaluronan is a large (up to 10^7 Da), linear polysaccharide belonging to the glycosaminoglycan group of carbohydrates and is found abundantly in vertebrate tissues. Other members of the group include chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin and keratan sulfate. Unlike the other members, hyaluronan is not sulfated or covalently attached to proteins. The versatile properties of hyaluronan have been exploited in medicine and new applications in drug delivery and tissue engineering are evolving (DeAngelis 2012).

In humans, hyaluronan is synthesized by three hyaluronan synthases and the growing hyaluronan chain is extruded into the exterior of cells. At the cell surface, hyaluronan may be bound by several receptors, the most important of which is CD44. Hyaluronan is degraded by hyaluronidases. Hyaluronan is mainly located in the extracellular matrix, where it fills the intercellular spaces by binding water molecules. The biological functions of hyaluronan extend far beyond its role as a space filler in tissues, as it is involved in embryogenesis (Camenisch 2000, Tien 2005), cell differentiation (Pasonen-Seppänen 2003), wound healing (Haider 2003, Tammi 2005) and inflammation (Jiang 2007, Jiang 2011). Hyaluronan also has size-dependent functions and its oligosaccharides have been reported to participate in inflammation (Stern 2006), angiogenesis (Gao 2008b, Gao 2010) and inhibition of drug resistance (Cui 2009b) and anchorage-independent growth (Ghatak 2002). In addition to its main location extracellularly, hyaluronan has also been found inside cells (Evanko 1999b, Pienimäki 2001, Tammi 2001, Evanko 2004), but the role of intracellular hyaluronan has remained controversial.

The content of hyaluronan is altered in many cancers, suggesting an important role for hyaluronan in tumorigenesis (Toole 2005, Heldin 2008). Increased hyaluronan content often correlates with poor patient outcome, *e.g.* in adenocarcinomas of the breast (Auvinen 2000), lung (Pirinen 2001) and prostate (Lipponen 2001). On the contrary, reduced hyaluronan content is associated with poor prognosis in squamous cell carcinomas (SCC) originating in the esophagus (Wang 1996b), larynx (Hirvikoski 1999) and mouth (Kosunen 2004). Hyaluronan levels are also decreased in cutaneous SCC (Karvinen 2003a) and localized melanoma (Karjalainen 2000), but the changes in the early phases or in the hyaluronan metabolizing enzymes have not been studied earlier in these tumors.

The present thesis concentrated on the function of the hyaluronan synthases and on the role of hyaluronan and its metabolism in UV-induced cutaneous tumors. In addition to conventional methods of cell biology and transient transfections, a novel method of microinjections was utilized to investigate various aspects of intracellular hyaluronan. For the analyses of the tissue specimens, histochemical stainings were performed.

The results presented in this thesis show that hyaluronan synthases are active at the plasma membrane and manage by themselves the translocation of the hyaluronan chain across the plasma membrane during synthesis. No specific cytosolic enrichment sites for hyaluronan or its oligosaccharides were found. This thesis also shows that hyaluronan content is increased during the early phases, but reduced in the advanced stages of cutaneous tumors, along with dynamic changes in the enzymes involved in its synthesis and degradation. The results presented in this thesis provide new information about hyaluronan synthesis and hyaluronan metabolism in cutaneous tumors, facilitating future research on new targets for cancer therapies.

2 Review of the literature

2.1 STRUCTURE AND PROPERTIES OF HYALURONAN MOLECULE

Hyaluronan was first isolated in 1934 from the vitreous of the bovine eye (Meyer 1934). The chemical structure of hyaluronan was revealed 20 years later by Weissmann and co-workers and it was shown to consist of repeating disaccharide units of N-acetylglucosamine and D-glucuronic acid linked with alternating β-1,4 and β-1,3 glycosidic bonds (Weissman 1954) (Figure 1). In normal physiological conditions, hyaluronan can include 2000–25000 disaccharides resulting in a relative molecular mass of 10^6–10^7 and polymer lengths of 2–25 μm (Toole 2004). Hyaluronan belongs to the glycosaminoglycan (GAG) group of polysaccharides, but unlike other GAGs, it is not sulfated or covalently linked to proteins (Fraser 1997). The linear hyaluronan molecule is very hydrophilic and makes an expanded random coil structure in water which is stabilized by hydrogen bonds (Scott 1989). In aqueous environments hyaluronan forms visco-elastic gels (Laurent 1992). Twists in the hyaluronan chain create hydrophobic patches which permit association with other hyaluronan chains and are also involved in interactions with proteins, lipids and membranes (Scott 1992). A hyaluronan network excludes large macromolecules and slows the diffusion of substances unable to penetrate the network (Laurent 1992).

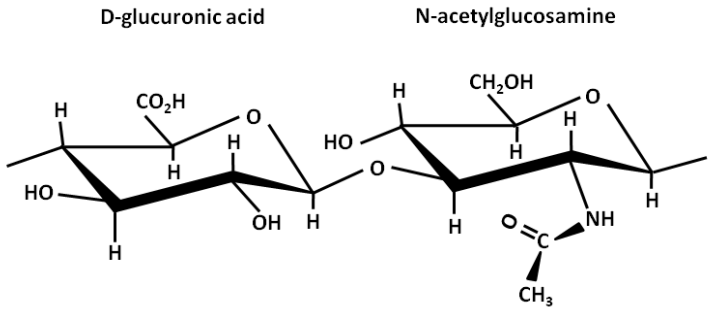

Figure 1. Repeating disaccharide unit of hyaluronan molecule consisting of D-glucuronic acid and N-acetylglucosamine, linked with alternating β-1,4 and β-1,3 glycosidic bonds. Modified from (Kultti 2009a).

2.2 SYNTHESIS OF HYALURONAN

2.2.1 Hyaluronan synthase genes

Hyaluronan is synthesized by hyaluronan synthases which are found in vertebrates, some bacteria and a virus (Weigel 2007). Mammalian cells have three distinct synthase genes, *HAS1-3*. The first hyaluronan synthase was cloned in Group A *Streptococcus pyogenes* and already then it was predicted to be an integral membrane protein (DeAngelis 1993). It was only a few years later that the eukaryotic hyaluronan synthases were found. A cDNA clone expressed in a mouse mammary carcinoma mutant cell line was shown to encode a 583-amino acid protein capable of hyaluronan synthesis (Itano 1996a). The first human *HAS* gene was isolated by two groups almost simultaneously as

Shyjan and co-workers used functional expression cloning in Chinese hamster ovary (CHO)-cells (Shyjan 1996) and Itano and Kimata screened a cDNA library of human fetal brain (Itano 1996b). Another human *HAS* (Watanabe 1996) and another murine *Has* gene (Spicer 1996) were cloned and observed to increase hyaluronan production in transfected cells. In 1997, the third mammalian *Has* was characterized (Spicer 1997a). Later, the frog protein DG42 (Differentially expressed in Gastrulation) was found to be a hyaluronan synthase although the role was unknown at its discovery in 1988 (Rosa 1988, DeAngelis 1996, Meyer 1996). Furthermore, a hyaluronan synthase has also been found in a virus infecting chlorella-like green algae (DeAngelis 1997). However, hyaluronan synthases have not been found in insects although Drosophila can produce hyaluronan when a mouse *Has2* gene is expressed in its tissues (Takeo 2004).

The three hyaluronan synthase proteins in humans are designated as HAS1, HAS2 and HAS3. They are well-conserved with highly homologous amino acid sequences, but located on separate chromosomes. *HAS1* resides in chromosome 19 at q13.3-13.4, *HAS2* is located in chromosome 8 at q24.12 and *HAS3* is in chromosome 16 at q22.1 (Spicer 1997b). *HAS1* gene has five exons, whereas *HAS2* and *HAS3* both have four (Monslow 2003). *HAS1* has been shown to have splice variants in Waldenström's macroglobulinemia (Adamia 2003), multiple myeloma (Adamia 2005) and bladder cancer (Golshani 2007). *HAS3* has two separate variant transcripts (4.9kb and 2.0kb) coding for proteins (Sayo 2002). *In silico*, the *HAS1* gene has 46 possible transcription-factor binding sites 500bp upstream of its promoter, *HAS2* has 54, *HAS3* variant 1 has 46 and variant 2 has 56, respectively (Monslow 2003).

2.2.2 Structure and function of hyaluronan synthases

Mammalian hyaluronan synthases are integral membrane proteins with 4-6 transmembrane domains in addition to 1-2 membrane-associated domains (Weigel 1997, Weigel 2007) (Figure 2). The synthase enzymes need Mg^{2+} or Mn^{2+} in addition to the sugar precursors uridine diphosphate (UDP)-glucuronic acid and UDP-N-acetylglucosamine to produce hyaluronan (Weigel 2007, Markovitz 1959) and the synthesis takes place at the inner surface of the plasma membrane (Prehm 1984). Human and mouse hyaluronan synthases add the precursor sugars to the reducing end of the growing polymer (Prehm 1983a, Asplund 1998, Prehm 2006), while *Xenopus laevis* hyaluronan synthase uses the nonreducing end (Bodevin-Authelet 2005) like the *Pasteurella multocida* hyaluronan synthase (DeAngelis 1999).

Already in 1983, it was suggested that the synthase enzyme does not require any primers for the synthesis of hyaluronan (Prehm 1983b). The adenosine triphosphate-binding cassette (ABC) transporters have been proposed to be important for hyaluronan export in fibroblasts (Schulz 2007), and a recent report suggests that this export of hyaluronan via ABC transporters, especially MRP5, depends on concurrent efflux of K^+ ions (Hagenfeld 2012). However, ABC transporters do not seem to contribute to the translocation of hyaluronan in breast cancer cells (Thomas 2010). In addition, it was demonstrated in *Streptococcus equisimilis* (*Se*) HAS reconstituted into proteoliposomes that the HAS protein produces hyaluronan in a combined process of synthesis and membrane translocation (Hubbard 2012). Moreover, the presence of an intraprotein pore in HAS and support for hyaluronan translocation by the synthase itself was presented in a recent study with liposomes containing purified *Se*-HAS (Medina 2012).

The mammalian HASs differ in their enzymatic properties and in their ability to produce a cell surface coat of hyaluronan. The K_m values of the precursor sugars are higher for HAS1 than for HAS2 or HAS3 (Itano 1999). COS-1 cells or rat fibroblasts transfected with any one of the three *Has* genes produce a hyaluronan coat, but the coat

produced by HAS1 is smaller than that produced by HAS2 or HAS3 (Itano 1999). In CHO cells, a minimum of >1000ng/ 1 x 10^5 cells/24h hyaluronan production was required in the HAS transfectants for the coat formation without proteoglycans (Brinck 1999). In rat fibroblasts, HAS1 and HAS2 produce larger hyaluronans of 2 x 10^5-2 x 10^6 Da compared with the polymer size of 1 x 10^5-1 x 10^6 Da from HAS3 (Itano 1999). HAS1 and HAS2 also produce hyaluronan faster than HAS3 (Itano 1999). For comparison, *Xenopus laevis* HAS has been measured to produce even larger hyaluronan chains of 3 x 10^6 – 2 x 10^7 Da (Pummill 1998). There are also other reports on the size of the hyaluronan chain produced by the mammalian HASs. In membrane preparations from CHO-cells transfected with mammalian HAS isoforms, HAS2 produced the largest hyaluronan (over 3.9 x 10^6 Da), HAS3 produced smaller hyaluronan (0.12-1 x 10^6 Da) and HAS1 the smallest polymer (0.12 x 10^6 Da), while in live cells all isoforms produced high molecular weight hyaluronan (3.9 x 10^6 Da) (Brinck 1999). On the other hand, in live rat arterial smooth muscle cells transduced with retroviral constructs of murine hyaluronan synthases, HAS1 and HAS2 produced high molecular weight hyaluronan (2-10 x 10^6 Da), whereas HAS3 produced lower molecular weight hyaluronan (~2 x 10^6 Da) (Wilkinson 2006). The size of the growing hyaluronan is increased or decreased by mutation of certain cysteine or serine amino acid in the HAS1 protein in *Xenopus laevis*, suggesting that the size of the hyaluronan chains are affected by the ability of the synthase to bind it (Pummill 2003).

The three HASs differ in their importance during embryogenesis. *Has2* knockout mice die at embryonic day 9.5 due to cardiovasculcar defects (Camenisch 2000), but mice deficient in *Has1* (Kobayashi 2010) or *Has3* (Bai 2005) are viable and fertile. Also mice with double knockout of *Has1* and *Has3* have been developed and they are phenotypically normal (Mack 2012).

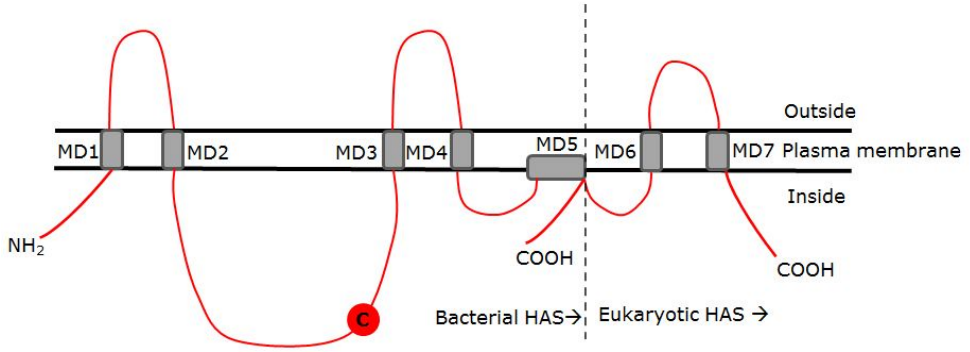

Figure 2. Proposed membrane topology for eukaryotic HAS proteins. There are 4-6 transmembrane domains in addition to 1-2 membrane associated domains. The N- and C-termini and the large central cytoplasmic domain between membrane domain (MD) 2 and MD3, probably containing the active site of HAS, are intracellular. Modified from (Weigel 1997).

2.2.3 Regulation of hyaluronan synthesis

Proper control of hyaluronan synthesis is important for the whole organism. For example, Shar Pei dogs have a thick, wrinkled skin due to overexpression or increased activity of HAS2, causing dermal accumulation of hyaluronan (Zanna 2009). Hyaluronan synthesis is regulated at all levels from transcription to modifications of the protein and substrate availability. The *HAS* genes are often regulated in parallel (Vigetti 2009b, Kultti 2009b) and the synthesis of hyaluronan reflects changes at the mRNA level (Pienimäki

2001, Jacobson 2000, Karvinen 2003b, Yamada 2004a). The *HAS2* promoter has been studied most widely. After the *HAS* genes had been cloned, it was found that dexamethasone suppresses the *HAS2* mRNA levels in fibroblasts and osteosarcoma cell lines (Zhang 2000). The suppressive effect of hydrocortisone (Jacobson 2000, Stuhlmeier 2004b) and dexamethasone (Stuhlmeier 2004b) on *HAS2* and *HAS3* mRNAs has also been demonstrated by others.

The *HAS* genes are also regulated by many growth factors, cytokines and other reagents (Table 1), but the isoforms seem to respond differently to external stimuli and the effects are highly dependent on the cell type or treatment conditions (Jacobson 2000). For example, transforming growth factor β (TGF-β), and to a lesser amount platelet derived growth factor (PDGF)-BB, increase *HAS2* mRNA and protein (Suzuki 2003). Likewise, TGF-β and interleukin (IL)-1 β are activators of *HAS1* transcription, but TGF-β is a suppressor for *HAS3* mRNA expression (Stuhlmeier 2004a).

The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is involved in interleukin-1β (IL-1β)-induced *HAS1* transcription in synoviocytes (Stuhlmeier 2005, Kao 2006). The NF-κB pathway is also involved in the induction of *HAS2* expression in IL-1β-, tumor necrosis factor (TNF)-α-, and TNF-β-treated endothelial cells (Vigetti 2010) and also in TNF-α-treated keratinocytes (Saavalainen 2007). Transcription factors specificity protein (SP) 1 and SP3 are also important in *HAS2* transcription regulation (Monslow 2006) in addition to signal transducer and activator of transcription 3 (STAT3) (Saavalainen 2005) and cyclic adenosine monophosphate (cAMP) response element binding protein 1 (CREB1) (Makkonen 2009). The human *HAS2* gene is also regulated by epidermal growth factor (EGF) and retinoic acid (RA) (Saavalainen 2005). Moreover, *HAS2* transcription is activated by adiponectin through an adenosine monophosphate kinase/peroxisome proliferator-activated receptor alpha-dependent pathway in human dermal fibroblasts (Yamane 2011). Also reactive oxygen species generated by NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) oxidase induce *Has2* expression and hyaluronan secretion in thrombin-treated murine vascular smooth muscle cells (Vendrov 2010). However, the changes in the *HAS* mRNA levels are highly dependent on the cell type.

Obviously, the synthesis of hyaluronan is also regulated by the substrate concentrations of the precursor sugars. A coumarin derivative, 4-methylumbelliferone (4-MU), has been shown to reduce hyaluronan synthesis in skin fibroblasts (Nakamura 1995, Nakamura 1997), skin keratinocytes (Rilla 2004), mesothelial cells (Rilla 2008) and melanoma cells (Kudo 2004). 4-MU causes its effects by depleting the UDP-GlcUA substrate pool of hyaluronan synthesis and reducing *HAS2* and *HAS3* mRNA levels (Kultti 2009b, Kakizaki 2004). Mannose can also reduce the amount of UDP-N-acetylhexosamines, leading to decreased hyaluronan synthesis (Jokela 2008a). In addition, the precursor sugars participate in the transcriptional regulation of the synthase genes, as O-linked-N-acetylglucosamine (O-GlcNAc) modification of SP1 and ying-yang 1 (YY1) is influenced by the cellular content of the UDP-N-acetylhexosamines, controlling *HAS2* expression (Jokela 2011). Hyaluronan synthesis is also regulated by modifications of the synthase proteins after translation. Phosphorylation and N-glycosylation of HAS or other targets modifying the function of HAS have been suggested to influence the activity of the synthase (Vigetti 2009a). HAS2 has been shown to form homodimers as well as heterodimers with HAS3 (Karousou 2010). HAS1 can exist in multimers of full length-HAS1 or its variants, formed by intermolecular disulfide bonds (Ghosh 2009). In addition, HAS2 is monoubiquitinated on its Lys-190 residue and this modification is important for the synthase activity, whereas polyubiqitinylation of Lys-48 or Lys-63 may be associated with a small pool of misfolded HAS2 proteins (Karousou 2010).

The microenvironment of hyaluronan synthase is also important for its activity. The bacterial HAS is phospholipid-dependent and cardiolipin is the best enzymatic activator (Weigel 2006). In mammalian cells, cholesterol might be important for mammalian HASs as depletion of cellular cholesterol by methyl-β-cyclodextrin (MβCD) suppresses hyaluronan synthesis, especially by down-regulating *HAS2* mRNA level (Kultti 2010), and this can be reversed by re-addition of cholesterol (Sakr 2008).

Table 1. Factors affecting HA synthesis. Modified from (Kultti 2009a).
↓ decreased, ↑ increased, - not changed, NE not expressed, empty not studied

Agent	Cell/tissue	HA	HAS1	HAS2	HAS3	Reference
full-length adiponectin	fibroblast	↑	NE	↑	-	(Akazawa 2011)
adiponectin	fibroblast	↑		↑		(Yamane 2011)
constitutively active PI3K transfection	mammary carcinoma cell	↑				(Misra 2005)
compound K	keratinocyte	↑	-	↑	-	(Kim 2004)
dehydroepiandrosterone	uterine fibroblast	↑				(Tanaka 1997)
EGF	fibroblast	↑				(Heldin 1989)
EGF	fibroblast	↑	↑	↑	↑	(Yamada 2004a)
EGF	keratinocyte	↑	-	↑	↑	(Pasonen-Seppänen 2003)
EGF	keratinocyte	↑		↑		(Saavalainen 2005)
EGF	oral mucosal cell	↑	↑	↑	↑	(Yamada 2004a)
EGF	neural crest cell	↑				(Erickson 1987)
EGF	mesothelial cell	↑				(Honda 1991)
EGF	cumulus cell	↑				(Tirone 1997)
EGF	lung adenocarcinoma cell	↑	NE	↑	↑	(Chow 2010)
17β-estradiol	uterine fibroblast	↑				(Tanaka 1997)
estrogen	endometrium	↑				(Tellbach 2002)
estrogen	uterine epithelium	↑				(Mani 1992)
bFGF	fibroblast	↑				(Heldin 1989)
FGF2	dental pulp	↑	↑	↑	-	(Shimabukuro 2005b)
FGF2	periodontal ligament	↑	↑	↑	-	(Shimabukuro 2005a)
FGF	fibroblast	↑	↑	↑	↑	(Kuroda 2001)
forskolin	orbital fibroblast	↑	↑	-	↑	(van Zeijl 2010)
forskolin	human embryonic kidney cell	↑		↑		(Makkonen 2009)
FSH	cumulus cell	↑				(Tirone 1997)
glucose	mesangial cell	↑				(Ren 2009)
HGF	epithelial cell	↑				(Zoltan-Jones 2003)

Agent	Cell/tissue	HA	HAS1	HAS2	HAS3	Reference
IFN-γ	keratinocyte	↑	-	NE	↑	(Sayo 2002)
IFN-γ	fibroblast	↑				(Sampson 1992)
IGF	fibroblast	↑	↑	↑	↑	(Kuroda 2001)
IGF	mesothelial	↑				(Honda 1991)
IL-1	fibroblast	↑				(Sampson 1992)
IL-1β	fibroblast	↑	↑	↑	↑	(Yamada 2004a)
IL-1β	fibroblast	↑	↑	↑	↑	(Kaback 1999)
IL-1β	uterine fibroblast	↑	↑	↑	↑	(Uchiyama 2005)
IL-1β	synoviocyte	↑				(Kawakami 1998)
IL-1β	synoviocyte	↑	-	-	↑	(Oguchi 2004)
IL-1β	orbital fibroblast	↑	↑	↑	↑	(van Zeijl 2010)
IL-1β	umbilical vein endothelial cell	↑	NE	↑	-	(Vigetti 2010)
IL-1β	lung adenocarcinoma cell	↑	NE	↑	↑	(Chow 2010)
IL-6	fibroblast	↑				(Duncan 1991)
KGF	keratinocyte	↑	-	↑	↑	(Karvinen 2003b)
KGF	keratinocyte	↑				(Jameson 2005)
leukemia inhibitory factor	osteoblast	↑	-	↑	NE	(Falconi 2007)
PTH	osteoblast	↑				(Midura 1994)
PDGF	fibroblast	↑				(Heldin 1989)
PDGF	mesothelial	↑				(Heldin 1992)
PDGF	mesothelial	↑	-	↑	-	(Jacobson 2000)
PDGF	vascular endothelial cell	↑		↑		(Suzuki 2003)
PDGF	vascular smooth muscle cell	↑		↑		(Evanko 2001)
PDGF	trabecular meshwork	↑		↑		(Usui 2003)
PDGF	fibroblast	↑	↑	↑	-	(Li 2007)
PDGF	cardiomyocyte	↑				(Hellman 2010)
PMA	fibroblast	↑				(Suzuki 1995)
poly I:C	smooth muscle cell	↑				(de la Motte 2003)
progesterone	uterine fibroblast	↑	↓	↓	↑	(Uchiyama 2005)
prostaglandin D2	orbital fibroblast	↑	↑	↑	↑	(Guo 2010)
prostaglandin J2	orbital fibroblast	↑				(Guo 2010)

Agent	Cell/tissue	HA	HAS1	HAS2	HAS3	Reference
prostaglandin E2	synoviocyte		↑			(Stuhlmeier 2007)
retinoic acid	epidermis	↑				(King 1981)
retinoic acid	epidermis	↑				(Tammi 1986)
retinoic acid	keratinocyte	↑		↑		(Saavalainen 2005)
retinoic acid	keratinocyte	↑	-	↑	↑	(Pasonen-Seppänen 2008)
retinyl retinoate	epidermis	↑		↑		(Kim 2010)
testosterone	rooster comb	↑				(Jacobson 1978)
TGF-β	fibroblast	↑				(Heldin 1989)
TGF-β	fibroblast	↑	↑	↑		(Sugiyama 1998)
TGF-β	keratinocyte	↑	↑	-		(Sugiyama 1998)
TGF-β1	vascular endothelial cell		-	↑	-	(Suzuki 2003)
TGF-β1	lung adenocarcinoma cell	-	NE	↑	-	(Chow 2010)
TGF-β	trabecular meshwork	↑	-	↑	-	(Usui 2003)
TGF-β	synoviocyte	↑	↑	-	-	(Oguchi 2004)
TGF-β	synoviocyte	↑	↑	-	↓	(Stuhlmeier 2004a)
TNF-α	synoviocyte	↑	-	-	↑	(Oguchi 2004)
TNF-α	fibroblast	↑				(Sampson 1992)
TNF-α	umbilical vein endothelial cell	↑	NE	↑	-	(Vigetti 2010)
TNF-β	umbilical vein endothelial cell	↑	NE	↑	-	(Vigetti 2010)
tunicamycin	smooth muscle cell	↑				(Majors 2003)
tunicamycin	smooth muscle cell	↑				(Lauer 2008)
benzbromarone	fibroblast	↓				(Prehm 2004)
5,7-dihydroxy-4-methylcoumarin	pancreatic cancer	↓				(Morohashi 2006)
6,7-dihydroxy-4-methylcoumarin	pancreatic cancer	↓				(Morohashi 2006)
dipyridamole	fibroblast	↓				(Prehm 2004)
estradiol	vascular smooth muscle cell	↓	↓	-	-	(Freudenberger 2011)
glucocorticoid	epidermis	↓				(Ågren 1995)
glucocorticoid	fibroblast			↓		(Zhang 2000)
glucocorticoid	synoviocyte	↓	-	↓	↓	(Stuhlmeier 2004b)

Agent	Cell/tissue	HA	HAS1	HAS2	HAS3	Reference
hydrocortisone	mesothelial cell	↓	-	↓	-	(Jacobson 2000)
indomethacin	fibroblast	↓				(August 1994)
indomethacin	fibroblast	↓				(Prehm 2004)
mannose	keratinocyte	↓				(Jokela 2008a)
MβCD	smooth muscle cell	↓				(Sakr 2008)
MβCD	breast cancer cell	↓	NE	↓	-	(Kultti 2010)
mefenamic acid	fibroblast	↓				(August 1994)
4-MU	fibroblast	↓				(Nakamura 1995)
4-MU	fibroblast	↓		↓	-	(Kakizaki 2004)
4-MU	uterine fibroblast	↓				(Tanaka 2007)
4-MU	keratinocyte	↓				(Rilla 2004)
4-MU	melanoma cell	↓				(Kudo 2004)
4-MU	melanoma cell	↓				(Yoshihara 2005)
4-MU	pancreatic cancer cell	↓				(Nakazawa 2006)
4-MU	breast cancer cell MCF-7	↓	NE	↓	-	(Kultti 2009b)
4-MU	breast cancer cell MDA-MB-361	↓	NE	NE	↓	(Kultti 2009b)
4-MU	melanoma cell	↓	NE	↓	↓	(Kultti 2009b)
4-MU	ovarian cancer cell	↓	NE	NE	↓	(Kultti 2009b)
4-MU	smooth muscle cell	↓	↓	↓	↓	(Vigetti 2009b)
4-MU	fibroblast	↓				(Edward 2010)
4-MU	breast cancer cell	↓	NE	↓	-	(Urakawa 2012)
progesterone	uterine fibroblast	↓				(Tanaka 1997)
S-decyl-glutathione	fibroblast	↓				(Prehm 2004)
TGF-β1	synoviocyte	↓	↓			(Kawakami 1998)
TGF-β	mesothelial cell		↑	↓	-	(Jacobson 2000)
TGF-β	keratinocyte	↓	-	↓	↓	(Pasonen-Seppänen 2003)
TGF-β	keratinocyte	↓	-	NE	↓	(Sayo 2002)
trequinsin	fibroblast	↓				(Prehm 2004)
vesnarinone	myofibroblast	↓				(Ueki 2000)
valspodar	fibroblast	↓				(Prehm 2004)
verapamil	fibroblast	↓				(Prehm 2004)
vitamin D	osteoblast	↓				(Takeuchi 1989)

2.3. DEGRADATION OF HYALURONAN

2.3.1. Hyaluronidases

The catabolism of hyaluronan in tissues is as important as its synthesis for homeostasis. The rate of hyaluronan catabolism varies between different tissues, as the half-life of hyaluronan can be almost three weeks in cartilage (Morales 1988), about one day in the skin (Tammi 1991a) and only 2.5-4.5 minutes in the plasma (Fraser 1981).

Hyaluronan is degraded by hyaluronidase (HYAL) enzymes. HYALs can be divided into three different subtypes based on their end-products in hyaluronan degradation. Some insect species present HYALs that are endo-β-glucuronidases and their end-products are tetra- and hexasaccharides. Bacterial HYALs yield disaccharides and are endo-β-N-acetyl-hexosaminidases specific for hyaluronan. Mammals also have endo-β-N-acetyl-hexosaminidases that function as hydrolases to produce hexa- and tetrasaccharides and can also degrade chondroitin sulfate (Stern 2004). The degradation of hyaluronan from a large molecule to its monosaccharides needs the exoenzymes β-D-glucuronidase and β-N-acetyl-D-hexosaminidase in addition to the hyaluronidase activity (Roden 1989) and recently a co-operative, redundant function of these enzymes was demonstrated in a mouse model (Gushulak 2012). The human HYALs share 30% identity with the bee venom hyaluronidase and according to crystal structure they resemble a classical alpha/beta-triose phosphate isomerase (TIM) barrel composed of seven strands, a general structure type among glycoside hydrolases that commonly represent eight strands (Markovic-Housley 2000, Rigden 2003).

The human genome contains six *HYAL* genes located on two chromosomes. *HYAL1*, *HYAL2* and *HYAL3* are found on chromosome 3p21.3, whereas the other three genes *HYAL4*, Hyaluronidase pseudogene 1 (*PHYAL1*) and Sperm adhesion molecule 1 (*SPAM1*) are clustered on chromosome 7q31.3 (Stern 2004, Csoka 1999).

HYAL1 is the main hyaluronidase in plasma (Afify 1993, Frost 1997), but it is also found in urine with much higher activity than in the plasma (Csoka 2001). HYAL1 is an acid-active lysosomal enzyme (Stern 2004). Mucopolysaccharidosis IX is a known lysosomal storage disease caused by deficiency of HYAL1 (Natowicz 1996, Triggs-Raine 1999) leading to short stature, cutaneous swelling, painful soft tissue masses over articular surfaces and bilateral joint effusions. Mice with *Hyal1* knockout are viable and fertile, but display osteoarthritis with loss of proteoglycans and accumulation of hyaluronan in joints (Martin 2008).

HYAL2 is the other major hyaluronidase in somatic tissues. HYAL2 is located at the plasma membrane by a glycosylphosphatidylinositol-anchor, and it is active in acidic pH and produces hyaluronan fragments of about 20 kDa in size (Stern 2004, Lepperdinger 1998, Lepperdinger 2001, Rai 2001, Müllegger 2002). *Hyal2*-deficient mice are viable and fertile, but they have lower survival, defects in craniovertebral bone formation, thrombocytopenia, hemolysis and increased plasma hyaluronan levels (Jadin 2008).

HYAL3 is found widely in different human tissues, but its levels are especially high in testis and bone marrow (Csoka 1999). HYAL3 may have an important role in stem cell regulation, because testis and bone marrow retain a stem cell-like state throughout the life (Csoka 2001). HYAL3 has also been suggested to have an activating effect on HYAL1 (Hemming 2008). In addition, a *Hyal3*-knockout mouse did not show any accumulation of hyaluronan as the mice were viable and fertile (Atmuri 2008), suggesting that HYAL3 is not involved in constitutive hyaluronan degradation.

HYAL4 appears to be a chondroitinase with no activity against hyaluronan (Csoka 2001, Stern 2003). PHYAL1 is transcribed but not translated in humans (Csoka 2001, Stern 2003). Mice have an additional hyaluronidase, HYAL5, involved in sperm penetration during fertilization (Kim 2005).

SPAM1, also known as PH20, is a sperm-associated hyaluronidase in testes (Stern 2003, Jones 1995) and it is also expressed in the human epididymis (Evans 2003). SPAM1 expression has also been found in the female mouse genital tract (Zhang 2003) and in human breast (Beech 2002), placenta and the fetal tissues (Csoka 1999). SPAM1 has enzymatic and signaling activities during fertilization (Cherr 2001).

Extracellular hyaluronan is bound to its receptors, such as the cluster of differentiation (CD) 44, lymph vessel endothelium receptor (LYVE)-1 and hyaluronan receptor for endocytosis (HARE)/Stabilin-2. The degradation of hyaluronan has been suggested to consist of its degradation at the cell surface by HYAL2, internalization of the fragments (Tammi 2001, Hua 1993, Culty 1992, Aguiar 1999) and further degradation in lysosomes by HYAL1 (Stern 2004). Hyaluronan is partly degraded locally in tissues, but it can be transferred via lymph or blood to lymph nodes, liver and spleen for eventual catabolism (Jadin 2012).

2.3.2. Regulation of hyaluronidases

The mechanisms of regulation of hyaluronan degradation are not yet clear, but they have a crucially important role in tissue homeostasis. Cellular expression of the alternative mRNA splice variants of the three main hyaluronidases, HYAL1-3, have been shown to regulate their activity (Lokeshwar 2002). *HYAL1* promoter region contains binding sites for several transcription factors, such as SP1, early growth response -1 (EGR-1), and activating protein-2 (AP-2) (Lokeshwar 2008). *HYAL1* expression is also regulated by methylation and the binding of EGR-1 and AP-2 to unmethylated promoter turns transcription on, while binding of SP1 to the methylated promoter turns transcription off. In line with this, cells expressing *HYAL1* have EGR-1 and AP-2 bound to the promoter, while the promoter is occupied by SP1 in cells not expressing *HYAL1* (Lokeshwar 2008). In dermal fibroblasts, PDGF-BB increases the mRNA level of *HYAL1* while the protein level is not changed and *HYAL2* levels are not affected (Li 2007). In the same study, TGF-β1 increased hyaluronidase activities (Li 2007). Bone morphogenetic protein-7 (BMP-7) suppresses *HYAL1* and *HYAL2* mRNA levels in renal proximal tubular cells (Selbi 2004). A recent study also observed repression of *Hyal1* and *Hyal2* gene expression levels during the first 24h after renal ischemia-reperfusion in rats (Decleves 2012).

HYAL1 expression increases in fibroblasts during chondrogenesis (Nicoll 2002). In chondrocytes, the mRNA levels of *HYAL2* and *HYAL3* are upregulated by cytokines like IL-1-β and TNF-α, while *HYAL1* is not (Flannery 1998). Ultraviolet B (UVB) irradiation of normal human keratinocyte cultures increases *HYAL1* mRNA, but decreases *HYAL2* and *HYAL3* mRNA levels 6h after exposure, and accordingly at 24h post-irradiation the HYAL1 protein level is increased, HYAL2 is decreased and HYAL3 is not changed (Kurdykowski 2011). However, in another study the mRNA levels of *Hyal1-3* were slightly increased at day 3 after UVB in mouse epidermis (Tobiishi 2011).

Inhibitors of hyaluronidases are present in the serum, but they are poorly characterized. The predominant hyaluronidase inhibitor in serum is a member of the inter-alpha-inhibitor (IαI) family, belonging to the acute-phase proteins released during stress reactions (Mio 2000, Mio 2002). Delayed degradation of hyaluronan may thus have an important role in stress reactions.

2.3.3. Other catabolic mechanisms in the degradation of hyaluronan

In addition to enzymatic breakdown, hyaluronan is decomposed by reactive oxygen species (ROS) and free radicals. One reason for the susceptibility of hyaluronan to depolymerization by ROS may be the lack of sulphate groups in its structure (Moseley

1995). Endogenous ROS contribute to the degradation of hyaluronan in human epidermis which has a high turn-over rate of hyaluronan (Ågren 1997). In human airway epithelium during inflammation, hyaluronan is degraded by ROS and also by ROS-induced Hyal2 (Monzon 2010). Hyaluronan degradation by ROS might have protective, but also harmful effects in the tissue. Already in 1988 it was suggested that excessive ROS are scavenged by hyaluronan degradation in synovial fluid of rheumatoid arthritis and this protects articular tissues from further damage (Sato 1988). On the contrary, in rabbit articular chondrocytes, ROS destroy hyaluronan-proteoglycan complexes, affecting cartilage structure (Panasyuk 1994). In breast cancer, nitric oxide, one type of ROS, stimulates the synthesis of hyaluronan which then acts as a scavenging molecule for the excessive free radicals by being degraded itself (Karihtala 2007). Many antioxidants have been suggested to be protective against ROS-induced breakdown of hyaluronan (Mendoza 2007). *In vitro*, hyaluronan can also be cleaved by hydrolysis, sonication and thermal degradation (Stern 2007).

2.4. HYALADHERINS

Hyaluronan can be bound by many molecules, some of which are receptor proteins having signaling properties. The link module superfamily of hyaladherins consists of aggrecan, versican, brevican, neurocan, link proteins, CD44, LYVE-1, tumor necrosis factor alpha stimulated gene-6 (TSG-6), and HARE (Day 2002). A common factor among the members of the link module superfamily is a hyaluronan-binding domain of about 100 amino acids (Day 2002, Day 1999). Other molecules capable of binding to hyaluronan include the receptor for hyaluronan-mediated motility (RHAMM), heavy chains (HC) of IαI, plasma hyaluronan binding protein, CDC37, hyaluronan binding protein (HABP)/P-32, intracellular hyaluronan binding protein 4 (IHABP4), CD38, a sialoprotein (SPACR) and a sialoproteoglycan (SPACRCAN) associated with cones and rods (Day 2002) and layilin (Bono 2001).

The minimum chain length needed by the different hyaladherins for binding of hyaluronan varies. A minimum of 10 sugars (HA10) is required for link proteins and proteoglycans (Seyfried 2005, Hardingham 1973, Hascall 1974), but for CD44 the size seems to vary depending on the cell line. Six sugars or more (>HA6) prevented the binding of hyaluronan to the surface of cultured SV-3T3 cells (Underhill 1979) and HA6 was also sufficient to inhibit assembly of the pericellular coat in chondrocytes (Knudson 1993). However, keratinocytes require HA10 or longer oligosaccharides for the displacement of cell surface hyaluronan (Tammi 1998). An optimal length of 6-10 sugars (HA6-HA10) to displace hyaluronan from CD44 has been shown in other studies too (Lesley 2000, Teriete 2004). Layilin needs HA20 to interfere with its binding to hyaluronan (Bono 2001), while HA8 is sufficient for LYVE-1 (Banerji 2010). The size requirements of the other hyaladherins have not been established.

2.4.1. CD44

CD44 is a transmembrane glycoprotein located at the cell surface (Underhill 1985, Underhill 1987). It is formed by a single chain that makes a single pass through the cell membrane (Toole 2009). The standard form of CD44 has a molecular weight of 85 kDa in SDS-PAGE, and is expressed in most cell types, but CD44 can be expressed in many variant forms resulting from alternative splicing of a single gene (Goldstein 1989, Screaton 1992). The expression of splice variants depends on cell type. For example human keratinocytes express high levels of several mRNA and protein variants of CD44,

however, their expression levels are downregulated at confluency and differentiation (Zhou 1999). The hyaluronan binding domain of CD44 is reasonably conserved among mammals and is located at the amino terminal distal extracellular region, whereas the rest of the extracellular part, the membrane-proximal region can be modified by O-linked glycosylation or addition of chondroitin sulphate or heparan sulphate (Isacke 2002). CD44 can also be modified by phosphorylation (Neame 1992), palmitoylation (Guo 1994) and proteolytic cleavage (Okamoto 1999, Thorne 2004) or oligomerization (Heldin 2008). The posttranslational modifications vary depending on the cell type and state of the cell. The heparan sulfate-glycosylated CD44 is the main form in normal epidermis (Tuhkanen 1997). On the other hand, in cultured melanocytes one of the two expressed CD44 variants is covalently modified by chondroitin sulphate (Herbold 1996).

Early after its discovery as a hyaluronan receptor, CD44 was noticed to participate in receptor-mediated uptake of hyaluronan (Hua 1993, Culty 1992). CD44 can bind ligands influencing cell behavior, act as a co-receptor mediating signaling cascades and form a link between the cell membrane and cytoskeleton (Ponta 2003). Cell surface CD44 is required for the survival and proliferation of tumor cells in the microenvironment of metastasis (Yu 1997). Inhibition of hyaluronan-CD44 contacts by oligosaccharides also attenuates signaling pathways activated by CD44. For example, hyaluronan oligosaccharides can inhibit tumor growth by disturbing the hyaluronan-CD44 interaction in melanoma cells (Zeng 1998) and glioma (Ward 2003), and hyaluronan fragments of HA6-HA36 size can induce cleavage of CD44, thereby promoting cell migration (Sugahara 2003). In addition, hyaluronan oligosaccharides and CD44 siRNA, both perturbing the contact of hyaluronan and CD44, can reverse hyaluronan-induced increase in the expression of cyclo-oxygenase-2, a mediator of cell-survival properties (Misra 2008). Hyaluronan oligosaccharides also disturb the complexes formed by hyaluronan and CD44, emmprin (extracellular matrix metalloproteinase inducer; CD147; basigin (Toole 2008)) and monocarboxylate transporters (MCT) leading to reduced lactate efflux in cancer cells (Slomiany 2009b). The activation of CD44 and its consequent signaling has been suggested to be affected by its posttranslational modifications, its location at certain cell membrane domains, interactions with the cytoskeleton and binding of hyaluronan (Toole 2009).

In human epidermis, CD44 and hyaluronan are present in the basal and spinous cell layers, but are both missing from the upper granular or cornified layers of the skin (Wang 1992). In keratinocytes, CD44 participates in the binding of hyaluronan in the pericellular coat, although other mechanisms have been suggested, like binding to the synthase or other hyaladherins (Tammi 1998, Pasonen-Seppänen 2012a). CD44 is also present in normal skin fibroblasts and its expression is substantially increased in hypertrophic scars (Messadi 1993). In psoriasis, in which the epidermis is hypertrophic, the main pattern of hyaluronan and CD44 stainings resembles that of normal skin, but in areas invaded by leukocytes the stainings are decreased (Tammi 1994). CD44 is consistently stained in different benign melanocytic lesions, while in malignant melanoma it shows heterogenous and decreased staining, correlating with the size, depth and invasion of the lesion (Karjalainen 2000, Leigh 1996).

2.4.2. Other cell-surface hyaladherins

Four cell-surface hyaladherins exist in addition to CD44: LYVE-1, HARE, layilin and toll-like receptor (TLR)-4. LYVE-1, also called cell surface retention sequence binding protein 1 (CRSBP-1), is a type I integral membrane protein containing the link module in its extracellular domain (Banerji 1999). The structure of LYVE-1 is homologous to CD44 (Banerji 1999). However, as the binding of hyaluronan and CD44 is mediated by

hydrogen bonds and hydrophobic interactions, LYVE-1 has also been suggested to bind hyaluronan by charge interactions (Banerji 2010). LYVE-1 is a lymph endothelium-specific hyaluronan receptor and it has been used widely as a marker for lymph vessels (Banerji 1999, Solis 2012). LYVE-1/CRSBP-1 knockout mice are normal and fertile, but they have distended lumens in lymph vessels and as a consequence, the lymph flow is constitutively increased (Huang 2006).

Stabilin-2, also known as HARE, has its gene located on chromosome 12 and encodes two isoforms of 190 and 315 kDa (Zhou 2003). HARE is abundant in the sinusoidal endothelial cells of human liver, spleen and lymph nodes (Zhou 2003), in which endocytosis of hyaluronan occurs via the clathrin-coated pit pathway (Zhou 2003, McGary 1989). In addition to hyaluronan, HARE can also endocytose chondroitin sulphates (Harris 2007) and heparin (Harris 2008). Binding of hyaluronan to HARE increases phosphorylation of both HARE and extracellular signal-regulated kinase (ERK) 1/2, leading to activation of ERK (Kyosseva 2008), suggesting that HARE is involved in cell signaling. In addition, HARE may have an important role in cancer cell spreading. Inhibition of HARE totally prevents formation of lymph node metastases in prostate cancer (Simpson 2012) and in Stabilin-2 knockout mice the number of lung metastasis was reduced possibly because the elevated serum hyaluronan levels prevented the attachment of the circulating melanoma cells to the lung tissue (Hirose 2012).

Layilin, a transmembrane protein expressed in membrane ruffles of adherent cells (Borowsky 1998), can bind to hyaluronan via its lectin-like domain (Bono 2001). Layilin may modulate the cytoskeleton because it can associate with talin, merlin and radixin (Bono 2001, Borowsky 1998, Bono 2005). Layilin is suppressed by IL-1β in chondrocytes and it has been suggested to participate in the functions of hyaluronan in arthritis (Murata 2012).

Small hyaluronan chains can activate TLR-4, a receptor complex associated with innate immunity (Termeer 2002). Contact of hyaluronan with TLR-4 initiates inflammatory responses in acute lung injury (Jiang 2005) and in chondrocyte cultures the addition of hyaluronan oligosaccharides up-regulates CD44 and TLR-4 and increases pro-inflammatory cytokines (Campo 2010).

2.4.3. Intracellular hyaluronan binding molecules

Intracellular hyaladherins include RHAMM, HABP/P-32 and CDC37. RHAMM was first described by Turley and colleagues in 1982 (Turley 1982). It was cloned in 1992 and found to be a cell-surface hyaluronan receptor involved in cell locomotion, but lacking the link module (Hardwick 1992). RHAMM has two isoforms that encode an alternatively spliced exon 4 (Hall 1995, Wang 1996a) and these variants determine its location in the cell (Zhang 1998). Although RHAMM was first located at the cell surface, it was also later discovered to be an intracellular protein (Hofmann 1998, Assmann 1998). The cell surface form of RHAMM is designated as CD168 (Maxwell 2008). RHAMM has two B(X_7)B motifs in its carboxyl terminal, required for HA binding (Yang 1994).

RHAMM associates with microtubules and –filaments in cells (Assmann 1999). It is suggested to be involved in cell division, localizing in centrosomes and stabilizing the mitotic spindle (Maxwell 2003). RHAMM also has signaling properties, as it can bind to ERK (Zhang 1998), among other kinases (Turley 2002). Furthermore, RHAMM is oncogenic as its hyaluronan-binding ability is required for transformation by *ras* oncogene (Hall 1995). RHAMM-negative fibroblasts display delayed wound healing and a similar phenomenon with decreased wound contraction and cell migration was also seen in RHAMM-deficient mice, although they were otherwise viable (Tolg 2006). RHAMM also controls entry into mitosis (Mohapatra 1996).

The second intracellular hyaladherin is a homodimer of two 34 kDa subunits (Gupta 1991). It was first isolated from rat liver and was also found on the surface of fibroblasts, initially named HABP (Gupta 1991). This HABP was later found to be identical with P-32, a protein co-purified with the human pre-mRNA splicing factor SF2 (Deb 1996) and to have sequence homology with the receptor for complement protein (gC1q-R) (Das 1997). It is phosphorylated in the presence of hyaluronan (Ranganathan 1995). Reduced levels of HABP on the surface of sperm cells is associated with loss of sperm motility (Ghosh 2002), suggesting a crucial role in fertilization. HABP/P-32 is located in mitochondria and also outside of them, for example in zymogen granules, ER and on the cell surface (Soltys 2000). It has also been shown to accumulate in the nucleus (Brokstad 2001).

The third intracellular hyaluronan-binding molecule is CDC37. It is a vertebrate homologue of a cell cycle control protein found in yeast and Drosophila (Grammatikakis 1995). CDC37 acts cooperately with chaperone Hsp90 to regulate protein kinases (Grammatikakis 1999) and both of them participate in a signaling complex containing also ErbB2, which is activated in many cancers (Ghatak 2005). The biological relevance of hyaluronan-CDC37 interaction remains unclear. By using the same antibody, yet another intracellular HABP, termed IHABP4, has been found and suggested to be located in the cytoplasm (Huang 2000).

Members of the ubiquitin specific protease subfamily 17 (USP17) are deubiquitinating enzymes that also contain a conserved hyaluronan binding motif (R/K)X_7(R/K) and are able to interact with hyaluronan *in vitro* based on precipitation assays (Shin 2006). In addition, USP17 deubiquitinates suppressor of defective silencing 3 (SDS3) protein in humans and also SDS3 contains motifs able to bind to hyaluronan (Ramakrishna 2012). These proteins are involved in the inhibition of anchorage-independent growth of tumors (Ramakrishna 2012).

2.4.4. Extracellular hyaluronan binding molecules

The extracellular matrix contains a wide array of molecules capable of binding to hyaluronan. These connections provide stability in the matrix as well as a variety of effects on cell behavior. Aggrecan, versican, neurocan and brevican are chondroitin sulphate proteoglycans that form a hyalectican family of hyaluronan binding molecules. Aggrecan is a large proteoglycan present in cartilage produced by chondrocytes. Aggrecan contains the hyaluronan-binding link module in addition to high amounts of carbohydrates like chondroitin sulphate, keratan sulphate and O- and N-linked oligosaccharides. Formation of aggregates of hyaluronan and aggrecan's link module produces a hydrophilic matrix providing the cartilage with load-bearing functions (Hardingham 1974, Hardingham 1992).

Versican (a versatile proteoglycan), first found in fibroblasts (Zimmermann 1989) but later shown to be present in many cells and tissues, contains sequence homology with known hyaluronan-binding molecules (LeBaron 1992). It can also bind to CD44 (Wu 2005). It has been shown to be upregulated in many cancers (Ricciardelli 2009) and it regulates lymphocyte traffic together with hyaluronan in inflamed tissues (Evanko 2012).

Neurocan is located in the brain tissue (Rauch 1992) being involved in tissue modeling and remodeling. Besides hyaluronan, it can bind other glycosaminoglycans, growth factors and structural matrix proteins (Rauch 2001). Brevican is another proteoglycan found in the brain (Yamada 1994).

TSG-6 is a secreted protein, the synthesis of which is increased by inflammatory signals (Lee 1992). It contains a hyaluronan-binding region, closely related to CD44 (Lee 1992). The IαI consists of two heavy chain protein chains covalently linked to a chondroitin sulphate of a proteoglycan, bikunin (Zhuo 2004). IαI and TSG-6 can form complexes

(Wisniewski 1994, Mukhopadhyay 2001) and these complexes are necessary intermediates in the transfer of the heavy chains to hyaluronan (Rugg 2005). IαI heavy chains associated with hyaluronan have been found in pericellular matrices of, for example, cultured mouse dermal fibroblasts (Yoneda 1990, Huang 1993). In addition to catalyzing the transfer of the heavy chains of IαI to hyaluronan (Rugg 2005), TSG-6 has been suggested to be a hyaluronan cross-linker (Baranova 2011). Concentration of TSG-6 may also affect the binding of hyaluronan to CD44 (Lesley 2004). TSG-6 has anti-inflammatory effects (Milner 2003) as indicated by vast inflammatory destruction of cartilage and bone observed in TSG-6 knockout mice (Szanto 2004). TSG-6 is also accumulated in the matrix of the preovulatory follicle during ovulation, possibly providing the matrix with stability (Mukhopadhyay 2001).

SPACR and SPACRCAN are both present in the eye, and contain a RHAMM-type domain for binding to hyaluronan (Acharya 1998, Acharya 2000). They are suggested to participate in the extracellular molecular scaffold in the retinal interphotoreceptor matrix (Chen 2004). The life cycle of hyaluronan is illustrated in Figure 3.

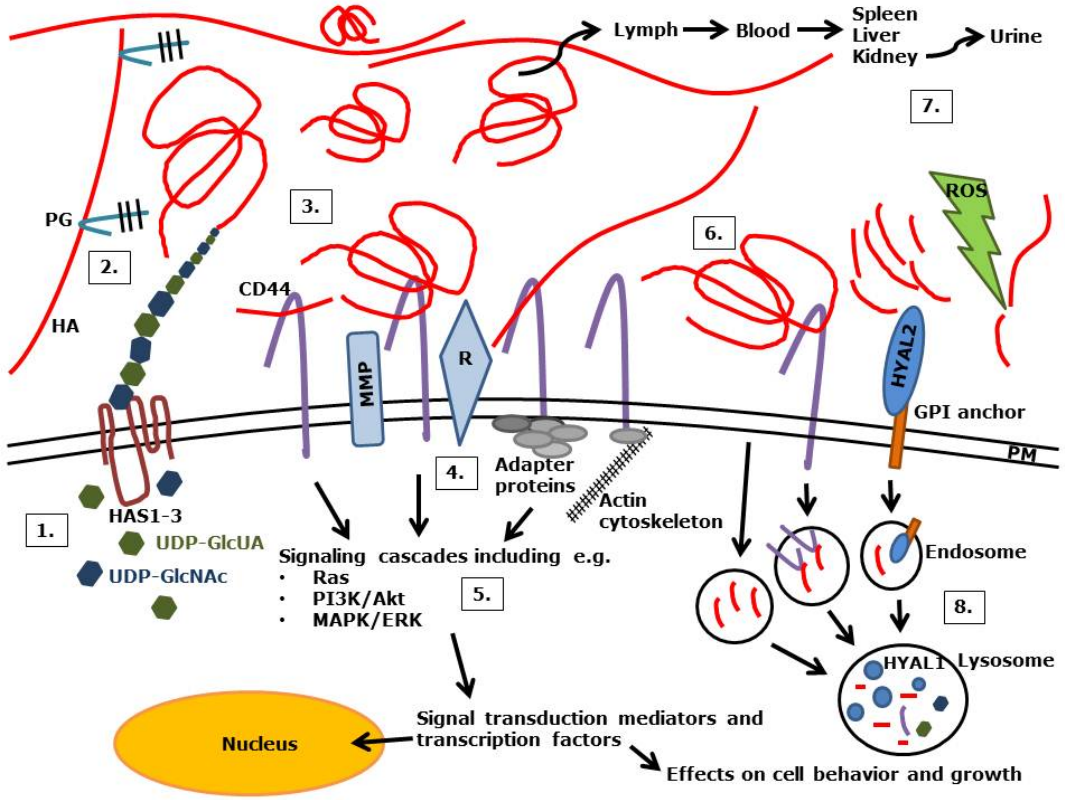

Figure 3. The life cycle of hyaluronan.

1. Hyaluronan is synthesized from cytosolic UDP-sugars by HASs 1-3 at the plasma membrane and the growing hyaluronan chain is extruded into the extracellular space.

2. Extracellular hyaluronan is either released into the matrix or bound by the synthases. Hyaluronan may also be bound by proteoglycans and other structural macromolecules. In the extracellular matrix, hyaluronan contributes to the mechanical properties of the tissue and it has an important structural role especially in cartilage. Pericellular HA expands intercellular spaces and affects migration, proliferation, tumor development and wound healing.

3. Extracellular hyaluronan is also bound by various cell surface receptors, e.g. CD44. Hyaluronan may elicit different responses based on its molecular size.

4. Hyaluronan binding by receptor proteins may lead to activation of various signaling cascades either directly by the receptor, or the receptor may act as a co-receptor interacting with various other receptors (e.g. ErbB, EGFR) or matrix metalloproteinases (MMP). In addition, hyaluronan binding by receptors may lead to formation of signaling complexes by adapter proteins near the cell surface. The composition of the complexes depends on the particular HA receptor and may vary between different cell types. CD44 also forms interactions with the actin cytoskeleton.
5. Hyaluronan binding by receptors activates signaling cascades and signal transduction mediators, leading to changes in protein synthesis, cell behavior and growth.
6. Hyaluronan may be endocytosed from the extracellular space by fluid endocytosis or by receptor-mediated endocytosis, reducing its content at the cell surface. HARE and LYVE-1 are endocytotic hyaluronan receptors in liver and lymph vessels.
7. Hyaluronan is removed from the extracellular matrix and synovial fluid to the lymph and blood, delivered to the liver, spleen and kidney and finally excreted in urine. Hyaluronan may also be degraded by reactive oxygen species or free radicals. At the cell surface, hyaluronan is locally degraded by HYAL2, which is located at the cell surface by a GPI-anchor. HYAL2 degrades hyaluronan into ~20 kDa polymers.
8. Hyaluronan is endocytosed with HYAL2 and the endosomes fuse with lysosomes. In lysosomes, hyaluronan is further degraded by HYAL1 and exoglycosidases.

2.5. BIOLOGICAL FUNCTIONS OF HYALURONAN

Hyaluronan is present ubiquitously in the extracellular matrix and it is not just a space filler. Hyaluronan can affect many aspects of cell shape and the growth of cells, and it also participates in situations when the cellular homeostasis is disrupted, like inflammation. Many of these properties of hyaluronan are especially valuable for normal tissue homeostasis and regeneration during wound healing and injury, but also cancer cells take advantage of them.

2.5.1. Pericellular hyaluronan coat

Abundant hyaluronan is retained around the cells after its synthesis by binding to its receptors, like CD44, making a fluffy coat surrounding the cells. The shape of cells and the space they take in tissues is affected by this pericellular hyaluronan. Dynamic fluctuations in the pericellular coat are important during limb morphogenesis (Knudson 1985). The Ppericellular hyaluronan coat has been demonstrated in fibrosarcoma cells by the red blood cell exclusion test (Goldberg 1984a) and has been shown to contain proteoglycans in addition to hyaluronan, especially in the pericellular coat of chondrocytes (Goldberg 1984b). The pericellular hyaluronan coat has been seen in many different cell types thereafter. For example, dividing or motile vascular smooth muscle cells have thick pericellular hyaluronan coats (Evanko 1999a). Some cell lines have hyaluronan coats naturally (Rilla 2008, Knudson 1993, Evanko 1999a, Heldin 1993), while other cell lines produce it in response to overexpression of hyaluronan synthase (Itano 1999, Brinck 1999, Kakizaki 2004, Li 2001, Kultti 2006). The pericellular hyaluronan coat can also be induced by growth factors like EGF (Pienimäki 2001) and PDGF (Evanko 2001).

Active hyaluronan synthesis at the plasma membrane induces the formation of microvilli up to 20 μm in length (Rilla 2008, Kultti 2006). These microvilli contain actin, but are not dependent on CD44 (Kultti 2006). The size of the hyaluronan coat has been shown to correlate with the rate of hyaluronan synthesis (Rilla 2008, Li 2001) and

inhibition of its synthesis with 4-MU reduces the coat (Kakizaki 2004, Kultti 2006). The hyaluronan coats are also affected by hyaluronan oligosaccharides. Human mesothelial cells have hyaluronan coats that are destabilized by hyaluronan oligosaccharides capable of interfering with hyaluronan-receptor interactions (Heldin 1993). In tumor cells, hyaluronan oligosaccharides prevent the formation of the pericellular hyaluronan matrix and inhibit tumorigenesis (Hosono 2007). The hyaluronan coat is required for the elongated, spindle-shape morphology of the smooth muscle cells, while cells treated with hyaluronan oligosaccharides loose the coat and gain a flattened, spread shape (Evanko 1999a).

In addition to the fluffy hyaluronan matrix, pericellular hyaluronan can take the form of cables that bind to leukocytes. Such cables have been induced with a viral mimic in colon smooth muscle cells (de la Motte 2003), with bone morphogenetic protein-7 in proximal tubular epithelial cells (Selbi 2004), with proinflammatory agents such as IL-1β in epidermal keratinocytes (Jokela 2008b) or with endoplasmic reticulum stress, as in colon smooth muscle cells (Majors 2003). IαI as well as versican have been shown to be important for the cable structure in human colon smooth muscle cells (de la Motte 2003) and renal proximal tubular epithelial cells (Selbi 2006), although it is not required for the cable formation in airway smooth muscle cells (Lauer 2009a). Although the cables generally associate with increased synthesis of hyaluronan, no correlation between the amount of secreted hyaluronan or the expression levels of *Has1-3* and cable formation was observed in rat keratinocytes (Jokela 2008b).

2.5.2. Epithelial to mesenchymal transition

One significant aspect of the role of hyaluronan in modulating cell shape is its ability to affect epithelial to mesenchymal transition (EMT), which is especially important during tissue development, healing processes and cancer progression. The association of hyaluronan and EMT has been demonstrated in several studies. Transformed properties and epithelial to mesenchymal transition is seen in phenotypically normal Madin-Darby canine kidney and human mammary epithelial cells when hyaluronan synthesis is stimulated with adenoviral expression of *Has2* (Zoltan-Jones 2003). *Has2* knockout prevents normal cardiac EMT in mice (Camenisch 2000). Hyaluronan oligosaccharides prevent cardiac EMT by inducing vascular endothelial growth factor (VEGF) (Rodgers 2006). Several other growth factors are also involved in hyaluronan-induced EMT. Increased synthesis of hyaluronan was connected to EMT in lung adenocarcinoma cells treated with a combination of TGF-β and IL-1β (Chow 2010). In line with this, inhibition of hyaluronan synthesis with 4-MU attenuates EMT in dermal fibroblasts (Meran 2007). Hyaluronan mediates its effects on EMT by binding to CD44. TNF-α has been shown to activate TGF-β signaling by promoting the formation of hyaluronan-CD44-moesin complexes required for EMT in retinal pigment epithelial cells (Takahashi 2010). The importance of CD44 in EMT has also been shown in another study with colon cancer cells, in which overexpression of CD44 increased EMT changes, whereas knockdown of CD44 decreased them (Cho 2012). Furthermore, TSG-6, in addition to CD44, has been suggested to be needed for EMT in proximal tubular epithelial cells (Bommaya 2011).

2.5.3. Effects of hyaluronan on apoptosis

Hyaluronan also affects cellular properties involved in cell viability. First, hyaluronan has been shown to influence apoptosis, but the results are controversial and may depend on the cell type. High molecular weight hyaluronan induces apoptosis in macrophages (Sheehan 2004) and in activated T cells (Ruffell 2008). In contrast to inducing apoptosis in

inflammation-associated cells, hyaluronan has been shown to have the opposite effect in other cell types. For example, high molecular weight hyaluronan protects the human corneal epithelial cells from apoptosis after UVB exposure (Pauloin 2009), and human aortic smooth muscle cells from apoptosis induced by 4-MU (Vigetti 2011). A similar protective effect of hyaluronan was found in hepatocellular carcinoma cells, in which inhibition of hyaluronan synthesis by 4-MU induced apoptosis (Piccioni 2012).

The effect of hyaluronan on apoptosis seems to be also size-dependent. Hyaluronan oligomers are proapoptotic by stimulating the tumor suppressor PTEN and inhibiting the phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway, leading to activation of pro-apoptotic mediators in mammary carcinoma cells (Ghatak 2002). Similar effect was later found in lymphoma cells (Alaniz 2006). Hyaluronan oligomers also inhibit phosphorylation of ErbB2 and assembly of ErbB2-signaling complex in colon carcinoma and mammary carcinoma cells, attenuating cell survival signaling (Ghatak 2005). The oligomers have been suggested to block the binding of hyaluronan to CD44, as an anti-CD44 antibody had similar effects (Ghatak 2002). This mechanism is also supported by the earlier notion of increased apoptosis in tumor cells treated with soluble CD44 (Yu 1997).

2.5.4. Cell proliferation

Hyaluronan influences cell growth, but the results have been contradictory and may depend on the different cell types. In rabbit synovial cells and 3T3 cells, the addition of hyaluronan to the culture medium inhibited proliferation, but the effect depended on the molecular weight and concentration of hyaluronan (Goldberg 1987). Hyaluronan can facilitate proliferation by activating several signaling routes in cells or by providing an adaptable matrix for cell division (Brecht 1986). Hyaluronan promotes the interaction of CD44 and epidermal growth factor receptor (EGFR) leading to activation of mitogen-activated protein kinase (MAPK)/ERK and further induction of cell proliferation in fibroblasts (Meran 2011). Melanoma cells have been shown to secrete growth factors that increase hyaluronan synthesis in fibroblasts, and the elevated hyaluronan content in turn stimulates melanoma cell proliferation (Willenberg 2012). Also in keratinocytes, there is a thick accumulation of hyaluronan in the cleavage furrow of the mitotic cells before separation of the daughter cells (Tammi 1991b) and inhibition of hyaluronan synthesis by 4-MU results in decreased proliferation (Rilla 2004). The role of the pericellular HA-coat for cell division was further shown in aortic smooth muscle cells, where dissolution of the hyaluronan coat by hyaluronan oligosaccharides was associated with reduced cell proliferation (Evanko 1999a).

Hyaluronan oligosaccharides have been shown to increase proliferation of endothelial cells under specific conditions, such as wound repair. The angiogenic effect of hyaluronan oligosaccharides, often associating with proliferation, has been known for a long time, both *in vivo* (West 1985) and *in vitro* (Montesano 1996). HA2-HA10 oligosaccharides enhance proliferation of endothelial cells and increase angiogenesis, possibly via RHAMM-dependent signaling pathways (Gao 2008b). In another study, HA6-HA10, but not HA4, stimulated proliferation of endothelial cells and increased angiogenesis in a membrane assay (Cui 2009a). The combination of proliferation and angiogenesis is especially useful in wound repair. Enhanced angiogenesis and lymphangiogenesis by HA oligosaccharides has been demonstrated in a murine excisional dermal wound model (Gao 2010).

Hyaluronan-rich pericellular matrices are also important for proliferation during morphogenesis. Many growth factors, such as TGF-β and basic fibroblast growth factor (bFGF), stimulate hyaluronan synthesis and cell proliferation in embryonic mesoderm

(Toole 1989). Hyaluronan accumulation is required at sites of cell proliferation and migration during development of limbs, while downregulation of hyaluronan synthesis is important during precartilage condensation of the skeletal elements (Li 2007b). Hyaluronan is also needed for the expansion of the cumulus cell-oocyte complex and for the extrusion of the oocyte (Salustri 1989, Salustri 1999), and it may also protect the oocyte from penetration by functionally deficient spermatozoa (Salustri 1999). Furthermore, the correct amount of hyaluronan is crucial for the proper organization of simple epithelia in tissues. Overexpression of HAS3 and consequent accumulation of hyaluronan in Madin-Darby canine kidney cells causes disturbed cell-cell contacts with aberrant polarization of cells during mitosis, eventually leading to formation of cysts with multiple lumina (Rilla 2012). Hyaluronan also functions in the differentiation of stratified epithelia, like skin. Hyaluronan synthesis correlates positively with epidermal proliferation and differentiation in an organotypic keratinocyte culture (Pasonen-Seppänen 2003) and removal of epidermal hyaluronan with *Streptomyces* hyaluronidase suppresses proliferation and accelerates differentiation of cells (Passi 2004).

2.5.5. Migration

Hyaluronan has been suggested to enhance cell migration in many different cell types, like smooth muscle cells of the ductus arteriosus (Boudreau 1991) and Ras-transformed fibroblasts (Turley 1991) or epidermal Langerhans cells (Mummert 2003). By increasing the volume of cell-free space in tissues, hyaluronan creates a pericellular environment that facilitates migration of cells. The promigratory effect of hyaluronan is especially valuable during wound healing. Smooth muscle cells accumulate hyaluronan and increase migration during wound healing (Savani 1995). Keratinocytes are surrounded by CD44-bound hyaluronan during the healing process (Oksala 1995), and epidermal hyaluronan content increases rapidly after the trauma concomitantly with upregulation of *Has2* and *Has3* mRNA (Tammi 2005). Inhibition of hyaluronan synthesis by 4-MU (Rilla 2004) or mannose (Jokela 2008a) results in reduced migration in keratinocytes. Interestingly, enhanced migration and faster wound closure was observed in *Has1* and *Has3* double knockout mice, indicating enhanced migration *in vivo* in the absence of these enzymes (Mack 2012).

The importance of hyaluronan in cell locomotion is clearly evident in embryogenesis. HAS2 is the only hyaluronan synthase expressed during embryonic days 8.5-9.5, the time period important for the development of the heart (Tien 2005). Normally, the cardiac jelly is rich in hyaluronan and the migrating cells express high levels of *Has2* mRNA, whereas *Has2* knockout mice die at embryonic day 9.5 because of multiple cardiovascular defects, due to the lack of hyaluronan-rich cardiac cushions (Camenisch 2000).

2.5.6. Anchorage-independent growth

Yet another way hyaluronan affects cell growth is its enhancement of anchorage-independent growth, important for cell invasion in tissues. Increased amount of hyaluronan due to expression of *HAS2* promotes anchorage-independent growth of fibrosarcoma cells (Kosaki 1999) and mesothelioma cells (Li 2001), while suppression of *HAS2* decreases it, as shown in breast cancer cells (Li 2007a). The binding of hyaluronan by CD44 is essential, as disturbing the contact with soluble CD44 reduces anchorage-independent growth in breast cancer cells (Peterson 2000). Hyaluronan oligosaccharides of 3-10 disaccharide units have been shown to inhibit anchorage-independent growth of tumor cells by disturbing the interaction of hyaluronan and CD44, leading to suppression of the PI3K/Akt cell survival pathway (Ghatak 2002). The elevated expression of

emmprin in breast cancer cells stimulates hyaluronan production, leading to the activation of the PI3K/Akt cell survival pathway and anchorage-independent growth (Marieb 2004).

2.5.7. Multidrug resistance

Hyaluronan can promote cancer cell growth by enhancing multidrug resistance. Increased production of hyaluronan has been shown to provide many different cancer cell lines with drug resistance, whereas hyaluronan oligosaccharides antagonize this effect (Misra 2003). Similarly, exogenously added hyaluronan promotes resistance to chemotherapy in head and neck squamous cell carcinoma (Wang 2006). The interaction of hyaluronan with CD44 is important for drug resistance (Ohwada 2008, Liu 2009), and the ability of hyaluronan oligosaccharides to exert their antagonizing effect depends on this interaction (Misra 2005). Osteopontin provides the cells with multidrug resistance by enhancing the binding of hyaluronan to CD44 (Tajima 2010). The interaction of hyaluronan and CD44 has been reported to lead to increased expression of multidrug resistance transporter 1 (MDR1) (Misra 2005) and multidrug resistance protein 2 (MRP2) providing the cells with chemoresistance (Ohashi 2007). Moreover, it has been shown that hyaluronan and CD44 can form complexes with multidrug transporters, and hyaluronan oligosaccharides inhibit these contacts (Slomiany 2009c, Slomiany 2009a). Moreover, high hyaluronan content around tumor cells prevents the binding of ErbB2 by trastuzumab, an anti-ErbB2 antibody used in breast cancer chemotherapy (Varadi 2012), providing one reason for the negative effect of hyaluronan on the outcome of breast cancer.

2.5.8. Effects of hyaluronan and hyaluronan fragments on inflammation

Many of hyaluronan's functions depend on its molecular size. Hyaluronan oligosaccharides of variable length often have opposite functions as compared to high molecular weight hyaluronan. This size-dependent effect of hyaluronan makes it a very versatile molecule in biological context. Hyaluronan fragments or oligosaccharides have functions in tumor growth and drug resistance, gene expression, inflammation, and they also affect angiogenesis.

High-molecular weight hyaluronan is anti-inflammatory (Delmage 1986), whereas the hyaluronan oligosaccharides have been claimed to be pro-inflammatory (Stern 2006). Elevated levels of hyaluronan have been observed in inflammatory conditions of many diseases, for example arthritis (Goldberg 1991), nephritis (Nakamura 2005), asthma (Cheng 2011), and meningitis (Laurent 1996). Hyaluronan is also accumulated in various injuries, such as skin (Tammi 2005) and lung injury (Jiang 2005). In skin, keratinocyte damage activates dendritic epidermal T cells to produce cytokines that stimulate *Has* expression and hyaluronan synthesis by keratinocytes (Jameson 2005). Upregulation of *HAS* expression and increased intercellular hyaluronan is also seen in inflammatory acute eczema with spongiosis (Ohtani 2009). During inflammation, hyaluronan takes the form of cables that bind to leukocytes (de la Motte 2003, Jokela 2008b, Selbi 2004, Lauer 2009b) and recruit inflammatory cells at the site of inflammation. Hyaluronan also affects inflammation by its actions on cell proliferation and migration.

At the site of inflammation, hyaluronan is degraded by HYAL2 or reactive oxygen species (Monzon 2010, de la Motte 2009, Soltes 2006). Hyaluronan fragments and their interaction with hyaluronan receptors during acute inflammation have been demonstrated to increase the transcription of several inflammatory cytokines resulting in amplified inflammation via activation of NF-κB (Campo 2010). Hyaluronan oligosaccharides have been reported to induce the expression of MMP-3 (Ohno 2005),

MMP-13 (Ohno 2006) and synthesis of nitric oxide (Iacob 2006) in chondrocytes. In addition, hyaluronan oligosaccharides increase synthesis of heat shock protein 72 under stress conditions such as hyperthermia (Xu 2002). However, some of the effects of the hyaluronan oligosaccharides in these studies may be explained by possible contaminants of the reagents, such as lipopolysaccharide, which is known to induce NF-κB (Yasuda 2011). TLR4 and TLR2 have been shown to mediate the responses of hyaluronan oligosaccharides in lung injury (Jiang 2005) and in the activation of dendritic cells in skin (Termeer 2002). In addition, TLR2, by increasing the production of TGF-β, promotes interaction of hyaluronan and RHAMM, leading to increased chemotaxis of macrophages in the lung (Foley 2012). Superoxide dismutase 3 (SOD3) has been shown to prevent degradation of hyaluronan and inhibit inflammation in lung injury (Gao 2008a). SOD3 has also been suggested to prevent inflammation in skin through its effects on TLR-4 (Kwon 2012).

2.5.9. Intracellular hyaluronan

Although hyaluronan is normally synthesized by the hyaluronan synthase at the plasma membrane and extruded into the extracellular space, hyaluronan is also frequently seen intracellularly. There are several reports suggesting functions for the intracellular hyaluronan, but its role remains controversial.

Several intracellular proteins capable of binding to hyaluronan have been found, such as RHAMM (Turley 1982), HABP/P32 (Gupta 1991), CDC37 (Grammatikakis 1995) and USP17 (Shin 2006) and it raises the possibility that these proteins have a function in hyaluronan binding within cells *in vivo*. However, the biological relevance of the ability of these proteins to bind to hyaluronan is not resolved.

Hyaluronan staining has been seen in smooth muscle cells and fibroblasts in the cytoplasm as a network-like pattern and in vesicles in addition to nuclear staining, suggesting a role for hyaluronan in chromosome condensation, nuclear matrix and cytoskeleton (Evanko 1999b). Intracellular hyaluronan staining has also resembled microtubule distribution (Evanko 2004). In contrast to smooth muscle cells, intracellular hyaluronan was seen in cytoplasmic vesicles without any nuclear staining in keratinocytes treated with EGF (Pienimäki 2001). Moreover, it was shown that the intracellular hyaluronan in keratinocytes has its origin at the cell surface and most of the intracellular hyaluronan is endocytosed soon after its synthesis at the plasma membrane, with a half-life of 2-3 h (Tammi 2001). Correspondingly, a large part of the intracellular hyaluronan in aortic smooth muscle cells also colocalizes with a lysosomal marker, suggesting that it is endocytosed and destined for degradation, although some of the hyaluronan was found in the nucleus (Evanko 2004). In the same study it was noticed that while endocytosed high molecular weight hyaluronan remains in larger vesicles, hyaluronan of 50 kD or 300 kD shows a diffuse, network-like pattern, often in the perinuclear area.

Intracellular activation of hyaluronan synthesis has been suggested to occur during conditions of ER stress, leading to the production of hyaluronan cables inside the cell (Hascall 2004). In addition, under hyperglycemic conditions intracellular hyaluronan synthesis can lead to hyaluronan cables that span through the cell (Ren 2009, Wang 2009a, Wang 2011). Different hyaluronan synthases have been proposed to be involved in the synthesis of intracellular hyaluronan. In multiple myeloma, B-cells expressing a splice variant of HAS1 were reported to produce intracellular, presumably cytosolic hyaluronan which modulates RHAMM and leads to the mitotic abnormalities in multiple myeloma (Adamia 2005). In the osteosarcoma cell line MG-63, intracellular hyaluronan produced

by HAS2 accumulates in proliferating osteoblasts, although the source and exact site of the cytoplasmic hyaluronan remained unclear (Nishida 2005).

2.6. HYALURONAN IN CANCER

2.6.1. Alterations of hyaluronan in cancers

The content of hyaluronan is changed in many tumors compared to normal tissues. Tumors originating from simple epithelia show increased hyaluronan content in cancer cells, while the normal epithelial tissue is devoid of hyaluronan. On the contrary, hyaluronan is abundant in stratified epithelia, but in poorly differentiated tumors the amount of hyaluronan in cancer cells is decreased (Tammi 2008). Stromal hyaluronan is increased in many tumors correlating with poor patient prognosis (Tammi 2008). The interplay between the tumor cells and the surrounding stroma makes the role of hyaluronan in cancers especially fascinating. For example, melanoma cells can induce hyaluronan synthesis in surrounding fibroblasts by secreting PDGF, leading to activation of fibroblast *HAS2* (Willenberg 2012, Pasonen-Seppänen 2012b) and as a consequence of the increased hyaluronan, melanoma cells increase their proliferation (Willenberg 2012).

Benign cutaneous nevomelanocytic lesions show intense staining of CD44, while the intensity is decreased in melanoma, inversely correlating with the size, depth and level of invasion (Leigh 1996). In localized cutaneous melanoma, reduced stainings of CD44 and hyaluronan is associated with poor patient prognosis (Karjalainen 2000). *In situ* carcinomas and well-differentiated squamous cell carcinomas of the skin show enhanced HA and CD44 staining compared to normal epidermis, while in poorly differentiated SCC and basal cell carcinoma the intensities are reduced (Karvinen 2003a). Hyaluronan and CD44 display similar changes in squamous cell carcinomas originating from different anatomical sites like the larynx (Hirvikoski 1999), mouth (Kosunen 2004), esophagus (Wang 1996b), vulva (Hämäläinen 2010) and lung (Pirinen 1998), as seen in SCC of skin. Reduced CD44 staining is also found in uterine high-grade intraepithelial neoplasia and invasive SCC (Davidson 1998). The irregular, reduced staining pattern of hyaluronan and CD44 associates with nodal involvement and poor patient outcome in laryngeal and oral SCC (Hirvikoski 1999, Kosunen 2004) and to distant metastasis in laryngeal SCC (Hirvikoski 1999). In nonmelanoma skin tumors, the depletion of CD44 associates with increased staining of MMP-7 (Hartmann-Petersen 2009).

Distribution of hyaluronan in adenocarcinomas is different compared to the squamous cell carcinomas described above. In benign breast lesions, hyaluronan is present in the stromal connective tissue and the ductal epithelial cells are negative for hyaluronan. In breast adenocarcinomas, the stromal hyaluronan is increased compared to normal tissue and hyaluronan is also detected in some of the tumor cells (Auvinen 1997), associating strongly with tumor progression and patient survival (Auvinen 2000, Auvinen 2013). Hyaluronant content in ductal carcinoma in situ (DCIS) with a microinvasive component is higher than in plain DCIS (Corte 2010), and the stroma around lymph node metastases is enriched with hyaluronan (Afify 2008). Interestingly, increased expression of CD44 in the stromal cells was recently found to correlate to large tumor size, poor differentiation, relapses and poor survival (Auvinen 2013).

Other adenocarcinomas show similar changes to breast cancer in hyaluronan and CD44 staining. These include ovarian cancer (Hiltunen 2002, Anttila 2000), endometrial carcinoma (Afify 2005), lung adenocarcinoma (Pirinen 2001), gastric cancers (Setälä 1999), prostate adenocarcinoma (Lipponen 2001, Lokeshwar 2001, Josefsson 2011) and bladder cancer (Hautmann 2001). In addition to solid tumors, hyaluronan content is altered in

hematological malignancies, like in the bone marrow of acute myeloid leukemia patients (Sundström 2005).

2.6.2. Hyaluronan synthases in cancer

As hyaluronan content is known to be changed in many cancers, possible alterations in the expression of hyaluronan synthases have been studied to explain the underlying mechanisms for it. Increased expression of HAS1 associates with poor patient survival in ovarian cancer (Yabushita 2004), colon cancer (Yamada 2004b), Waldenström's macroglobulinemia (Adamia 2003) and multiple myeloma (Adamia 2005). *HAS1* expression is also increased in bladder cancer, correlating with increased hyaluronan levels (Golshani 2007), and predicting metastasis (Kramer 2011). In serous ovarian tumors, *HAS1* has been shown to be very low or totally absent, whereas the levels of *HAS2* and *HAS3* mRNA or staining levels are not elevated compared to normal ovaries or benign tumors (Nykopp 2009). Interestingly, in endometrial carcinoma the immunoreactivity of all HAS proteins is increased without alterations in the mRNA levels of *HAS1-3*, suggesting that the reduced turnover of HAS participates in the accumulation of hyaluronan (Nykopp 2010).

Overexpression of *Has2* has been shown to increase tumor growth both *in vivo* and *in vitro* in an experimental rat colon carcinoma cell line (Jacobson 2002). Transgenic mice with overexpression of *Has2* in mammary glands developed poorly differentiated adenocarcinomas with increased intratumoral stroma in addition to enhanced angiogenesis and tumor growth (Koyama 2007). Moreover, colon carcinoma cells isolated from lymph node metastasis have more pericellular hyaluronan and *HAS3* mRNA compared to primary tumors, and inhibition of *HAS3* with antisense cDNA results in impaired anchorage-independent growth, emphasizing the importance of hyaluronan and HAS activity in tumor progression (Bullard 2003).

2.6.3. Hyaluronidases in cancer

Hyaluronidases are proposed to be both tumor promoters and tumor suppressors. This is partly explained by their localization to chromosome region 3p21.3, which often contains deletions in lung (Wistuba 2000), breast (Maitra 2001) and epithelial ovarian (Tuhkanen 2004) cancers. The effect of HYAL1 on cancer progression, whether suppressing or promoting, has been suggested to be concentration-dependent (Lokeshwar 2005).

Increased hyaluronidase levels have been reported in many cancers, like prostate cancer (Lokeshwar 1996), bladder cancer (Hautmann 2001), head and neck SCC (Franzmann 2003) and breast adenocarcinoma (Madan 1999, Udabage 2005), correlating with invasiveness, and the hyaluronidase level may be useful as a prognostic marker in some cancers. The immunohistochemical staining intensity of HYAL1 is an independent prognostic factor in prostate cancer (Posey 2003). Urine HYAL1 content combined with hyaluronan levels can be used as a sensitive and specific test to detect bladder cancer and its grade (Lokeshwar 2000), and *HYAL1* expression also predicts metastasis in bladder cancer (Kramer 2011). *HYAL1* is associated with poor prognosis in lung adenocarcinoma and SCC (de Sa 2012). On the contrary, decreased hyaluronidase levels are also observed in cancers. In serous ovarian cancer, the level of *HYAL1* is decreased, inversely correlating with hyaluronan content (Nykopp 2009). Similarly, in endometrial carcinoma the mRNA level of *HYAL1* and *HYAL2* were downregulated, correlating with increased hyaluronan in the tumor (Nykopp 2010).

Hyaluronidases produce hyaluronan fragments in tumor tissue and may facilitate tumor progression, as the fragments have their own biological activities, for example

cleavage of CD44 and enhancement of cell motility (Sugahara 2003) or enhanced angiogenesis (Liu 1996). On the other hand, overexpression of *Hyal1* in an experimental rat colon carcinoma cell line decreases tumor growth both *in vitro* and *in vivo*, and the tumors have larger necrotic areas than controls (Jacobson 2002). In line with this, intravenous hyaluronidase administered to mice bearing human breast carcinoma xenografts reduced tumor volume as well as hyaluronan staining (Shuster 2002). Hyaluronidase treatment has been shown to enhance the response to chemotherapy in several experimental cancer models like human melanomas implanted on mice and treated with regional vinblastine chemotherapy (Spruss 1995), and mammary carcinoma in a mouse model, treated with adriamycin (Beckenlehner 1992). Recently, a PEGylated human recombinant PH20 was tested in a mouse model of pancreatic ductal adenocarcinoma and it was shown to decrease tumoral hyaluronan, leading to increased intratumoral delivery of cancer drugs and macromolecular permeability of the tumors (Jacobetz 2012). Moreover, it has been suggested that the hyaluronan content of the tumor could be used as a biomarker predicting the response of the tumor to hyaluronan depletion by PEGylated PH20 (Jiang 2012).

2.7. CUTANEOUS MALIGNANCIES INDUCED BY ULTRAVIOLET RADIATION

2.7.1. Structure and function of human skin

Skin is the largest organ of the human body, accounting for about 15% of total body weight and providing important protection against chemical, physical and biological impacts. The thickness of the skin varies depending on body location. The most superficial layer of the skin is the epidermis, separated from the dermis by the dermo-epidermal junction, while hypodermis or subcutaneous tissue forms the bottom layer of the skin. Skin also contains epidermal appendages such as sweat glands, pilosebaceous glands, hair and nails, in addition to nerves and vasculature (Kanitakis 2002). The epidermis is of ectodermal origin, populated early in life by three cell types in addition to keratinocytes: bone-marrow-derived, antigen presenting Langerhans cells, neural crest-derived melanocytes and neuroendocrine Merkel cells probably originating from neural crest, while the dermis and hypodermis are mesodermal (Burgdorf 2009). The origin of the Merkel cell is unclear, as they have also been suggested to derive from epidermal keratinocytes (Freinkel 2001).

The epidermis is a multi-layered (stratified), self-renewing epithelium (Kanitakis 2002, Burgdorf 2009) (Figure 4). The main cell type (90-95% of all cells) of the epidermis is the keratinocyte (Kanitakis 2002). The keratinocytes differentiate terminally as they migrate from the basal layers up towards the surface of the skin, where they are shed away. During this process, cuboidal cells with large nuclei become flattened and anucleated (Kanitakis 2002). In psoriasis, the turnover time of the keratinocytes is decreased (Burgdorf 2009). The keratinocytes in the various stages of the differentiation process produce continuous layers in the epidermis: the deepest layer is the basal layer, containing dividing cells giving rise to new keratinocytes and primitive stem cells, followed by the prickle-cell or spinous layer (stratum spinosum), the granular layer and cornified layer (Kanitakis 2002). The skin stem cells are located in the basal layer of interfollicular epidermis, but also in the hair follicle and sebaceous gland (Fuchs 2008). The skin stem cells have been proposed to be involved in skin cancers, but their exact role in skin biology remains unresolved (Blanpain 2009, Arwert 2012).

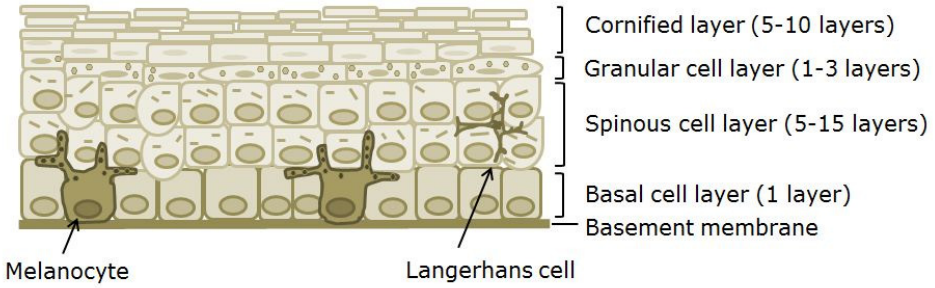

Figure 4. Layers of the epidermis.

The cytoskeleton of a keratinocyte is made of keratins, which are the major structural proteins of the epidermis (Kanitakis 2002, Fuchs 1995). Keratin filaments make stable intra- and intermolecular associations in the epidermis, providing keratinocytes with mechanical strength (Fuchs 1995). The basal layer keratinocytes are attached to the basement membrane below the cells by special structures called hemidesmosomes, whereas adjacent cells are held together by desmosomes containing keratin (K) bundles, which give this layer its spinous appearance (Kanitakis 2002, Freinkel 2001). The keratin filaments of the basal layer consist of K5 and K14, while K1 and K10 are expressed in the spinous layer (Fuchs 1995). The granular layer has keratohyalin granules, containing mainly profilaggrin and keratin, in the cytoplasm of the cells (Kanitakis 2002, Sandilands 2009). Lack of functional filaggrin protein due to mutations in the profilaggrin gene and the resulting defective skin barrier are associated with the pathogenesis of atopic eczema (Sandilands 2009). The granular layer cells also contain lamellar bodies (Kanitakis 2002). The proteins produced by the spinous and granular layer cells interconnect with the lipid lamellae in the cornified layer and form the cornified cell envelope important for the penetration barrier of the skin (Kanitakis 2002, Sandilands 2009, Morita 2003). Thus, the cornified layer of the skin consists of flat, hexagonal, terminally differentiated corneocytes containing a thick cornified envelope and a dense, filamentous keratin matrix. Eventually the corneocytes desquamate from the surface of the skin (Kanitakis 2002).

In addition to keratinocytes, melanocytes are present in the skin at a ratio of approximately 1 melanocyte for 4-10 basal keratinocytes (Kanitakis 2002). The function of melanocytes is the production and transfer of melanin, the main determinant of skin color (Burgdorf 2009). Melanocytes produce melanin from tyrosine and pack it in melanosomes that are transported along the dendritic processes of the melanocytes and further transferred to adjacent keratinocytes, forming a protective cover over the keratinocyte nucleus (Kanitakis 2002). The epidermal melanin unit consists of one melanocyte and the associated keratinocytes to which it provides with melanin (Kanitakis 2002). The melanin content of skin affects its resistance to UV light-induced damage, as light skin has 30-40-fold higher risk for skin cancer than dark skin (Hearing 2011).

The epidermis and dermis are separated by the dermo-epidermal junction, which is a sheet-like basement membrane zone consisting of extracellular matrix proteins. The basement membrane acts as an adhesion interface between the epidermal cells and the dermal connective tissue, provides a permeability barrier and controls cell behavior through mutual interactions between cell-surface receptors and the extracellular matrix molecules (Masunaga 2006). The superficial layer of the dermis is organized as dermal papillae, fingerlike upward projections that provide the skin with support, while the deeper part is reticular and contains coarser fiber bundles, and vascular and nerve plexuses (Kanitakis 2002). The dermis consists of collagen fibers, elastic fibers (elastin),

proteoglycans, fibroblasts and other connective tissue cells, and mast cells (Kanitakis 2002, Burgdorf 2009). Most of the dermal fibers (>90%) are made of type I and III collagens, but also type IV and VII collagens are present. In addition to fiber structure, the dermis also contains macromolecules such as glycoproteins and proteoglycans. Spindle-shaped fibroblasts are the most abundant cell type of the dermal connective tissue, capable of producing different types of fibers and macromolecules (Kanitakis 2002).

2.7.2. Squamous cell carcinoma

Non-melanoma skin cancers include squamous cell carcinoma (SCC) and basal cell carcinoma. Squamous cell carcinoma is the second most common skin cancer (LeBoit 2006). The NORDCAN database shows that the yearly incidence of non-melanoma skin cancers has increased by 4.8% in women and by 3.4% in men during the past 10 years in the Nordic countries. According to the Finnish Cancer Registry, there are approximately 1500 new cases of squamous cell carcinoma each year in Finland, and it is the 5th most common cancer in women and 4th most common cancer in men in Finland (Finnish Cancer Registry 2012). Squamous cell carcinoma arises from epidermal keratinocytes and includes the *in situ* form (Bowen disease) in addition to invasive SCC (Burgdorf 2009, LeBoit 2006). Actinic keratosis is a premalignant dysplasia often preceding SCC (Bonerandi 2011). The percentage of actinic keratosis developing to SCC varies in different studies, but an average of 10% has been suggested (Glogau 2000). Also the percentage of SCC arising from actinic keratosis varies. According to one source, most SCC's are suggested to arise from actinic keratosis (LeBoit 2006), whereas 20-27% is found elsewhere (Cabral 2011). Clinically, SCC typically presents raised infiltrating tumors with a budding and bleeding center (Bonerandi 2011).

Excessive exposure to UV radiation is the major risk factor for SCC (Melnikova 2005), but other risk factors include radiation therapy, previous skin burns, arsenic, coal tar, industrial carcinogens, immunosuppression, inflammatory lesions and chronic wounds (LeBoit 2006, Bonerandi 2011). Also human papilloma virus (HPV) infection has been suggested to be involved in skin carcinogenesis. Several HPV types have been found in skin samples from renal transplant patients presenting actinic keratosis and SCC (Berkhout 2000). In addition, mice overexpressing HPV oncogenes have been shown to develop lesions resembling human actinic keratosis and SCC when exposed to chronic UV irradiation (Viarisio 2011).

Mutations causing inactivation of the p53 tumor suppressor gene are especially important for the development of SCC and such mutations are typically found in epidermal keratinocytes exposed to solar radiation very early, before the actual tumor develops (de Gruijl 2008, Klein 2010). Brash and colleagues discovered in 1991 that the majority of human skin SCC's contain mutations in p53 and these mutations are dipyrimidine substitutions (C→T, CC→TT), which are the mutations caused by UV (Brash 1991). Sunlight-induced p53 mutations are also found in premalignant human actinic keratosis and the number of apoptotic, sunburn cells is reduced in p53-negative skin of mice (Ziegler 1994). In addition to creating new mutations, UVB exposure has been suggested to enhance the growth of pre-existing p53 mutant clones in the skin (Klein 2010). Moreover, p53 mutations cause upregulation of CD44 expression, indicating that the consequences of p53 loss in cancer might result from increased CD44 (Godar 2008).

In addition to p53, Fas/Fas-Ligand (Fas-L) interactions are needed for the apoptosis of cells containing UV-induced DNA damage (Hill 1999). In the same study, it was observed that the absence of Fas/Fas-L interaction results in accumulation of p53 mutations (Hill

1999). Chronic exposure to UV has been shown to decrease Fas/Fas-L in keratinocytes and loss of Fas-L expression is also seen in papillomas and SCC induced by chronic UV in mice (Ouhtit 2000). Also in human skin, the level of Fas-L is first increased at 14h after a single exposure to UV, but then decreased while Fas is continuously increased (Bachmann 2001). However, in human invasive SCC the expression of Fas-L is again increased, while Fas levels are reduced (Bachmann 2001), suggesting that Fas/Fas-L levels vary depending on the stage of the UV-induced lesion. Also aberrant activation of EGFR (Kolev 2008) and Fyn, a Src-family tyrosine kinase (Zhao 2009) have been found in human SCC, and these proteins decrease p53. Moreover, amplification and mutations of the *ras* oncogene have been found in SCC (Pierceall 1991, Ratushny 2012). Interestingly, keratinocyte growth factor (KGF), which stimulates migration and activates *Has2* and *Has3* in keratinocytes (Karvinen 2003b), suppresses the malignant phenotype of SCC (Toriseva 2012), partly perhaps by restoring the decreased hyaluronan content found in SCC.

Histologically, SCC consists of irregular malignant squamous cell colonies extending from the epidermis into the dermis, which often shows a marked inflammatory reaction. The cells have eosinophilic cytoplasm with a large nucleus, and keratinization and horn pearl production are often seen inside the nests. The tumors are graded by the degree of anaplasia (LeBoit 2006). SCC is usually only locally invasive and metastatic disease is rare (Bonerandi 2011, Cranmer 2010). Local SCC can be treated with surgery, while metastatic disease requires systemic therapies (Bonerandi 2011). More aggressive forms of SCC are seen in immunocompromised patients (LeBoit 2006).

2.7.3. Melanoma

Melanoma is the most fatal skin cancer with a rapidly increasing incidence worldwide (Garbe 2009, Simard 2012). According to the NORDCAN database, the yearly incidence of melanoma has increased by 4.3% both in women and men during the past 10 years in the Nordic countries. In Finland, it is the 6th most common cancer in women and 7th most common cancer in men, with about 1200 new cases each year (Finnish Cancer Registry. 2012). Most melanoma patients are relatively young adults (LeBoit 2006) and it is becoming a disease of young women (Zaidi 2012). Cutaneous melanoma arises from epidermal melanocytes. According to WHO classification, the main subtypes are lentigo maligna melanoma, superficial spreading melanoma, nodular melanoma and acral lentiginous melanoma. Lentigo maligna is melanoma *in situ* and when accompanied by dermal invasion, it is called lentigo maligna melanoma. Superficial spreading melanoma is the most common subtype in Caucasians and its prognosis is favorable. Nodular melanoma is the most aggressive form of melanoma. Acral lentiginous melanoma is often found in hands, feet or under the nails. Clinically, melanoma can have various forms, but the classical features include asymmetry and uneven pigmentation in a flat macular or nodular lesion. A variant of melanoma is amelanotic melanoma, in which the pigmentation is totally absent (LeBoit 2006). High amount of benign nevi and especially atypical, dysplastic nevi are associated with increased risk for melanoma (Gandini 2005), but most melanomas develop *de novo* on healthy skin (Seykora 1996). About 5-10% of melanomas are familial (Garbe 2010). Factors associated with melanoma risk include skin type, number of nevi, having atypical nevi and positive family history of skin cancer (LeBoit 2006). However, exposure to UV radiation is considered as the major risk factor for melanoma (Kanavy 2011).

Many different mutations are known to occur in melanoma. Although the p53 mutations are well-characterized mutations in SCC, no definitive UV-induced mutations have been observed in melanoma (Zaidi 2012). However, p53 expression in melanoma is

inversely correlated with a high number of nevi, and associate with a skin type that burns easily (Richmond-Sinclair 2008). Up to 40% of families with familial melanoma have mutations in the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene, encoding p16 and p14 in alternate reading frames involved in cell-cycle control (Goldstein 2007). Mutations in the CDKN2A gene are more probable in individuals with multiple primary melanoma (Helsing 2008). BRAF mutations are found in a variety of cancers, but the highest frequency is in melanoma, as 66% of primary melanomas present this mutation (Davies 2002). The mutated BRAF protein acts as an activated kinase and it can transform cells (Davies 2002). In addition to BRAF, *ras* oncogene mutations are found in melanoma (Ball 1994) and either one of these mutations can activate the Ras-Raf-MEK-ERK/MAPK signaling pathway (Omholt 2003). Interestingly, BRAF and Ras mutations do not seem to exist in the same lesions, indicating a complementary effect of them on tumor progression (Omholt 2003). UVB-induced mutations of PTEN tumor suppressor gene are present in melanoma (Guldberg 1997), and especially in patients with xeroderma pigmentosum (Wang 2009b), which is a genetic disorder with defective DNA repair, resulting in a 1000-fold increased risk for melanoma. Activating mutations in the KIT oncogene, a gene essential for melanocyte survival and development, are found in acral and mucosal melanoma as well as in melanomas of chronically sun-damaged skin (Curtin 2006). In addition, many small (~22nt), non-coding regulatory RNA molecules (microRNAs) are deregulated in melanoma (Glud 2012), further complicating the concept of cellular events leading to melanoma.

Melanomas are diagnosed by their architectural and cytological features and as many of these are shared by benign nevi, the diagnosis can be challenging. Histologically, melanocytes vary in size, degree of pigmentation and shape, which can be round, oval, spindle-shaped, or thin and dendritic. Melanocytes usually have only little of cytoplasm and their nuclei are angular and dark. Melanocytes can form nests of cells both in benign and malignant lesions. The growth phases of melanoma are divided as radial, commonly seen in melanoma *in situ*, and vertical (tumorigenic), in which the cells invade into the dermis and are able to proliferate (LeBoit 2006). Early melanoma lesions can often be cured with primary surgery, but the prognosis is poor in advanced stages, due to its therapy resistance (Garbe 2010). Metastatic melanoma is treated with surgery, accompanied with radiation therapy and adjuvant therapies including chemotherapy and immunotherapy (Garbe 2010).

2.7.4. Properties of ultraviolet radiation

Ultraviolet radiation (UVR) is part of the electromagnetic irradiation and its spectrum lies between visible light and X-rays. UVR is divided into three sections based on its wavelengths: UVA (320-400 nm), UVB (280-320 nm) and UVC (100-280 nm). Only UVB and UVA can reach the Earth's surface, as UVC is blocked by the ozone layer (Matsumura 2004). UVR is widely known for its role in carcinogenesis, and it is considered the main cause of skin SCC, melanoma and basal cell carcinomas (Armstrong 2001). UVB causes also erythema and skin burns, while UVA causes skin aging and wrinkling (Matsumura 2004). UVR causes local and systemic immunosuppression in addition to DNA damage, formation of oxygen radicals, lipid peroxidation and molecular isomerization (Kripke 1994).

2.7.5. Effects of ultraviolet radiation on skin hyaluronan

Many skin cancers present aberrant levels of hyaluronan. UV exposure affects skin hyaluronan both in human and mouse *in vivo* and in cell culture experiments, but the

results vary depending on the exposure time and dose used. Hairless mouse skin irradiated with sun lamps three times a week for 10 weeks show elastic fiber hyperplasia and increased hyaluronan, chondroitin sulphate and fibronectin quantities (Schwartz 1988). In another study, exposure of hairless mice to UV (16.3 J/cm^2) five times a week for 20 weeks resulted in a thickened epidermis and increased hyaluronan, chondroitin sulphate and dermatan sulphate contents in the skin and these accumulations could be prevented by hydrocortisone (Mitani 1999). However, when mice were exposed to 210 mJ/cm^2 UVB three times per week for a longer time period of 182 days, loss of dermal hyaluronan and down-regulation of *Has1-3* mRNA was reported (Dai 2007).

A single dose of UVA (10 J/cm^2) or UVB (1 J/cm^2) has been shown to decrease epidermal hyaluronan and CD44 in mouse skin 2 h after the UV exposure, but the levels are recovered in 24 h (Calikoglu 2006). In line with this mouse model, *HAS2* was decreased in human keratinocytes (HaCaT) 3 h after exposure to UVB (30 mJ/cm^2), while upregulation of all three hyaluronan synthases occurred 24 h post-irradiation (Averbeck 2007). Interestingly, *HAS1* was already increased after 3 h (Averbeck 2007). In another study, a single dose of UVB (10 mJ/cm^2) decreased the expression of *HAS2* and *HYAL2* in HaCat cells 6 h after irradiation (Hasova 2011). Hyaluronan synthesis in human keratinocytes shows a biphasic dose-response: low doses (10-30 mJ) of UVB stimulate the secretion of hyaluronan and the expression of *HAS2* and *HAS3*, while higher doses (>50 mJ) inhibit those (Kakizaki 2008). In the same study, a moderate UVA dose shows minor effects on hyaluronan synthesis, while high UVA doses slightly inhibit hyaluronan content and *HAS* expression (Kakizaki 2008). Similarly, Tobiishi and colleagues used a single low dose of 0.15 J/cm^2 UVB on hairless mice and found that *Has2* mRNA is increased on day 1 and *Has3* on days 1-2 after exposure (Tobiishi 2011).

It has been suggested that collagen fragments derived from UVB-induced collagen damage down-regulate *HAS2*, leading to reduced hyaluronan synthesis in fibroblasts (Röck 2011). In addition to hyaluronan synthases, the role of hyaluronidases has been studied in UV-induced hyaluronan metabolism. Using a reconstructed epidermis model irradiated once with 40 mJ/cm^2, *HYAL1* mRNA increased at 6 h and then decreased progressively until 48 h, while *HYAL2* and *HYAL3* mRNA levels were decreased at 6h and returned to basal level at 48 h (Kurdykowski 2011). In the same study, HYAL1 increased, while HYAL2 decreased and HYAL3 remained unchanged at the protein level (Kurdykowski 2011). UV exposure has also been shown to affect the molecular size of hyaluronan. The average molecular mass of HA is decreased from 1000 kDA to 100 kDa on day 3 after a single dose of 0.15 J/cm^2 UVB (Tobiishi 2011).

3 Aims of the study

Hyaluronan is an extracellular matrix molecule with a simple primary structure, but diverse functions. Hyaluronan is especially abundant in the skin and its content is often changed in tumors. Since the discovery of the hyaluronan synthase genes, knowledge on the synthesis of hyaluronan and its regulation has expanded enormously. However, the exact role of the synthases in UV-induced cutaneous tumors is not known, and the subcellular trafficking, plasma membrane targeting and regulation of their enzymatic activity remain poorly understood. In addition, the localization and possible functions of intracellular hyaluronan remain unresolved. The aim of this thesis was to investigate hyaluronan and proteins associated with its synthesis, binding and degradation, particularly in UV-induced squamous cell carcinoma and melanoma, and to examine the role of intracellular hyaluronan.

The specific aims of this thesis were:

1. To investigate the subcellular localization, trafficking and functions of HASs, utilizing normal and mutated HASs with a fluorescent tag.

2. To optimize a method for microinjection and to study how fluorescent hyaluronan and its fragments microinjected into the cytosol are distributed within cells, and whether they influence hyaluronan synthesis.

3. To study the influences of long-term, high-dose UVB irradiation on epidermal hyaluronan, CD44 and HAS1-3 in a mouse model using histochemical stainings.

4. To analyze the histochemical staining patterns of hyaluronan, CD44, HAS1-3 and HYAL1-2 in benign cutaneous lesions and malignant melanoma.

4 Materials and methods

4.1. MATERIALS

4.1.1. Cell lines

The cell lines used in this thesis are shown in Table 2. Specific culture conditions are presented in the original publications. When studying the pericellular hyaluronan coat in Has1-transfected cells (II), the cells were incubated with 1 mM glucosamine, 20 mM glucose, 10 ng/ml IL-1β, 10 ng/ml TNF-α and 10 ng/ml TGF-β.

Table 2. Cell lines used in the project.

Origin of cells	**Name of cells**	**Original publication**	**Reference**
Newborn rat skin keratinocytes	REK	I	(Baden 1983)
Human dermal fibroblast	Fibroblast	II	Dr. Michael Edward, Univ. of Glasgow, UK
Human epidermal keratinocyte	HaCaT	II	(Boukamp 1988)
African green monkey kidney	COS-1	II	(Gluzman 1981)
Human breast adenocarcinoma	MCF-7	II, III	(Soule 1973)
Human mesothelium	LP-9	III	Institute of Clinical Medicine/Clinical Pathology, Univ. of Eastern Finland
Human breast adenocarcinoma cell line with doxycycline-inducible overexpression of EGFP-HAS3	EGFP-HAS3-MCF-7	III	Dr. Genevieve Bart, Univ. of Eastern Finland

4.1.2. Tissue specimens

A short scheme of the study protocol used to investigate the effect of chronic UV exposure to hyaluronan metabolism in mouse skin is shown in Figure 5. The specific details of the study are presented in the original publication (IV) and (Kumlin 1998).

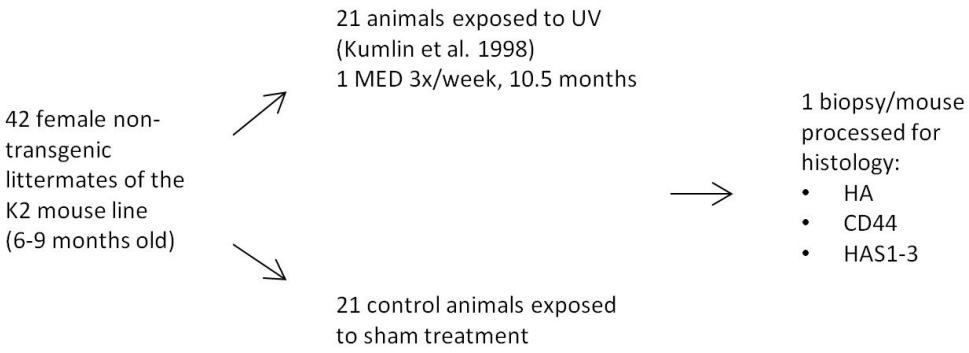

Figure 5. Study protocol of UV exposures.

A chart showing the specimen groups used to study hyaluronan metabolism in various human melanocytic lesions (V) is shown in Figure 6. The specific details of the study are presented in the original publication (V).

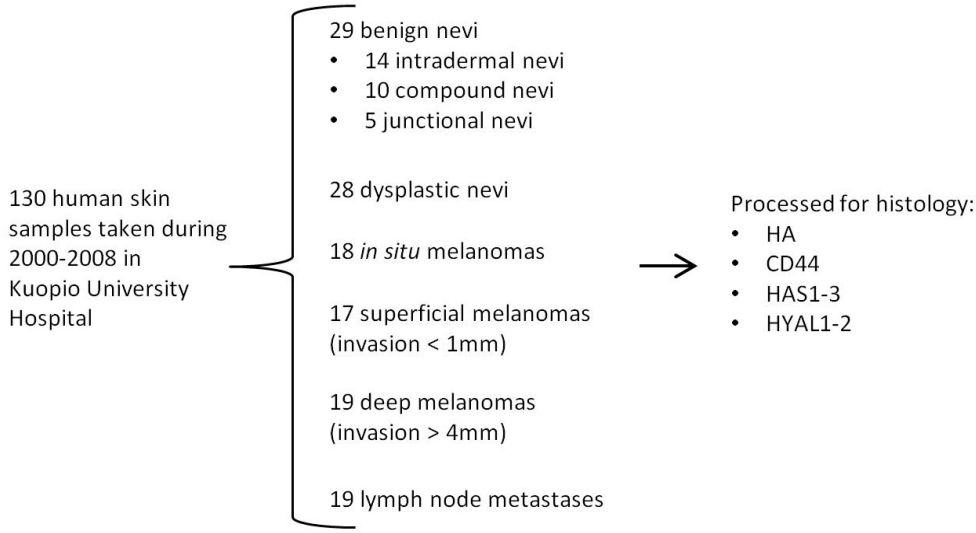

Figure 6. Groups of melanocytic lesions.

4.2. METHODS

The methods used in this thesis are listed in Tables 3-6. Specific details of every method are presented in the original publications (I-V). Table 3 itemizes the methods used to study the content and intracellular localization of hyaluronan. Table 4 lists the plasmids used for transfections. Table 5 shows the treatments of cells used for studying localization of HASs or hyaluronan synthesis. Table 6 shows the antibodies used for histological staining of tissue samples.

Table 3. Methods used to study hyaluronan. Specific details are presented in the original publications.

Purpose	**Method**	**Original publication**	**Reference**
Content of hyaluronan	Enzyme-linked sorbent assay for hyaluronan	I, II	(Hiltunen 2002)
Demonstration of hyaluronan in fixed cell cultures	Staining with biotinylated HABC (bHABC) for fluorescence microscopy	I, II, III	(Tammi 1994, Tammi 1998)
Demonstration of hyaluronan in live cells	Staining with fluorescent Hyaluronan Binding Complex (fHABC) for fluorescence microscopy	II, III	(Rilla 2008)
Demonstration of possible intracellular hyaluronan in live cells	Microinjection of fHABC	III	Method optimized in original publication III

Table 4. Plasmids used to study proteins. Specific details are presented in the original publications.

Purpose	**Method**	**Original Publication**	**Reference**
Overexpression of mouse *Has2* and *Has3*	Transient transfection with mouse *Has*-EGFPN-constructs	I	(Spicer 1999)
Overexpression of mouse *Has3* with an inactivating mutation	Transient transfection with mouse *Has3*-EGFPN-construct containing a missense mutation at D216A	I	(Spicer 1999)
Overexpression of mouse *Has3* with C-terminal truncations	Transient transfection with mouse *Has3*- EGFPN-constructs containing truncations (E539stop and E510stop)	I	(Spicer 1999)
Overexpression of human *HAS* with EGFP- construct	Transient EGFP-*HAS*-transfection	II, III	hHas3-pEGFP-C1-construct made by M.Sc. Hannu Karjalainen, Univ. of Eastern Finland and hHas1-pEGFP-C1-construct made by Dr. Genevieve Bart, Univ. of Eastern Finland
Overexpression of human *HAS* with Dendra-construct	Transient *HAS*-pDendra2-C transfection	II	HAS-pDendra2-C-constructs made by Dr. Juha Hyttinen, Univ. of Eastern Finland
Visualization of the Golgi apparatus	Transient GnTII- pcDNA3-mCerulean transfection	II	Dr. Sakari Kellokumpu, Univ. of Oulu

Table 5. Treatments used to study the localization of HASs or hyaluronan in cells. Specific details are presented in the original publications.

Purpose	**Treatment/method**	**Original publication**	**Reference**
Breakdown of microtubules	Nocodazole	I	Sigma-Aldrich, St. Louis, MO, USA, (De Brabander 1976)
Demonstration of fluid phase endocytosis	Alexa Fluor®568 hydrazide	I, III	Molecular Probes, Eugene, OR, USA
Removal of hyaluronan	*Streptomyces* hyaluronidase	I, II, III	Seikagaku Kogyo Co., Tokyo, Japan
Interference with vesicular traffic	Brefeldin A	I, II	Sigma-Aldrich, (Klausner 1992)
Inhibition of protein synthesis	Cycloheximide	I	Sigma-Aldrich, (Obrig 1971)
Inhibition of hyaluronan synthesis	Incubation of cells with 4-MU	I, II	(Rilla 2004)
Visualization of the Golgi	Staining with anti-58K antibody	I	Sigma-Aldrich
Visualization of the Golgi	Staining with trans-Golgi network antibody	I	Affinity Bioreagents, Golden, CO, USA
Visualization of the Golgi	Incubation of cells with Bodipy ® TR Ceramide or staining with anti-human Golgin 97 antibody	II	Molecular Probes, Eugene, OR, USA
Visualization of the ER	Staining with anti-calnexin antibody	I	StressGen Biotechnologies Corp., Victoria, British Columbia, Canada
Visualization of the ER	Incubation of cells with ER-Tracker®	II	Molecular Probes, Eugene, OR, USA
Interference with the binding of hyaluronan and its receptor	Incubation of cells with HA6 oligosaccharide	II	Seikagaku Kogyo Co., Tokyo, Japan, (Rilla 2008, Knudson 1996)
Interference with the binding of hyaluronan and CD44	Incubation of cells with Hermes1 (anti-CD44 monoclonal antibody)	II	Developmental Studies Hybridoma Bank, Iowa city, Iowa, USA, (Pasonen-Seppänen 2012a)
Interference with hyaluronan synthesis	Injection of glucose, mannose, hyaluronan oligosaccharides or *Streptomyces* hyaluronidase	III	(Jokela 2008a), injection method optimized in III

Table 6. Methods used to analyze hyaluronan, CD44, HAS1-3 and HYAL1-2 in tissue sections with light microscopy. Specific details are presented in the original publications.

Purpose	**Method**	**Original publication**	**Reference**
Demonstration of hyaluronan in tissue sections	Staining with bHABC	IV, V	(Wang 1992, Tammi 1994)
Demonstration of CD44 in mouse tissue sections	Staining with an IM7 antibody recognizing all splice variants of CD44	IV	Dr. Jayne Lesley, San Diego, CA, USA
Demonstration of CD44 in human tissue sections	Staining with a Hermes3 antibody recognizing all splice variants of CD44	V	Dr. Sirpa Jalkanen, University of Turku, Finland
Demonstration of HAS1 in tissue sections	Staining with a polyclonal antibody for HAS1	IV, V	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Demonstration of HAS2 in tissue sections	Staining with a polyclonal antibody for HAS2	IV, V	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Demonstration of HAS3 in tissue sections	Staining with a polyclonal antibody for HAS3	IV, V	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Demonstration of HYAL1 in tissue sections	Staining with a polyclonal antibody for HYAL1	V	Atlas Antibodies, Stockholm, Sweden
Demonstration of HYAL2 in tissue sections	Staining with a polyclonal antibody for HYAL2	V	Abcam, Cambridge, UK

5 Results

5.1. LOCALIZATION AND TRAFFIC OF EGFP-HAS2 AND EGFP-HAS3 (I)

5.1.1. Enzymatic activity and cellular distribution of EGFP-HAS fusion proteins

Rat epidermal keratinocytes transfected with enhanced green fluorescent protein (EGFP)-*Has2* or EGFP–*Has3* showed an increased production of hyaluronan compared to control cells transfected with the empty EGFP vector only. Production of hyaluronan was increased about 2.5-fold in cells transfected with EGFP-*Has2* and about 2-fold in cells transfected with EGFP-*Has3* compared to EGFP-transfected controls. The mutant constructs containing a missense mutation close to the enzyme active site (D216A) or C-terminal truncations (R539Stop or E510Stop) produced hyaluronan close to the level of the controls (I: Fig. 1). These results indicated that the EGFP-*Has* constructs were enzymatically active and capable of producing increased amounts of hyaluronan when overexpressed in keratinocytes.

The cellular localization of EGFP-HAS2 and EGFP-HAS3 was similar (I: Fig. 2A-E). Both showed a vesicular appearance around the nucleus, resembling the ER-Golgi complex. Nuclear localization was not seen. The EGFP-HAS2 and EGFP-HAS3 were also found at the plasma membrane, although EGFP-HAS3 produced a more intense signal at the cell surface. Both of these EGFP-proteins were located in protrusions of the plasma membrane at the cell surface and the tips of these extensions were especially strongly stained. The empty EGFP vector was diffusely spread in the cytoplasm without any enrichment sites (I: Fig. 2F).

5.1.2. EGFP-HAS colocalization with subcellular markers and hyaluronan

Plasma membrane residence of EGFP-HAS2 and EGFP-HAS3 was confirmed with anti-CD44 immunostaining. CD44 signal was found at the cell surface and colocalized with EGFP-HAS3 (I: Fig. 3A), demonstrating its localization at the plasma membrane. The ER marker calnexin was partially colocalized with EGFP-HAS3 in vesicles close to the nucleus, but to a lesser extent in the periphery of the cell (I: Fig. 3C). To confirm the intracellular localization of EGFP-HAS2 and EGFP-HAS3 to the ER-Golgi complex, experiments with subcellular markers were performed. To visualize the Golgi complex, three different methods were used. First, the cells transfected with EGFP-HAS3 were stained with the anti-58K antibody visualizing the Golgi and partial colocalization was observed (I: Fig. 3B). Similar results were obtained with a trans-Golgi antibody (data not shown). To further confirm the localization of EGFP-HAS3 in the Golgi, the cells were treated with nocodazole, an agent known to disrupt the Golgi complex. After treatment with nocodazole, the Golgi complex was dispersed and the EGFP-HAS3 signal was also located in small vesicles showing colocalization with the 58K Golgi marker, confirming that EGFP-HAS3 is substantially located in the Golgi (I: Fig. 3D). Similar colocalization in the ER and Golgi complex was obtained with EGFP-HAS2 (data not shown). To check if the EGFP-HAS3 at the cell surface was taken up by the cells through endocytosis into intracellular vesicles, the cells were incubated with a fluid phase endocytosis marker (Alexa Fluor hydrazide). As a result, colocalization of the endocytosed marker with EGFP-HAS3 in positive large, circular vesicles was observed intracellularly (I: Fig. 3E),

thus indicating that some of the intracellular EGFP-HAS3 was endocytosed from the plasma membrane.

Keratinocytes transfected with EGFP-*Has2* or EGFP-*Has3* showed increased amounts of cell surface hyaluronan close to the EGFP-HASs (I: Fig. 3F). Also intracellular hyaluronan that was not removed with *Streptomyces* hyaluronidase was detected, and it colocalized with intracellular EGFP-HAS3 to a variable extent (I: Fig. 3G-I). When the transfected cells were digested with *Streptomyces* hyaluronidase for 30 minutes, the level of intracellular hyaluronan was equal or even increased compared to non-digested cells (I: Fig. 3J). However, when the incubation with hyaluronidase was prolonged for 1-4 h, the amount of intracellular hyaluronan staining was gradually decreased and finally almost completely lost (I: Fig. 3K-L). These data suggest that intracellular hyaluronan originates from the cell surface and can be co-endocytosed with EGFP-HAS3. When the cell surface pool of hyaluronan is depleted with hyaluronidase digestion, endocytosis of partially degraded hyaluronan is first enhanced and the endocytosed hyaluronan later reduced.

The cellular localization of the mutated EGFP-HAS3 constructs differed from those of the intact EGFP-HAS3 and also from each other. The truncated form (R539Stop) mainly produced a reticular pattern in the cytoplasm (I: Fig. 4A) and showed co-localization with calnexin, the ER marker (I: Fig. 4B) in addition to some colocalization with the 58K Golgi marker (I: Fig. 4C). The missense-mutated form (D216A) was mainly located in large vesicles close to the nucleus and also produced a reticular pattern in the cytosol resembling ER (I: Fig. 4D). Unlike the truncated forms, the missense-mutated form showed partial co-localization with the 58K Golgi marker (I: Fig. 4F) and to a lesser extent with calnexin marking the ER (I: Fig. 4E). CD44 immunostaining did not show any colocalization with the mutated EGFP-HASs at the plasma membrane (I: Fig. 4G-H). Also colocalization with hyaluronan was absent (I: Fig. 4I).

5.1.3. Effect of Brefeldin A and cycloheximide on EGFP-HAS traffic

HAS traffic was inhibited with Brefeldin A (BFA), which interferes with the vesicular traffic from the trans-Golgi network to the plasma membrane and redistributes Golgi elements back to the ER. As a result of treatment of the EGFP-*Has3* transfected keratinocytes with BFA for 6 h, secretion of hyaluronan was 60-80% decreased. When the transfected cells were first pretreated with BFA for 2 h and then chased without it for 4 h, the amount of secreted hyaluronan recovered up to 70-95% of the hyaluronan level in untreated cells (I: Fig. 5A), indicating that the effect of BFA was reversible. To check if there was synthesis of new HAS proteins, or alternatively, redistribution of pre-existing HAS proteins blocked in their ER-Golgi pathway, the cells were also treated with cycloheximide, which inhibits protein synthesis. Treatment with cycloheximide alone decreased hyaluronan secretion ~50% (I: Fig. 5B). Cycloheximide with BFA did not impair hyaluronan synthesis any further than BFA alone. Moreover, when the cells were chased for 4 h after BFA, addition of cycloheximide into the chase medium did not affect the rate of the recovery of hyaluronan secretion (I: Fig. 5B). Thus, these results indicate that the recovery of hyaluronan synthesis after BFA block was mainly due to HAS already present in the secretory pathway and not because of newly synthesized HAS protein.

In addition to the effects on hyaluronan synthesis, BFA and cycloheximide also affected the cellular distribution of EGFP-HAS. In cells treated with BFA for 2 h, there was a diffuse reticular pattern of EGFP-HAS signal dispersed in the cytoplasm and resembling ER (I: Fig. 6A). The EGFP-HAS3 was not found at the plasma membrane and there was no colocalization of EGFP-HAS3 with CD44 (I: Fig. 6B). However, low to moderate levels of cell-associated hyaluronan was still found (I: Fig. 6C). When the cells were imaged for

0.5-2 h after removal of BFA (I: Fig. 6D-F), the EGFP-HAS3 signal gradually recovered and was found in the Golgi complex at 1 h time point (I: Fig. 6E) and at the plasma membrane after 2 h (I: Fig. 6F). To investigate the turnover time of EGFP-HAS3 in keratinocytes, the cells were incubated with cycloheximide and imaged with confocal microscopy. After 2 h with cycloheximide, there was no change observed (I: Fig. 6G). By 4 h, the signal of EGFP-HAS3 was located on the plasma membrane and small peripheral, cytoplasmic vesicles (I: Fig. 6H) and after 5 h the signal was very low (I: Fig. 6I). It can be concluded that when protein synthesis is inhibited by cycloheximide, the existing EGFP-HAS3 vanishes in 4-5 h.

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

The effect of hyaluronan synthesis inhibition by 4-MU on EGFP-HAS2 and EGFP-HAS3 hyaluronan secretion and cellular distribution was also tested. 4-MU caused 70-90% reductions in hyaluronan synthesis in 20 h (I: Fig. 7). Along with the reduced synthesis, the plasma membrane residence of EGFP-HAS3 was also decreased and the EGFP-HAS3 signal had returned back to the Golgi and cytoplasm (I: Fig. 6J). Thus, inhibition of hyaluronan synthesis inhibited the localization of EGFP-HAS3 on the plasma membrane (I: Fig. 6K). When the cells were treated with 4-MU for 24 h and then chased thereafter for 2h, the EGFP-HAS3 was restored at the plasma membrane (I: Fig. 6L).

5.2. EGFP-HAS1 LOCALIZATION AND TRAFFIC (II)

5.2.1. Cellular distribution of endogenous HAS1 and EGFP-HAS1 fusion protein

The localization of endogenous HAS1 was investigated in three cell lines with different levels of hyaluronan synthesis. Fibroblasts with high hyaluronan synthesis showed intense staining with HAS1 antibody close to the nucleus resembling the Golgi apparatus (II: Fig. 1A), while keratinocytes secreting less hyaluronan than fibroblasts showed lower signal for HAS1 (II: Fig. 1B). In COS-1 cells, a perinuclear staining of HAS1 was observed only occasionally (II: Fig.1C). The localization of HAS1 was mainly in the Golgi, as confirmed by double staining of HAS1 and an anti-human Golgin-97 antibody (II: Fig. 1D-F).

MCF-7 cells transfected with EGFP-*HAS1* or Dendra-*HAS1* presented similar intracellular pattern of HAS1 as the endogenous HAS1. The signal was intense and close to the nucleus, with diffuse, occasionally reticular staining in the cytoplasm (II: Fig. 2A-C, F, I). To confirm the localization of the transfected, fluorescent HAS1 in the Golgi, two methods were used. First, the cells were co-transfected with Dendra-*HAS1* and a plasmid coding for a medial Golgi enzyme N-acetylglucosaminyltransferase II (GnTII) labeled with Cerulean (II: Fig. 2C-E) and also by incubating the Dendra-*HAS1*-transfected cells with Bodipy ® TR Ceramide Golgi marker (II: Fig. 2F-H), both of these methods showed localization of HAS1 in the Golgi. Colocalization with an ER marker was also found, mostly in the periphery of the cytosol (II: Fig. 2I-K).

Immunostaining of endogenous HAS1 was observed at the plasma membrane in fibroblasts (II: Fig. 1A) and also occasionally in the *HAS1*-transfected MCF-7 cells (II: Fig. 2A). However, the plasma membrane signal of HAS1 was not as clear as with HAS2 and HAS3 (I) because the diffuse cytoplasmic fluorescence often prevented the observation of plasma membrane as a separate entity. MCF-7 cells transfected with *HAS1* did not produce any protrusions at the plasma membrane (II: Fig. 2B). No nuclear localization of HAS1 was observed.

5.2.2. Effect of substrates and cytokines on pericellular hyaluronan coat in Has1-transfected cells

EGFP-*HAS1*-transfected MCF-7 cells only produced a faint pericellular hyaluronan coat, when they were not stimulated by substrates or various cytokines (II: Fig. 3A). Increasing the concentrations of substrates for hyaluronan synthesis, such as providing the cells with 1 mM glucosamine (II: Fig. 3B) or 20 mM glucose (II: Fig. 3D), led to the production of clearly visible, thick pericellular coats around the EGFP-*HAS1*-transfected cells. Also various cytokines, such as 10 ng/ml of IL-1β (II: Fig. 3C), TGF-β (II: Fig. 3E) and TNF-α (II: Fig. 3F) increased the formation of the hyaluronan coat by HAS1. Interestingly, when the transfected cells were treated with both glucosamine and IL-1β (II: Fig. 3G), the hyaluronan coat was more intense and thicker than with either of the stimulants alone, suggesting an additional effect of glucosamine and IL-1β. Interestingly, IL-1β appeared to increase the pericellular hyaluronan coat in HAS1-overexpressing cells already after 1 h incubation (II: Fig. 3H). When the IL-1β-stimulated cells were treated with hyaluronan oligosaccharide 6 (HA6), the pericellular hyaluronan coat was reduced by 85% (II: Fig. 3 I, L), while HA6 decreased the coat by 70% in glucosamine-stimulated *HAS1*-transfected cells (II: Fig. 3L). To spesifically block the binding of hyaluronan to CD44, the IL-1β-treated HAS1-overexpressing cells were incubated with anti-CD44 antibody Hermes1, causing a similar decrease in the hyaluronan coat as HA6 (II: Fig. 3L). The hyaluronan coat produced by EGFP-HAS1 appeared curly and thick, very different from that in HAS3-overexpressing cells having microvillous protrusions (Rilla 2008, Kultti 2006). Few of the non-transfected cells showed hyaluronan at cell surface and treatments with glucosamine (II: Fig. 3J) or IL-1β (II: Fig. 3K) did not increase their coat.

5.2.3. Effect of Brefeldin A and 4-MU on pericellular hyaluronan coat in *HAS1*-transfected cells

To interfere with the vesicular traffic from Golgi to the plasma membrane, the EGFP-*HAS1*-transfected cells were treated with BFA. As a consequence, the pericellular hyaluronan coat was lost and the fluorescent EGFP-HAS1 signal was dispersed from the Golgi-like signal to a dispersed, ER-like pattern (II: Fig. 4A). Depletion of the substrates for hyaluronan synthesis by treatment of IL-1β-stimulated cells with 4-MU led to the disappearance of the pericellular coat in 2 h (II: Fig. 4B), while a 3 h chase after 22 h-treatment with 4-MU recovered the coat in most cells (II: Fig. 4C).

5.2.4. Intracellular hyaluronan in Has1-transfected cells

Nontransfected MCF-7 cells and those transfected with EGFP-*HAS1* or with the empty EGFP vector (data not shown) were treated with IL-1β with or without BFA. In EGFP-*HAS1*-transfected cells and control cells, intracellular hyaluronan was found in vesicular structures in the cytosol (II: Fig. 5A, E). When the cells were treated with BFA alone (II: Fig. 5B, F) or with IL-1β (Fig. 5D, H) the staining of intracellular hyaluronan was decreased and the EGFP-HAS1 appeared as a weak, diffuse, dispersed signal in the cytosol (II: Fig. 5D). IL-1β did not seem to change the amount of intracellular hyaluronan in EGFP-transfected and non-transfected cells (II: Fig. 5C, G).

5.2.5. Hyaluronan secretion in Has1-transfected cells

The secretion of hyaluronan in the growth medium of MCF-7 cells was similar in non-transfected cells and those transfected with the empty EGFP-vector (II: Fig. 6A). The effects of IL-1β with and without BFA and 1 mM glucosamine on hyaluronan secretion by

non-tranfected control cells and cells transfected with the empty vector were small and did not reach statistical significance (II: Fig. 6A). The average content of hyaluronan in the growth medium was 4.6-fold higher in the cultures transfected with EGFP-*HAS1* than in non-transfected cultures or those transfected with the empty vector (II: Fig. 6A). Incubation with IL-1β did not significantly increase the hyaluronan secretion of EGFP-*HAS1*-transfected cells compared to non-treated EGFP-HAS1-overexpressing cells. However, BFA significantly inhibited the secretion of hyaluronan in *HAS1*-transfected cells treated with IL-1β. In contrast to IL-1β, glucosamine showed a 3.4-fold, statistically significant increase of hyaluronan secretion in the EGFP-*HAS1*-transfected cells (II: Fig. 6A), close to the 6-fold increase found in *HAS1*-transfected COS-1-cells treated with 1 mM glucosamine for 6 h (Rilla 2013).

The contents of hyaluronan in the trypsinates, representing extracellular hyaluronan associated with the cell layer, were in the same range as those measured from the growth medium (II: Fig. 6B). The changes induced by the treatments were also mostly parallel to those in medium. On average, the *HAS1*-transfected cell surfaces had 3.6-fold more hyaluronan than controls. Again, IL-1β caused no significant change in the hyaluronan level of *HAS1*-transfected cells, while glucosamine caused a significant, 3.8-fold increase of hyaluronan in the trypsinate, and BFA dramatically reduced the level of trypsinate hyaluronan in IL-1β-treated EGFP-*HAS1*-transfected cells.

5.3. MICROINJECTION-BASED PROBING OF THE POSSIBLE EXISTENCE AND FUNCTIONS OF CYTOSOLIC HYALURONAN (III)

5.3.1. Endogenous intracellular hyaluronan in MCF-7 cells

Intracellular hyaluronan was investigated in MCF-7 cells using the traditional protocol of staining fixed cells and a novel method of introducing fluorescent hyaluronan binding complex into the cytosol by microinjection of living cells. With the traditional method, intracellular hyaluronan was found in vesicular structures of different sizes, while the cytosol and the nucleus were negative (III: Fig. 1A-B). In EGFP-*HAS3*-transfected MCF-7 cells, the intracellular hyaluronan was co-localized with the EGFP-HAS3 in some of the cells (III: Fig. 1B). Similar localization of intracellular hyaluronan was observed in LP-9 mesothelioma cells naturally expressing high levels of hyaluronan (III: Fig. 1C). Pretreatment of the fHABC with HA10 oligosaccharides prevented the staining of hyaluronan (III: Fig. 1E-G). To investigate the origin of the intracellular hyaluronan, the cells were incubated with a fluid phase endocytosis marker (Alexa Fluor hydrazide). The endogenous hyaluronan was mostly colocalized with this marker in the intracellular vesicles, confirming its origin at the cell surface (III: Fig. 1D, H-I).

To study intracellular hyaluronan with another method, microinjection of living cells was used. This method helped to overcome the possible loss of hyaluronan due to the fixation and permeabilization used with the traditional protocol. When MCF-7 cells were injected with fHABC, most of the cells showed a weak, diffuse fluorescent signal in the cytoplasm in addition to granular or vesicular structures of 0.2-2 μm in diameter (III: Fig. 2A, D). This staining pattern was considered to be dependent on the intrinsic property of hyaluronan binding complex proteins, since the microinjection marker with the same fluorescent label was evenly distributed in the cytosol without granular accumulations. The fHABC signal was sometimes close to the EGFP-HAS3 in the cells (III: Fig. 2D). Non-transfected LP-9 cells showed similar patterns of fHABC localization as the MCF-7 cells (III: Fig. 2G). Several methods were used to check whether the localization of fHABC depended on its binding to hyaluronan, or was nonspecific. Addition of HA10 oligosaccharide (III: Fig. 2B, E) or hyaluronidase (III: Fig. 2C, F) to the injection buffer

with fHABC did not affect the localization of fHABC in the cells, indicating that its localization was not due to hyaluronan. Possible colocalization of the fHABC vesicles with lysosomes was tested by adding a lysosomal marker (LysoTracker) to the medium after microinjections, but only occasional colocalization of fHABC and the lysosomal marker was observed in ~30% of MCF-7 and LP-9 cells. (III: Fig. 2H-I). The cells microinjected with fHABC were also stained for endogenous hyaluronan with the traditional method using bHABC, but no colocalization of fHABC and endogenous hyaluronan was observed, although the fixation and permeabilization caused some dispersion of the vesicular fHABC pattern (III: Fig. 2J). Moreover, to check if the fHABC was co-localized to microtubuli, MCF-7 cells were transfected with yellow fluorescent protein (YFP)-tagged α-tubulin and injected with fHABC. However, there was no colocalization of fHABC and microtubuli (III: Fig. 2K). These results indicate that the intracellular enrichment sites of microinjected fHABC are not associated to lysosomes, endosomes, Golgi, microtubules, or to endogenous hyaluronan or intracellular HAS.

5.3.2. Intracellular distribution of labeled hyaluronan and hyaluronan oligosaccharides

Distribution of intracellular hyaluronan and its fragments in nontransfected MCF-7 cells was tested with two methods: microinjection and endocytosis from the growth medium. The localization of fluorescent hyaluronan oligosaccharides after cytosolic microinjection was imaged with confocal microscopy 1.5 h and 6 h after microinjection. The localization was similar at both time points. AMAC-labeled GlcUA was located only in the cytoplasm where it made a speckled pattern, resembling vesicles (III: Fig. 3A), while AMAC-GlcNAc was distributed throughout the cell, including the nucleus (III: Fig. 3B). The microinjected HA4-HA28 oligosaccharides were evenly distributed in the cytosol and also in the nucleus, but excluded from membrane-bound organelles like ER, Golgi and mitochondria (III: Fig. 3C-H). Oligosaccharides longer than HA8 were excluded from intranuclear structures resembling nucleoli (III: Fig. 3E-H). A longer hyaluronan fragment (HA120) was evenly distributed in the cytosol, but it did not enter the nucleus at all (III: Fig. 3Q) and was not colocalized to lysosomes either (III: Fig. 3R).

MCF-7 cells were also allowed to endocytose AMAC-labeled hyaluronan monosaccharides and fragments (HA4-HA28) for 6h before imaging with confocal microscopy. The hyaluronan monosaccharides, GlcUA and GlcNAc labeled with AMAC were endocytosed from the medium. AMAC-GlcUA remained in intracellular vesicles like the oligosaccharides (III: Fig. 3I), but AMAC-GlcNAc was distributed diffusely in the cell and even in the nucleus (III: Fig. 3J). All hyaluronan oligosaccharides of the range HA4-HA28 were located in small intracellular vesicles, and no staining of the cytosol or nucleus was observed (III: Fig. 3K-P). The size and the amount of the vesicles was the same for all sizes of oligosaccharides. In conclusion, hyaluronan oligosaccharides endocytosed from the medium reside in vesicles and do not reach the cytosol. The possibility of AMAC-GlcNAc to penetrate membranes may be due to its stronger lipophilic character.

5.3.3. Effect of microinjected hyaluronan oligosaccharides, hyaluronidase and glucose on the hyaluronan coat size in MCF-7 cells

The size of the pericellular hyaluronan coat produced by EGFP- *HAS3*-transfected MCF-7 cells was used as an estimate of hyaluronan synthesis in microinjected cells. The pericellular hyaluronan coat was visualized with fHABC and its size (mean area and intensity) was measured from confocal images taken with fixed settings of the microscope at 4, 8 and 24 h after injections. HA10 and HA14 oligosaccharides did not

affect the hyaluronan coat (III: Fig. 5), but HA4 first caused an 18% decline in the coat at 4 h and then a 30% increase at the 24 h time point (III: Fig. 4D-F, Fig. 5). Injection of hyaluronidase into the cytosol did not have any effect on the hyaluronan coat at any time point investigated (III: Fig. 5). When the cells were injected with glucose, the pericellular hyaluronan coat was increased at every time point studied (III: Fig. 4G-I, Fig. 5), but cytosolic injection of mannose, a structurally close isomer of glucose used as a control, did not affect the coat size (III: Fig. 4J-L, Fig. 5).

5.4. HYALURONAN, CD44 AND HAS1-3 EXPRESSION IN MOUSE EPIDERMIS EXPOSED TO CHRONIC UV RADIATION (IV)

5.4.1. Hyaluronan and CD44 stainings

In normal mouse skin, the dermal staining of hyaluronan was intensive, while in the epidermis the signal was mostly limited to a weak staining around the orifices of hair follicles (IV: Table 2, Fig. 1a). Immunostaining of CD44 was located in the basal and suprabasal layers of the interfollicular epidermis, but was generally weak (IV: Table 2, Fig. 1b).

The epidermal stainings of hyaluronan and CD44 were increased in UVR-treated mice. The coverage of the hyaluronan staining in the interfollicular epidermis was over 66% in 18 out of 21 mice and the staining intensity was moderate in average (IV: Fig. 1c). The staining of CD44 covered over 66% of the epidermis in all samples and the intensity was mainly moderate (IV: Fig. 1d).

In samples presenting benign hyperplasia, there was moderate or strong staining of hyaluronan (IV: Fig. 1e) and CD44 (IV: Fig. 1f) in the epidermis and the number of positive cell layers was increased along with the degree of hyperplasia. There were positive correlations between the epidermal hyperplasia and the coverage and intensity of hyaluronan and CD44 stainings in the epidermis. However, in dysplastic areas and SCC, there were areas with decreased staining of hyaluronan (IV: Fig. 2b, e, h) and CD44 (IV: Fig. 2c, f, i) although in general they were moderately or strongly stained. In dermis, the intensities of hyaluronan and CD44 were mostly moderate and were not changed compared to controls (IV: Table 2).

5.4.2. HAS1-3 immunostainings

In normal mouse epidermis, positive HAS1-3 stainings were only found in a few cells. The intensity of the HAS1-3 stainings in the dermis was weak or moderate (IV: Fig. 3a, e, i). All HAS stainings presented increased staining intensity of epidermis and dermis in specimens exposed to UVR, although the highest induction appeared in HAS2. The HAS immunostainings were increased in hyperplastic areas (IV: Fig. 3c, g, k) and in the SCCs (IV: Fig. 3b, f, j). However, the staining pattern in the SCCs was nonhomogenous with areas showing decreased staining. The specificity of the HAS antibodies was tested by incubating the antibodies with peptides used in the immunization (IV: Fig. 3d, h, l).

5.5. HYALURONAN, CD44, HAS1-3 AND HYAL1-2 STAININGS IN BENIGN AND MALIGNANT CUTANEOUS MELANOCYTIC LESIONS (V)

Detailed analysis of each staining is presented in the original publication (V: Fig. 1-2 and Tables 1-3). A summary of the alterations observed in the stainings is presented in Table 7 for melanocytic cells and in Table 8 for stromal cells.

In benign melanocytic nevi, hyaluronan and its cell surface receptor CD44 were abundant and presented moderate to strong staining in the melanocytic cells and in the stroma surrounding the nevus. HYAL1 and HYAL2 were mainly weak or moderate in most of the melanocytes, while the stroma was almost negative. HAS1-3 were present in the melanocytic cells showing mainly weak staining with variable coverage. Few of the stromal cells stained for HAS2 and HAS3 while 26-50% in average were positive for HAS1 with moderate intensity of staining.

In dysplastic nevi, the intensity of stromal hyaluronan staining was reduced compared to benign nevi, while no difference was observed in the staining of melanocytic cells. The CD44 showed increased staining intensity in the melanocytes, but not in the stromal tissue. In the melanocytes the intensity and area of positive HYAL2 staining, and the intensity of the staining in the stroma were significantly increased in dysplastic nevi compared to benign nevi. The coverages of HAS1 and HAS2 stainings were higher in dysplastic melanocytes than in the benign nevi, in contrast to the stroma where HAS1 and HAS3 presented decreased stainining coverage and intensity.

In melanoma *in situ*, the intensities of hyaluronan and CD44 stainings were increased in the melanocytic cells, but decreased in the stroma. HYAL2 showed increased coverage and intensity of the staining in melanocytic cells. The coverage of HAS2 was also elevated in the melanocytic cells. HAS1 and HAS3 presented similar patterns of decreased stromal staining intensity as dysplastic nevi.

In the superficial melanoma, the staining intensities for hyaluronan, CD44 and all HASs in the stromal compartment were decreased compared to the benign nevi, while in the melanocytic cells no difference was observed. HYAL2 staining showed increased coverage and intensity in the melanocytic cells and increased intensity in the stromal cells compared to the benign nevi.

Of all groups, deep melanoma was the most altered in regard to histochemical stainings compared to benign nevi. Hyaluronan and CD44 stainings were reduced both in the melanocytic cells and in the stroma. In contrast, HYAL2 was significantly increased both in the melanocytic cells as well as in the stroma. All three HASs showed reduced staining intensities in the stromal tissue and the coverage of HAS1 and HAS3 was reduced, while no difference was observed in the melanocytic cells. The staining pattern of lymph node metastases was otherwise similar to deep melanoma, but the coverage and intensity of CD44 and the coverage of HAS1 were reduced in melanocytic cells in addition to decreased stromal intensity of CD44.

Table 7. Summary of hyaluronan, CD44, HYAL1-2 and HAS1-3 in melanocytic cells of cutaneous melanocytic lesions. The arrows indicate the direction of the change compared to benign nevi. Arrows for lymph node metastasis show the change compared to deep melanoma.

	HA	CD44	HYAL1	HYAL2	HAS1	HAS2	HAS3
Dysplastic nevus		↑		↑	↑	↑	
Melanoma *in situ*	↑	↑		↑		↑	
Superficial melanoma				↑			
Deep melanoma	↓	↓		↑			
Lymph node metastasis		↓			↓		

Table 8. Summary of hyaluronan, CD44, HYAL1-2 and HAS1-3 in stromal cells of cutaneous melanocytic lesions. The arrows indicate the direction of the change compared to benign nevi. Arrows for lymph node metastasis show the change compared to deep melanoma.

	HA	CD44	HYAL1	HYAL2	HAS1	HAS2	HAS3
Dysplastic nevus	↓			↑	↓		↓
Melanoma *in situ*	↓	↓			↓		↓
Superficial melanoma	↓	↓		↑	↓	↓	↓
Deep melanoma	↓	↓		↑	↓	↓	↓
Lymph node metastasis		↓					

6 Discussion

Hyaluronan is a very fascinating molecule. On one hand its structure is very simple, consisting of just a pair of alternating monosaccharides in a linear chain. On the other hand, it has a multitude of effects on cell behavior based on its size. The effects of the molecule can be both beneficial and detrimental, depending on the overall conditions. To be such a versatile molecule, its synthesis and degradation must be carefully regulated in cellular microenvironments.

The main purpose of this thesis was to investigate the intracellular localization, trafficking, function and site of activation of the hyaluronan synthases. Another purpose was to study the changes in hyaluronan and its metabolizing enzymes, especially synthases, in UV-induced cutaneous tumors.

6.1. HAS TRAFFICKING

Studies on the cellular traffic of overexpressed HAS1-3 showed that all of the HASs were mostly located close to the nucleus and within the Golgi apparatus. The more diffuse staining outside of the Golgi colocalized with the ER. HAS2 and HAS3 were especially clearly located at the plasma membrane (I), while a similar pattern for HAS1 was only occasionally observed at the cell surface (II). Interestingly, HAS1 was often seen in bright vesicular structures close to the plasma membrane (II), indicating that these cytoplasmic patches contained high amounts of the synthase. Similar spots of fluorescent HAS1 at the plasma membrane were found in Vero cells (Müllegger 2003). These differences between HASs may reflect the possibly divergent roles and activities of these synthases in various conditions, *e.g.* during embryogenesis (Tien 2005), responses to cytokines (Suzuki 2003, Vigetti 2010, Oguchi 2004) and activity in cancers (Yabushita 2004, Yamada 2004b, Bullard 2003, Yabushita 2005, Bernert 2011).

While our findings about the subcellular distribution of HAS2 and HAS3 were similar to those reported previously (Müllegger 2003), the findings on HAS1 distribution differed from those of (Ghosh 2009). In contrast to the present finding suggesting that HAS1 localized mainly in the Golgi, Ghosh and colleagues described the full length HAS1 to be diffusely spread in the cytosol in HeLa and multiple myeloma cells (Ghosh 2009). The differences in the localization of HAS1 may be due to different cell lines, gene constructs and detection methods. The variant forms of HAS1, which may exhibit different subcellular distribution from the full-length form (Ghosh 2009), were not studied in this thesis.

Inhibition of protein synthesis by cycloheximide suggested a total lifetime of 4-5h for HAS3 protein (I). The BFA experiments suggested that ER to Golgi translocation required 1 h and another 1 h from Golgi to the plasma membrane, where it stayed for 2 h. This time frame is in line with the time required for the synthesis of one hyaluronan molecule (Kitchen 1995). According to the experiments with cycloheximide (I), hyaluronan synthesis recovered from the BFA-block due to the return of the existing synthases from the ER to Golgi and back to the plasma membrane and not because of synthesis of new proteins. Also in earlier studies, a short term cycloheximide treatment did not inhibit hyaluronan synthesis in fibroblasts (Mapleson 1981) or the regeneration of the hyaluronan coat in rat fibrosarcoma cells after hyaluronidase treatment (Goldberg 1984a). These findings are in line with a reservoir of enzymes that can be activated on demand.

On the other hand, cycloheximide treatment in chondrocytes decreased hyaluronan synthesis (Bansal 1986), perhaps due to different enzyme kinetics.

Earlier results on the role of vesicular traffic in hyaluronan synthesis were inconsistent. In rat chondrosarcoma cells, BFA failed to affect hyaluronan synthesis during an 8 h incubation, suggesting that the synthesis of hyaluronan was independent of traffic through Golgi (Calabro 1994). On the contrary, BFA shifted the HAS signal in Vero cells into a reticular ER-like pattern and the signal returned to the Golgi complex and cell surface after removal of the block (Müllegger 2003). The results presented in this thesis support the findings of (Müllegger 2003), showing that the HAS proteins are transported via the secretory pathway from the ER and Golgi to the plasma membrane, where hyaluronan synthesis takes place. The lack of the inhibitory effect of BFA in rat chondrosarcoma cells may reflect a prolonged half-life and stable residence of HAS at the plasma membrane in these cells (Calabro 1994). In addition to the fluorescent HAS created by transient transfection, the endogenous HAS was found mainly in the Golgi (II), indicating that the results on transfected cells were not due to an artifact caused by the overexpression of the fluorescent protein. Moreover, as the fluorescent signal of the empty GFP vector was diffusely dispersed throughout the cell, the possibility of an effect of the fluorescent GFP tag *per se* on the localization was excluded.

The results indicated that there is an intracellular pool of HAS in the secretory pathway and synthesis of new HAS proteins is not necessarily required for the recovery of the hyaluronan synthesis. Such a large pool of inactive enzymes makes them a favorable target for posttranslational regulation. The HAS proteins in the latent pool could be activated by phosphorylation (Vigetti 2009a, Goentzel 2006), ubiquitinylation (Karousou 2010) and O-GlcNAcylation (Vigetti 2012).

Most interestingly, our results show that the active synthesis of hyaluronan keeps the HAS enzyme at the plasma membrane. Thus, treatment of the cells with 4-MU, an inhibitor of hyaluronan synthesis which depletes the intracellular pool of UDP-glucuronic acid (Kultti 2009b, Kakizaki 2004), reduced the plasma membrane signal of EGFP-HAS3, along with the reduced synthesis of hyaluronan (I). Similarly, mutated EGFP-HAS3 constructs, unable to produce hyaluronan, were found only in the ER or in the Golgi and not at the plasma membrane (I). The inhibition of hyaluronan synthesis may prevent the exit of HAS from Golgi, entry of HAS to the plasma membrane or the enzyme may be endocytosed for degradation or returned to the Golgi. In some cases HAS may even be shed into the extracellular space with the bound hyaluronan chain (Kitchen 1995, Mausolf 1990).

Interestingly, plasma membrane protrusions were observed in EGFP-HAS2 and EGFP-HAS3 overexpressing cells (I), while cells overexpressing EGFP-HAS1 did not produce any microvilli (II). This difference may be due to the different levels of hyaluronan synthesis among the enzymes, as the protrusions depend on active hyaluronan synthesis and a certain level of hyaluronan synthesis is needed for the formation of the protrusions (Rilla 2008, Kultti 2006). However, HAS1 did not produce any protrusions even if the cells were stimulated with GlcN to produce more hyaluronan (II). At present, the reasons for the lack of protrusions can only be speculated. As HAS1 was found often in vesicles close to the plasma membrane, it is possible that HAS1 produces hyaluronan in larger complexes at certain sites of the plasma membrane. HAS1 has been reported to form multimers stabilized by intermolecular disulfide bonds (Ghosh 2009). Such complexes of several HAS1 enzymes could be formed at the spots seen in our cells and earlier in Vero cells (Müllegger 2003). The spot-like enrichment sites of HAS1 resemble pits rather than protrusions. This organization obviously cannot create or support microvilli like the evenly dispersed HAS3 (Kultti 2006).

6.2. FUNCTION OF THE HAS PROTEINS

6.2.1. Synthesis of hyaluronan by different HAS isoenzymes

Rat epidermal keratinocytes overexpressing EGFP-tagged mouse HAS2-3 produced 2-2.5-fold more hyaluronan than EGFP-transfected control cells (I). The MCF-7 cells overexpressing EGFP-tagged human HAS1 produced 4.6-fold more hyaluronan than the controls transfected with empty EGFP vector (II). However, hyaluronan production by the different HASs cannot be directly compared between different cell lines. For example, overexpression of EGFP-HAS3 in MCF-7 cells increases hyaluronan secretion by 122-fold (Kultti 2006). Nevertheless, the HAS isoenzymes differ in their ability to synthesize hyaluronan, which may reflect different affinities to the substrates since there are significant differences in their K_m values (Itano 1999).

6.2.2. Regulation of the pericellular hyaluronan coat formation in HAS1-transfected cells by the cytokines

The EGFP-*HAS1*-transfected MCF-7 cells produced significantly more hyaluronan than controls without any additional stimulation, but formed pericellular hyaluronan coats only when treated with various cytokines or substrates (II). Interestingly, IL-1β did not significantly increase hyaluronan production in the *HAS1*-transfected cells, although the hyaluronan coat became clearly evident (II). At present, the underlying mechanism of the effect of IL-1β on the formation of the hyaluronan coat can only be speculated, and may act through adjacent proteins, such as receptors, or directly on HAS1.

It seems most likely that the cytokines affect cell surface receptors, like CD44, changing the structure of the pericellular hyaluronan into a more extended conformation. This hypothesis is supported by the clear inhibition by HA6 and Hermes1 on the pericellular hyaluronan coat produced by IL-1β-treated HAS1-overexpressing cells, and by the notion that the effect of IL-1β and GlcN on the pericellular coat appeared to be additive (II). The effect of GlcN can be explained by the substrate-induced increase in hyaluronan synthesis, the pericellular hyaluronan being bound to the synthase itself, while IL-1β might affect the way hyaluronan is bound to its receptors, without markedly affecting the activity of hyaluronan synthesis. The observation in keratinocytes that the formation of hyaluronan cables by IL-1β does not require changes in hyaluronan secretion (Jokela 2008b) is also consistent with an idea of changed hyaluronan coat structure.

The cytokines might modify the cell surface receptors, activating their binding to hyaluronan at the cell surface or formation of complexes with HAS1. Several cytokines activate monocyte CD44 ability to bind hyaluronan (Levesque 1997, Gee 2003) and TGF-β influences the post-translational modifications of CD44 in lung-derived epithelial tumor cells (Cichy 2000). In addition, CD44 O-glycosylation by IL-1β and glucose have been shown to increase CD44 binding to hyaluronan in proximal tubular cells (Jones 2003). Cytokines also induce CD44 gene expression (Tsubaki 2005, Foster 1998, Li 2012, Muthukumaran 2006), but the effect on the hyaluronan coat already in 1 h suggests post-translational modification at the protein level.

Another possibility is that the cytokines induce posttranslational modifications in HAS1 and thereby affect its stability, function or localization at the plasma membrane. Different cytokines, including IL-1β, TGF-β and TNF-α are known to regulate transcription or mRNA levels of HAS1-3 in several cell types (Yamada 2004a, Suzuki 2003, Stuhlmeier 2004a, Vigetti 2010, van Zeijl 2010, Uchiyama 2005, Oguchi 2004). In contrast, posttranslational regulation of the HAS1 protein by cytokines seems conceivable in the present experiments, since the MCF-7 cells do not express endogenous HAS1, but readily responded to cytokines when transfected with HAS1. The EGFP-*HAS1* or Dendra- *HAS1*

constructs contain the human *HAS1* gene with a fluorescent tag, but lack the normal promoter regions with binding sites of regulatory transcription factors. In addition, the pericellular hyaluronan coat response to IL-1β within 1h suggests regulatory actions at the protein level, either HAS itself or a receptor. The 1 h response time appears too short for originating from gene transcription.

Rapid post-translational phosphorylation of hyaluronan synthases in response to the tumor promoter phorbol-12-myristate-13 acetate (PMA) and IL-1β have been suggested earlier (Vigetti 2009a). The enzymes responsible for the phosphorylation might include protein kinase C, cAMP-dependent kinase or Ca^{2+}-dependent kinase (Klewes 1994). The activity of HAS2 is regulated posttranslationally also by ubiquitinylation (Karousou 2010) and O-GlcNAcylation (Vigetti 2012). However, these modifications have not been studied in HAS1. It is possible, that the posttranslational modifications of HAS1 might promote its ability to form multimers (Ghosh 2009).

The cytokines might also affect the cellular UDP-precursor sugar levels and thereby induce coat formation. TGF-β has been shown to increase the expression of UDP-glucose dehydrogenase, which oxidizes UDP-glucose to produce UDP-glucuronic acid (Clarkin 2011). However, since the hyaluronan secretion was not significantly increased by IL-1β, this explanation does not seem very likely. It is also possible that the cytokines increase the coat by slowing the turnover of cell surface hyaluronan.

6.2.3. Effect of substrates to hyaluronan synthesis

The precursor sugar contents are known to affect hyaluronan synthesis (Nakamura 1995, Nakamura 1997, Rilla 2004, Rilla 2008, Kudo 2004, Jokela 2008a). The substrate levels were manipulated with two methods in this thesis. The coumarine derivative 4-MU, decreasing the cellular levels of UDP-glucuronic acid (Kultti 2009b, Kakizaki 2004) inhibited hyaluronan synthesis in cells overexpressing HAS1-3 (I, II). Then, cytosolic injections of glucose and mannose were used to investigate the possibility of influencing hyaluronan synthesis with substrate precursors introduced directly into the cytosol (III). As expected, cytosolic injection of glucose increased the hyaluronan coat at every time point (4 h, 8 h and 24 h). Glucose is the substrate for the synthesis of the UDP-precursor sugars and rapidly (in less than 2 h) increases hyaluronan synthesis and formation of pericellular hyaluronan coat (Tammi 2011).

It was somewhat unexpected that mannose did not affect hyaluronan synthesis at any time point when injected into the cytosol, while it has been shown to rapidly inhibit hyaluronan synthesis by decreasing the cellular contents of UDP-N-acetylhexosamines when added to the growth medium (Jokela 2008a). Mannose can be directed to multiple metabolic pathways, including building of different glycoconjugates and glycolysis (Alton 1998). The exact mechanism of mannose to inhibit UDP-sugar precursor pools has remained unclear, but the glucosamine-6-phosphate deaminase has been suggested to be the target (Jokela 2008a, Cayli 1999). Alternatively, an as yet unidentified cell surface receptor could mediate the effects of mannose and explain the lack of the inhibition on hyaluronan synthesis when introduced directly into the cytosol.

6.2.4. Site of activation of hyaluronan synthesis

The main intracellular localization of all three HASs was in the Golgi complex (I, II). Since the precursor sugars are available in the cytosol, it would be basically possible for the synthases to start hyaluronan synthesis before reaching the plasma membrane.

The site of activation of hyaluronan synthesis was investigated in this thesis by performing several experiments on different aspects of this issue (I). In experiments with

EGFP-HAS2 and EGFP-HAS3 overexpressing cells, a portion of hyaluronan was found to reside within the cells and even colocalize with EGFP-HAS. Incubation of the cells for 1-4 h with *Streptomyces* hyaluronidase in the culture medium led to the almost complete disappearance of the intracellular hyaluronan and its colocalization with EGFP-HAS. Also the experiments with cells transfected with the mutated HAS3 constructs suggested that HAS is not active inside cells. Moreover, inhibition of vesicular traffic using BFA or inhibiting hyaluronan synthesis by 4-MU abolished the plasma membrane signal and hyaluronan secretion of EGFP-HAS3. These findings indicate a tight connection between plasma membrane location of HAS and activation of hyaluronan synthesis.

The possible synthesis of intracellular hyaluronan by HAS1 was also tested (II). The amount of the intracellular hyaluronan was reduced in cells treated with BFA, indicating that HAS1 was not producing hyaluronan when its traffic to the plasma membrane was inhibited. This is in line with earlier studies, in which the full-length HAS1 proteins were not producing hyaluronan inside of cells (Adamia 2005, Ghosh 2009).

In conclusion, no evidence for the intracellular synthesis of hyaluronan was found. However, the experiments performed in this thesis cannot rule out the possibility that intracellular synthesis of hyaluronan might occur in abnormal situations. Earlier reports have suggested that ER stress evoked by hyperglycemia (Ren 2009, Wang 2009a) might activate intracellular HAS, leading to formation of hyaluronan cables extending through and between adjacent cells capable of binding to monocytes. As these studies were carried out on fixed cells, similar cell-spanning cable structures in live cells are yet to be found and possible effects of fixation and permeabilization on the localization of molecules cannot be ruled out (Schnell 2012). In addition, a mechanism for the transfer of the hydrophilic hyaluronan chains from the cytosol through lipophilic membrane bilayers has not been demonstrated, and only a hypothetical model has been proposed (Wang 2011).

6.2.5. Translocation of the hyaluronan chain across the plasma membrane

Reports on the mechanism of the translocation of the hyaluronan chain through plasma membrane are controversial. While ABC transporters have been suggested to be involved in some studies (Schulz 2007, Prehm 2004, Ouskova 2004), there are several reports suggesting that only HAS protein is required for the translocation of hyaluronan (Thomas 2010, Hubbard 2012, Medina 2012). The methods used in these earlier transport studies mentioned above have been very complex and thus definite answers are difficult to obtain.

In the present study, we took advantage of microinjections of hyaluronan fragments to compete with the hyaluronan chain for binding to HAS or any accessory protein involved in the transport. Microinjections of HA10 and HA14 showed that the translocation apparatus is not accessible to hyaluronan fragments of this size at least when introduced from the cytosolic side. The finding also favors the alternative theory that accessory hyaluronan translocation molecules are not involved in the secretion.

Presently, the mechanisms of the early inhibition and late stimulation of hyaluronan coat by HA4 can only be speculated, whether it directly interfered with HAS or acted through indirect signals. The HA4 may be small enough to reach the active site of HAS, explaining the initial decrease in hyaluronan synthesis. Then later, degradation of HA4 may provide an extra source of the precursor sugars for hyaluronan synthesis, possibly explaining the coat enlargement at 24h. However, it would then remain obscure why HA10 or HA14 did not increase the synthesis of hyaluronan, given their similar structure. In general, the free N-linked oligosaccharides generated from misfolded glycoproteins in the ER are transported into the cytosol, processed to $Mannose_5GlcNAc$ and further

transported into lysosomes to be hydrolyzed to monomers (Suzuki 2006, Chantret 2008). Perhaps HA4 is also delivered into lysosomes for eventual degradation like Mannose$_5$GlcNAc. The HA10 and HA14 might be too large to enter lysosomes, or their degradation process might be slower. However, the hyaluronan oligosaccharides are not likely to exist naturally in the cytosol, as they were unable to escape the endocytic vesicles, as shown in this thesis (III).

Whether the pore for hyaluronan translocation through the plasma membrane is produced by the HAS proteins or another membrane channel was also probed by microinjecting hyaluronidase. Hyaluronidase injected into the cytosol did not affect the coat size, indicating that the growing HA chain is not accessible to degradation by hyaluronidase. A similar result has been recently obtained by Hubbard and colleagues showing that the newly formed HA is protected from enzymatic degradation (Hubbard 2012). In conclusion, these results support the conclusion that the synthesis of hyaluronan and its membrane translocation are coupled together by the HAS protein itself. However, it is likely that the translocation of hyaluronan is carried out by HAS oligomers, forming the pore together (Karousou 2010).

6.3. INTRACELLULAR HYALURONAN

6.3.1. Localization of endogenous intracellular hyaluronan

Several reports have suggested the presence of cytosolic hyaluronan (Evanko 1999b, Evanko 2004, Collis 1998) in addition to its location in endocytic vesicles destined for degradation (Tammi 2001, Hua 1993, Culty 1992), suggesting that intracellular hyaluronan may have roles other than simply being destined for degradation. The issue has, however, remained controversial. The possible existence of cytosolic hyaluronan was addressed in this thesis by comparing the distribution of the endogenous intracellular hyaluronan visualized by the normal staining protocol, and that obtained by direct cytosolic microinjection of fHABC. Furthermore, the distributions were compared for exogenous hyaluronan either endocytosed by the cells or introduced to cytosol by microinjection (III).

Endogenous intracellular hyaluronan in MCF-7 and LP-9 cells, visualized by traditional staining, was found in similar vesicles as earlier described in keratinocytes (Tammi 2001) and in smooth muscle cells and fibroblasts (Evanko 1999b). The intracellular hyaluronan of MCF-7 cells colocalized with a fluid-phase endocytosis marker, supporting the conclusion that the origin of the intracellular hyaluronan is at the cell surface rather than intracellular hyaluronan synthesis (III), like in keratinocytes (Tammi 2001). At present it is not known how much of the newly synthesized hyaluronan is degraded in MCF-7 cells, but in rat keratinocytes ~25% of the newly synthesized hyaluronan is endocytosed shortly after its synthesis (Tammi 2001). In breast cancer cell lines, only 0.5% of the labeled hyaluronan was degraded during 16 h (Heldin 1996).

In addition to the cytosolic vesicular structures, endogenous intracellular hyaluronan has been observed earlier as a diffuse pattern throughout the cytoplasm and also in the nucleus of aortic smooth muscle cells and fibroblasts during the mitosis (Evanko 1999b). To avoid possible loss and mislocalization of the cytosolic or nuclear hyaluronan during the fixation and staining process, fluorescent HABC was injected into live MCF-7 cells (III). It was found in granular cytosolic structures. Co-injection of the fHABC with HA10, or hyaluronidase did not affect the location of fHABC, indicating that it was not due to specific binding to hyaluronan. Supporting this conclusion, the injected fHABC did not show any significant colocalization with the endogenous intracellular hyaluronan in the injected cells. However, the exact localization of the fHABC in the cells remained

unresolved. Perhaps the injected fHABC was considered as an external material and was degraded in the cells. In any case, the location of fHABC was not due to the fluorescent tag, as the microinjection marker with a similar Alexa Fluor tag was evenly distributed in the cytosol.

In the present experiments the intracellular hyaluronan failed to colocalize with microtubules and the localization of the injected fHABC was not influenced by the cell division occasionally detected during microinjections, in contrast to an earlier study (Evanko 2004). Differences between cell lines or cell culture conditions may influence the presence and localization of the intracellular hyaluronan. For example, the hyperglycemic media used in the earlier studies has been reported to activate intracellular synthesis of hyaluronan in dividing cells (Ren 2009, Wang 2009a, Wang 2011). In the present study, observation of living cells was considered more physiologically relevant than using fixed cells, and it helped to avoid possible artefacts caused by the permeabilization and other additional steps in the staining procedure (Schnell 2012).

6.3.2. Distribution of exogenous hyaluronan inside the cells

The present results indicated that the endocytosed hyaluronan (≥ HA4) does not naturally escape from the endocytic or lysosomal vesicles into the cytosol, but remains in these membrane-bound compartments (III). In contrast, it was reported in earlier studies, that fluorescent Texas Red-hyaluronan (700 kDa) accumulated in the nucleus of ras-transformed fibroblasts after endocytosis (Collis 1998), and that smaller fluorescein-labeled hyaluronan fragments (50 kD and 300 kD) accumulated in the cytoplasm of aortic smooth muscle cells, while the high molecular weight hyaluronan (500-1000kD) remained in the endocytic vesicles (Evanko 2004). The specific conditions in these studies may have influenced the results. For example, Collis and colleagues suggested that the Ras transformation or activation of protein kinase C might facilitate the re-entry of large hyaluronan into cells (Collis 1998). It seems possible that endocytosed hyaluronan is capable of leaking into the cytosol in some cell types, possibly due to cell culture conditions. The fluorescent tag in the hyaluronan preparation may also enhance the membrane permeability. This probably happened with the fluorescent GlcNAc which was evenly distributed in the cytosol and also in the nucleus, whether it was endocytosed or injected into the cytosol, its lipophilic character allowed penetration of the membranes.

Supporting the conclusion that the intracellular membranes are impermeable for hyaluronan, the fluorescent hyaluronan oligosaccharides injected into the cytosol were excluded from the membrane-bound organelles such as ER, Golgi and mitochondria (III). In contrast, nuclear membrane pores apparently allowed the diffusion of smaller hyaluronan fragments into the nucleus, while excluding the largest, probably due to the presence of nuclear pore complexes. The size limit was between 5.6 (HA28) and 25 kDa (HA120). This is in line with the 25-40 kDa barrier for the passive diffusion of proteins into the nucleus (Tran 2006), as the hydrated and extended structure explains the lower mass limit observed for hyaluronan.

6.4. HYALURONAN METABOLISM IN UV-INDUCED CUTANEOUS TUMORS

Ultraviolet radiation is the main risk factor for cutaneous SCC (Melnikova 2005) and melanoma (Kanavy 2011). Many of the effects of hyaluronan (reviewed in chapter 2.5.) provide cancer cells with growth advantage and may facilitate metastasis. In this thesis, the immunohistochemical stainings of mouse skin SCC induced by UVB and human skin

melanoma samples were analyzed to investigate the levels of hyaluronan and proteins involved in its metabolism (IV, V). In both models the stainings of hyaluronan, CD44 and HAS1-3 were increased in the early lesions, *e.g.* in the hyperplastic and dysplastic areas of the UVR-treated mouse skin and in the dysplastic nevi and melanoma *in situ*, representing the first abnormalities in tumor progression. These findings suggest that the elevated levels of hyaluronan and CD44 are important features of the early phases of UV-induced cutaneous tumors. Similar results have been reported in a mouse model of melanoma, in which the amount of hyaluronan was increased during the early stages of invasion (Turley 1985). The staining of hyaluronan and CD44 correlated positively in epidermal hyperplasia of mouse skin, suggesting that increased hyaluronan in the early lesions may provide a growth advantage, perhaps by promoting cell division (Brecht 1986), or protecting from apoptosis (Pauloin 2009, Yasuda 2001). Hyaluronan may also enhance EMT (Takahashi 2010, Cho 2012), motility, adhesion (Kudo 2004, Kultti 2009b, Ichikawa 1999) and invasion (Edward 2010) of melanoma cells, as suggested by studies *in vitro*.

Both in melanoma samples and in the UVB induced SCC, changes in hyaluronan and CD44 occurred in parallel. Similar close association of hyaluronan and CD44 has been observed also in SCC of the skin (Karvinen 2003a), non-small cell lung cancer (Pirinen 2001) and laryngeal SCC (Hirvikoski 1999). The increased content of CD44 at the plasma membrane may retain hyaluronan at the cell surface. However, the role of CD44 is somewhat obscure, since it is also involved in the endocytosis of hyaluronan, reducing its amount on the cell surface (Tammi 2001, Hua 1993). UV radiation has been shown to decrease CD44 levels (Calikoglu 2006, Hasova 2011), while treatment with hyaluronan inhibits the release of soluble CD44 (Hasova 2011). The increased amount of CD44 in UVB-exposed skin may also be due to the mutated p53 in tumors, because normal p53 has been shown to inhibit the expression of CD44 (Godar 2008). It is thus possible, that hyaluronan and CD44 have a mutual regulation to stay at the cell surface together. Interaction of hyaluronan and CD44 also activates many signaling pathways in cancer, promoting tumor progression (Toole 2009, Ghatak 2005, Bourguignon 2001, Bourguignon 2007, Herishanu 2011, Bourguignon 2008).

Increased immunostaining of HAS1-3 was found in hyperplasia and dysplasia of UVB-treated mouse skin while increased immunostaining of HAS1 and HAS2 were found in the human dysplastic nevi and melanoma *in situ* lesions (IV, V). Activation of the synthases provides a reasonable explanation for the concomitant increase in the amount of hyaluronan. In line with the present results, increased HAS expression levels were found in fibroblasts and keratinocytes exposed to UVB (Averbeck 2007, Kakizaki 2008). UVB radiation is known to activate several pathways involving cytokines, growth factors and transcription factors, such as EGFR and ErbB2 (Madson 2007), KGF and IL-1β (Kakizaki 2008), STAT3 (Sano 2005, Ahsan 2005), MAPK family (Chen 2000, Katiyar 2007) and the PI3K/Akt pathway (Wunderlich 2008), known to be involved in the activation of *HAS* gene expression (Pienimäki 2001, Karvinen 2003b, Yamada 2004a, Saavalainen 2005, Pasonen-Seppänen 2012b). HAS2 immunostaining showed the most prominent increase in response to UV in mouse skin, while in melanoma samples HAS1 was increased in addition to HAS2. Similar observations were made in cultured keratinocytes and in the human skin (Averbeck 2007) as well as in oral SCC, where suppression of HAS2 was associated with decreased tumor cell migration and growth (Wang 2012). In addition, HAS2 was recently reported to enhance adhesion of cancer stem cells to endothelial cells and promote extravasation, further emphasizing its role in tumorigenesis (Okuda 2012).

In contrast to the increased staining of hyaluronan observed in the early lesions, the hyaluronan content was locally decreased in more advanced tumors, like the poorly differentiated SCC of mouse skin and the invasive melanoma. The results are in line with

several earlier reports of tumors originating from stratified epithelia like skin (Karvinen 2003a), mouth (Kosunen 2004), larynx (Hirvikoski 1999) and lung (Pirinen 2001), reporting similar decreased hyaluronan staining in advanced tumors. The increased stromal staining of HYAL2 in addition to decreased levels of stromal HAS1 and HAS3 in dysplastic nevi and HAS1-3 in melanomas (V) provide a reasonable explanation for the reduction of stromal hyaluronan observed in these tumors. It is possible that the increased expression of hyaluronidases cleave the high molecular weight hyaluronan to smaller oligosaccharides capable of promoting angiogenesis (Liu 1996) and tumor invasion (Fieber 2004). The clearance of hyaluronan may also provide the tissue with more space for inflammatory cells. The inhibitory effect of high extracellular hyaluronan on mast cell proliferation has been demonstrated in a cell culture model (Takano 2012). Mast cells among other inflammatory cells produce cytokines and chemokines (Gregory 2011, Ribatti 2012), which further stimulate the tumor cells. The tumor-promoting interplay between tumor cells and mast cells has been found in tissue samples of melanoma (Ribatti 2003) and SCC and melanoma cell lines (Artuc 2011).

In conclusion, elevated levels of hyaluronan and CD44 are important features for the onset of tumorigenesis and the enhanced expression of hyaluronan synthases is probably responsible for the increased production of hyaluronan. The amount of hyaluronan probably declined in advanced skin tumors due to decreased levels of HAS1-3 and upregulation of HYAL2. This may lead to the remodeling of the tumor microenvironment, making it more accessible to inflammatory cells and mast cells, and thereby enhancing tumor growth and metastasis through *e.g.* angiogenesis. Although SCC and melanoma originate from different cells and the sparse melanocytes are scattered in between the numerous keratinocytes comprising the stratified epidermis, there seems to be a general pattern of UV-induced tumorigenesis in the skin. A conceivable model could be that UV-exposure increases the levels of various cytokines in the skin, leading to activation of hyaluronan synthases and subsequent elevation in the hyaluronan content. Increased hyaluronan levels promote the growth of cells and enhance signaling cascades through interactions with cells surface receptors. These effects reinforce the transformation potential of the epithelial cells in concert with the multiple other simultaneous processes occurring in the tumor microenvironment.

7 Summary and Conclusions

The main findings of this thesis are:

- All HASs are transported to the plasma membrane along the ER-Golgi pathway.
- The HAS proteins reside at the cell surface only when active hyaluronan synthesis is possible. If the hyaluronan synthesis is inhibited by substrate depletion or interference of membrane traffic to the plasma membrane, the HAS protein ceases hyaluronan production.
- HAS1 appears to require cytokines or excess of substrates to form the pericellular hyaluronan coat. Stimulation of HAS1-overexpressing cells with cytokines leads to thick, receptor-bound hyaluronan coat. Cytokines probably induce post-translational modification of HAS1 or a receptor associated with it.
- Under physiological conditions, hyaluronan is not located freely in the cytosol. Intracellular hyaluronan is derived from the cell surface and does not enter the cytosol from the endocytic vesicles.
- During its synthesis, the hyaluronan molecule is not accessible to cytosolic hyaluronan binding proteins, oligosaccharides or enzymatic degradation, suggesting that HAS protein itself forms the pore for hyaluronan translocation through the plasma membrane.
- Hyaluronan and its receptor CD44 increase during UV-induced hyperplasia and dysplasia in the skin, but reduce in advanced SCC and melanoma. The initial upregulation of HAS1-3 and the subsequent reduction in HAS levels with increased HYAL2 expression explain the alterations of hyaluronan during tumor progression.

In the future, the functions of the HAS proteins should be investigated in more detail, revealing the possible effects of abnormal, but physiologically possible conditions on their activity. Future experiments should also look into the molecular mechanisms of cytokines in hyaluronan coat formation by HAS1. In tumor tissues, it would be interesting to compare the mRNA and protein levels to provide new information about the regulation of hyaluronan synthases, perhaps due to altered turnover of mRNA or protein during cancer progression. Also the possible role of the hyaluronan oligosaccharides in cancers should be addressed. In addition, future research should focus on the possible role of hyaluronan in the inflammatory model of tumor progression, and especially on the early events in the malignant transformation of cells.

8 References

Acharya S, Rodriguez IR, Moreira EF, Midura RJ, Misono K, Todres E, Hollyfield JG. SPACR, a novel interphotoreceptor matrix glycoprotein in human retina that interacts with hyaluronan. J Biol Chem 1998;273(47):31599-31606.

Acharya S, Foletta VC, Lee JW, Rayborn ME, Rodriguez IR, Young WS,3rd, Hollyfield JG. SPACRCAN, a novel human interphotoreceptor matrix hyaluronan-binding proteoglycan synthesized by photoreceptors and pinealocytes. J Biol Chem 2000;275(10):6945-6955.

Adamia S, Crainie M, Kriangkum J, Mant MJ, Belch AR, Pilarski LM. Abnormal expression of hyaluronan synthases in patients with Waldenstrom's macroglobulimenia. Semin Oncol 2003;30(2):165-168.

Adamia S, Reiman T, Crainie M, Mant MJ, Belch AR, Pilarski LM. Intronic splicing of hyaluronan synthase 1 (HAS1): a biologically relevant indicator of poor outcome in multiple myeloma. Blood 2005;105(12):4836-4844.

Afify AM, Stern M, Guntenhoner M, Stern R. Purification and characterization of human serum hyaluronidase. Arch Biochem Biophys 1993;305(2):434-441.

Afify AM, Craig S, Paulino AF, Stern R. Expression of hyaluronic acid and its receptors, CD44s and CD44v6, in normal, hyperplastic, and neoplastic endometrium. Ann Diagn Pathol 2005;9(6):312-318.

Afify A, McNiel MA, Braggin J, Bailey H, Paulino AF. Expression of CD44s, CD44v6, and hyaluronan across the spectrum of normal-hyperplasia-carcinoma in breast. Appl Immunohistochem Mol Morphol 2008;16(2):121-127.

Ågren UM, Tammi M, Tammi R. Hydrocortisone regulation of hyaluronan metabolism in human skin organ culture. J Cell Physiol 1995;164(2):240-248.

Ågren UM, Tammi RH, Tammi MI. Reactive oxygen species contribute to epidermal hyaluronan catabolism in human skin organ culture. Free Radic Biol Med 1997;23(7):996-1001.

Aguiar DJ, Knudson W, Knudson CB. Internalization of the hyaluronan receptor CD44 by chondrocytes. Exp Cell Res 1999;252(2):292-302.

Ahsan H, Aziz MH, Ahmad N. Ultraviolet B exposure activates Stat3 signaling via phosphorylation at tyrosine705 in skin of SKH1 hairless mouse: a target for the management of skin cancer? Biochem Biophys Res Commun 2005;333(1):241-246.

Akazawa Y, Sayo T, Sugiyama Y, Sato T, Akimoto N, Ito A, Inoue S. Adiponectin resides in mouse skin and upregulates hyaluronan synthesis in dermal fibroblasts. Connect Tissue Res 2011;52(4):322-328.

Alaniz L, Garcia MG, Gallo-Rodriguez C, Agusti R, Sterin-Speziale N, Hajos SE, Alvarez E. Hyaluronan oligosaccharides induce cell death through PI3-K/Akt pathway independently of NF-kappaB transcription factor. Glycobiology 2006;16(5):359-367.

Alton G, Hasilik M, Niehues R, Panneerselvam K, Etchison JR, Fana F, Freeze HH. Direct utilization of mannose for mammalian glycoprotein biosynthesis. Glycobiology 1998;8(3):285-295.

Anttila MA, Tammi RH, Tammi MI, Syrjanen KJ, Saarikoski SV, Kosma VM. High levels of stromal hyaluronan predict poor disease outcome in epithelial ovarian cancer. Cancer Res 2000;60(1):150-155.

Armstrong BK, Kricker A. The epidemiology of UV induced skin cancer. J Photochem Photobiol B 2001;63(1-3):8-18.

Artuc M, Guhl S, Babina M, Unger T, Steckelings UM, Zuberbier T. Mast cell-derived TNF-alpha and histamine modify IL-6 and IL-8 expression and release from cutaneous tumor cells. Exp Dermatol 2011;20(12):1020-1022.

Arwert EN, Hoste E, Watt FM. Epithelial stem cells, wound healing and cancer. Nat Rev Cancer 2012;12(3):170-180.

Asplund T, Brinck J, Suzuki M, Briskin MJ, Heldin P. Characterization of hyaluronan synthase from a human glioma cell line. Biochim Biophys Acta 1998;1380(3):377-388.

Assmann V, Marshall JF, Fieber C, Hofmann M, Hart IR. The human hyaluronan receptor RHAMM is expressed as an intracellular protein in breast cancer cells. J Cell Sci 1998;111 (Pt 12):1685-1694.

Assmann V, Jenkinson D, Marshall JF, Hart IR. The intracellular hyaluronan receptor RHAMM/IHABP interacts with microtubules and actin filaments. J Cell Sci 1999;112 (Pt 22):3943-3954.

Atmuri V, Martin DC, Hemming R, Gutsol A, Byers S, Sahebjam S, Thliveris JA, Mort JS, *et al.* Hyaluronidase 3 (HYAL3) knockout mice do not display evidence of hyaluronan accumulation. Matrix Biol 2008;27(8):653-660.

August EM, Nguyen T, Malinowski NM, Cysyk RL. Non-steroidal anti-inflammatory drugs and tumor progression: inhibition of fibroblast hyaluronic acid production by indomethacin and mefenamic acid. Cancer Lett 1994;82(1):49-54.

Auvinen PK, Parkkinen JJ, Johansson RT, Ågren UM, Tammi RH, Eskelinen MJ, Kosma VM. Expression of hyaluronan in benign and malignant breast lesions. Int J Cancer 1997;74(5):477-481.

Auvinen P, Tammi R, Parkkinen J, Tammi M, Ågren U, Johansson R, Hirvikoski P, Eskelinen M, *et al.* Hyaluronan in peritumoral stroma and malignant cells associates with breast cancer spreading and predicts survival. Am J Pathol 2000;156(2):529-536.

Auvinen P, Tammi R, Kosma VM, Sironen R, Soini Y, Mannermaa A, Tumelius R, Uljas E, *et al.* Increased hyaluronan content and stromal cell CD44 associate with HER2 positivity and poor prognosis in human breast cancer. Int J Cancer 2013;132(3):531-539.

Averbeck M, Gebhardt CA, Voigt S, Beilharz S, Anderegg U, Termeer CC, Sleeman JP, Simon JC. Differential regulation of hyaluronan metabolism in the epidermal and dermal compartments of human skin by UVB irradiation. J Invest Dermatol 2007;127(3):687-697.

Bachmann F, Buechner SA, Wernli M, Strebel S, Erb P. Ultraviolet light downregulates CD95 ligand and TRAIL receptor expression facilitating actinic keratosis and squamous cell carcinoma formation. J Invest Dermatol 2001;117(1):59-66.

Baden HP, Kubilus J. The growth and differentiation of cultured newborn rat keratinocytes. J Invest Dermatol 1983;80(2):124-130.

Bai KJ, Spicer AP, Mascarenhas MM, Yu L, Ochoa CD, Garg HG, Quinn DA. The role of hyaluronan synthase 3 in ventilator-induced lung injury. Am J Respir Crit Care Med 2005;172(1):92-98.

Ball NJ, Yohn JJ, Morelli JG, Norris DA, Golitz LE, Hoeffler JP. Ras mutations in human melanoma: a marker of malignant progression. J Invest Dermatol 1994;102(3):285-290.

Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol 1999;144(4):789-801.

Banerji S, Hide BR, James JR, Noble ME, Jackson DG. Distinctive properties of the hyaluronan-binding domain in the lymphatic endothelial receptor Lyve-1 and their implications for receptor function. J Biol Chem 2010;285(14):10724-10735.

Bansal MK, Mason RM. Evidence for rapid metabolic turnover of hyaluronate synthetase in Swarm rat chondrosarcoma chondrocytes. Biochem J 1986;236(2):515-519.

Baranova NS, Nileback E, Haller FM, Briggs DC, Svedhem S, Day AJ, Richter RP. The inflammation-associated protein TSG-6 cross-links hyaluronan via hyaluronan-induced TSG-6 oligomers. J Biol Chem 2011;286(29):25675-25686.

Beckenlehner K, Bannke S, Spruss T, Bernhardt G, Schonenberg H, Schiess W. Hyaluronidase enhances the activity of adriamycin in breast cancer models in vitro and in vivo. J Cancer Res Clin Oncol 1992;118(8):591-596.

Beech DJ, Madan AK, Deng N. Expression of PH-20 in normal and neoplastic breast tissue. J Surg Res 2002;103(2):203-207.

Berkhout RJ, Bouwes Bavinck JN, ter Schegget J. Persistence of human papillomavirus DNA in benign and (pre)malignant skin lesions from renal transplant recipients. J Clin Microbiol 2000;38(6):2087-2096.

Bernert B, Porsch H, Heldin P. Hyaluronan synthase 2 (HAS2) promotes breast cancer cell invasion by suppression of tissue metalloproteinase inhibitor 1 (TIMP-1). J Biol Chem 2011;286(49):42349-42359.

Blanpain C, Fuchs E. Epidermal homeostasis: a balancing act of stem cells in the skin. Nat Rev Mol Cell Biol 2009;10(3):207-217.

Bodevin-Authelet S, Kusche-Gullberg M, Pummill PE, DeAngelis PL, Lindahl U. Biosynthesis of hyaluronan: direction of chain elongation. J Biol Chem 2005;280(10):8813-8818.

Bommaya G, Meran S, Krupa A, Phillips AO, Steadman R. Tumour necrosis factor-stimulated gene (TSG)-6 controls epithelial-mesenchymal transition of proximal tubular epithelial cells. Int J Biochem Cell Biol 2011;43(12):1739-1746.

Bonerandi JJ, Beauvillain C, Caquant L, Chassagne JF, Chaussade V, Clavere P, Desouches C, Garnier F, *et al.* Guidelines for the diagnosis and treatment of cutaneous squamous cell carcinoma and precursor lesions. J Eur Acad Dermatol Venereol 2011;25 Suppl 5:1-51.

Bono P, Rubin K, Higgins JM, Hynes RO. Layilin, a novel integral membrane protein, is a hyaluronan receptor. Mol Biol Cell 2001;12(4):891-900.

Bono P, Cordero E, Johnson K, Borowsky M, Ramesh V, Jacks T, Hynes RO. Layilin, a cell surface hyaluronan receptor, interacts with merlin and radixin. Exp Cell Res 2005;308(1):177-187.

Borowsky ML, Hynes RO. Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. J Cell Biol 1998;143(2):429-442.

Boudreau N, Turley E, Rabinovitch M. Fibronectin, hyaluronan, and a hyaluronan binding protein contribute to increased ductus arteriosus smooth muscle cell migration. Dev Biol 1991;143(2):235-247.

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988;106(3):761-771.

Bourguignon LY, Zhu H, Shao L, Chen YW. CD44 interaction with c-Src kinase promotes cortactin-mediated cytoskeleton function and hyaluronic acid-dependent ovarian tumor cell migration. J Biol Chem 2001;276(10):7327-7336.

Bourguignon LY, Gilad E, Peyrollier K. Heregulin-mediated ErbB2-ERK signaling activates hyaluronan synthases leading to CD44-dependent ovarian tumor cell growth and migration. J Biol Chem 2007;282(27):19426-19441.

Bourguignon LY. Hyaluronan-mediated CD44 activation of RhoGTPase signaling and cytoskeleton function promotes tumor progression. Semin Cancer Biol 2008;18(4):251-259.

Brash DE, Rudolph JA, Simon JA, Lin A, McKenna GJ, Baden HP, Halperin AJ, Ponten J. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci U S A 1991;88(22):10124-10128.

Brecht M, Mayer U, Schlosser E, Prehm P. Increased hyaluronate synthesis is required for fibroblast detachment and mitosis. Biochem J 1986;239(2):445-450.

Brinck J, Heldin P. Expression of recombinant hyaluronan synthase (HAS) isoforms in CHO cells reduces cell migration and cell surface CD44. Exp Cell Res 1999;252(2):342-351.

Brokstad KA, Kalland KH, Russell WC, Matthews DA. Mitochondrial protein p32 can accumulate in the nucleus. Biochem Biophys Res Commun 2001;281(5):1161-1169.

Bullard KM, Kim HR, Wheeler MA, Wilson CM, Neudauer CL, Simpson MA, McCarthy JB. Hyaluronan synthase-3 is upregulated in metastatic colon carcinoma cells and manipulation of expression alters matrix retention and cellular growth. Int J Cancer 2003;107(5):739-746.

Burgdorf WHC, Plewig G, Wolff HH, Landthaler M editors. Braun-Falco's Dermatology. 3rd ed. Heidelberg: Springer Medizin Verlag; 2009.

Cabral LS, Festa Neto C, Sanches JA,Jr, Ruiz IR. Genomic instability in human actinic keratosis and squamous cell carcinoma. Clinics (Sao Paulo) 2011;66(4):523-528.

Calabro A, Hascall VC. Differential effects of brefeldin A on chondroitin sulfate and hyaluronan synthesis in rat chondrosarcoma cells. J Biol Chem 1994;269(36):22764-22770.

Calikoglu E, Sorg O, Tran C, Grand D, Carraux P, Saurat JH, Kaya G. UVA and UVB decrease the expression of CD44 and hyaluronate in mouse epidermis, which is counteracted by topical retinoids. Photochem Photobiol 2006;82(5):1342-1347.

Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A,Jr, Kubalak S, Klewer SE, *et al.* Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. J Clin Invest 2000;106(3):349-360.

Campo GM, Avenoso A, Campo S, D'Ascola A, Nastasi G, Calatroni A.. Small hyaluronan oligosaccharides induce inflammation by engaging both toll-like-4 and CD44 receptors in human chondrocytes. Biochem Pharmacol 2010;80(4):480-490.

Cayli A, Hirschmann F, Wirth M, Hauser H, Wagner R. Cell lines with reduced UDP-N-acetylhexosamine pool in the presence of ammonium. Biotechnol Bioeng 1999;65(2):192-200.

Chantret I, Moore SE. Free oligosaccharide regulation during mammalian protein N-glycosylation. Glycobiology 2008;18(3):210-224.

Chen Q, Cai S, Shadrach KG, Prestwich GD, Hollyfield JG. Spacrcan binding to hyaluronan and other glycosaminoglycans. Molecular and biochemical studies. J Biol Chem 2004;279(22):23142-23150.

Chen W, Bowden GT. Role of p38 mitogen-activated protein kinases in ultraviolet-B irradiation-induced activator protein 1 activation in human keratinocytes. Mol Carcinog 2000;28(4):196-202.

Cheng G, Swaidani S, Sharma M, Lauer ME, Hascall VC, Aronica MA. Hyaluronan deposition and correlation with inflammation in a murine ovalbumin model of asthma. Matrix Biol 2011;30(2):126-134.

Cherr GN, Yudin AI, Overstreet JW. The dual functions of GPI-anchored PH-20: hyaluronidase and intracellular signaling. Matrix Biol 2001;20(8):515-525.

Cho SH, Park YS, Kim HJ, Kim CH, Lim SW, Huh JW, Lee JH, Kim HR. CD44 enhances the epithelial-mesenchymal transition in association with colon cancer invasion. Int J Oncol 2012;41(1):211-218.

Chow G, Tauler J, Mulshine JL. Cytokines and growth factors stimulate hyaluronan production: role of hyaluronan in epithelial to mesenchymal-like transition in non-small cell lung cancer. J Biomed Biotechnol 2010;2010:485468.

Cichy J, Pure E. Oncostatin M and transforming growth factor-beta 1 induce post-translational modification and hyaluronan binding to CD44 in lung-derived epithelial tumor cells. J Biol Chem 2000;275(24):18061-18069.

Clarkin CE, Allen S, Kuiper NJ, Wheeler BT, Wheeler-Jones CP, Pitsillides AA. Regulation of UDP-glucose dehydrogenase is sufficient to modulate hyaluronan production and release, control sulfated GAG synthesis, and promote chondrogenesis. J Cell Physiol 2011;226(3):749-761.

Collis L, Hall C, Lange L, Ziebell M, Prestwich R, Turley EA. Rapid hyaluronan uptake is associated with enhanced motility: implications for an intracellular mode of action. FEBS Lett 1998;440(3):444-449.

Corte MD, Gonzalez LO, Junquera S, Bongera M, Allende MT, Vizoso FJ. Analysis of the expression of hyaluronan in intraductal and invasive carcinomas of the breast. J Cancer Res Clin Oncol 2010;136(5):745-750.

Cranmer LD, Engelhardt C, Morgan SS. Treatment of unresectable and metastatic cutaneous squamous cell carcinoma. Oncologist 2010;15(12):1320-1328.

Csoka AB, Scherer SW, Stern R. Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31. Genomics 1999;60(3):356-361.

Csoka AB, Frost GI, Stern R. The six hyaluronidase-like genes in the human and mouse genomes. Matrix Biol 2001;20(8):499-508.

Cui X, Xu H, Zhou S, Zhao T, Liu A, Guo X, Tang W, Wang F. Evaluation of angiogenic activities of hyaluronan oligosaccharides of defined minimum size. Life Sci 2009a;85(15-16):573-577.

Cui X, Zhou S, Xu H, Zhao T, Liu A, Guo X, Wang F. Reversal effects of hyaluronan oligosaccharides on adriamycin resistance of K562/A02 cells. Anticancer Drugs 2009b;20(9):800-806.

Culty M, Nguyen HA, Underhill CB. The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan. J Cell Biol 1992;116(4):1055-1062.

Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. J Clin Oncol 2006;24(26):4340-4346.

Dai G, Freudenberger T, Zipper P, Melchior A, Grether-Beck S, Rabausch B, de Groot J, Twarock S, *et al.* Chronic ultraviolet B irradiation causes loss of hyaluronic acid from mouse dermis because of down-regulation of hyaluronic acid synthases. Am J Pathol 2007;171(5):1451-1461.

Das S, Deb TB, Kumar R, Datta K. Multifunctional activities of human fibroblast 34-kDa hyaluronic acid-binding protein. Gene 1997;190(1):223-225.

Davidson B, Goldberg I, Gotlieb WH, Ben-Baruch G, Kopolovic J. CD44 expression in uterine cervical intraepithelial neoplasia and squamous cell carcinoma: an immunohistochemical study. Eur J Gynaecol Oncol 1998;19(1):46-49.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, *et al.* Mutations of the BRAF gene in human cancer. Nature 2002;417(6892):949-954.

Day AJ. The structure and regulation of hyaluronan-binding proteins. Biochem Soc Trans 1999;27(2):115-121.

Day AJ, Prestwich GD. Hyaluronan-binding proteins: tying up the giant. J Biol Chem 2002;277(7):4585-4588.

De Brabander MJ, Van de Veire RM, Aerts FE, Borgers M, Janssen PA. The effects of methyl (5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl) carbamate, (R 17934; NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured in vitro. Cancer Res 1976;36(3):905-916.

de Gruijl FR, Rebel H. Early events in UV carcinogenesis--DNA damage, target cells and mutant p53 foci. Photochem Photobiol 2008;84(2):382-387.

de la Motte C, Nigro J, Vasanji A, Rho H, Kessler S, Bandyopadhyay S, Danese S, Fiocchi C, *et al.* Platelet-derived hyaluronidase 2 cleaves hyaluronan into fragments that trigger monocyte-mediated production of proinflammatory cytokines. Am J Pathol 2009;174(6):2254-2264.

de la Motte CA, Hascall VC, Drazba J, Bandyopadhyay SK, Strong SA. Mononuclear leukocytes bind to specific hyaluronan structures on colon mucosal smooth muscle cells treated with polyinosinic acid:polycytidylic acid: inter-alpha-trypsin inhibitor is crucial to structure and function. Am J Pathol 2003;163(1):121-133.

de Sa VK, Olivieri E, Parra ER, Ab'Saber AM, Takagaki T, Soares FA, Carraro D, Carvalho L, *et al.* Hyaluronidase splice variants are associated with histology and outcome in adenocarcinoma and squamous cell carcinoma of the lung. Hum Pathol 2012;43(5):675-683.

DeAngelis PL, Papaconstantinou J, Weigel PH. Molecular cloning, identification, and sequence of the hyaluronan synthase gene from group A Streptococcus pyogenes. J Biol Chem 1993;268(26):19181-19184.

DeAngelis PL, Achyuthan AM. Yeast-derived recombinant DG42 protein of Xenopus can synthesize hyaluronan in vitro. J Biol Chem 1996;271(39):23657-23660.

DeAngelis PL, Jing W, Graves MV, Burbank DE, Van Etten JL. Hyaluronan synthase of chlorella virus PBCV-1. Science 1997;278(5344):1800-1803.

DeAngelis PL. Molecular directionality of polysaccharide polymerization by the Pasteurella multocida hyaluronan synthase. J Biol Chem 1999;274(37):26557-26562.

DeAngelis PL. Glycosaminoglycan polysaccharide biosynthesis and production: today and tomorrow. Appl Microbiol Biotechnol 2012;94(2):295-305.

Deb TB, Datta K. Molecular cloning of human fibroblast hyaluronic acid-binding protein confirms its identity with P-32, a protein co-purified with splicing factor SF2. Hyaluronic acid-binding protein as P-32 protein, co-purified with splicing factor SF2. J Biol Chem 1996;271(4):2206-2212.

Decleves AE, Caron N, Voisin V, Legrand A, Bouby N, Kultti A, Tammi MI, Flamion B. Synthesis and fragmentation of hyaluronan in renal ischaemia. Nephrol Dial Transplant 2012;27(10):3771-3781.

Delmage JM, Powars DR, Jaynes PK, Allerton SE. The selective suppression of immunogenicity by hyaluronic acid. Ann Clin Lab Sci 1986;16(4):303-310.

Duncan MR, Berman B. Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol 1991;97(4):686-692.

Edward M, Quinn JA, Pasonen-Seppänen SM, McCann BA, Tammi RH. 4-Methylumbelliferone inhibits tumour cell growth and the activation of stromal hyaluronan synthesis by melanoma cell-derived factors. Br J Dermatol 2010;162(6):1224-1232.

Erickson CA, Turley EA. The effects of epidermal growth factor on neural crest cells in tissue culture. Exp Cell Res 1987;169(2):267-279.

Evanko SP, Angello JC, Wight TN. Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 1999a;19(4):1004-1013.

Evanko SP, Wight TN. Intracellular localization of hyaluronan in proliferating cells. J Histochem Cytochem 1999b;47(10):1331-1342.

Evanko SP, Johnson PY, Braun KR, Underhill CB, Dudhia J, Wight TN. Platelet-derived growth factor stimulates the formation of versican-hyaluronan aggregates and pericellular matrix expansion in arterial smooth muscle cells. Arch Biochem Biophys 2001;394(1):29-38.

Evanko SP, Parks WT, Wight TN. Intracellular hyaluronan in arterial smooth muscle cells: association with microtubules, RHAMM, and the mitotic spindle. J Histochem Cytochem 2004;52(12):1525-1535.

Evanko SP, Potter-Perigo S, Bollyky PL, Nepom GT, Wight TN. Hyaluronan and versican in the control of human T-lymphocyte adhesion and migration. Matrix Biol 2012;31(2):90-100.

Evans EA, Zhang H, Martin-DeLeon PA. SPAM1 (PH-20) protein and mRNA expression in the epididymides of humans and macaques: utilizing laser microdissection/RT-PCR. Reprod Biol Endocrinol 2003;1:54.

Falconi D, Aubin JE. LIF inhibits osteoblast differentiation at least in part by regulation of HAS2 and its product hyaluronan. J Bone Miner Res 2007;22(8):1289-1300.

Fieber C, Baumann P, Vallon R, Termeer C, Simon JC, Hofmann M, Angel P, Herrlich P, *et al.* Hyaluronan-oligosaccharide-induced transcription of metalloproteases. J Cell Sci 2004;117(Pt 2):359-367.

Finnish Cancer Registry. 2012; Available at: www.cancerregistry.fi.

Flannery CR, Little CB, Hughes CE, Caterson B. Expression and activity of articular cartilage hyaluronidases. Biochem Biophys Res Commun 1998;251(3):824-829.

Foley JP, Lam D, Jiang H, Liao J, Cheong N, McDevitt TM, Zaman A, Wright JR, *et al.* TLR2, TGFbeta, hyaluronan and RHAMM are required for Surfactant Protein A-stimulated macrophage chemotaxis. J Biol Chem 2012;287(44):37406-37419.

Foster LC, Arkonac BM, Sibinga NE, Shi C, Perrella MA, Haber E. Regulation of CD44 gene expression by the proinflammatory cytokine interleukin-1beta in vascular smooth muscle cells. J Biol Chem 1998;273(32):20341-20346.

Franzmann EJ, Schroeder GL, Goodwin WJ, Weed DT, Fisher P, Lokeshwar VB. Expression of tumor markers hyaluronic acid and hyaluronidase (HYAL1) in head and neck tumors. Int J Cancer 2003;106(3):438-445.

Fraser JR, Laurent TC, Pertoft H, Baxter E. Plasma clearance, tissue distribution and metabolism of hyaluronic acid injected intravenously in the rabbit. Biochem J 1981;200(2):415-424.

Fraser JR, Laurent TC, Laurent UB. Hyaluronan: its nature, distribution, functions and turnover. J Intern Med 1997;242(1):27-33.

Freinkel R, Woodley D editors. *The Biology of the skin*. New York: The Parthenon Publishing Group; 2001.

Freudenberger T, Rock K, Dai G, Dorn S, Mayer P, Heim HK, Fischer JW. Estradiol inhibits hyaluronic acid synthase 1 expression in human vascular smooth muscle cells. Basic Res Cardiol 2011;106(6):1099-1109.

Frost GI, Csoka AB, Wong T, Stern R. Purification, cloning, and expression of human plasma hyaluronidase. Biochem Biophys Res Commun 1997;236(1):10-15.

Fuchs E. Keratins and the skin. Annu Rev Cell Dev Biol 1995;11:123-153.

Fuchs E. Skin stem cells: rising to the surface. J Cell Biol 2008;180(2):273-284.

Gandini S, Sera F, Cattaruzza MS, Pasquini P, Abeni D, Boyle P, Melchi CF. Meta-analysis of risk factors for cutaneous melanoma: I. Common and atypical naevi. Eur J Cancer 2005;41(1):28-44.

Gao F, Koenitzer JR, Tobolewski JM, Jiang D, Liang J, Noble PW, Oury TD. Extracellular superoxide dismutase inhibits inflammation by preventing oxidative fragmentation of hyaluronan. J Biol Chem 2008a;283(10):6058-6066.

Gao F, Yang CX, Mo W, Liu YW, He YQ. Hyaluronan oligosaccharides are potential stimulators to angiogenesis via RHAMM mediated signal pathway in wound healing. Clin Invest Med 2008b;31(3):E106-16.

Gao F, Liu Y, He Y, Yang C, Wang Y, Shi X, Wei G. Hyaluronan oligosaccharides promote excisional wound healing through enhanced angiogenesis. Matrix Biol 2010;29(2):107-116.

Garbe C, Leiter U. Melanoma epidemiology and trends. Clin Dermatol 2009;27(1):3-9.

Garbe C, Peris K, Hauschild A, Saiag P, Middleton M, Spatz A, Grob JJ, Malvehy J, *et al.* Diagnosis and treatment of melanoma: European consensus-based interdisciplinary guideline. Eur J Cancer 2010;46(2):270-283.

Gee K, Kozlowski M, Kumar A. Tumor necrosis factor-alpha induces functionally active hyaluronan-adhesive CD44 by activating sialidase through p38 mitogen-activated protein kinase in lipopolysaccharide-stimulated human monocytic cells. J Biol Chem 2003;278(39):37275-37287.

Ghatak S, Misra S, Toole BP. Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the phosphoinositide 3-kinase/Akt cell survival pathway. J Biol Chem 2002;277(41):38013-38020.

Ghatak S, Misra S, Toole BP. Hyaluronan constitutively regulates ErbB2 phosphorylation and signaling complex formation in carcinoma cells. J Biol Chem 2005;280(10):8875-8883.

Ghosh A, Kuppusamy H, Pilarski LM. Aberrant splice variants of HAS1 (Hyaluronan Synthase 1) multimerize with and modulate normally spliced HAS1 protein: a potential mechanism promoting human cancer. J Biol Chem 2009;284(28):18840-18850.

Ghosh I, Bharadwaj A, Datta K. Reduction in the level of hyaluronan binding protein 1 (HABP1) is associated with loss of sperm motility. J Reprod Immunol 2002;53(1-2):45-54.

Glogau RG. The risk of progression to invasive disease. J Am Acad Dermatol 2000;42(1 Pt 2):23-24.

Glud M, Gniadecki R. MicroRNAs in the pathogenesis of malignant melanoma. J Eur Acad Dermatol Venereol 2012; in press.

Gluzman Y. SV40-transformed simian cells support the replication of early SV40 mutants. Cell 1981;23(1):175-182.

Godar S, Ince TA, Bell GW, Feldser D, Donaher JL, Bergh J, Liu A, Miu K, *et al.* Growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. Cell 2008;134(1):62-73.

Goentzel BJ, Weigel PH, Steinberg RA. Recombinant human hyaluronan synthase 3 is phosphorylated in mammalian cells. Biochem J 2006;396(2):347-354.

Goldberg RL, Toole BP. Hyaluronate coat formation and cell spreading in rat fibrosarcoma cells. Exp Cell Res 1984a;151(1):258-263.

Goldberg RL, Toole BP. Pericellular coat of chick embryo chondrocytes: structural role of hyaluronate. J Cell Biol 1984b;99(6):2114-2122.

Goldberg RL, Toole BP. Hyaluronate inhibition of cell proliferation. Arthritis Rheum 1987;30(7):769-778.

Goldberg RL, Huff JP, Lenz ME, Glickman P, Katz R, Thonar EJ. Elevated plasma levels of hyaluronate in patients with osteoarthritis and rheumatoid arthritis. Arthritis Rheum 1991;34(7):799-807.

Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Bishop DT, Azizi E, Bergman W, *et al.* Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. J Med Genet 2007;44(2):99-106.

Goldstein LA, Zhou DF, Picker LJ, Minty CN, Bargatze RF, Ding JF, Butcher EC. A human lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. Cell 1989;56(6):1063-1072.

Golshani R, Hautmann SH, Estrella V, Cohen BL, Kyle CC, Manoharan M, Jorda M, Soloway MS, *et al.* HAS1 expression in bladder cancer and its relation to urinary HA test. Int J Cancer 2007;120(8):1712-1720.

Grammatikakis N, Grammatikakis A, Yoneda M, Yu Q, Banerjee SD, Toole BP. A novel glycosaminoglycan-binding protein is the vertebrate homologue of the cell cycle control protein, Cdc37. J Biol Chem 1995;270(27):16198-16205.

Grammatikakis N, Lin JH, Grammatikakis A, Tsichlis PN, Cochran BH. p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function. Mol Cell Biol 1999;19(3):1661-1672.

Gregory AD, Houghton AM. Tumor-associated neutrophils: new targets for cancer therapy. Cancer Res 2011;71(7):2411-2416.

Guldberg P, thor Straten P, Birck A, Ahrenkiel V, Kirkin AF, Zeuthen J. Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma. Cancer Res 1997;57(17):3660-3663.

Guo N, Baglole CJ, O'Loughlin CW, Feldon SE, Phipps RP. Mast cell-derived prostaglandin D2 controls hyaluronan synthesis in human orbital fibroblasts via DP1 activation: implications for thyroid eye disease. J Biol Chem 2010;285(21):15794-15804.

Guo YJ, Lin SC, Wang JH, Bigby M, Sy MS. Palmitoylation of CD44 interferes with CD3-mediated signaling in human T lymphocytes. Int Immunol 1994;6(2):213-221.

Gupta S, Batchu RB, Datta K. Purification, partial characterization of rat kidney hyaluronic acid binding protein and its localization on the cell surface. Eur J Cell Biol 1991;56(1):58-67.

Gushulak L, Hemming R, Martin D, Seyrantepe V, Pshezhetsky A, Triggs-Raine B. Hyaluronidase 1 and beta-hexosaminidase have redundant functions in hyaluronan and chondroitin sulfate degradation. J Biol Chem 2012;287(20):16689-16697.

Hagenfeld D, Borkenhagen B, Schulz T, Schillers H, Schumacher U, Prehm P. Hyaluronan Export through Plasma Membranes Depends on Concurrent K(+) Efflux by K(ir) Channels. PLoS One 2012;7(6):e39096.

Haider AS, Grabarek J, Eng B, Pedraza P, Ferreri NR, Balazs EA, Darzynkiewicz Z. In vitro model of "wound healing" analyzed by laser scanning cytometry: accelerated healing of epithelial cell monolayers in the presence of hyaluronate. Cytometry A 2003;53(1):1-8.

Hall CL, Yang B, Yang X, Zhang S, Turley M, Samuel S, Lange LA, Wang C, *et al.* Overexpression of the hyaluronan receptor RHAMM is transforming and is also required for H-ras transformation. Cell 1995;82(1):19-26.

Hardingham TE, Muir H. Binding of oligosaccharides of hyaluronic acid to proteoglycans. Biochem J 1973;135(4):905-908.

Hardingham TE, Muir H. Hyaluronic acid in cartilage and proteoglycan aggregation. Biochem J 1974;139(3):565-581.

Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. FASEB J 1992;6(3):861-870.

Hardwick C, Hoare K, Owens R, Hohn HP, Hook M, Moore D, Cripps V, Austen L, *et al.* Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. J Cell Biol 1992;117(6):1343-1350.

Harris EN, Kyosseva SV, Weigel JA, Weigel PH. Expression, processing, and glycosaminoglycan binding activity of the recombinant human 315-kDa hyaluronic acid receptor for endocytosis (HARE). J Biol Chem 2007;282(5):2785-2797.

Harris EN, Weigel JA, Weigel PH. The human hyaluronan receptor for endocytosis (HARE/Stabilin-2) is a systemic clearance receptor for heparin. J Biol Chem 2008;283(25):17341-17350.

Hartmann-Petersen S, Tammi RH, Tammi MI, Kosma VM. Depletion of cell surface CD44 in nonmelanoma skin tumours is associated with increased expression of matrix metalloproteinase 7. Br J Dermatol 2009;160(6):1251-1257.

Hascall VC, Heinegård D. Aggregation of cartilage proteoglycans. II. Oligosaccharide competitors of the proteoglycan-hyaluronic acid interaction. J Biol Chem 1974;249(13):4242-4249.

Hascall VC, Majors AK, De La Motte CA, Evanko SP, Wang A, Drazba JA, Strong SA, Wight TN. Intracellular hyaluronan: a new frontier for inflammation? Biochim Biophys Acta 2004;1673(1-2):3-12.

Hasova M, Crhak T, Safrankova B, Dvorakova J, Muthny T, Velebny V, Kubala L. Hyaluronan minimizes effects of UV irradiation on human keratinocytes. Arch Dermatol Res 2011;303(4):277-284.

Hautmann SH, Lokeshwar VB, Schroeder GL, Civantos F, Duncan RC, Gnann R, Friedrich MG, Soloway MS. Elevated tissue expression of hyaluronic acid and hyaluronidase validates the HA-HAase urine test for bladder cancer. J Urol 2001;165(6 Pt 1):2068-2074.

Hearing VJ. Determination of melanin synthetic pathways. J Invest Dermatol 2011;131(E1):E8-E11.

Heldin P, Laurent TC, Heldin CH. Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts. Biochem J 1989;258(3):919-922.

Heldin P, Asplund T, Ytterberg D, Thelin S, Laurent TC. Characterization of the molecular mechanism involved in the activation of hyaluronan synthetase by platelet-derived growth factor in human mesothelial cells. Biochem J 1992;283 (Pt 1):165-170.

Heldin P, Pertoft H. Synthesis and assembly of the hyaluronan-containing coats around normal human mesothelial cells. Exp Cell Res 1993;208(2):422-429.

Heldin P, Delatorre M, Ytterberg D, Bergh J. Differential synthesis and binding of hyaluronan by human breast cancer cell lines. Oncol Rep 1996;3(6):1011-1016.

Heldin P, Karousou E, Bernert B, Porsch H, Nishitsuka K, Skandalis SS. Importance of hyaluronan-CD44 interactions in inflammation and tumorigenesis. Connect Tissue Res 2008;49(3):215-218.

Hellman U, Malm L, Ma LP, Larsson G, Morner S, Fu M, Engstrom-Laurent A, Waldenstrom A. Growth factor PDGF-BB stimulates cultured cardiomyocytes to synthesize the extracellular matrix component hyaluronan. PLoS One 2010;5(12):e14393.

Helsing P, Nymoen DA, Ariansen S, Steine SJ, Maehle L, Aamdal S, Langmark F, Loeb M, *et al.* Population-based prevalence of CDKN2A and CDK4 mutations in patients with multiple primary melanomas. Genes Chromosomes Cancer 2008;47(2):175-184.

Hemming R, Martin DC, Slominski E, Nagy JI, Halayko AJ, Pind S, Triggs-Raine B. Mouse Hyal3 encodes a 45- to 56-kDa glycoprotein whose overexpression increases hyaluronidase 1 activity in cultured cells. Glycobiology 2008;18(4):280-289.

Herbold KW, Zhou J, Haggerty JG, Milstone LM. CD44 expression on epidermal melanocytes. J Invest Dermatol 1996;106(6):1230-1235.

Herishanu Y, Gibellini F, Njuguna N, Hazan-Halevy I, Farooqui M, Bern S, Keyvanfar K, Lee E, *et al.* Activation of CD44, a receptor for extracellular matrix components, protects chronic lymphocytic leukemia cells from spontaneous and drug induced apoptosis through MCL-1. Leuk Lymphoma 2011;52(9):1758-1769.

Hill LL, Ouhtit A, Loughlin SM, Kripke ML, Ananthaswamy HN, Owen-Schaub LB. Fas ligand: a sensor for DNA damage critical in skin cancer etiology. Science 1999;285(5429):898-900.

Hiltunen EL, Anttila M, Kultti A, Ropponen K, Penttinen J, Yliskoski M, Kuronen AT, Juhola M, *et al.* Elevated hyaluronan concentration without hyaluronidase activation in malignant epithelial ovarian tumors. Cancer Res 2002;62(22):6410-6413.

Hirose Y, Saijou E, Sugano Y, Takeshita F, Nishimura S, Nonaka H, Chen YR, Sekine K, *et al.* Inhibition of Stabilin-2 elevates circulating hyaluronic acid levels and prevents tumor metastasis. Proc Natl Acad Sci U S A 2012;109(11):4263-4268.

Hirvikoski P, Tammi R, Kumpulainen E, Virtaniemi J, Parkkinen JJ, Tammi M, Johansson R, Ågren U, *et al.* Irregular expression of hyaluronan and its CD44 receptor is associated with metastatic phenotype in laryngeal squamous cell carcinoma. Virchows Arch 1999;434(1):37-44.

Hofmann M, Fieber C, Assmann V, Gottlicher M, Sleeman J, Plug R, Howells N, von Stein O, *et al.* Identification of IHABP, a 95 kDa intracellular hyaluronate binding protein. J Cell Sci 1998;111 (Pt 12):1673-1684.

Honda A, Noguchi N, Takehara H, Ohashi Y, Asuwa N, Mori Y. Cooperative enhancement of hyaluronic acid synthesis by combined use of IGF-I and EGF, and inhibition by tyrosine kinase inhibitor genistein, in cultured mesothelial cells from rabbit pericardial cavity. J Cell Sci 1991;98(Pt 1):91-98.

Hosono K. Hyaluronan oligosaccharides inhibit tumorigenicity of osteosarcoma cell lines MG-63 and LM-8 in vitro and in vivo via perturbation of hyaluronan-rich pericellular matrix of the cells. Am J Pathol 2007;171(1):274.

Hua Q, Knudson CB, Knudson W. Internalization of hyaluronan by chondrocytes occurs via receptor-mediated endocytosis. J Cell Sci 1993;106(Pt 1):365-375.

Huang L, Yoneda M, Kimata K. A serum-derived hyaluronan-associated protein (SHAP) is the heavy chain of the inter alpha-trypsin inhibitor. J Biol Chem 1993;268(35):26725-26730.

Huang L, Grammatikakis N, Yoneda M, Banerjee SD, Toole BP. Molecular characterization of a novel intracellular hyaluronan-binding protein. J Biol Chem 2000;275(38):29829-29839.

Huang SS, Liu IH, Smith T, Shah MR, Johnson FE, Huang JS. CRSBP-1/LYVE-l-null mice exhibit identifiable morphological and functional alterations of lymphatic capillary vessels. FEBS Lett 2006;580(26):6259-6268.

Hubbard C, McNamara JT, Azumaya C, Patel MS, Zimmer J. The hyaluronan synthase catalyzes the synthesis and membrane translocation of hyaluronan. J Mol Biol 2012;418(1-2):21-31.

Hämäläinen K, Kosma VM, Eloranta ML, Tammi R, Tammi M, Leppänen M, Leppänen M, Heinonen S, *et al.* Downregulated CD44 and hyaluronan expression in vulvar intraepithelial neoplasia and squamous cell carcinomas. Acta Obstet Gynecol Scand 2010;89(1):108-119.

Iacob S, Knudson CB. Hyaluronan fragments activate nitric oxide synthase and the production of nitric oxide by articular chondrocytes. Int J Biochem Cell Biol 2006;38(1):123-133.

Ichikawa T, Itano N, Sawai T, Kimata K, Koganehira Y, Saida T, Taniguchi S. Increased synthesis of hyaluronate enhances motility of human melanoma cells. J Invest Dermatol 1999;113(6):935-939.

Isacke CM, Yarwood H. The hyaluronan receptor, CD44. Int J Biochem Cell Biol 2002;34(7):718-721.

Itano N, Kimata K. Expression cloning and molecular characterization of HAS protein, a eukaryotic hyaluronan synthase. J Biol Chem 1996a;271(17):9875-9878.

Itano N, Kimata K. Molecular cloning of human hyaluronan synthase. Biochem Biophys Res Commun 1996b;222(3):816-820.

Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, Imagawa M, Shinomura T, Hamaguchi M, *et al.* Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. J Biol Chem 1999;274(35):25085-25092.

Jacobetz MA, Chan DS, Neesse A, Bapiro TE, Cook N, Frese KK, Feig C, Nakagawa T, *et al.* Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer. Gut 2012;62(1):112-120.

Jacobson A, Brinck J, Briskin MJ, Spicer AP, Heldin P. Expression of human hyaluronan synthases in response to external stimuli. Biochem J 2000;348 Pt 1:29-35.

Jacobson A, Rahmanian M, Rubin K, Heldin P. Expression of hyaluronan synthase 2 or hyaluronidase 1 differentially affect the growth rate of transplantable colon carcinoma cell tumors. Int J Cancer 2002;102(3):212-219.

Jacobson B. Hyaluronic acid synthesis in rooster comb. Effect of testosterone on nucleotide sugar metabolism. Connect Tissue Res 1978;5(4):217-223.

Jadin L, Wu X, Ding H, Frost GI, Onclinx C, Triggs-Raine B, Flamion B. Skeletal and hematological anomalies in HYAL2-deficient mice: a second type of mucopolysaccharidosis IX? FASEB J 2008;22(12):4316-4326.

Jadin L, Bookbinder LH, Frost GI. A comprehensive model of hyaluronan turnover in the mouse. Matrix Biol 2012;31(2):81-89.

Jameson JM, Cauvi G, Sharp LL, Witherden DA, Havran WL. Gammadelta T cell-induced hyaluronan production by epithelial cells regulates inflammation. J Exp Med 2005;201(8):1269-1279.

Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, Prestwich GD, Mascarenhas MM, *et al.* Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nat Med 2005;11(11):1173-1179.

Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 2007;23:435-461.

Jiang D, Liang J, Noble PW. Hyaluronan as an immune regulator in human diseases. Physiol Rev 2011;91(1):221-264.

Jiang P, Li X, Thompson CB, Huang Z, Araiza F, Osgood R, Wei G, Feldmann M, *et al.* Effective targeting of the tumor microenvironment for cancer therapy. Anticancer Res 2012;32(4):1203-1212.

Jokela TA, Jauhiainen M, Auriola S, Kauhanen M, Tiihonen R, Tammi MI, Tammi RH. Mannose inhibits hyaluronan synthesis by down-regulation of the cellular pool of UDP-N-acetylhexosamines. J Biol Chem 2008a;283(12):7666-7673.

Jokela TA, Lindgren A, Rilla K, Maytin E, Hascall VC, Tammi RH, Tammi MI. Induction of hyaluronan cables and monocyte adherence in epidermal keratinocytes. Connect Tissue Res 2008b;49(3):115-119.

Jokela TA, Makkonen KM, Oikari S, Kärnä R, Koli E, Hart GW, Tammi RH, Carlberg C, *et al.* Cellular content of UDP-N-acetylhexosamines controls hyaluronan synthase 2 expression and correlates with O-linked N-acetylglucosamine modification of transcription factors YY1 and SP1. J Biol Chem 2011;286(38):33632-33640.

Jones MH, Davey PM, Aplin H, Affara NA. Expression analysis, genomic structure, and mapping to 7q31 of the human sperm adhesion molecule gene SPAM1. Genomics 1995;29(3):796-800.

Jones SG, Ito T, Phillips AO. Regulation of proximal tubular epithelial cell CD44-mediated binding and internalisation of hyaluronan. Int J Biochem Cell Biol 2003;35(9):1361-1377.

Josefsson A, Adamo H, Hammarsten P, Granfors T, Stattin P, Egevad L, Laurent AE, Wikstrom P, *et al.* Prostate cancer increases hyaluronan in surrounding nonmalignant stroma, and this response is associated with tumor growth and an unfavorable outcome. Am J Pathol 2011;179(4):1961-1968.

Kaback LA, Smith TJ. Expression of hyaluronan synthase messenger ribonucleic acids and their induction by interleukin-1beta in human orbital fibroblasts: potential insight into the molecular pathogenesis of thyroid-associated ophthalmopathy. J Clin Endocrinol Metab 1999;84(11):4079-4084.

Kakizaki I, Kojima K, Takagaki K, Endo M, Kannagi R, Ito M, Maruo Y, Sato H, *et al.* A novel mechanism for the inhibition of hyaluronan biosynthesis by 4-methylumbelliferone. J Biol Chem 2004;279(32):33281-33289.

Kakizaki I, Itano N, Kimata K, Hanada K, Kon A, Yamaguchi M, Takahashi T, Takagaki K. Up-regulation of hyaluronan synthase genes in cultured human epidermal keratinocytes by UVB irradiation. Arch Biochem Biophys 2008;471(1):85-93.

Kanavy HE, Gerstenblith MR. Ultraviolet radiation and melanoma. Semin Cutan Med Surg 2011;30(4):222-228.

Kanitakis J. Anatomy, histology and immunohistochemistry of normal human skin. Eur J Dermatol 2002;12(4):390-9; quiz 400-1.

Kao JJ. The NF-kappaB inhibitor pyrrolidine dithiocarbamate blocks IL-1beta induced hyaluronan synthase 1 (HAS1) mRNA transcription, pointing at NF-kappaB dependence of the gene HAS1. Exp Gerontol 2006;41(6):641-647.

Karihtala P, Soini Y, Auvinen P, Tammi R, Tammi M, Kosma VM. Hyaluronan in breast cancer: correlations with nitric oxide synthases and tyrosine nitrosylation. J Histochem Cytochem 2007;55(12):1191-1198.

Karjalainen JM, Tammi RH, Tammi MI, Eskelinen MJ, Ågren UM, Parkkinen JJ, Alhava EM, Kosma VM. Reduced level of CD44 and hyaluronan associated with unfavorable prognosis in clinical stage I cutaneous melanoma. Am J Pathol 2000;157(3):957-965.

Karousou E, Kamiryo M, Skandalis SS, Ruusala A, Asteriou T, Passi A, Yamashita H, Hellman U, *et al.* The activity of hyaluronan synthase 2 is regulated by dimerization and ubiquitination. J Biol Chem 2010;285(31):23647-23654.

Karvinen S, Kosma VM, Tammi MI, Tammi R. Hyaluronan, CD44 and versican in epidermal keratinocyte tumours. Br J Dermatol 2003a;148(1):86-94.

Karvinen S, Pasonen-Seppänen S, Hyttinen JM, Pienimäki JP, Törrönen K, Jokela TA, Tammi MI, Tammi R. Keratinocyte growth factor stimulates migration and hyaluronan synthesis in the epidermis by activation of keratinocyte hyaluronan synthases 2 and 3. J Biol Chem 2003b;278(49):49495-49504.

Katiyar SK, Meeran SM. Obesity increases the risk of UV radiation-induced oxidative stress and activation of MAPK and NF-kappaB signaling. Free Radic Biol Med 2007;42(2):299-310.

Kawakami M, Suzuki K, Matsuki Y, Ishizuka T, Hidaka T, Konishi T, Matsumoto M, Kataharada K, *et al.* Hyaluronan production in human rheumatoid fibroblastic synovial lining cells is increased by interleukin 1 beta but inhibited by transforming growth factor beta 1. Ann Rheum Dis 1998;57(10):602-605.

Kim E, Baba D, Kimura M, Yamashita M, Kashiwabara S, Baba T. Identification of a hyaluronidase, Hyal5, involved in penetration of mouse sperm through cumulus mass. Proc Natl Acad Sci U S A 2005;102(50):18028-18033.

Kim JE, Kim B, Kim H, Kim H, Lee JD, Kim HJ, Choi KY, Lee SH. Retinyl retinoate induces hyaluronan production and less irritation than other retinoids. J Dermatol 2010;37(5):448-454.

Kim S, Kang BY, Cho SY, Sung DS, Chang HK, Yeom MH, Kim DH, Sim YC, *et al.* Compound K induces expression of hyaluronan synthase 2 gene in transformed human keratinocytes and increases hyaluronan in hairless mouse skin. Biochem Biophys Res Commun 2004;316(2):348-355.

King IA. Characterization of epidermal glycosaminoglycans synthesized in organ culture. Biochim Biophys Acta 1981;674(1):87-95.

Kitchen JR, Cysyk RL. Synthesis and release of hyaluronic acid by Swiss 3T3 fibroblasts. Biochem J 1995;309(Pt 2):649-656.

Klausner RD, Donaldson JG, Lippincott-Schwartz J. Brefeldin A: insights into the control of membrane traffic and organelle structure. J Cell Biol. 1992;116(5):1071-1080.

Klein AM, Brash DE, Jones PH, Simons BD. Stochastic fate of p53-mutant epidermal progenitor cells is tilted toward proliferation by UV B during preneoplasia. Proc Natl Acad Sci U S A 2010;107(1):270-275.

Klewes L, Prehm P. Intracellular signal transduction for serum activation of the hyaluronan synthase in eukaryotic cell lines. J Cell Physiol 1994;160(3):539-544.

Knudson CB, Toole BP. Changes in the pericellular matrix during differentiation of limb bud mesoderm. Dev Biol 1985;112(2):308-318.

Knudson CB. Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. J Cell Biol 1993;120(3):825-834.

Knudson W, Aguiar DJ, Hua Q, Knudson CB. CD44-anchored hyaluronan-rich pericellular matrices: an ultrastructural and biochemical analysis. Exp Cell Res 1996;228(2):216-228.

Kobayashi N, Miyoshi S, Mikami T, Koyama H, Kitazawa M, Takeoka M, Sano K, Amano J, *et al.* Hyaluronan deficiency in tumor stroma impairs macrophage trafficking and tumor neovascularization. Cancer Res 2010;70(18):7073-7083.

Kolev V, Mandinova A, Guinea-Viniegra J, Hu B, Lefort K, Lambertini C, Neel V, Dummer R, *et al.* EGFR signalling as a negative regulator of Notch1 gene transcription and function in proliferating keratinocytes and cancer. Nat Cell Biol 2008;10(8):902-911.

Kosaki R, Watanabe K, Yamaguchi Y. Overproduction of hyaluronan by expression of the hyaluronan synthase Has2 enhances anchorage-independent growth and tumorigenicity. Cancer Res 1999;59(5):1141-1145.

Kosunen A, Ropponen K, Kellokoski J, Pukkila M, Virtaniemi J, Valtonen H, Kumpulainen E, Johansson R, *et al.* Reduced expression of hyaluronan is a strong indicator of poor survival in oral squamous cell carcinoma. Oral Oncol 2004;40(3):257-263.

Koyama H. Hyperproduction of hyaluronan in neu-induced mammary tumor accelerates angiogenesis through stromal cell recruitment: possible involvement of versican/PG-M. Am J Pathol 2007;170(3):1086.

Kramer MW, Escudero DO, Lokeshwar SD, Golshani R, Ekwenna OO, Acosta K, Merseburger AS, Soloway M, *et al.* Association of hyaluronic acid family members (HAS1, HAS2, and HYAL-1) with bladder cancer diagnosis and prognosis. Cancer 2011;117(6):1197-1209.

Kripke ML. Ultraviolet radiation and immunology: something new under the sun--presidential address. Cancer Res 1994;54(23):6102-6105.

Kudo D, Kon A, Yoshihara S, Kakizaki I, Sasaki M, Endo M, Takagaki K. Effect of a hyaluronan synthase suppressor, 4-methylumbelliferone, on B16F-10 melanoma cell adhesion and locomotion. Biochem Biophys Res Commun 2004;321(4):783-787.

Kultti A. Hyaluronan in cancer. Regulation and Consequences of Hyaluronan Biosynthesis.University of Kuopio; 2009a.

Kultti A, Rilla K, Tiihonen R, Spicer AP, Tammi RH, Tammi MI. Hyaluronan synthesis induces microvillus-like cell surface protrusions. J Biol Chem 2006;281(23):15821-15828.

Kultti A, Pasonen-Seppänen S, Jauhiainen M, Rilla KJ, Kärnä R, Pyöriä E, Tammi RH, Tammi MI. 4-Methylumbelliferone inhibits hyaluronan synthesis by depletion of cellular UDP-glucuronic acid and downregulation of hyaluronan synthase 2 and 3. Exp Cell Res 2009b;315(11):1914-1923.

Kultti A, Kärnä R, Rilla K, Nurminen P, Koli E, Makkonen KM, Si J, Tammi MI, *et al.* Methyl-beta-cyclodextrin suppresses hyaluronan synthesis by down-regulation of hyaluronan synthase 2 through inhibition of Akt. J Biol Chem 2010;285(30):22901-22910.

Kumlin T, Kosma VM, Alhonen L, Jänne J, Komulainen H, Lang S, Rytomaa T, Servomaa K, *et al.* Effects of 50 Hz magnetic fields on UV-induced skin tumourigenesis in ODC-transgenic and non-transgenic mice. Int J Radiat Biol 1998;73(1):113-121.

Kurdykowski S, Mine S, Bardey V, Danoux L, Jeanmaire C, Pauly G, Brabencova E, Wegrowski Y, *et al.* Ultraviolet-B irradiation induces differential regulations of hyaluronidase expression and activity in normal human keratinocytes. Photochem Photobiol 2011;87(5):1105-1112.

Kuroda K, Utani A, Hamasaki Y, Shinkai H. Up-regulation of putative hyaluronan synthase mRNA by basic fibroblast growth factor and insulin-like growth factor-1 in human skin fibroblasts. J Dermatol Sci 2001;26(2):156-160.

Kwon MJ, Han J, Kim BH, Lee YS, Kim TY. Superoxide dismutase 3 suppresses hyaluronic acid fragments mediated skin inflammation by inhibition of toll-like receptor 4 signaling pathway: superoxide dismutase 3 inhibits reactive oxygen species-induced trafficking of toll-like receptor 4 to lipid rafts. Antioxid Redox Signal 2012;16(4):297-313.

Kyosseva SV, Harris EN, Weigel PH. The hyaluronan receptor for endocytosis mediates hyaluronan-dependent signal transduction via extracellular signal-regulated kinases. J Biol Chem 2008;283(22):15047-15055.

Lauer ME, Erzurum SC, Mukhopadhyay D, Vasanji A, Drazba J, Wang A, Fulop C, Hascall VC. Differentiated murine airway epithelial cells synthesize a leukocyte-adhesive hyaluronan matrix in response to endoplasmic reticulum stress. J Biol Chem 2008;283(38):26283-26296.

Lauer ME, Fulop C, Mukhopadhyay D, Comhair S, Erzurum SC, Hascall VC. Airway smooth muscle cells synthesize hyaluronan cable structures independent of inter-alpha-inhibitor heavy chain attachment. J Biol Chem 2009a;284(8):5313-5323.

Lauer ME, Mukhopadhyay D, Fulop C, de la Motte CA, Majors AK, Hascall VC. Primary murine airway smooth muscle cells exposed to poly(I,C) or tunicamycin synthesize a leukocyte-adhesive hyaluronan matrix. J Biol Chem 2009b;284(8):5299-5312.

Laurent TC, Fraser JR. Hyaluronan. FASEB J 1992;6(7):2397-2404.

Laurent UB, Laurent TC, Hellsing LK, Persson L, Hartman M, Lilja K. Hyaluronan in human cerebrospinal fluid. Acta Neurol Scand 1996;94(3):194-206.

LeBaron RG, Zimmermann DR, Ruoslahti E. Hyaluronate binding properties of versican. J Biol Chem 1992;267(14):10003-10010.

LeBoit P, Burg G, Weedon D, Sarasin A. World Health Organization classification of tumours. Pathology and Genetics. Skin tumours. Lyon: IARC Press; 2006.

Lee TH, Wisniewski HG, Vilcek J. A novel secretory tumor necrosis factor-inducible protein (TSG-6) is a member of the family of hyaluronate binding proteins, closely related to the adhesion receptor CD44. J Cell Biol 1992;116(2):545-557.

Leigh CJ, Palechek PL, Knutson JR, McCarthy JB, Cohen MB, Argenyi ZB. CD44 expression in benign and malignant nevomelanocytic lesions. Hum Pathol 1996;27(12):1288-1294.

Lepperdinger G, Strobl B, Kreil G. HYAL2, a human gene expressed in many cells, encodes a lysosomal hyaluronidase with a novel type of specificity. J Biol Chem 1998;273(35):22466-22470.

Lepperdinger G, Müllegger J, Kreil G. Hyal2--less active, but more versatile? Matrix Biol 2001;20(8):509-514.

Lesley J, Hascall VC, Tammi M, Hyman R. Hyaluronan binding by cell surface CD44. J Biol Chem 2000;275(35):26967-26975.

Lesley J, Gal I, Mahoney DJ, Cordell MR, Rugg MS, Hyman R, Day AJ, Mikecz K. TSG-6 modulates the interaction between hyaluronan and cell surface CD44. J Biol Chem 2004;279(24):25745-25754.

Levesque MC, Haynes BF. Cytokine induction of the ability of human monocyte CD44 to bind hyaluronan is mediated primarily by TNF-alpha and is inhibited by IL-4 and IL-13. J Immunol 1997;159(12):6184-6194.

Li J, Zha XM, Wang R, Li XD, Xu B, Xu YJ, Yin YM. Regulation of CD44 expression by tumor necrosis factor-alpha and its potential role in breast cancer cell migration. Biomed Pharmacother 2012;66(2):144-150.

Li L, Asteriou T, Bernert B, Heldin CH, Heldin P. Growth factor regulation of hyaluronan synthesis and degradation in human dermal fibroblasts: importance of hyaluronan for the mitogenic response of PDGF-BB. Biochem J 2007;404(2):327-336.

Li Y, Heldin P. Hyaluronan production increases the malignant properties of mesothelioma cells. Br J Cancer 2001;85(4):600-607.

Li Y, Li L, Brown TJ, Heldin P. Silencing of hyaluronan synthase 2 suppresses the malignant phenotype of invasive breast cancer cells. Int J Cancer 2007a;120(12):2557-2567.

Li Y, Toole BP, Dealy CN, Kosher RA. Hyaluronan in limb morphogenesis. Dev Biol 2007b;305(2):411-420.

Lipponen P, Aaltomaa S, Tammi R, Tammi M, Ågren U, Kosma VM. High stromal hyaluronan level is associated with poor differentiation and metastasis in prostate cancer. Eur J Cancer 2001;37(7):849-856.

Liu CM, Chang CH, Yu CH, Hsu CC, Huang LL. Hyaluronan substratum induces multidrug resistance in human mesenchymal stem cells via CD44 signaling. Cell Tissue Res 2009;336(3):465-475.

Liu D, Pearlman E, Diaconu E, Guo K, Mori H, Haqqi T, Markowitz S, Willson J, *et al.* Expression of hyaluronidase by tumor cells induces angiogenesis in vivo. Proc Natl Acad Sci U S A 1996;93(15):7832-7837.

Lokeshwar VB, Lokeshwar BL, Pham HT, Block NL. Association of elevated levels of hyaluronidase, a matrix-degrading enzyme, with prostate cancer progression. Cancer Res 1996;56(3):651-657.

Lokeshwar VB, Obek C, Pham HT, Wei D, Young MJ, Duncan RC, Soloway MS, Block NL. Urinary hyaluronic acid and hyaluronidase: markers for bladder cancer detection and evaluation of grade. J Urol 2000;163(1):348-356.

Lokeshwar VB, Rubinowicz D, Schroeder GL, Forgacs E, Minna JD, Block NL, Nadji M, Lokeshwar BL. Stromal and epithelial expression of tumor markers hyaluronic acid and HYAL1 hyaluronidase in prostate cancer. J Biol Chem 2001;276(15):11922-11932.

Lokeshwar VB, Schroeder GL, Carey RI, Soloway MS, Iida N. Regulation of hyaluronidase activity by alternative mRNA splicing. J Biol Chem 2002;277(37):33654-33663.

Lokeshwar VB, Cerwinka WH, Isoyama T, Lokeshwar BL. HYAL1 hyaluronidase in prostate cancer: a tumor promoter and suppressor. Cancer Res 2005;65(17):7782-7789.

Lokeshwar VB, Gomez P, Kramer M, Knapp J, McCornack MA, Lopez LE, Fregien N, Dhir N, *et al.* Epigenetic regulation of HYAL-1 hyaluronidase expression. identification of HYAL-1 promoter. J Biol Chem 2008;283(43):29215-29227.

Mack JA, Feldman RJ, Itano N, Kimata K, Lauer M, Hascall VC, Maytin EV. Enhanced inflammation and accelerated wound closure following tetraphorbol ester application or full-thickness wounding in mice lacking hyaluronan synthases Has1 and Has3. J Invest Dermatol 2012;132(1):198-207.

Madan AK, Yu K, Dhurandhar N, Cullinane C, Pang Y, Beech DJ. Association of hyaluronidase and breast adenocarcinoma invasiveness. Oncol Rep 1999;6(3):607-609.

Madson JG, Hansen LA. Multiple mechanisms of Erbb2 action after ultraviolet irradiation of the skin. Mol Carcinog 2007;46(8):624-628.

Maitra A, Wistuba II, Washington C, Virmani AK, Ashfaq R, Milchgrub S, Gazdar AF, Minna JD. High-resolution chromosome 3p allelotyping of breast carcinomas and precursor lesions demonstrates frequent loss of heterozygosity and a discontinuous pattern of allele loss. Am J Pathol 2001;159(1):119-130.

Majors AK, Austin RC, de la Motte CA, Pyeritz RE, Hascall VC, Kessler SP, Sen G, Strong SA. Endoplasmic reticulum stress induces hyaluronan deposition and leukocyte adhesion. J Biol Chem 2003;278(47):47223-47231.

Makkonen KM, Pasonen-Seppänen S, Törrönen K, Tammi MI, Carlberg C. Regulation of the hyaluronan synthase 2 gene by convergence in cyclic AMP response element-binding protein and retinoid acid receptor signaling. J Biol Chem 2009;284(27):18270-18281.

Mani SK, Carson DD, Glasser SR. Steroid hormones differentially modulate glycoconjugate synthesis and vectorial secretion by polarized uterine epithelial cells in vitro. Endocrinology 1992;130(1):240-248.

Mapleson JL, Buchwald M. Effect of cycloheximide and dexamethasone phosphate on hyaluronic acid synthesis and secretion in cultured human skin fibroblasts. J Cell Physiol 1981;109(2):215-222.

Marieb EA, Zoltan-Jones A, Li R, Misra S, Ghatak S, Cao J, Zucker S, Toole BP. Emmprin promotes anchorage-independent growth in human mammary carcinoma cells by stimulating hyaluronan production. Cancer Res 2004;64(4):1229-1232.

Markovic-Housley Z, Miglierini G, Soldatova L, Rizkallah PJ, Muller U, Schirmer T. Crystal structure of hyaluronidase, a major allergen of bee venom. Structure 2000;8(10):1025-1035.

Markovitz A, Cifonelli JA, Dorfman A. The biosynthesis of hyaluronic acid by group A Streptococcus. VI. Biosynthesis from uridine nucleotides in cell-free extracts. J Biol Chem 1959;234:2343-2350.

Martin DC, Atmuri V, Hemming RJ, Farley J, Mort JS, Byers S, Hombach-Klonisch S, Csoka AB, *et al.* A mouse model of human mucopolysaccharidosis IX exhibits osteoarthritis. Hum Mol Genet 2008;17(13):1904-1915.

Masunaga T. Epidermal basement membrane: its molecular organization and blistering disorders. Connect Tissue Res 2006;47(2):55-66.

Matsumura Y, Ananthaswamy HN. Toxic effects of ultraviolet radiation on the skin. Toxicol Appl Pharmacol 2004;195(3):298-308.

Mausolf A, Jungmann J, Robenek H, Prehm P. Shedding of hyaluronate synthase from streptococci. Biochem J 1990;267(1):191-196.

Maxwell CA, Keats JJ, Crainie M, Sun X, Yen T, Shibuya E, Hendzel M, Chan G, *et al.* RHAMM is a centrosomal protein that interacts with dynein and maintains spindle pole stability. Mol Biol Cell 2003;14(6):2262-2276.

Maxwell CA, McCarthy J, Turley E. Cell-surface and mitotic-spindle RHAMM: moonlighting or dual oncogenic functions? J Cell Sci 2008;121(Pt 7):925-932.

McGary CT, Raja RH, Weigel PH. Endocytosis of hyaluronic acid by rat liver endothelial cells. Evidence for receptor recycling. Biochem J 1989;257(3):875-884.

Medina AP, Lin J, Weigel PH. Hyaluronan synthase mediates dye translocation across liposomal membranes. BMC Biochem 2012;13:2.

Melnikova VO, Ananthaswamy HN. Cellular and molecular events leading to the development of skin cancer. Mutat Res 2005;571(1-2):91-106.

Mendoza G, Alvarez AI, Pulido MM, Molina AJ, Merino G, Real R, Fernandes P, Prieto JG. Inhibitory effects of different antioxidants on hyaluronan depolymerization. Carbohydr Res 2007;342(1):96-102.

Meran S, Thomas D, Stephens P, Martin J, Bowen T, Phillips A, Steadman R. Involvement of hyaluronan in regulation of fibroblast phenotype. J Biol Chem 2007;282(35):25687-25697.

Meran S, Luo DD, Simpson R, Martin J, Wells A, Steadman R, Phillips AO. Hyaluronan facilitates transforming growth factor-beta1-dependent proliferation via CD44 and epidermal growth factor receptor interaction. J Biol Chem 2011;286(20):17618-17630.

Messadi DV, Bertolami CN. CD44 and hyaluronan expression in human cutaneous scar fibroblasts. Am J Pathol 1993;142(4):1041-1049.

Meyer K, Palmer JW. The polysaccharide of the vitreous humor. 1934;107:629-634.

Meyer MF, Kreil G. Cells expressing the DG42 gene from early Xenopus embryos synthesize hyaluronan. Proc Natl Acad Sci U S A 1996;93(10):4543-4547.

Midura RJ, Evanko SP, Hascall VC. Parathyroid hormone stimulates hyaluronan synthesis in an osteoblast-like cell line. J Biol Chem 1994;269(18):13200-13206.

Milner CM, Day AJ. TSG-6: a multifunctional protein associated with inflammation. J Cell Sci 2003;116(Pt 10):1863-1873.

Mio K, Carrette O, Maibach HI, Stern R. Evidence that the serum inhibitor of hyaluronidase may be a member of the inter-alpha-inhibitor family. J Biol Chem 2000;275(42):32413-32421.

Mio K, Stern R. Inhibitors of the hyaluronidases. Matrix Biol 2002;21(1):31-37.

Misra S, Ghatak S, Toole BP. Regulation of MDR1 expression and drug resistance by a positive feedback loop involving hyaluronan, phosphoinositide 3-kinase, and ErbB2. J Biol Chem 2005;280(21):20310-20315.

Misra S, Obeid LM, Hannun YA, Minamisawa S, Berger FG, Markwald RR, Toole BP, Ghatak S. Hyaluronan constitutively regulates activation of COX-2-mediated cell survival activity in intestinal epithelial and colon carcinoma cells. J Biol Chem 2008;283(21):14335-14344.

Misra S, Ghatak S, Zoltan-Jones A, Toole BP. Regulation of multidrug resistance in cancer cells by hyaluronan. J Biol Chem 2003;278(28):25285-25288.

Mitani H, Koshiishi I, Toyoda H, Toida T, Imanari T. Alterations of hairless mouse skin exposed to chronic UV irradiation and its prevention by hydrocortisone. Photochem Photobiol 1999;69(1):41-46.

Mohapatra S, Yang X, Wright JA, Turley EA, Greenberg AH. Soluble hyaluronan receptor RHAMM induces mitotic arrest by suppressing Cdc2 and cyclin B1 expression. J Exp Med 1996;183(4):1663-1668.

Monslow J, Williams JD, Norton N, Guy CA, Price IK, Coleman SL, Williams NM, Buckland PR, *et al.* The human hyaluronan synthase genes: genomic structures, proximal promoters and polymorphic microsatellite markers. Int J Biochem Cell Biol 2003;35(8):1272-1283.

Monslow J, Williams JD, Fraser DJ, Michael DR, Foka P, Kift-Morgan AP, Luo DD, Fielding CA, *et al.* Sp1 and Sp3 mediate constitutive transcription of the human hyaluronan synthase 2 gene. J Biol Chem 2006;281(26):18043-18050.

Montesano R, Kumar S, Orci L, Pepper MS. Synergistic effect of hyaluronan oligosaccharides and vascular endothelial growth factor on angiogenesis in vitro. Lab Invest 1996;75(2):249-262.

Monzon ME, Fregien N, Schmid N, Falcon NS, Campos M, Casalino-Matsuda SM, Forteza RM. Reactive oxygen species and hyaluronidase 2 regulate airway epithelial hyaluronan fragmentation. J Biol Chem 2010;285(34):26126-26134.

Morales TI, Hascall VC. Correlated metabolism of proteoglycans and hyaluronic acid in bovine cartilage organ cultures. J Biol Chem 1988;263(8):3632-3638.

Morita K, Miyachi Y. Tight junctions in the skin. J Dermatol Sci 2003;31(2):81-89.

Morohashi H, Kon A, Nakai M, Yamaguchi M, Kakizaki I, Yoshihara S, Sasaki M, Takagaki K. Study of hyaluronan synthase inhibitor, 4-methylumbelliferone derivatives on human pancreatic cancer cell (KP1-NL). Biochem Biophys Res Commun 2006;345(4):1454-1459.

Moseley R, Waddington R, Evans P, Halliwell B, Embery G. The chemical modification of glycosaminoglycan structure by oxygen-derived species in vitro. Biochim Biophys Acta 1995;1244(2-3):245-252.

Mukhopadhyay D, Hascall VC, Day AJ, Salustri A, Fulop C. Two distinct populations of tumor necrosis factor-stimulated gene-6 protein in the extracellular matrix of expanded mouse cumulus cell-oocyte complexes. Arch Biochem Biophys 2001;394(2):173-181.

Müllegger J, Lepperdinger G. Degradation of hyaluronan by a Hyal2-type hyaluronidase affects pattern formation of vitelline vessels during embryogenesis of Xenopus laevis. Mech Dev 2002;111(1-2):25-35.

Müllegger J, Rustom A, Kreil G, Gerdes HH, Lepperdinger G. 'Piggy-back' transport of Xenopus hyaluronan synthase (XHAS1) via the secretory pathway to the plasma membrane. Biol Chem 2003;384(1):175-182.

Mummert DI, Takashima A, Ellinger L, Mummert ME. Involvement of hyaluronan in epidermal Langerhans cell maturation and migration in vivo. J Dermatol Sci 2003;33(2):91-97.

Murata M, Yudoh K, Shimizu H, Beppu M, Nakamura H, Kato T, Masuko K. Layilin, a talin-binding hyaluronan receptor, is expressed in human articular chondrocytes and synoviocytes and is down-regulated by interleukin-1beta. Mod Rheumatol 2012; in press.

Muthukumaran N, Miletti-Gonzalez KE, Ravindranath AK, Rodriguez-Rodriguez L. Tumor necrosis factor-alpha differentially modulates CD44 expression in ovarian cancer cells. Mol Cancer Res 2006;4(8):511-520.

Nakamura H, Kitazawa K, Honda H, Sugisaki T. Roles of and correlation between alpha-smooth muscle actin, CD44, hyaluronic acid and osteopontin in crescent formation in human glomerulonephritis. Clin Nephrol 2005;64(6):401-411.

Nakamura T, Takagaki K, Shibata S, Tanaka K, Higuchi T, Endo M. Hyaluronic-acid-deficient extracellular matrix induced by addition of 4-methylumbelliferone to the medium of cultured human skin fibroblasts. Biochem Biophys Res Commun 1995;208(2):470-475.

Nakamura T, Funahashi M, Takagaki K, Munakata H, Tanaka K, Saito Y, Endo M. Effect of 4-methylumbelliferone on cell-free synthesis of hyaluronic acid. Biochem Mol Biol Int 1997;43(2):263-268.

Nakazawa H, Yoshihara S, Kudo D, Morohashi H, Kakizaki I, Kon A, Takagaki K, Sasaki M. 4-Methylumbelliferone, a Hyaluronan Synthase Suppressor, Enhances the Anticancer Activity of Gemcitabine in Human Pancreatic Cancer Cells. Cancer Chemother Pharmacol 2006;57(2):165-170.

Natowicz MR, Short MP, Wang Y, Dickersin GR, Gebhardt MC, Rosenthal DI, Sims KB, Rosenberg AE. Clinical and biochemical manifestations of hyaluronidase deficiency. N Engl J Med 1996;335(14):1029-1033.

Neame SJ, Isacke CM. Phosphorylation of CD44 in vivo requires both Ser323 and Ser325, but does not regulate membrane localization or cytoskeletal interaction in epithelial cells. EMBO J 1992;11(13):4733-4738.

Nicoll SB, Barak O, Csoka AB, Bhatnagar RS, Stern R. Hyaluronidases and CD44 undergo differential modulation during chondrogenesis. Biochem Biophys Res Commun 2002;292(4):819-825.

Nishida Y, Knudson W, Knudson CB, Ishiguro N. Antisense inhibition of hyaluronan synthase-2 in human osteosarcoma cells inhibits hyaluronan retention and tumorigenicity. Exp Cell Res 2005;307(1):194-203.

Nykopp TK, Rilla K, Sironen R, Tammi MI, Tammi RH, Hämäläinen K, Heikkinen AM, Komulainen M, *et al.* Expression of hyaluronan synthases (HAS1-3) and hyaluronidases (HYAL1-2) in serous ovarian carcinomas: inverse correlation between HYAL1 and hyaluronan content. BMC Cancer 2009;9:143.

Nykopp TK, Rilla K, Tammi MI, Tammi RH, Sironen R, Hämäläinen K, Kosma VM, Heinonen S, *et al.* Hyaluronan synthases (HAS1-3) and hyaluronidases (HYAL1-2) in the accumulation of hyaluronan in endometrioid endometrial carcinoma. BMC Cancer 2010;10:512.

Obrig TG, Culp WJ, McKeehan WL, Hardesty B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J Biol Chem 1971;246(1):174-181.

Oguchi T, Ishiguro N. Differential stimulation of three forms of hyaluronan synthase by TGF-beta, IL-1beta, and TNF-alpha. Connect Tissue Res 2004;45(4-5):197-205.

Ohashi R, Takahashi F, Cui R, Yoshioka M, Gu T, Sasaki S, Tominaga S, Nishio K, *et al.* Interaction between CD44 and hyaluronate induces chemoresistance in non-small cell lung cancer cell. Cancer Lett 2007;252(2):225-234.

Ohno S, Ohno-Nakahara M, Knudson CB, Knudson W. Induction of MMP-3 by hyaluronan oligosaccharides in temporomandibular joint chondrocytes. J Dent Res 2005;84(11):1005-1009.

Ohno S, Im HJ, Knudson CB, Knudson W. Hyaluronan oligosaccharides induce matrix metalloproteinase 13 via transcriptional activation of NFkappaB and p38 MAP kinase in articular chondrocytes. J Biol Chem 2006;281(26):17952-17960.

Ohtani T, Memezawa A, Okuyama R, Sayo T, Sugiyama Y, Inoue S, Aiba S. Increased hyaluronan production and decreased E-cadherin expression by cytokine-stimulated keratinocytes lead to spongiosis formation. J Invest Dermatol 2009;129(6):1412-1420.

Ohwada C, Nakaseko C, Koizumi M, Takeuchi M, Ozawa S, Naito M, Tanaka H, Oda K, *et al.* CD44 and hyaluronan engagement promotes dexamethasone resistance in human myeloma cells. Eur J Haematol 2008;80(3):245-250.

Okamoto I, Kawano Y, Tsuiki H, Sasaki J, Nakao M, Matsumoto M, Suga M, Ando M, *et al.* CD44 cleavage induced by a membrane-associated metalloprotease plays a critical role in tumor cell migration. Oncogene 1999;18(7):1435-1446.

Oksala O, Salo T, Tammi R, Hakkinen L, Jalkanen M, Inki P, Larjava H. Expression of proteoglycans and hyaluronan during wound healing. J Histochem Cytochem 1995;43(2):125-135.

Okuda H, Kobayashi A, Xia B, Watabe M, Pai SK, Hirota S, Xing F, Liu W, *et al.* Hyaluronan synthase HAS2 promotes tumor progression in bone by stimulating the interaction of breast cancer stem-like cells with macrophages and stromal cells. Cancer Res 2012;72(2):537-547.

Omholt K, Platz A, Kanter L, Ringborg U, Hansson J. NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. Clin Cancer Res 2003;9(17):6483-6488.

Ouhtit A, Gorny A, Muller HK, Hill LL, Owen-Schaub L, Ananthaswamy HN. Loss of Fas-ligand expression in mouse keratinocytes during UV carcinogenesis. Am J Pathol 2000;157(6):1975-1981.

Ouskova G, Spellerberg B, Prehm P. Hyaluronan release from Streptococcus pyogenes: export by an ABC transporter. Glycobiology 2004;14(10):931-938.

Panasyuk A, Frati E, Ribault D, Mitrovic D. Effect of reactive oxygen species on the biosynthesis and structure of newly synthesized proteoglycans. Free Radic Biol Med 1994;16(2):157-167.

Pasonen-Seppänen S, Karvinen S, Törrönen K, Hyttinen JM, Jokela T, Lammi MJ, Tammi MI, Tammi R. 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 2003;120(6):1038-1044.

Pasonen-Seppänen S, Hyttinen JM, Rilla K, Jokela T, Noble PW, Tammi M, Tammi R. Role of CD44 in the organization of keratinocyte pericellular hyaluronan. Histochem Cell Biol 2012a;137(1):107-120.

Pasonen-Seppänen S, Takabe P, Edward M, Rauhala L, Rilla K, Tammi M, Tammi R. Melanoma cell-derived factors stimulate hyaluronan synthesis in dermal fibroblasts by upregulating HAS2 through PDGFR-PI3K-AKT and p38 signaling. Histochem Cell Biol 2012b;138(6):895-911.

Pasonen-Seppänen SM, Maytin EV, Törrönen KJ, Hyttinen JM, Hascall VC, MacCallum DK, Kultti AH, Jokela TA, *et al.* All-trans retinoic acid-induced hyaluronan production and hyperplasia are partly mediated by EGFR signaling in epidermal keratinocytes. J Invest Dermatol 2008;128(4):797-807.

Passi A, Sadeghi P, Kawamura H, Anand S, Sato N, White LE, Hascall VC, Maytin EV. Hyaluronan suppresses epidermal differentiation in organotypic cultures of rat keratinocytes. Exp Cell Res 2004;296(2):123-134.

Pauloin T, Dutot M, Joly F, Warnet JM, Rat P. High molecular weight hyaluronan decreases UVB-induced apoptosis and inflammation in human epithelial corneal cells. Mol Vis 2009;15:577-583.

Peterson RM, Yu Q, Stamenkovic I, Toole BP. Perturbation of hyaluronan interactions by soluble CD44 inhibits growth of murine mammary carcinoma cells in ascites. Am J Pathol 2000;156(6):2159-2167.

Piccioni F, Malvicini M, Garcia MG, Rodriguez A, Atorrasagasti C, Kippes N, Piedra Buena IT, Rizzo MM, *et al.* Antitumor effects of hyaluronic acid inhibitor 4-methylumbelliferone in an orthotopic hepatocellular carcinoma model in mice. Glycobiology 2012;22(3):400-410.

Pienimäki JP, Rilla K, Fulop C, Sironen RK, Karvinen S, Pasonen S, Lammi MJ, Tammi R, *et al.* Epidermal growth factor activates hyaluronan synthase 2 in epidermal keratinocytes and increases pericellular and intracellular hyaluronan. J Biol Chem 2001;276(23):20428-20435.

Pierceall WE, Goldberg LH, Tainsky MA, Mukhopadhyay T, Ananthaswamy HN. Ras gene mutation and amplification in human nonmelanoma skin cancers. Mol Carcinog 1991;4(3):196-202.

Pirinen R, Tammi R, Tammi M, Hirvikoski P, Parkkinen JJ, Johansson R, Böhm J, Hollmen S, *et al.* Prognostic value of hyaluronan expression in non-small-cell lung cancer: Increased stromal expression indicates unfavorable outcome in patients with adenocarcinoma. Int J Cancer 2001;95(1):12-17.

Pirinen RT, Tammi RH, Tammi MI, Pääkkö PK, Parkkinen JJ, Ågren UM, Johansson RT, Viren MM, *et al.* Expression of hyaluronan in normal and dysplastic bronchial epithelium and in squamous cell carcinoma of the lung. Int J Cancer 1998;79(3):251-255.

Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003;4(1):33-45.

Posey JT, Soloway MS, Ekici S, Sofer M, Civantos F, Duncan RC, Lokeshwar VB. Evaluation of the prognostic potential of hyaluronic acid and hyaluronidase (HYAL1) for prostate cancer. Cancer Res 2003;63(10):2638-2644.

Prehm P. Synthesis of hyaluronate in differentiated teratocarcinoma cells. Characterization of the synthase. Biochem J 1983a;211(1):181-189.

Prehm P. Synthesis of hyaluronate in differentiated teratocarcinoma cells. Mechanism of chain growth. Biochem J 1983b;211(1):191-198.

Prehm P. Hyaluronate is synthesized at plasma membranes. Biochem J 1984;220(2):597-600.

Prehm P, Schumacher U. Inhibition of hyaluronan export from human fibroblasts by inhibitors of multidrug resistance transporters. Biochem Pharmacol 2004;68(7):1401-1410.

Prehm P. Biosynthesis of hyaluronan: direction of chain elongation. Biochem J 2006;398(3):469-473.

Pummill PE, Achyuthan AM, DeAngelis PL. Enzymological characterization of recombinant xenopus DG42, a vertebrate hyaluronan synthase. J Biol Chem 1998;273(9):4976-4981.

Pummill PE, DeAngelis PL. Alteration of polysaccharide size distribution of a vertebrate hyaluronan synthase by mutation. J Biol Chem 2003;278(22):19808-19814.

Rai SK, Duh FM, Vigdorovich V, Danilkovitch-Miagkova A, Lerman MI, Miller AD. Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation. Proc Natl Acad Sci U S A 2001;98(8):4443-4448.

Ramakrishna S, Suresh B, Bae SM, Ahn WS, Lim KH, Baek KH. Hyaluronan Binding Motifs of USP17 and SDS3 Exhibit Anti-Tumor Activity. PLoS One 2012;7(5):e37772.

Ranganathan S, Bharadwaj A, Datta K. Hyaluronan mediates sperm motility by enhancing phosphorylation of proteins including hyaluronan binding protein. Cell Mol Biol Res 1995;41(5):467-476.

Ratushny V, Gober MD, Hick R, Ridky TW, Seykora JT. From keratinocyte to cancer: the pathogenesis and modeling of cutaneous squamous cell carcinoma. J Clin Invest 2012;122(2):464-472.

Rauch U, Karthikeyan L, Maurel P, Margolis RU, Margolis RK. Cloning and primary structure of neurocan, a developmentally regulated, aggregating chondroitin sulfate proteoglycan of brain. J Biol Chem 1992;267(27):19536-19547.

Rauch U, Feng K, Zhou XH. Neurocan: a brain chondroitin sulfate proteoglycan. Cell Mol Life Sci 2001;58(12-13):1842-1856.

Ren J, Hascall VC, Wang A. Cyclin D3 mediates synthesis of a hyaluronan matrix that is adhesive for monocytes in mesangial cells stimulated to divide in hyperglycemic medium. J Biol Chem 2009;284(24):16621-16632.

Ribatti D, Vacca A, Ria R, Marzullo A, Nico B, Filotico R, Roncali L, Dammacco F. Neovascularisation, expression of fibroblast growth factor-2, and mast cells with tryptase activity increase simultaneously with pathological progression in human malignant melanoma. Eur J Cancer 2003;39(5):666-674.

Ribatti D, Crivellato E, Vacca A. Inflammation and antiangiogenesis in cancer. Curr Med Chem 2012;19(7):955-960.

Ricciardelli C, Sakko AJ, Ween MP, Russell DL, Horsfall DJ. The biological role and regulation of versican levels in cancer. Cancer Metastasis Rev 2009;28(1-2):233-245.

Richmond-Sinclair NM, Lee E, Cummings MC, Williamson R, Muller K, Green AC, Hayward NK, Whiteman DC. Histologic and epidemiologic correlates of P-MAPK, Brn-2, pRb, p53, and p16 immunostaining in cutaneous melanomas. Melanoma Res 2008;18(5):336-345.

Rigden DJ, Jedrzejas MJ, de Mello LV. Identification and analysis of catalytic TIM barrel domains in seven further glycoside hydrolase families. FEBS Lett 2003;544(1-3):103-111.

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

Rilla K, Tiihonen R, Kultti A, Tammi M, Tammi R. Pericellular hyaluronan coat visualized in live cells with a fluorescent probe is scaffolded by plasma membrane protrusions. J Histochem Cytochem 2008;56(10):901-910.

Rilla K, Pasonen-Seppänen S, Kärnä R, Karjalainen HM, Törrönen K, Koistinen V, Tammi MI, Tammi RH, *et al.* HAS3-induced accumulation of hyaluronan in 3D MDCK cultures results in mitotic spindle misorientation and disturbed organization of epithelium. Histochem Cell Biol 2012;137(2):153-164.

Rilla K, Oikari S, Jokela TA, Hyttinen JM, Kärnä R, Tammi RH, Tammi MI. Hyaluronan synthase 1 (HAS1) requires higher cellular UDP-GlcNAc concentration than HAS2 and HAS3. J Biol Chem. 2013; in press.

Roden L, Campbell P, Fraser JR, Laurent TC, Pertoft H, Thompson JN. Enzymic pathways of hyaluronan catabolism. Ciba Found Symp 1989;143:60-76; discussion 76-86, 281-5.

Rodgers LS, Lalani S, Hardy KM, Xiang X, Broka D, Antin PB, Camenisch TD. Depolymerized hyaluronan induces vascular endothelial growth factor, a negative regulator of developmental epithelial-to-mesenchymal transformation. Circ Res 2006;99(6):583-589.

Rosa F, Sargent TD, Rebbert ML, Michaels GS, Jamrich M, Grunz H, Jonas E, Winkles JA, *et al.* Accumulation and decay of DG42 gene products follow a gradient pattern during Xenopus embryogenesis. Dev Biol 1988;129(1):114-123.

Ruffell B, Johnson P. Hyaluronan induces cell death in activated T cells through CD44. J Immunol 2008;181(10):7044-7054.

Rugg MS, Willis AC, Mukhopadhyay D, Hascall VC, Fries E, Fulop C, Milner CM, Day AJ. Characterization of complexes formed between TSG-6 and inter-alpha-inhibitor that act as intermediates in the covalent transfer of heavy chains onto hyaluronan. J Biol Chem 2005;280(27):25674-25686.

Röck K, Grandoch M, Majora M, Krutmann J, Fischer JW. Collagen fragments inhibit hyaluronan synthesis in skin fibroblasts in response to ultraviolet B (UVB): new insights into mechanisms of matrix remodeling. J Biol Chem 2011;286(20):18268-18276.

Saavalainen K, Pasonen-Seppänen S, Dunlop TW, Tammi R, Tammi MI, Carlberg C. The human hyaluronan synthase 2 gene is a primary retinoic acid and epidermal growth factor responding gene. J Biol Chem 2005;280(15):14636-14644.

Saavalainen K, Tammi MI, Bowen T, Schmitz ML, Carlberg C. Integration of the activation of the human hyaluronan synthase 2 gene promoter by common cofactors of the transcription factors retinoic acid receptor and nuclear factor kappaB. J Biol Chem 2007;282(15):11530-11539.

Sakr SW, Potter-Perigo S, Kinsella MG, Johnson PY, Braun KR, Goueffic Y, Rosenfeld ME, Wight TN. Hyaluronan accumulation is elevated in cultures of low density lipoprotein receptor-deficient cells and is altered by manipulation of cell cholesterol content. J Biol Chem 2008;283(52):36195-36204.

Salustri A, Yanagishita M, Hascall VC. Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. J Biol Chem 1989;264(23):13840-13847.

Salustri A, Camaioni A, Di Giacomo M, Fulop C, Hascall VC. Hyaluronan and proteoglycans in ovarian follicles. Hum Reprod Update 1999;5(4):293-301.

Sampson PM, Rochester CL, Freundlich B, Elias JA. Cytokine regulation of human lung fibroblast hyaluronan (hyaluronic acid) production. Evidence for cytokine-regulated hyaluronan (hyaluronic acid) degradation and human lung fibroblast-derived hyaluronidase. J Clin Invest 1992;90(4):1492-1503.

Sandilands A, Sutherland C, Irvine AD, McLean WH. Filaggrin in the frontline: role in skin barrier function and disease. J Cell Sci 2009;122(Pt 9):1285-1294.

Sano S, Chan KS, Kira M, Kataoka K, Takagi S, Tarutani M, Itami S, Kiguchi K, *et al.* Signal transducer and activator of transcription 3 is a key regulator of keratinocyte survival and proliferation following UV irradiation. Cancer Res 2005;65(13):5720-5729.

Sato H, Takahashi T, Ide H, Fukushima T, Tabata M, Sekine F, Kobayashi K, Negishi M, *et al.* Antioxidant activity of synovial fluid, hyaluronic acid, and two subcomponents of hyaluronic acid. Synovial fluid scavenging effect is enhanced in rheumatoid arthritis patients. Arthritis Rheum 1988;31(1):63-71.

Savani RC, Wang C, Yang B, Zhang S, Kinsella MG, Wight TN, Stern R, Nance DM, *et al.* Migration of bovine aortic smooth muscle cells after wounding injury. The role of hyaluronan and RHAMM. J Clin Invest 1995;95(3):1158-1168.

Sayo T, Sugiyama Y, Takahashi Y, Ozawa N, Sakai S, Ishikawa O, Tamura M, Inoue S. Hyaluronan synthase 3 regulates hyaluronan synthesis in cultured human keratinocytes. J Invest Dermatol 2002;118(1):43-48.

Schnell U, Dijk F, Sjollema KA, Giepmans BN. Immunolabeling artifacts and the need for live-cell imaging. Nat Methods 2012;9(2):152-158.

Schulz T, Schumacher U, Prehm P. Hyaluronan export by the ABC transporter MRP5 and its modulation by intracellular cGMP. J Biol Chem 2007;282(29):20999-21004.

Schwartz E. Connective tissue alterations in the skin of ultraviolet irradiated hairless mice. J Invest Dermatol 1988;91(2):158-161.

Scott JE. Secondary structures in hyaluronan solutions: chemical and biological implications. Ciba Found Symp 1989;143:6-15; discussion 15-20, 281-5.

Scott JE. Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues. FASEB J 1992;6(9):2639-2645.

Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc Natl Acad Sci U S A 1992;89(24):12160-12164.

Selbi W, de la Motte C, Hascall V, Phillips A. BMP-7 modulates hyaluronan-mediated proximal tubular cell-monocyte interaction. J Am Soc Nephrol 2004;15(5):1199-1211.

Selbi W, de la Motte CA, Hascall VC, Day AJ, Bowen T, Phillips AO. Characterization of hyaluronan cable structure and function in renal proximal tubular epithelial cells. Kidney Int 2006;70(7):1287-1295.

Setälä LP, Tammi MI, Tammi RH, Eskelinen MJ, Lipponen PK, Ågren UM, Parkkinen J, Alhava EM, *et al.* Hyaluronan expression in gastric cancer cells is associated with local and nodal spread and reduced survival rate. Br J Cancer 1999;79(7-8):1133-1138.

Seyfried NT, McVey GF, Almond A, Mahoney DJ, Dudhia J, Day AJ. Expression and purification of functionally active hyaluronan-binding domains from human cartilage link protein, aggrecan and versican: formation of ternary complexes with defined hyaluronan oligosaccharides. J Biol Chem 2005;280(7):5435-5448.

Seykora J, Elder D. Dysplastic nevi and other risk markers for melanoma. Semin Oncol 1996;23(6):682-687.

Sheehan KM, DeLott LB, West RA, Bonnema JD, DeHeer DH. Hyaluronic acid of high molecular weight inhibits proliferation and induces cell death in U937 macrophage cells. Life Sci 2004;75(26):3087-3102.

Shimabukuro Y, Ichikawa T, Takayama S, Yamada S, Takedachi M, Terakura M, Hashikawa T, Murakami S. Fibroblast growth factor-2 regulates the synthesis of hyaluronan by human periodontal ligament cells. J Cell Physiol 2005a;203(3):557-563.

Shimabukuro Y, Ueda M, Ichikawa T, Terashi Y, Yamada S, Kusumoto Y, Takedachi M, Terakura M, *et al.* Fibroblast growth factor-2 stimulates hyaluronan production by human dental pulp cells. J Endod 2005b;31(11):805-808.

Shin JM, Yoo KJ, Kim MS, Kim D, Baek KH. Hyaluronan- and RNA-binding deubiquitinating enzymes of USP17 family members associated with cell viability. BMC Genomics 2006;7:292.

Shuster S, Frost GI, Csoka AB, Formby B, Stern R. Hyaluronidase reduces human breast cancer xenografts in SCID mice. Int J Cancer 2002;102(2):192-197.

Shyjan AM, Heldin P, Butcher EC, Yoshino T, Briskin MJ. Functional cloning of the cDNA for a human hyaluronan synthase. J Biol Chem 1996;271(38):23395-23399.

Simard EP, Ward EM, Siegel R, Jemal A. Cancers with increasing incidence trends in the United States: 1999 through 2008. CA Cancer J Clin 2012; in press.

Simpson MA, Weigel JA, Weigel PH. Systemic blockade of the hyaluronan receptor for endocytosis prevents lymph node metastasis of prostate cancer. Int J Cancer 2012;131(5):E836-40.

Slomiany MG, Dai L, Bomar PA, Knackstedt TJ, Kranc DA, Tolliver L, Maria BL, Toole BP. Abrogating drug resistance in malignant peripheral nerve sheath tumors by disrupting hyaluronan-CD44 interactions with small hyaluronan oligosaccharides. Cancer Res 2009a;69(12):4992-4998.

Slomiany MG, Grass GD, Robertson AD, Yang XY, Maria BL, Beeson C, Toole BP. Hyaluronan, CD44, and emmprin regulate lactate efflux and membrane localization of monocarboxylate transporters in human breast carcinoma cells. Cancer Res 2009b;69(4):1293-1301.

Slomiany MG, Dai L, Bomar PA, Knackstedt TJ, Kranc DA, Tolliver L, Maria BL,Toole BP. Abrogating drug resistance in malignant peripheral nerve sheath tumors by disrupting hyaluronan-CD44 interactions with small hyaluronan oligosaccharides. Cancer Res. 2009c;69(12):4992-4998.

Solis MA, Chen YH, Wong TY, Bittencourt VZ, Lin YC, Huang LL. Hyaluronan regulates cell behavior: a potential niche matrix for stem cells. Biochem Res Int 2012;2012:346972.

Soltes L, Mendichi R, Kogan G, Schiller J, Stankovska M, Arnhold J. Degradative action of reactive oxygen species on hyaluronan. Biomacromolecules 2006;7(3):659-668.

Soltys BJ, Kang D, Gupta RS. Localization of P32 protein (gC1q-R) in mitochondria and at specific extramitochondrial locations in normal tissues. Histochem Cell Biol 2000;114(3):245-255.

Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 1973;51(5):1409-1416.

Spicer AP, Augustine ML, McDonald JA. Molecular cloning and characterization of a putative mouse hyaluronan synthase. J Biol Chem 1996;271(38):23400-23406.

Spicer AP, Olson JS, McDonald JA. Molecular cloning and characterization of a cDNA encoding the third putative mammalian hyaluronan synthase. J Biol Chem 1997a;272(14):8957-8961.

Spicer AP, Seldin MF, Olsen AS, Brown N, Wells DE, Doggett NA, Itano N, Kimata K, *et al.* Chromosomal localization of the human and mouse hyaluronan synthase genes. Genomics 1997b;41(3):493-497.

Spicer AP, Nguyen TK. Mammalian hyaluronan synthases: investigation of functional relationships in vivo. Biochem Soc Trans 1999;27(2):109-115.

Spruss T, Bernhardt G, Schonenberger H, Schiess W. Hyaluronidase significantly enhances the efficacy of regional vinblastine chemotherapy of malignant melanoma. J Cancer Res Clin Oncol 1995;121(4):193-202.

Stern R. Devising a pathway for hyaluronan catabolism: are we there yet? Glycobiology 2003;13(12):105R-115R.

Stern R. Hyaluronan catabolism: a new metabolic pathway. Eur J Cell Biol 2004;83(7):317-325.

Stern R, Asari AA, Sugahara KN. Hyaluronan fragments: an information-rich system. Eur J Cell Biol 2006;85(8):699-715.

Stern R, Kogan G, Jedrzejas MJ, Soltes L. The many ways to cleave hyaluronan. Biotechnol Adv 2007;25(6):537-557.

Stuhlmeier KM, Pollaschek C. Differential effect of transforming growth factor beta (TGF-beta) on the genes encoding hyaluronan synthases and utilization of the p38 MAPK pathway in TGF-beta-induced hyaluronan synthase 1 activation. J Biol Chem 2004a;279(10):8753-8760.

Stuhlmeier KM, Pollaschek C. Glucocorticoids inhibit induced and non-induced mRNA accumulation of genes encoding hyaluronan synthases (HAS): hydrocortisone inhibits HAS1 activation by blocking the p38 mitogen-activated protein kinase signalling pathway. Rheumatology (Oxford) 2004b;43(2):164-169.

Stuhlmeier KM, Pollaschek C. Adenovirus-mediated gene transfer of mutated IkappaB kinase and IkappaBalpha reveal NF-kappaB-dependent as well as NF-kappaB-independent pathways of HAS1 activation. J Biol Chem 2005;280(52):42766-42773.

Stuhlmeier KM. Prostaglandin E2: a potent activator of hyaluronan synthase 1 in type-B-synoviocytes. Biochim Biophys Acta 2007;1770(1):121-129.

Sugahara KN, Murai T, Nishinakamura H, Kawashima H, Saya H, Miyasaka M. Hyaluronan oligosaccharides induce CD44 cleavage and promote cell migration in CD44-expressing tumor cells. J Biol Chem 2003;278(34):32259-32265.

Sugiyama Y, Shimada A, Sayo T, Sakai S, Inoue S. Putative hyaluronan synthase mRNA are expressed in mouse skin and TGF-beta upregulates their expression in cultured human skin cells. J Invest Dermatol 1998;110(2):116-121.

Sundström G, Dahl IM, Hultdin M, Lundström B, Wahlin A, Engström-Laurent A. Bone marrow hyaluronan distribution in patients with acute myeloid leukemia. Med Oncol 2005;22(1):71-78.

Suzuki K, Yamamoto T, Usui T, Suzuki K, Heldin P, Yamashita H. Expression of hyaluronan synthase in intraocular proliferative diseases: regulation of expression in human vascular endothelial cells by transforming growth factor-beta. Jpn J Ophthalmol 2003;47(6):557-564.

Suzuki M, Asplund T, Yamashita H, Heldin CH, Heldin P. Stimulation of hyaluronan biosynthesis by platelet-derived growth factor-BB and transforming growth factor-beta 1 involves activation of protein kinase C. Biochem J 1995;307(Pt 3):817-821.

Suzuki T, Funakoshi Y. Free N-linked oligosaccharide chains: formation and degradation. Glycoconj J 2006;23(5-6):291-302.

Szanto S, Bardos T, Gal I, Glant TT, Mikecz K. Enhanced neutrophil extravasation and rapid progression of proteoglycan-induced arthritis in TSG-6-knockout mice. Arthritis Rheum 2004;50(9):3012-3022.

Tajima K, Ohashi R, Sekido Y, Hida T, Nara T, Hashimoto M, Iwakami S, Minakata K, *et al.* Osteopontin-mediated enhanced hyaluronan binding induces multidrug resistance in mesothelioma cells. Oncogene 2010;29(13):1941-1951.

Takahashi E, Nagano O, Ishimoto T, Yae T, Suzuki Y, Shinoda T, Nakamura S, Niwa S, *et al.* Tumor necrosis factor-alpha regulates transforming growth factor-beta-dependent epithelial-mesenchymal transition by promoting hyaluronan-CD44-moesin interaction. J Biol Chem 2010;285(6):4060-4073.

Takano H, Furuta K, Yamashita K, Sakanaka M, Itano N, Gohda E, Nakayama K, Kimata K, *et al.* Restriction of mast cell proliferation through hyaluronan synthesis by co-cultured fibroblasts. Biol Pharm Bull 2012;35(3):408-412.

Takeo S, Fujise M, Akiyama T, Habuchi H, Itano N, Matsuo T, Aigaki T, Kimata K, *et al.* In vivo hyaluronan synthesis upon expression of the mammalian hyaluronan synthase gene in Drosophila. J Biol Chem 2004;279(18):18920-18925.

Takeuchi Y, Matsumoto T, Ogata E, Shishiba Y. 1,25-Dihydroxyvitamin D3 inhibits synthesis and enhances degradation of proteoglycans in osteoblastic cells. J Biol Chem 1989;264(31):18407-18413.

Tammi R, Tammi M. Influence of retinoic acid on the ultrastructure and hyaluronic acid synthesis of adult human epidermis in whole skin organ culture. J Cell Physiol 1986;126(3):389-398.

Tammi R, Saamanen AM, Maibach HI, Tammi M. Degradation of newly synthesized high molecular mass hyaluronan in the epidermal and dermal compartments of human skin in organ culture. J Invest Dermatol 1991a;97(1):126-130.

Tammi R, Tammi M. Correlations between hyaluronan and epidermal proliferation as studied by [3H]glucosamine and [3H]thymidine incorporations and staining of hyaluronan on mitotic keratinocytes. Exp Cell Res 1991b;195(2):524-527.

Tammi R, Paukkonen K, Wang C, Horsmanheimo M, Tammi M. Hyaluronan and CD44 in psoriatic skin. Intense staining for hyaluronan on dermal capillary loops and reduced expression of CD44 and hyaluronan in keratinocyte-leukocyte interfaces. Arch Dermatol Res 1994;286(1):21-29.

Tammi R, MacCallum D, Hascall VC, Pienimäki JP, Hyttinen M, Tammi M. Hyaluronan bound to CD44 on keratinocytes is displaced by hyaluronan decasaccharides and not hexasaccharides. J Biol Chem 1998;273(44):28878-28888.

Tammi R, Rilla K, Pienimäki JP, MacCallum DK, Hogg M, Luukkonen M, Hascall VC, Tammi M. Hyaluronan enters keratinocytes by a novel endocytic route for catabolism. J Biol Chem 2001;276(37):35111-35122.

Tammi R, Pasonen-Seppänen S, Kolehmainen E, Tammi M. Hyaluronan synthase induction and hyaluronan accumulation in mouse epidermis following skin injury. J Invest Dermatol 2005;124(5):898-905.

Tammi RH, Kultti A, Kosma VM, Pirinen R, Auvinen P, Tammi MI. Hyaluronan in human tumors: pathobiological and prognostic messages from cell-associated and stromal hyaluronan. Semin Cancer Biol 2008;18(4):288-295.

Tammi RH, Passi AG, Rilla K, Karousou E, Vigetti D, Makkonen K, Tammi MI. Transcriptional and postranslational regulation of hyaluronan synthesis. FEBS J 2011;278(9):1419-1428.

Tanaka K, Nakamura T, Takagaki K, Funahashi M, Saito Y, Endo M. Regulation of hyaluronate metabolism by progesterone in cultured fibroblasts from the human uterine cervix. FEBS Lett 1997;402(2-3):223-226.

Tanaka K, Taniguchi R, Higuchi T, Ozaki T, Mizunuma H, Takagaki K. 4-Methylumbelliferone inhibits hyaluronate synthesis in human uterine cervical fibroblasts. J Obstet Gynaecol Res 2007;33(6):772-776.

Tellbach M, Salamonsen LA, Van Damme MP. The influence of ovarian steroids on ovine endometrial glycosaminoglycans. Glycoconj J 2002;19(6):385-394.

Teriete P, Banerji S, Noble M, Blundell CD, Wright AJ, Pickford AR, Lowe E, Mahoney DJ, *et al.* Structure of the regulatory hyaluronan binding domain in the inflammatory leukocyte homing receptor CD44. Mol Cell 2004;13(4):483-496.

Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, Miyake K, Freudenberg M, *et al.* Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. J Exp Med 2002;195(1):99-111.

Thomas NK, Brown TJ. ABC transporters do not contribute to extracellular translocation of hyaluronan in human breast cancer in vitro. Exp Cell Res 2010;316(7):1241-1253.

Thorne RF, Legg JW, Isacke CM. The role of the CD44 transmembrane and cytoplasmic domains in co-ordinating adhesive and signalling events. J Cell Sci 2004;117(Pt 3):373-380.

Tien JY, Spicer AP. Three vertebrate hyaluronan synthases are expressed during mouse development in distinct spatial and temporal patterns. Dev Dyn 2005;233(1):130-141.

Tirone E, D'Alessandris C, Hascall VC, Siracusa G, Salustri A. Hyaluronan synthesis by mouse cumulus cells is regulated by interactions between follicle-stimulating hormone (or epidermal growth factor) and a soluble oocyte factor (or transforming growth factor beta1). J Biol Chem 1997;272(8):4787-4794.

Tobiishi M, Sayo T, Yoshida H, Kusaka A, Kawabata K, Sugiyama Y, Ishikawa O, Inoue S. Changes in epidermal hyaluronan metabolism following UVB irradiation. J Dermatol Sci 2011;64(1):31-38.

Tolg C, Hamilton SR, Nakrieko KA, Kooshesh F, Walton P, McCarthy JB, Bissell MJ, Turley EA. Rhamm-/- fibroblasts are defective in CD44-mediated ERK1,2 motogenic signaling, leading to defective skin wound repair. J Cell Biol 2006;175(6):1017-1028.

Toole BP, Munaim SI, Welles S, Knudson CB. Hyaluronate-cell interactions and growth factor regulation of hyaluronate synthesis during limb development. Ciba Found Symp 1989;143:138-45; discussion 145-9 281-5.

Toole BP. Hyaluronan: from extracellular glue to pericellular cue. Nat Rev Cancer 2004;4(7):528-539.

Toole BP, Zoltan-Jones A, Misra S, Ghatak S. Hyaluronan: a critical component of epithelial-mesenchymal and epithelial-carcinoma transitions. Cells Tissues Organs 2005;179(1-2):66-72.

Toole BP, Slomiany MG. Hyaluronan, CD44 and Emmprin: partners in cancer cell chemoresistance. Drug Resist Updat 2008;11(3):110-121.

Toole BP. Hyaluronan-CD44 Interactions in Cancer: Paradoxes and Possibilities. Clin Cancer Res 2009;15(24):7462-7468.

Toriseva M, Ala-aho R, Peltonen S, Peltonen J, Grénman R, Kähäri VM. Keratinocyte growth factor induces gene expression signature associated with suppression of malignant phenotype of cutaneous squamous carcinoma cells. PLoS One 2012;7(3):e33041.

Tran EJ, Wente SR. Dynamic nuclear pore complexes: life on the edge. Cell 2006;125(6):1041-1053.

Triggs-Raine B, Salo TJ, Zhang H, Wicklow BA, Natowicz MR. Mutations in HYAL1, a member of a tandemly distributed multigene family encoding disparate hyaluronidase activities, cause a newly described lysosomal disorder, mucopolysaccharidosis IX. Proc Natl Acad Sci U S A 1999;96(11):6296-6300.

Tsubaki H, Ogawa M, Hosoya N, Shimizu D, Obara M, Hirano H, Tanaka T. Expression of CD44 mRNA induced by interleukin-1beta in human cultured uterine cervical fibroblasts. Eur J Obstet Gynecol Reprod Biol 2005;122(2):156-161.

Tuhkanen AL, Tammi M, Tammi R. CD44 substituted with heparan sulfate and endo-beta-galactosidase-sensitive oligosaccharides: a major proteoglycan in adult human epidermis. J Invest Dermatol 1997;109(2):213-218.

Tuhkanen H, Anttila M, Kosma VM, Ylä-Herttuala S, Heinonen S, Kuronen A, Juhola M, Tammi R, *et al.* Genetic alterations in the peritumoral stromal cells of malignant and borderline epithelial ovarian tumors as indicated by allelic imbalance on chromosome 3p. Int J Cancer 2004;109(2):247-252.

Turley EA. Purification of a hyaluronate-binding protein fraction that modifies cell social behavior. Biochem Biophys Res Commun 1982;108(3):1016-1024.

Turley EA, Tretiak M. Glycosaminoglycan production by murine melanoma variants in vivo and in vitro. Cancer Res 1985;45(10):5098-5105.

Turley EA, Austen L, Vandeligt K, Clary C. Hyaluronan and a cell-associated hyaluronan binding protein regulate the locomotion of ras-transformed cells. J Cell Biol 1991;112(5):1041-1047.

Turley EA, Noble PW, Bourguignon LY. Signaling properties of hyaluronan receptors. J Biol Chem 2002;277(7):4589-4592.

Uchiyama T, Sakuta T, Kanayama T. Regulation of hyaluronan synthases in mouse uterine cervix. Biochem Biophys Res Commun 2005;327(3):927-932.

Udabage L, Brownlee GR, Nilsson SK, Brown TJ. The over-expression of HAS2, Hyal-2 and CD44 is implicated in the invasiveness of breast cancer. Exp Cell Res 2005;310(1):205-217.

Ueki N, Taguchi T, Takahashi M, Adachi M, Ohkawa T, Amuro Y, Hada T, Higashino K. Inhibition of hyaluronan synthesis by vesnarinone in cultured human myofibroblasts. Biochim Biophys Acta 2000;1495(2):160-167.

Underhill CB, Toole BP. Binding of hyaluronate to the surface of cultured cells. J Cell Biol 1979;82(2):475-484.

Underhill CB, Thurn AL, Lacy BE. Characterization and identification of the hyaluronate binding site from membranes of SV-3T3 cells. J Biol Chem 1985;260(13):8128-8133.

Underhill CB, Green SJ, Comoglio PM, Tarone G. The hyaluronate receptor is identical to a glycoprotein of Mr 85,000 (gp85) as shown by a monoclonal antibody that interferes with binding activity. J Biol Chem 1987;262(27):13142-13146.

Urakawa H, Nishida Y, Wasa J, Arai E, Zhuo L, Kimata K, Kozawa E, Futamura N, *et al.* Inhibition of hyaluronan synthesis in breast cancer cells by 4-methylumbelliferone suppresses tumorigenicity in vitro and metastatic lesions of bone in vivo. Int J Cancer 2012;130(2):454-466.

Usui T, Nakajima F, Ideta R, Kaji Y, Suzuki Y, Araie M, Miyauchi S, Heldin P, *et al.* Hyaluronan synthase in trabecular meshwork cells. Br J Ophthalmol 2003;87(3):357-360.

van Zeijl CJ, Fliers E, van Koppen CJ, Surovtseva OV, de Gooyer ME, Mourits MP, Wiersinga WM, Miltenburg AM, *et al.* Effects of thyrotropin and thyrotropin-receptor-stimulating Graves' disease immunoglobulin G on cyclic adenosine monophosphate and hyaluronan production in nondifferentiated orbital fibroblasts of Graves' ophthalmopathy patients. Thyroid 2010;20(5):535-544.

Varadi T, Mersich T, Auvinen P, Tammi R, Tammi M, Salamon F, Besznyak I,Jr, Jakab F, *et al.* Binding of Trastuzumab to ErbB2 Is Inhibited by a High Pericellular Density of Hyaluronan. J Histochem Cytochem 2012;60(8):567-575.

Vendrov AE, Madamanchi NR, Niu XL, Molnar KC, Runge M, Szyndralewiez C, Page P, Runge MS. NADPH oxidases regulate CD44 and hyaluronic acid expression in thrombin-treated vascular smooth muscle cells and in atherosclerosis. J Biol Chem 2010;285(34):26545-26557.

Viarisio D, Mueller-Decker K, Kloz U, Aengeneyndt B, Kopp-Schneider A, Grone HJ, Gheit T, Flechtenmacher C, *et al.* E6 and E7 from beta HPV38 cooperate with ultraviolet light in the development of actinic keratosis-like lesions and squamous cell carcinoma in mice. PLoS Pathog 2011;7(7):e1002125.

Vigetti D, Genasetti A, Karousou E, Viola M, Clerici M, Bartolini B, Moretto P, De Luca G, *et al.* Modulation of hyaluronan synthase activity in cellular membrane fractions. J Biol Chem 2009a;284(44):30684-30694.

Vigetti D, Rizzi M, Viola M, Karousou E, Genasetti A, Clerici M, Bartolini B, Hascall VC, *et al.* The effects of 4-methylumbelliferone on hyaluronan synthesis, MMP2 activity, proliferation, and motility of human aortic smooth muscle cells. Glycobiology 2009b;19(5):537-546.

Vigetti D, Genasetti A, Karousou E, Viola M, Moretto P, Clerici M, Deleonibus S, De Luca G, *et al.* Proinflammatory cytokines induce hyaluronan synthesis and monocyte adhesion in human endothelial cells through hyaluronan synthase 2 (HAS2) and the nuclear factor-kappaB (NF-kappaB) pathway. J Biol Chem 2010;285(32):24639-24645.

Vigetti D, Rizzi M, Moretto P, Deleonibus S, Dreyfuss JM, Karousou E, Viola M, Clerici M, *et al.* Glycosaminoglycans and glucose prevent apoptosis in 4-methylumbelliferone-treated human aortic smooth muscle cells. J Biol Chem 2011;286(40):34497-34503.

Vigetti D, Deleonibus S, Moretto P, Karousou E, Viola M, Bartolini B, Hascall VC, Tammi M, *et al.* Role of UDP-GlcNAc and O-GlcNAcylation of hyaluronan synthase 2 in the control of chondroitin sulfate and hyaluronan synthesis. J Biol Chem 2012;287(42):35544-35555.

Wang A, Hascall VC. Hyperglycemia, intracellular hyaluronan synthesis, cyclin D3 and autophagy. Autophagy 2009a;5(6):864-865.

Wang A, de la Motte C, Lauer M, Hascall V. Hyaluronan Matrices in Pathobiological Processes. FEBS J 2011;278(9):1412-1418.

Wang C, Tammi M, Tammi R. Distribution of hyaluronan and its CD44 receptor in the epithelia of human skin appendages. Histochemistry 1992;98(2):105-112.

Wang C, Entwistle J, Hou G, Li Q, Turley EA. The characterization of a human RHAMM cDNA: conservation of the hyaluronan-binding domains. Gene 1996a;174(2):299-306.

Wang C, Tammi M, Guo H, Tammi R. Hyaluronan distribution in the normal epithelium of esophagus, stomach, and colon and their cancers. Am J Pathol 1996b;148(6):1861-1869.

Wang SJ, Bourguignon LY. Hyaluronan and the interaction between CD44 and epidermal growth factor receptor in oncogenic signaling and chemotherapy resistance in head and neck cancer. Arch Otolaryngol Head Neck Surg 2006;132(7):771-778.

Wang SJ, Earle C, Wong G, Bourguignon LY. Role of hyaluronan synthase 2 to promote CD44-dependent oral cavity squamous cell carcinoma progression. Head Neck 2012; in press.

Wang Y, Digiovanna JJ, Stern JB, Hornyak TJ, Raffeld M, Khan SG, Oh KS, Hollander MC, *et al.* Evidence of ultraviolet type mutations in xeroderma pigmentosum melanomas. Proc Natl Acad Sci U S A 2009b;106(15):6279-6284.

Ward JA. Perturbation of hyaluronan interactions inhibits malignant properties of glioma cells. Am J Pathol 2003;162(5):1403.

Watanabe K, Yamaguchi Y. Molecular identification of a putative human hyaluronan synthase. J Biol Chem 1996;271(38):22945-22948.

Weigel PH, Hascall VC, Tammi M. Hyaluronan synthases. J Biol Chem 1997;272(22):13997-14000.

Weigel PH, Kyossev Z, Torres LC. Phospholipid dependence and liposome reconstitution of purified hyaluronan synthase. J Biol Chem 2006;281(48):36542-36551.

Weigel PH, DeAngelis PL. Hyaluronan synthases: a decade-plus of novel glycosyltransferases. J Biol Chem 2007;282(51):36777-36781.

Weissman B, Meyer K. The structure of hyalobiuronic acid and of hyaluronic acid from umbilical cord. J Am Chem Soc 1954(76):1753-1757.

West DC, Hampson IN, Arnold F, Kumar S. Angiogenesis induced by degradation products of hyaluronic acid. Science 1985;228(4705):1324-1326.

Wilkinson TS, Bressler SL, Evanko SP, Braun KR, Wight TN. Overexpression of hyaluronan synthases alters vascular smooth muscle cell phenotype and promotes monocyte adhesion. J Cell Physiol 2006;206(2):378-385.

Willenberg A, Saalbach A, Simon JC, Anderegg U. Melanoma cells control HA synthesis in peritumoral fibroblasts via PDGF-AA and PDGF-CC: impact on melanoma cell proliferation. J Invest Dermatol 2012;132(2):385-393.

Wisniewski HG, Burgess WH, Oppenheim JD, Vilcek J. TSG-6, an arthritis-associated hyaluronan binding protein, forms a stable complex with the serum protein inter-alpha-inhibitor. Biochemistry 1994;33(23):7423-7429.

Wistuba II, Behrens C, Virmani AK, Mele G, Milchgrub S, Girard L, Fondon JW,3rd, Garner HR, *et al.* High resolution chromosome 3p allelotyping of human lung cancer and preneoplastic/preinvasive bronchial epithelium reveals multiple, discontinuous sites of 3p allele loss and three regions of frequent breakpoints. Cancer Res 2000;60(7):1949-1960.

Wu YJ, La Pierre DP, Wu J, Yee AJ, Yang BB. The interaction of versican with its binding partners. Cell Res 2005;15(7):483-494.

Wunderlich L, Paragh G, Wikonkal NM, Banhegyi G, Karpati S, Mandl J. UVB induces a biphasic response of HIF-1alpha in cultured human keratinocytes. Exp Dermatol 2008;17(4):335-342.

Xu H, Ito T, Tawada A, Maeda H, Yamanokuchi H, Isahara K, Yoshida K, Uchiyama Y, *et al.* Effect of hyaluronan oligosaccharides on the expression of heat shock protein 72. J Biol Chem 2002;277(19):17308-17314.

Yabushita H, Noguchi M, Kishida T, Fusano K, Noguchi Y, Itano N, Kimata K, Noguchi M. Hyaluronan synthase expression in ovarian cancer. Oncol Rep 2004;12(4):739-743.

Yabushita H, Kishida T, Fusano K, Kanyama K, Zhuo L, Itano N, Kimata K, Noguchi M. Role of hyaluronan and hyaluronan synthase in endometrial cancer. Oncol Rep 2005;13(6):1101-1105.

Yamada H, Watanabe K, Shimonaka M, Yamaguchi Y. Molecular cloning of brevican, a novel brain proteoglycan of the aggrecan/versican family. J Biol Chem 1994;269(13):10119-10126.

Yamada Y, Itano N, Hata K, Ueda M, Kimata K. Differential regulation by IL-1beta and EGF of expression of three different hyaluronan synthases in oral mucosal epithelial cells and fibroblasts and dermal fibroblasts: quantitative analysis using real-time RT-PCR. J Invest Dermatol 2004a;122(3):631-639.

Yamada Y, Itano N, Narimatsu H, Kudo T, Morozumi K, Hirohashi S, Ochiai A, Ueda M, *et al.* Elevated transcript level of hyaluronan synthase1 gene correlates with poor prognosis of human colon cancer. Clin Exp Metastasis 2004b;21(1):57-63.

Yamane T, Kobayashi-Hattori K, Oishi Y. Adiponectin promotes hyaluronan synthesis along with increases in hyaluronan synthase 2 transcripts through an AMP-activated protein kinase/peroxisome proliferator-activated receptor-alpha-dependent pathway in human dermal fibroblasts. Biochem Biophys Res Commun 2011;415(2):235-238.

Yang B, Yang BL, Savani RC, Turley EA. Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein. EMBO J 1994;13(2):286-296.

Yasuda M, Tanaka Y, Fujii K, Yasumoto K. CD44 stimulation down-regulates Fas expression and Fas-mediated apoptosis of lung cancer cells. Int Immunol 2001;13(10):1309-1319.

Yasuda T. Hyaluronan inhibits Akt, leading to nuclear factor-kappaB down-regulation in lipopolysaccharide-stimulated U937 macrophages. J Pharmacol Sci 2011;115(4):509-515.

Yoneda M, Suzuki S, Kimata K. Hyaluronic acid associated with the surfaces of cultured fibroblasts is linked to a serum-derived 85-kDa protein. J Biol Chem 1990;265(9):5247-5257.

Yoshihara S, Kon A, Kudo D, Nakazawa H, Kakizaki I, Sasaki M, Endo M, Takagaki K. A hyaluronan synthase suppressor, 4-methylumbelliferone, inhibits liver metastasis of melanoma cells. FEBS Lett 2005;579(12):2722-2726.

Yu Q, Toole BP, Stamenkovic I. Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumor cell surface CD44 function. J Exp Med 1997;186(12):1985-1996.

Zaidi MR, De Fabo EC, Noonan FP, Merlino G. Shedding light on melanocyte pathobiology in vivo. Cancer Res 2012;72(7):1591-1595.

Zanna G, Docampo MJ, Fondevila D, Bardagi M, Bassols A, Ferrer L. Hereditary cutaneous mucinosis in shar pei dogs is associated with increased hyaluronan synthase-2 mRNA transcription by cultured dermal fibroblasts. Vet Dermatol 2009;20(5-6):377-382.

Zeng C, Toole BP, Kinney SD, Kuo JW, Stamenkovic I. Inhibition of tumor growth in vivo by hyaluronan oligomers. Int J Cancer 1998;77(3):396-401.

Zhang H, Martin-DeLeon PA. Mouse Spam1 (PH-20) is a multifunctional protein: evidence for its expression in the female reproductive tract. Biol Reprod 2003;69(2):446-454.

Zhang S, Chang MC, Zylka D, Turley S, Harrison R, Turley EA. The hyaluronan receptor RHAMM regulates extracellular-regulated kinase. J Biol Chem 1998;273(18):11342-11348.

Zhang W, Watson CE, Liu C, Williams KJ, Werth VP. Glucocorticoids induce a near-total suppression of hyaluronan synthase mRNA in dermal fibroblasts and in osteoblasts: a molecular mechanism contributing to organ atrophy. Biochem J 2000;349(Pt 1):91-97.

Zhao L, Li W, Marshall C, Griffin T, Hanson M, Hick R, Dentchev T, Williams E, *et al.* Srcasm inhibits Fyn-induced cutaneous carcinogenesis with modulation of Notch1 and p53. Cancer Res 2009;69(24):9439-9447.

Zhou B, McGary CT, Weigel JA, Saxena A, Weigel PH. Purification and molecular identification of the human hyaluronan receptor for endocytosis. Glycobiology 2003;13(5):339-349.

Zhou J, Haggerty JG, Milstone LM. Growth and differentiation regulate CD44 expression on human keratinocytes. In Vitro Cell Dev Biol Anim 1999;35(4):228-235.

Zhuo L, Hascall VC, Kimata K. Inter-alpha-trypsin inhibitor, a covalent protein-glycosaminoglycan-protein complex. J Biol Chem 2004;279(37):38079-38082.

Ziegler A, Jonason AS, Leffell DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T, *et al.* Sunburn and p53 in the onset of skin cancer. Nature 1994;372(6508):773-776.

Zimmermann DR, Ruoslahti E. Multiple domains of the large fibroblast proteoglycan, versican. EMBO J 1989;8(10):2975-2981.

Zoltan-Jones A, Huang L, Ghatak S, Toole BP. Elevated hyaluronan production induces mesenchymal and transformed properties in epithelial cells. J Biol Chem 2003;278(46):45801-45810.

Hanna Siiskonen

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