UNIVERSITY OF TURKU

EXPRESSION AND ROLE OF DECORIN IN HUMAN EPITHELIAL CANCERS

– With Special Reference to Bladder, Colon and Vulva Carcinomas

Marie Nyman

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. D OSA - TOM. 1424 | MEDICA - ODONTOLOGICA | TURKU 2019

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To My Family

ABSTRACT

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Expression and role of decorin in human epithelial cancers
- with special reference to bladder, colon and vulva carcinomas

University of Turku, Faculty of Medicine, Department of Medical Biochemistry and Genetics, Turku Doctoral Programme of Molecular Medicine (TuDMM)

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Today, the extracellular matrix (ECM) is not only known for its structural role but also for its many functional roles that are critical in the pathogenesis of various diseases including carcinogenesis. This is also true for decorin, an essential proteoglycan of the ECM. Decorin is especially recognized for its potent oncosuppressive activity, e.g. via its ability to bind to and to restrict the expression and activity of various growth factors, growth factor receptors, and receptors of the immune system. However, the expression of decorin and its cellular origin in different malignancies has not been explicitly defined.

In this thesis, the expression and role of decorin in human bladder, colon and vulva carcinomas were examined. The first aim of this thesis was to clarify, whether malignant cells of the above carcinomas express decorin. Next, this thesis aimed to examine the effects of adenovirus-mediated decorin cDNA (Ad-DCN) transduction on the behaviour of carcinoma cell lines *in vitro*. Finally, potential mechanisms mediating the effects of Ad-DCN transduction on the carcinoma cells were evaluated. Particularly, the effects of Ad-DCN transduction on the ErbB/HER family of receptors were studied.

The results showed that malignant cells of human bladder, colon and vulva carcinomas lack decorin expression *in vivo* and *in vitro*. Ad-DCN transduction of the cell lines was shown to potently suppress the malignant behaviour of the cells. Moreover, the results also defined downregulation of ErbB2/HER2 as a potential mechanism leading to decorin provoked oncosuppressive effects on human epithelial cancer cells, specifically on vulva carcinoma cells. In conclusion, the results of this thesis reinforce the relevance of developing novel, decorin-based adjuvant therapies for human epithelial cancers such as bladder, colon and vulva carcinomas.

KEYWORDS: decorin, epithelial cancer, bladder carcinoma, colon carcinoma, vulva carcinoma, oncosuppression, adenovirus, transduction, ErbB2/HER2

TIIVISTELMÄ

Marie Nyman

Dekoriinin ilmentyminen ja merkitys ihmisen epiteeliperäisissä syövissä - erityiskohteena rakon, paksusuolen ja vulvan epiteelisyövät.

Turun yliopisto, Lääketieteellinen tiedekunta, Lääketieteellinen biokemia ja genetiikka, ja Molekyylilääketieteen tohtoriohjelma (TuDMM)

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Sidekudoksella on kudosten rakennetta ylläpitävän ominaisuutensa lisäksi tärkeä toiminnallinen tehtävä, mikä tulee esille mm. erilaisten sairauksien kuten syövän synnyssä. Tämä pätee myös sidekudoksen keskeiseen proteoglykaaniin dekoriiniin, jolla on osoitettu olevan syöpien kasvua estävä vaikutus. Toistaiseksi dekoriinin alkuperä erilaisissa syövissä on osittain epäselvä.

Tämän väitöskirjatyön tarkoituksena oli selvittää dekoriinin ilmentymistä ja merkitystä ihmisen rakko-, paksusuoli- ja vulvasyövissä. Tutkimuksen ensimmäisenä tavoitteena oli selvittää, ilmentävätkö ko. syövissä malignit solut dekoriinia. Seuraavaksi tutkittiin, miten virusvälitteinen dekoriinin komplementaarinen DNA (Ad-DCN) vaikuttaa syöpäsolujen käyttäytymiseen. Lopuksi selvitettiin mahdollisia mekanismeja, joihin saadut tulokset perustuivat.

Tutkimuksen tulokset osoittivat kiistatta, etteivät yllä mainituissa syövissä malignit solut ilmennä lainkaan dekoriinia. Ad-DCN transduktio alensi merkittävästi syöpäsolujen jakaantumista ja elinkykyä sekä kykyä muodostaa kasvainpesäkkeitä. Yhdeksi mahdolliseksi vaikutusmekanismiksi paljastui epidermaalisen kasvutekijän reseptorin ErbB2/HER2 alentunut ilmentyminen ja aktiivisuus. Tämän väitöskirjatutkimuksen tulokset kannustavat jatkamaan dekoriiniin perustuvien, uusien adjuvanttihoitojen kehitystyötä ihmisen epiteeliperäisten syöpien voittamiseksi tulevaisuudessa.

AVAINSANAT: dekoriini, epiteeliperäinen syöpä, rakkosyöpä, paksusuolisyöpä, vulvasyöpä, syövän esto, adenovirus, transduktio, epidermaalisen kasvutekijän reseptori

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ABBREVIATIONS

Ad-DCN	Adenovirus-mediated decorin cDNA
Ad-LacZ	Adenovirus-mediated *E-coli ß*-galoctosidase gene
Akt	V-akt murine thymoma viral oncogene homolog
ATCC	American Type Culture Collection
BSA	Bovine serum albumine
CS	Chondroitin sulphate
CS-4S	Chondroitin 4-sulphate
CS-6S	Chondroitin 6-sulphate
CTGF	Connective tissue growth factor
DEPC	Diethyl pyrocarbonate
DS	Dermatan sulphate
DIG	Digoxigenin
DMEM	Dulbecco´s Modified Eagle Medium
ECD	Extracellular domain
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ErbB	V-erb b avian erytroblastic leukemia viral oncogene homolog
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAG	Glycosaminoglycan
HA	Hyaluronan
HER 1-4	Human epidermal growth factor receptor 1-4
HIF-1α	Hypoxia-inducible factor 1α
HPV	Human papillomavirus
hRPL19	Human ribosomal protein L19
IGF-1	Insulin-like growth factor-1
IGF-R1	Insulin-like growth factor-1 receptor

ICD	Intracellular domain
IHC	Immunohistochemistry
IL-12	Interleukin 12
ISH	In situ hybridization
LRR	Leucine-rich repeat
LacZ	*E-coli ß*-galoctosidase gene
MAPK	Mitogen activated protein kinase
Met	Hepatocyte growth factor receptor
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	Non-small cell lung carcinoma
p62/SQSTM1	Ubiquitin-binding protein p62
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDCD4	Programmed cell death protein 4
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PEG3	Paternally-expressed gene 3
PG	Proteoglycan
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
PI3K	Phosphoinositol-3 kinase
RTK	Receptor tyrosine kinase
RT-qPCR	Quantitative reverse transcriptase polymerase chain reaction
SCC	Squamous cell carcinoma
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLRP	Small leucine-rich proteoglycan
SSC	Saline-sodium citrate
TBS	Tris buffered saline
TGF-ß	Transforming growth factor-ß

TIMP	Tissue inhibitor of matrix metalloproteinase
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TMA	Tissue microarray
TMD	Transmembrane domain
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
VSCC	Vulvar squamous cell carcinoma

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals I – III. In addition, previously unpublished data are presented.

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

II Nyman MC, Sainio AO, Pennanen MM, Lund RJ, Vuorikoski S, Sundström JT, Järveläinen HT: Decorin in human colon cancer: Localization in vivo and effect on cancer cell behavior in vitro. Journal of Histochemistry & Cytochemistry 2015; 63:710-20

III Nyman M, Jokilammi A, Boström P, Kurki S, Sainio A, Grenman S, Orte K, Hietanen S, Elenius K, Järveläinen H: Decorin expression in human vulva carcinoma – oncosuppressive effect of decorin cDNA transduction on carcinoma cells. Journal of Histochemistry & Cytochemistry 2019; doi: 10.1369/0022155419845373

1. INTRODUCTION

Epithelial cancers, termed carcinomas, are collectively the most common types of cancer, comprising approximately 90% of all malignancies. Carcinomas are derived from cells of epithelial origin that line or cover every organ of the body.

The extracellular matrix (ECM) is a non-cellular meshwork of structural and functional molecules surrounding all cells in the body. The ECM functions as a scaffold, in which cells are embedded. In addition to its structural role, the ECM also plays a significant functional role in the maintenance of tissue homeostasis. Moreover, today the involvement of the ECM in different disease processes including carcinogenesis is indisputably recognized. Indeed, it is generally accepted that malignant cells are not only guided by their genetic code, but also by the ECM surrounding them.

Decorin, the prototype member of the small leucine-rich proteoglycan (SLRP) family, is known both for its structural role in the ECM and also for its multiple functional roles in regulation of cell behaviour. As such, decorin has been titled the "guardian from the matrix".

In this study, the expression and role of decorin in human epithelial cancers, specifically in bladder, colon and vulva carcinomas, were elucidated. The study sought to clarify whether malignant cells of the above carcinomas express decorin. Next, the effects of adenovirus-mediated decorin cDNA (Ad-DCN) transduction on the behaviour of cancer cells representing the above carcinomas were investigated *in vitro*. Finally, efforts to find potential mechanisms behind the effects of Ad-DCN transduction on the carcinoma cell lines *in vitro* were made. Particularly, the effects of Ad-DCN transduction on the expression and activity of the members of the EGF receptor family were examined.

2. REVIEW OF LITERATURE

2.1 Cancer

Cancer, originating from the Greek word *karkinos* (crab), is the term for a group of related malignant diseases, and the second leading cause of death worldwide (WHO, 2018). Cancers are genetic diseases caused by germline or somatic mutations or epigenetic modifications (Kumar and Robbins, 2013). Cancer is not merely one disease but a group of more than 200 distinct diseases that can be divided into different types depending on which organ they originate from, and into different categories based on the specific cell type from which the cancer has emerged (NIH, 2018). These categories include carcinomas, sarcomas, myelomas, leukemias, lymphomas, melanoma and brain and spinal cord tumours (NIH, 2018). At the time of diagnosis, cancer is further graded and staged depending on the growth and spread of the tumour. Most cancers, around 90%, are derived from epithelial cells and are called carcinomas or epithelial cancers.

Human bladder cancer is the tenth most common cancer with around half a million new diagnoses worldwide in 2018 (World Cancer Research Fund, 2018). Its incidence is highest in North America, Europe and parts of Western Asia (Cumberbatch et al., 2018). Its incidence is also much higher in men than in women (Cumberbatch et al., 2018). Smoking is considered the most important risk factor for bladder cancer (Cumberbatch et al., 2018). Urothelial cell carcinomas of the bladder are derived from epithelial cells and constitute around 90% of all bladder cancers (El Behi et al., 2013).

Colorectal cancer is one of the most frequent types of cancer in both genders worldwide. It is the second most common type of cancer in females and the third most common type in men worldwide (World Cancer Research Fund, 2018). The global incidence and mortality rate of colorectal cancer is expected to increase by 60% by the year 2030 (Arnold et al., 2017). Most colorectal cancers arise through the adenoma-carcinoma sequence via accumulation of genetic and epigenetic mutations (Fearon and Vogelstein 1990, Vogelstein et al.,2013). Almost all colorectal malignancies are of epithelial origin and can be classified as adenocarcinomas (Fleming et al., 2012).

Vulva carcinoma, the fourth most common gynaecological malignancy, is a relatively rare form of cancer representing only 5% of all female genital cancers (Alkatout et al., 2015). The vast majority (about 95%) of vulva carcinomas are squamous cell carcinomas (VSCC), while adenocarcinoma, melanoma, sarcoma and basalioma comprise the remaining 5% of vulva cancer cases (Alkatout et al., 2015). VSCC arise thorough human papilloma virus

(HPV)-dependent or HPV-independent pathways (Bloss et al., 1991, Hørding et al., 1994, Rakislova et al., 2017, Weberpals et al., 2017). The incidence of vulva carcinomas in younger women, often HPV-associated, is increasing worldwide (Judson et al., 2006, Meltzer-Gunnes et al., 2017). Vaccination programs for HPV are estimated to lead to a decline in HPV-associated vulva carcinomas but this improvement is estimated to take place no sooner than approximately 20 years from now (Hellner and Münger, 2011).

2.1.1 Carcinogenesis

The maintenance of homeostasis in normal tissues is imperative and highly dependent on the control mechanisms guarding cell growth, proliferation and apoptosis. Carcinogenesis is a process where normal cells transform into malignant cells that are able to grow and proliferate past these control mechanisms. It is a multistep process consisting of initiation, progression and metastasis (Hanahan and Weinberg, 2000). The course of cancer progression has been compared to the Darwinian evolution, the concept of "evolution by natural selection" first introduced by Charles Darwin in 1859 (Fischer et al., 2004).

The distinct characteristics of cancer, universally accepted as "the hallmarks of cancer", represent various traits acquired by the cancer cells, leading to the development and progression of cancer (Hanahan and Weinberg, 2000). These characteristics enable cancer cells to evolve past the control mechanisms that restrict normal growth and homeostasis in healthy cells. Cancer cells have acquired these traits mainly through genome instability and mutations causing activation of oncogenes and silencing of tumour suppressor genes, that in turn allow clonal expansion (Fischer et al., 2004). These distinct characteristics are represented by the cancer cell´s unlimited ability to divide, escape growth suppression, resist apoptosis, maintain growth stimulation, invade and metastasise and to induce angiogenesis (Hanahan and Weinberg, 2000). In addition to the original hallmarks of cancer, the "Emerging" and "Enabling" hallmarks were added in 2011 (Hanahan and Weinberg, 2011). These additional hallmarks describe the cancer cell´s ability to avoid immune destruction, modify their metabolism as well as characteristics enabling tumourigenesis by immune instability and tumour-promoting inflammation (Esteller 2011, Hanahan and Weinberg, 2011).

A malignant tumour consists of the malignant transformed cells (the parenchyma) and the host derived non-neoplastic stroma composed of con-

nective tissue, blood vessels and inflammatory cells (Connolly et al., 2003). The parenchyma and the stroma collectively forms the tumour microenvironment (TME) that encompasses all cellular and non-cellular components in the tumour including cancer cells, cancer stem cells, cancer associated fibroblasts, endothelial cells, immune cells, inflammatory cells, pericytes and the extracellular matrix (ECM) (Wang et al., 2017). In addition to the cancer cells themselves, carcinogenesis is also highly dependent on and influenced by the TME (Kumar and Robbins, 2013, Wang et al., 2017). The impact of the TME on tumour cells is fundamental, and influences all stages of cancer progression from initiation to progression and metastasis (Wang et al., 2017, Pickup et al., 2014).

2.2 The extracellular matrix (ECM)

All cells in solid tissues and organs are surrounded by a complex meshwork of non-cellular components called the extracellular matrix (ECM). In the past, the ECM was considered a stable, supportive structure that preserved tissue morphology. Today, the ECM is known as a dynamic structure that has both biomechanical and biochemical properties. The ECM functions as a scaffold and supports the cells in all tissues. It also forms a space in-between the cells keeping the cells separate. Besides the structural role of the ECM, it also has various cellular functions (Theocharis et al., 2016). The ECM is a dynamic network that constantly undergoes remodelling to maintain tissue homeostasis (Frantz et al., 2010).

The ECM consists of water and a diverse number of structural and functional components. The components of the ECM can be divided into fibrous and non-fibrous molecules. The fibrous molecules of the ECM include collagens, fibronectins, elastin and laminins, whereas the non-fibrous molecules of the ECM are mainly represented by proteoglycans (PGs) and hyaluronan (HA). (Järveläinen et al., 2009, Frantz et al., 2010). The molecular composition of the ECM is specific for each tissue type, giving every tissue its distinct characteristics (Frantz et al., 2010). The quantity of the ECM also varies in different tissues and is higher in connective tissues than in the epithelium and muscle tissues (Seidler and Dreier, 2008).

The ECM can be divided into interstitial and pericellular matrices. These two types of matrices are distinct in composition, structure and location. The pericellular matrix is in close contact with the cells and comprises the basement membrane, whereas the interstitial matrix surrounds the cells (Lu et al., 2012, Theocharis et al., 2016). The pericellular matrix is a rather compact structure primarily composed of hyaluronan and also collagen type

IV networks, laminins, fibronectins, specific PGs such as perlecan and integrins (Evanko et al., 2007, Theocharis et al., 2016). The basement membrane, which separates the epithelium and the endothelium from the stroma, is composed of for example collagen type IV, laminins, nidogen and PGs such as perlecan and agrin (Lu et al., 2012, Theocharis et al., 2016). The composition of the interstitial matrix is more porous and includes collagen fibrils, elastin and is rich in PGs such as decorin (Lu et al., 2012, Theocharis et al., 2016).

Remodelling, i.e. renewal and repair, of the ECM takes place during development, wound healing and is also important for normal tissue homeostasis. Tissue homeostasis depends on the equilibrium between the synthesis and degradation of the ECM. Proteases such as the matrix metalloproteinases (MMPs) and adamalysins are mainly responsible for the degradation of the ECM (Bonnans et al., 2014). In contrast, the family of tissue inhibitors of metalloproteinses (TIMPs) inhibits the activity of MMPs and adamalysins and thereby regulates excessive tissue degradation (Bonnans et al., 2014).

Cells are constantly communicating with the ECM surrounding them and there is a feedback loop between the cells and the surrounding ECM (Lu et al., 2012). The components of the ECM are capable of initiating cellular responses, e.g. via interacting with their specific receptors called integrins and also via variously binding to different growth factors and growth factor receptors.

Dysregulation of the ECM is associated with several pathological conditions. In fact, dysregulation of the ECM at some level has been considered to be involved in all diseases (Järveläinen et al., 2009, Sainio and Järveläinen, 2018b). Indeed, there is presumably no disease without changes in the ECM. Diseases caused or affected by dysregulation of the ECM range from rare hereditary genetic disorders such as Ehlers–Danlos syndrome and Marfan´s syndrome to common multifactorial disorders such as cardiovascular diseases and cancer (Sainio and Järveläinen, 2018b, Lu et al., 2012, Meester et al., 2017).

2.2.1 ECM and cancer

The ECM is a significant part of the TME and its importance in carcinogenesis is recognized (Hanahan and Weinberg, 2011, Pickup et al., 2014). In fact, the involvement of the ECM in carcinogenesis and cancer progression is so profound that it is considered to modulate all of the hallmarks of cancer (Pickup et al., 2014). Besides directly affecting cancer progression by regulat-

ing cellular transformation and metastasis, the ECM also contributes to carcinogenesis via regulation of for example angiogenesis and inflammation (Lu et al., 2012)

The ECM continuously undergoes remodelling to maintain tissue homeostasis. Dysregulation of the ECM turnover and aberrant ECM remodelling are hallmarks of cancer (Hanahan and Weinberg 2011, Lu et al., 2012). ECM remodelling enzymes like MMPs are frequently deregulated in human cancers and MMP-mediated degradation of the ECM has been associated with the metastatic potential (Kessenbrock et al, 2010, Liotta et al., 1980). For example, cell migration is regulated both by the proteolysis of the ECM releasing biological active components and by altering the structure of the ECM (Egeblad et al., 2010). Tumour cells that are able to synthesize their own ECM proteins are highly metastatic (Pickup et al., 2014).

The progression of cancer alters the architecture of the ECM surrounding the tumour cells (Walker et al., 2018). The ECM in tumours is usually stiffer than in normal tissues (Gkretsi and Stylianopoulos, 2018). This can be related to the fact that tumours have been compared to wounds that do not heal, because the altered and stiffer ECM of tumours resembles that of wounds (Dvorak et al., 2008). Tumour stiffness is a consequence of ECM composition and accumulation of ECM molecules such as hyaluronan, and structural changes in collagen fibres, i.e. fibrosis (Tammi et al., 2018). Collagen fibres are thicker and more linear in tumours compared to normal tissues (Egeblad et al., 2010, Provenzano et al., 2006). A stiff ECM enhances tumour growth and metastasis, as it increases cell growth and survival, and stimulates cell migration (Lo et al., 2000, Levental et al., 2009). Furthermore, tumour stiffness and fibrosis can disrupt drug delivery (Egeblad et al., 2010).

A desmoplastic reaction is an accumulation of ECM components, mainly collagen fibers, myofibroblasts and PGs, around a neoplastic cell population (Sainio and Järveläinen, 2013). Desmoplasia is found in both malignant and benign neoplasms and it is frequently found in breast, pancreatic, and colorectal cancers (Abbas and Mahalingam, 2011, Sainio and Järveläinen, 2013, Coulson-Thomas et al., 2011, Merika et al., 2012, Walker, 2001). The definite role of the desmoplastic reaction in carcinogenesis is not known (Sainio and Järveläinen, 2013). It has been suggested that it is a defence mechanism by the normal host cells against the invading tumour (Abbas and Mahalingam, 2011). However, desmoplasia has also been found in normal tissues of the breast and has been considered a pre-existing condition that favours tumour development (DeClerck, 2012). The presence of a desmoplastic reaction in cancer tissues functions as a biophysical barrier

against therapeutic agents and has been associated with poor prognosis (Cannon et al., 2018).

2.3 Proteoglycans of the ECM

Proteoglycans (PGs) are a group of structural and functional macromolecules composed of a core protein with one or more glycosaminoglycan (GAG) chains attached to the core protein. The GAG chains are linear polysaccharides composed of repeating disaccharide units consisting of an amino sugar and uronic acid (either glucuronic or iduronic acid) or galactose (Lindahl et al., 2017). PGs are present inside the cell, on the cell surface and in the ECM (Lindahl et al., 2017). PGs are ubiquitous in the ECM and perform important biological roles via interactions with other ECM molecules. PGs can be secreted into the ECM, embedded in or associated with the plasma membrane or stored inside the cell in secretory granules (Nikitovic et al., 2018). PGs are able to interact through their core protein or GAG chains with growth factors, cell surface receptors and other molecules of the ECM, and they play an important role in cell signalling in both health and diseases including cancer (Nikitovic et al., 2018, Theocharis et al., 2016).

PGs can be divided into different classes depending on their distribution, structure and function (Lindahl et al., 2017). On the basis of their distribution, PGs are divided into intracellular, cell surface, pericellular and extracellular PGs (Iozzo and Schaefer, 2015). While the intracellular group only contains one PG, namely serglycin, the biggest group of PGs are the extracellular PGs comprising 25 genes, including the group of small leucine-rich proteoglycans (SLRPs) (Iozzo and Schaefer, 2015). PGs can further be classified on the basis of their GAG side chain (Iozzo and Schaefer, 2015). GAGs can be divided into sulphated GAGs, consisting of chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and heparin, and non-sulphated GAG represented by HA (Iozzo and Schaefer, 2015).

2.3.1 Small leucine-rich proteoglycans

The small leucine-rich proteoglycans (SLRPs) are a class of PGs located and ubiquitously expressed in most ECMs (Iozzo and Schaefer, 2015). The SLRP gene family constitutes the largest family of PGs, including 18 members grouped into five different classes based on their structure and biological functions (Table 1.) (Iozzo and Schaefer, 2015, Nastase et al., 2014). The 18 genes of the human SLRP gene family are clustered to seven chromosomes

(Schaefer and Iozzo, 2008). The presence of chromosomal clusters of SLRPs suggests that they have arisen during evolution as a result of gene duplication (Schaefer and Iozzo, 2008).

SLRPs are characterized by their relatively small protein core and the presence of tandem leucine-rich repeats (LRRs) in their central domain (Lindahl et al., 2017, Iozzo and Schaefer, 2015). The majority of the members of the classes I-III carry one or more GAG chains whereas the class IV and V members lack the GAG chain (Nastase et al., 2014). The class I-III members also contain an "ear repeat", considered to be a hallmark of a typical SLRP, composed of a C-terminal motif formed by a disulphide bond between two cysteine residues. (McEwan et al., 2006, Schaefer and Iozzo, 2008).

SLRPs are important structural molecules and are variously involved in the regulation of matrix assembly through their ability to bind to and interact with other matrix macromolecules, such as certain collagen species, fibronectin and thrombospondin (Chen and Birk, 2013, Winnemöller et al., 1991, Davies et al., 2001). The spatial and temporal expression of SLRPs during development regulates tissue specific matrix assembly and thereby determines tissue function (Chen and Birk, 2013).

Although SLRPs were originally thought to be merely structural components of the ECM, they are now also recognized as important molecules in cell signalling, with fundamental impacts on cellular functions such as cell viability, differentiation, proliferation, adhesion and migration. Furthermore, they have a role in the regulation of inflammation, autophagy and mitophagy (Iozzo and Schaefer, 2010, Schaefer et al., 2017).

The leucine-rich repeats (LRRs) of their protein core are important for protein-protein interactions and are responsible for the involvement of SLRPs in many important biological functions (Iozzo and Schaefer, 2015). Mutations in certain SLRP encoding genes have been found to lead to skin fragility, osteoporosis and cardiovascular diseases, and to disorders causing ocular abnormalities (Schaefer and Iozzo, 2008). Some functional overlaps and the existence of rescuing and compensating mechanisms have been found between different members of the SLRP family (Ameye and Young, 2002).

Decorin and biglycan are closely related class I SLRPs, with >65% homology (Iozzo and Schaefer, 2015). The molecular weights of their core protein are around 40kDa (Lindahl et al., 2017). The biggest structural difference between these two SLRPs is in their GAG chains. Decorin carries only one chondroitin sulphate (CS)/dermatan sulphate (DS) GAG chain, whereas biglycan carries two CS/DS side chains, whereby it was given its eponym, meaning two GAG chains (Fisher at al., 1989). Decorin and biglycan can also be distinguished by their different localization in the ECM. Decorin is local-

ized to the ECM where it binds to e.g. collagen type I, while biglycan can be found closely around the cells (Bianco et al., 1990). Both decorin and biglycan are known for their structural role and for their role as signalling molecules (Schaefer et al., 2017).

Table 1. Small leucine rich proteoglycans (SLRPs). Modified from (Nastase et al., 2014).

Name	Class (I-V)	GAG chains	Molecular weight (kDa)
Class I	Decorin	CS/DS	39,7
	Biglycan	CS/DS	41,6
	Asporin	no GAG	43,2
	ECM-2	no GAG	79,8
	ECMX	no GAG	64,2
Class II	Keratocan	KS	40,5
	PRELP	KS	43,8
	Osteoadherin	KS	49,5
	Lumican	KS	38,4
	Fibromodulin	KS	43,2
Class III	Osteoglycin	KS	33,9
	Epiphycan	CS/DS	36,6
	Opticin	no GAG	37,3
Class IV	Tsukushi	no GAG	37,8
	Nyctalopin	no GAG	52
	Chondroadherin	no GAG	40,5
Class V	Podocan	no GAG	69
	Podocan-like	no GAG	56,5

CS, chondroitin sulphate; DS, dermatan sulphate; KS, keratan sulphate; GAG, glycosaminoglycan; ECM-2, extracellular matrix protein-2; ECMX, ECM-2-like protein x chromosome; PRELP, proline/arginine-rich repeat protein.

2.4 Decorin

Decorin, originally named PG40, PGII, DSPGI and PG-S2, is a class I SLRP and the prototype member of the SLRP gene family. The name decorin was derived from the discovery that decorin binds to and "decorates" collagen type I fibers. Today, in addition to collagen, decorin is known to bind to and interact with various categories of ligands, such as members of the ECM, growth factors and cell surface receptors (Figure 1) (Gubbiotti et al., 2016).

The human decorin gene was cloned and sequenced in 1986 and has been mapped to chromosome 12q23 (Krusius and Ruoslahti, 1986, Danielson et al., 1993). The transcription of the decorin gene is induced by quiescence and repressed by tumour necrosis factor-α (TNF- α) (Mauviel et al., 1995).

Decorin is expressed in a wide range of connective tissues. Originally decorin was thought to be synthesized by all cells of the body. However, today decorin is known to be synthesized by specific cell types, mainly fibroblasts and smooth muscle cells but also stressed vascular endothelial cells and macrophages (Järveläinen et al., 1992).

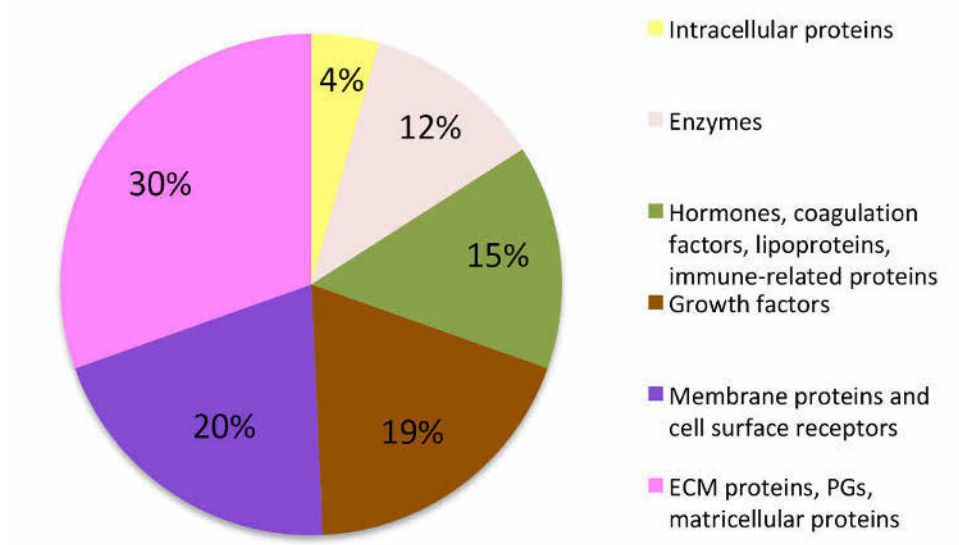

Figure 1. Various categories of decorin binding partners. The diverse molecular groups of decorin interactions including other ECM proteins, cell surface receptors and growth factors, presented as percentages. The interactions have been identified in the literature and in interaction databases by Gubbiotti et al 2016. Modified from (Gubbiotti et al., 2016).

2.4.1 Molecular structure of decorin

Decorin comprises a 396 amino acid long core protein of approximately 40 kDa consisting of 12 LRRs, with up to three N-linked oligosaccharides attached, flanked by two cysteine-rich regions with a single glycosaminoglycan (GAG) side chain attached to it (Figure 2) (Krusius and Ruoslahti, 1986, Mann et al., 1990, Scott et al., 2004, Sainio and Järveläinen, 2018a). The structure of decorin is divided into four domains. The first domain consists of a 14 amino acid long signal peptide and a 16 amino acid long propeptide (Sainio and Järveläinen, 2018a). Both the signal peptide and the propeptide are cleaved before the mature decorin is secreted into the ECM, and thus are not present in mature decorin (Sainio and Järveläinen, 2018a). The second domain is a negatively charged cysteine-rich domain with a single GAG

chain, consisting of chondroitin sulphate or dermatan sulphate, attached to Ser^4 (Sainio and Järveläinen 2018a). The third domain of decorin consists of 12 about 24 amino acid long LRRs consisting of an α-helix and a ß-turn (Kobe and Deisenhofer, 1993). LRR 11, referred to as the "ear repeat", is the longest repeat, projecting laterally out from the core protein. The "ear repeat" is thought to participate in the protein folding of decorin and also to contribute to ligand recognition (McEwan et al., 2006). The carboxyl terminal domain of decorin is the fourth domain containing two cysteine residues and a conserved disulphide loop (Sainio and Järveläinen, 2018a).

Although decorin has earlier been suggested to form dimers in solution, dimerization of decorin has been shown to be relatively weak and reversible and biologically active decorin is now known to act as a monomer in solution (Goldoni et al., 2004, Scott et al., 2003, Islam et al., 2013). Decorin interacts with multiple molecules and is capable of regulating cellular functions both via the LRRs of the core protein and via its GAG side chain (Seidler et al., 2006).

The decorin protein core "decoron" is important both for the structural and the functional roles of decorin. The 12 LRRs of the core protein gives decorin its shape. The LRRs are aligned with their α-helices on the outer convex surface and their ß-sheets on its inner concave surface giving decorin its curved shape (Weber et al., 1996, McEwan et al., 2006, Bella et al., 2008, Scott et al., 2004). Decorin was initially thought to be shaped as a horseshoe but is now known to have a solenoid shape (Bella et al., 2008).

The decorin protein core is responsible for most of decorin´s interactions. The inner concave side of decorin was initially believed to hold one single triple helix of collagen but is now known to be able to accommodate up to six triple helices of collagen and also to bind to and interact with receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR) or the vascular endothelial growth factor receptor-2 (VEGFR-2) (Weber et al., 1996, Scott et al., 2004, Moscatello et al., 1998, Khan et al., 2011). Specific LRRs of the core protein are responsible for decorin´s interactions. For example, LRRs 4-5 are known to be high affinity binding sites for collagen type I, LRRs 3-5 for TGF-ß, LRR 5 for VEGFR-2 and LRR 6 for EGFR (Svensson et al., 1995, Schönherr et al., 1998, Khan et al., 2011, Santra et al., 2002). The theory that decorin acts as a dimer in solution was undermined partly because of the fact that dimerization would make the LRRs of its protein core inaccessible for interactions (Islam et al., 2013, Goldoni et al., 2004, Gubbiotti et al., 2016).

The GAG chain of decorin is a linear sulphated polysaccharide consisting of multiple disaccharide repeats linked by glycosidic bonds attached by the N terminus (Lindahl et al., 2017, Sofeu Feugaing et al., 2013). The di-

saccharide repeats consist of N-acetylgalactosamine (GalNAc) and uronic acid (UA). The UA can be either glucuronic acid as in chondroitin sulphate (CS) or iduronic acid as in dermatan sulphate (DS) (Lindahl et al., 2017, Sofeu Feugaing et al., 2013). The decorin GAG side chain is composed of both condroitin and dermatan sulphates and is therefore referred to as a co-polymer of CS/DS (Sofeu Feugaing et al., 2013). The composition of the GAG side chain, the length of the chain and its sulphation pattern vary depending on tissue type and age (Seidler and Dreier, 2008, Viola et al., 2006). For example, both the number of sulphated GAGs and the size of the GAG chains are significantly reduced in human aged skin (Li et al., 2013).

While most of the interactions of decorin go through its core protein, decorin is also capable of interacting with collagen, specific enzymes and growth factors through its GAG chain (Gubbiotti et al., 2016). Decorin is capable of simultaneously binding to collagen molecules through the core protein and the dermatan sulphate of the GAG chain (Gubbiotti et al., 2016). In fact, decorin which lacks its GAG chain has been shown to bind more weakly to collagen than intact decorin (Nareyeck et al., 2004). Moreover, the composition of the GAG chain affects matrix assembly as the length of the GAG chain dictates the distance between collagen fibrils (Iozzo, 1999). The GAG chain reduces fibrillar diameter in early collagen fibrillogenesis (Rühland et al., 2007). The importance of the GAG chain and its significance for normal matrix assembly is demonstrated in a progeroid phenotype of Ehlers-Danlos syndrome, where 30-70% of the decorin molecules lack the GAG chain (Kresse et al., 1987, Seidler and Dreier, 2008).

In addition to pathologies such as Ehlers-Danlos syndrome, changes in the composition of the GAG chain and post-translational modifications of the GAG chain of decorin have also been identified in various malignancies such as colon and gastric cancers (Theocharis, 2002, Theocharis et al., 2003). Interestingly, post-translational modifications in the GAG chain of decorin have been associated with the malignant phenotype in pancreatic and laryngeal cancers (Skandalis et al., 2006a, Skandalis et al., 2006b,). The GAG chain of endothelial decorin also acts as a receptor for the surface proteins of *Borrelia burgdorferi* and is thus involved in the pathogenesis of Lyme disease (Lin et al., 2017).

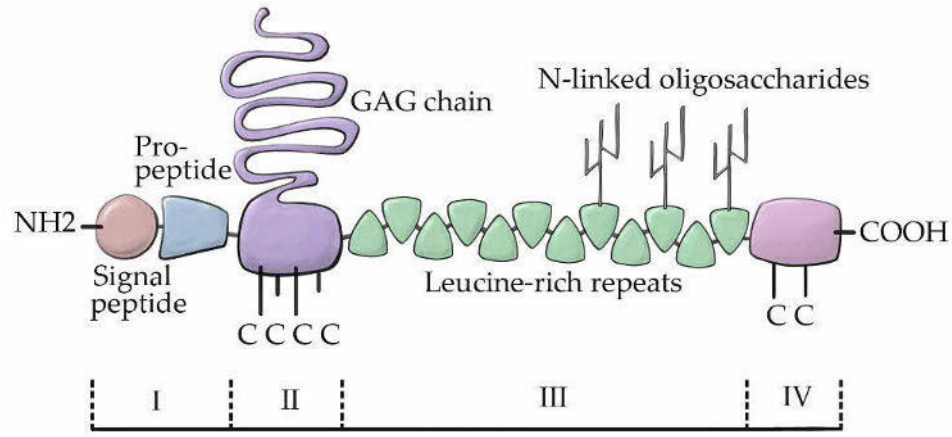

Figure 2. **Schematic structure of decorin.** The core protein of decorin is divided into four domains (I-IV) further described in the text. Modified from (Sainio and Järveläinen, 2018a).

2.5 Structural role of decorin: interactions with other ECM macromolecules

Decorin plays an important structural role in the ECM and has a regulatory role in collagen fibrillogenesis and matrix assembly. By binding to various molecules of the ECM, decorin is capable of regulating fibrillogenesis, matrix assembly and cell behaviour (Table 2).

Decorin was initially recognized for its structural role in the ECM and for binding to collagen type I fibres. Today, decorin is also known to bind to collagens II, III, IV, V, VI, XII and XIV (Gubbiotti et al., 2016). Decorin is capable of binding to and interacting with four to six collagen molecules simultaneously (Orgel et al., 2009). The interaction of decorin with collagen is important for both fibril formation and fibrillar spacing (Gubbiotti et al., 2016).

In addition to collagens, decorin also interacts with other macromolecules of the ECM affecting both matrix assembly and cell behaviour. For example, by interacting with trombospondin-1, decorin inhibits endothelial migration and interferes with cell attachment (Davies et al., 2001, Winnemöller et al., 1992). By interacting with fibronectin, decorin is capable of inhibiting cell adhesion (Winnemöller et al., 1991). Furthermore, interactions of decorin with dermatopontin, fibrillin-1, microfibril-associated glycoprotein (MAGP) and tenasin X all affect matrix assembly (Okamoto et al., 1996, Takeda et al., 2002, Trask et al., 2000, Elefteriou et al., 2001, Gubbiotti et al., 2016).

The structural role of decorin is demonstrated by its importance in skin morphology and wound healing (Danielson et al., 1997, Järveläinen et

al., 2006). Decorin knockout mice develop skin fragility and abnormal collagen morphology (Danielson et al., 1997). Furthermore, there is a significant delay in wound healing in decorin deficient mice (Järveläinen et al., 2006). Decorin also stimulates muscle differentiation and repair in humans (Li et al., 2007). Exercise leads to a rise in decorin expression in muscle and decorin production has recently been shown to be stimulated by growth hormone in humans (Bahl et al., 2018).

Table 2. **Decorin interactions with other ECM macromolecules**. Examples of decorin interacting macromolecules, and effects on ECM structure and cell behaviour. Modified from (Sofeu Feugaing et al., 2013, Chen and Birk, 2013).

ECM molecule	Binding via	Effects	References
Collagen I	Core protein/ GAG chain, LRRs 4-5	Collagen fibril organization, matrix assembly	Brown and Vogel, 1989, Pringle and Dodd, 1990, Svensson et al., 1995
Collagen II	Core protein/GAG chain	Collagen fibril organization, matrix assembly	Douglas et al., 2006
Collagen III	Core protein/GAG chain	Collagen fibril organization, matrix assembly	Douglas et al., 2006
Collagen V	Core protein	Collagen fibril organization, matrix assembly	Whinna et al., 1993
Collagen VI	Core protein	Collagen fibril organization, matrix assembly	Bidanset et al., 1992
Collagen XII	GAG chain	Collagen fibril organization, matrix assembly	Font et al., 1996
Collagen XIV	GAG chain	Collagen fibril organization, matrix assembly	Font et al., 1993
Dermatopontin	Core protein	Acceleration of collagen fibrillogenesis, alteration of collagen diameter	Okamoto et al., 1996, Takeda et al., 2002
Fibrillin-1	Core protein	Matrix assembly	Trask et al., 2000
Fibronectin	Core protein	Inhibition of cell adhesion	Winnemöller et al., 1991
Microfibril-associated glycoprotein-1	Core protein	Matrix assembly	Trask et al., 2000
Tenascin X	GAG chain	Preservation of mechanical properties of the ECM and effects on interfibrillar spacing	Elefteriou et al., 2001, Gubbiotti et al., 2016
Thrombospondin-1	Core protein	Inhibition of endothelial migration, interference with cell attachment	Davies et al., 2001, Winnemöller et al., 1992

2.6 Functional role of decorin: interactions with growth factors, growth factor receptors and receptors of the immune system

Besides having an important structural role, decorin is also known for its multiple functional roles via its capability to bind to and interact with growth factors, growth factor receptors and receptors of the immune system (Table 3). Decorin was the first PG shown to act as a growth suppressor via its ability to bind to and inhibit the activity of transforming growth factor-ß (TGF-ß) (Yamaguchi and Ruoslahti, 1988, Yamaguchi et al., 1990). Today, various PGs including decorin are known to bind to and sequester various growth factors in the ECM (Gubbiotti et al., 2016). In addition to TGF-ß, the decorin interactome also includes receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR or ErbB1), vascular endothelial growth factor receptor-2 (VGFR-2) and insulin-like growth factor-1 receptor (IGF-R1) (Buraschi et al., 2013, Schönherr et al., 2005, Csordas et al., 2000). In fact, decorin is known as a pan-RTK inhibitor (Sofeu Feugaing et al., 2013). In addition to interacting with growth factors and growth factor receptors, decorin also interacts with receptors of the immune system. Through the above interactions decorin is capable of regulating numerous cellular processes such as inflammation, autophagy, mitophagy, fibrosis, angiogenesis and wound healing (Järveläinen et al., 2006, Merline et al., 2011, Buraschi et al., 2013, Border et al., 1992). However, the interaction of decorin with various receptors is context dependent. In other words, interactions of decorin with a receptor can result in different outcomes depending on the cellular milieu (Gubbiotti et al., 2016).

2.6.1 Decorin and growth factors

As mentioned above, decorin was initially found to bind to and be a natural inhibitor of TGF-ß, a growth factor that regulates many fundamental physiological processes such as proliferation, migration and fibrosis (Table 3) (Yamaguchi et al., 1990). Decorin is known to be capable of binding to and interacting with all three different isoforms of TGF-ß (TGF-ß1-3) through its core protein (Hildebrand et al., 1994, Yamaguchi et al., 1990). Decorin binds to TGF-ß through two binding sites in its protein core and thereby inhibits its activity (Yamaguchi et al., 1990, Takeuchi et al., 1994). The interaction of decorin with TGF- ß can also take place when decorin is bound to collagen, which is thought to prevent TGF- ß from binding to its receptors by isolating it in the ECM (Gubbiotti et al., 2016, Kolb et al., 2001, Hildebrand et al.,

1994). By interacting with TGF-ß, decorin is capable of suppressing tumourigenesis and fibrosis (Ständer et al., 1998, Kolb et al., 2001). Moreover, because TGF-ß is known to regulate angiogenesis, it has also been speculated that decorin partly regulates angiogenesis through this mechanism of action (Järveläinen et al., 2015).

In addition to TGF-ß, decorin has also been found to interact with other growth factors such as connective tissue growth factor (CTGF), the platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) (Table 3) (Vial et al., 2011, Nili et al., 2003, Mohan et al., 2011, Iozzo et al., 2011).

2.6.2 Decorin and growth factor receptors

In addition to growth factors, decorin also binds to growth factor receptors of the RTK family such as the receptor for hepatocyte growth factor (Met), the vascular endothelial growth factor receptor-2 (VEGFR-2), the insulin-like growth factor-1 receptor (IGF-R1), the platelet-derived growth factor receptor (PDGFR) and the ErbB family of RTKs (Table 3). These receptors regulate most cellular functions such as cell cycle progression, proliferation, apoptosis, differentiation and cell migration (Lemmon and Schlessinger, 2010). Hence, their regulation is fundamental for normal cell function, and dysregulation of RTKs leads to various diseases including cancer (Lemmon and Schlessinger, 2010, Du and Lovly, 2018).

The Met receptor is known as an oncogenic driver of tumour growth, and hepatocyte growth factor (HGF)/Met signalling contributes to aggressiveness and metastasis (Cooper et al., 1984, Cecchi et al., 2011, Comoglio et al., 2018). Moreover, the Met receptor has been found to be overexpressed in various mesenchymal and epithelial cancers (Cooper et al., 1984, Comoglio et al., 2018, Cecchi et al., 2011). Met is considered to be the key receptor for decorin and decorin binds to Met with high affinity (kd = 1,5 nM) (Neill et al., 2016, Goldoni et al., 2009). Binding of decorin to Met suppresses tumourigenesis through three different pathways. The binding of decorin to Met leads to phosphorylation and degradation of the receptor resulting in downregulation of ß-catenin and Myc levels, ultimately leading to tumour growth suppression (Goldoni et al., 2009, Buraschi et al., 2010). Decorin also reduces angiogenesis by binding to Met, which leads to the inhibition of hypoxia-inducible factor-1α (HIF-1α) (Neill et al., 2012). Furthermore, the binding of decorin to Met also causes mitophagy in breast cancer cells via peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1α) (Neill et al., 2014).

The vascular endothelial growth factor receptor-2 (VEGFR-2) is the receptor for endothelial cell signalling and regulates angiogenesis and cell viability. Decorin acts as a VEGFR-2 antagonist and binds to VEGFR-2 through LRR 5 in its core protein in a binding site overlapping with the ligand binding site (Khan et al., 2011). Decorin inhibits the signalling of VEGFR-2 by competing with VEGFA, the endogenous ligand of VEGFR-2 (Lala et al., 2012). Furthermore, by binding to VEGFR-2, decorin has been shown to induce autophagy in endothelial cells, ultimately leading to suppression of angiogenesis (Buraschi et al., 2013).

The insulin-like growth factor-1 receptor (IGF-R1) is a receptor essential for cell growth and is involved in the development and progression of various types of cancer such as pancreatic, breast and ovarian cancers (Denduluri et al., 2015). Decorin is capable of binding to both IGF-R1 and its ligand IGF-1 (Iozzo et al., 2011). Decorin binds to a region of the IGF-R1 that does not overlap with the binding site of its natural ligand IGF-1 (Iozzo et al., 2011). Depending on the context, decorin can act as either an agonist or an antagonist for IGF-R1 (Theocharis, Karamanos, 2017). In transformed cells, decorin functions as an IGF-R1 antagonist. For example, in bladder cancer, decorin inhibits migration and invasion of malignant cells through the interaction with IGF-R1 leading to the attenuation of Akt and MAPK (Iozzo et al., 2011). However, in non-transformed cells decorin acts as an agonist for IGF-R1 and regulates phosphorylation of downstream signalling (Morrione et al., 2013, Schönherr et al., 2005).

The platelet-derived growth factor receptor (PDGFR) family consists of four receptors involved in the control of growth of connective tissue cells such as fibroblasts and smooth muscle cells (Chen et al., 2013). Decorin has been shown to bind to PDGF and thereby to regulate PDGFR-α and PDGFR-ß (Baghy et al., 2013). In a model of arterial injury, decorin binds to PDGF, the natural ligand of PDGFR, and prevents PDGF stimulated PDGFR-ß phosphorylation inhibiting vascular smooth muscle cell function and reducing intimal hyperplasia after arterial injury (Nili et al., 2003). In hepatocellular carcinoma decorin was found to bind to PDGF and to interfere with PDGFR-α signalling by attenuating its activity (Baghy et al., 2013, Horváth et al., 2014). Whether decorin directly interacts with PDGFR still remains to be resolved.

2.6.3 Decorin and the epidermal growth factor (EGF) receptor family

The original finding that decorin binds to the EGF receptor with high affinity and downregulates its activity has established decorin as an anti-

oncogenic molecule and has resulted in a multitude of studies on decorin as a signalling molecule in cancer (Seidler et al., 2006, Csordas et al., 2000, Moscatello et al., 1998).

The epidermal growth factor (EGF) receptor family, also known as the human epidermal growth factor receptor (HER) family, consists of four distinct members, namely EGFR (also known as ErbB1/HER1), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4) (Roskoski, 2014). All the receptors of the EGF receptor family have a similar structure containing an extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular domain (ICD) (Roskoski, 2014). The ECD consists of four domains including two cysteine-rich domains (Roskoski, 2014). The ICD consists of a tyrosine kinase domain and a tyrosine containing C-terminal tail (Roskoski, 2014).

The members of the EGF receptor family can adopt an open "active" or closed "inactive" conformation. The receptors exist as monomers in the inactive state and dimerize when activated to form homodimers or heterodimers (Wieduwilt and Moasser, 2008). ErbB1, ErbB3 and ErbB4 adopt a closed conformation, unless activated by ligand binding, while ErbB2 is constantly in an open conformation and ready to dimerize. ErbB2 does not bind to any known ligand and ErbB3 does not have an active tyrosine kinase domain, and thus these receptors are not fully functional in their homomeric form (Wieduwilt and Moasser, 2008). In order to be fully functional receptors, ErbB2 and ErbB3 must bind to and form heterodimers with other members of the EGF receptor family (Wieduwilt and Moasser, 2008). After receptor homo- or heterodimerization, the C-terminal domain of the receptors are phosphorylated, whereafter tyrosine residues activate signalling pathways including the RAS/MAPK and the PI3K/Akt pathways (Wieduwilt and Moasser, 2008).

Decorin binds to a narrow region in the L2 domain of the EGFR/ErbB1 through LRRs of its protein core (Santra et al., 2002). The binding site is different from the EGF-binding epitope but partially overlaps with it (Santra et al., 2002). The central LRR of the decorin protein core, LRR6, is especially important in the interaction between decorin and EGFR/ErbB1 because it is required for a proper binding with the L2 domain of EGFR/ErbB1 (Santra et al., 2002). Binding of decorin to EGFR/ErbB1 causes receptor dimerization, internalization and ultimately leads to receptor degradation (Zhu et al., 2005). Decorin binding causes internalization of EGFR/ErbB1 through an endocytotic pathway (Zhu et al., 2005). This caveola-mediated endocytotic pathway causes a sustained internalization of EGFR/ErbB1, which reduces the number of EGF-binding sites on the cell surface leading to continued growth suppression (Zhu et al., 2005, Csordas et al., 2000, Seidler et al.,

2006). This pathway differs from the clathrin dependent endocytotic pathway caused by EGF-binding to EGFR/ErbB1, where some of the receptors are recycled and transported back to the cell surface (Zhu et al., 2005).

Decorin also interacts with other members of the EGF receptor family such as ErbB2 and ErbB4. Decorin has been shown to downregulate ErbB2 in human breast cancer cells (Santra et al., 2000). The downregulation of ErbB2 in these cells is considered to be caused by the interaction between decorin and ErbB4, preventing the formation of heterodimers between ErbB4 and ErbB2 and therefore suppressing the ErbB2 kinase activity (Santra et al., 2000). Decorin has also been found to bind to and suppress the activity of ErbB4 in the central nervous system (Minor et al., 2011).

The decorin EGFR/ErbB1 interaction seems to be a conserved biological pathway in ErbB signalling as Kekkon 1, a homologous to decorin in *Drosophila melanogaster*, binds to mammalian members of the EGF receptor family and suppresses their signalling pathways (Ghiglione et al., 2003).

2.6.4 Decorin and receptors of the immune system

Besides interacting with growth factors and growth factor receptors, decorin is also capable of influencing the immune system via interactions with receptors of the immune system. Decorin is capable of binding to receptors of the family of toll-like receptors (TLRs), key receptors in the regulation of innate immunity and inflammation (Vidya et al., 2018). These transmembrane receptors are located on the cell surface or intracellular compartments and they activate the NF-κB and MAP kinase pathways, leading to the enrolment of pro-inflammatory cytokines and other pro-inflammatory molecules (Vidya et al., 2018). Thus far, decorin has been found to bind with high affinity to TLR2 and TLR4 on macrophages (Merline et al., 2011). Binding of decorin to TLR2/4 activates p38, MAPK and NF-κB pathways leading to synthesis and secretion of cytokines such as TNF-α and IL-12, which in turn results in a pro-inflammatory response (Merline et al., 2011). By influencing the signalling of TLR2/4, decorin also stimulates the production of the tumour suppressor programmed cell death 4 (PDCD4) (Merline et al., 2011).

Table 3. Decorin interactome. The major decorin binding ligands and some of the ligand mediated effects. Modified from (Sofeu Feugaing et al., 2013, Theocharis and Karamanos, 2017).

Decorin ligand		Binding via	Binding region	Ligand mediated effects	References
Growth factor	CTGF	Core protein	LRR 12	Inhibition of receptor activity	Vial et al., 2011
	IGF-1	Core protein		Inhibition of migration and invasion	Schönherr et al., 2005, Iozzo et al., 2011
	PDGF			Inhibition of cell proliferation, migration and collagen synthesis	Nili et al., 2003
	TGF-ß	Core protein	Between LRR 3 and LRR 5	Growth inhibition, suppression of fibrosis	Yamaguchi and Ruoslahti, 1988, Yamaguchi et al., 1990, Hildebrand et al., 1994, Schönherr et al., 1998, Kolb et al., 2001
	VEGF			Suppression of tumour cell mediated angiogenesis	Mohan et al., 2011, Grant et al., 2002
RTK	EGFR	Core protein	LRR 6	Cell cycle arrest, growth inhibition, apoptosis	Moscatello et al., 1998, Iozzo et al., 1999b, Santra et al., 2000, Seidler et al., 2006
	ErbB2	Core protein		Cell cycle arrest, suppression of growth, decreased metastsis	Santra et al., 2000, Reed et al., 2005
	ErbB4	Core protein		Cell cycle arrest, growth suppression	Santra et al. 2000
	IGF-R1	Core protein		Inhibition of migration and invasion	Iozzo et al., 2011
	Met	Core protein		Growth inhibition, inhibition of migration, increased mitophagy, angiostasis	Goldoni et al., 2009, Buraschi et al., 2010, Neill et al., 2012, Neill et al., 2014
	VEGFR-2	Core protein	LRR 5	Inhibition of angiogenesis, stimulation of autophagy	Khan et al., 2011, Buraschi et al., 2013, Goyal et al., 2014
TLR	TLR2			Inflammation, tumour growth inhibition	Merline et al., 2011
	TLR4			Inflammation, tumour growth inhibition	Merline et al., 2011

CTGF, connective tissue growth factor; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; TGF- ß, transforming growth factor-ß; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; ErbB2, v-erb b avian erytroblastic leukemia viral oncogene homolog 2; ErbB4, v-erb b avian erytroblastic leukemia viral oncogene homolog 4; IGF-R1, insulin-like growth factor-1 receptor; Met, hepatocyte growth factor receptor; VEGFR-2, vascular endothelial growth factor receptor-2, TLR2, toll-like receptor-2; TLR4, toll-like receptor-4.

2.7 Decorin in cancer

2.7.1 Decorin expression in cancer

The expression of decorin has generally been considered to be decreased in cancer tissues compared to normal tissues. This has been shown in various types of cancer including colorectal, bladder and breast cancers (Augoff et al., 2008, Bi et al., 2008, Suhovskih et al., 2015, Dyrskjøt et al., 2004, Iozzo et al., 2011, Boström et al., 2013). Furthermore, the absence of decorin from tumour stroma is considered to be a prognostic marker for the invasiveness and metastatic potential of different cancers. This has particularly been shown to be true for breast and lung cancers (Troup et al., 2003, Goldoni et al., 2008, Oda et al., 2012, Biaoxue et al., 2011).

Even though decorin expression is generally found to be decreased in most types of cancer, the expression of decorin has also been shown to be increased in certain cancer types such as pancreatic cancer and hepatocellular carcinoma (Skandalis et al., 2006b, Jia et al., 2012). In pancreatic cancer, the amount of decorin has been found to be 7-fold higher than in normal pancreas (Skandalis et al., 2006b). Furthermore, the increased amount of decorin has been shown to be associated with a highly malignant phenotype of pancreatic cancer (Skandalis et al., 2006b). In laryngeal squamous cell carcinoma, a stage related accumulation of decorin has been found in the tumour-associated stroma (Skandalis et al., 2006a). One explanation for the accumulation of decorin in the tumour stroma could be the presence of a desmoplastic reaction.

2.7.2 Epigenetic regulation of decorin expression in cancer

Epigenetic modifications causing changes in gene expression are common in cancer. These epigenetic modifications are frequently caused by methylation, a covalent addition of a methyl group to cytosine (Dor and Cedar, 2018). In fact, the degree of aberrant methylation in normal tissues correlates with the development of cancer (Klutstein et al., 2017). It has previously been shown that the expression of decorin is affected by methylation in human cancer tissues and cells. For example, hypomethylation of the decorin gene in stromal cells has been found to lead to increased expression of decorin in human colon carcinoma tissues (Adany et al., 1990, Adany and Iozzo, 1991). Furthermore, decorin expression has been found to be reduced in highly metastatic non-small cell lung cancer (NSCLC) cells compared to

low-metastatic NSCLC cells due to the methylation of the leader sequence (5′-UTR) of decorin (Qian et al., 2014).

2.8 Decorin as an oncosuppressive molecule

Decorin is by far the most studied SLRP, partly due to its involvement in carcinogenesis and its anti-proliferative capabilities. Decorin was initially associated with cancer when it was discovered that p53 and decorin double knockout mice developed tumours faster than p53 knockout mice (Iozzo et al., 1999a). Furthermore, decorin deficient mice were shown to spontaneously develop intestinal tumours (Bi et al., 2008). Moreover, the incidence of tumours and tumour size were increased when the mice were fed a Western style high-risk diet (Bi et al., 2008). These tumourigenic effects were associated with decreased cell differentiation and increased cell proliferation linked to downregulation of $p21^{WAF1/cip1}$, $p27^{kip1}$, intestinal trefoil factor and E-cadherin and to the upregulation of ß-catenin signalling (Bi et al., 2008). Accordingly, decorin deficiency was suggested to be permissive for tumourigenesis (Bi et al., 2008). Following the discoveries of decorin´s involvement in carcinogenesis and tumourigenesis, a plethora of studies have focused on the significance of decorin in cancer. Based on these studies decorin is mainly considered to be a growth suppressing, anti-proliferative molecule and has been named a "guardian of the matrix" because of its ability to counteract pro-tumourigenic signalling via interactions with various growth factors and growth factor receptors (Figure 3) (Neill et al., 2012).

Decorin is synthesised and secreted by fibroblasts, smooth muscle cells, stressed vascular endothelial cells and macrophages, and therefore acts in a paracrine fashion in cancer signalling. Decorin signalling in cancer is mediated through its many binding partners, mainly through the interactions with TGF-ß, Met, IGF-R1, VEGFR-2, the EGF family of receptors and TLR2/4 (Table 3). Through these interactions decorin has been found to be involved in all stages of cancer progression and to regulate tumour growth, apoptosis, angiogenesis and metastasis. In addition, decorin is also known to affect carcinogenesis by regulating inflammation, autophagy and mitophagy.

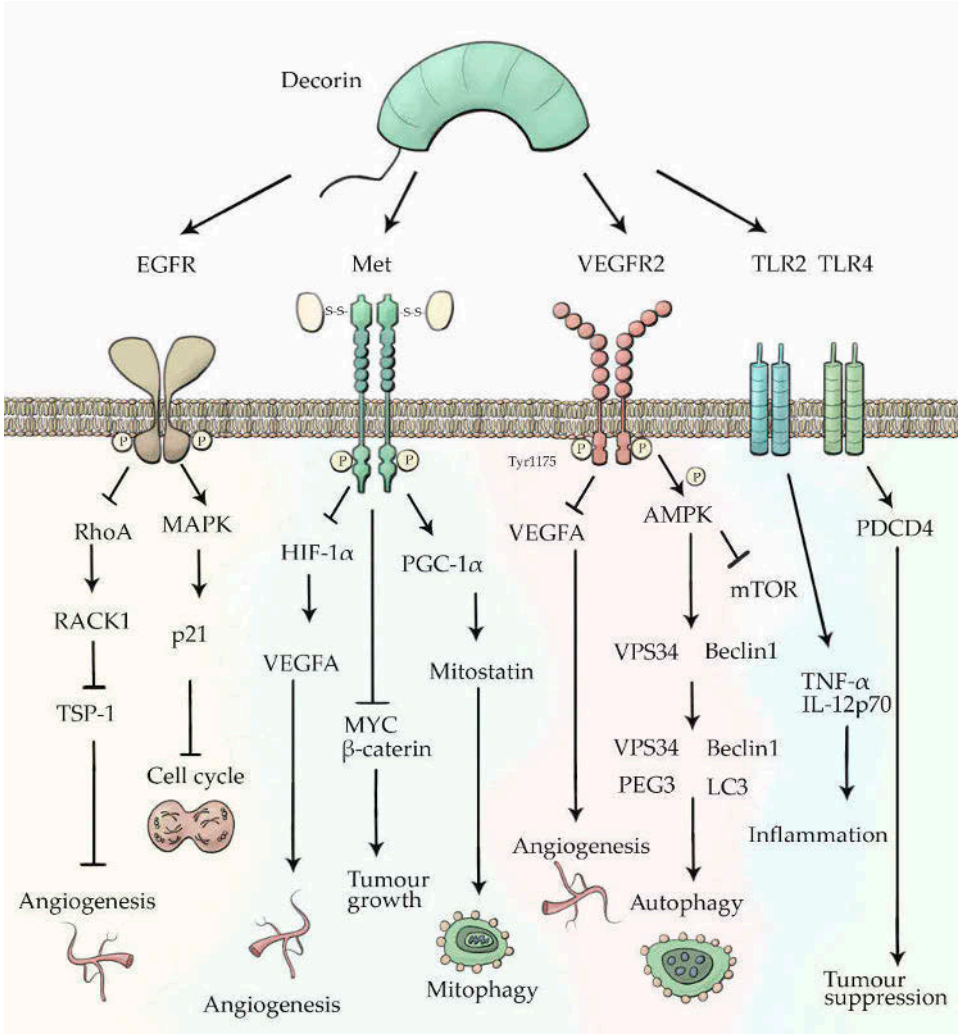

Figure 3. Decorin´s main interactions in cancer signalling. A schematic illustration of decorin´s interactions with cell surface receptors and downstream signalling. Decorin is capable of regulating tumourigenesis and tumour progression via its interactions with for example epidermal growth factor receptor (EGFR), the receptor for hepatocyte growth factor (Met), vascular endothelial growth factor receptor-2 (VEGFR-2), and the toll-like receptors 2 and 4 (TLR2/4). These interactions lead to alterations in tumour growth, angiogenesis, autophagy, mitophagy and inflammation. Modified from (Schaefer et al., 2017).

2.8.1 Decorin and tumour growth

Decorin has been shown to impede cancer cell and tumour growth both in cell lines and in animal models as mentioned earlier. Decorin is capable of

regulating tumour growth by binding to TGF-ß, RTKs such as the EGFR, Met and TLR2/4.

Decorin is capable of suppressing tumour growth and causing sustained growth suppression by its interactions with the EGFR, ErbB2 and ErbB4 (Santra et al., 2000, Csordas et al., 2000, Zhu et al., 2005). The role of the interaction of decorin with TGF-ß in tumour growth is complicated by the fact that the TGF-ß signalling pathway suppresses cell growth in early-stage tumours, but promotes tumour growth in late-stage tumours (Akhurst and Derynck, 2001). Moreover, because TGF-ß stimulates the synthesis of decorin, decorin is thought to regulate TGF-ß activity through a negative feedback loop (Ruoslahti and Yamaguchi, 1991). By binding to Met, decorin causes phosphorylation and degradation of the receptor leading to down-regulation of ß-catenin and Myc levels, and thereby to tumour growth suppression (Goldoni et al., 2009, Buraschi et al., 2010, Yamaguchi and Ruoslahti, 1988). By interacting with TLR2/4, decorin stimulates the production of proinflammatory molecules such as programmed cell death 4 (PDCD4) in macrophages, ultimately leading to suppression of tumour growth (Merline et al., 2011). On the other hand, lack of decorin has been shown to have a tumour growth promoting effect. The absence of decorin in the intestinal mucosa causes proliferation leading to intestinal tumour growth in decorin deficient mice (Bi et al., 2008).

2.8.2 Decorin and apoptosis in cancer

Decorin is known for its oncosuppressive action and also for its capability of causing apoptosis in transformed cells (Tralhão et al., 2003). In contrast, decorin expression in fibroblasts, endothelial cells, hepatocytes and macrophages protects cells from programmed cell death (Schönherr et al., 1999, Xaus et al., 2001, Tralhão et al., 2003). Decorin causes apoptosis in cancer cells through binding with the EGFR and ErbB2/HER 2 receptors. Binding of decorin to EGFR or ErbB2 leads to an activation of the MAPK signalling pathway, resulting in upregulation of the cyklin-dependent kinase inhibitor p-21 and cleavage and activation of caspase 3, ultimately leading to cell cycle arrest and apoptosis (Santra et al., 2000, Seidler et al., 2006). For example, systemic delivery of decorin protein core to tumour xenografts downregulates EGFR and triggers apoptosis via activation of the MAPK signalling pathway, activation of caspase-3 and induction of p21 (Seidler et al., 2006).

2.8.3 Decorin, angiogenesis and cancer

Angiogenesis, the formation of new blood vessels from existing ones, allows tumours to grow beyond the bounds of their original blood supply. The role of decorin in angiogenesis is context dependent and decorin can be regarded as either a pro- or anti-angiogenic molecule (Sainio and Järveläinen, 2018a, Järveläinen et al., 1992, Davies et al., 2001). Decorin supports angiogenesis in a normal non-tumourigenic environment, but is considered to be anti-angiogenic in tumourigenesis (Zhang et al., 2018).

Decorin affects angiogenesis through binding directly to cell surface receptors such as the EGFR, Met and VEGFR-2 (Neill et al., 2012). Decorin has been shown to downregulate EGFR and to disrupt formation of EGFR/ErbB2 dimers in breast cancer leading to growth suppression and diminished distant metastasis (Goldoni et al., 2008). Decorin is also capable of regulating angiogenesis by binding to the VEGFR-2 in endothelial cells (Buraschi et al., 2013).

Decorin´s anti-angiogenic role is also associated with its capability of regulating matrix assembly. Matrix metalloproteinases (MMPs) are enzymes known to promote angiogenesis by degrading the ECM and by setting free pro-angiogenic factors from the ECM, and they are abundantly expressed in all human cancers (Rundhaug, 2005). Decorin stimulates the synthesis of tissue inhibitors of matrix metalloproteinases TIMP-2 and TIMP-3, that both decrease angiogenesis by inhibiting MMPs (Neill et al., 2012).

In addition, it has also been speculated that decorin can regulate angiogenesis through its interactions with TGF-ß (Järveläinen et al., 2015). Whether the interaction with TGF-ß influences angiogenesis in cancer remains to be elucidated.

2.8.4 Decorin, inflammation and cancer

The role of inflammation in carcinogenesis was put forward already in 1863 and is today well established (Hanahan and Weinberg, 2011). Inflammation is considered to be one of the enabling hallmarks of cancer (Hanahan and Weinberg, 2011). However, the role of inflammation in cancer is dual. Besides triggering neoplastic transformation and tumour growth, inflammation can also be tumour growth inhibiting (Mantovani et al., 2008). The ECM influences both inflammation and tumour-associated inflammation by affecting immune cell behaviour (Sorokin, 2010, Lu et al., 2012). PGs are secreted at the inflammation site by machrophages (Uhlin-Hansen et al., 1993). Generally, decorin´s role in inflammation is both pro- and anti-inflammatory

(Sainio and Järveläinen, 2018a). It has been suggested that intact decorin, consisting of both its protein core and its GAG chain, can provoke pro inflammatory signalling while the decorin protein core alone inhibits inflammation (Merline et al., 2011). However, in cancer, decorin has been shown to be pro-inflammatory.

Decorin has been shown to provoke pro-inflammatory signalling in cancer, leading to suppressed tumour growth through interactions with TGF-ß and TLR2/4 (Merline et al., 2011). By binding to TLR2/4 on macrophages, decorin activates the p38, MAPK and NF-κB pathways leading to synthesis of pro-inflammatory cytokines TNF-α and IL-12 (Merline et al., 2011). By binding to TLR2/4, decorin also stimulates the production of programmed cell death protein 4 (PDCD4), a regulator of tumourigenesis and inflammation (Merline et al., 2011). Furthermore, by inactivating TGF-ß, decorin also prevents the translational repression of PDCD4 by reducing microRNA-21 (Merline et al., 2011). A decorin induced increase in PDCD4 results in PDCD4 mediated suppression of anti-inflammatory IL-10 (Merline et al., 2011). Collectively, these decorin provoked effects lead to an inflammatory response, resulting in tumour suppression (Merline et al., 2011).

2.8.5 Decorin, autophagy and mitophagy in cancer

Autophagy is a "self-eating" process through which the cell forms double membrane vesicles called autophagosomes. These vesicles engulf products from the cytosol and fuse with lysosomes, where their contents are degraded (Dikic and Elazar, 2018). Mitophagy is a distinct type of autophagy, where specifically mitochondria are degraded (Youle and Narendra, 2011). The process of autophagy is an evolutionary conserved pathway that maintains cellular homeostasis (Dikic and Elazar, 2018). Consequently, dysregulation of this process is involved in many diseases including cancer (Dikic and Elazar, 2018). The role of autophagy in cancer has been shown to be either tumour-suppressive or tumourigenic depending on cancer type and stage (Singh et al., 2018). Decorin has been shown to be involved in the regulation of both autophagy and mitophagy (Buraschi et al., 2013, Neill et al., 2014). Decorin causes autophagy in endothelial cells via activation of PEG3 (Paternally expressed gene3) and by inhibition of the anti-autophagic Akt/mTOR/p70S6K and the concurrent activation of the AMPK-mediated pro-autophagic pathway (Buraschi et al., 2013, Goyal et al., 2014). Furthermore, the soluble decorin protein core also causes mitophagy by binding to Met and by activating mitostatin (Neill et al., 2014, Buraschi et al., 2017).

2.8.6 Decorin and metastasis

One of the characteristics distinguishing malignant from benign tumours is their capability to metastasize. For a tumour to spread, malignant cells must invade the ECM and disseminate to the vasculature. Malignant cells are in close contact with the ECM at various stages of metastasis (Lu et al., 2012). Both the decorin protein core and its GAG chain have been shown both to reduce the metastatic spread and to inhibit metastasis (Stock et al., 2011, Reed et al., 2005). Treatment with decorin protein core has been shown to reduce primary tumour growth and to eliminate metastasis in an orthotropic breast cancer model (Reed et al., 2005). These effects were associated with the downregulation of the activity and expression of the ErbB2/HER2 (Reed et al., 2005). Treatment of melanoma cells with the decorin CS-6S or CS-4S GAG chain has been demonstrated to inhibit the invasion of melanoma cells by intracellular acidification (Stock et al., 2011).

2.9 Decorin-based therapies in cancer treatment

The numerous anti-oncogenic functions of decorin, referred to above, make decorin a viable candidate for the development of decorin-based adjuvant therapies against human epithelial cancers (Sainio and Järveläinen, 2018a). Initially, ectopic expression of decorin was shown to cause growth suppression in neoplastic cells of various histogenic origins (Santra et al., 1997). Subsequently, the effects of ectopic expression of decorin and systemic or adenovirus-mediated delivery of the decorin protein core or gene have been examined in several different studies of various types of cancers such as breast, lung and colorectal cancers (Sainio and Järveläinen, 2018a). Collectively, all these studies show oncosuppressive effects of decorin treatments, such as decreased and inhibited tumour growth, increased tumour cell death, decreased progression and reduced metastasis (Reed et al., 2002, Goldoni et al., 2008, Dawoody Nejad et al., 2017, Bi et al., 2012). The development of decorin-based therapies for various diseases has commenced. A fusion molecule called CAR-DCN, an engineered variant of decorin with a vascular-homing and cell-penetrating peptide cloned to the C-terminus of decorin, is being developed for the treatment of human diseases (Järvinen and Ruoslahti, 2018). Furthermore, adenovirus-based decorin gene transfer has been used in several studies to treat various cancers.

2.9.1 Adenovirus-mediated decorin gene therapy

A number of studies have used adenovirus-based decorin treatments to investigate the effects of decorin on carcinogenesis and tumourigenesis *in vivo* and *in vitro* in different types of cancer. Both oncolytic and non-oncolytic adenoviruses have been used for this purpose. The concept of using adenovirus-mediated decorin gene transfer to treat human cancers was first introduced in colon carcinoma and squamous carcinoma tumour xenografts, where *in vivo* treatment with adenovirus-mediated decorin was shown to cause significant growth retardation (Reed et al., 2002). Thereafter, tumour injection of a lung adenocarcinoma xenograft model with adenovirus-mediated decorin was shown to suppress both local and distant growth of cancer cells (Tralhão et al., 2003). Resembling these results, adenovirus-mediated decorin was shown to both suppress primary growth and growth of pulmonary metastasis in primary breast cancer in an orthotropic mammary carcinoma model (Reed et al., 2005). Adenovirus-mediated decorin was also shown to decrease progression in a highly metastatic breast cancer cell line (Dawoody Nejad et al., 2017). Oncolytic decorin adenoviruses have also been used in various studies. In pancreatic cancer with desmoplasia, an oncolytic decorin adenovirus was shown to both degrade the ECM and induce apoptosis in orthotopic pancreatic tumours and in pancreatic cancer patient derived tumour spheroids (Li et al., 2018). The efficacy of oncolytic adenovirus-mediated decorin in the treatment of bone metastasis has also been studied in breast and prostate cancers. A study using an oncolytic adenovirus-mediated decorin showed a significant inhibition of progression of bone metastasis, reduced tumour burden and inhibited bone destruction in a mouse xenograft breast cancer bone metastasis model (Yang et al., 2015). Also, in a mouse model of human prostate cancer, systemic delivery of an oncolytic adenovirus expressing decorin has been shown to inhibit bone metastasis (Xu et al., 2015). Furthermore, the efficacy of oncolytic adenoviruses co-expressing decorin and another tumour suppressing agent has also been studied. For example, an oncolytic adenovirus co-expressing decorin and cMet-specific short-hairpin RNA (shMet) has been shown to induce tumour cell death in an orthotropic lung tumour model (Yoon et al., 2016). Moreover, oncolytic decorin adenoviruses encoding both decorin and granulocyte macrophage colony stimulating factor have been shown to inhibit tumour growth in a colorectal tumour model (Liu et al., 2017). Also, in an orthotopic breast cancer model, an oncolytic adenovirus co-expressing interleukin-12 and decorin was demonstrated to induce a potent anti-tumour immune response (Oh et al., 2017).

Collectively these studies show growth-inhibiting and anti-metastatic effects of both oncolytic and non-oncolytic adenovirus-mediated decorin transduction. Furthermore, both specific and distant effects were demonstrated (Tralhão et al., 2003, Reed et al., 2005). Moreover, the results from these studies demonstrate that adenovirus-mediated decorin gene transfer is highly specific to tumour cells, and therefore exhibit low toxicity to normal cells (Tralhão et al., 2003). The results from all of these studies suggest that the development of adenovirus-based decorin gene therapy for the treatment of cancer is reasonable.

3. AIMS OF THE STUDY

Malignant cells in tumours are surrounded and markedly influenced by the tumour microenvironment (TME). The ECM, a complex network of both structural and functional molecules, is a significant part of the TME, and its role in carcinogenesis is increasingly being acknowledged. Decorin, the prototype member of the SLRP family, is recognized for its involvement in carcinogenesis. This is based on the findings regarding decorin´s multiple oncosuppressive actions.

The purpose of this study was to investigate the involvement of decorin in human epithelial cancers, particularly in human bladder, colon and vulva carcinomas. The specific aims of the present study were as follows:

1. To localize decorin expression *in vivo* in tissue samples of human bladder, colon and vulva carcinomas and to examine whether malignant cells in these tissue samples express decorin.

2. To examine whether cell lines representing the above carcinomas express decorin *in vitro*.

3. To study the effects of adenovirus-mediated decorin cDNA transduction on the behaviour of human bladder, colon and vulva carcinoma cell lines *in vitro*.

4. To investigate potential mechanisms responsible for the effects of adenovirus-mediated decorin cDNA transduction on the above cell lines *in vitro*.

4. MATERIALS AND METHODS

4.1 Tissue samples

In this study, tissue samples from human bladder, colon and vulva carcinomas were used to analyze decorin expression *in vivo* in paraffin embedded tissue samples (Table 4). All samples were obtained from the archives of Turku University Hospital and Auria Biobank, Turku, Finland.

Table 4. Tissue samples.

Number of samples	Origin	Histologic grade	Study
9	Human bladder carcinoma	WHO grade I	I
71	Human bladder carcinoma	WHO grade II	I
119	Human bladder carcinoma	WHO grade III	I
3	Human colon adenocarcinoma	WHO grade I	II
5	Human colon adenocarcinoma	WHO grade II	II
3	Human colonic tubulovillous adenoma	low grade dysplasia	II
1	Human colonic tubulovillous adenoma	high grade dysplasia	II
3	Human colonic tubular adenoma	low grade dysplasia	II
1	Human colonic serrated adenoma	low grade dysplasia	II
1	Human colonic neuroendocrine tumour	WHO grade I	II
1	Human colonic neuroendocrine tumour	WHO grade III	II
36	Human vulva SCC	WHO grade I	III
39	Human vulva SCC	WHO grade II	III
17	Human vulva SCC	WHO grade III	III
2	Human vulva SCC	unknown	III
2	Human vulva SCC in situ	unknown	III
1	Human vulva SCC microinvasive	WHO grade I	III
2	Human vulva SCC microinvasive	WHO grade III	III
1	Human vulva verrucous carcinoma	WHO grade I	III

SCC, squamous cell carcinoma; WHO, World Health Organization

4.2 Cell lines

Human bladder, colon and vulva carcinoma cell lines used in this study are presented in Table 5. The human bladder carcinoma cell lines RT-4, 5637 and T24, and the human colon carcinoma cell lines CO-115, HCT-116, DLD-1, HT-29, Vaco-5, LS-180, SW- 620 and RKO were all derived from the American Type Culture Collection (ATCC). The human primary vulva carcinoma cell lines UM-SCV-1A, UM-SCV-1B, UM-SCV-4, UM-SCV-7 and the endo-

metrial carcinoma cell lines UT-EC-1 and UT-EC-3 were a gift from Professor Seija Grenman, Turku University Hospital, Turku, Finland (Grenman et al., 1990, Raitanen et al., 2004, Grenman et al., 1988, Rantanen et al., 1992). The human vulva carcinoma cell line A431 was derived from ATCC.

Table 5. Cell lines.

Cell line	Origin	Histologic grade	Study
RT-4	human bladder carcinoma	I	I
5637	human bladder carcinoma	II	I
T24	human bladder carcinoma	III	I
CO-115	human colorectal adenocarcinoma	Dukes C	II
HCT-116	human colorectal carcinoma	Dukes D	II
DLD-1	human colorectal adenocarcinoma	Dukes C	II
HT-29	human colorectal adenocarcinoma	I	II
Vaco-5	human colonic carcinoma	unknown	II
LS180	human colorectal adenocarcinoma	Dukes B	II
SW620	human colorectal adenocarcinoma	Dukes C	II
RKO	human colonic carcinoma	unknown	II
UM-SCV-1A	human vulva carcinoma	I-III	III
UM-SCV-1B	human vulva (pleural effusion)	III	III
UM-SCV-4	human vulva carcinoma	I	III
UM-SCV-7	human vulva carcinoma	I-III	III
A431	human vulva carcinoma	unknown	III
UT-EC-1	human endometrial carcinoma	II	
UT-EC-3	human endometrial carcinoma	III	

4.3 Primary antibodies

Primary antibodies used for immunohistochemistry (IHC) and Western blot analyses are presented in Table 6.

Table 6. Primary antibodies.

Molecule	Name /#	Manufacturer	Type	Applica-tion	Dilu-tion	Study
Akt	#2920	CST	Mouse monoclonal	WB	1:2000	III
Anti-ß-Actin	AC-15	Sigma-Aldrich	Mouse monoclonal	WB	1:10000	III
Biglycan	L-15	Santa Cruz	Goat polyclonal	IHC	1:200	I-III
Decorin	H-80	Santa Cruz	Rabbit polyclonal	IHC	1:400	I-III
EGFR	#2232	CST	Rabbit polyclonal	WB	1:10000	III
ErbB2	#2165	CST	Rabbit monoclonal	WB	1:10000	III
ErbB3	#4754	CST	Rabbit monoclonal	WB	1:10000	III
Ki-67	30-9	Ventana/ Roche	Rabbit monoclonal	IHC	1:200	I, III
P62/SQSTM1	NBP1-48320	Novus Biologicals	Rabbit polyclonal	WB	1:1000	III
Phospho-AKT	#4060	CST	Rabbit monoclonal	WB	1:2000	III
Phospho-EGFR	#2220	CST	Rabbit polyclonal	WB	1:1000	III
Phospho-ErbB2	#2243	CST	Rabbit monoclonal	WB	1:1000	III
Phospho-ErbB3	#4791	CST	Rabbit monoclonal	WB	1:1000	III
Synaptofysin	SP-11	Ventana/ Roche	Rabbit monoclonal	IHC		II

CST, Cell Signaling Technology; IHC, immunohistochemistry; WB, Western blotting. For other abbreviations see Abbreviations section.

4.4 Primers and probes for quantitative reverse transcriptase PCR (RT-qPCR) analyses

The sequences for the primers and probes used for RT-qPCR analyses are presented in Table 7. Specific probes were used for TaqMan RT-qPCR. Decorin, biglycan and hRPL19 were analyzed using SYBR Green, and EGFR, ErbB2, ErbB3, ErbB4 and GADPH were analyzed with TaqMan RT-qPCR.

Table 7. Primers and TaqMan probes used for RT-qPCR analyses.

Gene	Primer/Probe	Sequence	Study
decorin	primer forward	5´-GGA ATT GAA AAT GGG GCT TT-3´	I-III
	primer reverse	5´-GCC ATT GTC AAC AGC AGA GA-3´	I-III
decorin A1	primer forward	5´-CAG GTG TGG AAA GGA GGA GG-3´	I-II
	primer reverse	5´-GTG TCA GCC GGA TTG TGT TC-3´	I-II
decorin A2	primer forward	5´-AGT CCT CAC CTG AAC CCT GA-3´	I-II
	primer reverse	5´-GAA AGC AGC ATC TTG CCT GG-3´	I-II
decorin B-E	primer forward	5´-CTG CAT CCC ACT CAC CCA AA-3´	I-II
	primer reverse	5´-TTC CTG ATG ACC GCG ACT TC-3´	I-II
biglycan	primer forward	5´-GGA CTC TGT CAC ACC CAC CT-3´	I-III
	primer reverse	5´-CTT GTT GTT CAC CAG GAC GA-3´	I-III
hRPL19	primer forward	5´-AGG CAC ATG GGC ATA GGT AA-3´	I-III
	primer reverse	5´-CCA TGA GAA TCC GCT TGT TT-3´	I-III
EGFR	primer forward	5´-CCA CCT GTG CCA TCC AAA CT-3´	III
	primer reverse	5´-GGC GAT GGA CGG GAT CTT-3´	III
	probe	5´-CCA GGT CTT GAA GGC TGT CCA ACG AAT-3´	III
ErbB2	primer forward	5´-AGC CTT GCC CCA TCA ACT G-3´	III
	primer reverse	5´-ATT GCC AAC CAC CGC AGA-3´	III
	probe	5´-CCA CTC CTG TGT GGA CCT GGA TGA CA-3´	III
ErbB3	primer forward	5´-CCC TGC CAT GAG AAC TGC AC-3´	III
	primer reverse	5'-TCA CTG TCA AAG CCA TTG TCA GAT-3´	III
	probe	5´-GTT TGT CCT AAA CAG TCT TGA AGC TCT GGT C-3´	III
ErbB4 (JM-a)	primer forward	5´-CCA CCC ATG CCA TCC AAA-3´	III
	primer reverse	5´-CCA ATT ACT CCA GCT GCA ATC A-3´	III
	probe	5´-CAT GGA CGG GCC ATT CCA CTT TAC CA-3´	III
GAPDH	primer forward	5´-AGC CAC ATC GCT CAG ACA C-3´	III
	primer reverse	5´-GCC CAA TAC GAC CAA ATC C-3´	III
	probe	universal fluorescent probe #60 (Universal ProbeLibrary, Roche)	III

hRPL19, human ribosomal protein L19; EGFR, epidermal growth factor receptor; ErbB2/3/4, v-erb b avian erytroblastic leukemia viral oncogene homolog 2/3/4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

4.5 IST Online and Human Protein Atlas databases for analyses of decorin expression and immunoreactivity (I-II)

Two publicly available in silico databases were used for data analyses of decorin expression and immunoreactivity in human epithelial cancers. The IST Online database is a database of the human transcriptome (Kilpinen et al., 2008). The Human Protein Atlas database contains data on the distribution of various proteins in normal and malignant tissue samples (Uhlen et

al., 2010). The IST Online (http://www.ist.medisapiens.com/) (formerly known as GeneSapiens database, (http://www.genesapiens.org) was used to analyze previously published results on decorin expression in human bladder and colon carcinomas included in this database. Data on decorin expression in human bladder carcinoma samples (*n*=174) were compared with samples representing human normal bladder tissue (*n*=20). The database was also used to analyze decorin expression in tissue samples representing human colorectal adenocarcinomas (*n*=32), human colon carcinomas (*n*=251), and human colon adenocarcinomas (*n*=497). The Human Protein Atlas database (http://www.proteinatlas.org) was used to analyze the location of decorin expression in high resolution images of previously immunohistochemically analyzed tissues of human colon adenocarcinoma (*n*=10), human adenocarcinoma derived from rectum (*n*=11) and healthy colon (*n*=4).

4.6 Immunohistochemistry (IHC) (I-III)

The immunohistochemistry analyses were performed on 5 μm tissue sections of paraffin embedded tissue samples. The sections were dewaxed in xylene and rehydrated in descending concentrations of ethanol, and washed with phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by treatment of the sections with 0.3% H_2O_2 in H_2O for 15 minutes at room temperature, after which the sections were washed in PBS. Nonspecific binding was blocked by treatment with 2% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. The sections were incubated with primary antibodies diluted in 2% BSA in PBS at 4°C overnight. On the following day, the sections were washed with PBS and incubated with secondary antibodies diluted in 2% BSA in PBS for 1 hour at room temperature. Next, the sections were washed with PBS and incubated with avidine-peroxidase complex solution (Vector Laboratories) for 35 minutes at room temperature, whereafter the sections were washed with PBS and stained with diaminobenzidine (DAB) (Vector Laboratories) for visualization of the signal. Finally, the sections were washed in H_2O and counterstained with Papanicolau hematoxylin and mounted with Aquamount (BDH Laboratory supplies). Positive controls were used and the stained sections were viewed by a pathologist.

4.7 In situ hybridization (ISH) (I-III)

Anti-sense and sense single-stranded RNA riboprobes were constructed for probing of decorin. A 533 bp fragment containing human decorin cDNA was cloned into the Eco RI/Hind III site of a pGEM-4Z transcription vector (provided by Dr. Liliana Schaefer, University of Frankfurt, Frankfurt am Main, Germany). QIA quick PCR purification kit (Qiagen) was used to purify the linearized plasmid. Thereafter, digoxigenin- (DIG) labelled probes were synthesized with SP6 (sense) and T7 (anti-sense) polymerases using the DIG RNA labelling kit (Roche, Applied Science). To quantify the probe, a DIG nucleic acid detection kit (Roche Applied Sciences) was used.

In situ hybridization was performed on 5 μm sections of paraffin embedded tissue samples. The tissue sections were dewaxed in xylene, rehydrated in descending concentrations of ethanol and air dried. Next, the sections were pre-treated with 0.2 M HCl for 20 minutes at room temperature to remove background noise and to improve the signal, and the sections were washed with saline-sodium citrate (SSC) twice for three minutes. The cell membranes were permeabilized with proteinase K in 10 mM Tris-HCl (pH 7.4) containing 2mM $CaCl_2$ at 37°C for 15 minutes. Next, the sections were washed in diethyl pyrocarbonate (DEPC) H_2O and in 2 g/l glycine in PBS twice for three minutes to stop the protease reaction. Acetylation of the sections was performed with 0.1 M triethanolamine containing 0.40% acetic anhydride. Thereafter, the sections were washed in DEPC H_2O twice, dehydrated in a rising concentrations of ethanol and air dried. Subsequently, the sections were rehydrated in PBS. The sections were pre-treated with ficin (Digest –All, Zymed Lab Inc) for 20 minutes at 37°C, and washed with PBS and SSC. Next, the sections were prehybridized with a prehybridization mixture of 50% deionized formamide and 10% dextran sulphate in 4 x SSC, 1 x Denhardt´s solution with 2 nM EDTA and 0.5 mg/ml denatured salmon sperm DNA in a moisture chamber for 2 hours at 42°C. Thereafter, the sections were hybridized with prehybridization mixture containing anti-sense and sense RNA probes in a moisture chamber at 42°C overnight. The sections were washed twice with 2 x SSC, three times for 5 minutes with 60% formamide in 2 x SSC at 37°C, and twice with 2 x SSC. Sections were washed in Tris buffered saline (TBS) and blocked with 3% BSA in TBS for 30 minutes at room temperature in a moisture chamber. Following, DIG label detection was performed on the sections with anti-DIG fab fragments (Roche, Applied sciences) at a concentration of 1:2000 in blocking buffer for two hours at room temperature. The sections were washed twice for 5 minutes in 0.05% Triton X-100 in TBS, 5 minutes in TBS and 2 minutes in detection buffer containing 100 mM Tris pH 9,5, 100 mM NaCl and 50 mM

$MgCl_2$. Thereafter, the signal was visualized by incubation of the sections in detection buffer containing 0.34 mg/ml nitroblue tetrazolium and 0.18 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in a moisture chamber for 3 hours at room temperature, followed by an overnight incubation at 4°C and a two hour incubation at room temperature. Finally, the sections were washed twice in a buffer containing 10 mM Tris (pH 8.0) and 1 mM EDTA, and counterstained with Mayer´s hematoxylin. The sections were mounted using Aquamount (BDH Laboratory supplies).

4.8 Imaging (I-III)

A virtual microscope and a Pannoramic Digital Slide scanner (The Pannoramic 250 Flash, 3D HISTECH, Ltd., Hungary) were used for imaging of the tissue samples. The dotslide (.slide) System (Soft Imaging System, Olympus Company) and the Pannoramic Viewer were used to view and edit the images.

4.9 Cell cultivation (I-III)

The cells used in this study were cultured in Dulbecco´s Modified Eagle Medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS, Biochrom AG), and penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich). The cells were grown at 37°C with 5% CO_2.

4.10 Adenoviral vector (I-III)

The adenoviral vector dcn-pxc1c-1, a recombinant replication-deficient adenoviral vector, was used for decorin cDNA transduction of human bladder, colon and vulva carcinoma cells. In the adenoviral vector decorin cDNA is under the control of the cytomegalovirus (CMV) promoter. The full-length decorin cDNA was cloned into pGEM standard cloning vector and inserted into pxcJL-1 (Fisher et al., 1989). The adenoviral vectors were constructed by cotransfection of HEK293 cells with backbone plasmid pBHG10 (Figure 4). Ad-LacZ, a vector that harbours the *E coli ß*-galoctosidase gene (lacZ) under the control of the CMV 1E promoter, was used as a control (Wilkinson and Akrigg, 1992). The control vector was purchased from the Virus Vector facility at the Centre for Biotechnology of the University of Turku, Turku Finland.

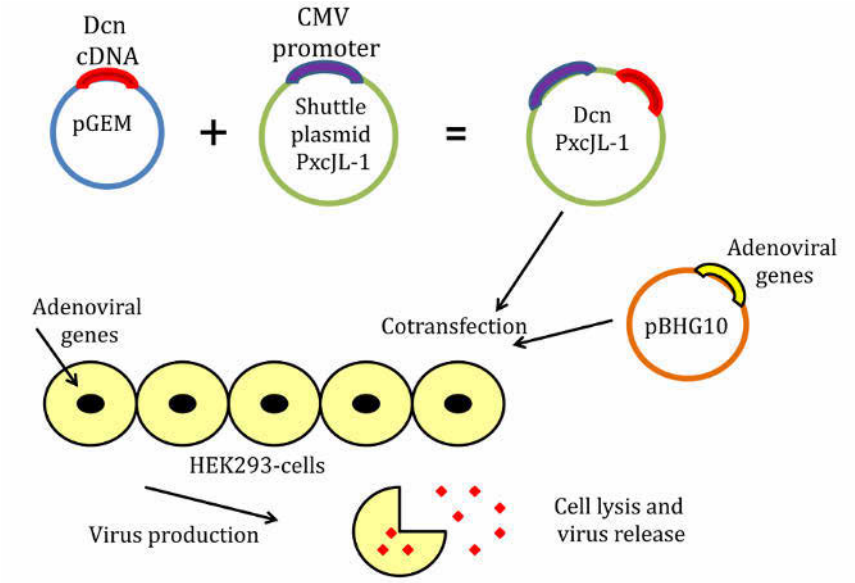

Figure 4. Construction of the adenoviral vector dcn-pxc1c-1 (Ad-DCN). See the text for details.

4.11 Decorin cDNA transduction (I-III)

Carcinoma cells were cultured as described above. The cells were counted and seeded to wells, plates or culture flasks depending on the experiment. After 24 hours, the cells were transduced with culture medium containing 10, 100 or 1000 plaque forming units (pfu)/cell of the decorin cDNA containing vector (Ad-DCN) or the empty control vector (Ad-LacZ). In this study, cells were transduced once or twice. The second transduction was performed 24 hours after the first transduction. 24 hours after the first or second transduction the medium was changed and the cells were grown for an additional 24 hours. Thereafter, the cells were counted and collected for further analyses.

4.12 Soft agar colony formation assay (II)

The soft agar colony formation assay was used to study the colony forming capability of colon carcinoma cells. 6-well plates were coated with a layer (1 ml) of DMEM containing 10% FBS, penicillin (100 IU/ml), streptomycin (100 mg/ml and 0.5% agar (Bacto-agar, Difco). When the first layer had solidified, a second layer (1 ml) containing 60 000 cells in DMEM containing 10% FBS,

penicillin (100 IU/ml), streptomycin (100 mg/ml and 0.35% agar (Bacto-agar, Difco) was added on top of the first layer. After the second layer was solidified 2 ml of culture medium (DMEM containing 10% FBS, penicillin (100 IU/ml), streptomycin (100mg/ml)) was added to the wells and the cultures were grown at 37°C with 5% CO_2. The cultures were maintained for eight days and the medium was changed after four days. The cultures were fixed with 4% paraformaldehyde on day eight and the number of colonies was counted using a light microscope. To determine the number of colonies formed, five fields of view in three layers of three replicate cultures were counted.

4.13 Methylation analysis (I-II)

To analyse the methylation status of the decorin gene promotor in human bladder and colon carcinoma cells automated MethylCap and MeDIP assays were performed using epigenetic sample preparation robot SX-8G IP-Star (Diagenode). Total DNA was extracted from the cells using the QIAamp genomic DNA kit (Qiagen) and MethylCap and MeDIP assays were performed followed by RT-qPCR. Methylated DNA fragments were purified, after which RT-qPCR was performed. Three different decorin isoforms (A1, B1, B-E) were analyzed and each sample was run in four replicates. The qPCR primers are shown in Table 7. The protocol is described in detail in (I).

4.14 RT-qPCR (I-III)

Total RNA from the cells was isolated using the Nucleospin RNA II kit (Marchery-Nagel) according to the manufacturer's protocol. RNA concentrations were determined using a Nano-drop spectrophotometer (Thermo Fisher Scientific). The SensiFast cDNA synthesis kit (Bioline) was used to synthesize cDNA from 1 μg of RNA according to the manufacturer´s instructions. RT-qPCR analyses of gene expression were performed with DyNAmo Flash SYBR Green qPCR kit (Thermo Fisher Scientific). hRPL19 was used for normalization. RT-qPCR analyses for EGFR/HER1, ErbB2/HER2, ErbB3/HER3, ErbB4/HER4 (JM-a) isoform and the housekeeping gene GAPDH expression were performed with TaqMan primers and probes. All samples were run in triplicates. Thermal cycling was performed with QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). Primers and probes are presented in Table 7. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method (Livak, Schmittgen, 2001).

4.15 Protein extraction (III)

For the preparation of cell lysates for Western blotting, the cells were washed with PBS and lysed with lysis buffer (1% Triton X-100, 10 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA with added Protease and Phospatase Inhibitor (Thermo Scentific). Lysed cells were centrifuged at 14 000 x g for 10 minutes at +4°C. The protein concentration in the supernatants was measured with Bradford protein assay (Bio-Rad).

4.16 SDS-PAGE and Western blotting (III)

Western blotting was used to analyze protein expression and activity of the EGF receptor family and the effects of decorin cDNA transduction on the expression and activity of these receptors. SDS–PAGE loading buffer was added to equal amounts of protein sample and the samples were denatured for 5 minutes at 95°C. Equal amounts of the protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% non- fat milk, 1% BSA in TBS for 30 minutes at room temperature. Membranes were analyzed with the primary antibodies EGFR (#2232, Cell Signaling Technology), HER2/ErbB2 (#2165, Cell Signaling Technology), HER3/ErbB3 (#4754, Cell Signaling technology), phospho-EGFR (#2220 Cell Signaling Technology), phospho-HER2/ErbB2 (#2243, Cell Signaling Technology), phospho-HER3/ErbB3 (#4791, Cell Signaling Technology). Actin was used as a loading control. The Western blots were developed using the WesternBright Quantum Western blotting detection kit (Advansta).

4.17 Statistical analyses

The Student´s two-tailed *t*-test was used for statistical analyses. All values $p<0,05$ were considered statistically significant.

5. RESULTS

5.1 Decorin expression and immunoreactivity in human epithelial cancers *in vivo*

The expression of decorin was examined *in vivo* in three different types of human epithelial cancers, namely in human bladder, colon and vulva carcinomas, using the IST Online (formerly known as GeneSapiens database) and Human Protein Atlas databases as well as ISH and IHC analyses of tissue samples from these malignancies.

5.1.1 In silico analyses of decorin expression in human bladder and colon carcinomas

The IST Online database revealed that decorin is abundantly expressed in human bladder carcinoma *in vivo*. However, its expression was lower in carcinoma tissues compared to normal bladder tissues (I: Fig. 1). Regarding decorin expression in colorectal tissues the IST Online database showed an upregulation of decorin expression in colorectal adenocarcinoma, colon carcinoma, colon adenocarcinoma and mucinous colon adenocarcinoma tissues in comparison to human healthy colon (II: Fig. I). On the other hand, analyses of decorin expression in 22 colorectal cancer cell lines included in the IST Online database revealed that only one of the cell lines expressed decorin. The Human Protein Atlas database localized decorin immunoreactivity to cancer cells in 8 of 21 tissue samples.

5.1.2 ISH and IHC analyses of decorin expression in human bladder, colon and vulva carcinoma tissues in vivo

Decorin mRNA expression and immunoreactivity in human bladder carcinoma tissue samples from 199 patients were analysed using ISH and IHC for decorin. The analyses revealed that malignant cells of all the carcinoma samples lacked decorin expression (Figure 5) and that the expression of decorin could only be detected in the original stroma of the samples (Figure 5). The results were identical independently on the grade, histologic type or invasiveness of the carcinomas (I: Fig. 2, 3). On the contrary, malignant cells of these samples were positive for biglycan, a PG closely related to decorin (I: Fig. 4).

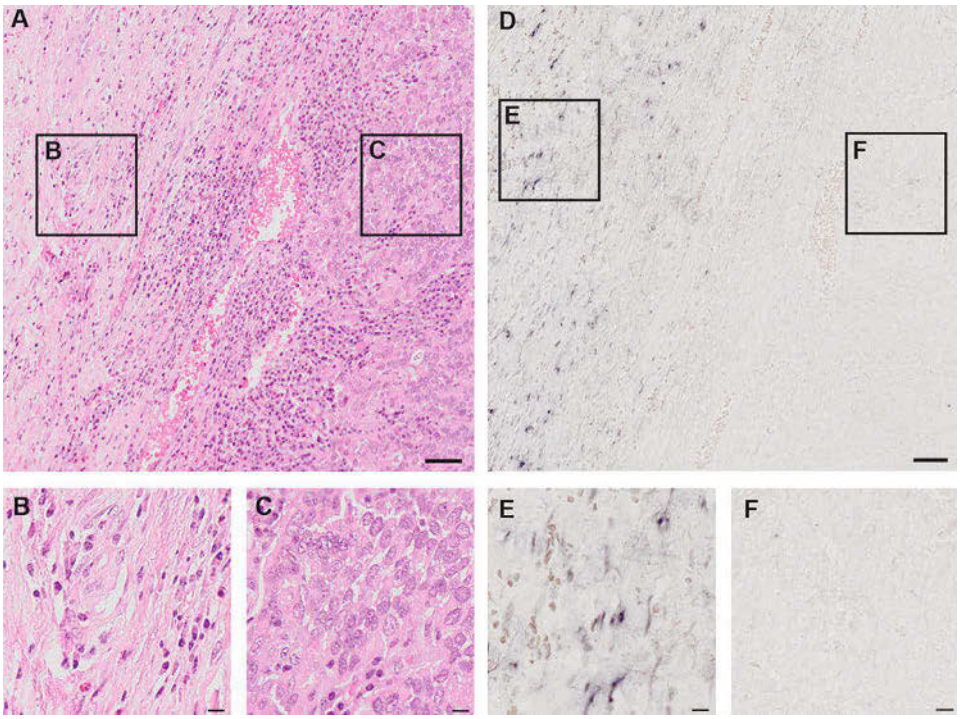

Figure 5. Representative image of decorin expression in human bladder cancer tissues. Panels showing serial sections of a human bladder carcinoma tissue stained with HE (A-C) and analyzed with ISH for decorin (D-F). Left frames in A and D represent non-malignant stromal tissue areas, and are magnified below in B and E. Right frames in A and D represent carcinoma tissue areas populated by malignant cells, and are magnified below in C and F. Decorin mRNA, seen in purple, can only be detected in the non-malignant stromal tissue areas. Scale bars in A and D is 50 μm and in B, C, E and F 20 μm.

Subsequently, decorin mRNA expression and immunoreactivity were examined in tissue samples representing different types and grades of human colon carcinoma. The analyses of human colon carcinoma tissues representing WHO grade I and II colon adenocarcinomas revealed that malignant cells of these tissue samples were devoid of both decorin mRNA expression and immunoreactivity (Figure 6). This was also true for adenoma forming cells including serrated adenoma (II: Fig. 3). Furthermore, malignant cells of human tissue samples representing low grade (WHO grade I) and high grade (WHO grade III) neuroendocrine tumours were found to lack decorin expression (II: Fig. 4).

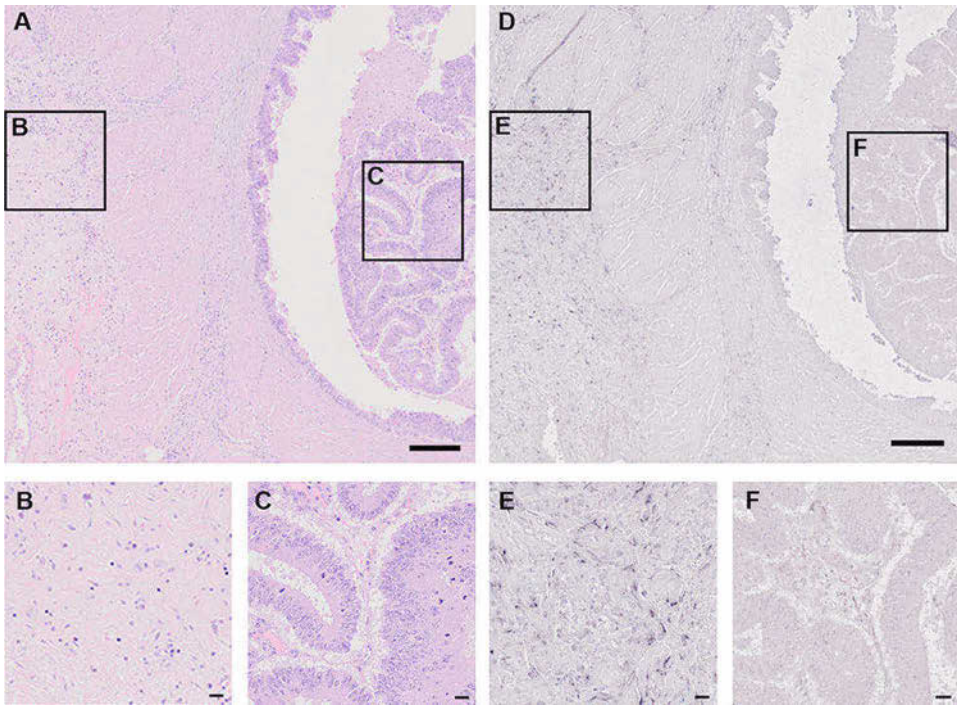

Figure 6. Representative image of decorin expression in human colon adenocarcinoma tissues. Panels showing serial sections of a human colon adenocarcinoma tissue stained with HE (A-C) and analyzed with ISH for decorin (D-F). Left frames in A and D represent non-malignant stromal tissue areas, and are magnified below in B and E. Right frames in A and D represent carcinoma tissue areas populated by malignant cells, and are magnified below in C and F. Decorin mRNA, seen in purple, can only be detected in the non-malignant stromal areas as seen in E. Scale bars in A and D is 200 μm and in B, C, E and F 20 μm.

Finally, the expression of decorin was analyzed in vulva carcinoma tissue samples from 100 patients. The material included samples from WHO grade I, II and III vulva carcinomas. The results were consistent with the previous studies. Decorin mRNA expression or immunoreactivity could not be found in the malignant cells of these tissue samples (Figure 7). In contrast, the malignant cells of these tissues were positive for biglycan immunoreactivity (Figure 7).

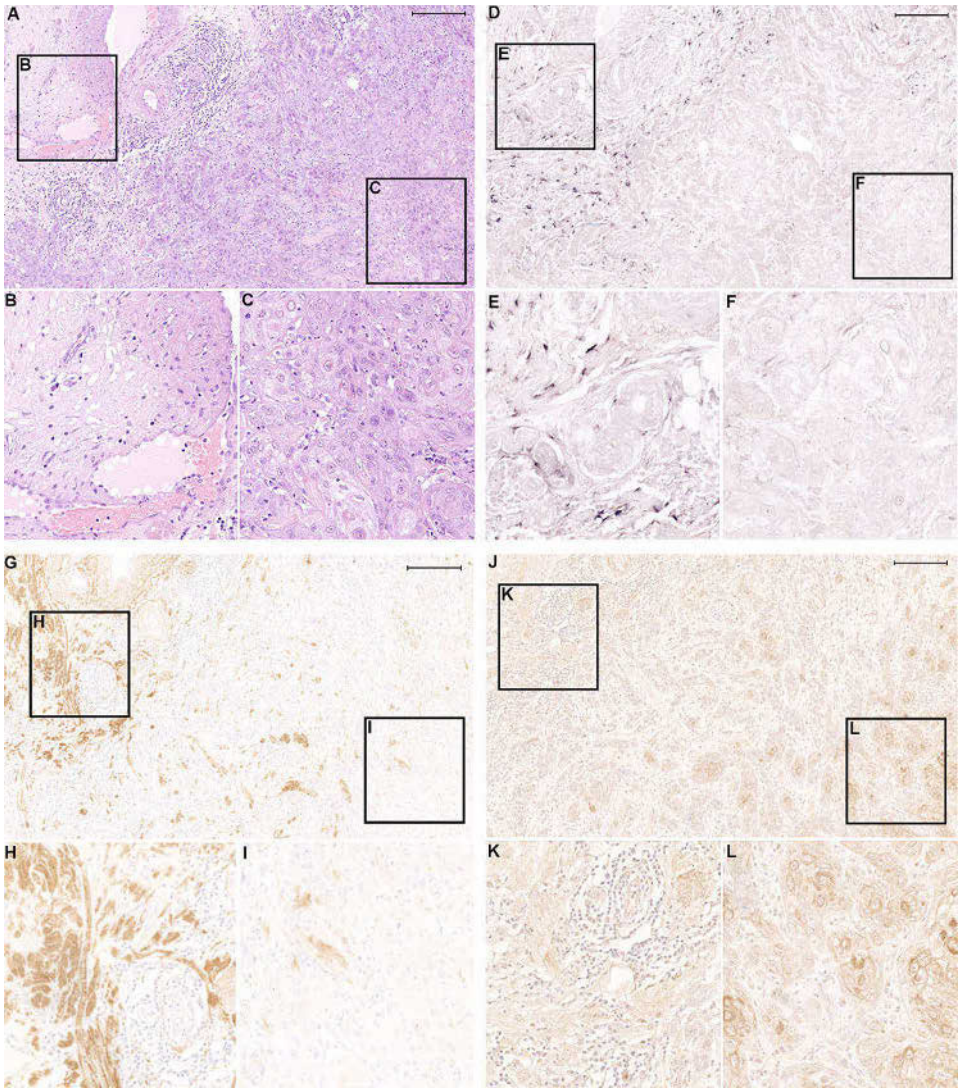

Figure 7. Representative image of decorin and biglycan expression in human vulva carcinoma tissues. Panels showing serial sections of a human vulva carcinoma tissue analyzed with HE (A-C), ISH for decorin (D-F), IHC for decorin (G-I) and IHC for biglycan (J-L). Left frames in A, D, G and J represent non-malignant stromal tissue areas, and are magnified below in B, E, H and K. Right frames in A, D, G and J represent carcinoma tissue areas populated by malignant cells, and are magnified below in C, F, I and L. Decorin mRNA (seen in purple colour) (E), and immunoreactivity (in brown colour) (H) can only be detected in the non-malignant stromal areas, while immunoreactivity for biglycan (in brown colour) can be detected also in carcinoma tissue areas (L). Scale bars in A, D, G and J is 500 μm and in B, C, E, F, H, I, K and L 200 μm.

5.2 Decorin expression in human carcinoma cell lines *in vitro*

Decorin expression *in vitro* was analyzed in carcinoma cell lines representing the above malignancies using RT-qPCR. In the analyses, 3 human bladder carcinoma cell lines, 8 human colon and colorectal carcinoma cell lines, 5 human vulva carcinoma cell lines and two human endometrial carcinoma cell lines were included (Table 5). The results from these analyses showed that none of the carcinoma cell lines expressed decorin. On the other hand, the analyses of biglycan expression in human vulva and endometrial carcinoma cell lines showed that biglyan was moderately expressed by these cells *in vitro*.

5.3 Methylation status of the decorin gene promotor in human bladder and colon carcinoma cells *in vitro*

The methylation status of the decorin gene promotor was studied to explore a potential explanation for the lack of decorin expression in human bladder and colon carcinoma cells. The analyses did not detect methylation in the decorin gene promotor in any of the bladder carcinoma cell lines (I: Fig. 5). In contrast, in the colon carcinoma cell lines, the decorin gene promotor isoforms were methylated (II: Fig. 5).

5.4 Effects of adenovirus-mediated decorin cDNA transduction on human carcinoma cell behaviour *in vitro*

The effects of decorin cDNA transduction on the behaviour of human bladder, colon and vulva carcinoma cells were studied *in vitro* by transducing the cells with Ad-DCN and investigating the effects of the transduction on the proliferation, colony forming capability and viability of the cells.

Ad-DCN transduction was found to significantly decrease the cell number of bladder carcinoma cells compared to Ad-LacZ transduction. This decrease in cell number was due to a significant decrease in the proliferation of the cells (I: Fig. 6).

The soft agar colony formation assay was used to study the effects of decorin cDNA transduction on the malignant behaviour of human colon carcinoma cells. The results using this method clearly showed that Ad-DCN transduction significantly reduced the colony forming capability of the cells.

Ad-DCN transduction significantly decreased both the number (II: Fig. 6 B) and the size (II: Fig. 6 C) of the colonies formed by the cells in soft agar. This was true independently of their original colony forming capability, i.e. for carcinoma cells with high or low colony forming capability.

Regarding human vulva carcinoma cells Ad-DCN transduction affected the viability of these cells. The cell number after Ad-DCN transduction was significantly lower in Ad-DCN transduced cultures compared to AD-LacZ transduced cultures (III: Fig. 2 C). The number of dead cells was higher in Ad-DCN transduced cultures compared to Ad-LacZ transduced cultures (III: Fig. 3). However, decorin cDNA transduction did not affect the proliferation rate of human vulva carcinoma cells (III: Fig. 4).

The mRNA expression of biglycan was also examined in Ad-DCN transduced cells vulva carcinoma cells using RT-qPCR. Interestingly, the expression level of biglycan was reduced in response to Ad-DCN transduction (Figure 8).

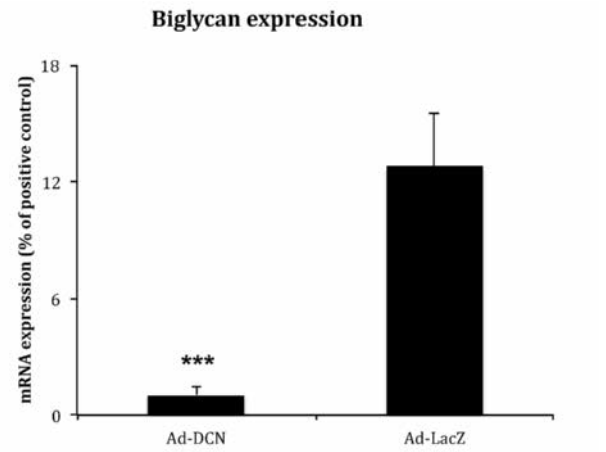

Figure 8. Biglycan mRNA expression is significantly reduced in Ad-DCN transduced human vulva carcinoma cells. The mRNA expression of biglycan as % of positive control in Ad-DCN and Ad-LacZ transduced UM-SCV-7 vulva carcinoma cells.

5.5 Expression of EGF receptor family members in human carcinoma cells

The expression and activity of EGF receptor family members were studied before and after Ad-DCN transduction to explore a potential mechanism behind the Ad-DCN induced effects on human vulva carcinoma cells.

5.5.1 *mRNA expression of EGF receptor family members before and after Ad-DCN transduction of human vulva and endometrial carcinoma cells*

The basal mRNA expression level of EGF receptor family members (EGFR/HER1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4) was first analyzed in human primary vulva carcinoma (UM-SCV-7) and endometrial carcinoma (UT-EC-1) cell lines. The results showed that detectable amounts of EGFR/HER1, ErbB2/HER2 and ErbB3/HER3 were expressed in both cell lines, while no significant expression of ErbB4/HER4 could be detected (Figure 9A, C). The expression level of EGFR/HER1 was the highest in these cells (Figure 9A, C). Subsequently, the expression levels of the above three detectable receptors were studied at the mRNA level after decorin cDNA transduction. The RT-qPCR analyses of cDNA transduced vulva and endometrial carcinoma cells showed a significant downregulation of the ErbB2/HER2 (Figure 9B, D).

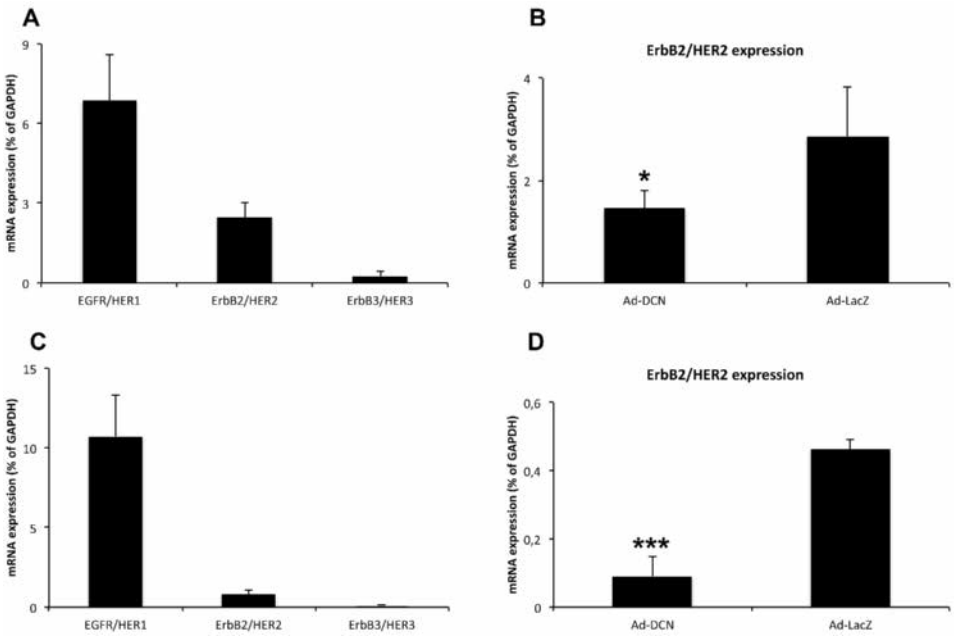

Figure 9. ErbB2/HER2 mRNA expression is downregulated in Ad-DCN transduced human vulva and endometrial carcinoma cell lines. Histograms showing EGFR/HER1, ErbB2/HER2 and ErbB3/HER3 mRNA expression before (A, C) and ErbB2 expression after (B, D) Ad-DCN transduction in human vulva (A, B) and endometrial (C, D) carcinoma cells. *p<0,05, ***p<0,001, Student´s t-test.

5.5.2 *Effects of decorin cDNA transduction on protein amount and activity of EGF receptor family members in human vulva carcinoma cells*

The protein amount and the activity of EGFR/HER1, ErbB2/HER2 and ErbB3/HER3 were also analyzed in the primary human vulva carcinoma cell lines UM-SCV-1B and UM-SCV-7, and in the A431 human vulva carcinoma cell line before and after Ad-DCN transduction. In agreement with the results from mRNA expression analyses of these receptors, both the protein amount and the activity of ErbB2/HER2 were found to be downregulated in all of the above cell lines in response to Ad-DCN transduction (III: Fig. 6, III: Supplementary Fig. 1, III: supplementary Fig. 2). However, no universally consistent effect of Ad-DCN transduction on the protein amount and the activity of the EFGR/HER1 and ErbB3/HER3 was detected in these cell lines (III: supplementary Figure 1).

The protein amount of Akt in vulva carcinoma cells was studied to explore the effects of Ad-DCN downstream of ErbB2/HER2. The results showed that Akt was downregulated in Ad-DCN transduced compared to Ad-LacZ transduced human primary (UM-SCV-7) vulva carcinoma cells (III: Fig. 7).

5.5.3 *Effects of decorin cDNA transduction on protein amount of p62*

The protein amount of the autophagy marker p62/SQSTM1 was studied in Ad-DCN and Ad-LacZ transduced human primary UM-SCV-7 vulva carcinoma cells. The results showed no changes in the expression level of p62/SQSTM1 in Ad-DCN transduced compared to Lac-Z transduced cells (III: supplementary Fig. 3).

6. DISCUSSION

The significant role of the ECM in carcinogenesis has been acknowledged (Hanahan and Weinberg, 2011). Decorin is an essential PG of the ECM known both for its structural and functional roles and for its involvement in carcinogenesis (Schaefer et al., 2017, Sainio and Järveläinen, 2018a). Ever since the oncosuppressive potential of decorin was discovered, its expression has been studied in various types of cancer with mesenchymal or epithelial origin. A plethora of studies have shown decorin expression to be reduced or increased in various epithelial cancers. However, the cellular origin of decorin in epithelial cancers has remained obscure. Particularly, it is not exactly known whether malignant cells in epithelial cancers (carcinomas) express decorin.

In this study, the IST Online (formerly known as the GeneSapiens database) and the Human Protein Atlas databases were used to analyze the previous results on decorin expression in human bladder and colon carcinomas. Decorin expression was also analyzed at the cellular level *in vivo* in these carcinomas using ISH and IHC. Decorin expression *in vivo* was also analyzed in human vulva carcinoma tissues using these methods. Furthermore, cell lines representing the above malignancies were analyzed for decorin expression. The methylation status of the decorin gene promotor was investigated in human bladder and colon carcinoma cells to explore a potential mechanism regulating decorin expression by these cell lines. Moreover, this study examined the effects of adenovirus-mediated decorin cDNA (Ad-DCN) transduction on the malignant behaviour of all the above cell lines. Specifically, the effects on proliferation, colony forming capability and cell viability of the cell lines were studied. Finally, potential mechanisms behind the effects of Ad-DCN transduction on the cell lines were investigated by analyzing the expression and the activity of EGF receptor family members before and after Ad-DCN transduction.

6.1 Decorin expression in human carcinomas

As mentioned above, decorin expression has generally been shown to be decreased in human epithelial cancers such as ovarian, endometrial, prostate, breast and lung carcinomas (Shridhar et al., 2001, Smid-Koopman et al., 2000, Banerjee et al., 2003, Boström et al., 2013, McDoniels-Silvers et al., 2002). On the other hand, decorin expression has also been demonstrated to be upregulated in certain cancers such as pancreatic cancer (Skandalis et al.,

2006b). Nevertheless, as brought up above the exact cellular origin of decorin expression in diverse malignant tissues has remained somewhat unclear. Furthermore, decorin expression in tissues from rare malignancies such as vulva carcinoma has not been previously examined.

Regarding decorin in bladder carcinoma, its expression has been found to be decreased independently on the grade or invasiveness of this malignancy (Dyrskjøt et al., 2004, Sanchez-Carbayo et al., 2006, Iozzo et al., 2011, Appunni et al., 2017). Interestingly, the circulatory levels of decorin have also been found to be lower in bladder carcinoma patients than in healthy controls (Appunni et al., 2017). On the other hand, a strong decorin expression has been found in the muscular layers of superficial bladder carcinomas and in the *tunica muscularis* of the original stroma of these tumours (Iozzo et al., 2011). Also in colon carcinoma, the majority of studies have found decorin expression to be decreased, although increased decorin expression has also been described (Bi et al., 2008, Augoff et al., 2008, Suhovskih et al., 2015, Adany et al., 1990).

The results of this study invariably demonstrated that malignant cells in tissues of human bladder, colon and vulva carcinomas and in human cell lines representing these malignancies lack decorin expression. In contrast, the expression of decorin in these epithelial cancer tissues was shown to be localized to tissue areas populated by non-malignant stromal cells. Therefore, it can be concluded that the decrease or increase in decorin expression reported in the earlier studies is a reflection of how much non-malignant stromal cells is included in the analyzed tissues. Also, the possibility of a desmoplastic reaction influencing the magnitude of decorin expression has to be taken into account. Nevertheless, when analyzing decorin expression *in vivo* in tumour tissue samples, it is crucial to distinguish malignant cells from non-malignant stromal cells. Here, decorin expression was detected at the cellular level *in vivo* using ISH for decorin. With this technique it is possible to exactly localize the expression of a molecule of interest, such as decorin in this study. The use of IHC as the only method is not suitable for this purpose. This is due to the fact that molecules secreted by cells can be distributed to a wide tissue area. The previously published results included in the IST Online and Human Protein Atlas databases do not fulfil these criteria. This explains the obvious discrepancy between the results of this study and the previous results of others. The IST Online database is based on gene expression array data of tissue samples including different types of cells. Consequently, the *in silico* analyses using this database do not specify the exact cellular origin of gene expression. The Human Protein Atlas database consists of IHC analyses and therefore also depends on the specificity of the antibody used in the analyses.

Biglycan, another SLRP that is structurally closely related to decorin, was detected in tissue areas populated by malignant cells in bladder and vulva carcinoma samples. RT-qPCR further demonstrated that human vulva and endometrial carcinoma cell lines express biglycan. These results suggest that opposite to decorin, human carcinoma cells express biglycan. This assumption is further strengthened by previous studies showing that biglycan expression is increased in several epithelial cancers such as colorectal, bladder, endometrial, prostate and ovarian carcinomas (Gu et al., 2012, Liu et al., 2014, Appunni et al., 2017, Jacobsen et al., 2017, Pan et al., 2009). Furthermore, biglycan has previously been found to be overexpressed in endometrial cancer and its expression levels have been found to correlate with the histopathological grade and Figo stage (Liu et al., 2014). Interestingly, the *in vitro* analyses of this study showed that biglycan expression in decorin cDNA transduced vulva carcinoma cells was decreased compared to control cells. This implies that Ad-DCN transduction regulates biglycan expression in these cells and partially explains its oncosuppressive effect.

Besides being structurally closely related, the functions of decorin and biglycan also seem to be somewhat overlapping. This has been demonstrated in decorin and biglycan double knockout mice that have a more severe phenotype than decorin knockout mice (Corsi et al., 2002). However, despite their structural resemblance and their partially overlapping functions, their roles in carcinogenesis are diverse. Decorin is known for its tumour-suppressing capabilities while biglycan functions as a promotor of tumourigenesis (Sun et al., 2016, Gu et al., 2012, Jacobsen et al., 2017, Recktenwald et al., 2012). This has been demonstrated for example in endometrial carcinoma, where biglycan increases the migration and invasion of endometrial cancer cells (Sun et al., 2016). The results of this study further reinforce the opposite roles of these closely related SLRPs in carcinogenesis.

6.2 Role of methylation as a regulator of decorin expression in human carcinomas

Methylation of CpG islands of the 5′ promoter region is common in genes that are not expressed in certain tissues (Dor and Cedar, 2018). Furthermore, epigenetic modifications in general, including methylation, are common in cancer (Esteller, 2011). Therefore, the methylation status of the decorin gene promoter was studied in human bladder and colon carcinoma cell lines to investigate whether this epigenetic mechanism might cause the lack of decorin expression by these cells. The results showed that the decorin gene

promoter was methylated in human colon carcinoma cells but not in human bladder carcinoma cells. These results indicate that epigenetic regulation of decorin expression is carcinoma specific. However, methylation as an epigenetic regulator of SLRP expression in carcinogenesis is important. In agreement with this study, three different sites in the 5′ region of the decorin gene have previously been found to be fully methylated in colon carcinoma cells (Adany and Iozzo, 1991). Furthermore, regarding biglycan, its expression has been shown to be upregulated in tumour endothelial cells through demethylation of its promoter region (Maishi et al., 2016). In addition to methylation, other important epigenetic regulatory mechanisms of gene expression also exist, such as histone modification (Esteller, 2011). Regarding the lack of decorin expression in bladder carcinoma cells, it is probably caused by alternative epigenetic modifications and their role has to be further examined (Esteller, 2011).

6.3 Effects of decorin cDNA transduction on human carcinoma cells

The effects of adenovirus-mediated decorin cDNA transduction on human carcinoma cell lines representing human bladder, colon and vulva carcinomas were investigated. Adenovirus-mediated decorin cDNA transduction was shown to lead to oncosuppression of the cells. The proliferation rate, colony formation capability and viability of the cells were significantly decreased in response to Ad-DCN transduction. These results are in line with previous results from a vast majority of studies, in which ectopic expression of decorin or systemic and adenovirus-mediated delivery of the decorin core protein or gene have been shown to cause oncosuppressive effects on various types of cancer (Sainio and Järveläinen, 2018a). For example, de novo expression of decorin has been shown to suppress the malignant phenotype of human colon carcinoma cells (Santra et al., 1995). Furthermore, A431vulva carcinoma cells, genetically engineered to express decorin, grow tumour xenografts at lower rates than wild type A431 cells (Csordas et al., 2000). In addition, in vivo treatments with adenovirus-mediated decorin cDNA have been shown to suppress tumour growth and metastasis in various malignancies such as colon, breast and lung carcinomas (Reed et al., 2002, Reed et al., 2005, Tralhão et al., 2003).

6.4 Mechanisms of decorin induced oncosuppressive effects on human carcinoma cells

This study investigated some of the potential mechanisms behind the Ad-DCN induced oncosuppressive effects on human epithelial carcinoma cells *in vitro*. Specifically, the effects of Ad-DCN transduction on the expression and activity of the EGF receptor family members were investigated in human vulva and endometrial carcinoma cells. The results showed that the ErbB2/HER2 was downregulated both at the mRNA and protein level in response to Ad-DCN transduction. A downregulation of EGFR/HER1 and ErbB3/HER3 at the protein level could also be detected, but this finding was not consistent. In agreement with this study, decorin has previously been shown to downregulate ErbB2/HER2 (Santra et al., 2000). Decorin is also known to bind to and downregulate EGFR and trigger apoptosis via caspase-3 activation in A431 vulva carcinoma cells (Moscatello et al., 1998, Iozzo et al., 1999b, Seidler et al., 2006). One explanation for the variations in the effects of Ad-DCN transduction on EGFR/HER1 and ErbB3/HER3 seen in this study can be based on the fact that ErbB2/HER2 generally forms heterodimers with these receptors (Wieduwilt and Moasser, 2008).

In this study Ad-DCN transduction caused decreased viability of human vulva carcinoma cells without affecting the proliferation of these cells. The two main signalling pathways downstream of the ErbB2/HER2 are the MAPK pathway that stimulates cell proliferation and the PI3K-Akt pathway that promotes tumour cell survival (Baselga and Swain, 2009). Therefore, to further explore the mechanism behind the decreased cell viability in response to Ad-DCN transduction, the expression of Akt was analyzed in human vulva carcinoma cells. The results showed that the protein expression of Akt was decreased in response to Ad-DCN transduction. This implies that the downregulation of Akt is at least partially involved in the Ad-DCN caused decrease in cell viability. However, the decrease in viability of the vulva carcinoma cells might also be caused by caspase-3 dependent apoptosis (Seidler et al., 2006). Furthermore, because decorin binds to various growth factors and growth factor receptors, it is likely that the oncosuppressive effects of decorin seen in this study in addition to ErbB2 are also mediated through other growth factors and growth factor receptors. Therefore the downregulation of Akt might also be a result of an interaction with for example IGF-R1, another RTK that signals via the PI3K-Akt pathway (Iozzo et al., 2011). Furthermore, autophagy should also be taken into consideration as a potential mechanism of Ad-DCN induced effects seen in this study. Decorin has been shown to induce autophagy and mitophagy in for example breast cancer and glioma cells (Neill et al., 2014, Yao et al., 2016). In this

study the protein expression of the well established autophagy marker p62/SQSTM1 was not effected by Ad-DCN transduction (Bjørkøy et al., 2009, Katsuragi et al., 2015). However, the effects of Ad-DCN transduction on other markers of autophagy were not investigated. Therefore, the molecular mechanisms behind the decorin cDNA induced effects of this study need to be further investigated.

The members of the EGF receptor family are aberrantly expressed in various carcinomas and are involved in both the initiation and progression of cancer. Consequently, these receptors have become targets for cancer therapies (Arteaga and Engelman, 2014). Both monoclonal antibodies such as trastuzumab (Herceptin) and tyrosine kinase inhibitors such as erlotinib and geftinib are currently in use for targeted cancer therapy (Hudziak et al., 1989, Slamon et al., 2001, Arteaga and Engelman, 2014). ErbB2/HER2 overexpression has been found in various types of human malignancies including human breast, gastric and ovarian carcinomas. Decorin has been shown to downregulate the activity of ErbB2/HER by 40% and to almost completely eliminate its phosphorylation in mammary tumour cells (Santra et al., 2000). The effects of decorin on ErbB2/HER2 expression in breast carcinoma have been compared to traztuzumab (Herceptin) (Santra et al., 2000). The results of this study suggest that decorin could be used as a natural antagonist in the treatment of cancers overexpressing ErbB2/HER2.

6.5 Future perspectives

The influence of the ECM on all stages of carcinogenesis and tumourigenesis is well accepted. Decorin, a significant SLRP of the ECM has been acknowledged for its oncosuppressive capacity and has been given the name “guardian from the matrix” for its capability to bind to various growth factors, growth factor receptors and components of ECM, and thereby oppose protumourigenic cues (Neill et al., 2012). While decorin is a naturally occurring, non-toxic molecule in the human body, it is also an oncosuppressive molecule deleterious to malignant cells of epithelial origin. Based on these facts and the results of this study, it is reasonable to continue developing decorin-based targeted therapies for the treatment of epithelial cancers.

7. SUMMARY AND CONCLUSION

The importance of the ECM in health and disease including a variety of malignancies is currently indisputable. This is also true for the ECM proteoglycan decorin, the prototype member of the SLRP family that among other functions is well known for its oncosuppressive activity. However, it has remained obscure whether malignant cells of epithelial origin express it. Thus, the first aim of this study was to examine decorin expression in three different human epithelial cancers, namely in bladder, colon and vulva carcinomas, using tissue samples and cell lines of these malignancies. Next, this study aimed to examine whether adenovirus-mediated decorin cDNA transduction is capable of altering the malignant behaviour of the above cell lines. Thereafter, potential mechanisms mediating the effects of decorin cDNA transduction on these cell lines were evaluated. Particularly, the effects of decorin cDNA transduction on the EGF receptor family members of human vulva carcinoma cell lines were studied.

The main findings were:

1. Malignant cells in human bladder, colon and vulva carcinoma tissue samples as well as the cell lines representing these malignancies did not express decorin. In colon carcinoma cells, this lack of decorin expression was associated with the methylation of the decorin gene promoter. In contrast, in human bladder cancer cells decorin gene promoter was not methylated, indicating that the epigenetic mechanisms regulating decorin expression in malignant cells are carcinoma specific.

2. Adenovirus-mediated decorin cDNA transduction of human bladder, colon and vulva carcinoma cell lines was shown to cause a marked suppression in the malignant behaviour of studied cell lines. This oncosuppressive activity of decorin cDNA transduction was shown to be mediated via various effects on cell functions, e.g. via its effect on the proliferation, viability and colony formation capability of the cell lines.

3. A mechanism responsible for the oncosuppressive effect of decorin cDNA transduction on carcinoma cell lines, specifically on vulva carcinoma cells, was shown to be mediated via the decreased expression and activity of the EGF receptor family members, particularly ErbB2/HER2.

In conclusion, the above results collectively encourage the development of novel, decorin-based adjuvant therapies, specifically targeting the malignant cells of human epithelial cancers such as bladder, colon and vulva carcinomas.

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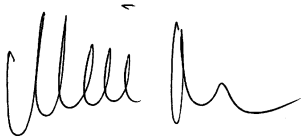

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