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UNIVERSITY OF
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ABSTRACT

Hyaluronan is an abundant high-molecular weight polysaccharide in the extracellular matrix of various human tissues. Its accumulation is observed in many pathological conditions, including several types of malignancies of epithelial origin. Changes in hyaluronan content are a result of its metabolic dysregulation due to altered function and balance of hyaluronan synthesis and degradation enzymes. In human ovarian carcinoma, increased stromal hyaluronan is associated with poor outcome. Similarly, stromal hyaluronan accumulation has been observed in endometrial adenocarcinoma. In this study, the mechanism of hyaluronan accumulation in these gynecological malignancies was investigated by analyzing the expression of hyaluronan synthases (HAS1-3) and hyaluronidases (HYAL1-2), and their roles in tumorigenesis and invasion were clarified.

Increased expression of HAS1-3 proteins was observed in ovarian and endometrial carcinomas without corresponding changes in mRNA levels, suggesting reduced protein turnover or altered post-transcriptional regulation. *HYAL1* mRNA was significantly downregulated, which correlated with its enzymatic activity in ovarian serous carcinoma. Transcription of *HYAL1* and *HYAL2* mRNA was downregulated and correlated with decreased protein levels. The decreased *HYAL1* mRNA levels were associated with increased tumoral hyaluronan content in both of these carcinoma types.

The expression of HYAL1 and HYAL2 protein in a larger set of histopathological samples of normal endometria, precancerous lesions, and endometrial adenocarcinomas were further examined. Increased HYAL2 expression was associated with the proliferative phase of the menstrual cycle. Decreased HYAL1 expression was associated with high carcinoma grade, deep myometrial invasion, large tumor size, lymphovascular invasion, and lymph node metastasis. HYAL1 was also an independent marker of early disease recurrence. In addition, the decreased expression of HYAL1 correlated with decreased tumoral E-cadherin levels, suggesting a potential role of HYAL1 in epithelial-to-mesenchymal transition (EMT).

These results indicate that tumoral hyaluronan accumulation can be a consequence of decreased hyaluronidase function. The present results also indicate the importance of HYAL1 in endometrial cancer tumorigenesis, as its downregulation is associated with aggressive disease.

TIIVISTELMÄ

Hyaluronaani on soluväliaineen suurikokoinen sokerimolekyyli, jonka on todettu kertyvän soluväliaineeseen epiteliaalista alkuperää olevissa syövissä. Hyaluronaanin kertymisen taustalla on yleensä sen metabolian säätelyhäiriö, missä hyaluronaania rakentavien ja hajottavien entsyymien toiminta on epätasapainossa. Munasarjan epiteliaaliset pahanlaatuiset kasvaimet muodostavat huonoennusteisimman gynekologisten syöpien ryhmän. Niissä hyaluronaanin kertyminen soluväliaineeseen liittyy aggressiiviseen taudinkuvaan. Kohdun limakalvon syövässä, joka edustaa parempiennusteista gynekologista syöpää, on myös todettu hyaluronaanin määrän lisääntyvän. Tämän tutkimuksen tarkoituksena oli selvittää näissä kahdessa gynekologisessa syöpätaudissa niitä mekanismeja jotka aiheuttavat hyaluronaanin kertymisen soluväliaineeseen tutkimalla hyaluronaanisyntaasien (HAS1-3) ja hyaluronidaasien (HYAL1-2) ilmentymistä.

Munasarja- ja kohtusyövässä voitiin vaihtelevasti todeta hyaluronaanisyntaasien ilmentymisen lisääntyneen proteiinitasolla, mutta ilman korrelaatiota vastaaviin lähetti-RNA pitoisuuksiin. *HYAL1* ali-ilmentyi ja korreloi hyaluronidaasi aktiivisuuden kanssa seroosissa munasarjasyövässä. Kohtusyövässä sekä *HYAL1*, että *HYAL2* ali-ilmentyivät voimakkaasti ja tämä korreloi myös vastaavien geenien proteiinitasojen kanssa. Sekä munasarja- että kohtusyövässä, alentunut *HYAL1* lähetti-RNA taso korreloi kasvaneen hyaluronaanipitoisuuden kanssa.

Koska tuloksemme osoittivat että hyaluronaanin kertymisen taustalla voi olla sen alentunut hajotus, HYAL1 ja HYAL2 proteiinien ilmentymistä tutkittiin laajemmassa kohtusyöpäaineistossa. Näissä tuloksissa HYAL2 liittyi enemmänkin eri kuukautiskierron vaiheisiin. Kohtusyövässä HYAL1 oli ali-ilmentynyt ja korreloi vahvasti syövän erilaistumisasteen kanssa. HYAL1 assosioitui aggressiiviseen kohtusyöpään liittyviin piirteisiin, kuten syvään lihasinvaasioon, kasvaimen suureen kokoon, veri-imutieinvaasioon ja imusolmukemetastaaseihin. Monimuuttuja-analyysissä alentunut HYAL1:n ilmentyminen toimi itsenäisenä ennustekijänä kohtusyövän varhaisen uusiutumisen suhteen. HYAL1:n ali-ilmentyminen liittyi myös epiteeli-mesenkyymi-transitioon, sillä se korreloi alentuneen E-kadheriini tason kanssa.

Tämän väitöskirjatyön tulokset osoittivat että hyaluronaanin kertyminen syöpäkudoksiin voi johtua sen alentuneesta hajotuksesta. Lisäksi tuloksemme osoittivat HYAL1:n ennusteellisen merkityksen kohtusyövässä, sillä sen ali-ilmentyminen liittyi aggressiiviseen tautiin.

"…we recognize that, unlike Beowulf at the hall of Hrothgar, we have not slain our enemy, the cancer cell, or figuratively torn the limbs from his body. In our adventures, we have only seen our monster more clearly and described his scales and fangs in new ways – ways that reveal a cancer cell to be, like Grendel, a distorted version of our normal selves. May this new vision and the spirit of tonight´s festivities inspire our band of biological warriors to inflict much greater wounds tomorrow."

Harold E. Varmus – speech at the Nobel Banquet, December 10, 1989

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Kuopio, June 2015

Timo K. Nykopp

List of the original publications

This dissertation is based on the following original publications:

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

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

III   Nykopp TK, Pasonen-Seppänen S, Tammi MI, Tammi RH, Kosma VM, Anttila M, Sironen R. Decreased hyaluronidase 1 expression is associated with early disease recurrence in human endometrial carcinoma. *Gynecol Oncol 137:152-9, 2015.*

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In addition, some unpublished data is presented.

Contents

Abbreviations

AMPK	AMP-activated protein kinase	EMT	Epithelial-to-mesenchymal transition
ARIDIA	AT-rich interactive domain 1A	ErbB	Erythroblastosis oncogene B
bHABC	Biotinylated hyaluronan-binding complex	ERK	Extracellular signal-regulated kinase
BRCA	Breast cancer susceptibility gene	FAM	6-carboxyfluorescein
		FBXW7	F-box/WD repeat-containing protein 7
BSA	Bovine serum albumin		
CD44	Cluster of differentiation 44	FGF	Fibroblast growth factor
		FIGO	International Federation of Gynecology and Obstetrics
CDC37	Cell division cycle 37		
cDNA	Complementary DNA		
CI	Confidence interval	GAG	Glycosaminoglycan
CTNNB1	Catenin-associated protein, beta 1	GlcNAc	N-acetyl-D-glucosamine
		GlcUA	D-glucuronic acid
CXCR4	Chemokine receptor type 4	HA	Hyaluronan
		HABP	Hyaluronan binding protein
CXCL12	C-X-C motif chemokine 12		
Da	Dalton	HARE	Hyaluronan receptor for endocytosis
DLBCL	Diffuse large B-cell lymphoma		
		HAS	Hyaluronan synthase
ECM	Extracellular matrix	HER-2	Human epidermal growth factor receptor 2
EDTA	Ethylenediaminetetra-acetic acid		
		HGSC	High-grade serous carcinoma
EES	Epithelial expression score		

HMW-HA	High-molecular weight hyaluronan		No-template negative control
HPRT	Hypoxanthine phosphoribosyltransferase	O-GlcNAc	O-linked β-N-acetyl-glucosamine
HR	Hazard ratio	OSE	Ovarian surface epithelium
HNPCC	Hereditary non-polyposis colorectal cancer	PB	Phosphate buffer
HYAL	Hyaluronidase	PEGPH20	Pegylated human recombinant hyaluronidase
HYAL-P1	Hyaluronidase pseudogene 1		
IgG	Immunoglobulin G	15-PDGH	15-hydroxyprostaglandin dehydrogenase
KRAS	Kirsten rat sarcoma viral oncogene homolog	PIK3	Phosphatidylinositol 3-kinase
LGSC	Low-grade serous carcinoma	PMS2	Postmeiotic segregation increased 2
LMW-HA	Low-molecular weight hyaluronan	PTEN	Phosphatase and tensin homolog
LYVE-1	Lymph vessel endothelial hyaluronan receptor 1	RASSF1	Ras association domain-containing protein 1
MLH1	MutL homolog 1	RHAMM	Receptor for hyaluronan-mediated motility
MGB	Minor groove binder		
MMP	Matrix metalloproteinases	RNA	Ribonucleic acid
MMR	Mismatch repair genes	ROS	Reactive oxygen species
mRNA	Messenger RNA	RPL22	Ribosomal protein L22
MSH2	MutS protein homolog 2	RT-PCR	Reverse transcription polymerase chain reaction
MSI / MI	Microsatellite instability		
NAD	Nicotinamide adenine dinucleotide NTC	SBLT	Serous borderline tumor
		SCC	Squamous cell carcinoma

sHA	Small hyaluronan oligosaccharides
SPAM1	Sperm adhesion molecule 1
STIC	Serous tubal intraepithelial carcinoma
TGFβ	Transforming growth factor β
TP53	Tumor protein p53
TSG-6	Tumor necrosis factor alpha stimulated gene-6
WHO	World Health Organization
WWOX	WW domain-containing oxidoreductase

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1 Introduction

Cancer, the Emperor of all maladies (Mukherjee 2010), lives silently in our society, affecting human lives of all ages. There were 14.1 million new cancer cases, 8.2 million cancer deaths, and 32.6 million people living with cancer worldwide in 2012 (GLOBOCAN 2012). Among gynecological malignancies, ovarian cancer is the leading cause of death in most Western countries, and the eighth most common type of cancer and the seventh most common cause of cancer-related death among women (Jemal et al. 2011). Endometrial cancer is the sixth most common cancer in women worldwide and the most common gynecological malignancy in developed countries. In contrast to ovarian cancer, most cases are diagnosed at an early stage and have a good prognosis (Siegel, Naishadham & Jemal 2013, Howlader et al. 2011).

A major component of the local cancer cell microenvironment is extracellular matrix. This complex network of macromolecules has distinctive physical, biochemical, and biomechanical properties and plays a crucial role in cancer development and metastasis (Lu, Weaver & Werb 2012). Hyaluronan is a glycosaminoglycan (a linear polysaccharide) and one of the most abundant and ubiquitous components of the vertebrate extracellular matrix (Laurent, Fraser 1992). Hyaluronan synthases (HAS1-3) contribute to the biosynthesis of hyaluronan polymers that occurs transiently for cell division and motility (Itano et al. 1999). After synthesis, hyaluronan is rapidly degraded by endocytic uptake and hydrolyzed by hyaluronidases (Csoka, Frost & Stern 2001, McAtee, Barycki & Simpson 2014). In many human cancers, this normal hyaluronan metabolism is altered. In carcinomas of epithelial origin, hyaluronan accumulates in the tumor stroma, which can have devastating consequences (Tammi et al. 2008).

In ovarian carcinoma, the high stromal hyaluronan level is significantly associated with poor differentiation, serous histological type, advanced stage, and large primary residual tumor. High stromal hyaluronan content is also an independent prognostic factor for short disease-free and overall survival (Anttila et al. 2000). Increased hyaluronan accumulation has also been shown in endometrial cancer (Afify et al. 2005).

In this thesis, the expression of hyaluronan synthesis (HAS1-3) and degradation (HYAL1-2) enzymes in serous ovarian adenocarcinomas and endometrial carcinomas was examined. By analyzing these genes, the possible alterations in hyaluronan metabolism that cause the increased hyaluronan content in tumors were investigated. Because the results suggest that decreased degradation could lead to tumoral hyaluronan accumulation, we analyzed the expression of hyaluronidases in a larger series of histopathological samples to elucidate their role in precancerous and malignant processes.

2 Review of the Literature

2.1 OVARIAN CANCER

2.1.1 Epidemiology and risk factors

Ovarian cancer is the leading cause of death from all gynecological cancers in most Western countries, with more than 140,000 women dying from this disease each year worldwide. Thus, ovarian cancer is the eighth most common type of cancer and the seventh most common cause of cancer-related death among women. Ovarian cancer is uncommon before the age of 40, and the incidence increases until the age of 70-74 (Jemal et al. 2011, Lowe et al. 2013).

In Finland, ovarian cancer is the eleventh most common type of cancer in women, with 434 new ovarian cancer cases diagnosed in 2013. During the same period, 293 women died due to ovarian cancer, making it the fifth most common cause of cancer death among women in Finland. For ovarian cancer, the age-adjusted incidence rate was 8.0 per 100 000 and mortality rate 4.1 per 100 000 (www.cancerregistry.fi).

Nulliparity is associated with an increased risk of ovarian cancer, whereas pregnancy, lactation, oral contraceptive use, and tubal ligation are associated with reduced risk (Beral et al. 2008, Jordan et al. 2010, Hankinson et al. 1993). The most important risk factor for ovarian cancer is a strong family history (i.e., one or more first degree relatives with ovarian or breast cancer diagnosed under the age of 50) (Soegaard et al. 2009, Cannistra 2004). An identifiable genetic predisposition is present in 5-15% of cases (Boyd et al. 2000). Inherited mutations in tumor suppressor genes *BRCA1* and *BRCA2* are the most common cause of hereditary ovarian cancer. *BRCA1* and *BRCA2* are located on chromosomes 17q and 13q, respectively, and their gene products are involved in DNA repair (King et al. 2003). In population-based studies, the lifetime risk for ovarian cancer in *BRCA1* and *BRCA2* mutation carriers is 24-39% and 8-22%, respectively (Chen et al. 2006, Risch et al. 2006). Approximately 15-30% of sporadic cases exhibit epigenetic hypermethylation of the *BRCA1* promoter, leading to decreased protein expression (Baldwin et al. 2000).

Lynch syndrome, also known as hereditary non-polyposis colorectal cancer syndrome (HNPPC), is a second familial disorder that carries an increased risk of ovarian cancer. It is an autosomal dominant cancer-susceptibility disorder caused by germline mutations in four mismatch repair (MMR) genes. Nearly 90% of the mutations are located in *MLH1* and *MSH2*, and approximately 10% are located in *MSH6* and *PMS2.1* Carriers of *MMR* gene mutations are at high risk of early-onset colorectal, endometrial, and ovarian cancer. The Lynch syndrome spectrum also includes tumors of the small bowel, urothelium, biliary tract, and stomach (Lynch, de la Chapelle 2003). For ovarian cancer, the estimated cumulative cancer risk by 70 years

of age is 20% for *MLH1*, 24% for *MSH2*, and 1% for *MSH6* mutations (Bonadona et al. 2011).

2.1.2 Pathogenesis

Ovarian cancers are classified by histological appearance. Serous histology accounts for up to 80 - 85% of ovarian cancers. Mucinous and endometrioid tumors are less common (~10% each), followed by clear cell tumors and transitional, squamous, mixed, and undifferentiated subtypes (Soslow 2008). Borderline ovarian tumors are considered to be semi-malignant and are atypically proliferative without stromal invasion. Different histology is observed, but the majority of borderline tumors are serous or mucinous types (Silverberg et al. 2004). Classically, ovarian carcinomas are divided into three classes of histological nuclear grading, but a two class system (high-grade and low-grade) is nowadays used, especially for serous carcinomas (Malpica et al. 2004).

Recent morphological and molecular studies have changed the theory that ovarian cancer arises from the epithelium of the ovarian surface. Ovarian cancer is a heterogeneous disease. Serous tumors are thought to arise from the fallopian tube. Mucinous tumors are cystic tumors with a smooth lining of mucin-secreting epithelial cells resembling either endocervical or colonic epithelium, whereas endometrioid and clear cell lesions are thought to arise from dysregulated endometriosis (Wei et al. 2013).

The major histological subtypes of ovarian tumors are divided into type I and type II based on differences in histopathological features supported by molecular genetic changes. Type I tumors include low-grade serous carcinomas (LGSC), low-grade endometrioid carcinomas, clear cell carcinomas, Brenner tumors, and mucinous carcinomas. Type II tumors include high-grade serous carcinomas (HGSC), high-grade endometrioid carcinomas, malignant mixed mesodermal tumors, and undifferentiated carcinomas (Shih, Kurman 2004, Hennessy, Coleman & Markman 2009).

LGSCs comprise a minority of epithelial ovarian carcinomas; at presentation, 70-80% of patients have high-grade disease (Della Pepa et al. 2015). LGSCs have different epidemiology, histopathology, associated molecular changes, and clinical course than HGSCs (Diaz-Padilla et al. 2012). LGSCs arise in a stepwise manner from serous borderline tumors (SBLTs), in which *KRAS* or *BRAF* mutations are often present (Singer et al. 2003) or precede the development of SBLTs (Ho et al. 2004). Papillary tubal hyperplasia has been suggested to be a preceding lesion of SBLT closely related to an aberration in *KRAS* signaling that occurs very early in tumorigenesis (Kurman et al. 2011). Interestingly, in advanced stage LGSC, *BRAF* mutations are more rare (Wong et al. 2010) and may even prevent LGSC progression to more aggressive disease (Grisham et al. 2013).

Low-grade endometrioid and clear cell carcinomas arise from dysregulated endometriosis, possibly due loss of tumor suppressor *PTEN* (Sato et al. 2000).

Mutations or inactivation of *ARID1A* are also associated with the development of endometrioid and clear cell carcinomas (Wiegand et al. 2010).

The majority of ovarian carcinomas present as HGSC, harboring *TP53* mutations in over 95% of cases, but mutations that are common in low-grade tumors are very rarely detected in high-grade tumors (Kurman 2013). HGSCs were first suggested to arise de novo in epithelial inclusion cysts, but their origin has been shown to be the fallopian tubes instead of the ovarian epithelium (Kurman 2013). The first evidence was found in gene expression studies demonstrating that the expression profiles of ovarian HGSCs more closely resemble fallopian tube epithelium (FTE) than ovarian surface epithelium (OSE) (Tone et al. 2008). In another study, investigators found that non-invasive tubal carcinomas are associated with serous carcinomas, and these neoplasms were designated as serous tubal intraepithelial carcinoma (STIC) (Kindelberger et al. 2007). STICs and concordant HGSCs involving the ovary have been shown to have identical *TP53* mutations, supporting a clonal relationship (Kuhn et al. 2012). Moreover, studies of the human cancer genome atlas network showed this *TP53* association in more than 97% of cases (Cancer Genome Atlas Research Network 2011).

2.1.3 Clinical features

The symptoms of ovarian cancer are non-specific (e.g., nausea, general weakness, abdominal fullness), and most patients with early stage disease are asymptomatic. At the time of diagnosis, 70% of ovarian cancer patients have advanced disease presenting as stages III-IV. Ovarian cancer is staged on the basis of imaging, macroscopic findings in primary surgery, and histopathological samples taken for staging. The TNM and International Federation of Gynecology and Obstetrics (FIGO) criteria and classification of staging are presented in Table 1 (Edge et al. 2010b).

The rates of long-term survival (> 5 years) in patients with early-stage disease (stages I-II) are 71-90%, 19-47% with advanced disease (stages III-IV) (Heintz et al. 2006). In early-stage disease, the stage, rupture of the ovarian capsule, grade, histology, age, and pelvic fluid cytology have the best prognostic significance (Hennessy, Coleman & Markman 2009). In advanced stages, the presence of residual tumor and its size after surgical debulking has the strongest prognostic significance (du Bois et al. 2009). Stage, histology, age, grade, and lymph node status are other important factors predicting survival in advanced disease (Hennessy, Coleman & Markman 2009).

Table 1. TNM and FIGO Classifications for Ovarian Cancer 2010

Primary tumor (T)

TNM	FIGO	Definition
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
T1	I	Tumor limited to the ovaries (1 or both)
T1a	IA	Tumor limited to 1 ovary; capsule intact, no tumor on ovarian surface; no malignant cells in ascites or peritoneal washings
T1b	IB	Tumor limited to both ovaries; capsules intact, no tumor on ovarian surface; no malignant cells in ascites or peritoneal washings
T1c	IC	Tumor limited to 1 or both ovaries with any of the following: capsule ruptured, tumor on ovarian surface, malignant cells in ascites or peritoneal washings
T2	II	Tumor involves 1 or both ovaries with pelvic extension
T2a	IIA	Extension and/or implants on the uterus and/or tube(s); no malignant cells in ascites or peritoneal washings
T2b	IIB	Extension to other pelvic tissues; no malignant cells in ascites or peritoneal washings
T2c	IIC	Pelvic extension (T2a or T2b) with malignant cells in ascites or peritoneal washings
T3	III	Tumor involves 1 or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis
T3a	IIIA	Microscopic peritoneal metastasis beyond the pelvis (no macroscopic tumor)
T3b	IIIB	Macroscopic peritoneal metastasis beyond the pelvis 2 cm or less in greatest dimension
T3c	IIIC	Macroscopic peritoneal metastasis beyond the pelvis > 2 cm in greatest dimension and/or regional lymph node metastasis

Regional lymph nodes (N)

TNM	FIGO	Definition
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1	IIIC	Regional lymph node metastasis

Distant metastasis (M)

TNM	FIGO	Definition
M0		No distant metastasis
M1	IV	Distant metastasis (excludes peritoneal metastasis)

The presence of nonmalignant ascites is not classified. The presence of ascites does not affect staging unless malignant cells are present.

Liver capsule metastasis is T3/stage III; liver parenchymal metastasis, M1/stage IV. Pleural effusion must have positive cytology for M/stage IV.

2.2 ENDOMETRIAL CANCER

2.2.1 Epidemiology and risk factors

Endometrial cancer is the sixth most common cancer in women worldwide and the most common gynecologic malignancy in developed countries, with an incidence of 15-25 per 100,000 women annually. The majority of cases are diagnosed at an early stage of disease and have a good prognosis, as indicated by the overall 5-year survival rate of 80% (Siegel, Naishadham & Jemal 2013, Howlader et al. 2011).

In Finland, endometrial cancer is the fifth most common type of cancer in women and 868 new endometrial cancer cases were diagnosed in 2013. During the same period, 189 women died due to endometrial cancer, making it the ninth most common cause of cancer death among women in Finland. For endometrial cancer, the age-adjusted incidence rate was 13.8 per 100 000 and the mortality rate 2.1 per 100 000 (www.cancerregistry.fi).

The majority of endometrial cancers are estrogen-related, and estrogen-promoting factors increase the risk of disease. Unopposed exposure to estrogen is likely to cause endometrial hyperplasia and be a major risk factor for type I endometrial cancer. Increased risk can be significantly reduced by concomitant administration of a progestin (Voigt et al. 1991). Other risk factors are age, obesity and metabolic syndrome, diabetes, nulliparity, high years of menstruation, and tamoxifen (Amant et al. 2005).

A familial tendency for endometrial cancer is found in first degree relatives (Lucenteforte et al. 2009). Interestingly, women with first degree relatives with colorectal cancer have a higher risk of developing endometrial cancer than those without a family history (Win, Reece & Ryan 2015). Women with Lynch syndrome have a high risk of endometrial cancer and are likely to develop the disease at a young age. With Lynch syndrome, the lifetime risk of endometrial cancer in women with *MLH1* or *MLH2* mutations is as high as 60%, with a median age of 49 years. Women with *MSH6* mutations have a similar risk of endometrial cancer but a later age of diagnosis (Lu, Broaddus 2005, Lu, Daniels 2013).

2.2.2 Pathogenesis

Approximately 80% of endometrial cancers are endometrioid adenocarcinomas. Serous and clear cell carcinomas account for 1-5% and 5-10% of endometrial cancers, respectively. Mucinous, squamous cell, transitional cell, and small cell carcinomas compromise less than 2% of endometrial cancers (Clement, Young 2002, Boruta et al. 2004). Endometrioid adenocarcinomas are divided into three classes in the histological nuclear grading system. Serous and clear cell carcinomas are classified as high-grade by definition (Prat 2004).

There are two major subdivisions of endometrial cancer with different histological and genetic profiles. The most common carcinomas (type I) are estrogen-dependent, endometrioid-type, and present with a low histological grade (grades 1-2). Type I tumors usually develop in postmenopausal women and coexist with or are preceded

by complex and atypical endometrial hyperplasia. Women with atypical hyperplasia will have an approximately 50% risk of endometrial carcinoma (Bokhman 1983, Amant et al. 2005, Horn et al. 2004).

Approximately 10% of endometrial carcinomas are type II tumors, which occur mainly in older postmenopausal women and are not dependent on estrogen. Most type II tumors are associated with endometrial atrophy, are more aggressive, and are mainly high-grade serous or clear cell carcinomas (non-endometrioid cancers) or poorly differentiated endometrioid carcinoma (grade 3). Type II carcinomas normally arise *de novo*, but occasionally they are associated with serous endometrial intraepithelial carcinoma in an atrophic endometrium or endometrial polyp. Forty percent of non-endometrial cancers are mixed tumors with an endometrioid component (Matias-Guiu, Prat 2013, Amant et al. 2005, Bokhman 1983).

In type I carcinomas, mutations has been found in the PTEN, K-RAS, PIK3CA, and CTNNB1 genes, and tumors exhibit microsatellite instability (MI) (Matias-Guiu, Prat 2013, Mutter et al. 2000, Kong et al. 1997, Lagarda et al. 2001, Rudd et al. 2011, Machin et al. 2002). In endometrial cancers associated with Lynch syndrome, MI has been reported in 75% of cases; MI has also been reported in 25-30% of sporadic carcinomas, occurring more frequently in type I cancers (Duggan et al. 1994).

In contrast to type I carcinomas, 90% of type II non-endometrial carcinomas are associated with alterations in p53, and 80-90% of tumors have markedly reduced E-cadherin expression. The expression of *c-erb-B2 (HER-2)* is also reduced, alterations in *STK15* (mitotic spindle checkpoint) regulation are observed, and heterozygosity is lost at multiple loci, reflecting chromosomal instability (Lax et al. 2000, Tashiro et al. 1997, Hayes, Ellenson 2010, Morrison et al. 2006).

Comprehensive genomic and transcriptomic analyses of endometrioid and serous carcinomas have revealed a new genomic classification for these endometrial carcinomas (Cancer Genome Atlas Research Network et al. 2013). On the basis of the integration of mutation spectra, copy number alterations, and MI status, endometrial carcinomas of endometrioid and serous histology are categorized into four genomic classes: 1) POLE (ultramutated) tumors characterized by very high mutation rates and hotspot mutations in the exonuclease domain of POLE (a subunit of DNA polymerase ε that plays a role in DNA replication), few copy-number aberrations, increased frequency of C $\rightarrow$ A transversions, mutations in *PTEN*, *PIK3R1*, *PIK3CA*, *FBXW7*, and *KRAS*, and favorable outcome; 2) an MI group of endometrioid tumors characterized by MI due to *MLH1* promoter methylation, high mutation rates, few copy-number aberrations, recurrent RPL22 frameshift deletions, and *KRAS* and *PTEN* mutations; 3) low copy-number (endometrioid) tumors, comprising microsatellite-stable grade 1 and 2 endometrioid cancers with low mutation rates characterized by frequent *CTNNB1* mutations; and 4) high copy-number (serous-like) tumors characterized by extensive copy-number aberrations and low mutation rates, recurrent *TP53*, *FBXW7*, and *PPP2R1A* mutations, infrequent *PTEN* and *KRAS* mutations, and poor outcome (Murali, Soslow & Weigelt 2014, Cancer Genome Atlas Research Network et al. 2013).

2.2.3 Clinical features

Abnormal uterine bleeding in postmenopausal women is the most frequent symptom of endometrial cancer. Diagnosis is usually made histologically from endometrial biopsy or curettage (Amant et al. 2005).

Prognostic parameters for endometrial carcinoma can be divided into uterine and extrauterine factors. Uterine factors include histological type, histological grade, depth of myometrial invasion, vascular invasion, presence of atypical endometrial hyperplasia, cervical involvement, DNA ploidy and S-phase fraction, and hormone receptor status. Extrauterine factors include positive peritoneal cytology, adnexal involvement, pelvic and para-aortic lymph node metastasis, and peritoneal metastasis (Prat 2004). Endometrial cancer is staged on the basis of findings during primary surgery and in pathological samples. The TNM and FIGO (International Federation of Gynecology and Obstetrics) criteria and classification of staging are presented in Table 2 (Edge et al. 2010a).

FIGO stage is the single strongest prognostic parameter of endometrial carcinoma. The 5-year disease-free survival has been reported to be 85% for stage I, 75% for stage II, 45% for stage III, and 25% for stage IV (Amant et al. 2005). In low-stage endometrial carcinomas, myometrial invasion is an independent predictor of outcome. The previous iteration of the FIGO system subdivided stage I tumors into IA, IB, and IC tumors. Stage IA tumors were confined to the endometrial complex, stage IB tumors invaded only the inner half of the myometrium (<50% of the depth of the myometrium), and stage IC tumors invaded the outer half of the myometrium (≥50% of the depth of the myometrium). In the 2009 revised FIGO staging system, tumors confined to the endometrium and those invading the inner half of the myometrium are designated as stage IA tumors, and tumors invading the outer half of the myometrium are designated as stage IB tumors (Edge et al. 2010a). According to older FIGO classification for myometrial invasion, 5-year survival for low-grade IA lesions is 95%, whereas high-grade IC lesions had only 42% survival (Grigsby et al. 1992, Creutzberg et al. 2004, Amant et al. 2005).

Histological type plays a critical role; type II cancers account for roughly 10% of all endometrial cancers but involve more than 50% of disease recurrence and deaths. The 5-year survival rates for serous, clear cell, squamous, and undifferentiated carcinomas vary from 30% to 70% (Amant et al. 2005, Prat 2004). Histological grading of endometrioid endometrial carcinomas is prognostically important; the 5-year overall survival is 94% for grade 1 tumors, 84% for grade 2 tumors, and 72% for grade 3 tumors (Zaino et al. 1991).

Table 2. TNM and FIGO Classifications for Endometrial Cancer 2010

Primary tumor (T)

	TNM	FIGO	Definition
	TX		Primary tumor cannot be assessed
	T0		No evidence of primary tumor
	Tis*		Carcinoma in situ (preinvasive carcinoma)
	T1	I	Tumor confined to corpus uteri
	T1a	IA	Tumor limited to endometrium or invades less than one half of the myometrium
	T1b	IB	Tumor invades one half or more of the myometrium
	T2	II	Tumor invades stromal connective tissue of the cervix but does not extend beyond uterus**
	T3a	IIIA	Tumor involves serosa and/or adnexa (direct extension or metastasis)
	T3b	IIIB	Vaginal involvement (direct extension or metastasis) or parametrial involvement
		IIIC	Metastases to pelvic and/or para-aortic lymph nodes
	T4	IV	Tumor invades bladder mucosa and/or bowel mucosa, and/or distant metastases
		IVA	Tumor invades bladder mucosa and/or bowel mucosa (bullous edema is not sufficient to classify a tumor as T4)
Regional lymph nodes (N)			
	NX		Regional lymph nodes cannot be assessed
	N0		No regional lymph node metastasis
	N1	IIIC1	Regional lymph node metastasis to pelvic lymph nodes
	N2	IIIC2	Regional lymph node metastasis to para-aortic lymph nodes, with or without positive pelvic lymph nodes
Distant metastasis (M)			
	M0		No distant metastasis
	M1	IVB	Distant metastasis (includes metastasis to inguinal lymph nodes, intraperitoneal disease, or lung, liver, or bone metastases; it excludes metastasis to para-aortic lymph nodes, vagina, pelvic serosa, or adnexa)

*FIGO no longer includes stage 0 (Tis)

**Endocervical glandular involvement should only be considered as stage I and no longer as stage II

2.3. HYALURONAN

2.3.1 Structure and biochemical properties of hyaluronan

Hyaluronan (hyaluronic acid or hyaluronate) is a high molecular mass linear glycosaminoglycan (GAG). Hyaluronan is found predominantly in the extracellular matrix (ECM) between cells but can be found intracellularly and on the cell surface. Hyaluronan is a ubiquitous polymer consisting of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) with the repeating disaccharide structure of (–1,3-*N*-acetyl-D-glucosamine–1,4-D-glucuronic acid–)*n* (Figure 1).
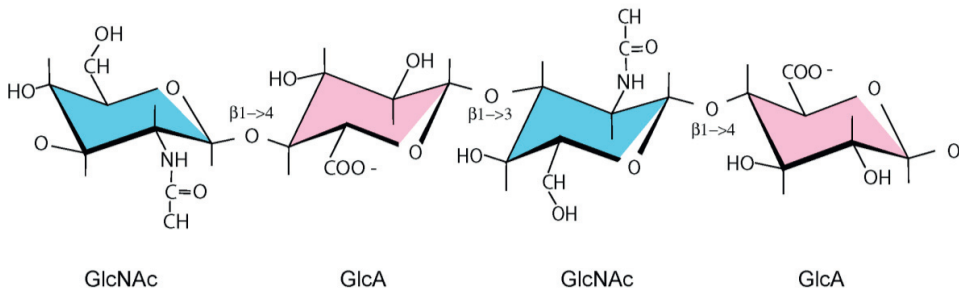
Figure 1. Repeating disaccharide structure of the hyaluronan chain with its N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) repeating units linked via alternating β1->4 and β1->3 glycosidic bonds.

The number of repeating units varies but can reach up to 25,000, corresponding to a molecular mass of 10 million Da (Stern 2008a). Hyaluronan has one carboxyl group, four hydroxyl groups, and an acetyl amine group per disaccharide repeating unit, making it a polyelectrolyte with a negative charge at neutral pH. Due to hydrogen bonding and mutual electrostatic repulsion between carboxyl groups, hyaluronan has unique hydrodynamic properties, such as the capacity to bind large amounts of water and form viscous gels at relatively low concentrations. In more concentrated solutions, hyaluronan molecules form a continuous but porous meshwork that can act as a filter, facilitating the diffusion of small molecules and excluding large molecules. When retained at the cell surface, this voluminous pericellular matrix has been termed the "glycocalyx" and is involved in structural and mechanochemical properties, the regulation of cell division and motility, and in cancer progression and metastasis. However, hyaluronan is more than a structural component of the ECM, as it also regulates cell behavior by interacting with cell surface receptors and initiating signaling pathways (Toole 2004, Tammi et al. 2008, Evanko et al. 2007, Itano, Kimata 2002).

2.3.2 Biosynthesis of hyaluronan

Although hyaluronan belongs to the family of GAGs that includes heparan sulfate and chondroitin sulfate, their synthesis differs greatly. Other GAGs are made as proteoglycans, but hyaluronan is synthesized as a free polysaccharide and not covalently bound to a protein core (Toole 2004). Hyaluronan is synthesized by one of the three hyaluronan synthase proteins (HAS 1-3), which are integral membrane proteins containing seven putative membrane-spanning regions. The large predicted cytoplasmic loop contains the UDP-binding motif and the catalytic sites for glycosyltransferases (Itano, Kimata 2002). Each HAS is capable of transferring the sugars from both UDP-GlcA and UDP-GlcNAc substrates in the presence of Mg^{2+} or Mn^{2+}, and each is able to synthesize hyaluronan alone (Weigel, DeAngelis 2007). Hyaluronan is synthesized on the inner face of the plasma membrane, and the growing chain is extruded or translocated to the extracellular space (Itano, Kimata 2002).

HAS isoenzymes are highly homologous and independently active in hyaluronan synthesis, but they still differ from each other (Itano, Kimata 2002). The kinetic properties of the isoenzymes also differ. The affinity of HAS1 for its substrates is lower than that of HAS2, and HAS3 has the highest affinity implying a lower synthesis rate of hyaluronan. In cell culture, HAS1 transfectants have smaller pericellular coats than those of HAS2 and HAS3 (Itano et al. 1999). Furthermore, HAS1 requires a higher UDP-sugar concentration than HAS2 and HAS3, and *HAS1* expression correlates with cellular UDP-sugar supply (Rilla et al. 2013). Interestingly, the cellular UDP-GlcNAc content controls *HAS2* expression. High cellular UDP-GlcNAc decreases *HAS2* expression in keratinocytes, and a low cellular UDP-GlcNAc concentration increases *HAS2* expression (Jokela et al. 2011).

In mammals, HAS genes are highly homologous but located in different chromosomes. In humans, *HAS1* is located on chromosome 19 q13.4, *HAS2* on chromosome 8q24.12, and *HAS3* on chromosome 16q22.1 (Spicer et al. 1997). *HAS2* is the most important gene in the regulation of hyaluronan synthesis, as large changes in the rate of synthesis are noted with the use of stimulants that affect its expression (Tammi et al. 2011, Tien, Spicer 2005). In addition, deletion of only *HAS2* results in a lethal phenotype in the knockout mouse model (Camenisch et al. 2000). The expression of HAS genes changes rapidly during embryonic development, and in adult tissues hyaluronan synthesis is stimulated by injury, inflammation, and neoplastic tumors (Tammi et al. 2011). Multiple growth factors and cytokines are involved in the transcriptional regulation of HAS genes, but the response depends on the cell type and treatment conditions (Tammi et al. 2011). Hyaluronan synthesis is also sensitive to prostaglandins and hormones. *HAS2* expression is upregulated and the pericellular formation of hyaluronan increased by prostaglandins (Sussmann et al. 2004). Corticosteroids have been shown to suppress *HAS2* expression (Zhang et al. 2000). In the uterine cervix of pregnant mice, progesterone increases *HAS3* expression, whereas *HAS1* and *HAS2* expression are downregulated (Uchiyama, Sakuta &

Kanayama 2005). Moreover, the subcutaneous injection of estrogen in mice increases dermal hyaluronan with the induction of *HAS3* (Rock et al. 2012).

Post-translational regulation of hyaluronan synthases has also been reported. Cytokines, growth factors, and protein kinase C activators have immediate influence on HAS activity in addition to their stimulation of *HAS* transcription (Tammi et al. 2011). Phosphorylation has been shown to influence HAS activity. Hyaluronan secretion is increased when HAS protein is phosphorylated using phorbol ester or interleukin (IL)-1beta (Vigetti et al. 2009). Activation of ERK1/2 and protein kinase-C has also been shown to activate HAS proteins (Bourguignon, Gilad & Peyrollier 2007, Wang, Hascall 2004). On the other hand, phosphorylation of HAS2 by AMKP reduces hyaluronan synthesis in human aortic smooth muscle cells (Vigetti et al. 2011). O-GlcNacylation is a reaction in which β-*N*-acetylglucosamine (O-GlcNAc) is linked to the side chain hydroxyl group of serine or threonine. After glucosamine treatments, HAS2 is O-GlcNacylated, increasing its activity in isolated membranes and cell cultures (Vigetti, Passi 2014, Vigetti et al. 2012). Proteins are led to proteosomal degradation when they are labeled with polyubiquitin chains. Mono-ubiquination is required for HAS2 activity, as its enzymatic activity is lost with site-directed lys190Arg mutation (Lys 190 is normally mono-ubiquinated) (Karousou et al. 2010). Intracellular trafficking can also control hyaluronan synthase function; Rab10-mediated endocytosis has been shown to control HAS3 levels in the plasma membrane and change the cell surface hyaluronan coat and hyaluronan secretion (Deen et al. 2014).

2.3.3 Degradation of hyaluronan

In human tissues, approximately 33% of total body hyaluronan turns over daily. This rapid process is mostly the result of hyaluronidases, enzymes that are primarily involved in the degradation of hyaluronan (Stern 2004). Alone or with hyaluronidase, hyaluronan can also be fragmented by reactive oxygen species (ROS) and free radicals (Agren, Tammi & Tammi 1997, Monzon et al. 2010a). Tight regulation of hyaluronan catabolism is crucial for normal homeostasis and embryonic development, wound healing, tissue regeneration, and repair (Stern, Jedrzejas 2006). Approximately 20% of total body hyaluronan is degraded in peripheral tissues, where the rest 80% goes to the lymphatic system, mainly to be degraded in the lymph nodes (85%). Fifteen percent of this lymphatic hyaluronan goes to the liver via the blood circulation where it is degraded in minutes (Laurent, Dahl & Reed 1991, Stern 2004, Jadin, Bookbinder & Frost 2012).

Hyaluronan catabolism involves the binding of hyaluronan to a specific cell-surface receptor, such as CD44 in the peripheral tissues, LYVE-1 in the lymphnodes, and HARE in the liver. High molecular weight hyaluronan is fragmented by the membrane-associated HYAL-2. Hyaluronan is internalized by hyaluronan receptors or fluid phase endocytosis for lysosomal catabolism and fragmentation. The fragments are taken into vesicles, and eventually lysosomes, for complete degradation to monosaccharides (Stern 2004). HYAL-1 is a key enzyme involved in the lysosomal

degradation of hyaluronan by exoglycosidases (hexosaminodase and glucuronidase) (Gushulak et al. 2012).

Hyaluronidases are endoglycosidases, as they break the β-*N*-acetyl-D-glucosamine linkages in hyaluronan polymer (Figure 2). Hyaluronidases predominantly degrade hyaluronan, but they also have a limited ability to degrade chondroitin sulfate and chondroitin.

In the human genome, there are six hyaluronidase genes, and they are linked in two different clusters. *HYAL-1*, *HYAL-2,* and *HYAL-3* are located in chromosome 3p21.3, and *HYAL-4*, hyaluronidase pseudogene 1 (*HYAL-P1*), and *PH-20* (SPAM1) are located in chromosome 7q31.3 (Csoka, Frost & Stern 2001). These two clusters of hyaluronidase genes may have arisen through gene duplication events, as they also share significant amino acid identity. Among the six mammalian hyaluronidases, HYAL-1, HYAL-2, and PH-20 are well characterized. HYAL-1 and HYAL-2 are the two major hyaluronidases involved in the degradation of hyaluronan (Stern, Jedrzejas 2006).
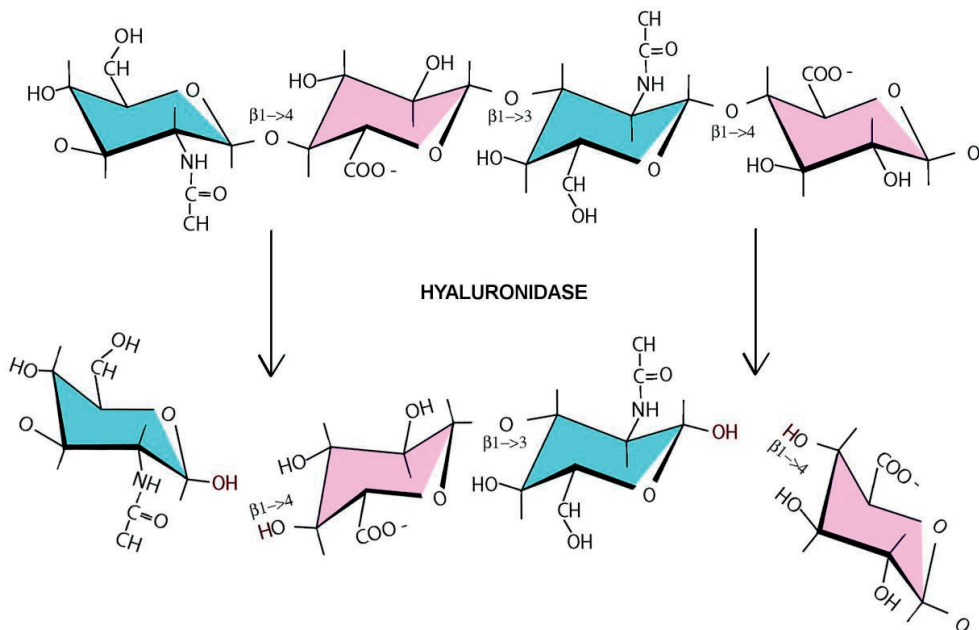
Figure 2. Hyaluronidase cleaves N-acetyl-D-glucosamine β(1->4) glycoside bonds in hyaluronan polymer

HYAL-1 was the first hyaluronidase isolated and characterized from human plasma (Afify et al. 1993). HYAL-1 is generated by two endoprotease reactions that form a 57-kDa single polypeptide glycoprotein or a processed 45 kDa form. Both isoforms occur in urine and tissue extracts, but only the high-molecular weight isoform is present in circulation, with a low concentration of 60 ng/ml (Girish,

Kemparaju 2007, Stern, Jedrzejas 2006). Knockdown of Hyal-1 results in an 80% decrease in total acid hyaluronidase activity in the mouse liver, confirming that HYAL-1 plays a key role in HA catabolism in this organ (Boonen et al. 2014). HYAL-1 has high specific activity for the degradation of hyaluronan and is in an active form at acidic pH.

HYAL-1 degrades high-molecular weight hyaluronan down to hexa- and tetrasaccharides in lysosomes (Stern 2004). HYAL-1 precursor traffics to endosomes via a mannose 6-phosphate-independent secretion/recapture mechanism involving the mannose receptor. Inside the endosomes, the precursor protein is processed into a form with a smaller molecular mass and transported to lysosomes, suggesting that non-covalent associations support the lysosomal activity of HYAL-1 (Puissant et al. 2014).

In humans, HYAL-1 deficiency can cause a lysosomal storage disease known as mucopolysaccharidosis IX (Triggs-Raine et al. 1999). *HYAL-1* knockout mice are viable and fertile without elevated hyaluronan levels in the serum or non-skeletal tissues. As in mucopolysaccharidosis IX, *HYAL-1*-deficient mice develop osteoarthritis and exhibit hyaluronan accumulation in the joints (Martin et al. 2008). HYAL-1 also plays a role in the regulation of ovarian follicle development, showing an inter-relationship between this enzyme and the follistatin/activin/Smad3 pathway and the apoptotic process (Dumaresq-Doiron et al. 2012). High HYAL1 activity can result in apoptosis by increasing the expression of WOX1 (WW domain-containing oxidoreductase, WWOX) (Lokeshwar et al. 2005). WOX1 causes mitochondrial permeabilization and is an essential partner of p53 in cell death (Chang et al. 2001). Hyaluronidase can also cause apoptosis by inducing NAD+-linked 15-hydroxyprostaglandin dehydrogenase (15-PDGH), an enzyme that degrades prostaglandins and promotes apoptosis in lung carcinoma cells (Ding et al. 2005). Ectopic expression of HYAL1 has also been demonstrated to induce granulosa cell apoptosis (Dumaresq-Doiron et al. 2012).

HYAL-2 is a glycosylphosphatidylinositol-anchored, lipid raft-associated hyaluronidase that is active under acidic conditions at the cell surface (Andre et al. 2011). HYAL-2 has been suggested to initiate the degradation of hyaluronan, processing it to approximately 20 kDa products (50 disaccharide units), as HYAL-1 and exoglycosidases (hexaminodase) continue degradation the protein into smaller particles in lysosomes (Stern 2004, Gushulak et al. 2012). HYAL-2 can also act as a receptor on the cell surface for oncogenic sheep retroviruses, but it does not play a crucial role in cancer induction (Miller 2008). Studies in knockout mice have shown an essential role of HYAL-2 in hyaluronan catabolism. *HYAL-2* knockout mice are viable and fertile despite a 10-fold increase in plasma hyaluronan levels and 2-fold increase in plasma hyaluronidase activity. No global accumulation of hyaluronan occurs in the tissues, though liver sinusoidal cells seem overloaded with high-molecular weight hyaluronan. Mice also exhibit localized congenital defects in frontonasal and vertebral bone formation and suffer from mild thrombocytopenia and chronic hemolysis (Jadin et al. 2008). Outbred *HYAL-2* knockout mice exhibit a more severe phenotype with increased mortality and an accumulation of extracellular

hyaluronan, leading to dramatic cardiopulmonary dysfunction. Serum hyaluronan levels in these *HYAL-2* knockout mice increase continuously, reaching an average 27-fold increase by the time of euthanasia (Chowdhury et al. 2013).

The major *HYAL-3* transcript is enzymatically inactive and appears to play only a supportive role in *HYAL-1* expression (Hemming et al. 2008). HYAL-3 knockout mice also do not display any evidence of hyaluronan accumulation (Atmuri et al. 2008). Very little is known about HYAL-4, but its expression is limited, and it appears to be a chondroitinase with no activity against hyaluronan (Stern, Jedrzejas 2006). HYAL-P1 is a pseudogene with active transcription but no translation in humans (Stern, Jedrzejas 2006).

PH-20 is glycosylphosphatidylinositol-anchored hyaluronidase with activity at acidic and neutral pH. It is mainly a sperm-associated testicular hyaluronidase, but it is also expressed epididymis, breast, placenta, and fetal tissues (Stern, Jedrzejas 2006). PH-20 plays an important role during ovum fertilization, as it facilitates penetration of the sperm through the cumulus oophorus and zona pellucida of the ovum, and it also works as a receptor during fertilization (Cherr, Yudin & Overstreet 2001).

There are different approaches in the regulation of hyaluronidases, as little is known about the exact mechanisms. Alternative splicing resulting in enzymatically inactive proteins is one mechanism of *HYAL-1* regulation, and one of the splice variants (HYAL1-v1) has been shown to act as a negative regulator of tumor growth, invasion, and angiogenesis (Lokeshwar et al. 2002, Lokeshwar et al. 2006). *HYAL-1* expression has also been shown to be regulated epigenetically by the binding of different transcription factors (SP1, EGR-1, and AP-2) to the methylated and unmethylated *HYAL-1* promoter (Lokeshwar et al. 2008). In human airway epithelial cells, pro-inflammatory cytokines (TNF-alpha and IL-1beta) have been shown to increase *HYAL1-3* expression (Monzon et al. 2008), and ROS increase HYAL2 expression and activity (Monzon et al. 2010b). In chondrocytes, HYAL-2 expression is constitutive and does not appear to respond to cytokines, growth factors, or cellular mediators (Chow, Knudson 2005).

2.3.4 Hyaluronan binding proteins and receptors

As hyaluronan is secreted to the cell surface and extracellular matrix, it interacts with different molecules and hyaluronan binding proteins, including receptors with signaling properties. This group of proteins is called hyaladherins. Most hyaladherins belong to the link module superfamily, with a hyaluronan binding domain of 100 amino acids as a common feature. Hyaladherins can be extracellular, intracellular, or localize to the cell surface. In addition to the link proteins, aggrecan, versican, brevican, neurocan, tumor necrosis factor alpha stimulated gene-6 (TSG-6), are members of the link protein superfamily are located extracellularly. Sialoprotein (SPACR) and sialoproteoglycan (SPACRCAN) also have extracellular locations, but they are not part of the link family. CD44, LYVE-1, and HARE, which are cell surface receptors for hyaluronan, belong to the link module superfamily (Day, Prestwich 2002), whereas transmembrane hyaluronan receptor laylin does not have the link

module (Bono et al. 2001). Intracellular hyaluronan binding proteins include HABP)/P32 (hyaluronan binding protein), CDC37, and RHAMM (receptor for hyaluronan-mediated motility), and the latter is also found on the cell surface (Day, Prestwich 2002). As CD44 and RHAMM are the major receptors in cancer, they will be discussed in more detail.

CD44 is by far the most characterized cell surface receptor for hyaluronan. It is a single-pass transmembrane glycoprotein expressed in most cell types (Toole 2009). *CD44* is located in the short arm of chromosome 11. The regulation of CD44 is well known, occurring through promoter methylation, mRNA transcription, post-translational glycosylation and phosphorylation, variable splicing, and ligand binding (Bourrguignon et al. 1995, Naor, Sionov & Ish-Shalom 1997). Hyaluronan is the principal ligand for CD44, but many other molecules, such as osteopontin, FGF, or selectin, have been shown to be able to bind CD44 (Toole 2009). With the help of CD44 receptor, hyaluronan can anchor to the cell surface and form a pericellular matrix with associated aggregating proteoglycans (Evanko et al. 2007). CD44 is also involved in the endocytosis of hyaluronan (Thankamony, Knudson 2006). Most importantly, hyaluronan can activate intracellular signaling cascades associated with migration, proliferation, and invasion through its interaction with CD44 (Bourguignon 2008).

RHAMM is located in chromosome 5, and the transcript is alternatively spliced into intracellular and cell surface variants (Zhang et al. 1998). RHAMM does not belong to the link module superfamily, as it uses special RHAMM-type hyaluronan binding motifs to interact with hyaluronan (Yang et al. 1994). RHAMM can be present in the cytoplasm, nucleus, or cell surface. In normal adult human tissues, RHAMM mRNA and protein expression is low. RHAMM expression is increased in wound repair (Samuel et al. 1993), and its genetic deletion results in slow healing skin wounds (Tolg et al. 2006). RHAMM has been suggested to control microtubule assembly during mitosis (Maxwell, McCarthy & Turley 2008). The hyaluronan-RHAMM interaction can initiate signaling cascades and activate CD44; moreover, RHAMM can co-operate with CD44 signaling through ERK1/2 and promote cancer cell motility (Toole 2009).

2.4 HYALURONAN AND CANCER

2.4.1 Altered tissue hyaluronan content and cancer

Alterations in hyaluronan metabolism occur in many cancer types. Tumoral hyaluronan content can decrease or increase depending on the origin of the neoplastic process. A stratified epithelium that normally covers the hyaluronan-rich stroma goes through a dramatic change in hyaluronan content when carcinomas arise from its squamous cells (Tammi et al. 2008). This hyaluronan depletion has been shown with squamous cell carcinoma (SCC) of the mouth (Kosunen et al. 2004), larynx (Hirvikoski et al. 1999), lung (Pirinen et al. 1998), esophagus (Wang et al. 1996), vulva (Hamalainen et al. 2010), and skin (Karvinen et al. 2003).

In contrast, hyaluronan accumulation has been observed in carcinomas arising from epithelial cells that are normally devoid of hyaluronan and often surrounded by hyaluronan-poor connective tissue. High stromal hyaluronan levels have been found in many tumors of epithelial origin and correlate with poor prognosis (Tammi et al. 2008). In ovarian carcinoma, the high stromal hyaluronan level is significantly associated with poor differentiation, serous histological type, advanced stage, and large primary residual tumor. Furthermore, high stromal hyaluronan content was found to be an independent prognostic factor for short disease-free survival and overall survival, even when a patient subgroup consisting of FIGO I and FIGO II stages was examined separately (Anttila et al. 2000). In the endometrium, the stromal hyaluronan level increases during the progression from hyperplasia to adenocarcinoma (Afify et al. 2005). Similarly, hyaluronan accumulation occurs in adenocarcinoma of the breast (Auvinen et al. 1997), lung (Pirinen et al. 2001), stomach (Setala et al. 1999), prostate (Lipponen et al. 2001), and pancreas (Cheng et al. 2013). The stromal accumulation of hyaluronan in adenocarcinomas is associated with more aggressive growth and poor differentiation, which is related to the increased capability to penetrate connective tissue capsules and invade lymph vessels and nerves (Tammi et al. 2008, Sironen et al. 2011). Hyaluronan accumulation is also involved in urothelial carcinoma of the bladder (Hautmann et al. 2001), B-cell lymphomas (Bertrand et al. 2005), and acute myeloid leukemia (Sundstrom et al. 2005).

2.4.2 Hyaluronan biosynthesis in cancer

Accumulation of hyaluronan in cancer is due to its discoordinated metabolism. Increased hyaluronan levels can result from increased synthesis, decreased degradation, or both. Many studies have attempted to explain possible alterations in hyaluronan content due to altered synthesis.

HAS1 mRNA expression is increased in bladder cancer and associated with metastases (Kramer et al. 2011). Elevated *HAS1* mRNA levels have also been noted in renal cell carcinomas (Chi et al. 2012) and colon carcinoma (Yamada et al. 2004). In breast cancer, the HAS1-3 proteins are upregulated, and increased HAS1 and HAS3 levels in stromal cells are associated with poor patient survival (Auvinen et al. 2014). Increased HAS1 protein levels are also associated with poor patient survival in ovarian cancer (Yabushita et al. 2004). *HAS1* is aberrantly spliced in multiple myeloma and Waldenstrom macroglobulinemia (Adamia et al. 2013). Furthermore, inherited polymorphisms in *HAS1* predict the risk for systemic B-cell malignancies, but not for solid tumors (Kuppusamy et al. 2014).

Overexpression of *HAS2* in the mammary glands of a transgenic mouse model induces hyaluronan overproduction in the intratumoral stroma, accelerated tumor angiogenesis, and poorly differentiated adenocarcinomas (Koyama et al. 2007). This hyaluronan-rich tumor-associated stroma has also been shown to promote intratumoral lymphangiogenesis in a tumor xenograft model (Koyama et al. 2008). In previous studies, overexpression of *HAS2* in fibrosarcoma (Kosaki, Watanabe &

Yamaguchi 1999) and *HAS 3* in prostate cancer (Liu et al. 2001) and mesothelioma (Li, Heldin 2001) cell lines resulted in hyaluronan accumulation and increased the tumorigenic ability of the cells. Other studies in osteosarcoma (Nishida et al. 2005), prostate (Simpson, Wilson & McCarthy 2002, Simpson et al. 2002), and breast cancer (Udabage et al. 2005b) cell lines showed that, when *HAS2* and/or *HAS3* expression is suppressed, hyaluronan production decreased and the tumorigenic potential was reduced (Itano, Kimata 2008). Moreover, in pancreatic cancer, hyaluronan accumulation by HAS3 favors cancer growth and modulates the pancreatic cancer microenvironment, decreasing epithelial cell adhesion (Kultti et al. 2014). HAS3 induces the accumulation of hyaluronan in kidney cell cultures, resulting in possible premalignant changes, such as intraluminal invasion and disturbed organization of the epithelium likely mediated by mitotic spindle misorientation (Rilla et al. 2012). However, opposite effects have been seen in other models, suggesting that the effect of HAS overexpression depends on the cellular context. Thus, HAS2 overexpression reduces the tumorigenic potential of glioma cells (Enegd et al. 2002) and overexpression of all HAS isoforms reduces CHO cell migration (Brinck, Heldin 1999).

2.4.3 Role of hyaluronidases in cancer

Altered HYAL1 expression is found in many different cancer types, and its role as a biomarker has been widely studied. HYAL1 can be increased or decreased depending on the cell types arising from the neoplastic process. Interestingly, in prostate cancer, HYAL1 is a tumor promoter or suppressor depending on the concentration (Lokeshwar et al. 2005).

HYAL1 expression is increased in prostate adenocarcinoma (Lokeshwar et al. 2001), and its expression in biopsy samples predicts its recurrence (Gomez et al. 2009). Blocking HYAL1 expression in an urothelial carcinoma cell line resulted in decreased cell growth (Lokeshwar, Cerwinka & Lokeshwar 2005). In line with this finding, HYAL1 was increased and its overexpression predicted metastasis and mortality in a human urothelial bladder carcinoma (Kramer et al. 2011). Importantly, high HYAL1 expression correlated with muscle invasion, which is a crucial marker for radical surgery and poor prognosis (Kramer et al. 2010). HYAL1 is also an accurate urinary diagnostic marker for bladder cancer (Eissa et al. 2012).

In human colorectal cancer, HYAL1-2 expression and hyaluronidase activity is increased, especially in advanced disease (Bouga et al. 2010). In contrast, findings in a rat colon carcinoma cell line and tumor transplantable rat model showed that HYAL1 overexpression suppresses the growth rate of tumor cells (Jacobson et al. 2002).

In breast cancer cells, *HYAL1* mRNA and protein levels are overexpressed, and in human invasive ductal cancer tissues HYAL1 exhibits stronger staining compared to normal breast tissue. Blocking HYAL1 expression in breast cancer cells reduces tumor growth and invasion (Tan et al. 2011a). Moreover, in nude mice, forcing HYAL1 expression induces breast cancer cell xenograft tumor growth and angiogenesis (Tan et al. 2011b). Interestingly, in a mouse syngenic breast tumor model, HYAL1 overexpression promotes lung metastasis without affecting the accumulation of small

hyaluronan oligosaccharides (Schmaus, Sleeman 2015). In contrast, decreased *HYAL1* expression is also associated with short distant metastasis-free survival in human breast cancer (Heldin et al. 2013).

HYAL1 mRNA and protein expression is decreased in non-small cell carcinomas of the lung (Anedchenko et al. 2008, Wang et al. 2008) and kidney clear cell carcinoma (Chi et al. 2012, Wang et al. 2008). A recent study indicated that weak expression of HYAL1 is associated with poor survival in pancreatic ductal adenocarcinoma (Cheng et al. 2013).

HYAL2 expression is also associated with cancer progression. HYAL2 overexpression has been found in premalignant and malignant melanomas (Siiskonen et al. 2013) and is associated with the invasiveness of breast cancer (Udabage et al. 2005a). In contrast, *HYAL2* is deleted in human non-small cell lung cancer (Li et al. 2007). *HYAL2* expression is also decreased in B-cell non-Hodgkin lymphoma (Bertrand et al. 2005).

Interestingly, the methylation profile of *HYAL2* is also altered in breast cancer, with a strong association between decreased *HYAL2* methylation in the peripheral blood and an increased risk of breast cancer (Yang et al. 2015). Alterations in peripheral blood *HYAL2* methylation status have also been found in head and neck squamous cell carcinoma (Langevin et al. 2012).

2.4.4. Degradation products of hyaluronan and cancer

Native hyaluronan (HMW-HA) is a large molecule, corresponding to a molecular mass of up to 10 million Da. (Stern 2008a). However, hyaluronan synthases can also produce end products with an altered size as a possible consequence of mutations to its gene or different availability of UDP-sugar precursors (Weigel, Baggenstoss 2012, Vigetti et al. 2014). The degradation of high-molecular weight hyaluronan by HYAL2 yields intermediate-sizes particles ranging in size from 25 to 1000 disaccharides (LMW-HA), and HYAL1 creates HA-oligosaccharides (sHA) ranging from 2 to 25 disaccharides in length. ROS degrade hyaluronan to intermediate-sized fragments (Agren, Tammi & Tammi 1997), perhaps in the tumor environment as well (Stern et al. 2007).

Differently sized hyaluronan fragments and HMW-HA possess different biological properties in different diseases in a concentration-dependent manner (Schmaus, Bauer & Sleeman 2014). The accumulation of very high-molecular weight hyaluronan nearly double the average size of hyaluronan found in humans and other rodents was recently shown to be associated with exceptional longevity and cancer resistance in the naked mole rat. The ability to produce and accumulate this very high-molecular weight hyaluronan is due to decreased hyaluronidase activity and overexpression of *HAS2* specific to the naked mole rat. Interestingly, when knocking down HAS2 or overexpressing HYAL2, naked mole rat cells become susceptible to malignant transformation and readily form tumors (Tian et al. 2013).

sHA has been shown to have angiogenic potential *in vitro* (Cui et al. 2009, West, Kumar 1989); *in vivo*, it has been shown to increase blood vessels beneath the

epidermis where it is applied to the skin of rats (Sattar et al. 1994). sHA also increases endothelial cell proliferation, but only at low concentrations, whereas high concentrations of sHA have no effect on proliferation or are antiproliferative (Gao et al. 2010, Cui et al. 2009, Lokeshwar, Selzer 2000). Competition between HMW-HA and sHA for cellular hyaluronan receptors has also been suggested (Schmaus, Bauer & Sleeman 2014), as treatment with sHA results in decreased pericellular hyaluronan retention, leading to destruction and remodeling of the hyaluronan-rich pericellular matrices (Hosono et al. 2007, Knudson et al. 2000). Interestingly, HMW-HA-induced clustering of CD44 can be reversed by the addition of sHA (Yang et al. 2012). HMW-HA has also been shown to promote complex formation between CXCR4 and CD44, leading to enhanced signaling, migration, and angiogenesis. These effects are blocked in the presence of sHA (Fuchs et al. 2013).

Hyaluronan fragments play a role in inflammation during tumor development and progression, as they may be involved in activating the innate immune system and stimulate immune cells. Hyaluronan fragments also induce the expression of various cytokines that provide growth and survival signals for tumor cells (Schmaus, Bauer & Sleeman 2014). For example, sHA can induce cytokine expression in melanoma and breast tumor cells (Voelcker et al. 2008, Bourguignon et al. 2011). Controversially, cytokine expression was reported to be induced by HYAL2-cleaved intermediate-sized hyaluronan in monocytes (de la Motte et al. 2009). Thus, a broad size range of hyaluronan may be pro-inflammatory. However, the hyaluronidases used in these studies were purified from animal tissues and contain endotoxins and other unrelated proteins. In a recent study, neither highly purified recombinant human hyaluronidase (rHuPH20) nor its directly generated hyaluronan catabolites had inflammatory properties (Huang et al. 2014).

Matrix metalloproteinases (MMPs) are endopeptidases that can degrade components of the extracellular matrix and enable cells to invade surrounding tissues. They can also stimulate growth factors and cytokines and induce epithelial-to-mesenchymal transition (EMT). Different sizes of hyaluronan fragments can also induce MMP expression (Orlichenko, Radisky 2008, Schmaus, Bauer & Sleeman 2014). The expression of MMP-9 and MMP-13 can be induced by sHA fragments, as well as intermediate-sized hyaluronan fragments, suggesting that metastasis-associated hyaluronan degradation in tumors can promote invasion by inducing MMP expression (Fieber et al. 2004). Controversially, *in vivo* studies have shown that, in wounded tissue, sHA represses the transcription of MMPs (Gao et al. 2010), suggesting that the effects of hyaluronan fragments are specific and context-dependent.

Hyaluronan fragments play an important role in tumorigenesis, when hyaluronan accumulation and variation in hyaluronidase expression are often present. However, the results of studies concerning tumors and hyaluronan fragments have been inconsistent, as the fragments have been reported to be tumor-promoting or inhibiting (Schmaus, Bauer & Sleeman 2014). In pancreatic carcinoma cells, sHA enhances CD44 cleavage and tumor cell motility (Sugahara et al. 2006). In thyroid carcinoma cells,

sHA promotes cell proliferation and migration, inducing CXCR7 expression, and intratumorally injected sHA increases tumor growth *in vivo* (Dang et al. 2013). In contrast, sHA fragments can inhibit anchorage-dependent growth and promote apoptosis *in vitro* (Ghatak, Misra & Toole 2002). Hyaluronan oligosaccharides have been shown to suppress the progression of bone metastasis in breast carcinoma by interrupting the endogenous HA-CD44 interaction (Urakawa et al. 2012). Interestingly, sHA but not HMW-HA has been reported to reduce the growth of colon carcinoma cells; the effect was suggested to be achieved, in part, by stimulation of the immune system (Alaniz et al. 2009).

2.4.5 Hyaluronan and epithelial-mesenchymal transition

To acquire the ability to invade, tumor cells need to progress to an EMT. During this process, epithelial cells lose their polarity and cell-cell contacts and acquire a migratory phenotype, which results in a mesenchymal-like gene expression program (Colas et al. 2012). E-cadherin is a central cell adhesion molecule and plays a critical role in the suppression of tumor invasion and metastases. A critical molecular feature of EMT is the downregulation of E-cadherin expression (Thiery 2003). Several transcription factors capable of regulating this process have been identified, but Twist and Snail have emerged as the most promising candidates (Barrallo-Gimeno, Nieto 2005, Yang et al. 2004).

In endometrial cancer, downregulation of E-cadherin is often associated with high-grade, non-endometrioid carcinomas (Holcomb et al. 2002, Moreno-Bueno et al. 2003). E-cadherin expression is also associated with tumor dedifferentiation and deep myometrial invasion (Sakuragi et al. 1994), possibly due to hypermethylation of the promoter region of its gene (Saito et al. 2003). E-cadherin has been shown to be an independent prognostic factor for disease progression and mortality in stage I-III endometrial cancer (Mell et al. 2004).

Several findings support the conclusion that hyaluronan may facilitate the EMT. HAS2 knock-out prevents normal cardiac EMT in mice (Camenisch et al. 2000) via hyaluronan-augmented activation of ErbB2-ErbB3 receptors (Camenisch et al. 2002), whereas hyaluronan oligosaccharides prevent cardiac EMT via vascular endothelial growth factor (VEGF) activation (Rodgers et al. 2006). Increased hyaluronan content can also induce EMT in normal epithelial cells (Zoltan-Jones et al. 2003). Increased synthesis of hyaluronan is associated with the EMT in lung adenocarcinoma cells (Chow, Tauler & Mulshine 2010), and in pancreatic cancer cells, the accumulation of hyaluronan is associated with loss of E-cadherin. Interestingly, pegylated human recombinant hyaluronidase (PEGPH20) inhibits these changes (Kultti et al. 2014). In HAS2 transgenic mice, hyaluronan overproduction causes rapid development of aggressive breast carcinoma with a high incidence (Koyama et al. 2007); in further studies, this excess hyaluronan production was shown to drive cells towards the EMT. In particular, hyaluronan production made the plastic cancer cell population revert to stem cell states (Chanmee et al. 2014).

Overexpression of hyaluronan receptor CD44 was shown to downregulate E-cadherin expression and induce EMT changes in colon carcinoma, whereas knockdown of CD44 reduced these events, suggesting that the influence of hyaluronan is mediated by this receptor (Cho et al. 2012). In contrast to these previous studies, Porsch et al. and Heldin et al. showed that transforming growth factor β (TGFβ)-induced HAS2 expression plays a regulatory role in EMT independent of the hyaluronan-producing activity of the HAS and hyaluronan receptor CD44 expressed in these cells (Heldin et al. 2014, Porsch et al. 2013).

3 Aims of the study

Stromal accumulation of hyaluronan has been well documented in human ovarian and endometrial carcinomas, but the underlying mechanisms are not fully understood. The aim of this doctoral thesis was to elucidate the mechanisms of tumoral hyaluronan accumulation by investigating the expression and activity of the key enzymes affecting hyaluronan turnover.

The specific aims of this thesis were:

1. To investigate the expression of hyaluronan synthase (*HAS1-3*) and hyaluronidase genes (*HYAL1-2*) in ovarian and endometrial carcinomas and study the correlation between possible changes in expression and tumoral hyaluronan accumulation.

2. To examine the expression of HYAL1 and HYAL2 proteins in normal endometria, precancerous lesions, and endometrial carcinomas and elucidate their role in the pathology of endometrial cancer.

4 Materials and Methods

4.1 PATIENTS AND TISSUE SAMPLES (Studies I-III)

4.1.1 Study I

Thirty-nine ovarian tissue specimens from 39 patients were divided into five groups: normal ovaries (n = 5), serous cystadenomas (n = 10), serous borderline tumors (n = 4), low grade (grades 1 and 2) serous cystadenocarcinomas (n = 10), and high grade (grade 3) (n = 10) serous cystadenocarcinomas (Study I Table 1). All patients were diagnosed and treated at Kuopio University Hospital between 2002 and 2004. Histopathological tissue specimens for light microscopy were processed according to standard clinical protocol in the Pathology Department of Kuopio University Hospital. All samples were collected and handled identically. Tissue aliquots were 1) placed in RNAlater® (Ambion, Austin, TX) for mRNA analyses, 2) fixed in 10% buffered formalin, embedded in paraffin, or 3) homogenized in 1 mM sodium EDTA containing 1 mM benzamidine-HCl, 1 mM saccharic acid 1,4-lactone, 1 mM β-mercaptoethanol, 1 mM iodoacetate, and 0.5% Triton X-100 clarified by centrifugation at 4°C (1,000 × g for 15 min and 10,000 × g for 30 min). The extracts were stored at -70°C until assayed.

4.1.2 Study II

Thirty-five endometrial tissue specimens from 35 patients were divided into five groups: proliferative and secretory endometrium (n = 10), post-menopausal proliferative endometrium (n = 5), complex atypical hyperplasia (n = 4), grade 1 (n = 8) endometrioid adenocarcinomas, and grade 2+3 (n = 8) endometrioid adenocarcinomas. All patients were diagnosed and treated at Kuopio University Hospital between 2000 and 2006. Normal endometrium tissue specimens were obtained from hysterectomies for non-malignant diseases (e.g., leiomyoma or uterine prolapse). The tissue specimens collected in the operating room were prepared and evaluated by an experienced pathologist. All samples were collected and handled identically. Tissue aliquots were placed in RNAlater® (Ambion, Austin, TX) for mRNA analyses or fixed in 10% buffered formalin, and embedded in paraffin.

4.1.3 Study III

Endometrial tissue specimens were collected from 343 patients, including normal, atrophic, or premalignant (complex atypical hyperplasia) endometria and endometrial carcinomas (Study III Table 1). All patients were diagnosed and treated at Kuopio University Hospital between 2000 and 2012. The non-malignant endometrial samples were obtained from hysterectomies (e.g., due to leiomyoma or uterine prolapse). All patients with endometrial carcinoma underwent surgery. The

surgery included peritoneal cytology, total hysterectomy, bilateral salpingo-oophorectomy, and pelvic and para-aortic lymph node sampling when considered necessary. No patient received chemotherapy or radiotherapy before surgery.

Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Representative samples of carcinomas and hyperplastic endometria were cut into 3-µm-thick sections for immunohistochemical analyses. Samples of normal endometria representing different phases of the menstrual cycle or atrophic endometrium were evaluated using tissue microarrays. For tissue microarrays, three regions of the endometrium were chosen from each sample and incorporated into microarrays (core diameter, 1.3 mm) with a tissue microarray I device (Beecher Instruments, Silver Spring, MD, USA).

4.1.4 Histology (I-III)

Histological typing and tumor grading were performed in Studies I-III according to WHO classifications (Kurman et al. 2003) and staged according to the International Federation of Gynecology and Obstetrics (FIGO) guidelines (Edge et al. 2010a).

4.2 ANALYSIS OF *HAS1–3* AND *HYAL1–2* mRNA EXPRESSION (STUDIES I–II)

4.2.1 RNA extraction and cDNA preparation
Samples were frozen using liquid nitrogen and pulverized under pressure using a stainless steel cylinder and piston. Total RNA was isolated using Trizol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, quantified spectrophotometrically, and its integrity confirmed by agarose electrophoresis based on the appearance of the 18S and 28S RNA bands. First-strand cDNA was synthesized from 2.5 µg of total RNA using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol in a final volume of 50 µl.

4.2.2 Quantitative real-time RT-PCR
The PCR primers and fluorogenic probes for all target genes (*HYAL1*, *HYAL2*, *HAS1–3*) and the endogenous control hypoxanthine phosphoribosyltransferase 1 (HPRT1) were purchased as TaqMan® Gene Expression Assays (Applied Biosystems): Hs00201046_m1 (HYAL1); Hs00186841_m1 (HYAL2); Hs00758053_m1 (HAS1); Hs00193435_m1 (HAS2); Hs00193436_m1 (HAS3); and Hs99999909_m1 (HPRT). The assays were supplied as a 20× mix of PCR primers and TaqMan MGB (minor groove binder) probes labeled with a 6-FAM dye and a non-fluorescent quencher at the 3' end of the probe. The primers were designed to span an exon-exon junction, eliminating the possibility of detecting genomic DNA.

For each amplification, 6 µl of cDNA equivalent to 30 ng of total RNA was mixed with 1 µl of 20× Primer and Probe Mix and 10 µl of 2× TaqMan Universal Master Mix

in a final volume of 20 μl. Each sample was quantified using standard curves established by six series of 4-fold serial dilutions of cDNA obtained by reverse transcription of 2.5 μg Universal Human Reference RNA (Stratagene, La Jolla, CA). Standard curves and no-template negative controls (NTCs) were made for every plate. Triplicate reactions were used for each sample and each point of the standard curve. The reactions were performed in 96-well plates on the MX3000P real-time instrument (Stratagene, La Jolla, CA). The PCR conditions were as follows: 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

HPRT1 was used for normalization as an accurate reference for quantitative gene expression assays in clinical tumor samples (de Kok et al. 2005). Relative gene expression values were calculated as the ratio between the target gene and *HPRT1* obtained for each sample from the standard curves. Finally, these values were divided by the mean value for normal ovaries. C_T values were used to roughly compare the relative amount of *HYAL1* and *HYAL2* mRNA.

4.3 HYALURONIDASE ASSAY (STUDY I)

Hyaluronidase enzyme activities in tissue extracts were determined by the release of biotinylated hyaluronan coupled to the bottom of 96-well plates in triplicate reactions as described previously (Hiltunen et al. 2002). Briefly, aliquots of the tissue extracts and 0.001–10 units of hyaluronidase standards [Bovine Testes, type IV-S, H-3884 (pH 6.0); Sigma] were diluted in incubation buffers [0.1 M Na-acetate (pH 6.0) for standards and 0.2 M NaCl in 0.1 M formate (pH 3.7 and pH 7.0) for tissue extracts] and kept in hyaluronan-coated wells at 37°C for 2 h. The standards contained the same concentrations of protease inhibitors as the samples. The wells were washed with 0.05% Tween-PBS and the biotinylated hyaluronan remaining in the wells was quantitated using the avidin-biotin detection system. The hyaluronidase activity (mU) of each tissue extract was calculated using a logarithmic standard curve and the results normalized to protein concentration.

4.4 HYALURONAN STAINING (STUDIES I-II)

The level of hyaluronan accumulation in the present set of ovarian and endometrial tumors was scored in tissue sections using a biotinylated probe that specifically binds hyaluronan. This histological assay is closely correlated with biochemical quantitation of hyaluronan in ovarian tissues (Hiltunen et al. 2002). Deparaffinized 5-μm sections were stained for hyaluronan using our own preparation of biotinylated hyaluronan-binding complex (bHABC) as described previously (Wang et al. 1996). Briefly, deparaffinized sections were rehydrated, washed with 0.1 M sodium PB (pH 7.4), treated with 1% hydrogen peroxide for 5 min to inactivate peroxidases, and blocked with 1% BSA in PB. The sections were incubated in bHABC (2.5 μg/ml, diluted in 1%

BSA) overnight at 4°C, washed with PB, and treated with avidin-biotin-peroxidase (ABC Vectastain Elite kit; Vector Laboratories). The sections were washed with PB and the color developed with 0.05% diaminobenzidine tetrahydrochloride (Sigma) and 0.03% hydrogen peroxide in PB. The slides were counterstained with Mayer's hematoxylin. Staining specificity was controlled by digesting some of the sections with *Streptomyces* hyaluronidase in the presence of protease inhibitors before staining or by pre-incubating the bHABC probe with hyaluronan oligosaccharides.

All samples were scored by an observer blinded to the clinical data (M.A.). The intensity of hyaluronan positivity in the epithelium and stroma was graded into three categories (1, weak; 2, moderate; or 3, strong) and the percentage area of the strongest hyaluronan expression in the whole tumor section evaluated and used as an indicator of hyaluronan accumulation.

4.5 IMMUNOHISTOCHEMISTRY (STUDIES I–III)

4.5.1 HAS1-3 immunostaining (Studies I-II)

Antigen retrieval was performed for HAS2 staining by microwave treatment (700 W, 3 × 5 min) in citrate buffer. All deparaffinized sections were treated for 5 min with 1% H_2O_2 to block endogenous peroxidase, washed with 0.1 M Na-phosphate buffer pH 7.4 (PB), and incubated in 1% bovine serum albumin (BSA) in PB for 30 min to block non-specific binding. The sections were then incubated overnight at 4°C with polyclonal antibodies for HAS1 (1:100 dilution in 1% BSA, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), HAS2 (1:50, Santa Cruz), or HAS3 (1:100, Santa Cruz), followed by a 1 h incubation with biotinylated antigoat antibody (1:1000, Vector Laboratories). The bound antibodies were visualized using the avidin-biotin peroxidase method (1:200, Vectastain Kit, Vector Laboratories, Burlingame, CA). The sections were incubated for 5 min in 0.05% diaminobenzidine (Sigma) and 0.03% hydrogen peroxide in PB. After washing, the sections were counterstained with Mayer's hematoxylin for 1 min, washed, dehydrated, and mounted in DPX (Gurr, BDH Laboratory Supplies, Poole, U.K.).

All samples were scored by an observer blinded to the clinical data (K.R.). For study I, the percentage of area positive for each HAS was estimated in the stroma and epithelium for HAS1 and HAS3. The staining intensity for HAS2 in the epithelium was estimated by grading in three categories: 1, weak; 2, moderate; or 3, strong.

For study II, the staining intensity of HAS1, HAS2, and HAS3 in the epithelium was graded into three categories: negative (n.d.), weak, or moderate. The intensity in the stroma was graded into two categories: negative (n.d.) or weak. The percentage of area positive for each HAS was estimated in both the stroma and epithelium.

4.5.2 HYAL1-2 immunostaining (Study III)

Deparaffinized sections were incubated in 10 mM citrate buffer (pH 6.0) for 15 min in a pressure cooker at 120°C, washed with phosphate-buffered saline (PBS), and treated for 5 min with 1% H_2O_2 to block endogenous peroxidase activity. The sections were then incubated in 1% BSA, 0.05% Tween-20, and 0.1% gelatin (Sigma G-2500, Sigma) in PBS for 30 min to block non-specific binding. The sections were incubated with polyclonal primary antibodies against HYAL1 and HYAL2 overnight at 4°C, diluted in 1% BSA (HYAL1: HPA002112 Atlas Antibodies, Stockholm, Sweden, dilution 1:100; and HYAL2: Ab68608 Abcam, Cambridge, UK, dilution 1:100). This incubation was followed by 1-h incubation with biotinylated anti-rabbit antibody (1:200 dilution in 1% powdered milk in PBS, Vector Laboratories, Burlingame, CA) at room temperature. Next, sections were washed with PBS, incubated with avidin-biotin peroxidase complexes (1:200, Vecta stain ABC Kit, Vector Laboratories, Burlingame, CA) for 1 h at room temperature (RT), and then washed again with PBS. The color was developed for 5 min with 0.05% diaminobenzidine (DAB; Sigma, St. Louis, MO) containing 0.03% H_2O_2. Next, the sections were washed with distilled water and counterstained with Mayer's hematoxylin for 1 min, washed, dehydrated, and mounted in DPX (BDH Laboratory Supplies, Poole, UK).

4.5.3 E-cadherin immunostaining (Study III)

Deparaffinized and rehydrated sections were heated in a microwave oven in EDTA buffer (pH 8.0) for 2 × 5 min, and then incubated in the EDTA buffer for 18 min and washed twice in PBS for 5 min. Endogenous peroxidase activity was blocked by incubating the sections with 5% H_2O_2 for 5 min and then washing the sections twice in water for 5 min and twice in PBS for 5 min. Non-specific binding was blocked by incubating the sections with 1.5% normal horse serum in PBS for 45 min. The sections were incubated overnight at 4°C with the primary antibody for E-cadherin (mouse monoclonal anti-human E-cadherin, clone HECD-1; Invitrogen, California, USA; 1:100 dilution). The negative control was incubated with 1% BSA in PBS without the primary antibody. Next, the sections were washed twice in PBS for 5 min and then incubated with the biotinylated secondary antibody (anti-mouse IgG; ABC Vectastain Elite kit, Vector Laboratories) for 45 min at RT. The sections were then washed twice in PBS for 5 min, incubated for 50 min in preformed avidin-biotinylated peroxidase complex (ABC Vectastain Elite kit, Vector Laboratories), washed, developed for color, counterstained, and mounted as described above.

4.5.4 Evaluation of HYAL1-2 and E-cadherin staining (Study III)

Two independent observers (TKN, RS) evaluated the sections for staining intensity and coverage in the epithelia and stroma. For the tissue microarray, triplicate cores were analyzed for each sample and median intensities and staining scores calculated. Specimens with less than two representative cores were excluded from the analysis. The stained portion of each section was estimated based on a five-level scoring system where 1 = less than 5% of positive cells, 2 = 6-25% of positive cells, 3 = 26-50% of

positive cells, 4 = 51-75% of positive cells, and 5 = 76-100% of positive cells. The intensity of the most prominently stained area was estimated based on a four-point scale of 0 to 3, where 0 = negative, 1 = weak, 2 = moderate, and 3 = strong (Siiskonen et al. 2013). In this study, negative and weak staining intensities were combined into one subgroup (score = 1). Epithelial expression scores (EESs) were calculated by multiplying the intensity score by the score corresponding to the proportion of positively stained cells. The final calculated EES ranged from 1 to 15. In the survival analysis, HYAL1 and HYAL2 expression levels were considered negative if EES ≤ 5 and positive if EES > 5.

4.6 STATISTICAL ANALYSES (STUDIES I-III)

4.6.1 Study I
Statistical analyses were carried out using SPSS 11.5 for Windows (SPSS, Chicago, IL). Differences between groups were analyzed by non-parametric Kruskal-Wallis test, followed by a non-parametric Mann-Whitney U-test for paired comparisons between the patient groups if the intial analysis was significant. Chi-square tests were used to evaluate HAS2 epithelial staining. Correlations between HAS3, HYAL1, and HYAL2 gene expression, hyaluronidase activity, hyaluronan, and HAS staining were analyzed by Spearman's correlation test. A p-value ≤ 0.05 was considered significant.

4.6.2 Study II
Statistical analyses were carried out using SPSS 16.0 for Windows (SPSS, Chicago, IL). Differences between the patient groups were analyzed by a non-parametric Kruskal-Wallis test, followed by a non-parametric Mann-Whitney U-test for further comparisons between the patient groups if the initial analysis was significant. Correlations between gene expression data, hyaluronan staining, and immunostaining scores were analyzed by Spearman's correlation test. Chi-square tests were used to analyze the association between hyaluronan staining and immunostaining scores. A p-value ≤ 0.01 was considered significant.

4.6.3 Study III
Statistical analyses were carried out using SPSS version 19 (SPSS, Chicago, IL). Associations between staining scores and clinicopathological parameters were evaluated using chi-square, Kruskal-Wallis, or Mann-Whitney U-tests. Correlations between E-cadherin, HYAL1, and HYAL2 EES values were determined using Spearman's rank correlation test. Recurrence-free survival was calculated from the date of diagnosis to the date of recurrence. Univariate analyses for overall survival were conducted using the Kaplan–Meier method, and the significance of differences between groups was assessed by the log-rank test. Multivariate analyses were conducted with the Cox regression model. A p-value < 0.05 was considered significant, except in pair-wise comparisons, where a p-value < 0.01 was preferred.

4.7 ETHICAL CONSIDERATIONS (STUDIES I-III)

The ethics committee of Kuopio University Hospital approved the study protocol, and all patients provided signed informed consent.

5 Results

5.1 HYALURONAN IN OVARIAN AND ENDOMETRIAL TUMORS (STUDIES I-II)

The hyaluronan content of benign ovarian cystadenomas was close to that of normal ovarian tissue. Malignant tumors had markedly increased levels (malignant tumors vs. other lesions, p = 0.00026) (Figure 3 and Study I Figure 1A)

The epithelial and stromal hyaluronan intensity scores were significantly elevated (p = 0.0001 and p = 0.006, respectively) in endometrioid endometrial tumors compared to normal endometrium (Figure 4, Study II Figure 3 G, H, and Study II Table 3).
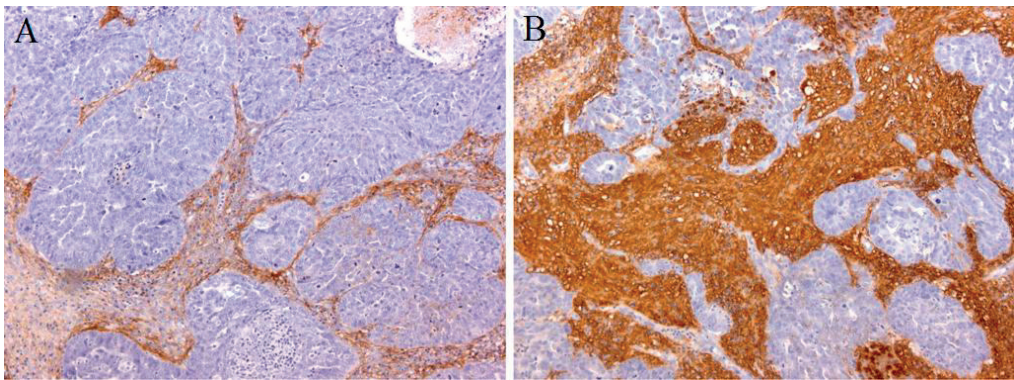
Figure 3. Hyaluronan accumulation in ovarian serous carcinoma. An example of the biological heterogeneity among tumors is demonstrated with a low stromal hyaluronan intensity in *a* and high stromal hyaluronan staining in *b*.
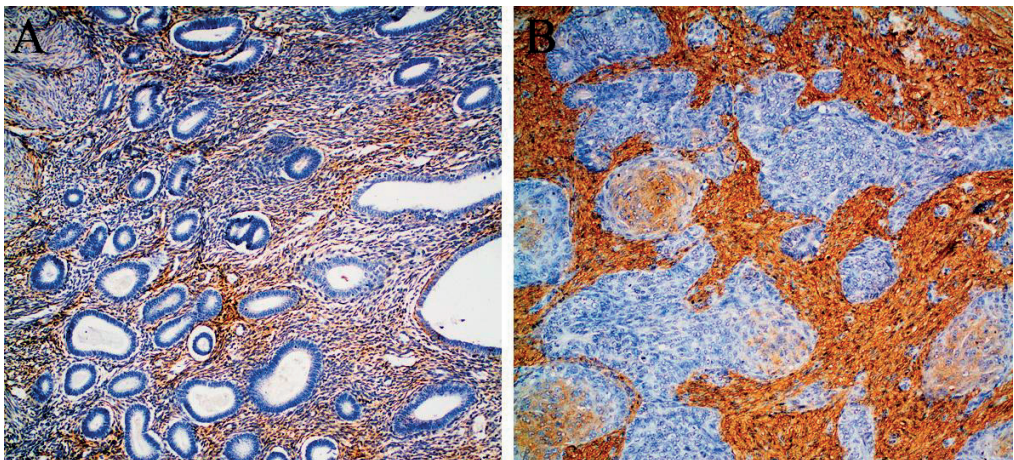
Figure 4. Hyaluronan accumulation in endometrial adenocarcinoma. Low tumoral hyaluronan staining is present in *a* and high hyaluronan staining in *b*.

5.2 EXPRESSION OF HYALURONAN SYNTHASES (STUDIES I-II)

5.2.1 Expression of *HAS1-3* mRNA

In both ovarian and endometrial tissue specimens, *HAS1* transcripts were detected at such a low level that reliable quantitation was not possible.

The expression of HAS2 was similar between benign and borderline ovarian tumors (Study I Figure 1B). The median for *HAS2* mRNA was 51–61% higher in malignant tumors compared to normal ovaries, but the variance between individual tumors was extensive (Study I Figure. 1B). Overall, *HAS2* mRNA expression was not significantly different between the groups (p = 0.387). *HAS3* expression was increased in benign tumors compared to normal ovaries (median +60%, p = 0.0039) and mRNA expression tended to be decreased in high grade (grade III) carcinomas (-44%) (Study I Figure 1C). No correlations were found between the levels of *HAS2* or *HAS3* mRNA and hyaluronan content in normal, benign, or borderline ovaries or serous ovarian carcinomas (Table 3).

HAS2 expression remained unaltered in postmenopausal or hyperplastic tissues of the endometria and malignant endometrioid adenocarcinomas compared to premenopausal endometria (Study II Figure 1A). *HAS3* expression was increased more than 4-fold in postmenopausal endometrium (p = 0.003) compared to premenopausal endometrium (Study II Figure 1B). Moreover, *HAS3* expression was elevated (1.5-fold increase) in grade I endometrioid adenocarcinomas compared to normal endometrium (p = 0.033) (Study II Figure 1B). No correlations were found between *HAS2* or *HAS3* mRNA levels and the hyaluronan content of normal or hyperplastic endometria and endometrioid adenocarcinoma (Table 3).

5.2.2 HAS1-3 immunostaining (Studies I-II)

No HAS1-positive cells were detected in the epithelia or stroma of normal ovaries, and staining remained very low in benign and borderline ovarian tumors. However, 41% of ovarian carcinomas exhibited a low level of epithelial HAS1-positivity (Study I Figure 3 and Table 2). The percentage of HAS1-positive cells did not correlate with the *HAS1* mRNA levels, hyaluronan content, histological types, or carcinoma grades (Table 3). The anti-HAS2-antibody resulted in more widespread staining and, in contrast to HAS1 and HAS3 antibodies, stained both epithelial and stromal cells (Study I Figure 3). All epithelial cells exhibited weak HAS2 staining in normal ovaries. The HAS2 signal in the epithelial cells of carcinomas was more variable. In addition to samples with weakly stained epithelial tissue similar to normal ovaries, 64% of tumor samples exhibited more intense epithelial HAS2 immunostaining. The highest HAS2 staining intensity was detected in benign tumors and grade I serous carcinomas, and the intensities were significantly different in the histological subgroups (p = 0.003). Of the stromal cells, 25–37% were HAS2-positive in both normal ovaries and tumor specimens, but the proportion of HAS2-positive stromal cells did not correlate with the *HAS2* mRNA level, hyaluronan staining intensity, or histological groups (Table 3 and Study I Table 2). No positive HAS3 signal was observed in normal

ovaries, but 46% of serous ovarian carcinomas presented with generally low numbers of HAS3-positive cancer epithelial cells (Study I Figure 3 and Table 2). An analysis including both normal and different tumor specimens indicated that the proportion (%) of HAS3-positive cells among all epithelial cells correlated with hyaluronan staining in the stroma (r = 0.424, p = 0.008). HAS3 immunostaining did not correlate with *HAS3* mRNA levels, histological type, or tumor grade (Table 3).

Table 3. Expression of hyaluronan synthases (HAS1-3) and their correlation to hyaluronan accumulation (I, II)

| | Hyaluronan synthase mRNA and protein* expression | | | | | | Hyaluronan (HA) staining | |
	HAS1		HAS2		HAS3			
Ovarian specimens (n = 39)								
Normal ovary	n.q.	○	○	○	○	○	○	No correlations between HAS1-3 mRNA and protein levels
Serous cystadenoma	n.q.	○ ***	○	↑	↑	○ ***	○	
Serous borderline tumor	n.q.	○ ***	○	○	○	○ ***	○	HAS3-positive epithelial cells correlate with strong stromal hyaluronan percentage (r = 0.424, p = 0.008)
Serous adenocarcinoma, grade I+II	n.q.	↑	○	↑	○	↑	↑	
Serous adenocarcinoma, grade III	n.q.	↑**	○	○	↓	↑**	↑	
Endometrial specimens (n = 35)								
Normal pre-menopausal endometrium	n.q.	○	○	○	○	○	○	No correlations between HAS1-3 mRNA and protein levels
Post-menopausal endometrium	n.q.	○	○	○	↑	○	○	
Complex atypical hyperplasia	n.q.	○	○	○	○	○	○	HAS2 epithelial staining intensity correlate with epithelial hyaluronan (p = 0.009)
Endometrioid adenocarcinoma (grade I)	n.q.	↑	○	↑	↑	↑	↑	
Endometrioid adenocarcinoma (grade II+III)	n.q.	↑	○	↑	○	↑	↑	

n.q. = not quantified, * HAS1-3 immunohistochemistry, ** low (≤ 10%) percentage of positive cells, *** very low (1-5%) percentage of

○ = No significant changes, ↑ = Significant increase, ↓ = Significant decrease

Positive HAS1 staining in the epithelium and stroma of normal endometrium was observed in a minority of cases and was completely absent in postmenopausal endometria. HAS1 staining was also generally negative or weak in atypical hyperplasia, but more consistent and significantly intense staining was noticed in endometrial adenocarcinomas compared to normal endometria (Study II Figure 3 A, B, and Study II Table 2) (p = 0.001). HAS1 immunostaining did not correlate with *HAS1* mRNA, tumor grade, or hyaluronan accumulation (Table 3). Epithelial and stromal HAS2 staining was negative or weak in premenopausal and postmenopausal endometria, and staining patterns were similar in hyperplastic endometria. However, epithelial HAS2 immunostaining was more intense in endometrioid adenocarcinomas compared to normal endometrium (Study II Figure 3 C, D and Study II Table 2) (p = 0.004), and a correlation was also found with epithelial hyaluronan staining (p = 0.009). Despite signs of a connection to hyaluronan accumulation, HAS2 did not correlate with *HAS2* mRNA levels or tumor grade (Table 3). HAS3 had a similar staining pattern as HAS1 and HAS2 in normal and hyperplastic endometria. The epithelial

intensity of HAS3 (Study II Figure 3 E, F) immunostaining was significantly stronger in endometrial adenocarcinomas compared to normal endometrium (p = 0.003) (Study II Table 2). The HAS3 staining intensities did not correlate with tumor grade, and no significant correlations were found between *HAS3* mRNA levels and hyaluronan accumulation (Table 3).

5.3 HYALURONIDASE ACTIVITY (STUDY I)

Hyaluronidase activity is present at pH 3.7 in ovarian tissues, with a tendency to decrease in malignant tumors (Hiltunen et al. 2002). The median hyaluronidase activity in borderline and malignant tumors was 58-40% lower than in normal ovary, but the difference did not reach significance (p = 0.076) (Study I Figure 2A). However, decreased hyaluronidase activity correlated inversely with hyaluronan accumulation (r = -0.5, p = 0.003) (Table 4).

5.4 HYALURONIDASE EXPRESSION (STUDIES I-II)

As two ubiquitous hyaluronidases, *HYAL1* and *HYAL2*, likely account for the hyaluronidase activity, we quantified mRNA levels by real-time RT-PCR. A gradual decline in *HYAL1* expression was measured in benign and borderline tumors compared to normal ovaries (Study I Figure 2B), with significant differences between the groups (p = 0.022). A pair-wise analysis indicated decreased *HYAL1* in all non-benign tumors compared to normal ovaries (borderline: -58% (median), p = 0.05; grades I+II: -79%, p = 0.05; grade III: -69%, p = 0.01). Malignant grade I+II and grade III tumors also expressed significantly less *HYAL1* than benign tumors (p = 0.034 and p = 0.028, respectively) (Table 4 and Study I Figure 2B). *HYAL2* mRNA expression was increased in benign ovarian cystadenomas compared to normal ovaries (+76%, p = 0.037), whereas a decrease was noted in grade III ovarian serous carcinomas (p = 0.0156) (Study I Figure 2C). *HYAL1* transcript levels correlated with hyaluronan content (r = -0.4; p = 0.025) and hyaluronidase activity (r = 0.5; p = 0.006), suggesting that *HYAL1* dominated the differences in hyaluronidase activity and contributed to the accumulation of hyaluronan in ovarian cancers (Table 4). Interestingly, *HYAL2* expression did not correlate with hyaluronidase activity, even though its mRNA level was two to three orders of magnitude higher than that of *HYAL1*, as suggested by real-time RT-PCR. A correlation with hyaluronan accumulation was not found with *HYAL2*.

Table 4. Expression of hyaluronidases (HYAL1 and HYAL2) and their correlation to hyaluronan accumulation (I, II)

	Hyaluronidase mRNA and protein* expression or activity				
	HYAL1	**HYAL2**	**Hyaluronidase activity**		**Hyaluronan (HA) staining**
Ovarian specimens (n = 39)					
Normal ovary	○	○	○	HYAL1 mRNA correlate with hyaluronidase activity (r = 0.5, p = 0.006)	○
Serous cystadenoma	○	↑	○		○
Serous borderline tumor	↓	○	↓		○
Serous adenocarcinoma, grade I+II	↓	○	↓		↑
Serous adenocarcinoma, grade III	↓	↓	↓		↑

HA staining (ovarian): HYAL1 mRNA and hyaluronidase activity inversely correlate with HA accumulation (r = -0.4, p = 0.025 and r = -0.5, p = 0.003 respectively)

	HYAL1 mRNA / protein		**HYAL2** mRNA / protein		**Hyaluronidase activity**	**Hyaluronan (HA) staining**
Endometrial specimens (n = 35)						
Normal pre-menopausal endometrium	○	○	○	○		○
Post-menopausal endometrium	○	○	○	○	HYAL1 mRNA correlate with HYAL1 protein levels (p = 0.001)	○
Complex atypical hyperplasia	○	○	○	○		○
Endometrioid adenocarcinoma (grade I)	↓	↓	↓	↓	HYAL2 mRNA correlate with HYAL2 protein levels (p = 0.045)	↑
Endometrioid adenocarcinoma (grade II+III)	↓	↓	↓	↓		↑

HA staining (endometrial): HYAL1 mRNA correlate inversely with epithelial (r = -0.6, p = 0.001) and stromal HA (r = -0.4, p = 0.01). HYAL1 protein correlate inversely to epithelial (p = 0.021) and stromal (p = 0.013) HA staining. HYAL2 mRNA and protein correlate inversely with epithelial HA staining (r = -0.4, p = 0.01 and p = 0.005)

* Epithelial staining intensity of HYAL1 or HYAL2 immunohistochemistry, ○ = No significant changes, ↑ = Significant increase
↓ = Significant decrease, ↓ = Borderline significant decrease

A significant decline in *HYAL1* expression was measured from normal endometrium to endometrioid adenocarcinoma (p = 0.002). A 10-fold higher expression of *HYAL1* mRNA was found in the normal endometrium compared to both grade I and grade II + III endometrial adenocarcinomas (p = 0.004 and p = 0.006, respectively). Values more than 15-fold higher were measured in normal post-menopausal endometrium (p = 0.002) (Study II Figure 2A). A similar trend to *HYAL1* was noted for *HYAL2* expression in grade I and grade II + III adenocarcinomas compared to normal endometrium (p = 0.020) (Study II Figure 2B). *HYAL1* transcript levels significantly inversely correlated with epithelial (r = -0.6, p = 0.001) and stromal hyaluronan staining (r = -0.4, p = 0.01). For endometrial material, we also analyzed HYAL1 and HYAL2 protein levels using immunohistochemistry. HYAL1 epithelial staining intensity correlated significantly with HYAL1 mRNA levels (p = 0.0009, n = 33) and inversely correlated with epithelial and stromal hyaluronan staining (p = 0.021 and p = 0.013, respectively) (unpublished result). *HYAL2* transcript levels correlated with epithelial hyaluronan staining (r = -0.4, p = 0.01). HYAL2 epithelial staining intensity correlated with *HYAL2* mRNA levels (p = 0.045, n = 33) and inversely correlated with epithelial hyaluronan staining (p = 0.005) (unpublished result).

A significant correlation was found between *HYAL1* and *HYAL2* mRNA levels in ovarian and endometrial tissue specimens (r = 0.5, p = 0.0013 and r = 0.8, p = 0.0001, respectively). The HAS3 epithelial staining intensity was also found to correlate inversely with HYAL1 mRNA in both ovarian and endometrial samples (r = -0.438, p = 0.005 and r = -0.5, p = 0.004, respectively).

5.5 HYAL1-2 IN ENDOMETRIUM AND ENDOMETRIAL TUMORS (STUDY III)

Because our previous results suggested that hyaluronan accumulation can be a result of decreased hyaluronan degradation, we analyzed HYAL1 and HYAL2 protein expression in a larger set of samples representing tissues from normal and precancerous endometria and endometrial carcinomas.

In premenopausal endometria, both HYAL1 and HYAL2 were predominantly expressed in the cytoplasm of glandular epithelial cells. Endometrial stromal cells were negative for HYAL1 and HYAL2, regardless of the phase of the endometrial cycle. This expression pattern was also evident in atrophic postmenopausal endometria and a similar distribution was observed in atypical complex hyperplasia and endometrial carcinomas, regardless of the carcinoma type or grade (Study III Table 2 and Figure 1). HYAL1 and HYAL2 localized mainly in the cytoplasm, but we also occasionally observed plasma membrane staining (Study III Figure 1).

The epithelial HYAL1 staining intensity was strong in the majority of premenopausal endometria (42/52, 80.8%), regardless of the phase of the endometrial cycle. Similarly, HYAL1 staining was strong in atrophic endometria (13/14, 92.9%) and atypical complex hyperplasia (21/26, 80.8%). In contrast, the intensity of HYAL1 staining and EES were reduced in the epithelia of endometrioid adenocarcinomas. Moreover, HYAL1 staining intensity was significantly negatively associated with the tumor grade ($p < 0.0001$). Thus, none of the poorly differentiated (grade III) endometrioid adenocarcinomas (0/33) exhibited strong HYAL1 staining, and in 21/33 (63.6%) cases it was negative or weak. In serous carcinomas, 9/12 (75%) cases exhibited a negative or weak HYAL1 staining intensity (Figure 5 and Study III Table 2).

In contrast to HYAL1 expression, premenopausal endometria exhibited strong HYAL2 intensity only in 5/52 (9.6%) samples. Furthermore, the expression of HYAL2 varied according to the phases of the endometrial cycle; the HYAL2 staining intensity was significantly stronger in the proliferative phase than in the secretory phase (Figure 5 and Study III Table 2). The HYAL2 staining intensity was not significantly different in normal endometria, complex atypical hyperplasia, or any grade of endometrioid adenocarcinoma. However, HYAL2 expression was significantly stronger in atrophic endometria than endometrioid endometrial carcinomas. In serous carcinoma, 4/11 (36.4%) cases had strong HYAL2 epithelial staining, which was significantly different from the proportion of strong staining found in normal endometria ($p = 0.0044$) (Figure 5 and Study III Table 2).
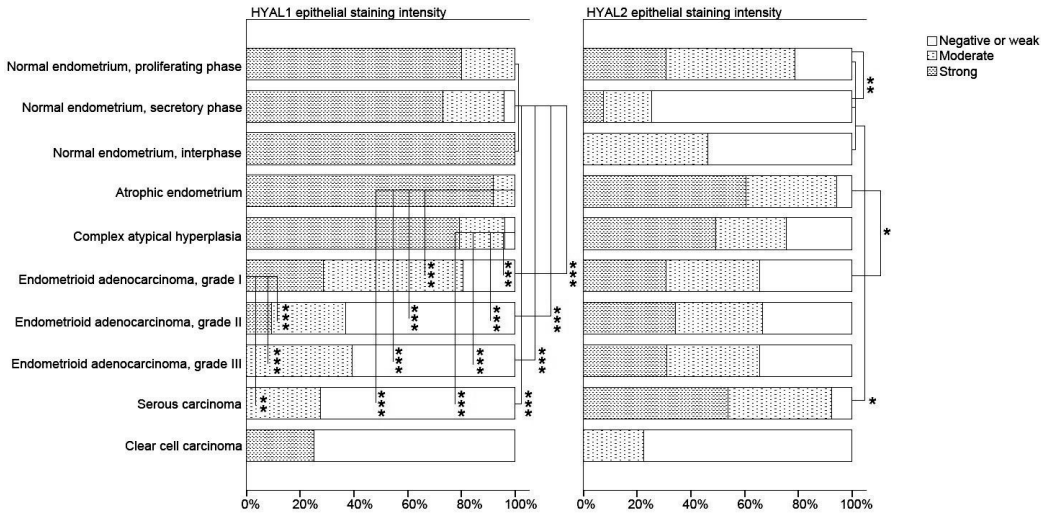
Figure 5. Distribution of different HYAL1 and HYAL2 epithelial staining intensities in the endometrium and its tumors (total n = 343). Significant differences between groups are indicated by asterisks: * p < 0.01, ** p < 0.001, *** p < 0.0001.

5.6 HYAL1-2 AND CLINICOPATHOLOGICAL FACTORS (STUDY III)

Reduced HYAL1 epithelial staining intensity and EES were significantly associated with large tumor size, lymphovascular invasion, and lymph node metastases in endometrial carcinoma (Study III Table 3). In particular, reduced HYAL1 expression strongly correlated with deep myometrial invasion (i.e., tumor invasion of one half or more of the myometrium) (p < 0.0001). In addition, HYAL1 staining intensity and EES were associated with the absolute depth of invasion. In tumors with negative or weak staining intensity, the median depth of invasion was 8.0 mm (range: 0-31 mm). Invasion was less profound for moderate or strong staining intensity [4.0 mm (range: 0-22 mm) and 1.5 mm (range: 0-15 mm), respectively; p < 0.0001]. In tumors with negative (EES ≤ 5) or positive (EES > 5) HYAL1 expression profiles, the absolute invasion depth was 7.5 mm (range: 0-31 mm) and 3.0 mm (range: 0-22 mm), respectively (p < 0.0001).

Weak HYAL2 staining intensity and EES were also associated with deep myometrial invasion, but not with the absolute depth of invasion or the other above-mentioned clinicopathological factors (Study III Table 3).

Follow-up data were available from 173 patients in this study. The patients were followed up for a maximum of 48 months (median 37 months, range: 16-48 months). Recurrence occurred in 13/173 (7.5%) patients, and the median disease-free survival

was 17 months (range: 5-23 months). In univariate analysis, the HYAL1 epithelial staining intensity and HYAL1 expression were associated with disease-free survival (Study III Figure 2). Because no recurrent events were observed in the group with strong epithelial staining, we subjected the EES to multivariate analysis. In the multivariate Cox regression model, age, lymphovascular invasion, myometrial invasion, cervical invasion, and tumor grade (low-grade vs. high-grade) were included as variables. We found that negative HYAL1 expression was an independent prognostic factor for early disease recurrence (HR 5.13, 95% CI 1.131 – 23.270, p = 0.034).

5.7 E-CADHERIN AND HYAL1 EXPRESSION (STUDY III)

Loss of E-cadherin expression is a key event in EMT. Therefore, we used E-cadherin as an indicator of EMT and analyzed its expression in endometrial cancer samples to determine whether it is associated with the previously reported reduction in HYAL1 expression.

Reduced E-cadherin expression was associated with high-grade endometrioid adenocarcinomas (Study III Table 4). The E-cadherin EES also exhibited a strong association with deep myometrial invasion, lymphovascular invasion, cervical invasion, and tumor size in endometrial carcinoma (Study III Table 3). We noted a significant positive correlation between HYAL1 and E-cadherin epithelial staining intensity (p<0.0001). Thus, in 21/25 (84%) cases with negative or weak E-cadherin epithelial staining intensity, the HYAL1 intensity was also negative or weak; conversely, in 32/34 (94.1%) cases with strong HYAL1 intensity, E-cadherin also had a strong pattern of epithelial staining. A significant correlation was also found between the EES for E-cadherin and HYAL1 (r = 0.5, p < 0.0001).

6 Discussion

6.1 ROLE OF HYALURONAN SYNTHASES IN HYALURONAN ACCUMULATION

6.1.1 HAS1-3 in serous ovarian carcinoma (Study I)

Little *HAS1* mRNA and no consistent upregulation of *HAS2* were observed in the cancers, and the median levels of *HAS3* mRNA were actually lower in cancers than controls. Immunohistochemical staining of HAS proteins revealed a low level of HAS1, a slightly elevated level of HAS3 in the tumor epithelia, and a variable elevation of HAS2 immunostaining in tumor epithelial cells, which is in agreement with the mRNA assays. In stromal cells, no difference was observed with the HAS2 immunoreactivity in normal ovaries and malignant tumors, an unexpected result considering the strong accumulation of hyaluronan in serous adenocarcinomas.

As *HAS2* and *HAS3* did not exhibit a consistent increase in their expression in serous ovarian cancers and *HAS1* mRNA was virtually absent, changes in the transcriptional activity of the HAS genes seemed to not be the main factor in the increased hyaluronan content of these tumors. A few of the serous adenocarcinoma specimens had high *HAS2* expression levels, but most of the cancers had no elevation in the expression of any of HAS genes.

Immunohistochemical staining confirmed that the HAS1 and HAS3 levels were relatively low in ovarian cancers, whereas the signal for HAS2 was more widespread, which is in line with the real-time RT-PCR analysis. Though stromal cells were positive for HAS2, the staining intensity did not correlate with that of stromal hyaluronan or the tumor type or grade. Unexpectedly, the HAS2 antibody stained normal, benign, and malignant ovarian epithelial cells, all of which were largely negative when using the hyaluronan probe. Taken together, these findings suggest that the epithelium somehow contributes to stromal hyaluronan. Theoretically, this would be possible if the epithelial cells were unable to hold and take up the synthesized hyaluronan via a receptor like CD44. In support of this idea, the expression of CD44 was reported to be reduced in high-grade ovarian cancers (Sillanpaa et al. 2003), and the released hyaluronan can be trapped in the stroma by complexing with versican (Voutilainen et al. 2003). Even if the epithelial HAS contributes to stromal hyaluronan, it would not explain the hyaluronan accumulation in high-grade tumors because the epithelial HAS2 staining intensity was highest in benign and well-differentiated tumors.

Taken together, the data suggest that, although a high HAS2/HAS3 level may contribute to hyaluronan accumulation in some ovarian tumors, stromal hyaluronan accumulation is not explained by the increased expression of any of the HASs in the majority of cases, particularly high-grade tumors.

6.1.2 HAS1-3 in endometrial adenocarcinoma (Study II)

Except for a trend of increased *HAS3* expression in grade 1 carcinomas, we did not find a clear pattern of increased *HAS1-3* mRNA in endometrial cancer compared to normal endometrium. This finding is somewhat similar to the enhanced *HAS3* mRNA level observed in benign, but not malignant, ovarian tumors in study I. Our results suggest that transcriptional upregulation of *HAS* expression is not the main contributor to the increased hyaluronan content in these tumors.

Despite minor changes in mRNA levels, the immunoreactivity for all HASs was stronger in cancer cells than normal endometrium, although the density of staining did not significantly correlate with tumor grade. A similar discordance between the levels of *HAS* mRNA and HAS immunoreactivity was found in ovarian cancer in study I. Interestingly, the significance of the cancer-associated increase in HAS immunoreactivity was strongest for HAS1, though real-time RT-PCR suggested very low transcription of this gene. Similar to the present study, Yabushita et al. (Yabushita et al. 2005) found that HAS1 immunoreactivity is strongly associated with endometrial cancer. Moreover, among all HASs, HAS1 had the strongest prognostic power for short survival in breast cancer (Auvinen et al. 2014).

HAS2 is the only HAS gene in which deletion causes a clear (lethal) phenotype (Camenisch et al. 2000), and it has been suggested to be the most important gene for hyaluronan synthesis, at least in fibroblasts (Kobayashi et al. 2010). In line with this idea, the immunohistochemical signal of epithelial HAS2 correlated with the epithelial hyaluronan staining score in the endometrium.

Because the expression of HAS genes in tumor tissues poorly correlated with the levels of the respective HAS proteins, the turnover of HAS proteins must be slower in cancer cells. One explanation for this is the availability of hyaluronan precursor sugar, UDP-ClcNAc. After glucosamine treatments, UDP-ClcNAc increases, HAS2 is O-GlcNacylated, and its activity is increased in isolated membranes and cell cultures (Vigetti, Passi 2014, Vigetti et al. 2012). Interestingly, cellular UDP-GlcNAc content controls *HAS2* mRNA expression. High cellular UDP-GlcNAc decreases HAS2 expression in keratinocytes, and a low cellular UDP-GlcNAc concentration increases its expression (Jokela et al. 2011). Recent results have shown that UDP-GlcNAc concentrations in breast and ovarian cancer tissues are elevated over 10-fold compared to normal tissues (Tammi M, personal communication). The duration of the functional work life of HAS proteins in ovarian and endometrial carcinomas is not known, but if extended, it may have a major influence on hyaluronan synthesis.

6.2 HYALURONIDASES IN HYALURONAN ACCMULATION AND CANCER

6.2.1 Altered HYAL 1-2 expression (Studies I-III)

In study I, *HYAL1* mRNA expression was consistently decreased in serous ovarian carcinoma specimens with a concomitant trend of reduced hyaluronidase enzyme activity and an inverse correlation to hyaluronan accumulation. In terms of the cellular mRNA content, the dominant hyaluronidase in these tissues was HYAL2. However, only *HYAL1* mRNA correlated with the measured hyaluronidase activity, and it inversely correlated with hyaluronan accumulation, suggesting higher enzymatic activity of HYAL1 and more importance in hyaluronan catabolism. There was also a trend of low *HYAL2* expression in the most aggressive grade 3 tumors, similar to diffuse large B-cell lymphomas (DLBCLs) (Bertrand et al. 2005). As in ovarian cancer, hyaluronidase activity in DLBCL tissue extracts was found to not correlate with *HYAL2* expression. In line with this, the ability of HYAL2 to degrade hyaluronan is less than that of HYAL1 (Vigdorovich, Miller & Strong 2007).

As in serous ovarian carcinomas, we found similar but more pronounced signs of *HYAL1* mRNA downregulation in endometrial endometrioid carcinoma in study II. We found a more than 10-fold decrease in *HYAL1* mRNA in endometrioid adenocarcinomas compared to normal endometria, and the expression inversely correlated with hyaluronan accumulation. *HYAL2* mRNA expression also declined in cancerous tissues, and there was a strong correlation between *HYAL1* and *HYAL2* mRNA levels. The expression of *HYAL1* and *HYAL2* also correlated with the protein levels determined by immunohistochemistry, suggesting that their expression is transcriptionally regulated.

Our results suggested that hyaluronan accumulation in endometrial endometrioid adenocarcinomas results from decreased degradation of hyaluronan; thus, we further analyzed HYAL1 and HYAL2 protein expression in a large sample representing cases from normal, precancerous, and cancerous endometria in study III. This study showed that HYAL2 expression was altered in healthy endometria during the phases of the menstrual cycle, whereas HYAL1 expression remained strong and constant. Our finding that HYAL2 expression was strong in the proliferative phase and declined in the secretory phase was consistent with a previous report describing increased hyaluronan content during the secretory phase (Afify et al. 2005). The data suggest that HYAL2 enzymatic activity regulates endometrial hyaluronan content during the menstrual cycle.

In endometrial adenocarcinomas, HYAL2 expression generally remained stable, and we found no signs of altered expression compared to normal endometrium or precancerous hyperplastic lesions. However, in serous endometrial cancer, HYAL2 expression was increased compared to normal endometrium. In addition, atrophic endometrium had a stronger HYAL2 staining pattern than normal samples. Because endometrial intraepithelial carcinoma typically arises from a background of atrophic endometrium (Acharya et al. 2005), this raises the question of whether HYAL2 plays

a role in malignant progression from atrophic endometrium, which is to be clarified in future studies.

The fact that HYAL1 staining was generally strong in normal, atrophic, and hyperplastic endometria but reduced in all types of cancers (endometrioid, serous, and clear cell) suggested that HYAL1 can significantly contribute to the progression of malignancy. This hypothesis was further supported by HYAL1 levels significantly decreasing as the tumor grade increases from I to III.

The exact mechanism of decreased HYAL1 and HYAL2 expression is not completely understood, but genomic alterations, transcriptional regulation, or epigenetic regulation may play a crucial role. Allelic imbalance and deletions are frequent in the tumor suppressor gene region of 3p21.3 containing *HYAL1* and *HYAL2*, suggesting that this site is important in ovarian (Tuhkanen et al. 2004) and other cancers (Csoka, Frost & Stern 2001, Stern 2008b). The positive correlation that existed between the expression of *HYAL1* and *HYAL2* may be explained by concomitant deletion of these closely mapped genes. Epigenetic regulation of this tumor suppressor gene cluster flanking RASSF1 has been studied in epithelial breast cancer cell lines; after demethylation treatment, *HYAL1* was significantly overexpressed, demonstrating that epigenetic repression is involved in the downregulation of its expression (da Costa Prando, Cavalli & Rainho 2011). Interestingly, hypermethylation of RASSF1 and its promoter element has also been shown in endometrial cancer (Visnovsky et al. 2013, Fiolka et al. 2013) and is associated with aggressive disease. The methylation status of HYAL1 has not been examined in endometrial cancer.

6.2.2 Hyaluronidase and clinicopathological factors

In study III, reduced HYAL1 expression in endometrial cancer was associated with lymphovascular invasion, large tumor size, lymph node metastasis, and deep myometrial invasion, all of which are properties of a more aggressive phenotype of cancer. Furthermore, as a possible consequence of this aggressive phenotype, decreased HYAL1 expression is associated with early disease recurrence in endometrial carcinoma.

The present findings of reduced *HYAL1* expression are consistent with findings in cancers of the lung (Anedchenko et al. 2008, Wang et al. 2008) and kidney (Chi et al. 2012, Wang et al. 2008). Interestingly, Yoffou et al. (2011) showed subtype-specific overexpression of *HYAL1* mRNA in ovarian carcinomas representing clear cell or mucinous histology. However, in line with our findings, HYAL1 expression was more likely to decline in serous carcinomas, but the decrease was not significant (Yoffou et al. 2011). Also in line with our findings are results in pancreatic ductal adenocarcinoma; weak HYAL1 expression was an independent factor of survival together with hyaluronan accumulation (Cheng et al. 2013). *In silico* mRNA studies have also revealed that decreased HYAL1 expression in breast cancer is associated with distant metastasis-free survival (Heldin et al. 2013).

In a strong contrast to our results, increased *HYAL1* expression in poorly differentiated prostate and bladder tumors is associated with advanced disease and unfavorable prognosis (Lokeshwar et al. 2005, Kramer et al. 2010). Malignancies arising from different cell types appear to utilize distinct strategies to survive and progress. Increased hyaluronan may contribute to tumor growth and invasiveness by providing an expanded, loose matrix for cancer cells, protecting the tumor from immune reactions and apoptosis, stimulating tumor cell migration, and increasing cell proliferation (Tammi et al. 2008). The relative importance of the opposite roles of hyaluronidase function in a particular type of cancer may determine the outcome. The exact expression level is also important; transfection of *HYAL1* can either promote or suppress malignant growth in a single cell type depending on the resulting enzyme activity (Lokeshwar et al. 2005).

6.2.3 Decreased HYAL1 expression and invasion

In study III, decreased HYAL1 expression was associated with deep and absolute myometrial invasion in endometrial carcinoma. Invasion over half of the myometrium is a well-known predictor of recurrence and usually a marker for adjuvant treatments. Recently, the absolute depth of invasion was suggested to have better predictive value for aggressive disease. An optimal cut of 4 mm has been presented (Geels et al. 2013). As our results show, endometrial tumors with strong HYAL1 staining have a median depth of invasion of only 1.5 mm.

As decreased HYAL1 expression was associated with invasive features in endometrial carcinoma, we further elucidated its role in EMT, a process that initiates invasion and metastasis in cancer. Because decreased E-cadherin expression is the key event in EMT, we analyzed and correlated its expression levels with HYAL1. Decreased E-cadherin was associated with tumor grade, and we found a strong significant association with deep myometrial invasion, lymphovascular invasion, cervical invasion, and tumor size. Our findings are quite similar to previous reports of E-cadherin and endometrial cancer (Holcomb et al. 2002, Moreno-Bueno et al. 2003, Sakuragi et al. 1994). In line with our hypothesis, decreased E-cadherin was associated with decreased HYAL1 expression in study III. One explanation for this could be the accumulation of hyaluronan due to decreased HYAL1 expression. Increased hyaluronan content can induce EMT in normal epithelial cells (Zoltan-Jones et al. 2003). Recently, the accumulation of hyaluronan in pancreatic cells was associated with a loss of E-cadherin. Interestingly, pegylated human recombinant hyaluronidase (PEGPH20) inhibits these changes (Kultti et al. 2014).

6.2.4 Hyaluronan degradation products

Because our results suggest that HYAL1 expression is decreased in endometrial carcinomas, the accumulating hyaluronan in tumor stroma may predominantly be HMW-HA, or in some cases intermediate-sized hyaluronan cleaved by HYAL2, due to impaired function of HYAL1 protein. We can also hypothesize that the amount of

small hyaluronan oligosaccharides is relatively low in carcinomas with decreased HYAL1.

HMW-HA accumulation in tumor stroma can cause dramatic changes in the cancer cell microenvironment purely by its chemical properties. HMW-HA has the capacity to bind a large amount of water and form viscous gels at relatively low concentrations. In more concentrated solutions, hyaluronan molecules can form a continuous but porous meshwork that can act as a filter, facilitating the diffusion of small molecules and excluding large molecules (Stern 2008b). This hyaluronan barrier can decrease the uptake of chemotherapeutic drugs and be one cause of chemoresistance in cancer. Baumgartner et al. (Baumgartner et al. 1998) first showed that hyaluronidase treatments can enhance the actions of various chemotherapeutic agents, especially when used locally. Hyaluronan has been shown to be the primary matrix determinant for these barriers, forming desmoplastic reactions and causing high interstitial fluid pressures in the tumor microenvironment (Provenzano et al. 2012). Using synthetic hyaluronidase (PEGPH20), this stromal hyaluronan was degraded, causing normalization of tumor IFP and re-expansion of the microvasculature (Provenzano et al. 2012). The same results were achieved in another study in which an animal model of HMW-HA accumulation in tumor stroma in pancreatic cancer was degraded by PEGPH20, improving vascular patency and increased chemotherapeutic delivery. In the same study, combination therapy with PEGPH20 and gemcitabine inhibited tumor growth and significantly extended survival (Jacobetz et al. 2013). However, there is more than one hyaluronan "barrier"; CD44 signaling promotes drug resistance, and disruption of this endogenous hyaluronan-induced signaling suppresses cell resistance to chemotherapeutics (Misra et al. 2003).

sHA is known to promote angiogenesis, which is a key component of tumor growth and progression (Toole 2004). However, administration of sHA to several types of tumor xenografts inhibited rather than stimulated tumor growth (Ghatak, Misra & Toole 2002, Zeng et al. 1998). Chemokine CXCL12 and its receptor CXCR4 are known to promote tumor growth and stimulate angiogenesis (Kryczek et al. 2007). Interestingly, HMW-HA and sHA were recently shown to have opposing effects on CXCR-induced signaling (Fuchs et al. 2013). In particular, HMW-HA promotes CXCL12-induced CXCR4 activation, whereas sHA inhibits it. This significant finding supports our results and suggests that carcinomas that accumulate HMW-HA, possibly due to decreased HYAL1, induce CXCR4 signaling, promoting tumor cell dissemination, angiogenesis, and potentially the growth of tumors and their metastatic spread.

The exact size or molecular weight of hyaluronan that accumulates in cancer is difficult to determine. The measurable sHA concentration can be low, as it is rapidly "thrown" to the systemic circulation or is locally endocytosed. Histologically detectable hyaluronan may be HMW-HA, as small fragments escape fixation and the affinity of the hyaluronan probe is weaker towards hyaluronan fragments (Yuan et al. 2013). Schmaus et. al. (Schmaus, Sleeman 2015) first hypothesized that accumulation of sHA accounts for the association between HYAL1 expression and metastasis, and

their study showed that HYAL-1 expression promotes lung metastasis in mouse tumor models, but without sHA accumulation.

Despite the downregulation of *HYAL1*, we can assume that some hyaluronidase function is left to produce low concentrations of sHA. Low concentrations of sHA can increase endothelial cell proliferation, but high levels of sHA can be antiproliferative (Gao et al. 2010, Lokeshwar, Selzer 2000, Cui et al. 2009). Intermediate-sized hyaluronan can also induce cytokine expression to provide stimulating growth signals to tumor cells (de la Motte et al. 2009). Concerning our results, this could occur in serous endometrial carcinomas, where the concentrations of intermediate-sized hyaluronan can be even higher due to HYAL2 upregulation.

It would be interesting to know the consequences that restoration of HYAL1 function could have on HYAL1-depleted carcinomas. Increasing sHA concentration could slow down proliferation and further reduce tumor growth as shown in colon carcinoma (Alaniz et al. 2009). Increased sHA could also silence CXCR signaling (Fuchs et al. 2013). Suppression of cancer progression could be achieved by interrupting endogenous HA-CD44 interactions via sHA as shown in bone metastasis of breast cancer (Urakawa et al. 2012). Furthermore, the HMW-HA that occupies cell surface CD44 receptors may maintain p-Akt- and PI3K-dependent signals that prevent cancer cell apoptosis, returning hyaluronidase function, and the sHA created by hyaluronidase could block these cell survival signals (Ghatak, Misra & Toole 2002).

7 Summary and Conclusions

The main findings of this thesis are that:

- Hyaluronan synthases (HAS1-3) are associated with ovarian and endometrial tumors without a correlation with their mRNA levels.

- *HYAL1* mRNA levels are downregulated in serous ovarian carcinoma and associated with decreased hyaluronidase activity. Hyaluronan accumulation is related to decreased expression of *HYAL1* but not to the increased expression of hyaluronan synthases (*HAS1-3*).

- *HYAL1* and *HYAL2* mRNA and protein levels evaluated by immunohistochemistry are downregulated in endometrioid endometrial carcinoma and inversely correlate with hyaluronan accumulation.

- Decreased HYAL1 expression is associated with aggressive features of endometrial carcinoma and is an independent prognostic marker of early disease recurrence.

- Decreased HYAL1 in endometrial cancer may be associated with epithelial-mesenchymal transition, as its expression correlates with decreased E-cadherin expression.

This thesis provides new knowledge about genes affecting hyaluronan metabolism and their role in hyaluronan accumulation. We initially identified decreased hyaluronidase expression as a possible mechanism or cause of increased hyaluronan in ovarian and endometrial cancer, and further elucidated the clinical consequences. This thesis opens new directions for research concerning hyaluronan metabolism in diagnosis and treatment. In the future, we may study the use of HYAL1 as a marker in endometrial biopsies to aid in clinical decision making for adjuvant treatments. This thesis also encourages the investigation of recombinant hyaluronidase in hyaluronidase-depleted cancers to answer questions such as: Is there a way to manipulate hyaluronan metabolism to stop or prevent cancer invasion and keep the tumor localized for surgical therapies and better prognosis?

8 References

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Expression of Hyaluronan Synthases and Hyaluronidases in Gynecological Malignancies

Hyaluronan is an abundant high-molecular weight polysaccharide in the extracellular matrix. Its accumulation is observed in several types of malignancies. In this study, the mechanism of hyaluronan accumulation was clarified by analyzing the expression of hyaluronan synthesis (HAS1-3) and degradation (HYAL1-2) enzymes in normal, precancerous and cancerous states of human ovaries and endometria. Decreased HYAL1 levels were associated with increased tumoral hyaluronan content in ovarian and endometrial carcinomas. Furthermore the decreased expression of HYAL1 was associated with more aggressive type of endometrial carcinoma and predicted early disease recurrence.

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