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THE ROLE OF EXTRACELLULAR MATRIX MACROMOLECULES IN CANCER AND DIABETIC MACROANGIOPATHY

- WITH SPECIAL REFERENCE TO DECORIN AND HYALURONAN

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To My Friends and Family

ABSTRACT

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The role of extracellular matrix macromolecules in cancer and diabetic macroangiopathy – with special reference to decorin and hyaluronan

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The central role of extracellular matrix (ECM) macromolecules in diseases such as cancer and atherosclerotic vascular diseases including diabetic macroangiopathy is indisputable. Decorin and hyaluronan (HA) represent vital ECM macromolecules in the microenvironment of cells and are centrally involved in human cancer and cardiovascular biology. In cancer, decorin is considered to play a tumor suppressive role. However, there is some discrepancy whether malignant cells express it. Regarding HA, its contribution to the development of atherosclerotic vascular diseases has been well established. Nevertheless, the precise role of HA in arterial narrowing associated with diabetes is not known.

The present study focused on two vital ECM macromolecules, namely decorin and HA. First, decorin expression was studied in human tumorigenesis. Furthermore, the effect of adenovirus-mediated decorin transduction on selected cancer cell lines was investigated. The results invariably showed that cancer cells completely lacked decorin expression. The study also demonstrated that transducing cancer cells with decorin adenoviral vector markedly inhibited their malignant behavior. In line with this, a strong induction of decorin expression in normal human embryonic stem cells (hESCs), but not in abnormal hESCs was observed during their differentiation. Secondly, the significance of HA in the development of diabetic macroangiopathy in response to hyperglycemia was evaluated. Results showed that the synthesis of HA by vascular smooth muscle cells was significantly increased in response to high glucose concentration. This increase was associated with the diminished ability of the cells to contract collagen-rich matrix suggesting that HA participates in the disturbed vascular remodeling of diabetic patients. The results of this study support endeavours to develop novel ECM macromolecule -based therapies targeting cancer and cardiovascular diseases.

Key words: Extracellular matrix, decorin, hyaluronan, cancer, diabetic macroangiopathy

TIIVISTELMÄ

Annele Sainio

Soluväliaineen makromolekyylit syövässä ja diabeettisessa makroangiopatiassa – erityiskohteina dekoriini ja hyaluronaani

Turun yliopisto, lääketieteellinen tiedekunta, biolääketieteen laitos,
lääketieteellinen biokemia ja genetiikka, sekä
Turun yliopiston molekyylilääketieteen tohtoriohjelma (TuDMM)

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Soluväliaineen makromolekyylien keskeinen merkitys erilaisissa sairauksissa kuten syövässä ja ateroskleroottisissa verisuonisairauksissa mukaan lukien diabeettinen makroangiopatia on kiistaton. Dekoriini ja hyaluronaani (HA) ovat kaksi keskeistä solujen mikroympäristön makromolekyyliä, joilla on merkittävä tehtävä ihmisen syöpä- ja kardiovaskulaariobiologiassa. Syövässä dekoriinilla ajatellaan olevan sen kasvua estävä vaikutus. On kuitenkin kiistanalaista, ilmentävätkö malignit solut dekoriinia. Toisaalta HA:n merkitys ateroskleroottisen verisuonitaudin kehittymisessä on ilmeinen tosiasia, mutta sen merkitys ja toiminta diabetekseen liittyvässä valtimoiden ahtautumisessa on jäänyt epäselväksi.

Tämän tutkimus keskittyi kahteen keskeiseen sidekudoksen makromolekyyliin, dekoriiniin ja HA:han. Ensinnäkin tutkittiin dekoriinin ilmentymistä ihmisen tuumorigeneesin aikana. Lisäksi testattiin dekoriinin adenovirusvälitteisen siirrostamisen vaikutusta valikoiduissa syöpäsolulinjoissa. Tulokset osoittivat kiistatta, etteivät syöpäsolut ilmennä dekoriinia lainkaan. Tutkimus myös paljasti, että kun syöpäsoluihin siirrostettiin dekoriinin sisältävä adenovirusvektori, solujen maligni käyttäytyminen estyi merkittävästi. Lisäksi havaittiin, että normaalit ihmisen alkiokantasolut alkavat ilmentää dekoriinia erilaistumisensa alkuvaiheessa. Samanlaista dekoriinin ilmentymistä ei havaittu epänormaalien kantasolujen erilaistuessa. HA:n merkitystä arvioitiin hyperglykemian aiheuttaman diabeettisen makroangiopatian kehittymisessä. Tulokset osoittivat, että HA:n synteesi verisuonten sileissä lihassoluissa lisääntyi huomattavasti korkeassa glukoosipitoisuudessa. Tämä johti solujen heikentyneeseen kykyyn kontraktoida soluväliainetta. Nämä tulokset viittaavat siihen, että HA:lla on tärkeä asema sokeritautia sairastavien potilaiden verisuonten seinämän paksuuntumisessa. Tulosten perusteella soluväliaineen molekyylien ilmentymisen säätelyyn pohjautuvat hoidot syövän ja ateroskleroosin estossa ovat kehittämisen arvoisia.

Avainsanat: Soluväliaine, dekoriini, hyaluronaani, syöpä, diabeettinen makroangiopatia

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ABBREVIATIONS

CD44	cluster of differentiation 44
CKI	cyclin-dependent kinase inhibitor
CkPan	broad spectrum CK antibody
CS	chondroitin sulfate
DCIS	ductal carcinoma in situ
DM	diabetes mellitus
DS	dermatan sulfate
EC	endothelial cell
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
FGFR	fibroblast growth factor receptor
GAG	glycosaminoglycan
GCT	germ cell tumor
GlcNac	N-acetylglucosamine
GlcUA	glucuronic acid
HA	hyaluronan
HABPs	hyaluronan-binding proteins
HAS1-3	hyaluronan synthases 1-3
hESC	human embryonic stem cell
HGF	hepatocyte growth factor
HMW HA	high molecular weight HA
HS	heparan sulfate
(HU)VSMC	(human) vascular smooth muscle cell
HYAL	hyaluronidase
IDC	invasive ductal carcinoma
IGF-I	insulin-like growth factor I
IL	interleukin
KS	keratan sulfate
LRR	leucine-rich repeat
Met	hepatocyte growth factor receptor
MMP	matrix metalloproteinase

Pan-RTK	multi-receptor tyrosine kinase
PDGF	platelet derived growth factor
PEG 3	paternally expressed gene 3
PG	proteoglycan
PLAP	placental alkaline phosphatase
ROS	reactive oxygen species
SLRP	small leucine-rich proteoglycan
SMC	smooth muscle cell
SPARC	secreted protein, acidic and rich in cysteine
TGCT	testicular germ cell tumor
TGF-β	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinase
TLR	toll-like receptor
TME	tumor microenvironment
TNF-α	tumor necrosis factor alfa
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred in the text by their Roman numerals I-V:

I Salomäki HH*, Sainio AO*, Söderström M, Pakkanen S, Laine J, Järveläinen, HT: Differential expression of decorin by human malignant and benign vascular tumors. J Histochem Cytochem 2008; 56:639-646. *Equal contribution.

II Boström P*, Sainio A*, Kakko T, Savontaus M, Söderström M, Järveläinen H: Localization of decorin gene expression in normal human breast tissue and in benign and malignant tumors of the human breast. Histochem Cell Biol 2013; 139:161-171. *Equal contribution.

III Sainio A*, Nyman M*, Lund R, Vuorikoski S, Boström P, Laato M, Boström PJ, Järveläinen H: Lack of decorin expression by human bladder cancer cells offers new tools in the therapy of urothelial malignancies. PlosOne 2013; 8:e76190. * Equal contribution.

IV Sainio A, Pennanen M, Söderström M, Laine J, Lund R, Järveläinen H: Expression of small leucine-rich proteoglycans in human embryonic stem cells *in vitro* and embryonic tumors *in vivo*, with special reference to decorin. Manuscript.

V Sainio A, Jokela T, Tammi MI, Järveläinen H: Hyperglycemic conditions modulate connective tissue reorganization by human vascular smooth muscle cells through stimulation of hyaluronan synthesis. Glycobiology 2010; 20:1117-1126.

1. INTRODUCTION

The extracellular matrix (ECM) is a dynamic and heterogeneous tissue-specific scaffold of molecular and cellular constituents. Indeed, each tissue has its own unique ECM composition with a complex 3-dimensional network, which is constantly being remodeled by enzymatic and non-enzymatic modifications resulting in versatile microenvironments. This in turn regulates the behavior of the cells. The main function of the ECM is to maintain homeostasis and normal architecture of each particular tissue. However, this homeostasis is broken virtually in every disease and subsequently quantitative and/or qualitative changes can be found in the ECM.

The ECM macromolecules can be divided into collagens, elastin and microfibrillar proteins, non-collagenous matrix glycoproteins, proteoglycans (PGs) and hyaluronan (HA). PGs comprise a versatile group of molecules which have common structure of glycosaminoglycan chain/s covalently bound to a specific core protein. HA is an exception. It has no core protein but exists as a free form.

Decorin is a multifaceted regulatory PG belonging to a group of small leucine-rich PGs. It has a single chondroitin sulfate or dermatan sulfate glycosaminoglycan (GAG) side chain attached to its core protein. The function of decorin has been indicated in numerous physiological and pathological processes where it is able to directly or indirectly regulate the activity and function of other ECM macromolecules, growth factors and cytokines. Decorin has also been shown to bind to specific receptors like epidermal growth factor receptor (EGFR) and insulin-like growth factor I receptor (IGF-IR). As a result of these functions, decorin has been shown to be involved in different physiological processes like angiogenesis and cellular differentiation. Thus, it can act as a regulator e.g. in tumorigenesis.

HA represents the only GAG without a core protein. It can be polymerized by three hyaluronan synthase isoenzymes (HAS1, HAS2, and HAS3) as a linear polysaccharide of repeating alternating units of glucuronic acid and N-acetylglucosamine. Despite of HA´s simple structure, it has been shown to be involved in a diverse set of biological functions via complex interactions with other ECM macromolecules as well as with the ECM resident cells. Due to its unique hydrophilic properties, HA is a vital molecule in the maintenance of tissue homeostasis and structure. Like decorin, HA is able to modulate tissue remodeling, angiogenesis, and it can act as a pro- and / or anti-angiogenic and –tumorigenic molecule. It has been also suggested to be crucially involved in the development of atherosclerotic vascular diseases.

The aim of this study was to examine the role of decorin in cancer and HA in diabetic macroangiopathy in man, respectively. The expression of decorin was studied in relation

to various benign and malignant tumors. First, the involvement of decorin in tumor angiogenesis was investigated using different benign and malignant human vascular tumors. Secondly, the expression of decorin was localized in various human epithelial tumors, namely breast and bladder tumor samples. Also the effect of targeted decorin adenoviral transduction was examined on human breast and bladder cancer cell lines. Thirdly, the expression of decorin was investigated during the early differentiation of human embryonic stem cells. Additionally, decorin expression was localized in different human embryonic germ cell tumors. Regarding HA, its expression was studied in human vascular smooth muscle cells (HUVSMC) after treatment of high glucose concentration. High glucose concentration mimicked the condition of a diabetic patient in poor glycemic condition.

This study focused on examining the function and the role of two central ECM macromolecules, namely decorin and HA. More precisely, this study defines the modulatory action of decorin during angiogenesis and tumor development, and confirms its role as an anti-tumorigenic molecule. This study also explores the effect of high glucose concentration and the role of HA in the process of ECM remodeling by vascular smooth muscle cells and in diabetes related complications such as macroangiopathy.

2. REVIEW OF THE LITERATURE

2.1 The extracellular matrix (ECM)

Initially, the ECM was believed to provide only a physical scaffold to maintain organ and tissue structure. Now, it is well recognized that the ECM is a highly dynamic structure which is under constant enzymatic and non-enzymatic remodeling processes. Furthermore, its abundant molecular components face a myriad of post-translational modifications. Every tissue has its own constitution of ECM macromolecules with various cellular components e.g. fibroblasts, smooth muscle cells, and endothelial and epithelial cells (Frantz et al., 2010). The ECM macromolecules do not exist in isolation, but usually form heterogeneous supramolecular structures containing different molecular species (Mouw et al., 2014). In addition to different macromolecules, the ECM acts as a reservoir or a "sink" for various growth factors and cytokines as well as for ECM-remodeling enzymes (Mott and Werb, 2004; Hynes, 2009; Martino et al., 2015; Takawale et al., 2015). The ECM remodeling is transmitted via among other things in response to signals transmitted by ECM receptors or ECM-modifying enzymes (Daley et al., 2008; Van Doren, 2015). In addition to macromolecules and stromal cells which comprise the so-called interstitial matrix, the ECM also contains specialized ECM structure, the basement membrane, which separates endothelium or epithelium from the stroma (Lu et al., 2012). The basement membrane has a more compact and less porous structure rich in type IV collagen in comparison to the interstitial matrix (Egeblad et al., 2010).

In mammals, the so-called core matrisome comprises of approximately 300 proteins forming the full complement of ECM proteins (Hynes and Naba, 2012; Naba et al., 2015). This vast array of molecules like collagens, glycoproteins and proteoglycans represents the vital function of ECM molecules and their complex interactions (Naba et al., 2012). Overall, the ECM forms the local microenvironment, "niche", for the embedded cells (Lu et al., 2012). The ECM structures and proteins are bioactive and possess capability to modulate fundamental cellular events such as differentiation, adhesion, migration, proliferation, and survival (Daley et al., 2008; Hynes, 2009). Among other things, the so-called matricellular proteins are involved in the regulation of cell functions like cell-matrix interactions without direct contribution to the structural elements of the ECM (Bornstein and Sage, 2002; Murphy-Ullrich and Sage, 2014). In addition to their normal functions as intact, full-length molecules, most ECM molecules can also act as biologically active fragments. These so-called matricryptins are formed by enzymatic cleavage (Davis et al., 2000; Ricard-Blum and Ballut, 2011; Ricard-Blum and Salza, 2014). In normal tissues, ECM proteolysis is strictly controlled, but in tumors it is often dysregulated (Egeblad et al., 2010). Nevertheless, it is important to recognize that despite of the remodeling of the ECM and its molecules, ECM is strictly organized and that this

organization determines the bioactivity of the ECM. Also the precise orchestration of different remodeling events is vital for the normal function of the ECM (Kass et al., 2007; Egeblad et al., 2010). In fact, changes in the cellular phenotype and in cell-matrix interactions can be the result of a minor alteration like a single amino acid substitution in a single ECM component. Ultimately, these changes can modulate tissue function and thereafter lead to the development of a certain disease. It can be assumed that there is no disease without quantitative and/or qualitative changes in the ECM. However, it is essential to understand which specific changes in the ECM cause the disease and what are the changes that develop during the disease process (Järveläinen et al., 2009). In addition to fibrotic (Karsdal et al., 2015) and cardiovascular diseases (Shami et al., 2014), the development of cancer and its progression is a central example of the loss of normal tissue homeostasis, altered ECM structure and remodeling (Malik et al., 2015). During tumorigenesis, interaction between the tumor cells and the ECM is highlighted by the fact that remodeled tumor ECM facilitates tumor cell invasion while the malignant cells further manipulate their microenvironment to ensure their survival and to enhance their capability to form metastases (Cox and Erler, 2011; Fullar et al., 2015; Malik et al., 2015). As a result, the dysregulation of the ECM dynamics can be considered one of the hallmarks of cancer (Lu et al., 2012; Pickup et al., 2014). Regarding the progression of cancer, the ECM has a critical role in functions necessary for tumor progression and metastasis (Belotti et al., 2011; Du et al., 2014; Neve et al., 2014). Subsequently, the tumor stroma offers a potential therapeutic target for cancer treatment (Järveläinen et al., 2009; Sounni and Noel, 2013; Werb and Lu, 2015).

2.2 ECM macromolecules

The ECM macromolecules can be divided into four main groups, namely collagens, elastin and microfibrillar proteins, non-collagenous glycoproteins, proteoglycans (PGs), and hyaluronan (HA) (Hynes, 2009). Next, these macromolecules are introduced more closely.

2.2.1 Collagens

The collagens are the most abundant proteins in mammals, with 28 members of the collagen superfamily (collagens I-XXVIII) each containing at least one triple-helical domain (Gordon and Hahn, 2010). On the basis of their molecular structure and assemblies, they can further be divided into subgroups like fibrillary collagens (~90% of the total collagen), collagens associated with banded fibrils (FACITs), network-forming collagens, transmembranous collagens, endostatin precursor collagens and other collagens (Gelse et al., 2003; Brodsky and Persikov, 2005; Gordon and Hahn, 2010). The molecular diversity becomes even higher when taking into account the existence

of several α chains, molecular isoforms and the use of alternative gene promoters as well as alternative splicing (Ricard-Blum, 2011). In addition to these "true" collagens, also proteins containing a collagen –like domain can be considered to belong to the collagen family, *sensu lato* (Hynes and Naba, 2012). Fibrillar collagens represent the most abundant group of collagens and they participate in the structural maintenance of tissue architecture, shape and mechanical properties (Gelse et al., 2003). As a group, collagens possess critical roles in the regulation of cell differentiation, migration and growth by interacting with various cellular receptors, particularly the integrin receptor family (Hynes, 2009; Ricard-Blum, 2011; Hamaia and Farndale, 2014; Heino, 2014). Additionally, collagens can be cleaved into bioactive fragments via controlled proteolysis or cryptic sites can be unmasked (Ricard-Blum and Ballut, 2011). Collagenolytic enzymes include for example different matrix metalloproteinases (MMPs) and cathepsin K (Fields, 2013; Van Doren, 2015). Subsequently, these fragments can also regulate different cell processes taking place e.g. in development, angiogenesis and tumorigenesis (Nyberg et al., 2005; Cretu et al., 2007; Egeblad et al., 2010; Agrawal et al., 2011).

2.2.2 Elastin and microfibrillar proteins

Besides collagens, elastin represents another major ECM macromolecule. Elastic fibers are formed from several tropoelastin molecules which are covalently bound to each other via cross-links of their lysine residues (Debelle and Tamburro, 1999; Wise and Weiss, 2009). The second component of elastin fibers is microfibrils, mainly microfibrillins (fibrillin 1 and 2), which are vital for the integrity of the elastin fiber (Wise and Weiss, 2009). Elastin acts as a versatile ECM macromolecule providing elasticity for tissues such as arterial wall imparting resilience and recoil. Elastin also possesses vital role as a signaling molecule in vascular morphogenesis and disease (Karnik et al., 2003; Wise and Weiss, 2009; Wise et al., 2014). Similarly to collagens, elastin can be cleaved proteolytically by various MMPs and these fragments are able to influence cell function and to enhance cellular chemotaxis, proliferation and adhesion (Rodgers and Weiss, 2005; Van Doren, 2015). Elastin peptide fragments are indicated specifically in processes like neovascularization, atherosclerosis, and tumorigenesis (Wells et al., 2015).

2.2.3 Non-collagenous glycoproteins

The non-collagenous glycoproteins form the third cluster of ECM macromolecules. They contain molecules like fibronectin, laminins, SPARC (secreted protein, acidic and rich in cysteine)/osteonectin, tenascins, thrombospondins and nidogen. This group is considered to act as bridging molecules between structural ECM molecules, cells and the ECM. Furthermore they attach soluble molecules to the matrix thus enhancing the development of a network-like ECM (Mouw et al., 2014). For instance, fibronectin is first assembled into functional fibrillary matrix around cells via selective binding to

various cell surface receptors, namely different integrins and syndecans, where after other ECM proteins can directly interact with the formed fibrils or use the fibrillar matrix as a scaffold for their own fibril formation (Mao and Schwarzbauer, 2005; Couchman, 2010; Singh et al., 2010; Schwarzbauer and DeSimone, 2011). Fibronectin can interact with several other ECM macromolecules including collagen type I, thrombospondin and tenascin C (Singh et al., 2010; Kostourou and Papalazarou, 2014). Together with laminins and nidogen, fibronectin represents a vital ECM molecule also for the assembly and organization of basement membranes (Hallmann et al., 2005; Hohenester and Yurchenco, 2013). As a group, non-collagenous glycoproteins are currently emerging as a vital regulators particularly in blood vessel morphogenesis and tumorigenesis (Kostourou and Papalazarou, 2014; Masli et al., 2014; Bishop, 2015).

2.2.4 Proteoglycans

Mammalian proteoglycans (PGs) include four major classes; intracellular, cell surface, pericellular and extracellular (Iozzo and Schaefer, 2015). The extracellular PGs form the largest class comprising of 25 members including hyalectans (HA-binding PGs) such as aggrecan and versican, small leucine-rich PGs (SLRPs) including decorin and biglycan and SPOCK (acronym of SPARC/Osteonectin CWCV and Kazal-like domains proteoglycan, previously known as testican 1-3) (Iozzo and Schaefer, 2015). These molecules are mostly formed by core protein with one or more covalently linked glycosaminoglycan (GAG) side chain. HA is an exception. It has no core protein but exits as a free form. PGs can be grouped on the basis of their GAG type; chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and heparin and heparan sulfate (HS) (Kjellen and Lindahl, 1991). However, the group also contains so-called part-time PGs similar in structural homology and function but without a GAG chain/s (Esko et al., 2009; Iozzo and Schaefer, 2015). Furthermore, PGs can vary in their structure, e.g. number of GAG chains, thus further increasing the diversity of ECM macromolecules (Iozzo and Murdoch, 1996; Iozzo, 1998). The molecular diversity of the extracellular PGs can also be seen at the functional level of its members (Kjellen and Lindahl, 1991; Iozzo, 1998; Järveläinen et al., 2009; Iozzo and Schaefer, 2015).

SLRPs

The SLRPs form the largest subgroup within the extracellular PGs with 18 members (Nastase et al., 2014). As their name predicts, the molecular structure of SLRPs consists of a small core protein with a leucine-rich repeat (LRR) flanked by cysteine-rich clusters (Hocking et al., 1998; Park et al., 2008). The SLRPs contain five classes of molecules (I-V) described in Table 1.

Table 1. Members of the SLRP gene family: classification and GAG chain type. Modified from (Schaefer and Schaefer, 2010; Nastase et al., 2014).

Class I	Class II	Class III	Class IV	Class V
Decorin, CS/DS	Fibromodulin, KS	Epiphycan, CS/DS	Chondroadherin, KS	Podocan, no GAG
Biglycan, CS/DS	Lumican, KS	Opticin, no GAG	Nyctalopin, no GAG	Podocan-like protein I, no GAG
Asporin, no GAG	PRELP, KS	Osteoglycan, KS	Tsukushi, no GAG	
ECM2, no GAG	Keratocan, KS			
ECMX, no GAG	Osteoadherin, KS			

CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; KS, keratan sulfate; ECM2, extracellular matrix protein 2; ECMX, ECM2-like protein, X chromosome; PRELP, proline/arginine-rich end leucine-rich repeat protein.

The SLRPs have a vital role in multiple cellular processes and interactions both in health and disease (Theocharis et al., 2010; Nastase et al., 2014; Ni et al., 2014; Theocharis et al., 2015). Almost all SLRPs bind collagen (Chen and Birk, 2013) and so far, interactions have been mapped into the LRR domain of their core protein (Kalamajski and Oldberg, 2010). Via regulation of the collagen fibrillogenesis, i.e. collagen fibril growth and organization, SLRPs are significant and vital structural regulators of the ECM assembly (Bidanset et al., 1992; Ezura et al., 2000; Ameye and Young, 2002; Zhang et al., 2009; Chen et al., 2014). Together with other molecules, SLRPs can also protect collagen fibrils from collagenolytic proteases (Geng et al., 2006).

In addition to having a structural function, SLRPs can bind and sequester various morphogens such as bone morphogenic protein (Chen et al., 2004; Moreno et al., 2005; Morris et al., 2007) and growth factors including transforming growth factor beta (TGF-β) ((Hildebrand et al., 1994). On the other hand, SLRPs can also induce the expression of various cytokines, e.g. tumor necrosis factor-α (TNFα) (Moreth et al., 2012) thus regulating signal transduction on multiple pathways and leading to modulation of cellular phenotype, proliferation and migration (Schaefer and Iozzo, 2008; Schaefer and Schaefer, 2010). Additionally, acting as ligands for versatile PG receptors, SLRPs are able to further modulate cell behavior, phenotype and the development of various pathologies (Neill et al., 2015). As such, SLRPs are involved in a vast selection of specific processes including neural development (Dellett et al., 2012), chondrogenesis (Tillgren et al., 2015), inflammation (Sjöberg et al., 2009; Moreth et al., 2012; Schaefer and Iozzo, 2012), innate immunity (Frey et al., 2013), angiogenesis (Jian et al., 2013; Nikitovic et al., 2014; Järveläinen et al., 2015), and tumorigenesis (Iozzo and Schaefer, 2010; Theocharis et al., 2010; Afratis et al., 2012).

2.3 Decorin

2.3.1 Molecular structure of decorin

Decorin is the prototype member of the SLRP gene family. Its molecular structure is comprised of an approximately 40 kDa core protein with one negatively charged

glycosaminoglycan (GAG) side chain attached to serine residue 4 in the amino terminal end (Chopra et al., 1985; Krusius and Ruoslahti, 1986; Mann et al., 1990). Although the decorin core protein and the GAG can have different functions in various physiological and pathological situations, they should be seen as one functional unit (Seidler, 2012). The core protein of decorin consists of four distinct domains (Iozzo, 1997) (Figure 1).

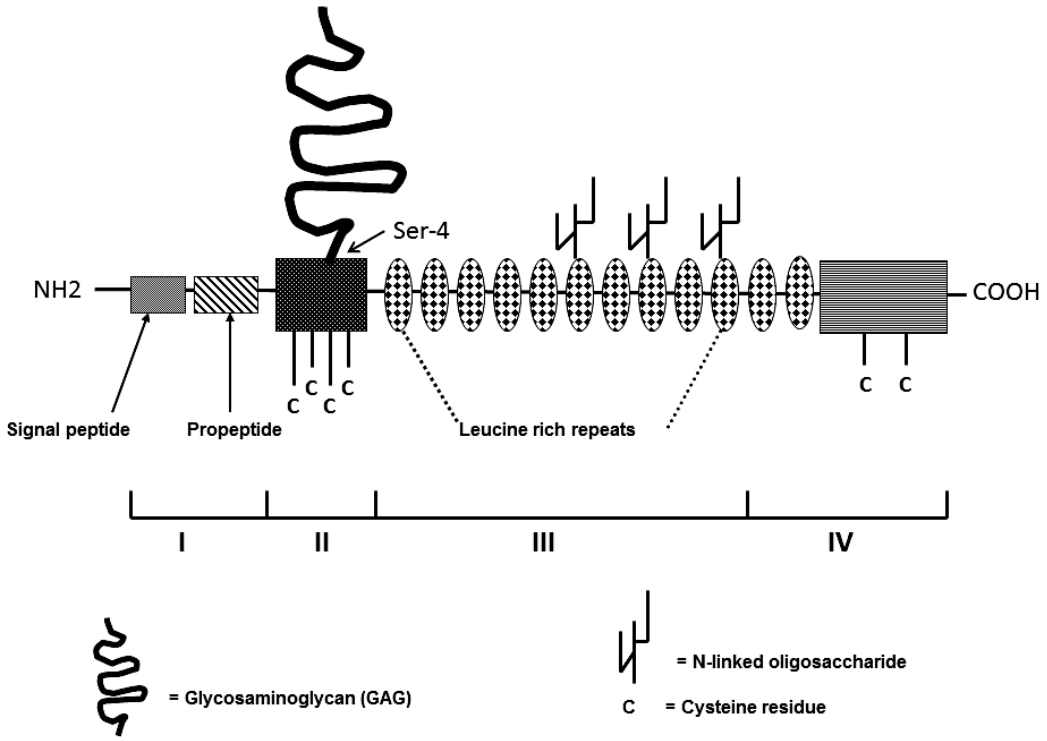

Figure 1. The molecular structure of decorin. Roman numbers I-IV indicate the domains of decorin core protein. For details, see the text.

The first domain contains a 14-amino acid signal peptide and a 16-amino acid propeptide which are not detected in the mature decorin and are thus cleaved before the secretion of this PG to the ECM. The second domain is negatively charged and rich in cysteine and it is the GAG carrying domain. The single GAG side chain of decorin is either chondroitin sulfate (CS) or dermatan sulfate (DS) consisting of repeating disaccharides; glucuronic acid-(β-1,3)-N-acetyl-galactosamine-(β-1,4) in CS and iduronic acid-(α-1,3)-N-acetyl-galactosamine-(β-1,4) in DS. However, because the GAG of decorin contains various amounts of both disaccharide types, decorin is in reality a co-polymer of CS and DS (Viola et al., 2006; Karousou et al., 2008; Sofeu Feugaing et al., 2013). Decorin can also be significantly modified at the post-translational level including the sulfation pattern of its GAG chains, e.g. in normal colon tissue CS/DS chains are mainly composed of 4-sulfated disaccharides while colon cancer cells exhibit large proportions of non- and

6-sulfated disaccharides (Theocharis, 2002; Suhovskih et al., 2015). In specific cases, decorin has even been shown to be synthezised as a protein core without a GAG chain (Kresse et al., 1987; Seidler et al., 2006a). Therefore, the precise structural features of decorin and modifications of DS/CS can vary depending on e.g. age, physiological and pathological situation, and tissue type (Mann et al., 1990; Laremore et al., 2010). The third domain contains twelve tandem leucine-rich repeats (LRRs) of about 24 amino acids in length flanked by the cysteine-rich regions of domains II and IV. Previously, it was shown that the LRRs formed a horseshoe-shaped structure where the outer convex surface is formed by the α-helixes and the inner concave surface is shaped by curved β-sheets (Kobe and Deisenhofer, 1993; Weber et al., 1996; Bella et al., 2008). However, the construction of a crystal structure of decorin core revealed that in fact decorin monomers form a solenoid fold with a parallel β-sheet inside and create a more open structure than previously thought (McEwan et al., 2006; Bella et al., 2008). The LRRs have been shown to be vital in protein-protein interactions in all proteins containing these motifs (Kobe and Deisenhofer, 1994; Kobe and Deisenhofer, 1995; Hocking et al., 1998). Each LRR possesses specific characterized properties in these interactions, e.g. LRR VII is involved in the direct binding of decorin to type I collagen (Kalamajski et al., 2007) and area between LRR 3 and 5 has a high affinity for TGF-β, respectively (Iozzo, 1997; Schönherr et al., 1998). In addition to the LRRs, this domain has also attachment sites for three N-linked oligosaccharides bound to asparagine residues (Scott and Dodd, 1990; Iozzo, 1997). The fourth domain of decorin is the carboxyl terminal domain with two cysteine residues and a conserved disulfide loop.

2.3.2 Decorin gene and its expression

As mentioned above, decorin represents the prototype molecule of class I SLRPs and it is the most studied member of this gene family (Iozzo, 1998; Iozzo and Schaefer, 2010; Iozzo and Schaefer, 2015). Previously also known as DS-PG II, PG-40 and PG-S2, decorin was first cloned from a fibroblast cDNA library and was found to be consisted of 329 amino acids (Rosenberg et al., 1985; Krusius and Ruoslahti, 1986; Oldberg et al., 1989). Thereafter its gene was mapped to human chromosome 12 (12q23) (Danielson et al., 1993). Subsequently, the intron-exon junctions of decorin gene were revealed and its promoter sequenced with two alternatively spliced leader exons, Ia and Ib, in the 5′untranslated region (Vetter et al., 1993; Santra et al., 1994). The promoter can be divided into proximal and distal regions, which contain various regulatory elements such as CAAT and TATA boxes in the proximal promoter and AP-1 and AP-5 binding sites in the distal promoter (Santra et al., 1994; Iozzo, 1997). The promoter also contains a bimodal regulatory element between residues -188 and -140 which mediates the repression of decorin expression via binding of TNF-α and on the other hand, induction of gene expression via binding of interleukin 1 (IL-1) (Santra et al., 1994; Mauviel et

al., 1995; Mauviel et al., 1996). Induction of decorin expression has also been shown to result from cell quiescence (Yamaguchi and Ruoslahti, 1988).

Decorin is expressed primarily by cells of mesenchymal origin such as fibroblasts, smooth muscle cells and chondrocytes, but in specific situations associated with angiogenesis, decorin expression can also be induced in endothelial cells (ECs) (Bianco et al., 1990; Järveläinen et al., 1991; Järveläinen et al., 2015). Interestingly, also mouse ECs derived from different vascular beds, e.g. heart and lung were shown to produce detectable amounts of decorin thus further extending the concept of endothelial heterogeneity (Calabrese et al., 2011).

2.3.3 Interactions of decorin

The aforementioned structural features of decorin form the basis for its multiple interactions with other ECM macromolecules, growth factors, cytokines, and various receptors (Iozzo, 1997; Sofeu Feugaing et al., 2013). Originally, decorin was found to interact with collagen type I fibrils and was thereafter named after its capability to "decorate" collagen fibrils (Pogany and Vogel, 1992; Kalamajski and Oldberg, 2010; Reese et al., 2013). Besides type I collagen, decorin is known to bind also other fibrillary collagens, namely types II, III and VI, thus further verifying its function as a vital regulator of fibrillogenesis (Bidanset et al., 1992; Danielson et al., 1997; Nareyeck et al., 2004; Orgel et al., 2009; Reese et al., 2013). In addition to controlling fibrillogenesis, decorin has been shown to regulate collagen turnover hence contributing to the regulation of tissue homeostasis (Danielson et al., 1997; Sofeu Feugaing et al., 2013). Apart from fibrillogenesis, decorin is also involved in several other physiological processes like myogenesis (Li et al., 2007; Brandan et al., 2008), gonad differentiation (Miqueloto and Zorn, 2007), tendon development (Kuo et al., 2008), chondrogenic differentiation (Twomey et al., 2014) and intestinal cell maturation (Bi et al., 2008).

Regarding decorin as a regulator of growth and cell proliferation, it was the first PG identified to be able to directly bind growth factors. Decorin was originally regarded as a regulator of cell proliferation via binding to TGF-β (Yamaguchi et al., 1990), but now the list of decorin-growth factor interactions encompasses several others including vascular endothelial growth factor (VEGF) (Mohan et al., 2011b), insulin-like growth factor I (IGF-I) (Schönherr et al., 2005; Iozzo et al., 2011), and platelet derived growth factor (PDGF) (Nili et al., 2003; Baghy et al., 2013). However, the binding between growth factors and decorin is not necessarily stable and permanent because for example TGF-β can be released after degradation of decorin by various proteases (Imai et al., 1997; Boivin et al., 2012).

As decorin is able to exist as a matrix-bound or soluble form, it can also directly regulate the intercellular signaling of various growth factors by binding to their receptors thus

inhibiting their subsequent activation. So far, decorin has been found to bind to EGFR (Moscatello et al., 1998; Iozzo et al., 1999a), Met, which is the receptor for hepatocyte growth factor (HGF) (Goldoni et al., 2009; Kristensen et al., 2013), IGF-IR (Schönherr et al., 2005; Iozzo et al., 2011; Morrione et al., 2013), VEGFR-2 (Khan et al., 2011), and PDGFR-α/β (Horvath et al., 2014). In addition to being able to bind growth factors and their receptors, decorin has been shown to be involved in the modulation of inflammation via interaction with toll-like receptors (TLR) 2 and 4 in macrophages (Merline et al., 2011; Moreth et al., 2012).

2.3.4 Epigenetic regulation of decorin

Besides binding to growth factor receptors, an interesting regulatory mechanism of decorin expression particularly in cancer is the methylation of the decorin gene promoter. Both hypo- and hypermethylation are common in many human diseases including different cancers (Esteller, 2007). Previously, it has been shown that methylation is likely to be differentially associated with the regulation of decorin expression in cancers like colon cancer (Adany et al., 1990; Nyman et al., 2015). Differences in the methylation pattern are encountered also in germ cell tumors (GCTs), but the methylation status of decorin in these tumors remains to be explored (Wermann et al., 2010).

2.4 Decorin in cancer

2.4.1 Function of decorin in tumorigenesis

Decorin has a valid reputation as an anti-tumorigenic molecule. It was originally shown that animals which lack decorin expression do not spontaneously develop tumors (Danielson et al., 1997). However, subsequently it was shown that decorin -/- mice were susceptible to intestinal tumor formation when they were exposed to certain diet conditions (Bi et al., 2008). Thus, decorin deficiency was suggested to act as permissive for tumorigenesis and the mechanism of action was associated among other things with the down-regulation of cyclin-dependent kinase (CKD) inhibitors p21 (WAF1/cip1) and p27 (kip1) and the up-regulation of β-catenin (Bi et al., 2008; Goldoni and Iozzo, 2008). Furthermore, experiments with mice lacking both decorin and p53 have revealed that these genes can co-operate; double knock-outs develop aggressive lymphomas faster than mice lacking only decorin or p53 (Iozzo et al., 1999b). As p53 represents an established tumor-suppressor gene named as the guardian of the genome (Lane, 1992), decorin in turn could be named as the guardian from the matrix (Neill et al., 2012a). Because decorin is primarily expressed by cells of mesenchymal origin, and not by epithelial cells which are the origin of most cancers (80-90%), the effects of decorin on cancer cells are exerted mostly in paracrine fashion (Sofeu Feugaing et al., 2013; Horvath et al., 2014).

In addition, decorin also potentiates immune response of the host by reducing the abundance of anti-inflammatory molecules and by increasing the amount of pro-inflammatory molecules resulting in decreased tumor growth (Merline et al., 2011; Moreth et al., 2012). Despite the fact that the mechanisms between inflammation and immune response with tumor development and progression are not very well understood, it is clear that tumor-promoting inflammatory environment has a vital place in the list of hallmarks of cancer (Raposo et al., 2015).

Regarding the structure of decorin, the ratio of CS compared to DS is balanced in normal tissues (Theocharis et al., 2010). Despite the fact that the effect of decorin should be examined as a sum effect of the core protein together with GAG side chain, one has to recognize the possible variations within GAG structure due to transcriptional, post-transcriptional and post-translational modifications (Theocharis, 2002; Skandalis et al., 2006a; Theocharis et al., 2010). For instance, during tumor development and progression the GAG content of tumor microenvironment is significantly altered in favor of CS (Theocharis et al., 2010; Afratis et al., 2012). This can be seen in e.g. gastric carcinoma (Theocharis et al., 2003) as well as in pancreatic cancer (Skandalis et al., 2006a). Interestingly, decorin carrying DS chain has been shown to be markedly more efficient in inhibiting osteosarcoma cell migration *in vitro* than decorin with CS chain (Merle et al., 1999). In addition to changes in the GAG chain, also decorin core protein can encounter marked modifications e.g. in laryngeal cancer where non-glycanated protein cores are present (Skandalis et al., 2006b).

2.4.2 Decorin as a regulator of tumor angiogenesis

Angiogenesis is the formation of new vascular vessels which can occur e.g. from sprouts of pre-existing vessels (Folkman and Shing, 1992). It is a vital process in both healthy body and various disease conditions like cancer, and it is normally strictly controlled via the balance of different pro- and anti-angiogenic factors (Carmeliet, 2000). In addition to regulating cell proliferation, decorin has gained a role as a modulator of angiogenesis (Järveläinen et al., 2015) and together with other specified ECM macromolecules, decorin forms the “backbone” of vascular endothelial glycocalyx covering the vascular endothelium luminally (Reitsma et al., 2007).

Decorin has been reported to possess both pro-angiogenic and anti-angiogenic effects in the regulation of angiogenesis. A lot of data on the function of decorin in different physiological and pathological conditions including angiogenesis has been gained after the generation of decorin-null mice (Danielson et al., 1997). Despite the fact that these mice have normal vasculature, the deficiency of decorin has since been shown to cause impaired angiogenesis for example in injured mouse cornea (Schönherr et al., 2004). In human tissues, decorin expression has been shown to be present in the

plaque neovasculature of atherosclerotic coronary arteries (Gutierrez et al., 1997) where angiogenesis is known to occur (Ross, 1999; Wragg et al., 2014) as well as in inflamed temporal artery wall (Nelimarkka et al., 2001).

Interestingly, decorin can also act as an anti-angiogenic molecule. For instance, decorin-expressing tumor xenografts have been shown to exhibit markedly lower neovascularization *in vivo* (Grant et al., 2002). Decorin is also centrally involved in the regulation of corneal transparency (Mohan et al., 2011a) where targeted decorin gene transduction has resulted in retarded corneal neovascularization in rabbit model *in vivo* (Mohan et al., 2011b). In addition to whole decorin molecules, even fragment of decorin LRR core, LRR5, has been shown to possess an anti-angiogenic action (Sulochana et al., 2005).

What are the mechanisms behind the observed effects of decorin on tumor angio-genesis? One has to remember, that angiogenesis is one of the central examples of the cell-matrix interactions; ECs regulate ECM assembly and ECM macromolecules influence EC phenotype while ECM remodeling influences development of EC angiogenic phenotype (Fiedler and Eble, 2009; Edgar et al., 2014). Also organ-specific EC differentiation and differential gene expression patterns depending on the EC type have been suggested (Kallmann et al., 2002).

Regarding interactions between decorin and growth factors, VEGF is likely to represent the most important growth factor in angiogenesis. In various situations, both pro-angiogenic and anti-angiogenic effects of decorin have been reported to be regulated via modulation of VEGF expression and function (Grant et al., 2002; Sulochana et al., 2005; Santra et al., 2008; Mohan et al., 2011b; Dil and Banerjee, 2012; Nayak et al., 2013). Additionally, decorin has been shown to bind directly to VEGFR-2 (Khan et al., 2011). Besides growth factors, decorin is able to modulate cell-matrix interactions via integrin adhesion receptors (Fiedler et al., 2008; Fiedler and Eble, 2009). Integrins form a physical connection from outside to inside the cells, thus mediating ECM originated signals which modulate cell behavior (Seguin et al., 2015). Decorin has been shown to modulate the activity of α2β1 integrin (Fiedler and Eble, 2009; Jungmann et al., 2012) and even to regulate its expression (Jungmann et al., 2012). However, direct interaction between the most angiogenic integrin, αvβ3 (Brooks et al., 1994; Weis and Cheresh, 2011), has not yet been demonstrated.

In addition to the mechanisms mentioned above, decorin has a role in EC targeted apoptosis and autophagy whereby it is able to modulate angiogenesis (Järveläinen et al., 2015). Regarding apoptosis, decorin was first believed to possess only anti-apoptotic effect on ECs (Schönherr et al., 1999). However, as subsequent studies have shown totally opposite effects, conclusion can be made that the effect of decorin on the regulation of apoptosis is likely to be context-dependent (Dimmeler and Zeiher, 2000; Hamid et al., 2013; Yu et al.,

2014). Furthermore, a new mechanism of decorin modulating angiogenesis via inducing autophagy in ECs has been discovered (Buraschi et al., 2013; Neill et al., 2013; Neill et al., 2014a). Autophagy is a vital cellular mechanism in maintaining tissue homeostasis; it dynamically recycles degraded proteins and damaged or excess organelles into energy and new material for cellular renovation (Mizushima and Komatsu, 2011). Autophagy has also been associated with inhibition of angiogenesis (Ramakrishnan et al., 2007) and cancer progression (Neill et al., 2014a). Decorin, in turn, has been shown to induce autophagy in both macro- and microvascular ECs via interacting with the VEGFR-2 thus ultimately resulting in inhibition of angiogenesis (Buraschi et al., 2013; Neill et al., 2013). This inhibition was subsequently revealed to be mediated by the activation of 5' adenosine monophosphate-activated protein kinase (AMPK) situated downstream of VEGFR-2 and induction of paternally expressed gene 3 (Peg3) (Buraschi et al., 2013; Goyal et al., 2014). Peg 3 has previously been identified as a tumor suppressor inhibiting e.g. glioma growth (Jiang et al., 2010).

In addition to inducing EC autophagy, decorin has been shown to be able to induce the selective degradation of tumor cell mitochondria by autophagy in a process called mitophagy (Neill et al., 2014b; Gubbiotti et al., 2015). To be precise, decorin was found to evoke mitostatin expression via the Met receptor activation leading to mitostatin-dependent mitophagy (Neill et al., 2014b). Mitostatin, meaning mitochondrial protein with oncostatic activity, has previously been identified as one of the up-regulated genes in tumor cells after decorin transfection (Vecchione et al., 2009). Furthermore, the mitostatin-dependent mitophagy has been shown to act as an inhibitor of tumor angiogenesis via negative feedback control of VEGFA transcription (Neill et al., 2014b).

In conclusion, one has to remember the importance of the local microenvironment as a dynamic entity. As discussed above, decorin´s role as a regulator of angiogenesis seems to be context-dependent. Angiogenic effect of decorin can also be influenced by the cell type producing it as different cell types differ in their post-translational processes leading to altered length or sulfation patterns of the GAG moiety (Fiedler and Eble, 2009). However, considering the importance of angiogenesis in the development and progression of tumors, the impact of decorin at least in tumorigenesis-associated angiogenesis can be considered as an inhibitory one (Järveläinen et al., 2015). The following chapter will introduce decorin and growth factor interactions specifically during tumorigenesis.

2.4.3 Decorin and growth factor interactions in cancer

As previously described, decorin is able to bind to different growth factor receptors such as EGFR (Iozzo et al., 1999a). This binding transiently activates EGFR signaling leading to concurrent activation of mitogen-activated protein (MAP) kinase, and induces cell cycle arrest and growth inhibition via up-regulation of the cyclin-dependent kinase

inhibitor (CKI), p21 (De Luca et al., 1996; Santra et al., 1997; Moscatello et al., 1998). Decorin binding to EGFR has also been shown to induce EGFR dimerization, and subsequent internalization via caveolar-mediated endocytosis ultimately leading to its lysosomal degradation (Zhu et al., 2005). As the down-regulation of p21 and p27 was subsequently shown to be tumor-permissive in decorin -/- mice, the anti-oncogenic role of decorin would require the up-regulation of these and other CKIs. Indeed, in addition to p21, decorin has recently been shown to be able to up-regulate also the expression of p57, which is down-regulated in several types of cancers (Hamid et al., 2013). Induction of p27 by decorin in cancers has not been observed but in normal macrophages decorin is able to increase the expression of both p21 and p27 which protect cells from apoptosis and inhibit proliferation, respectively (Xaus et al., 2001). Regarding other members of the ErbB gene family, decorin has been shown to suppress the activity of ErbB2 and ErbB4 via degradation (Santra et al., 2000; Reed et al., 2005). This is probably achieved indirectly by inhibiting heterodimerization of ErbB2 with ErbB4 (Santra et al., 2000; Iozzo and Schaefer, 2015). In addition to binding to various growth factors and their receptors, decorin has also been shown to be able to induce apoptosis of tumor cells via activation of caspase 3 (Seidler et al., 2006b).

2.4.4 Decorin expression in different cancers

Different cancer types can be classified according to their cellular origin. Epithelial tumors, which represent the majority of malignant growths in human, originate from epithelial cells, while sarcomas develop from mesenchymal cells. Decorin expression and immunoreactivity have been reported to be either decreased or totally lacking in the stroma of various cancer types of epithelial origin (Bozoky et al., 2014; Iozzo and Schaefer, 2015). For example, decorin expression has been found to be significantly decreased in breast carcinomas (Leygue et al., 2000), ovarian tumors (Nash et al., 2002; Grisaru et al., 2007), colon cancers (Augoff et al., 2008; Nyman et al., 2015; Suhovskih et al., 2015), and hepatic carcinomas (Duncan, 2013; Horvath et al., 2014). Also in bladder carcinoma, both decorin expression and immunoreactivity have been observed to be significantly decreased compared to normal preexisting stroma adjacent to malignant tissue (Dyrskjøt et al., 2004; Sanchez-Carbayo et al., 2006; Iozzo et al., 2011).

Opposite views on decorin expression have also been reported for instance in pancreatic cancer, where increased amount of decorin has been associated with the malignant phenotype (Skandalis et al., 2006a). When evaluating amounts of various molecules in tumors and their microenvironment, one has to recall the possibility of a desmoplastic reaction which means the accumulation of different ECM macromolecules like PGs in the vicinity of cancer cells (Sainio and Järveläinen, 2013). Desmoplastic reaction has been detected in several cancer types including pancreatic cancer (Merika et al., 2012), liver cancer (Kocabayoglu and Friedman, 2013) and prostate cancer (Coulson-

Thomas et al., 2011). Of course, in addition to desmoplasia, other explanations exist for the observed differential expression levels of decorin. Similarly to the regulation of angiogenesis, decorin may have opposite effects on cancer progression depending on the type of cancer and its microenvironment. Interestingly, e.g. in oral cancer decorin expression is significantly up-regulated in chemoresistant cancer cell lines (Yamano et al., 2010) while low decorin expression is related to increased chemosensitivity and thus improved patient prognosis (Kasamatsu et al., 2015).

Teratomas and teratocarcinomas represent tumors originating from germ cells. These germ cell tumors (GCTs) appear often at gonadal sites such as in the testis, and represent the most frequent solid malignant neoplasms in young men (Bosl and Motzer, 1997; Ulbright, 2005; Litchfield et al., 2015). Cancer cells and stem cells are also known to share striking similarities such as pluripotency and the ability of self-renewal (Reya et al., 2001). Recently, increased expression of decorin associated with differentiation of carcinoma cells has gained a lot of interest (Gasimli et al., 2013; Gasimli et al., 2014; Ma et al., 2014). Inducing a more differentiated state in cancer cells offers an auspicious strategy to decrease malignant behavior of the cells (Hultman et al., 2014). However, factors regulating differentiation of stem cells are still poorly understood (He et al., 2009), and particularly the role of ECM macromolecules in the development of embryonic tumors is not known (Diez-Torre et al., 2004; Diez-Torre et al., 2010).

Nevertheless, the level of decorin expression has been suggested to function as a beneficial biomarker in the evaluation of tumor aggressiveness. Correlation has been found between reduced decorin expression and poor disease-free survival e.g. in soft tissue tumors (Matsumine et al., 2007) and node-negative invasive breast cancer (Troup et al., 2003).

2.4.5 Decorin gene therapy in cancer

The anti-oncogenic action of decorin has been tested in different ways, by delivering either decorin core or decorin gene into malignant tumors. Regarding breast cancer, decorin has also been shown to possess the capability to prevent metastatic spreading of the tumor in addition to acting as pro-apoptotic factor and inhibitor of primary cancer growth (Reed et al., 2005; Goldoni et al., 2008). In addition, using an orthotopic breast carcinoma xenograft model, decorin core protein has been indicated to modulate the microenvironment of breast carcinoma resulting in specific stromal gene signatures consisting of various tumor suppressive genes (Buraschi et al., 2012). By introducing decorin to cancer cells via systematic delivery of decorin, either exogenously (Seidler et al., 2006b; Li et al., 2008) or by viral transduction (Reed et al., 2002; Tralhão et al., 2003; Xu et al., 2015) marked effects including decreased cell proliferation, decreased mitosis, and increased apoptosis have been achieved. Recently, significant anti-oncogenic effects

have also been reported using a decorin expressing oncolytic recombinant adenovirus in the treatment of bone metastasis in breast cancer (Yang et al., 2015) and pancreatic cancer (Na et al., 2015). Taken into account the versatile functionality of decorin as an anti-oncogenic molecule, it could represent a powerful molecule in ECM-based gene therapy in the future (Sainio and Järveläinen, 2014). Some of these desired effects of decorin on tumors are schematically presented in Figure 2.

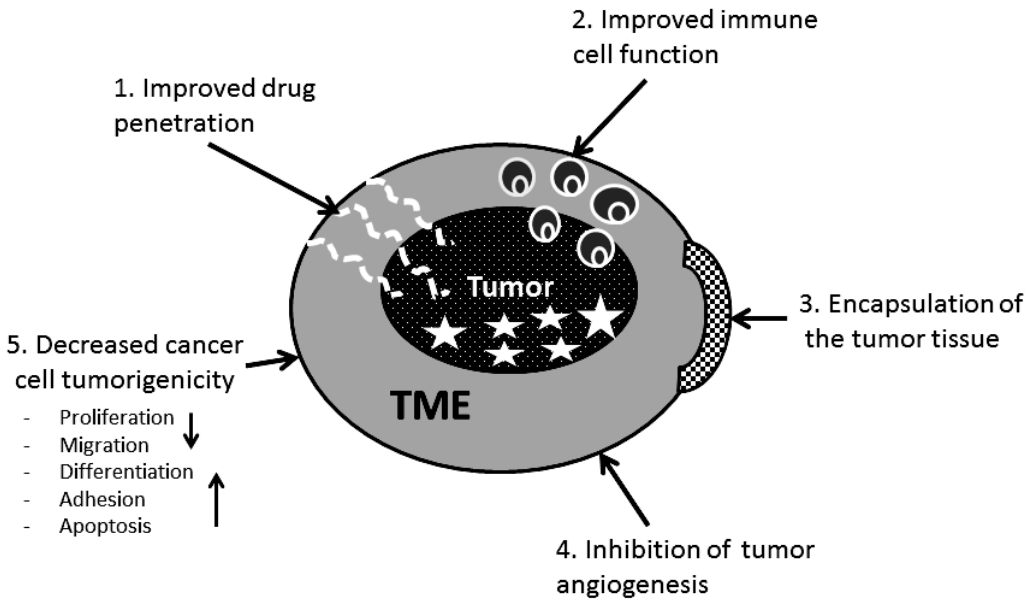

Figure 2. Anti-tumoral effects of decorin gene therapy. 1. Remodeling or degradation of ECM would improve drug penetration to tumor tissue. 2. Remodeling of ECM would also improve the action of immune cells. 3. Accumulation of decorin surrounding tumor would decrease tumor progression and metastasis. 4. Decreased angiogenesis would prevent tumor progression and could cause tumor tissue necrosis. 5. Inhibitory effects of decorin on tumor cell behavior. TME = tumor microenvironment. Modified from (Sainio and Järveläinen, 2014).

This chapter has discussed the role of decorin as an anti-oncogenic molecule. Next, another versatile ECM macromolecule, hyaluronan, and its functions will be presented in vascular biology, particularly in diabetic macroangiopathy.

2.5 Hyaluronan (HA)

2.5.1 Molecular structure of HA

Despite its seemingly simple structure, HA represents a versatile multifunctional ECM macromolecule. It was identified already in 1934 as hyaluronic acid (Meyer and Palmer, 1934) and subsequently renamed hyaluronan (Balazs et al., 1986). HA is a GAG that has no core protein. It is composed of unsulfated and unbranched polysaccarides of

repeating glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNac) disaccaride units linked together with β 1-3 linkage between GlcUA and GlcNac and a bond β 1-4 between GlcNac and GlcUA (Figure 3) (Weissmann and Meyer, 1954; Meyer, 1958).

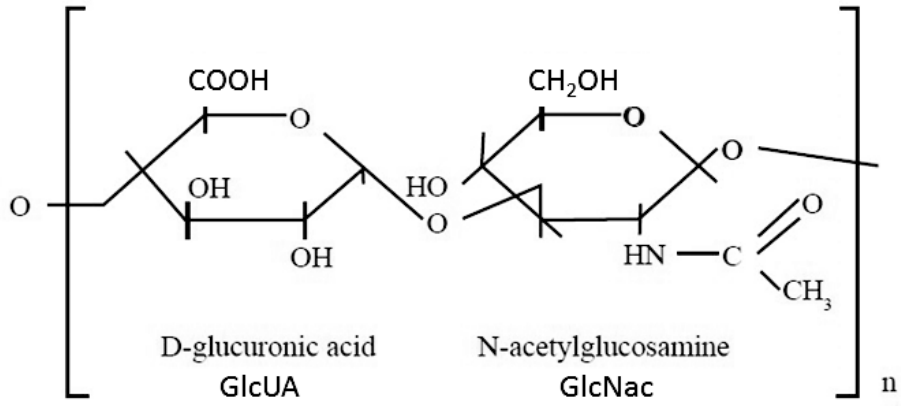

Figure 3. Molecular structure of HA. For details, see the text. Modified from (Bansal et al., 2010).

2.5.2 HA synthases and HA expression

Contrary to PGs, which are synthesized in the Golgi apparatus, HA is synthesized in the inner face of cell plasma membrane in mammals (Philipson and Schwartz, 1984) by three hyaluronan synthase (HAS) isoforms, namely HAS1, HAS2 and HAS3 (Itano and Kimata, 1996; Fulop et al., 1997; Spicer et al., 1997a; Weigel and DeAngelis, 2007). The chromosomal localizations of HASes have been identified; *HAS1* was localized to chromosome 19q13.3-q13.4 boundary, *HAS2* to 8q24.12, and *HAS3* to 16q22.1, respectively (Spicer et al., 1997b). Also the genomic structures of HASes have been further characterized (Monslow et al., 2003). HASes are glycosyltransferases that catalyze the polymerization of HA de novo with GlcNAc, GlcUA, and Mg2+ (Weigel and DeAngelis, 2007). Every HAS produces HA chains of different lengths and they differ in their matrix formation capability (Itano and Kimata, 1996; Itano, 2008; Vigetti et al., 2015). For example, HAS1 and HAS2 are able to produce HA up to 2000 kDa whereas HAS3 produces lower molecular mass HA of 100-1000 kDa (Weigel et al., 1997; Itano et al., 1999; Itano and Kimata, 2002; Weigel and DeAngelis, 2007). The HASes also differ in the regulation of their catalytic efficiency, e.g. only HAS2 is regulated by covalent modifications like ubiquitination (Karousou et al., 2010), while the activity of HAS3 is regulated via Rab10-mediated endocytosis (Deen et al., 2014). However, the precise physiological role of these different isoforms is not yet fully understood (Vigetti et al., 2014a). It is known that the expression level of HAS isoforms differs depending on the physiological and biological state in the body (Weigel et al., 1997) referring to a spatial and temporal regulation of HA synthesis (Weigel et al., 1997; Dicker et al., 2014).

HASes are also known for their response to different growth factors such as EGF, IGF-I and PDGF, various cytokines and cellular stress (Syrokou et al., 1999; Itano et al., 2008).

HA is found ubiquitously and abundantly in humans within the pericellular region and in the ECM of every tissue, but it can sometimes also exist intracellularly (Evanko and Wight, 1999; Hascall et al., 2004; Evanko et al., 2007). It is expressed by various cell types, including epithelial cells, endothelial cells, fibroblasts and smooth muscle cells (Jiang et al., 2011).

2.5.3 HA binding proteins and receptors

The amount of HA in the body is regulated by refined balance between its synthesis and turnover (Fraser et al., 1997; Dicker et al., 2014). Over 30% of the total amount of HA in the body is degraded every day (Stern, 2004). Significant amounts of HA can be found e.g. in vitreous humor, blood vessels and cartilage (Pardue et al., 2008; Cowman et al., 2015). HA forms the backbone of the jelly-barrier on different cell surfaces and thus regulates the accessibility of the cell surface (Ebid, 2015). Based on HA´s unique molecular structure, it has distinct biomechanical characteristics (Coleman, 2005). Particularly via its interactions with other ECM proteins and cell membrane receptors, the so-called hyaladherins (hyaluronan-binding proteins, HABPs), HA is capable of exerting its modulatory action on pericellular and extracellular matrices (Toole, 1990). It can also modify different aspects of cell behavior including cell migration, cell-cell adhesion and cellular differentiation (Knudson and Knudson, 1993; Turley et al., 2002; Vigetti et al., 2014a).

The HABPs can be divided into two groups on the basis of their binding motifs; the link module superfamily and the non-link module hyaladherins (Day and Prestwich, 2002; Dicker et al., 2014). The first group contains PGs such as aggrecan (Watanabe et al., 1997), versican (LeBaron et al., 1992) and neurocan (Rauch et al., 1992). This group also contains the ubiquitous HA receptor, a cluster of differentiation 44 (CD44) (Underhill, 1992; Isacke and Yarwood, 2002). In fact, the CD44 is a family of transmembrane proteins consisting of around nine different isoforms which are named CD44v2-v10 (Negi et al., 2012). These are present in various amounts in different cell types, particularly in inflammation and cancer associated cells (Aruffo et al., 1990; Underhill, 1992; Sherman et al., 1994; Negi et al., 2012). Indeed, the main signaling pathways activated by interaction of HA and CD44 are associated with inflammation process, cancer cell migration, and wound healing (Day and de la Motte, 2005; Tolg et al., 2014a; Misra et al., 2015). In addition to CD44, the link module superfamily contains members like lymphatic vessel endothelial receptor 1 (LYVE-1) (Gale et al., 2007; Johnson et al., 2007).

The receptor for hyaluronan mediated motility (RHAMM) (Turley, 1982; Sherman et al., 1994; Misra et al., 2015) represents the non-link module proteins which uses a two basic

amino acids flanking a seven amino acid stretch, the so-called B(X7)B motif, in binding to HA (Yang et al., 1994; Wang et al., 1996). Similarly to CD44, several isoforms of RHAMM are produced through alternative splicing (Entwistle et al., 1996). Normally, RHAMM resides inside the cell and its transport to the ECM is not yet understood (Dicker et al., 2014). However, in the ECM, RHAMM is known to associate with CD44 and HA and regulate intracellular signaling cascades (Turley et al., 1991). As the name reveals, RHAMM is associated with cell migration and plays an important role in inflammation (Zaman et al., 2005; Garcia-Posadas et al., 2014), tissue repair (Gao et al., 2008; Tolg et al., 2014b) and cancer (Misra et al., 2015). In addition to CD44 and RHAMM, another receptor types central in inflammation are the toll like receptors (TLRs), and HA has been shown to interact with TLR 2 and 4 and regulate their signaling (Jiang et al., 2007; Jiang et al., 2011). More on different HA receptors, their signaling cascades and different cellular and tissue functions can be found in recently published reviews (Jiang et al., 2011; Vigetti et al., 2014a; Viola et al., 2015).

2.5.4 Molecular size of HA

Different signaling cascades in response to interactions between HA and HABPs have been shown to depend strongly on the cell type in question and on molecular weight of the HA molecule (Turley et al., 2002; Stern et al., 2006). Because even as small as 6 saccharides of HA has been demonstrated to be able to bind to HABP, in theory one single chain of high molecular weight HA can interact with 1000 protein molecules (Stern et al., 2006). In addition to the complex HA-protein aggregates, huge superstructures of high molecular weight HA chains with various architectures and functional activities are known to be generated via crosslinking (Lapcik L Jr and et al., 1998; Day and de la Motte, 2005). These are indicated especially during inflammation where they are thought to sequester pro-inflammatory mediators inhibiting their receptor binding and thus act as inflammation limiting molecules (de la Motte et al., 2003; Day and de la Motte, 2005; Frey et al., 2013).

As mentioned in the beginning, HA is the target of frequent turnover and degradation. Originally, HA is synthetized as a high molecular weight HA (HMW HA) by different HASes, and subsequently either retained as a pericellular coat surrounding cells or released and incorporated into the ECM as a structural component (Monslow et al., 2015). HA can also exist as a soluble form in the vasculature and lymphatics (Stern et al., 2006). The signaling properties of HA are strongly dependent on its molecular mass. In general it can be said that HMW HA is a extracellular form and mainly exhibits regulatory and structural roles, while the smaller fragments of HA have angiogenic, immunostimulatory and inflammatory activities (Stern et al., 2006; Jiang et al., 2011; Bollyky et al., 2012; Petrey and de la Motte, 2014).

2.5.5 High molecular weight HA

In tissue homeostasis, HA has typically a high molecular weight of over 1000 kDa and represents the so-called native HA (Itano and Kimata, 2002; Monslow et al., 2015). This HMW HA is able to retain large quantities of water and it is able to act as a hydrating, lubricating and space-filling molecule in connective tissue and joints (Coleman et al., 1999; Dicker et al., 2014). It also creates microenvironment which indirectly influences the behavior of cells within the ECM via sequestering and releasing growth factors (Pardue et al., 2008; Dicker et al., 2014). The native HMW HA is of central importance during embryonic development where it facilitates cellular growth and tissue differentiation e.g. in the developing heart (Camenisch et al., 2000) and limb (Matsumoto et al., 2009). The promoting effect of HMW HA on chondrogenic differentiation has also been shown in murine chondrocytic cell line *in vitro* (Sato et al., 2014). Interestingly, a contrasting effect of HA of 900-1200 kDa in size has just recently been shown to inhibit osteoblast differentiation (Kaneko et al., 2015). In different wound healing models, HMW HA mediated signaling has proven to enhance contraction of collagen by vascular smooth muscle cells (VSMCs) (Travis et al., 2001) and to promote their actin stress-fiber arrangement (Goueffic et al., 2006). Also in myocardial repair after infarction (Chen et al., 2013) and wound healing in genetically diabetic mice (Galeano et al., 2011), the function of HMW HA has been shown to be beneficial. The amount of HA in tissues undergoing wound repair can be elevated for several weeks after injury (Dicker et al., 2014) and the binding of HA to its principle ligand, CD44, has been shown to be required for proper healing process (Jordan et al., 2015). This interaction has also been demonstrated to induce mesenchymal stem cell (MSC) homing to injured tissue, thus influencing tissue regeneration (Herrera et al., 2007; Bian et al., 2013). In addition to the effects on different tissue healing processes, HMW HA can also restrict cellular interactions and binding of signaling molecules to their receptors by forming a pericellular sheaths surrounding the cells (Pardue et al., 2008). It has also been suggested that HMW HA exhibits cancer suppressive functions (Tian et al., 2013; Fisher, 2015).

As a summary, the primary functions of HMW HA are the maintenance of tissue homeostasis in various ways and after injury, exertion of protective and healing effects. This can occur via suppression of inflammatory response e.g. by restricting monocyte and neutrophil infiltration (Liu et al., 2008) or decreasing the TLR signaling by regulating their accessibility (Ebid et al., 2014), and on the other hand, by enhancing immunosuppression (Asari et al., 2010).

2.5.6 HA fragments and their function

Fragmentation of HA occurs during tissue remodeling, in both normal and different disease conditions like wound healing and tumorigenesis, and this drastically alters the

effect and function of HA (Stern et al., 2006; Tolg et al., 2014b). This is an identical effect that can be observed also with extracellular matrikines, e.g. collagen-derived fragments during tissue remodeling in different conditions (Monboisse et al., 2014). The HA fragments can be categorized as intermediate i.e. medium MW HA (MMW HA) of 250-1000 kDa, and short HA of low MW HA (LMW) of 10-250 kDa (Monslow et al., 2015). Even smaller fragments than LMW HA exist and they are called HA oligomers (Stern et al., 2006). There is no consensus considering the grouping of HA MWs. The HA MW distrubution also varies between different tissues and ratio of different groups changes e.g., during disease progression (Monslow et al., 2015). HA fragmentation can result from the action of various hyaluronidases (HYALs), which in mammals are HYAL-1, -2, -3, -4, P1 and PH20 (Csoka et al., 2001). The HYAL-1 and -2 are the major hyaluronidases in somatic tissues and they work sequentially; first HYAL-2 (extracellular) degrades HA into fragments of up 20 kDa and then HYAL-1 (intracellular) reduces them into fragments up to 800 Da (Csoka et al., 2001; Vigetti et al., 2014a). More precisely, HYAL-2 is linked by a glycosylphosphatidylinositol (GPI) -anchor to the outer cell membrane and digests HMW HA into large HA fragments in unique acidic endocytic vesicles (Csoka et al., 2001; Tammi et al., 2001). Subsequently, these fragments are transported intracellularly via endosomal system and further digested in lysosomes to LMW HA fragments by HYAL-1 together with the exoglycosidase β-hexosaminidase (Csoka et al., 2001; Tammi et al., 2001; Schulze et al., 2009; Gushulak et al., 2012). Previously mentioned HA-binding receptor CD44 has also been shown to be a prerequisite in the uptake and degradation of HA as the fragments are usually transferred into cells via CD44-mediated action (Culty et al., 1992; Vigetti et al., 2014a). Different HYALs and their functions have been comprehensively reviewed by Stern and Jedrzeras (Stern and Jedrzejas, 2006). Fragments of HA can also be the consequence of the release of reactive oxygen species (ROS) subsequent to oxidative stress during tissue injuries (Agren et al., 1997; Soltes et al., 2006).

As the HMW HA is produced in homeostatic tissues and is anti-inflammatory, the HA fragments are abundantly present in sites of tissue catabolism and promote inflammation (Stern et al., 2006; Jiang et al., 2011; Bollyky et al., 2012; Petrey and de la Motte, 2014). Furthermore, HA fragments possess angiogenic properties and are able to induce endothelial differentiation and proliferation, and capillary formation through e.g. CD44- and RHAMM-mediated signaling (Takahashi et al., 2005; Matou-Nasri et al., 2009; Afratis et al., 2012).

On the other hand, HA fragments have also been found to possess anti-tumorigenic properties (Monslow et al., 2015). For instance, MMW HA is able to induce apoptosis in glioma via CD44-mediated signaling (Yang et al., 2002). In parallel, LMW HA is able to inhibit tumor cell proliferation in colorectal cancer and to stimulate immune response (Alaniz et al., 2009). Interestingly, very small HA oligosaccharides have been

reported to be able to either promote or inhibit tumor progression probably due to the vast heterogeneity of different tumors (Stern et al., 2006). In addition, LMW HA has also been associated with attenuation of osteoclast activity thus suggesting possible anti-osteoporotic effects (Lee et al., 2014).

However, very few studies have concentrated on the determination of the molecular size of HA and its fragments during tissue remodeling *in vivo*. On the other hand, *in vitro* studies have demonstrated that in fact the biological effect of HA size heterogeneity is more likely related to the capability of different receptors to bind various sizes of HA (Tolg et al., 2014a). Based on the binding abilities, it can be said that in general CD44 binds HMW HA, while RHAMM binds smaller fragments of HA and TLR 2 and 4 even smaller fragments, respectively (Vigetti et al., 2014a). Smaller fragments of HA have also been suggested to inhibit the binding of HMW HA to CD44 in addition to binding to the receptors themselves, thus further influencing the integrity of the tissues (Urakawa et al., 2012; Ruppert et al., 2014). Additionally, for example various skin cancer cells and tissues have also shown to be able to express different CD44 variant isoforms in addition to the CD44 standard form (CD44s) (Dietrich et al., 1997). These receptors are subsequently activated by small HA fragments resulting in inflammation and cancer cell proliferation which leads to tumor progression (Bourguignon and Bikle, 2015).

As discussed above, HA is involved in the regulation of various structural and physiological functions of tissues and contributes to several biological processes including morphogenesis, tissue remodeling and angiogenesis (Pardue et al., 2008; Dicker et al., 2014). In addition, similarly to the role of decorin, HA also has a versatile pathological role in various diseases, e.g. in cancer and cardiovascular diseases (Itano, 2008; Monslow et al., 2015). HA-associated excessive and detrimental vascularization occurs particularly in cancer where it promotes tumor growth and metastasis, as well as in proliferative diabetic retinopathy and atherosclerosis (Slevin et al., 2007). The effect of HA in cancer and vascular diseases including diabetic macroangiopathy and atherosclerosis are discussed further in the next two chapter.

2.6 HA in cancer

2.6.1 Function of HA in tumorigenesis

As discussed in the beginning (see chapter of the Extracellular matrix), tumorigenesis requires the development of pro-tumorigenic microenvironment. Both malignant and non-transformed cells including stromal cells participate in the remodeling of the ECM leading to the development of disorganized and deregulated tumor microenvironment (TME) (Balkwill et al., 2012; Lu et al., 2012). HA represents one of the major ECM

macromolecules in various epithelial and mesenchymal human tumors (Toole et al., 2002; Stern et al., 2006; Itano et al., 2008; Kouvidi et al., 2014). Excess HA is produced by cancer cells themselves or by stromal cells harnessed for HA production by the malignant cells (Stern, 2005; Dicker et al., 2014). Specifically, cancer associated fibroblasts have been reported to express all three HASes (Auvinen et al., 2014). Accumulation of HA in addition to other ECM macromolecules in the pericellular matrix of various tumors is called desmoplasia (Sainio and Järveläinen, 2013). Furthermore, abundant HA is often also present in the invasive front of tumors like breast cancers (Bertrand et al., 1992; Sainio and Järveläinen, 2013). As discussed above, HA is among other things able to increase cellular proliferation and migration, and to decrease apoptosis. Cancer cells can also create a HA-rich pericellular matrix to prevent the cytotoxic effects of immune system and on the other hand, huge cable-like HA aggregates of HMW HA are able to bind tissue macrophages and alter their behavior favoring tumor growth (Tammi et al., 2008; Dicker et al., 2014).

2.6.2 Accumulation of HA

The effect of excess HA can differ depending on the tissue type of the tumor origin. In other words, in malignancies developing from cells and stroma which normally are low in HA content, like single layered epithelium, the accumulation of HA strongly correlates with progression and poorer prognosis of the disease (Tammi et al., 2008; Sironen et al., 2011). These diseases include cancer types such as colon and gastric cancers and also prostate, ovarian and breast cancers (Tammi et al., 2008). Furthermore, in colon and gastric cancer, accumulation of HA occurs in the epithelium itself, whereas in prostate, ovarian and breast cancer, the volume of HA increases in the stroma (Tammi et al., 2008). However, tumors which develop from stratified epithelia, exhibit quite opposite trends. They already display moderate or substantial HA production and cell-associated HA decreases during tumor progression (Tammi et al., 2008; Sironen et al., 2011). This happens in cancers like squamous cell carcinomas (SCCs) of mouth, skin, and larynx.

The effect of HA accumulation can be predicted by the quality of the excess HA. Opposite effect on the accumulation of pro-inflammatory fragmented HA is provided by HMW HA, which in addition to the previously mentioned anti-inflammatory and anti-angiogenic functions, also possesses evident protective effect on tumorigenesis (Monslow et al., 2015). For example, HMW HA has been demonstrated to reduce the regrowth of human colon carcinoma xenografts after chemotherapy (Mueller et al., 2010) and to decrease the migration capacity of fibrosarcoma cells (Berdiaki et al., 2009). Furthermore, the production of extremely HMW HA (6-12 MDa) has been demonstrated to mediate significant cancer resistance in the naked mole rat (Tian et al., 2013; Fisher, 2015).

2.6.3 Mechanisms regulating the amount of HA in cancer

Mechanisms behind the increased or reduced amounts of HA in different cancers are complex and not yet fully understood. As discussed in the beginning of the chapter, different HASes are known to be responsible for HA synthesis. Interestingly, various growth factors have been observed to influence HAS activation. For example in breast cancer, the activation of fibroblast growth factor receptor (FGFR) has been shown to induce HA accumulation via HAS2 upregulation (Bohrer et al., 2014). Similar upregulation of both HAS1 and HAS2 leading to HA accumulation has been achieved in fibrosarcoma cells by FGF, PDGF and TGF-β (Berdiaki et al., 2009). In various cancer cells, also aberrant post-transcriptional splicing of HAS1 has been shown to increase both extracellular and intracellular HA promoting tumorigenesis (Adamia et al., 2014).

Alternatively, different HYALs are known to regulate the amount of HA and its fragmentation. They are also known to act as tumor promoters in several cancer types such as bladder (Kramer et al., 2010) and breast cancer (Tan et al., 2011). Subsequently, HA fragments are able to induce inflammation and cancer progression. Regarding inflammation, e.g. in breast cancer, high number of macrophages have been shown to correlate with hyaluronan accumulation and poor outcome indicating stimulation of inflammatory response and cancer progression (Tiainen et al., 2015).

2.7 HA in vascular diseases

2.7.1 Function of HA in vascular biology

In addition to other ECM macromolecules such as decorin, HA is known to play a pivotal role in vascular biology (West and Kumar, 1989; Vigetti et al., 2008). Furthermore, similarly to decorin, HA is an important part of the glycocalyx of the vascular endothelium and the ECM of the underlying tissue (Reitsma et al., 2007; Weinbaum et al., 2007; Gao and Lipowsky, 2010). Via maintaining the intact glycocalyx, HA is able to provide an endothelial surface with a non-adherent shield (Lennon and Singleton, 2011). Subsequently, the glycocalyx is able to regulate many actions such as anti-inflammatory and anti-thrombotic actions by inhibiting leukocyte (Constantinescu et al., 2003) and platelet adhesion (Vink et al., 2000), and to influence the overall vascular permeability (Henry and Duling, 1999; Rahbar et al., 2015). Altogether, maintenance of the vascular integrity is one of the fundamental roles of HA (Lennon and Singleton, 2011).

In normal vessels, HA is present only in low amounts, but its amount is dramatically increased in vascular diseases (Vigetti et al., 2008; Wight, 2008). The effect of HA on angiogenesis, similarly to its effects on other biological processes, depends on its molecular weight. The HMW HA acts as an anti-angiogenic molecule by forming a HA-rich pericellular sheaths surrounding the cells and thus, it restricts cellular interactions

and binding of signaling molecules to their receptors (Pardue et al., 2008). On the other hand, degraded HA and particularly HA oligosaccharides have been shown to be pro-angiogenic and able to stimulate EC proliferation, migration and neovascularization (West et al., 1985; Lokeshwar and Selzer, 2000; Takahashi et al., 2005; Genasetti et al., 2008; Jiang et al., 2011). This effect of HA on EC behavior has been established to be mediated via interaction with CD44 (Cao et al., 2006; Genasetti et al., 2008). In certain conditions also opposite effects have been detected; HMW HA has been shown to act as pro-angiogenic factor and HA oligosaccharides have revealed anti-angiogenic features (Fuchs et al., 2013). Additionally, the effect of HA on angiogenesis has been shown to be context dependent, i.e. depending particularly on the growth factors and chemokines of the microenvironment (Fuchs et al., 2013).

2.7.2 Atherosclerosis and HA

Atherosclerosis is a condition of chronic vascular wall inflammation that can lead to the narrowing of the vessel lumen due to accumulation of ECM macromolecules, which then trap lipoproteins and various inflammatory factors and growth factors from the circulation (Vijayagopal et al., 1996; Ross, 1999; Ramji and Davies, 2015). Subsequently, the core of the atheromatous plaque is formed by cholesterol crystals with foam cells surrounded by remodeled ECM (Yu et al., 2013). Lipoproteins trapped in the arterial wall undergo modifications like oxidation and are able to stimulate HA synthesis (Viola et al., 2013). Indeed, atherosclerosis is characterized by marked changes in the distribution and content of HA (Toole et al., 2002; Otsuka et al., 2015).

The onset of atherosclerosis is characterized by the migration of aortic smooth muscle cells towards the intima layer of the vessel wall (Wight, 2008), and the formation of HA-rich pericellular matrix has been shown to be a requirement for the migration and proliferation of VSMCs (Evanko and Wight, 1999; Evanko et al., 2007). In atherosclerosis, HA is able to form HA aggregates which can bind tissue macrophages thereby promoting leucocyte retention in the ECM (Wilkinson et al., 2006). Thus, it is not surprising that HA is found in atherosclerotic areas which also contain inflammatory cells like lymphocytes and macrophages (Toole et al., 2002). In early atherosclerotic lesions HA is usually accompanied with CD44 (Lennon and Singleton, 2011) and HABPs like versican (Wolf et al., 1994; Toole et al., 2002; Karangelis et al., 2010). Furthermore, accumulation of HA in the tunica media and subsequent thinning of the elastic lamellae in the arterial wall have been shown to increase e.g. the mechanical stiffness of the aortic wall and thus accelerate the progression of atherosclerosis (Chai et al., 2005). In addition, HA interacts with platelets (van Gils et al., 2009; Ombrello et al., 2010) which cleave HMW HA into smaller fragments that subsequently promote inflammation (Sadowitz et al., 2012). Indeed, the function of HMW HA, LWM HA and HA metabolism have been indicated as central regulators of atherosclerotic plaque stability (Bot et al., 2008; Bot et al., 2010).

2.7.3 Diabetes and HA

Diabetes mellitus (DM) is a group of metabolic diseases of different etiology where the body is unable to properly use and store glucose leading to hyperglycemia. Subsequently, hyperglycemia causes damages to vascular integrity leading to macro- and microangiopathy which are the main complications of the disease (Fowler, 2008; Lennon and Singleton, 2011). Both quantitative and qualitative changes can be observed in the vascular wall in response to diabetes, e.g. increase in intimal area and accumulation of HA (McDonald et al., 2007). The accumulation of HA has also been shown to contribute to insulin resistence (Kang et al., 2013). In addition to HA, the amount of CD44 has been found to be increased in a mouse model of diabetes (Campo et al., 2010). Furthermore, hyperglycemia has been shown to induce formation of HA structures that promote monocyte adhesion (Wang and Hascall, 2004). Interestingly, the accumulation of HA has just recently been suggested to act also as a mediator of inflammation in pancreatic islets leading to β-cell loss in both type 1 and 2 diabetes (Hull et al., 2015).

Earlier, serum of type 1 and 2 diabetic patients has been demonstrated to stimulate HA synthesis of HUVSMCs (Järveläinen et al., 1986; Järveläinen et al., 1987). On the other hand, increased plasma levels of hyaluronidase has also been observed in type 1 (Nieuwdorp et al., 2007) and type 2 diabetic patients (Broekhuizen et al., 2010). Furthermore, vascular dysfunction has been found in association with elevated HA serum levels in type 1 (Nieuwdorp et al., 2007) and type 2 diabetes (Morita et al., 2014). Indeed, the serum HA level has been suggested to be useful as a marker of diabetic angiopathy (Mine et al., 2006).

In healthy blood vessel, the vascular integrity is maintained among other things by endothelial glycocalyx (Lennon and Singleton, 2011), which forms a physical barrier between the vascular endothelium and the flowing blood (Lemkes et al., 2012). Hyperglycemia has been shown to be able to increase the shedding of the glycocalyx of diabetic patients and to increase their vascular permeability (Nieuwdorp et al., 2006; Broekhuizen et al., 2010). The damaging mechanisms are still unknown, but e.g. increased formation of reactive oxygen species (ROS) has been suggested (Singh et al., 2013) as well as the action of different serine proteases in addition to HYALs (Becker et al., 2015). Furthermore, shedding of the glycocalyx has been shown to produce LWM HA which is then able to promote inflammation as mentioned above. As a summary, endothelial dysfunction and changes in vessel wall permeability, blood flow and pressure are acknowledged causes for diabetes associated angiopathy (Perrin et al., 2007; Lemkes et al., 2012). However, despite the vast research on diabetic complications, the specific mechanisms behind diabetic macroangiopathy are still not exactly known.

3. AIMS OF THE STUDY

Macromolecules of the ECM have increasingly been shown to act as crucial mediators and regulators in the development and progression of various malignant and cardiovascular diseases. The purpose of this study was to examine the importance of two essential ECM macromolecules, decorin and HA in cancer and diabetic macroangiopathy, respectively. The specific aims of this study were:

1. To investigate the involvement of decorin in tumor angiogenesis comparing its expression between benign and malignant vascular growths in man *in vivo.*

2. To localize decorin expression in human epithelial tumors, namely in breast and bladder tumors *in vivo*, and to examine, whether adenovirus-mediated decorin transduction influences the behavior of these cancer cells *in vitro*.

3. To examine decorin expression during the early differentiation phase of hESCs into embryonic bodies *in vitro*, and to localize decorin expression in different human GCTs *in vivo*.

4. To investigate the effect of high glucose concentration on the gene expression profile of HUVSMCs and subsequently its ability to influence HUVSMC-mediated remodeling of the ECM.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Tissue samples

Table 2. Tissue samples used in the studies.

Tissue	Histology	Number of samples	Study
Vascular tumors	Cavernous hemangioma	4	I
	Capillary hemangioma	4	I
	Kaposi´s sarcoma	7	I
	Angiosarcoma	7	I
Breast tissues	Normal breast tissue	3	II
	Intraductal papilloma	3	II
	Ductal breast cancer	49	II
	Lobular breast cancer	11	II
	Breast cancer subtypes	9	II
Bladder cancers	Urothelial carcinoma	191	III
	Squamous cell carcinoma	5	III
	Adenocarcinoma	3	III
Testis tissues	Normal testis tissue	1	IV
	Benign teratoma	2	IV
	Malignant teratoma	6	IV
	Embryonal carcinoma	1	IV
	Mixed germ cell tumor	2	IV

Tissue samples for the study I were obtained from the archives of the Department of Pathology, Turku University Hospital, Turku, Finland and for the studies II-IV from Auria Biopank, Turku, Finland, respectively (see original publications for details).

4.1.2 Cell lines

Human cancer cell lines (Table 3) used in the studies II-III were purchased from American Type Culture Collection (ATCC). VSMCs used in the study V were derived from the human umbilical cord (Ihalainen et al., 2007). Cell lines were cultured as presented in the original publications.

Table 3. Cell line specifics used in different studies.

Cell line	Cell type	Species	Study
HUVSMC	vascular smooth muscle cells	human	V
MCF7	breast adenocarcinoma cells	human	II
RT-4	urinary bladder cancer cells	human	III
T24	urinary bladder cancer cells	human	III
5637	urinary bladder cancer cells	human	III

4.1.3 Primary antibodies

Table 4. Primary antibodies used in IHC stainings.

Molecule	Trade name/ obtained from	Species	Dilution	Study
Biglycan	L-15 / Santa Cruz	goat polyclonal	1:200/1:100	III
CD30	Ber-2 / Ventana, Roche	mouse monoclonal	ready-to-use	IV
CD31	BioGenex	mouse monoclonal	1:20	I
CkPan	AE1/AE3/PCK26 / Ventana, Roche	mouse monoclonal	ready-to-use	IV
Decorin	LF-136/ Dr. Larry Fisher, NIDR, USA	rabbit polyclonal	1:400	I
Decorin	H-80 / Santa Cruz	rabbit polyclonal	1:400 / 1:50	III / IV
EGFR	sc-03 / Santa Cruz	rabbit polyclonal	1:50	I
Estrogen receptor	SP1 / Ventana, Roche	rabbit monoclonal	ready-to-use	II
HER-2/neu	4B5 / Ventana, Roche	rabbit monoclonal	ready-to-use	II
Ki-67	30-9 / Ventana, Roche	rabbit monoclonal	ready-to-use / 1:200	II / III
p63	4A4 / Ventana, Roche	mouse monoclonal	ready-to-use	II
PLAP	NB10 / Ventana, Roche	mouse monoclonal	ready-to-use	IV
Progesteroni	1E2 / Ventana, Roche	rabbit monoclonal	ready-to-use	II
Type I collagen	LF-67 / Dr. Larry Fisher, NIDR, USA	rabbit polyclonal	1:100	I

4.1.4 Primers in real-time quantitative PCR

Table 5. Primer pair used in RT-qPCR analyses.

Target	5´-> 3´ sequence	Study
Decorin	Rev GAGTTGTGTCAGGGGGAAGA For GGACCGTTTCAACAGAGAGG	II, III
GNB2L1	Rev GCTTGCAGTTAGCCAGGTTC For GAGTGTGGCCTTCTCCTCTG	II, III
hHAS1	Rev TAAGAACGAGGAGAAAGCAG For CAAGATTCTTCAGTCTGGAC	V
hHAS2	Rev CAGAATCCAAACAGACAGTTC For TAAGGTGTTGTGTGTGACTG	V
hHAS3	Rev CTTAAGGGTTGCTTGCTTGC For GTTCGTGGGAGATAAGGAA	V
hArpo	Rev AGATGCAGCAGATCCGCAT For GTGGTGATACCTAAAGCCTG	V

4.2 Methods

Methods used in this study are presented in detail in the original publications. A summary of the different methods is listed in table 6.

Table 6. List of methods used in the studies I-V.

Method	Study
Adenoviral decorin transduction	II, III
Actin staining	V
Collagen gel contraction assay, CGC	V
DNA extraction	III, IV
DNA methylation analysis	III, IV
Enzyme-linked sorbent assay	V
GeneSapiens database analysis	II, III
Immunohistochemistry, ICC / IHC	I / II-IV
In situ hybridization, ISH	I-IV
IST Online database analysis	IV
Particle-exclusion test	V
Real-time quantitative PCR, RT-qPCR	II, III,V
RNA extraction	II, III,V
RNA microarray analysis	V
Trypan blue exclusion test	V

5. RESULTS

5.1 Localization of decorin gene expression and immunoreactivity in different human tumors of mesenchymal and epithelial origin (I-III)

5.1.1 Decorin in benign and malignant human vascular tumors (I)

With the aim to examine whether decorin is involved in tumor angiogenesis, its expression and immunoreactivity were localized in various human vascular tumors representing benign cavernous and capillary hemangiomas, Kaposi´s sarcoma and angiosarcoma. Figure 4 below is a panel of images representing cavernous hemangioma where decorin expression and immunoreactivity can be seen within the tumor mass, particularly in the stromal area between the intratumoral blood vessels. Identically, the tumor tissue in capillary hemangioma samples showed positivity to both the expression and the immunoreactivity for decorin (see study I: Figure 3).

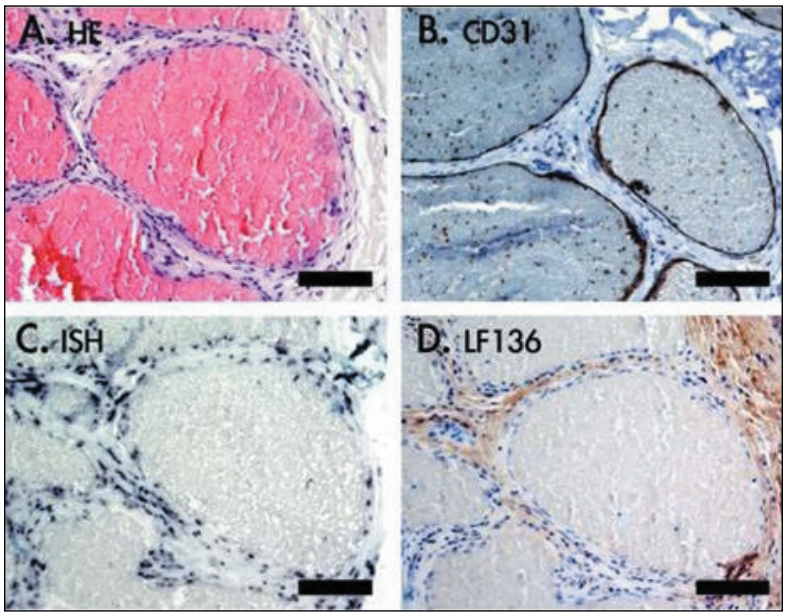

Figure 4. Stromal tissue in cavernous hemangioma is positive for decorin expression and immunoreactivity. A. HE staining of a representative human cutaneous cavernous hemangioma tissue. **B.** IHC for endothelial cell marker, CD31. **C.** In situ hybridization (ISH) for decorin mRNA. **D.** IHC for decorin with a LF-136 antiserum for decorin. Positive ISH reactions can be seen in purple, and IHC reactions in brown, respectively. Scale bar in A-D = 100 μm.

Instead, no detectable decorin mRNA expression or decorin immunoreactivity were present in tissue samples representing malignant human vascular tumors, namely Kaposi´s sarcoma (see study I: Figure 1) or angiosarcoma (see study I: Figure 2). In

these malignant vascular tumors, decorin was expressed merely in the connective tissue stroma lining the sarcoma tissue. In addition, strong desmoplastic reaction was observed particularly in tissue samples of Kaposi´s sarcoma (Figure 5).

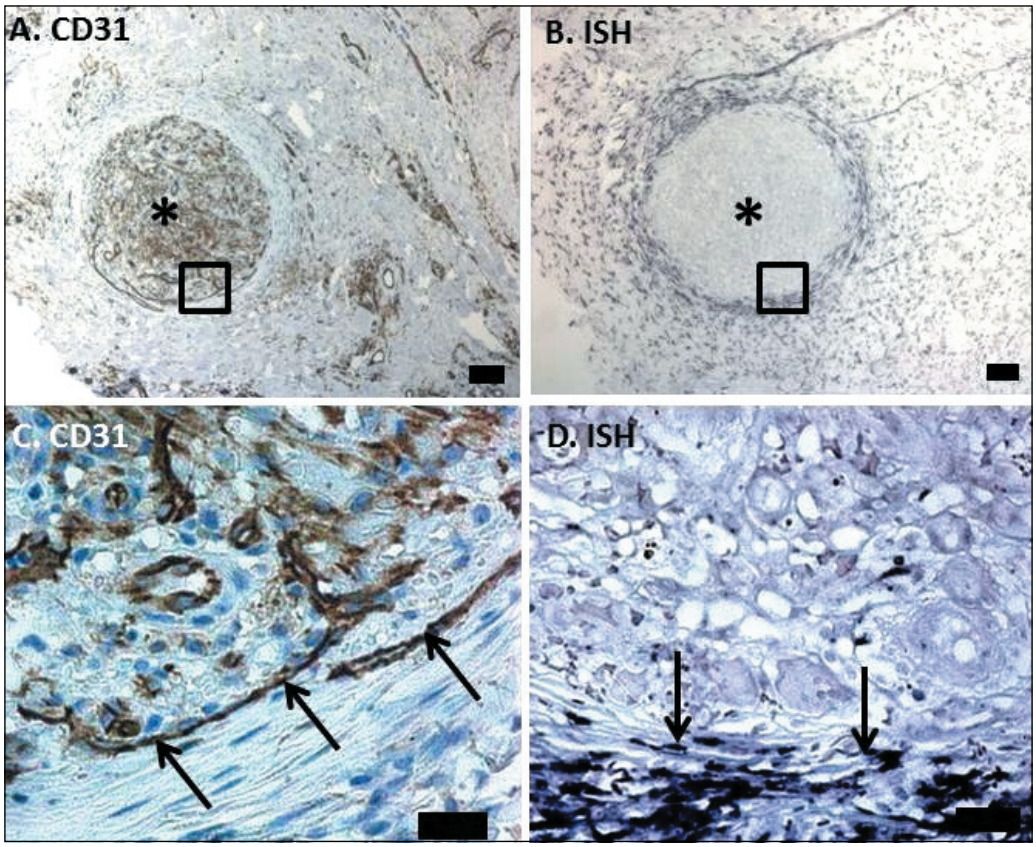

Figure 5. Kaposi´s sarcoma tissue lacks both decorin expression and decorin immunoreactivity. A. IHC for endothelial cell marker CD31 staining of a representative Kaposi´s sarcoma tissue sample. **B.** ISH for decorin. **C.** IHC for CD31. **D.** ISH for decorin. Images C and D are magnified areas from the indicated areas in image A and B, respectively. Positive IHC reaction can be seen in brown and positive DIG reaction in ISH in purple. The asterisks in A and B indicate areas of Kaposi´s sarcoma tissue and the arrows in C and D indicate the border between the sarcoma and its surrounding tissue. Note that Kaposi´s sarcoma is totally devoid of decorin expression. Scale bar in A and B = 100 µm and in C and D = 25 µm.

As a summary, decorin expression and immunoreactivity were detected only in benign hemangioma samples where angiogenesis has ceased. However, in malignant vascular tumor tissue samples which exhibit powerful angiogenesis, no decorin expression or immunoreactivity was observed. This indicates that decorin is likely to possess an anti-angiogenic effect on human tumor angiogenesis *in vivo*. Decorin-rich desmoplastic reaction surrounding particularly Kaposi's sarcoma together with the lack of decorin in the malignant tissue provides also an applicable tool for using decorin as a biomarker between healthy and malignant vascular tissue.

5.1.2 Decorin in human breast cancer (II)

Decorin expression was also studied in various breast tissue samples representing normal human breast tissue, intraductal papilloma, ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC) and invasive mucinous carcinoma. Preliminary analyses using GeneSapiens database suggested that decorin expression in different human breast cancers is decreased compared to healthy breast tissue but is still at a significant level in all types of human breast cancer (see study II: Figure 1). However, Figure 6 below demonstrates that decorin gene expression is totally lacking in the areas of human DCIS tissue. Identical results were obtained from all benign and malignant human breast tumor samples (see study II: Figures 2-5).

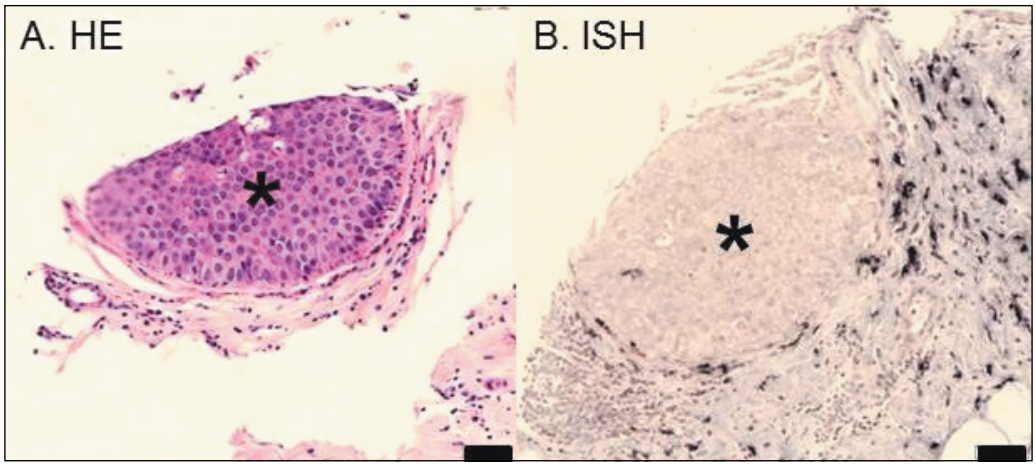

Figure 6. Human ductal carcinoma in situ (DCIS) cells do not express decorin mRNA. A. HE staining. **B.** ISH for decorin. Positive DIG reaction can be seen in ISH in purple. The asterisks indicate the area of cancer tissue. Note that malignant cells totally lack decorin expression. Scale bar in A and B = 50 µm.

From these results it can be concluded that decorin expression takes place solely in the cells of the original stroma, and not at all in the areas of benign or malignant breast tumor tissue.

5.1.3 Decorin in human bladder cancer (III)

Decorin expression was also studied in invasive and non-invasive human bladder cancer tissue samples representing urothelial carcinoma, squamous cell carcinoma and adenocarcinoma. Similarly to human breast cancer, preliminary screening using GeneSapiens database suggested that decorin expression is decreased in bladder cancer compared to normal bladder tissue (see study III: Figure 1). However, as seen in Figure 7 below, non-invasive in situ bladder cancer cells do not express decorin at all. Identical results were obtained from invasive bladder cancer tissue samples (see study III: Figure 2).

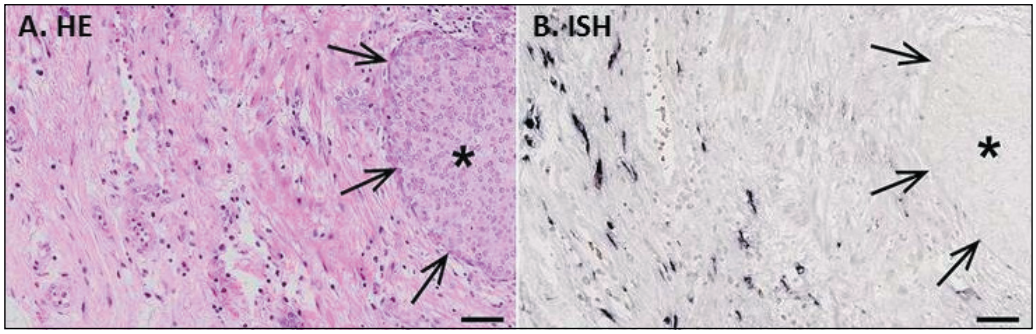

Figure 7. Non-invasive human in situ bladder cancer cells do not express decorin. A. HE staining. **B.** ISH for decorin. Positive DIG reaction can be seen in ISH in purple. The arrows mark the lining between healthy and cancerous tissue while the asterisks indicate the area of cancer. Note that the malignant cells totally lack decorin expression. Scale bar in A and B = 50 µm.

Parallel to the results obtained from human breast tumor samples, in human bladder cancer samples decorin is expressed only by the cells of the original stroma, and not by the cancer cells.

5.2 Decorin expression in different human cancer cells lines and the effect of adenoviral decorin transduction on their behavior (II-III)

5.2.1 Human breast adenocarcinoma cells (II)

First, a widely used human breast adenocarcinoma cell line, MCF7, was shown not to express decorin *in vitro*. Thereafter, these cells were transduced with a decorin adenovirus resulting in that they exhibited a markedly changed growth pattern including the loss of cohesion (Figure 8). Decorin-transduced cells also exhibited increased apoptosis and a significant decrease in their proliferation rate (see study II: Figure 6).

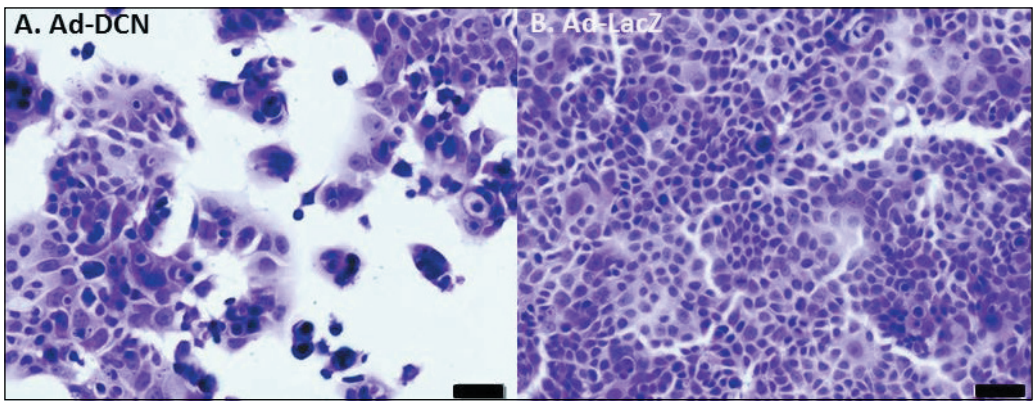

Figure 8. Adenovirus-mediated decorin gene transduction markedly modulates the growth pattern of MCF7 cells. HE staining of cells transduced with human decorin adenoviral vector (Ad-DCN) (**A**) and LacZ gene adenoviral vector (Ad-LacZ) (**B**). Note that decorin transduced cells exhibit a marked decrease in cellular cohesion. Scale bar in A and B = 50 µm.

As a conclusion, transduction of decorin-negative MCF7 cells with an adenoviral decorin vector markedly modulates their growth pattern. Thus, decorin may represent a potential ECM molecule in adenoviral-based gene therapy in the future.

5.2.2 Human bladder cancer cells (III)

Similarly to breast cancer cell line, human bladder cancer cell lines (RT-4, 5637, and T24) were shown not to express decorin *in vitro*. Subsequently to decorin adenoviral transduction, bladder cancer cells exhibited considerable changes in their growth pattern including a marked decrease in cohesion and cell number (Figure 9). Also the proliferation index of the cells was significantly decreased in response to decorin transduction (see study III: Figure 6). In an attempt to examine mechanisms behind the lack of decorin expression in bladder cancer, decorin gene promoter methylation was analyzed in these cells. However, no methylation of the decorin gene promoter was detected (see study III: Figure 1). This suggests that methylation does not play a role in the regulation of decorin expression in human bladder cancer cells.

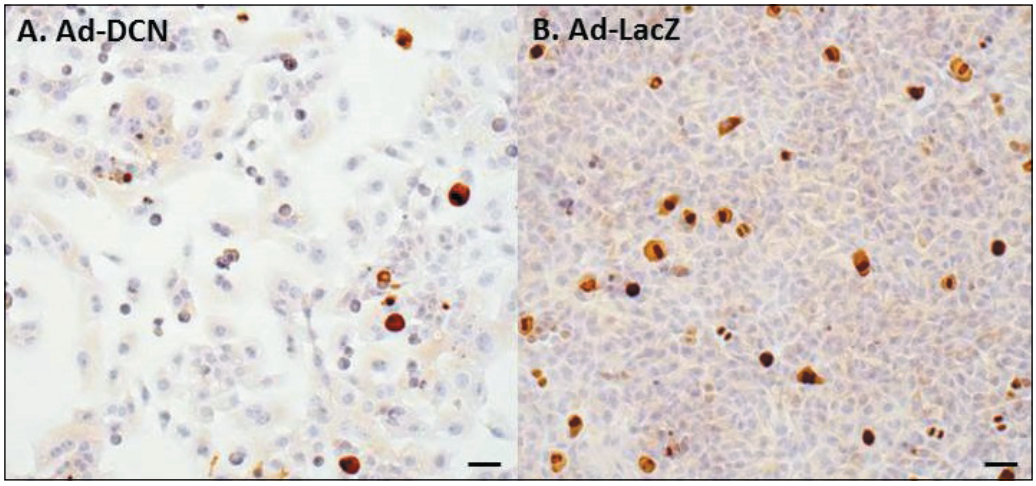

Figure 9. T. Transduction with a decorin gene markedly modulates the growth pattern of T24 bladder cancer cells. Ki-67 staining of cells transduced with human decorin adenoviral vector (Ad-DCN) (**A**) and LacZ gene adenoviral vector (Ad-LacZ) (**B**). Brown color indicates Ki-67 positive cells. Note that decorin transduction resulted in a marked decrease in both cohesion and cell number. Scale bar in A and B = 50 µm.

Taken together, the observed powerful anti-proliferative effect of adenovirus-mediated decorin gene transduction on decorin-negative bladder cancer cell lines offers new potential therapeutical tools in the treatment of human urothelial malignancies.

5.3 Decorin expression in human embryonic stem cells and in embryonal germ cell tumors (IV)

5.3.1 Human embryonic stem cells (hESCs)

Decorin expression is not detected in undifferentiated hESCs in either normal or abnormal cell types *in vitro* (Enver et al., 2005a). However, during the differentiation of normal hESCs into embryonic bodies (EBs, three-dimensional aggregates of hESCs), decorin expression is markedly induced (see study V: Figure 1). No similar induction can be observed during the differentiation of culture-adapted, so-called abnormal hESCs.

5.3.2 Human embryonic germ cell tumors (GCTs)

Preliminary screening using IST Online (updated version of Gene Sapiens database) showed that the expression of decorin is significantly higher in benign teratoma tissue samples than in healthy testis or in embryonal carcinomas. To investigate this notion *in vivo*, decorin expression was examined using normal human testis tissues and different benign and malignant human embryonic tumors. In healthy human testis, decorin expression was seen in stromal myofibroblasts surrounding the seminiferous tubules and in Leydig cells residing in the loose connective tissue between the tubules (Figure 10).

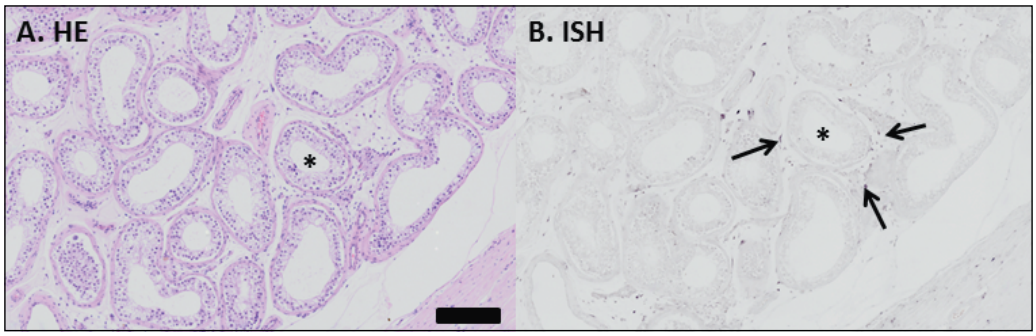

Figure 10. Decorin expression in healthy human testis. A. HE staining. **B.** ISH for decorin. Positive DIG reaction can be seen in ISH in purple. The asterisk in A and B mark a seminiferous tubule and the arrows in image B indicate decorin positive Leydig cells surrounding the tubule. Scale bar in A and B = 200 µm.

Decorin expression was also located in benign and malignant human embryonal germ cell tumors (GCTs) derived from testis tissues. In benign teratoma tissues, stromal cells were positive for decorin throughout the tumor samples (see study V: Figure 4). However, in malignant teratomas, embryonal carcinomas and mixed germ cell tumors decorin expression was totally lacking from all malignant cells. This was particularly clear in the tissue sample representing embryonal carcinoma tissue (Figure 11). Embryonal carcinoma tissue was characterized using established IHC markers for placental alkaline

phosphatase (PLAP), CD30 and low-molecular-weight keratins (broad spectrum CK antibody, CkPan) (Figure 11).

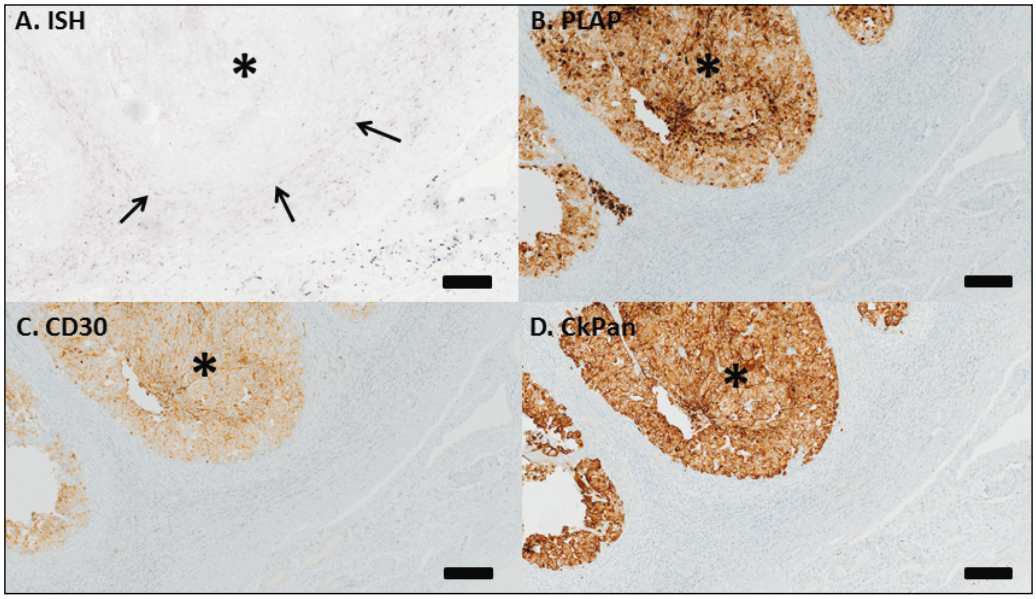

Figure 11. Decorin expression in human embryonal carcinoma. A. ISH for decorin. **B.** IHC for PLAP. **C.** IHC for CD30. **D.** IHC for CkPan. The asterisks mark the area of embryonal carcinoma and the arrows in image A indicate the border of the carcinoma tissue. Note that embryonal carcinoma cells are totally devoid of decorin expression. Scale bar in A-D = 200 µm.

These results support the previous observations that decorin expression is associated with the differentiation process of embryonal cells. Furthermore, the results provide evidence for the anti-oncogenic role of decorin in different malignant embryonic testicular tumors in man.

5.4 High glucose concentration stimulates hyaluronan synthesis in human vascular smooth muscle cells (HUVSMCs) (V)

5.4.1 High glucose concentration modulates the ability of HUVSMCs to re-organize collagen-rich matrix

With the aim of examining whether high glucose concentration can modulate the capacity of HUVSMCs to reorganize the ECM, the cells were first divided into two groups: normal glucose concentration (NG, 5 mmol/l) was considered as the physiological condition of a healthy person, and high glucose concentration (HG, 25 mmol/) mimicked the condition of diabetic patient in poor glycemic control. Results of the CGC assay clearly demonstrated that compared to the cells of NG group, the cells exposed to high glucose

concentration had a significantly decreased capability to contract collagen-rich matrix (see study I: Figure 1). This effect was also seen at the cytoskeletal level as a weaker staining of the F-actin of cells of the HG group (see study I: Figure 2).

5.4.2 High glucose concentration increases HA production in HUVSMCs and modulates their behavior

The mRNA profiles of HUVSMCs of NG and HG groups were studied using a whole genome RNA microarray (Illumina, Inc., San Diego, CA, USA). When focusing on the expression of ECM macromolecules, particularly hyaluronan synthase 2 (HAS2) was shown to exhibit a significant increase after treatment of cells with high glucose. This result was verified with RT-qPCR. In addition to HAS2, the expressions of HAS1 and HAS3 were also increased (see study V: Figure 4). Subsequently, increased production of HA by HUVSMCs was detected in the medium of HG cultures (see study V: Figure 5). The HA-rich pericellular matrix in HG-cultures was visualized by particle-exclusion test (Figure 12 below). When the cells of HG group were treated with hyaluronidase (degrades HA) or 4-methylumbelliferone (HA synthesis inhibitor), the capability of the cells to contract collagen gel was restored. This confirmed the central role of HA in the modulation of HUVSMC behavior.

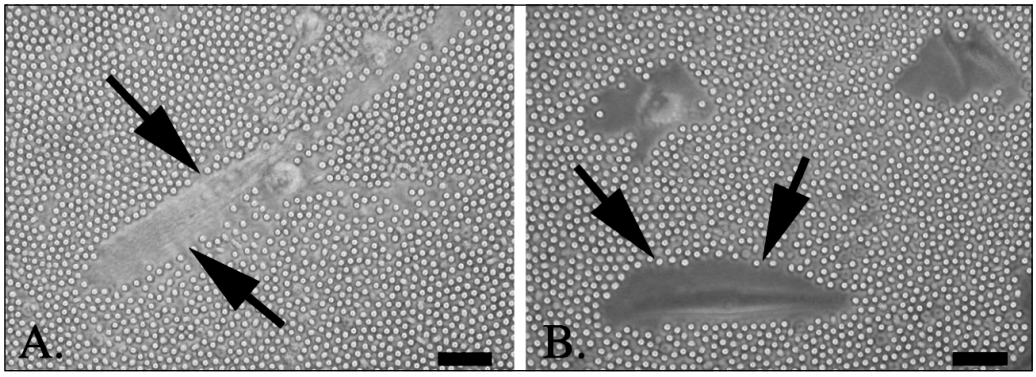

Figure 12. HA-rich pericellular matrix. A. HUVSMCs grown in normal glucose concentration. **B.** HUVSMCs grown in high glucose concentration. Note that HA-rich pericellular matrix surrounds HUVSMCs grown in high glucose concentration. The arrows indicate the border of the pericellular matrix. Scale bar in A and B = 100 µm. For technical details on the particle-exclusion test see original publication (study V).

6. DISCUSSION

The ECM macromolecules including decorin and HA are emerging as vital modulators of fundamental aspects in a number of malignant and cardiovascular diseases (Sofeu Feugaing et al., 2013; Otsuka et al., 2015; Theocharis et al., 2015). Regarding decorin, its expression and immunoreactivity have been found to be variously decreased and even totally missing in different types of cancer tissues (Bozoky et al., 2014; Iozzo and Schaefer, 2015). However, the exact cellular origin of decorin expression has often remained unresolved. In this study, the importance of decorin in human tumorigenesis was investigated in cancers of mesenchymal, epithelial and embryonic origin. Specifically, the involvement of decorin in tumor angiogenesis was investigated comparing its expression between benign and malignant human vascular tumors *in vivo*. Furthermore, decorin expression and decorin immunoreactivity were localized in human breast and bladder tumors *in vivo*. Using breast and bladder cancer cell lines, the effect of adenoviral mediated decorin transduction on these cells was examined *in vitro*. With the attempt to clarify factors regulating decorin expression in cancer, the methylation of decorin gene promoter in these human epithelial cancer cell lines was also evaluated. Lastly, the induction of decorin expression was studied during the early differentiation phase of normal and culture-adopted hESCs *in vitro* and decorin expression and immunoreactivity were investigated in different human GCTs *in vivo*. As for HA, it represent an important macromolecule in the progression of diabetes and its complications including diabetic macroangiopathy (Dicker et al., 2014; Hull et al., 2015). In this study, the production of HA was studied in HUVSMCs grown in high glucose concentration. This mimicked the situation of a diabetic patient in poor glycemic control. Also the effect of increased HA production on the behavior of HUVSMCs was examined.

6.1 Decorin in cancers of mesenchymal origin and in tumor angiogenesis

Decorin has a recognized role as an anti-tumorigenic molecule. However, only few studies have been executed focusing on decorin in sarcomas, i.e. cancers originating from mesenchymal cells. Previously, chondrosarcoma tissues (Söderström et al., 2002) and osteosarcoma cells (Zafiropoulos et al., 2008) have both been reported to express and produce decorin. In addition to being an anti-oncogenic molecule, decorin has also gained repute as an anti-angiogenic molecule, spesifically in tumorigenesis related angiogenesis (Järveläinen et al., 2015). Originally, decorin was shown to inhibit EC tube-like structure formation in culture (Davies Cde et al., 2001) and thereafter decorin-expressing tumor xenografts were described to exhibit significantly lower neovascularization (Grant et al., 2002). In the present study, expression and immunoreactivity of decorin were studied in different benign and malignant human vascular tumors, namely in hemangiomas,

Kaposi´s sarcomas and angiosarcomas. Results showed that both capillary and cavernous hemangiomas expressed decorin mRNA and were also positive for decorin immunoreactivity. However, their malignant counterparts, namely Kaposi´s sarcomas and angiosarcomas, were completely decorin negative. It is known that these vascular tumors exhibit different rate of angiogenesis; in benign hemagiomas neovascularization has attenuated, while in malignant sarcomas angiogenesis is frequent. Previously, decorin expression has been shown to be up-regulated in cases such as quiescence of vascular smooth muscle cells (Asundi and Dreher, 1992). In contrast, decorin is rarely expressed by actively proliferating cells (Iozzo, 1997). The data of this study support the idea that decorin displays an inhibitory role in human tumor angiogenesis *in vivo*. Previously, identical results have been reported in e.g. harmful foreign body reactions where decorin possesses anti-angiogenic properties (Järveläinen et al., 2006).

Furthermore, results of this study revealed a strong decorin expression in the border of malignant tissue, specifically in Kaposi´s sarcoma tissue. This kind of stromal reaction is called desmoplasia meaning the accumulation of a dense fibrotic tissue rich in collagen and other ECM macromolecules including specific species of PGs (Sainio and Järveläinen, 2013). The exact role of desmoplastic reaction associated with cancer development is not currently known, but it can be clearly observed in different cancers like pancreatic cancer (Merika et al., 2012). Stromal desmoplasia has been suggested to represent either a tumor restricting or promoting phenomenon (Angeli et al., 2009; Coulson-Thomas et al., 2011).

Taken together, total lack of decorin expression within sarcoma tumors and a strong decorin expression surrounding the tumor mass together offer a possibility to use decorin as a cancer biomarker. The results of this study as brought up above also support the idea of developing decorin-based therapies in inhibiting tumor angiogenesis.

6.2 Decorin in cancers and cancer cell lines of epithelial origin

In epithelial cancers like colon (Nyman et al., 2015; Suhovskih et al., 2015), breast (Leygue et al., 2000), bladder (Iozzo et al., 2011; Sanchez-Carbayo et al., 2006) and hepatic carcinomas (Duncan, 2013; Horvath et al., 2014), the expression of decorin has been reported to be significantly decreased. However, there has been somewhat conflicting results whether cancer cells in these malignancies express decorin. In this study, preliminary examination on decorin expression in different breast and bladder cancer was conducted using GeneSapiens and IST Online databanks. The results showed that based on the publicly available microarray data, the expression of decorin is higher in healthy breast and bladder tissues than in cancerous tissues. The results also showed that there is a marked expression level of decorin in cancer tissues. However, one has to remember that the mentioned databanks use total RNA extracted from tumor tissue

which usually represents only a section of the tumor mass and can also contain various amounts of surrounding original normal stroma. Thus, these analyses are unable to reveal the precise cellular origin of a molecule of interest.

Previously, decorin expression in breast cancer has been reported to be either decreased (Leygue et al., 2000; Eshchenko et al., 2007; Gu et al., 2010) or increased (Brown et al., 1999). There are also reports to demonstrate that reduced stromal decorin expression is associated with the progression of breast cancer, i.e. the amount of stromal decorin decreases during cancer progression (Oda et al., 2012). Furthermore, patients with reduced periductal decorin have been shown to have a higher risk of ductal carcinoma in situ recurrence (Van Bockstal et al., 2013). On the contrary, in in situ breast carcinomas with malignant-appearing microcalcifications, the expression of decorin has been found to be increased (Skandalis et al., 2011). In addition, using proteomic analyses, high decorin expression has been associated with among other things higher number of positive lymph nodes and a worse overall survival in breast cancer (Cawthorn et al., 2012).

Regarding decorin in bladder tissue, its expression has been shown to be especially prominent in subepithelial layers of urinary bladder during organ development and tissue differentiation in mice (Scholzen et al., 1994). In humans, strong decorin immunoreactivity has been observed in the muscular layer of superficial bladder cancer and in the original stroma adjacent to bladder cancer tissue (Iozzo et al., 2011). Additionally, decreased decorin expression has been reported in both in situ and invasive bladder cancers (Dyrskjøt et al., 2004; Sanchez-Carbayo et al., 2006). However, in *in vitro* studies decorin overexpression has been shown to be the requirement of invasiveness for human bladder carcinoma cell line (El Behi et al., 2013).

The earlier studies mentioned above give a somewhat confusing data on decorin expression and immunoreactivity in human breast and bladder cancers. Nevertheless, ISH and IHC results of this thesis have now uniformly showed that neither human breast cancer nor bladder cancer cells *in vivo* express decorin. These malignant cells were also shown to be negative for decorin immunoreactivity. All detected decorin expression and immunoreactivity were localized in the areas of original, non-malignant stroma. The data of this study strongly suggest the idea that decorin is not expressed by any malignant cells of epithelial origin *in vivo*.

Identically to the *in vivo* results, human breast adenocarcinoma cells (MCF7) and human bladder cancer cell lines (RT-4, 5637, T24) were shown not to express decorin *in vitro*. When MCF7 cells were transduced with a decorin cDNA carrying adenovirus, they exhibited a less cohesive growth pattern, statistically lower mitosis rate and apoptotic features. Similarly, bladder cancer cells reacted to decorin transduction with a decreased cell count and with a statistically significant decrease in the proliferation index.

Previously, the systemic delivery of decorin core protein has been shown to have drastic anti-oncogenic effects on orthotopic squamous carcinoma (Seidler et al., 2006b) and breast carcinoma xenograft models (Reed et al., 2005; Goldoni et al., 2008; Buraschi et al., 2012). Parallel to this, ectopic expression of decorin (Pucci-Minafra et al., 2008) and adenoviral decorin transduction (Xu et al., 2015) have resulted in decreased malignant behavior of breast cancer cells and even in inhibition of bone metastasis in prostate cancer, respectively. Adenovirus mediated decorin transfer has even been reported to result in selective and distant killing of cancer cells *in vivo* (Tralhão et al., 2003). The results of this study are in full 2 agreement with the previously demonstrated results mentioned above and thus support the role of decorin as an anti-oncogenic molecule. Furthermore, the results endorse the idea of developing a decorin adenoviral gene vector as an adjuvant therapy in the treatment of cancer.

In an attempt to discover regulatory mechanisms behind the observed lack of decorin expression in bladder cancer cells, methylation of decorin gene promoter was studied. Previously, methylation of decorin gene promoter has been shown to play a role in the regulation of decorin expression in colon cancer (Adany et al., 1990; Nyman et al., 2015). However, in this study no methylation of the decorin gene promotor was observed in bladder cancer cell lines. Thus, epigenetic regulation of decorin expression in epithelial cancer cells seems to be cancer type specific.

6.3 Decorin in embryonic stem cells and tumors of germ cell origin

6.3.1 Human embryonic stem cells

During culturing, human embryonic stem cells (hESCs) can alter their behavior towards more cancer-like cell types with promoted self-renewal and lesser differentiation and apoptosis (Enver et al., 2005b; Lund et al., 2012). In normal tissue, homeostasis is maintained via cellular differentiation which inhibits cancer (Bissell and Hines, 2011). Therefore, attempt to induce a more differentiated state of cancer cells represents an intriguing possibility to decrease the malignant behavior of the cells (Gasimli et al., 2013).

In this study, expression of decorin *in silico* was examined during the early differentiation of normal and abnormal (culture-adopted) hESCs into embryonic bodies (EBs). The results revealed a significant induction of decorin expression during the differentiation of normal hESCs whereas no similar rise was detected in abnormal cells. As decorin has been shown to be centrally involved in various differentiation processes, the results suggest that abnormal hESCs are not able to differentiate similarly to normal hESCs at the molecular level. At the same time, because abnormal hESCs exhibit a more cancer-

like behavior than normal hESCs, the results also support the role of decorin as an anti-oncogenic molecule.

Changes in the glycosaminoglycan pathway have been shown to be closely linked with differentiation of hESCs towards early mesoderm and endoderm lineages (Gasimli et al., 2014). This increase in differentiation has been demonstrated to be accompanied with a highly significant increase in decorin expression after retinoic acid treatment on human pluripotent cells (Gasimli et al., 2013). Previously, decorin has also been shown to be one of the most down-regulated genes in murine teratocarcinoma cell lines compared to normal murine embryonic stem cell lines (Heffron et al., 2007). In brain cancer, adenoviral decorin transduction has been shown to induce cell differentiation and to alter the expression of a number of vital proteins involved in transcription, chemotherapy resistance, mitosis, apoptosis, and fatty acid metabolism (Ma et al., 2014). In normal physiological differentiation, decorin has been reported to influence various processes including myogenesis (Li et al., 2007; Brandan et al., 2008), gonad differentiation (Miqueloto and Zorn, 2007), and intestinal cell maturation (Bi et al., 2008).

Various factors regulating the differentiation of hESCs are still poorly understood (He et al., 2009). Thus, identification of different molecules and mechanisms contributing to the regulation are of vital importance for the optimization of clinical utilization of hESCs (Zhou et al., 2014). The differentiation of hESCs has been shown to be controlled by intracellular transcriptional regulatory networks which respond to various extracellular signaling stimuli (Huang et al., 2015). Decorin represents one of the key extracellular PGs in mediating signals from the ECM into the cells (Iozzo, 1998; Merline et al., 2009; Iozzo and Schaefer, 2015). Nevertheless, the role of ECM macromolecules like decorin in the development of embryonic tumors is not known (Diez-Torre et al., 2004; Diez-Torre et al., 2010). The present study provides new information on the differences of gene expression profiles between normal and abnormal hESCs during their early differentiation process.

6.3.2 Decorin and germ cell tumors

Teratomas and teratocarcinomas represent embryonic tumors originating from germ cells. They often appear at gonadal sites like in the testis. Testicular germ cell tumors (TGCTs) are the most frequent neoplasms in young men (Bosl and Motzer, 1997; Ulbright, 2005; Litchfield et al., 2015). In this study, preliminary screening of decorin expression was performed *in silico* in normal human testis, teratoma tissues and embryonal carcinomas using IST Online analysis. The results showed that decorin expression is highest in the teratoma tissues, whereas healthy testis tissue and embryonal carcinomas express significantly lower level of decorin.

As discussed in the beginning of the chapter, decorin has a valid role as an anti-angiogenic and anti-oncogenic molecule. Regarding angiogenesis, decorin has been shown to possess a dual role in the suppression of tumor angiogenesis; decorin is able to down-regulate potent pro-angiogenic agents and simultaneously induce the expression of endogenous anti-angiogenic factors (Neill et al., 2012b). As an anti-oncogenic molecule, stromal decorin has been named as the "guardian of the matrix" reflecting its versatile innate capabilities to repress and attenuate cancer cell proliferation, migration, survival, and also angiogenesis (Neill et al., 2012a). They also indicate differentiating effect of decorin on hESCs during their development into EBs, and thus emphasize the importance of decorin as a versatile regulatory macromolecule of the ECM already in the beginning of embryonal cellular differentiation. Very little is known about the microenvironment of germ cell tumors and the role of different stromal factors in the initiation and progression of tumorigenesis (Diez-Torre et al., 2004; Diez-Torre et al., 2010). Therefore, this study sheds much needed light on the role of decorin expression, and particularly the lack of its expression affecting and/or regulating the undifferentiated state of malignant germ cells.

6.4 The role of HA in the modulation of vascular smooth muscle cell behavior

Hyperglycemia is recognized as a major causal agent in the development of endothelial dysfunction in diabetes (Hadi and Suwaidi, 2007). Hyperglycemia causes damages to vascular integrity leading to macro- (coronary artery disease, peripheral arterial disease, and stroke) and microvascular (nephropathy, neuropathy, and retinopathy) complications (Fowler, 2008). In addition, also the function of VSMCs has been shown to be impaired in diabetes and this contributes to the higher incidence of vascular complications (Li et al., 2015).

In early diabetes, changes in the composition and assembly of the ECM are known to be one of the most prominent structural abnormalities of the vascular system (Zhuang and Yin, 2013). The arterial ECM contains several molecules of which particularly HA and versican are greatly involved in vascular remodeling (Wight, 2008; Moretto et al., 2015). In normal blood vessels, HA is present in low amounts, but in vascular diseases it has been shown to be dramatically increased (Vigetti et al., 2008; Wight, 2008). Increase in intimal area as well as accumulation of HA have been associated with diabetes (McDonald et al., 2007). Also elevated serum HA and hyaluronidase levels have been linked to vascular dysfunction in diabetic patients (Nieuwdorp et al., 2007; Broekhuizen et al., 2010; Morita et al., 2014).

In this study, hyperglycemic condition was shown to inhibit the contraction of collagen-rich gels by HUVSMCs *in vitro*. It was also shown that this inhibition of contraction is due to increased HAS1-3 expression and a concomitant increase of HA production in HUVSMCs. However, the signaling pathways and the mechanisms leading to the increased

HA production were not explored. In hyperglycemia, excess glucose has been shown to enter in the hexosamine biosynthetic pathway (Buse, 2006) and to lead to increase in UDP-N-acetylhexosamine in muscles (Rossetti et al., 1995) and primary chondrocytes (Qu et al., 2007). Previously, also the amount of O-linked N-acetylglucosamine (O-GlcNAc) has been shown to be increased in skeletal muscle after insulin and glucosamine infusions *in vivo* (Yki-Jarvinen et al., 1998). Additionally, the O-GlcNAcylation has been reported to be increased in hyperglycemia (Slawson et al., 2010). These together induce an activation of HA synthesis and affect particularly the expression of HAS2 (Vigetti et al., 2014c; Vigetti et al., 2012). Hyperglycemia has also been shown to activate protein kinase C (PKC) pathway as well to increase the formation of advanced glucation end-products (AGEs), both of which induce inflammation within the vascular wall and thereby may lead to vascular complications (Ross, 1999; Brownlee, 2001).

Interestingly, also epigenetic mechanisms have been shown to regulate HA metabolism in VSMCs in hyperglycemic conditions (Maier et al., 2010; Stein et al., 2014; Vigetti et al., 2014b). Specifically, miRNA-21 has been shown to be located in VSMCs and its expression is up-regulated in atherosclerotic arteries compared to normal arteries (Wang et al., 2011). Another molecule known to be up-regulated in hyperglycemia and in vascular disease is thrombospondin 1 (Maier et al., 2010). Furthermore, the effect of thrombospondin 1 has been shown to be functionally dependent on miRNA-21(Stein et al., 2014).

Taken together, the findings of this study that high glucose concentration leads to a disturbance in ECM remodeling via increased HA production by VSMCs provide a new possible mechanism leading to macroangiopathy in diabetic patients. Thus, therapeutics targeting the regulation of HA production will provide new potential tools in the treatment and prevention of diabetic complications in the future. The results of this study also underline the great importance of hyperglycemia as an independent causal factor in the development of diabetic macroangiopathy as has recently been emphasized by the results of the DCCT/EDIC study (Nathan and DCCT/EDIC Research Group, 2014).

6.5 Future perspectives

The results of this study collectively support the idea to develop novel anti-tumorigenic and anti-atherosclerotic therapies that are targeted to the modulation of specific ECM macromolecules such as decorin and HA. Recently, studies to explore the effects of adenoviral decorin vector have been initiated using 3D cancer xenografts *in vitro*. Furthermore, preliminary studies have been performed using orthotopic cancer animal models *in vivo*. Previously, hyperglycemia has primarily been considered as the cause of microvascular complications associated with diabetes. However, in the future hyperglycemia must be also considered as an important factor behind diabetic macrovascular complications.

7. SUMMARY AND CONCLUSIONS

Based on the results of the present study, the following conclusions can be drawn:

1. The expression of decorin was shown to be significantly different in human benign and malignant vascular tumors. Spesifically, decorin expression was detected in benign hemangiomas, whereas malignant vascular tumors totally lacked its expression. This suggests that decorin likely displays an inhibitory role in tumor angiogenesis in man as has previously been shown to be true in various animal models. Thus, the development of decorin-based therapies in the inhibition of tumor angiogenesis is highly justified. Also strong decorin expression associated with desmoplastic reaction particularly in Kaposi´s sarcoma samples was observed. This in turn supports the idea to use decorin expression as a cancer biomarker in identifying healthy and malignant tissue.

2. Benign or malignant human breast tumors did not express decorin. Also human bladder cancers totally lacked decorin expression. In both tumor types, decorin expression was present only in the areas of original, non-malignant stroma. Additionally, human breast cancer and bladder cancer cell lines did not express decorin. After adenoviral decorin transduction, both cancer cell types exhibited changes towards a less malignant phenotype; breast cancer cells had a lower mitosis rate, lesser cohesion and increased apoptic features and bladder cancer cells experienced a significant decrease in their proliferation and cell count. These results support the idea to develop novel decorin-based adjuvant therapies for breast and bladder cancers as well as to other cancers of epithelial origin in the future.

3. Normal hESCs were able to express decorin in early phases of embryonic differentiation whereas abnormal hESCs did not express decorin. This suggests that decorin crucially participitates in the differentiation of benign hESCs and that the lack of its expression allows the development of malignant behavior. This also strongly indicates that the gene expression profiles of normal and abnormal hESCs are quite different during the differentiation of the cells. Malignant cells of germ cell tumors *in vivo* were also shown not to express decorin. This finding is identical to those obtained from studies using malignant vascular tumor, and breast and bladder cancer tissue samples. The results collectively support the notion that malignant cells, despite of their origin, do not express decorin and that this lack of decorin expression may be visible already at very early phase of their differentiation.

4. Treatment of HUVSMCs with high glucose concentration was able to modulate the ability of cells to reorganize collagen-rich matrix. The treatment also markedly altered the gene expression profile of the cells leading to up-regulation of HASes expression and subsequently to an increase in HA production. These results provide a new mechanistical explanation behind macrovascular complications in diabetes due to hyperglycemia. Thus, therapeutics targeting the regulation of HA production may provide novel tools in the treatment and prevention of diabetic complications in the future.

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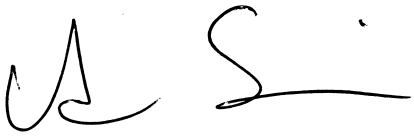

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