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# **The Biology of Decorin in the Tumor Microenvironment**

By

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Thesis submitted to the University of Warwick for the degree of  
Doctor of Philosophy by Published Work in Medical Sciences

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## Abbreviations

Akt/PKB, protein kinase B  
AMPK, AMP-activated protein kinase  
Bcl-2, B-cell lymphoma 2  
CS, chondroitin sulfate  
CSCD, congenital stromal corneal dystrophy  
DS, dermatan sulfate  
EC, endothelial cell  
ECM, extracellular matrix  
EGF, epidermal growth factor  
EGFR, epidermal growth factor receptor  
ErbB, erythroblastic leukemia viral oncogene homolog  
ERK, extracellular signal-regulated kinase  
FN, fibronectin  
GAG, glycosaminoglycan  
GFP, green fluorescent protein  
GSK-3 $\beta$ , glycogen synthase kinase 3  $\beta$   
HA, hyaluronic acid  
HGF, hepatocyte growth factor  
HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$   
HSPG, heparan sulfate proteoglycan  
HUVEC, human umbilical vein endothelial cell  
IGF-1, insulin-like growth factor 1  
IGF-IR, insulin-like growth factor 1 receptor  
KS, keratin sulfate  
LC3, microtubule-associated light chain protein 3  
LRR, leucine-rich repeat  
MDEC, microvascular dermal endothelial cells  
Met, hepatocyte growth factor receptor  
MMP, matrix metalloproteinase  
mtDNA, mitochondrial DNA  
mTOR, mammalian target of rapamycin  
Myc, avian myelocytomatosis virus oncogene cellular homolog  
OXPHOS, oxidative phosphorylation  
p21WAF1, cyclin-dependent kinase inhibitor 1  
Peg3, paternally expressed gene 3  
PGC1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$   
PI3K, phosphoinositide 3-kinase  
PINK1, PTEN induced kinase 1  
RTK, receptor tyrosine kinase  
SHP-1, src homology region 2 domain-containing phosphatase-1  
SLRP, small leucine-rich proteoglycan  
Su9, subunit 9 of mitochondrial ATPase  
TCHP, trichoplein keratin filament binding

TFEB, transcription factor EB  
TGF- $\beta$ , transforming growth factor  $\beta$   
Thr, threonine  
TIMP3, tissue inhibitor of metalloproteinase 3  
Tyr, tyrosine  
VEGFA, vascular endothelial growth factor A  
VEGFR2, vascular endothelial growth factor receptor 2  
VPS34, vacuolar sorting protein 34  
WNT, wntless-type  
 $\Delta\psi_m$ , mitochondrial membrane potential

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## Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy by Published Work, and it has been composed by myself. The submitted material as a whole is not substantially the same as published material that I have previously submitted for a degree. The original articles submitted for consideration are published in international peer-review journals. The following articles have been previously included in two doctoral theses:

1. T.Neill, H.Painter, S.Buraschi, R.T.Owens, M.P.Lisanti, L.Schaefer et al., Decorin antagonizes the angiogenic network. Concurrent inhibition of Met, hypoxia inducible factor-1 $\alpha$  and vascular endothelial growth factor A and induction of thrombospondin-1 and TIMP3, J. Biol. Chem. 287 (2012) 5492-5506.

*This work is in partial fulfillment of T. Neill doctoral thesis in Cell and Developmental Biology, Thomas Jefferson University.*

2. S.Buraschi, T.Neill, A.Goyal, C.Poluzzi, J.Smythies, R.T.Owens et al., Decorin causes autophagy in endothelial cells via Peg3, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) E2582-E2591.

*This work is in partial fulfillment of the doctoral theses of T. Neill and A. Torres in Cell and Developmental Biology, Thomas Jefferson University.*

3. T.Neill, A.Torres, S.Buraschi, R.T.Owens, J.Hoek, R.Baffa et al., Decorin induces mitophagy in breast carcinoma cells via peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) and mitostatin, J. Biol. Chem. 289 (2014) 4952-4968.

*This work is in partial fulfillment of the doctoral theses of T. Neill and A. Torres in Cell and Developmental Biology, Thomas Jefferson University.*

## Summary

Decorin is a ubiquitous secreted small-leucine rich proteoglycan associated with all major collagen-rich matrices. Initially described for its roles in maintaining the structural integrity of various organs and in collagen fibrillogenesis, decorin has gained notoriety for its involvement in regulating physiological and pathological processes such as wound healing, inflammation, cell adhesion, and carcinogenesis. Decorin exerts these functions through its ability to bind with high affinity to multiple receptor tyrosine kinases (RTKs). In cancer, decorin has been described as a paracrine tumor repressor and anti-angiogenic molecule. However, a detailed description of its mechanism of action in the tumor microenvironment was unknown. Understanding the role of the tumor stroma in modulating the signaling of cancer cells, tumor angiogenesis, and metastatic spreading is critical and of clinical relevance. This thesis unscrambles the biology of decorin in the tumor microenvironment and describes a new double mechanism of action where decorin alters signaling cascades of both cancer cells and surrounding stromal cells via selective binding to different RTKs, ultimately elucidating its tumor suppressor activity. The key observations of my studies are: **[1]** the discovery that physical interaction of decorin with the Met receptor on cancer cells causes a protracted downregulation of  $\beta$ -catenin and its downstream effector Myc both at the transcriptional and post-translational levels (chapter 3); **[2]** the discovery that systemic delivery of soluble decorin in vivo causes genotypic changes in the stroma of orthotopic breast carcinoma xenografts and the identification of Peg3 as a novel decorin-induced tumor suppressor in the tumor microenvironment (chapter 5); **[3]** the observation that decorin evokes autophagy in endothelial cells via binding to VEGFR2 and induction of Peg3 (chapter 6). Thus, here I define how decorin dually affects the tumor proper by suppressing Met activity and the tumor vasculature by inducing unrestrained autophagy. Decorin could tie the interplay between extracellular signaling and intracellular catabolic pathways that leads to angiostasis and

tumor growth suppression and could be an innovative therapeutic alternative against cancer.

## **CHAPTER 1**

### **Introduction**

#### **1.1 The extracellular matrix**

The extracellular matrix (ECM) is a complex acellular network of macromolecules that function as a scaffold for the growth, biochemical support, and homeostatic development of multicellular organisms (1-4). This three-dimensional meshwork of proteins defines cell polarity and tissue boundaries but also serves as a reservoir for growth factors and a versatile substrate for cell migration. Two main classes of macromolecules comprise the ECM: fibrillary molecules, such as collagen, elastin, fibronectin (FN), and laminin and non-fibrillar components such as proteoglycans, and hyaluronan (HA) (4;5). Based on structure and composition, two major types of ECMs: pericellular matrices and interstitial matrices, can be identified. The basement membrane is a specialized type of pericellular matrix found between epithelial cells and underlying connective tissue. Most basement membranes are composed by sheet-like layers of matrix proteins (mostly collagen IV, laminins, and proteoglycans) that anchor and support the epithelium, and is found basolateral to all cell monolayers in the body (6;7). In contrast, the interstitial matrix is the most abundant reservoir of ECM components in the body and is found in interstitial connective tissue. It is characterized by the presence of FN, proteoglycans, elastin, and types I, III, V, VI, VII, and XII collagens (4). Proteoglycans fill the interstitial space with a hydrated gel-like polysaccharide composed by repeated units of disaccharides called glycosaminoglycans (GAGs). Proteoglycans exert multiple functions in the matrix, from binding and sequestering various growth factors to providing hydration by trapping water

molecules for gel formation to confer mechanical support. Proteoglycans also interact with fibrous proteins to generate molecular structures that allow the forming matrix to withstand compressive forces (8-10). However, the most abundant components of the ECM are the fibrous proteins collagen, elastin, FN, and laminin. The majority of collagens form a triple-stranded helix that aggregates into molecular complexes that function as structural elements of the ECM. They regulate cell adhesion, provide tensile strength, and are involved in migration and development (5). Several types of collagens have been identified in connective tissues that either form fibrils or bind to collagen fibrils to link them together. Moreover, collagen associates with elastin fibers, which are abundant in those connective tissues undergoing repetitive stretch. Tight associations between elastin fibers and collagen fibrils modulate various properties of tissue elasticity (4). Another key fibrous protein, FN is a dimeric glycoprotein that serves as a primary adhesion molecule of the connective tissue. With binding sites for both GAGs and collagens, FN crosslinks these ECM members, as well as other growth factors and cell adhesion receptors (11). Lastly, laminin is a key structural component of the basal membrane that can interact with collagen, proteoglycans, and cell surface receptors (12). Although the ECM is most abundant in connective tissues, each tissue has a unique ECM signature and ultrastructure characterized by varying ratios of ECM gene expression that dictate the structure and function of the tissue. Cartilage, for example, is composed of a firm layer of highly concentrated polysaccharides that confers mechanical resistance. Tendons are, instead, characterized by a higher expression of fibrous proteins (13). In healthy tissue, non-activated fibroblasts secrete and organize collagen fibrils, various proteoglycans like decorin, HA, FN and elastin, all of which accumulate in the forming matrix and provide ECM functionality and impart tissues with structural integrity. Importantly, in most tissues, the ECM undergoes constant enzymatic or non-enzymatic remodeling through finely balanced cycles of degradation, synthesis, and deposition. This “dynamic reciprocity” (14) characterizes the mechanical properties of each

tissue, such as elasticity or stiffness, and confer protection by maintaining extracellular homeostasis and water retention (15-17).

A large majority of ECM proteins actively communicate with the surrounding cells through binding to various cell surface receptors such as integrins, discoidin domain receptors, and receptor tyrosine kinases (RTKs) to initiate signaling cascades that control gene transcription, cell growth, differentiation, and survival in healthy and diseased tissues (4). In this context, the ECM is the primary provider of external stimuli that control cellular behavior and function. The bidirectional dialogue between the ECM network and embedded cells is essential during tissue development to maintain organ function and homeostasis. Dysregulation of this interplay can lead to maladaptive changes in ECM composition and architecture (5). As the ECM signature dynamically changes in response to stress or injury, the ratio of the expression of different ECM components is actively modulated. In cancer, ECM remodeling, and aberrant deposition by cancer-associated fibroblasts increase the stiffness of the tumor compared to the surrounding tissue and induce inflammation (18). The release of various growth factors such as vascular endothelial growth factor (VEGFA) promotes neovascularization, fostering a malignant phenotype (19). Recently, advances in high-throughput genomic and proteomic techniques allowed molecular characterization of the global ECM in healthy and diseased tissues. The so-called matrisome, is the collection of ECM glycoproteins (FN, laminins, tenascins, etc.), collagens, proteoglycans, and ECM-affiliated proteins (secreted factors, enzymes, etc.) whose differential expression can be analyzed in various tissues (3). With this method, the elucidation of the total composition of different ECMs in healthy and disease tissues could lead to the identification of unique biomarkers and novel therapeutic targets.

## 1.2 Small leucine-rich proteoglycans

The small leucine-rich proteoglycans (SLRPs) represent the largest and most diverse family of proteoglycans, which is composed of 18 distinct macromolecules abundantly found in the ECM (10). Each member is characterized by a relatively small protein core of 36-42 kDa formed by a central region of 10-12 leucine-rich repeats (LRR). SLRPs are ubiquitously expressed and are synthesized and secreted into the pericellular space, which then gets incorporated into the tissue extracellular matrix. During development, SLRPs are widely expressed in all key membranes such as the meninges, pericardium, and pleura that surrounds all major organs. The 18 SLRPs known today are divided into five classes based on protein homology, intron-exon organization of their genes, chromosomal organization, evolutionary conservation, and the presence of N-terminal cysteine-rich clusters of four characteristic cysteine residues with defined spacing (10;20). The heterogeneous nature of the GAG chains, which are post-translationally and covalently attached to the N-terminus of the core protein of some SLRPs, also differentiates the five classes. The first distinction comes from the presence or absence of the GAG chain. Only members belonging to class I-III possess a GAG chain and are therefore considered canonical SLRPs. Almost all class I SLRPs have chondroitin sulfate (CS) or dermatan sulfate (DS) chains flanking their LRRs at the N-termini. Class II members are, instead, composed of poly-lactosamine or keratan sulfate (KS) chains. Class III members are more heterogeneous and contain a mixture of either CS/DS, KS, or no GAG chains may be present at all. Finally, classes IV and V are considered non-canonical as members of these classes do not undergo post-translational modification in the form of GAG chain addition. Still, they share structural homology with other SLRPs. At the genomic level, chromosomal clustering of the 18 SLRP genes on seven different chromosomes suggests they developed by chromosomal duplication. Although the significance of SLRP gene clusters is currently unknown, it indicates a degree of genetic redundancy and potential functional compensation (20;21).

The key distinguishing structural feature and reason for this eponymously named class of ECM molecules is the variable numbers of LRRs present in the central region of the protein. Each LRR is composed of a conserved hydrophobic motif where multiple leucines can be substituted by isoleucine, valine, or other hydrophobic amino acids. These LRRs form characteristically curved solenoidal structures, where each repeat functions as a single turn of the solenoid. Whether each individual LRR constitutes a functional unit of the SLRP or clusters of them confer function, akin to zinc fingers in a transcription factor, remains to be elucidated. Regardless of their classification, all SLRPs share similar biological functions, including the ability to bind to different collagens and cell surface receptors to modulate various intracellular signaling pathways (9;21;22). Class I SLRPs include decorin, biglycan, asporin, and ECM2. A distinctive feature of this class is the unique N-terminal Cys-rich consensus sequence that form two disulfide bonds and differs from any other class of SLRPs (23). Decorin and its closest relative, biglycan, share ~65% overall homology at the amino acid level and both are encoded by similar genes of eight exons with highly conserved intron/exon junctions (23). Biglycan contains two Ser-Gly attachment sites at the N-terminus for two CS/DS chains, hence its name. Conversely, asporin, lacks the typical Ser-Gly sequence required for glycanation and, therefore, does not contain a covalently attached GAG chain. However, asporin is still considered a Class I SLRP as it has a similar exonic organization with highly conserved intron/exon junctions similar to decorin and biglycan. ECM2 is the latest addition to the Class I SLRPs. The protein core of ECM2 is 35% identical to that of decorin. Moreover, both asporin and ECM2 are linked on chromosome 9 (20).

### **1.3 Decorin: structure and function**

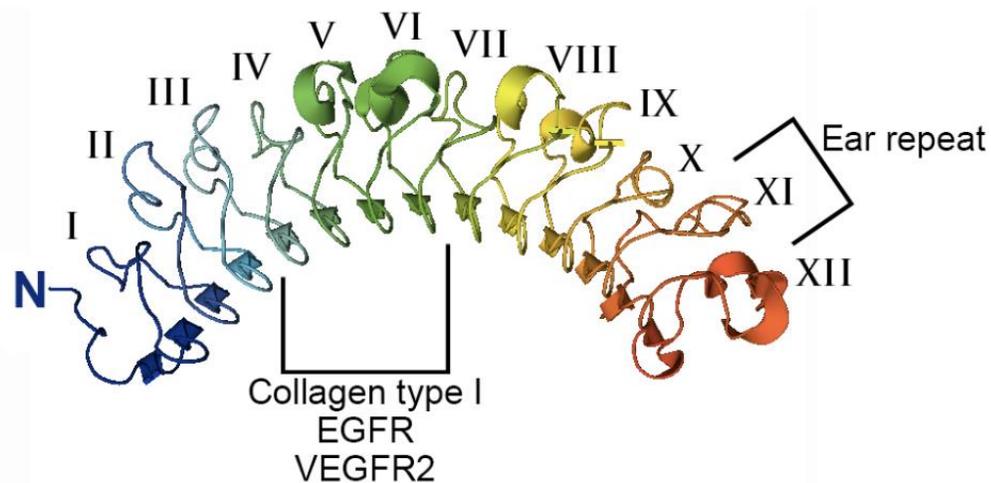
An extensively studied member of the canonical Class I SLRPs is decorin, whose gene is located on chromosome 12 (24). Originally investigated in the context of collagen fibrillogenesis (25), decorin owes its name to the unique

ability to bind and “decorate” collagen fibrils. Synthesized primarily by fibroblasts and myofibroblasts, decorin is ubiquitously expressed in most extracellular matrices. During development, its primary localization is in the membranes that envelop all primary organs, suggesting a role in regulating organ formation (1;26). Decorin was the first secreted proteoglycan to be linked to the control of cell growth via blockage of transforming growth factor  $\beta$  (TGF $\beta$ ), opening new and unexplored research avenues in the field of matrix biology, especially as it relates to human diseases (27). Since then, decorin has been described not only as a structural entity but also as one of the most versatile proteoglycans able to regulate a vast range of biological functions (27). Decorin has crucial roles in the control of angiogenesis (28), myocardial infarct (29), innate immunity (30), inflammation (31), fibrosis (32), wound healing (33), regulation of corneal wound healing (34), and autophagy (35). This repertory of functions arises from the ability of decorin to interact with numerous ligands in the ECM, mainly growth factors, and to evoke intracellular signaling cascades through binding to cellular receptors (36). These intricate interactions engage both decorin GAG chain as well as specific binding motifs in its LRR rich protein core (Fig. 1). The crystal structure of decorin shows the proteoglycan shaped like a horseshoe with the inner concave surface structurally defined by 14 curved  $\beta$ -sheets and the outer convex surface by multiple  $\alpha$ -helices (37) (Fig. 1). It is the concave surface of the arc-shaped decorin protein core, with leucine placed in conserved positions as predominant hydrophobic residues that mediates the physiological ability of decorin to bind ECM components and receptors, with high affinity (38). Each LRR, composed of an average of 24 amino acids, has a unique biological function and binding constant. The primary physiological function of decorin in normal tissue is the control of collagen fibrillogenesis (36). Site-directed mutagenesis studies of decorin LRR IV-VII revealed a direct collagen-binding site (39;40). Decorin binds to a region of collagen type I, close to the intermolecular cross-linking site at its C-terminus. Stoichiometrically, a singular molecule of decorin can bind to four to six collagen molecules at the D period of

the collagen type I molecule (41). This non-covalent, and presumably reversible binding regulates collagen fibril assembly and cross-linking with delayed assembly and constriction of the final diameter of the collagen fibers (42). Decorin-deficient mice are characterized by non-uniform, irregularly shaped collagen fibrils in the skin (43). This anomalous collagen network assembly allows for full-body development but leads to skin fragility and an irregular healing process. Notably, the N-terminal O-linked DS GAG chain of decorin can also simultaneously bind to adjacent collagen fibers and participate in collagen fibril formation during development (44). Decorin can also bind collagens II-XII and XIV (36). Other members of the SLRP family are involved in the regulation of collagen fibril assembly in connective tissue, as shown in various SLRP knockout animal models. For instance, biglycan null animals show a propensity for osteoarthritis and bone mass reduction (45). Osteoarthritis is also found in mice lacking fibromodulin, whereas lumican knockout causes corneal opacity and a skin fragility phenotype akin to decorin (46).

A crucial function of decorin and other SLRPs is the binding of growth factors and the subsequent regulation of their downstream signaling activities. Dysregulation of TGF $\beta$  is the hallmark of fibrotic diseases characterized by excessive and aberrant matrix deposition. Decorin binds and neutralizes TGF $\beta$  activity, ultimately preventing rampant fibrosis (16;47). Moreover, through LRR IV-VI, decorin binds and antagonizes multiple RTKs, such as the vascular endothelial growth factor receptor 2 (VEGFR2) in extravillous trophoblasts through LRR V/VI (48;49), and the epidermal growth factor receptor (EGFR) (50). A structural feature of decorin, which is shared by different SLRPs, is the presence of the so-called ear repeat at the C-terminal LRR XI (Fig. 1). The ear repeat extends laterally from the solenoid structure of the main protein and consists of a cysteine in LRR XI that forms a disulfide bond with another cysteine in the terminal LRR XII (51). This results in LRR XI being longer (30 amino acids) than the other LRRs in the decorin protein core. The ear repeat is believed to participate in protein folding, as shown in a mouse model of congenital stromal

corneal dystrophy (CSCD). This genetic disease is characterized by the truncation of the last 33 amino acid residues in the C-terminal domain, which includes the ear repeat. The mutation leads to intracellular accumulation of misfolded decorin and activation of the ER stress response, ultimately leading to visual impairment and corneal opacification in mice (52). Corneal specimens of patients affected by CSCD showed abnormal stroma and dysregulated fibrillar collagen structures due to the intracellular accumulation of truncated decorin (38). Decorin naturally undergoes dimerization, although it is the monomeric form that is responsible for most of its interactions, as dimerization prevents exposure of the core binding regions found on the concave surface of the protein (53). In this aspect, decorin alternates between forming homodimers and binding to collagen type I as a monomer (53). Importantly, dimerization of the protein core of decorin was found to be not necessary for decorin stability (53). Through its interactive network, decorin sequesters growth factors in the ECM (54;55), governs receptor-signaling events (56), and modifies the processing of other critical components of the matrix (57). The ability of decorin to alter intracellular signaling pathways from the matrix has tremendous implications in clinical oncology as decorin may function as a valid therapeutic approach to fight the development and spreading of the disease.



**Figure 1.** Graphical representation of the crystal structure of monomeric bovine decorin created via Pymol v1.7 (PDB accession number [1XKU](#)) depicting its curved appearance and leucine rich repeats. LRR 4–6 are important for binding of decorin to collagen and receptor tyrosine kinases, including VEGFR2 and EGFR. The ear repeat at the C-terminal domain is important for correct protein folding. Modified from Gubbiotti et al. (18)

#### 1.4 Decorin expression in solid tumors

To better understand the biological effects of decorin in cancer, it is necessary to investigate its expression patterns and localization within the tumor compartments. There are several incongruences in the literature when it comes to decorin expression levels in different types and grades of tumors. In invasive breast carcinoma, loss of decorin in the tumor microenvironment is associated with a poor prognosis (58). Moreover, a query of the Human Protein Atlas Database demonstrated a marked reduction in the expression levels of decorin in the stroma of various tumors, including breast, colon, cervical, and ovary carcinomas (59). In the stroma of low- and high-grade bladder cancers, decorin is significantly reduced within the tumor stroma (60) as well as in cases of esophageal squamous cell carcinoma (61) and colon cancer (62). However, other studies reported elevated amounts of decorin in the stromal compartment of certain tumors, including colon and breast carcinomas (63). Given the ample

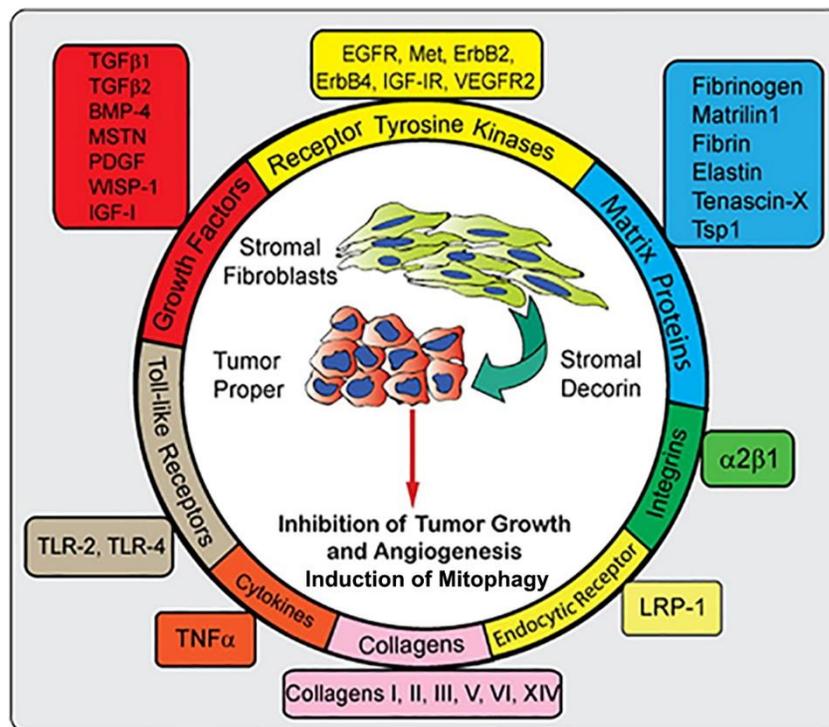
range of components of the extracellular matrix that binds to decorin, we can argue that its overexpression in the stroma of these tumors may negatively regulate growth by sequestering growth factors and downregulating RTKs present on the adjacent tumor cells. Pertinent to the tumor parenchyma, different studies have shown complete loss of decorin expression by the cancer cells of prostate, urothelial, and hepatic malignancies (64). In some neoplasms, the decorin promoter results hypermethylated, thus allowing unchecked tumor progression through silencing of decorin (64). Further evidence for the oncostatic role of decorin comes from genetic models of decorin ablation (43). If fed with a high-fat diet, decorin null mice develop spontaneous intestinal tumors (65). Moreover, a combined knockout mouse model of decorin and p53 results in the development of aggressive lymphomas (66), whereas reintroduction of decorin via adenoviral delivery or systemic administration of exogenous decorin significantly counteracts the tumorigenic and angiogenic growth in a variety of solid tumors (67;68). Collectively, these studies provide robust support for a general tumor-suppressive role of decorin in multiple solid tumors.

### **1.5 Decorin: a guardian from the matrix**

A significant part of the studies on the molecular mechanisms of decorin in tumorigenesis and angiogenesis is based on decorin's avid binding to RTKs on the cell surface of cancer cells. Decorin has been described as a guardian from the matrix (64) for its innate ability to oppose cancer growth via the sequestration of growth factors in the ECM and via the direct antagonism of several RTKs (36;69). This definition is based on multiple key observations derived from the plethora of decorin-binding molecules (Fig. 2). For many years, EGFR, the first-ever described RTK to bind decorin, has been believed to be the only receptor on cancer cells whose signaling was modulated by decorin interaction (70). Decorin antagonizes EGFR activity by triggering dimerization

of the receptor and internalization through the caveosomes, ultimately leading to degradation in the lysosomes and induction of p21WAF1 coincident with cell cycle arrest (71). Decorin competes with EGF, the natural ligand of EGFR, and exerts a potent physiological down-regulation of EGFR on cancer cells, affecting the growth of several types of solid tumors in which EGFR play a key role (72). In 2009, a new study revealed an additional RTK affected by decorin. In an RTK phosphotyrosine array, Met, the receptor for the hepatocyte growth factor, was found phosphorylated in quiescent cells stimulated with decorin for 15 min (73). In HeLa cells, decorin-evoked degradation of Met happens through association to caveolin-1 and concomitant lysosomal degradation. This is in contrast with the canonical endosomal sorting and recycling to the plasma membrane of Met upon binding to the hepatocyte growth factor (HGF), suggesting a selective mechanism of action of decorin that dictates a different biological outcome. Additional members of the epidermal growth factor receptor family of RTKs (ErbBs) have been shown to interact with decorin. Significantly, ErbB4 signaling is directly antagonized by the binding of decorin to the receptor (74). Decorin prevents ErbB4 to dimerize with ErbB2, ultimately causing growth inhibition of breast carcinoma cells (74). Decorin-binding to various RTKs usually stimulates receptor internalization and degradation. However, this is not the case for the insulin growth factor receptor type I (IGF-IR). Differently from the other RTKs studied, IGF-IR is not internalized and degraded by decorin in urinary bladder carcinoma, but the binding of decorin to the receptor and its natural ligand IGF-I leads to inhibition of migration (60). Lastly, decorin antagonizes VEGFR2 in trophoblastic (EVT) cells (48) and in endothelial cells (ECs) (35), resulting in downstream attenuation of ERK1/2 pathways and induction of autophagy (35), a biological process which will be discussed later. Importantly, Met is the primary receptor for decorin's activity in tumor cells. This conclusion is based primarily on the binding constants for decorin on various cell surface RTKs. Empirically, the  $K_d$  of decorin/Met interactions is tighter than the other known constants, such as decorin/EGFR, decorin/VEGFR2, and decorin/IGF-IR (36). Indeed,

decorin exhibits a hierarchical affinity for its various RTK targets. Functionally, pharmacological blockage of Met kinase activity, but not of EGFR activity, abolishes decorin-evoked downregulation of  $\beta$ -catenin in HeLa cells (73). Decorin might evoke intracellular cross-talk signaling events via multiple receptor binding, which ultimate in tumor growth arrest. This mechanism is in perfect accordance with the emerging body of evidence that stresses the action of soluble decorin as a paracrine tumor repressor whose absence is permissive for tumor neovascularization and cancer growth.



**Figure 2.** Decorin interactome, encompassing different cellular and extracellular components to which decorin physically binds through high-affinity interactions and negatively or positively regulates. Modified from Neill et al. (34)

## **CHAPTER 2**

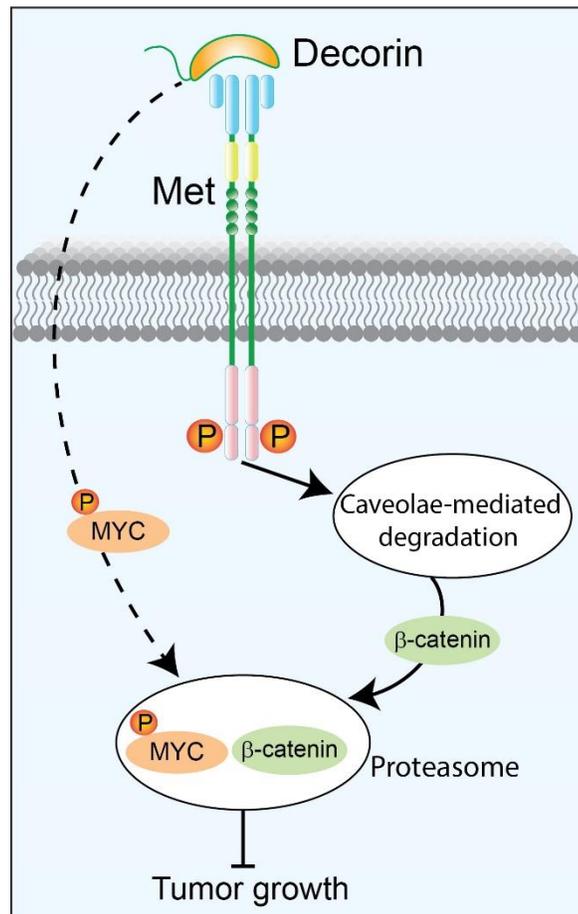
### **Research question**

In this thesis, I sought to determine the mechanism of action of decorin within the tumor microenvironment, a complex biological system that governs cancer initiation, recurrence, and metastasis. The tumor microenvironment encompasses extracellular matrix proteins, and stromal cells like fibroblasts, immune cells, and ECs essential for tumor vascularization. The balance between pro- and anti-angiogenic factors is finely regulated by synergy among the components of the tumor microenvironment and the proper cancer cells. In this context, the lack of an in-depth analysis of the role of decorin in regulating tumor-stroma interactions, as well as the composition of the tumor microenvironment, represented a significant gap in the study of decorin anti-angiogenic and anti-tumorigenic activity. Moreover, until recently, most of the emphasis on cancer research has focused on the biology of primary tumors rather than on the tumor microenvironment. Although most cancer-related deaths are the result of metastatic disease, some deaths are due to other factors, such as an overwhelming tumor stroma response. The factors contributing to cancer-related mortality require further exploration to facilitate the development of novel targeted treatments. Thus, studies to determine how the microenvironment can be targeted to prevent tumor initiation, progression, and metastasis are warranted. Decorin, a secreted proteoglycan physiologically bound to collagen, serves as a primary therapeutic candidate given that its absence in the tumor microenvironment favors cancer growth. With my studies, I dissected the mechanism of action of decorin on both the tumor stroma and cancer cells, further elucidating its biology. By hitting both compartments, decorin commences an anti-angiogenic and pro-autophagic program that explains its oncosuppressive effects and posit it as an innovative therapeutic strategy.

## **CHAPTER 3**

### **Decorin antagonizes Met receptor activity**

My initial studies explored the discovery that decorin binds to Met receptor with higher affinity than EGFR (KD circa 2 nM) (73). I became interested in this subject as this observation opened new research possibilities for the interpretation of the role of decorin in tumorigenesis and angiogenesis (75). Indeed, Met and its natural ligand, the hepatocyte growth factor (HGF), are essential to embryonic development. Deregulation of their signaling is often associated with tumorigenesis and metastasis, usually through overexpression of the receptor. Met interaction with decorin causes receptor degradation through translocation into caveolin-1 positive endosome post-recruitment of c-Cbl to Met at Tyr1003 phosphorylation site (73). This mechanism ensures a protracted cessation of Met oncogenic signaling similar to the effects exerted by decorin on EGFR signaling and in net contrast with HGF/Met canonical signaling. My contribution to this line of research lies in the discovery that decorin's antagonistic activity on Met causes downregulation of two key oncogenes,  $\beta$ -catenin and its downstream effector Myc via the 26S proteasome (76). Myc, a well-characterized critical oncogene involved in the pathogenesis of multiple types of cancers (77), is markedly down-regulated by decorin treatment both at the transcriptional and post-translational levels in cancer cells. Dissection of this mechanism explained decorin anti-tumorigenic activity and generated new insights into the use of decorin as a novel therapeutic modality against cancer (Fig. 3).



**Figure 3.** Tumor-suppressive action of decorin on cancer cells upon direct binding to Met. Decorin binding to Met causes a rapid phosphorylation of the receptor followed by caveolar endocytosis and degradation, and cessation of Met oncogenic signaling. Moreover, decorin triggers proteasomal degradation of  $\beta$ -catenin under Met control, as this effect can be abolished by blocking the kinase activity of the receptor. Decorin also phosphorylates Myc at Thr58 and targets it for degradation by the proteasome. This latter effect could be mediated by the antagonism of decorin to different RTKs on the surface of cancer cells.

### 3.1 Decorin/ $\beta$ -catenin/Myc signaling axis

Of clinical relevance is the fact that the binding of decorin to Met results in the reduction of primary tumor growth and metastatic spreading. Suppression of  $\beta$ -catenin and Myc levels lead to inhibition of multiple pathways under the control of Met, such as proliferation, migration, survival, and scattering. In particular, downregulation of Myc protein levels occurs via decorin-induced phosphorylation of Myc at Thr58 (78), a known phosphorylation site that evokes Myc degradation via the proteasome. Interestingly, Myc targets AP4, a transcriptional repressor of p21WAF148, a cyclin-dependent kinase inhibitor whose expression is induced by decorin in cancer cells (79). Moreover, the transcriptional downregulation of Myc, further boosts decorin oncostatic effects. Decorin might also be utilizing Met-independent pathways in suppressing Myc levels in HeLa cells, as reducing the expression of Met with specific siRNA is not sufficient for Myc downregulation, suggesting that Myc regulation is Met independent (73). Of note is that decorin silencing of  $\beta$ -catenin signaling occurs in a non-canonical manner independent of GSK-3 $\beta$ . Decorin targets  $\beta$ -catenin for proteasomal degradation by inducing phosphorylation via Met, causing an overall stall in tumor growth (73). To corroborate this, *in vivo* studies utilizing orthotopic squamous cell and mammary carcinoma models showed a significant reduction of tumor growth three weeks after systemic administration of exogenous decorin (58;67). Importantly, decorin shows a specific tropism *in vivo* for cancer cells. Indeed, infrared-labeled decorin injected in mice bearing xenografts targets explicitly the tumor mass for up to 68 hours post-injection. Except for the liver and the kidneys where decorin is metabolized and cleared, no other organs showed intake of the protein. My studies strengthen the potential use of soluble decorin as a therapeutic agent for solid malignancies and are a critical contribution to the field as they highlight the direct oncosuppressive mechanism of decorin action. Notably, systemic or adenovirus-mediated delivery of decorin in animals bearing various types of malignant xenografts markedly suppresses tumor growth and metastasis in

several independent studies (58;80-84). Collectively, these results describe for the first time a link between decorin, a secreted proteoglycan, and key oncogenic signaling proteins such as Met,  $\beta$ -catenin and the multifunctional Myc transcription factor. Furthermore, non-canonical governance of  $\beta$ -catenin by decorin may underlie the mechanism of intestinal tumor formation following decorin ablation. Intestinal epithelium turnover and maturation are propelled by  $\beta$ -catenin (85). Met is constitutively activated in colon carcinoma and directly influences  $\beta$ -catenin signaling (86). Given that global loss of decorin derepresses multiple RTKs in two distinct chemically induced models of hepatocellular carcinoma (87), malignant transformation of the intestinal epithelium (or other solid malignancies) is triggered by interfering with the Met/ $\beta$ -catenin axis. Lefebvre et al., 2012 described how Met degradation is an attractive novel strategy for tumor therapy given its overexpression and transforming activity in cancer cells. In this context, by preventing Met/HGF binding, and thus activation of the receptor, decorin forestalls Met-induced cell transformation (88). Baghy et al., 2016 also noticed that decorin could serve as a tumor suppressor in liver cancer given the ability to downregulate  $\beta$ -catenin and Myc. Notably, lower levels of decorin mRNA are reported in liver cancer, supporting the role of decorin as an inhibitor of cancer growth (54).

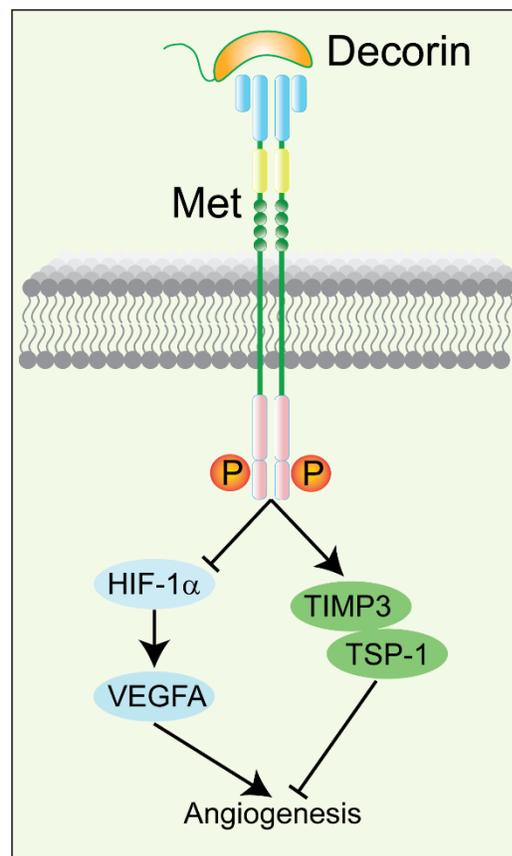
## **CHAPTER 4**

### **Decorin antagonizes the angiogenic network**

The description of the signaling events evoked by decorin through binding to Met has advanced the matrix biology research field, as it reinforces the role of the ECM in restraining tumor progression (18;89;90). A critical cancer hallmark and an important factor contributing to the growth and spreading of cancer cells is tumor angiogenesis. In this context, Met targeting by decorin is key for suppression of tumor angiogenesis in cervical and breast carcinomas, where it blocks HGF-induced vascularization in vivo (91). These initial findings have influenced my colleague Dr. Neill to investigate and reveal a more intricate signaling cascade upon decorin binding to Met that supports its antiangiogenic effects. He found that decorin exerts a robust angiostatic activity to counteract neo-vascularization by inducing two anti-angiogenic proteins, Thrombospondin 1 and TIMP3, and by concurrently inhibiting two influential pro-angiogenic factors, the hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and the vascular endothelial growth factor A (VEGFA) (91;92). Mechanistically, decorin transcriptionally represses HIF-1 $\alpha$  as well as the already mentioned oncogenes  $\beta$ -catenin and Myc under normoxic conditions, further contributing to angiostasis. Ultimately, the suppression of these genes critically impairs Met-evoked VEGFA transcription. This is critical as, in a feedback loop, loss of HIF-1 $\alpha$  inhibits expression of Met (93), compromising the ability of cancer cells to respond to the scatter factor HGF. Thus, decorin, by binding to Met, significantly mitigates important mechanisms of cancer progression and tumor angiogenesis that sustain cellular growth and proliferation. Moreover, decorin induces two key angiogenic inhibitors, TIMP3, and thrombospondin-1 in the tumor microenvironment, thus decreasing tumor growth by actively inhibiting intracellular signaling events and by hindering blood supply to tumor cells altering the tumor secretome and microenvironment (Fig. 4). This study functionally links decorin and the Met signaling axis to the regulation of

pathological VEGF-mediated angiogenesis and provides a mechanism of action for decorin in inhibiting a key cancer hallmark. By acting on the tumor cells, decorin suppresses VEGFA expression at both transcriptional and post-transcriptional levels, thus inhibiting de novo formation of blood vessels in the surrounding microenvironment. In particular, the concurrent reduction of matrix metalloproteinase 2 (MMP-2) activity coupled with induction of thrombospondin-1 and TIMP3 induces angiostasis in the matrix, acting to further restrict matrix-bound VEGFA from engaging cognate receptors (e.g., VEGFR2) on the surface of tumor ECs. Importantly, thrombospondin-1 expression is negatively regulated by Myc. The accumulated data revealed a mechanism where decorin inhibits tumor growth in multiple ways. This study is an important contribution to cancer, signal transduction, protease, and proteoglycans fields because it described a potent angiostatic effect for decorin in the initial phases of tumor angiogenesis. At this stage of tumor progression, before the activation of the angiogenic switch, tumor hypoxia is incapable of inducing tumor vascularization. The action of soluble decorin in this context delineates a compelling mechanism for this secreted proteoglycan as a modulator of early angiogenic events. The relevant role of the ECM in regulating tumor angiogenesis is well documented. Specific proteoglycans tightly interact with vascular cells in the ECM and can alter their phenotype, control vascular disease, and act as a molecular rheostat to inhibit or promote angiogenesis (19;94;95). The sprouting of novel blood vessels to supply oxygen to malignant tumors is orchestrated by crosstalk between migrating ECs and cancer cells. In this context, the vascular basement membrane, a type of ECM enriched in collagen type IV, laminin, perlecan, and other molecules, guides both physiological and malignant angiogenesis (27). Under normal conditions, angiogenesis, the process of new blood vessel formation from pre-existing ones, is strictly managed by a balance of activators and inhibitors. It is essential for embryonic development, and physiologically occurs during wound healing and the menstruation cycle. The initial step involves the release of pro-angiogenic triggers such as VEGFA, fibroblast

growth factor (FGF), platelet-derived growth factor (PDGF), etc. following tissue hypoxia or inflammation (96). ECs respond to this stimulus by enzymatically degrading the basement membrane through the activation of MMPs and other proteinases to clear the path for EC migration and proliferation (97). Once ECs have assembled in new vessels, they become metabolically quiescent phalanx-like cells that form stable interactions with the surrounding pericytes through a matured vascular basement membrane. In stark contrast, in cancer the balance between anti- and pro-angiogenic factors is compromised and is highly skewed to favor the latter. Hypoxic tumor cells excessively secrete pro-angiogenic factors (VEGFA, FGF, etc.), and cytokines which activate ECs, and remodel the ECM to favor growth (7). In this environment, tumor ECs lose their phalanx-like properties, coverage by pericytes, and become excessively and aberrantly



**Figure 4.** Schematic representation of decorin angiostatic activity in cancer cells mediated by direct binding to the Met receptor.

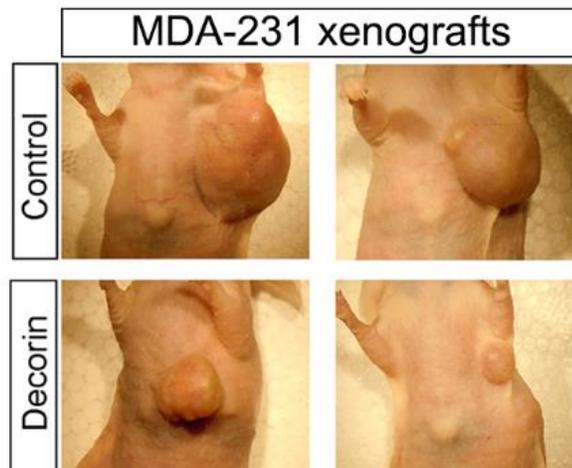
fenestrated. Proteases break down the basement membrane allowing exposure of soluble factors that can further stimulate malignant proliferation of tumor ECs towards the cancer mass (98). Compared to healthy ECs, tumor ECs present increased tube-formation abilities, chromosomal anomalies, and are resistant to senescence due to VEGFR2 overexpression and various other cytokine receptors (99). Moreover, the vascular basement membrane becomes loosely attached to tumor ECs and pericytes, allowing for vascular permeability and tumor intravasation (99). The anti-oncogenic properties of decorin have been confirmed in various preclinical (59;80-82;84;100-103) and genetic studies utilizing *Dcn*<sup>-/-</sup> mice where lack of decorin has been shown to be permissive for tumorigenesis (65;66;87;104) strengthening our hypothesis that decorin could have broad clinical applications as an angiostatic and tumor-suppressive agent.

## **CHAPTER 5**

### **Decorin affects the gene expression profile of the tumor microenvironment**

Decorin-evoked angiostasis through direct binding to RTKs on the tumor cell membrane and consequent inhibition of endogenous VEGFA expression presented decorin as a stromal-derived tumor repressor. My work showed the mechanism that allows decorin to target for degradation essential oncoproteins, such as  $\beta$ -catenin and Myc, thereby counteracting changes in the gene expression signature that would otherwise foster cancer growth. Since decorin is a secreted proteoglycan and acts as a soluble paracrine factor on the tumor proper, my next research question was to identify potential alterations evoked by decorin in the gene expression profile of the tumor microenvironment. This intricate compartment constantly evolves and interacts with the cancer cells to permit growth and neovascularization. To dissect the relationship between tumor stroma and parenchyma, I utilized a well-known human triple-negative breast carcinoma model and investigated gene expression changes, not only in the cancer cells but specific to the tumor microenvironment as well, following systemic administration of decorin. My experiments demonstrated that soluble decorin, acting in a paracrine fashion, altered the gene signature of the tumor-stromal compartment, as evidenced by the differential modulation of 374 stromal genes (68). My work further elucidates the relevance of the stromal matrix in cancer progression and how alteration to the stromal compartment can interfere with cancer growth. The initial observation was that mice bearing MDA-MB-231 xenografts and treated with decorin every other day for a total of 23 days showed a decreased growth rate compared to the untreated control group of mice and a smaller tumor volume at the end of the experiment (Fig. 5).

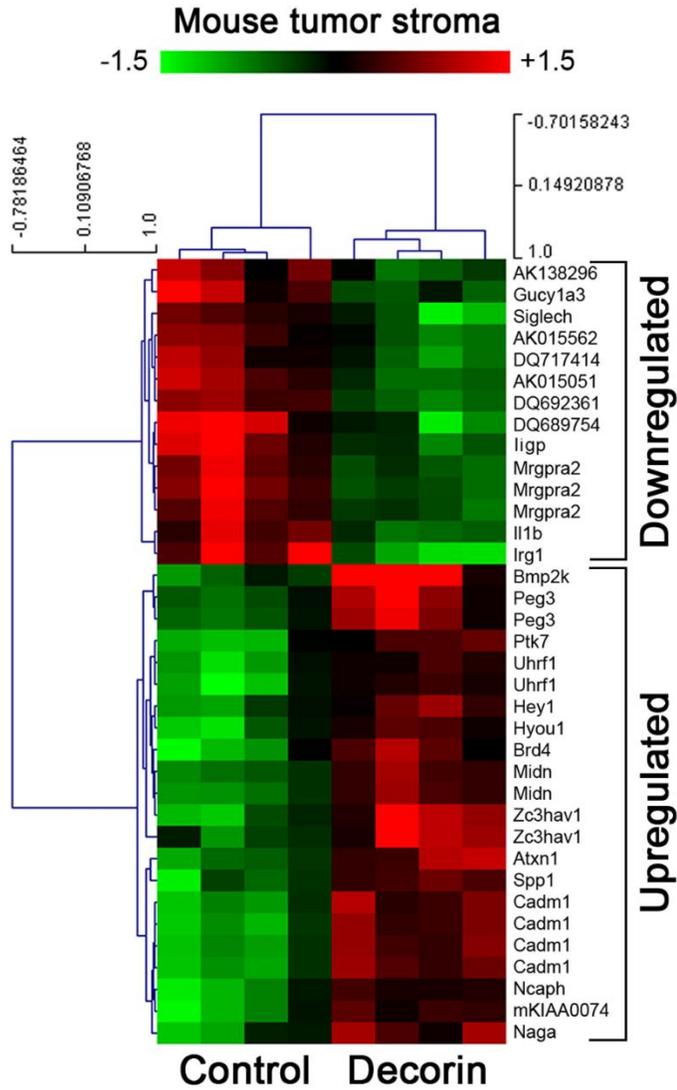
I decided to analyze the tumor tissues with the NimbleGen microarray to measure simultaneously the expression of transcripts derived from both the mouse (i.e., the stroma) and the human (i.e., the tumor cell line, MDA-MB-231). At that time, this innovative technology, in contrast with conventional microarrays, allowed to differentiate and profile 720,000 transcripts from both *Homo sapiens* and *Mus musculus* origin in the same sample and on the same array. The advancement of this investigation lies in the prevailing view of the tumor stroma as a central compartment heavily activated by cancer cells to promote growth and neovascularization. Thus, exploring genes that modulate the tumor stroma itself could provide novel targets for therapeutic intervention. With this technology, I identified stromal gene expression signatures altered by systemic delivery of decorin and, therefore, explored the activity of soluble decorin as a paracrine factor in the microenvironment of a growing human tumor. My analysis revealed that most of the stromal genes suppressed by decorin belonged to the immune response, whereas in the upregulated group of genes, I found many cell adhesion and tumor suppressor genes. Independent validation of the top 12 genes demonstrating >2-fold up- or down-regulatory changes identified *Mrgpra2*, *Siglech*, *Irg1*, and *Il1b* to be suppressed concurrent with induction of *Zc3hav1*, *Peg3*, and *Bmp2k* (68) (Fig. 6). These data propose



**Figure 5.** Representative macroscopic photographs of control and decorin-treated animals bearing MDA-231 orthotopic xenografts at day 23. Modified from Buraschi et al. (39)

decorin as a tumor repressor able to alter the tumor secretome overall, attenuating inflammation and metastatic spreading. My followed up studies focused on *Peg3* as it possesses unique and distinctive characteristics: the paternally expressed gene 3 is an imprinted gene whose paternal allele is exclusively responsible for expressing a molecule which contains 12 Krüppel-type zinc finger domains with two proline-rich periodic repeat domains and is often inactivated in several malignancies (105). Multiple studies of this gene described a role for *Peg3* as a tumor suppressor. Moreover, the N-terminal SCAN domain of *Peg3* mediates direct binding to  $\beta$ -catenin and promotes 26S proteasomal degradation that is independent of GSK3 $\beta$  (106). These studies are highly interesting because they recapitulate the pathway utilized by decorin, in cancer cells, for the non-canonical, GSK3 $\beta$ -independent antagonism and inhibition of  $\beta$ -catenin signaling (76) and could provide a vital link between decorin-evoked attenuation of RTK signaling, via Met, and non-canonical  $\beta$ -catenin antagonism. Moreover, an analysis of *PEG3* expression in publicly available databases of published microarrays, revealed a statistically significant decrease of *PEG3* mRNA in invasive ductal breast carcinoma, confirming its role as a tumor suppressor and providing further clinical validation of the critical role of *PEG3* in, at least, human ductal breast carcinomas. Most importantly, for my research area, these studies provide significant relevance for the newly discovered decorin-evoked induction of *Peg3* in the microenvironment of breast carcinoma and provide a new mechanism for decorin in inhibiting tumorigenesis. Validation of the mixed microarray data, as it pertains to stromal gene changes, via several experimental methodologies performed *in vitro* and *in vivo*, confirmed involvement for *Peg3* in breast carcinomas. Future studies may reveal direct functions for *Peg3* in modulating the surrounding tumor environment upon decorin stimulation. These findings provide a new model for decorin protein core in controlling the tumor microenvironment as a fundamental biological mechanism with implications for curbing tumorigenic growth by the induction of tumor suppressor genes within the stroma, and for the discovery of

novel gene signatures that could eventually help clinical assessment and prognosis. The role of exogenous decorin in altering the gene expression signature of the tumor stroma could also be investigated in different types of tumors where decorin expression is usually suppressed to find common pathways for decorin-evoked oncosuppression. Bozoky et al., 2014 explored decorin expression levels in different human tissues compared to the corresponding tumors. Decorin was highly expressed in the stroma of the



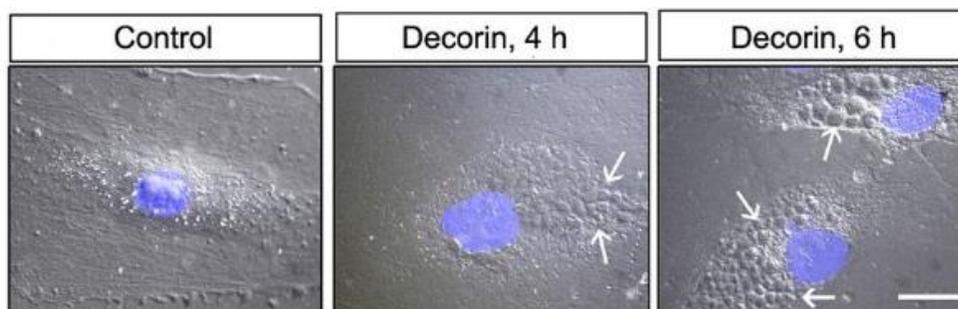
**Figure 6.** Heat map representing the top 36 genes downregulated (n=12) or upregulated (n=15) in the mouse tumor stroma in control and decorin-treated mice. Modified from Buraschi et al. (39)

healthy tissues examined but was absent or significantly reduced in the tumors. Moreover, a parallel comparison of three additional small leucine-rich proteoglycans, whose structures are very similar to the one of decorin, revealed a different pattern of expression (59). Indeed, asporin and biglycan showed the opposite pattern, with negative levels of expression in healthy tissue controls and elevated levels in the stroma of the relative tumors. In addition, Yamada et al., 2019 found a lack of decorin mRNA in three different lung cancer cell lines (107). Therefore, decorin lacks in the tumor microenvironment of different solid tumors where its absence allows dysregulated cancer growth. Re-introduction of this proteoglycan could be a valid therapeutic strategy as it sequesters important growth factors and alters the stromal gene expression signature and the tumor secretome, ultimately hindering cancer growth.

## **CHAPTER 6**

### **Decorin induces autophagy in endothelial cells and causes angiostasis**

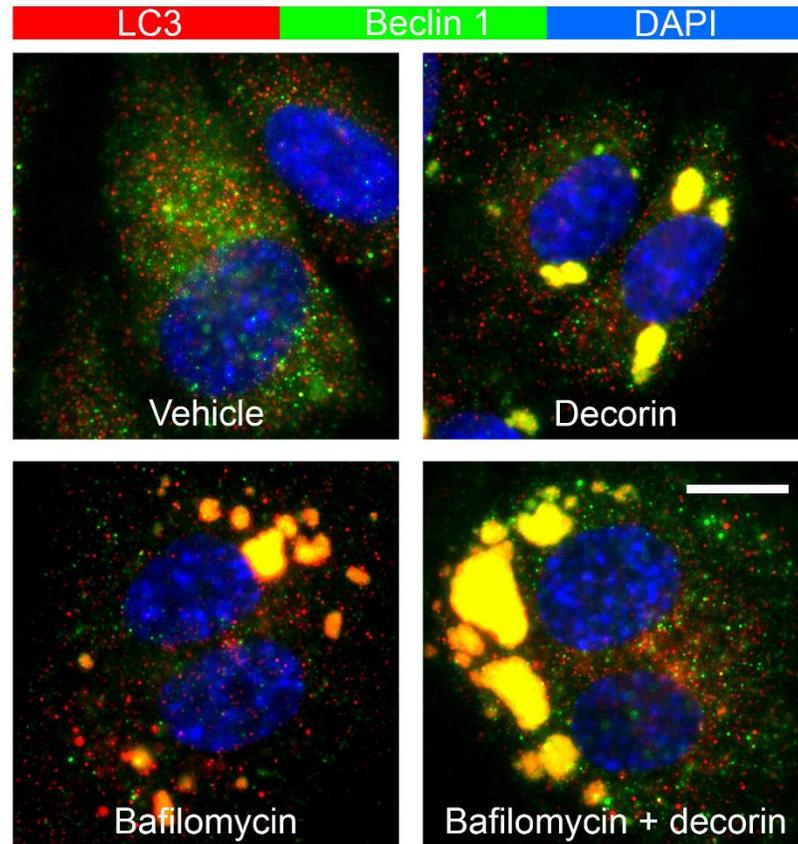
A highly significant contribution to the matrix biology field and a breakthrough in my research efforts is the serendipitous discovery that decorin evokes excessive autophagy in ECs, independently of nutrient deprivation, through partial agonistic activity on VEGFR2. In the last few years, the interest in the molecular machinery that regulates autophagy has tremendously increased. No one has previously shown that a secreted proteoglycan can modulate the autophagic program. This initial discovery jump-started an investigation that revealed a much more complex interplay between decorin and catabolism. Autophagy, an evolutionarily conserved eukaryotic process, promotes nonspecific turnover of cytosolic components, proteins, membranes, and cellular organelles that are no longer needed to the cell. ECs, a surrogate for the tumor microenvironment, are the primary cell type responsible for neovascularization (108). These cells are highly sensitive to decorin that, via binding to VEGFR2, markedly suppresses VEGFA expression, the main EC survival factor (64). The first line of evidence came from the empirical observation that stimulation of human umbilical vein endothelial cells (HUVEC) or mouse microvascular dermal endothelial cells (MDEC) with decorin caused the formation of large vacuoles reminiscent of autophagosomes (Fig. 7). Immunofluorescence analysis of these structures showed expression and co-localization of the autophagic markers microtubule-associated protein 1 light chain 3 alpha (LC3) and Beclin 1. Moreover, decorin induced autophagic flux (109), a widely known measurement of the cellular autophagic degradation activity (Fig. 8). As previously mentioned, Peg3 is a gene upregulated by decorin in the tumor stroma. Therefore, I sought to test its expression in ECs stimulated with decorin and found that it localized to the autophagosomes, and its expression pattern overlapped those of Beclin 1 and LC3.



**Figure 7.** Representative DIC/fluorescence images of MDEC treated with 200 nM decorin for 4 and 6 h. White arrows indicate autophagosomes fully formed in the cytoplasm of the cells upon exposure to decorin. Nuclei were stained with DAPI. (Scale bar:  $\sim 10 \mu\text{m}$ ). Modified from Buraschi et al. (17)

This discovery described, for the first time, a function for a secreted proteoglycan, in the regulation of autophagy. Given the affinity of decorin for a wide range of RTKs, I wanted to investigate the mechanism that allowed decorin to induce autophagy in ECs. The most prominent receptor that stimulates EC proliferation, migration, and survival is VEGFR2. Previous studies have shown that decorin is a VEGFR2 binding antagonist in human extravillous trophoblasts (48). I, therefore sought to determine the binding affinity of decorin to VEGFR2 with solid-phase binding assays and found an interaction of the two molecules, which I then confirmed in pull-down experiments (35). Moreover, my study showed that VEGFR2 was required for decorin-evoked Beclin 1 and LC3 expression as well as for Peg3 induction in ECs. Hence, the signaling cascade emanating from the decorin/VEGFR2 interaction leads to excessive autophagy in ECs with consequent inhibition of angiogenesis. These studies provided evidence that a soluble matrix molecule can regulate an intracellular catabolic program and described a novel role for *PEG3* in this fundamental cell process. *PEG3* is, indeed, required for decorin-evoked *BECN1* and *LC3* induction and is moreover responsible for maintaining Beclin 1 under basal conditions (35;110). Furthermore, de novo expression of Peg3 is sufficient to drive *BECN1* expression, confirming Beclin 1 as a direct target of Peg3 (111). The pro-autophagic program evoked by decorin under the control of Peg3, Beclin 1, and LC3, precludes BCI-2 from this complex, a known autophagic inhibitor (112). Of

note is that the initiation of this newly described autophagic signaling cascade by decorin is exclusively dependent on VEGFR2, the cardinal RTK for ECs, as it happens in full-serum conditions, unlike other autophagic stimuli such as nutrient deprivation or mTOR inhibition by rapamycin or Torin-1 (Fig. 9). Indeed, I showed that pharmacological inhibition of VEGFR2 via SU5416 abrogates decorin-induced EC autophagy. The binding of decorin to VEGFR2 differs from the previously discovered decorin/RTKs interactions insofar decorin does not induce internalization of VEGFR2. Decorin functions as a partial agonist of VEGFR2 by evoking non-canonical signaling cascades which are essential for autophagic activation. Two main pathways regulate autophagy: the pro-autophagic AMPK/Vps34/ULK1/2/Beclin 1 cascade and the anti-autophagic

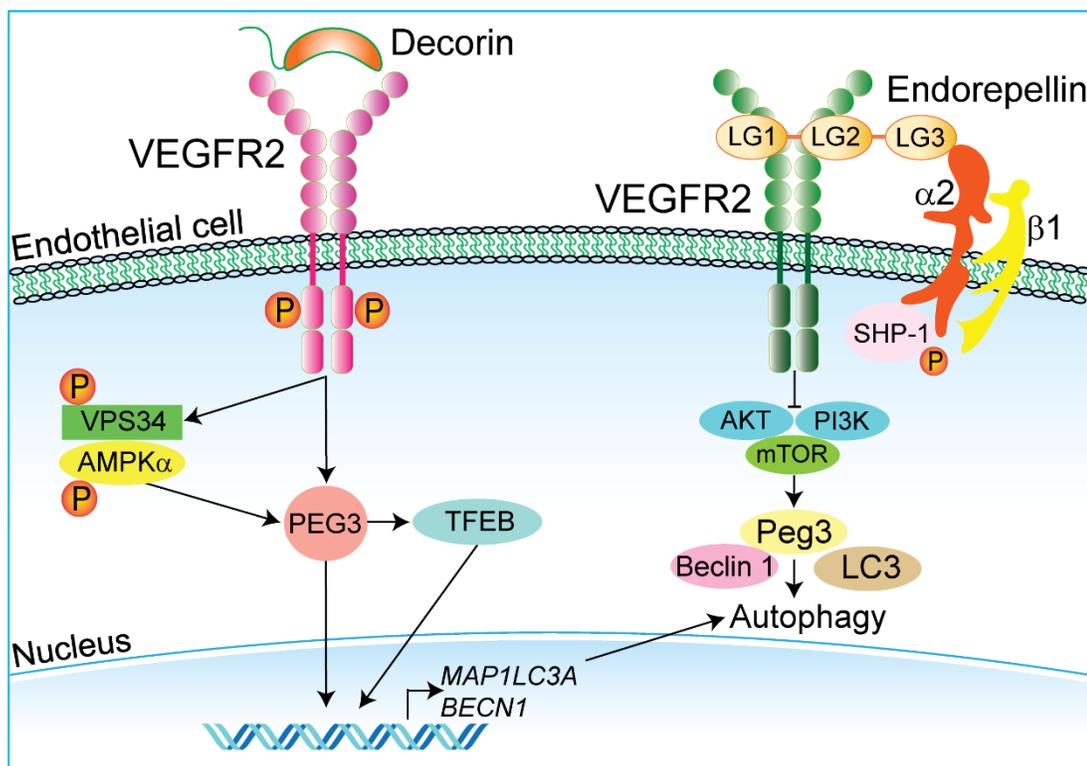


**Figure 8.** Representative images of HUVEC cells treated with either decorin (200 nM) or bafilomycin (500 nM) alone or in combination and stained for LC3 (red) and Beclin 1 (green). Nuclei, blue. (Scale bar: ~10  $\mu$ m).

PI3K/Akt/mTOR/p70S6K pathway (113;114). Goyal et al., 2014 showed that the binding of decorin to VEGFR2 induces adenosine monophosphate kinase AMPK $\alpha$  phosphorylation, thereby activating autophagy in an RTK-dependent manner. Mechanistically, depletion of VEGFR2 abolishes decorin-induced AMPK phosphorylation (115). This was the first study that linked VEGFR2 to the regulation of AMPK $\alpha$  and autophagy as a whole. At the same time, decorin protractedly attenuates the PI3K/Akt/mTOR/p70S6K pathway, although it remains to be elucidated if this latter signaling cascade also depends on decorin binding to VEGFR2. Moreover, decorin promotes formation of pro-autophagic Vps34/Beclin 1 complexes, which in turn increase the levels of Peg3. The depletion of Vps34 prevents decorin-induced upregulation of Peg3 and induction of autophagy in ECs (115). Although the importance of the decorin/VEGFR2 interaction for sustained AMPK activation has been documented, no direct association between VEGFR2 and Vps34 or VEGFR2 and AMPK has thus far been found. It is possible that the recruitment and /or activation of both AMPK and Vps34 is mediated by an intermediate kinase under the direct control of VEGFR2. Another possibility lies in the crosstalk among different RTKs, although these studies were performed exclusively in ECs, where RNAi-mediated depletion of VEGFR2 was functionally (and statistically) sufficient to abolish the effect of decorin. Together, these studies underscore the complexity of decorin signaling. Importantly, the requirement of VEGFR2 for AMPK-mediated activation of autophagy reveals a new potential target for the control of catabolism to suppress tumorigenesis. Following this study, Neill et al., 2017 identified the transcription factor EB TFEB, a master transcription factor for lysosomal biogenesis, as a key downstream molecule within the novel decorin/VEGFR2/AMPK $\alpha$ /Peg3 cascade (116). In ECs, TFEB expression is induced by decorin, and Peg3 is necessary and sufficient for driving TFEB induction. Increasing concentration of Peg3, or decorin, drive TFEB expression, and depletion of Peg3 is sufficient to inhibit decorin-mediated TFEB expression (116). Taken together, these studies have described a function for decorin in

reprogramming the tumor stroma via direct induction of autophagy with consequent inhibition of EC migration and capillary morphogenesis, overall acting to combat tumor angiogenesis. Moreover, I have assigned a novel role to Peg3 in controlling this process downstream of VEGFR2 engagement. The induction of Peg3 expression by decorin is critical for the activation of the autophagic program. This represents a significant improvement in the study of the biology of decorin as the previously described decorin-induced downregulation of  $\beta$ -catenin could also be explained by induced expression of Peg3. In a previous study, zebrafish embryos injected with Peg3 mRNA showed noticeable defects in tail development (106) which are strikingly similar to those of embryos injected with decorin (117). The noncanonical wingless-type MMTV integration site family (Wnt) pathway is known to regulate convergent extension (117). Thus, Peg3 and decorin could actively modulate the Wnt pathway during development and, in the same fashion, during tumor progression, causing suppression of  $\beta$ -catenin in cancer cells and enhanced autophagy in the endothelial compartment. Although autophagy is essential for normal development (118) and has been linked to cancer cell survival (119-121), induction of excessive autophagy can have profound deleterious effects on cancer growth and progression (122-126), leading to self-eating and self-killing (127;128). These studies undoubtedly influenced the field of Matrix Biology as they expanded understanding at the molecular level of how decorin functions as a tumor suppressor/anti-angiogenic factor, and stress the possibility that this multifaceted matrix constituent might be of therapeutic benefit in hindering the growth of the capillaries that are needed to bring nutrients to solid tumors. Following the publication of my studies in the *Journal of the Proceedings of the National Academy of Science (PNAS)* (35) (<https://f1000.com/prime/718025940>), other proteoglycans have been studied in the context of autophagy, and different signaling pathways have been elucidated. Biglycan is, like decorin, a member of the family of small leucine-rich proteoglycans and plays roles in angiogenesis (129) and inflammation (130). Emerging data suggest now that

biglycan can act in a pro-autophagic manner in cardiomyocytes subjected to reperfusion after ischemia (131), and in M1-type macrophages (132). On the contrary, perlecan, a basement membrane heparan sulfate proteoglycan, acts in an anti-autophagic manner (133). Studies involving conditional *Hspg<sup>-/-</sup>* mice revealed increased numbers of autophagosomes in muscle lacking perlecan and exhibits inhibition of the mTOR pathway when compared with their wild-type counterparts (133). However, endorepellin, the domain V of perlecan, undertakes a wholly different function in autophagy. Following my study on the role of decorin in autophagy, endorepellin was found to bind similarly and signal through VEGFR2 to induce the pro-autophagic intermediaries AMPK, Beclin 1,



**Figure 9.** Schematic representation of the signaling events evoked by decorin and endorepellin in endothelial cells upon high-affinity binding to VEGFR2. Decorin causes autophagy through induction of Peg3 and transcription of the autophagic genes Beclin 1 and LC3. Endorepellin activity, instead, requires both the  $\alpha 2\beta 1$  integrin and VEGFR2. Endorepellin evokes a physical down-regulation of both the  $\alpha 2\beta 1$  integrin and VEGFR2, with concurrent activation of the tyrosine phosphatase SHP-1, downstream attenuation of VEGFA transcription, and induces autophagy by inhibiting mTOR.

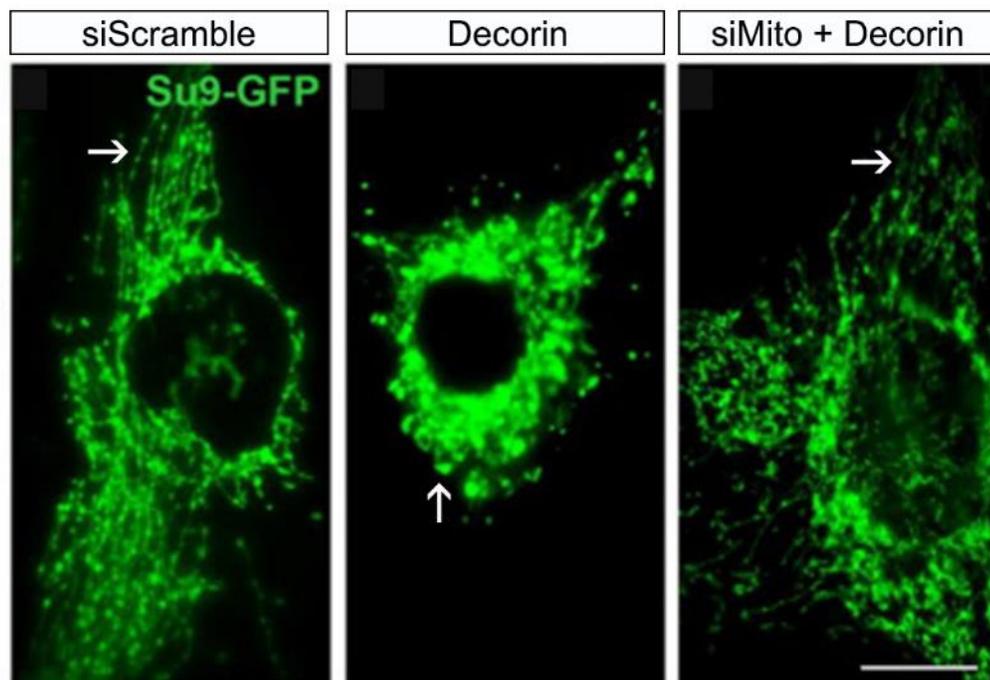
LC3, and p62 (134) while contemporaneously inhibiting mTOR. However, unlike decorin, endorepellin is unique in that it engages in dual receptor antagonism by binding simultaneously to VEGFR2 and  $\alpha 2\beta 1$  integrin (135) (Fig. 9). Thus, a single heparan sulfate proteoglycan shows opposing roles in finely regulating autophagy to prevent excessive or insufficient catabolism necessary for normal cellular homeostasis. These novel findings open new possibilities for future studies on the role of other matrix-associated molecules in the context of autophagy and angiogenesis. Notably, this work was cited by Klionsky et al., in the “*Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition)*”, *Autophagy*, 2016 (109). Dr. Klionsky, with this periodical review, defines the acceptable standard criteria and assays for the study and understanding of autophagy among the international scientific community. Citing my work, the author recognizes decorin as an autophagic inducer, the first proteoglycan shown to be involved in this catabolic process, and PEG3 as a decorin-induced genomically imprinted tumor suppressor gene required for macroautophagy in ECs.

## CHAPTER 7

### **Decorin evokes mitophagy in breast carcinoma cells via Met**

The functional consequences of induction of autophagy via VEGFR2 and Peg3 are of substantial interest as they could modulate vital cellular functions such as cell migration and mitochondrial membrane integrity through the loss of mitochondrial membrane potential ( $\Delta\psi_m$ ). Together with autophagy, mitophagy, the catabolic process that disrupts damaged mitochondria plays a crucial role in cell homeostasis. Although mitophagy can indicate initiation of apoptosis (136), it can also serve, in conjunction with autophagy, as a physiological survival mechanism that the cell adapts to avoid mutagenic events caused by reactive oxygen species that could trigger tumorigenesis. Dr. Neill, based on my studies on the role of decorin in autophagy, decided to investigate how decorin could affect cancer cell metabolism, a process strictly dependent on mitochondria. Supporting this line of research, and consistent with the activity of decorin to induce the tumor suppressor gene PEG3 is the previously described ability of decorin to induce mitostatin, an oncostatic mitochondrial protein (137). Mitochondria are vital biosynthetic hubs that provide macromolecules for cancer progression and angiogenesis (138). Moreover, mitochondrial biogenesis is known to be controlled by nuclear mitochondrial genes, which are also regulated by the proto-oncogene Myc (139), a target of decorin bioactivity. With these premises, a detailed study of the role of decorin on mitochondrial turnover in cancer cells was needed to elucidate decorin ability to suppress tumorigenesis under normoxia and to induce autophagy in the tumor stroma. Dr. Neill and I decided to utilize the triple-negative breast cancer cells, MDA-MB-231, that are highly responsive to decorin treatment through Met receptor, and interrogate their mitochondria activity. We found that decorin treatment of these cells resulted in mitochondrial DNA (mtDNA) depletion and prolonged complex respiratory turnover, two interpretive signs of mitochondrial autophagy (mitophagy) (140). We discover that in a mechanism similar to

VEGFR2 on ECs, decorin engages Met and induces mitophagy in triple-negative and luminal breast carcinoma cells (140) (Fig.10). As for EC autophagy, tumor cell mitophagy depends on a decorin-inducible tumor suppressor gene. This essential decorin-induced gene is TCHP renamed mitostatin, a mitochondrial protein that shows numerous hallmarks of a traditional tumor suppressor gene such as inhibiting tumor cell migration, growth and proliferation, and simultaneous triggering of pro-apoptotic pathways (137;141). Mitostatin lacks in ~35% of human prostate cancers (141), and its decreased expression is associated with advanced cancer stages. Similarly to Peg3, mitostatin is indeed often lost or mutated in bladder and breast carcinomas (137), preventing its ability to induce mitophagy through



**Figure 10.** Gallery of fluorescence images of MDA-MB-231 cells stably expressing the Su9-GFP transgene for mitochondrial network visualization. Cells were super-transfected with RNAi against mitostatin to determine the effect on the mitochondrial network as shown for the siScramble transfectants, treatment with decorin (200 nM), or decorin following mitostatin depletion. Arrows point at the healthy tubular mitochondrial network (siScramble), which aggregates and collapses upon decorin-treatment (middle panel). Depletion of mitostatin prevents decorin-induced mitochondrial fragmentation (right panel). All images were taken with the same exposure, gain, and intensity. (Scale bar ~ 10  $\mu$ m). Modified from Buraschi et al. (40)

mitochondrial fragmentation of the cancer cells and highlighting its role as a powerful oncostatic protein. Upon decorin binding to Met on the cell surface of MDA-MB-231 breast carcinoma cells, PGC1 $\alpha$ , a critical mitochondrial biogenesis factor (142;143) is mobilized and stabilizes TCHP mRNA for mitostatin accumulation. This process can be blocked by siRNA-mediated abrogation of either PGC-1 $\alpha$  or Met (140). Furthermore, depletion of mitostatin prevented decorin- or rapamycin-evoked mitophagy increased VEGFA expression, and inhibited decorin-evoked VEGFA suppression (140). These novel findings are highly significant because they establish mitostatin as a key regulator of tumor cell mitophagy and angiostasis. Elucidating this pathway, we described unique molecular cooperation between a mitophagic effector and a proto-oncogene orchestrated by the specific direct binding of decorin to RTKs on the surface of cancer cells. Decorin induces fragmentation of the mitochondrial network via induction of mitostatin and loss of the mitochondrial membrane potential ( $\Delta\Psi_m$ ), an early sign of mitochondrial dysfunction, and parkin recruitment (140). Altogether, this might cause an efflux of Ca<sup>2+</sup> from the endoplasmic reticulum to the mitochondrial matrix, evoking mitophagy. Mitostatin may indirectly stimulate Parkin expression, and the PTEN induced kinase 1 (PINK1) activity for targeting and ubiquitination of mitochondrial proteins (144). Collectively, we demonstrated that decorin-evoked Met signaling in breast cancer cells is essential for the induction of mitophagy and consequent cell growth arrest. Mitophagy of the tumor parenchyma, in conjunction with stromal autophagy, depicts a double mechanism of action for decorin that directly suppresses cancer growth and tumor neovascularization. Nanomolar concentrations of soluble decorin are sufficient to reprogram the signaling signature of the tumor stroma towards a protracted oncosuppressive microenvironment. The mitochondrial oxidative phosphorylation system, OXPHOS, is crucial for cancer cell proliferation, survival, migration, angiogenesis, and metastasis (145). Based on our novel observation that decorin compromises the OXPHOS system via a signaling pathway that

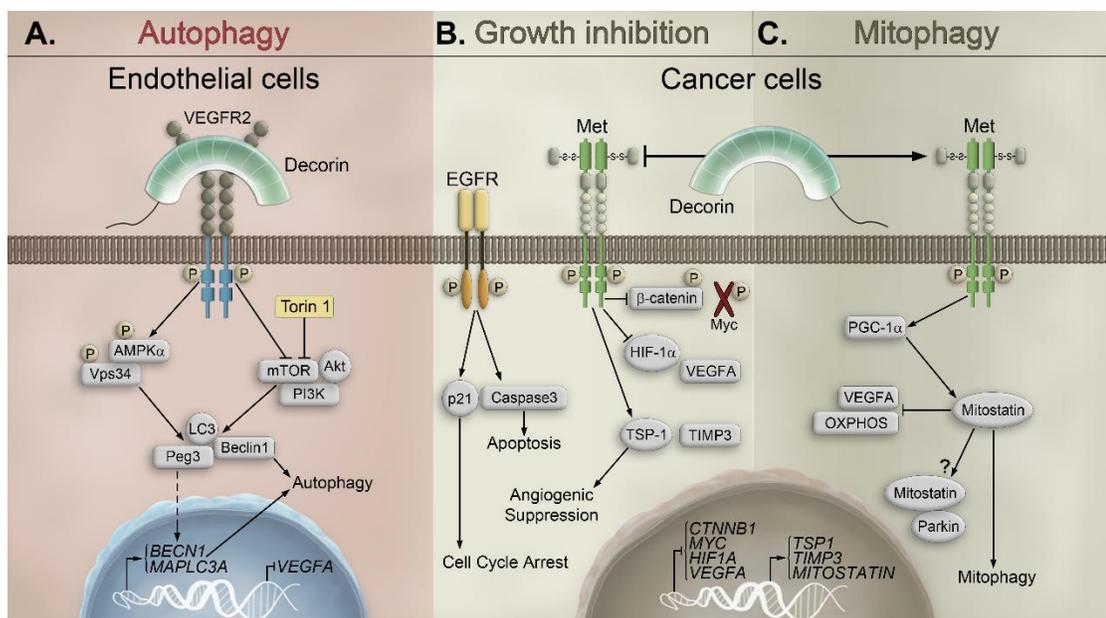
requires mitostatin (140), we hypothesize that decorin-evoked tumor cell mitophagy could contribute to its oncosuppressive and anti-metastatic properties. Further, the PINK1/Parkin pathway is directly involved in respiratory chain turnover (146), a central signaling pathway regulated by decorin and mitostatin. Our results provide the first evidence that a soluble constituent of the extracellular matrix can affect the intracellular digestive machinery, and could lead to the development of new therapeutics concurrently targeting the tumor stroma and cancer cells. We have unveiled for the first time a dual mechanism for a secreted proteoglycan in inducing autophagy in ECs and mitophagy in breast carcinoma cells resulting in tumor growth inhibition.

## CHAPTER 8

### **Significance and conclusions**

Breast cancer remains the most common malignancy in women in the UK and the world, with a steadily increasing incidence (147;148). Further delineation of the molecular underpinnings linking tumor initiation, progression, and metastases could lead to improved treatment modalities for breast cancer. Since angiogenesis and autophagy are both hallmarks of tumor initiation and progression from signals originating in the matrix, we sought to understand the role of a significant player of the extracellular matrix, decorin in shaping these events. My studies provide a novel concept whereby decorin, a soluble extracellular and circulating proteoglycan, regulates tumor initiation by modulating the tumor microenvironment. Because of its highly diverse and broad binding repertoire, decorin could be considered a “guardian from the matrix.” Decorin functions as a ‘guardian’ in the context of constraining the activity of multiple RTKs, thereby acting on both the cancer proper (via EGFR and Met) and the tumor stroma (via VEGFR2 in ECs). The concept of a master regulator of gene expression, such as p53, a guardian of the genome, and Myc, a gene that affects ~1,500 genes in cancer progression, is widely accepted. The described studies challenge current paradigms and provide a new concept whereby decorin affects autophagy, independently of nutrient deprivation. Three areas make these research studies highly innovative and groundbreaking: **[1]** The finding that soluble decorin antagonizes Met receptor activity and downregulates  $\beta$ -catenin and Myc levels counteracting tumor growth. Besides, decorin prevents migration of bladder cancer cells by binding with high affinity to IGF-I and IGF-IR. **[2]** The discovery that stromal-derived decorin evokes an excessive pro-autophagic program in ECs by activating AMPK $\alpha$ , an energy sensor kinases, downstream of VEGFR2 and concurrently inducing TFEB and Peg3, a novel master regulator of autophagy. This is the first description that a secreted proteoglycan affects AMPK $\alpha$  independently of

nutrient deprivation. Inducing the autophagic response could potentiate the anti-angiogenic effects of decorin on ECs, even in the absence of nutritional stress. **[3]** The discovery that decorin induces mitophagy in breast carcinoma cells via a partial agonistic activity on Met, unraveling a novel mechanism of action that involves a direct interaction between the decorin-inducible gene mitostatin and PGC-1 $\alpha$ . Metabolic reprogramming is one of the hallmarks of cancer, as it provides survival opportunities for cancer cells undergoing stress. Decorin stabilizes the expression of mitostatin through PGC-1 $\alpha$ . This is significant as it is the first-ever described link between metabolic reprogramming and decorin. Thus, decorin employs both the stroma (vascular endothelium) and the tumor proper via VEGFR2 and Met to exert profound signaling activities (Fig. 11). Decorin reprograms the tumor microenvironment and tumor parenchyma toward a pro-catabolic state by evoking EC autophagy and tumor cell mitophagy. This discovery came full circle as autophagic stimuli regulate decorin itself (149;150). These concerted research lines should firmly establish the functional roles of decorin in tumor angiogenesis and oncosuppression. The



**Figure 11.** Schematic representations delineating (A) the autophagic pathway evoked by decorin in endothelial cells via binding to VEGFR2 and (B) the classic growth inhibitory functions and (C) novel prometaphagic activities of decorin in tumor cells. Modified from Neill et al.(33)

discovery of dual decorin activity opens new possibilities for anti-angiogenic and anti-oncogenic therapies. Moreover, the mounting evidence that decorin targets the tumor vasculature suggests that decorin might be an ideal adjuvant candidate in the treatment of solid malignancies. There are several advantages of using decorin as primary or adjuvant therapy. Being a soluble protein, decorin could concurrently interact with many tumor cells at distant sites and should not trigger an immune response. Long term goals could focus on understanding the mode of action of decorin at the molecular level, decipher the decorin-induced signaling network operating during oncogenesis and tumor angiogenesis, and investigate its novel function as a soluble pro-autophagic molecule. These newly established concepts of the molecular mechanisms of action of decorin have significantly advanced the matrix and cancer biology fields, especially as they relate to understanding growth regulation, cross-talk between cancer and stroma, and tumor angiogenesis, all central issues in tumor progression and metastasis (18;90). Decorin's bioactivity would aid in signal integration, as circulating decorin (61;151-153) would modulate multiple RTKs with varying kinetics as a mechanism to subdue cancer growth. In conclusion, the extracellular matrix is becoming a key hotspot of potential diagnostic indicators, prognostic markers, and therapeutic targets, particularly when it comes to proteoglycans. Decorin is extremely attractive as an innovative therapeutic agent given its crucial active roles in both angiogenesis and autophagy, two biological processes highly linked to tumorigenesis, metastasis, and chemoresistance (154). The behavioral pattern of decorin, in the extracellular matrix, is directly related to the etiology and pathobiology of tumor progression and metastasis, and could potentially lead to novel avenues of cancer treatment by employing an anti-oncogenic protein capable of targeting various solid tumors and their metastases, particularly those enriched in RTKs. In conclusion, a deeper understanding of the molecular mechanisms of action of decorin and, perhaps, other matrix components could help us design novel strategic adjuvant therapies that will bring us closer to bridging the bench to bedside gap.

This thesis comprises eight published articles enclosed in Appendix B. I was involved in the experimental design and execution of the relative research projects at all stages. Table 1 summarizes my specific contributions in terms of figures I designed based on my experimental work.

<b>Published papers</b>	<b>List of figures designed by the candidate based on experiments he conceptualized and performed</b>
<i>Buraschi et al., JBC 2010</i>	Figures 1; 2; 3; 4; 5; 9
<i>Iozzo et al., JBC 2011</i>	Figures 1; 2; 3 A-C; 4; 6 B
<i>Neill et al., JBC 2011</i>	Figures 4 F-G; 6 D-E
<i>Buraschi et al., PLoS ONE 2012</i>	Figures 1; 6; 7; 8
<i>Buraschi et al., PNAS 2013</i>	Figures 1; 3; 4; 5 A-D; 8
<i>Neill et al., Autophagy 2013</i>	Figure 1 B
<i>Neill et al., JBC 2014</i>	Figure 9
<i>Buraschi et al., Matrix Biol. 2019</i>	Figure 1

**Table 1.** Table detailing the specific contributions made by the candidate to each paper comprising the thesis. Listed are the figures to which the candidate contributed the most, based on the amount of data generated by him in the laboratory.

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## **Appendix A**

Statements of authorship

I declare that all co-authors of the eight publications submitted for the Degree of Ph.D. by Published Work, where possible, were contacted via email. However, at the time of submission of this thesis, I only received the statements here enclosed.



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January 15, 2020

University of Warwick  
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**RE: STATEMENT OF AUTHORSHIP OF PUBLICATION**

To the Chair of the Board of Graduate Studies,

This letter confirms Simone Buraschi's contribution to a published manuscript submitted for the Degree of Ph.D. by Published Work. The following article is part of Simone Buraschi's application:

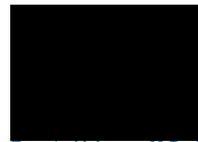
[J Biol Chem](#), 2010 Dec 31;285(53):42075-85. doi: 10.1074/jbc.M110.172841. Epub 2010 Oct 25.

**Decorin antagonizes Met receptor activity and down-regulates  $\beta$ -catenin and Myc levels.**

[Buraschi S](#), [Pal N](#), [Tyler-Rubinstein N](#), [Owens RT](#), [Neill T](#), [Iozzo RV](#).

Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the paper and design of the figures. This work was performed and completed entirely in my laboratory under my supervision. Should you have any further requests, please do not hesitate to contact me.

Best personal regards,



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J Biol Chem. 2011 Oct 7;286(40):34712-21. doi: 10.1074/jbc.M111.262766. Epub 2011 Aug 12.

**Decorin antagonizes IGF receptor I (IGF-IR) function by interfering with IGF-IR activity and attenuating downstream signaling.**

Iozzo RV, Buraschi S, Genua M, Xu SQ, Solomides CC, Peiper SC, Gomella LG, Owens RC, Morrione A.

Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the paper and design of the figures. This work was performed and completed entirely in my laboratory under my supervision. Should you have any further requests, please do not hesitate to contact me.

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J Biol Chem. 2012 Feb 17;287(8):5492-506. doi: 10.1074/jbc.M111.283499. Epub 2011 Dec 22.

**Decorin antagonizes the angiogenic network: concurrent inhibition of Met, hypoxia inducible factor 1 $\alpha$ , vascular endothelial growth factor A, and induction of thrombospondin-1 and TIMP3.**

Neill T, Painter H, Buraschi S, Owens RT, Lisanti MP, Schaefer L, Iozzo RV.

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PLoS One. 2012;7(9):e45559. doi: 10.1371/journal.pone.0045559. Epub 2012 Sep 19.

**Decorin protein core affects the global gene expression profile of the tumor microenvironment in a triple-negative orthotopic breast carcinoma xenograft model.**

Buraschi S, Neill T, Owens RT, Iniguez LA, Purkins G, Vadigepalli R, Evans B, Schaefer L, Peiper SC, Wang ZX, Iozzo RV.

Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the paper and design of the figures. This work was performed and completed entirely in my laboratory under my supervision. Should you have any further requests, please do not hesitate to contact me.

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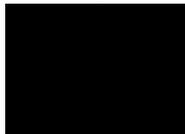
Proc Natl Acad Sci U S A. 2013 Jul 9;110(28):E2582-91. doi: 10.1073/pnas.1305732110. Epub 2013.

**Decorin causes autophagy in endothelial cells via Peg3.**

Buraschi S, Neill T, Goyal A, Poluzzi C, Smythies J, Owens RT, Schaefer L, Torres A, Iozzo RV.

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**Decorin has an appetite for endothelial cell autophagy.**

Neill T, Torres A, Buraschi S, Iozzo RV.

Simone actively contributed to the conceptualization, and writing of the paper and design of the figures. This work was performed and completed entirely in my laboratory under my supervision.  
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J Biol Chem, 2014 Feb 21;289(8):4952-68. doi: 10.1074/jbc.M113.512566. Epub 2014 Jan 8.

**Decorin induces mitophagy in breast carcinoma cells via peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) and mitostatin.**

Neill T<sup>1</sup>, Torres A, Buraschi S, Owens RT, Hoek JB, Baffa R, Iozzo RV.

Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the paper and design of the figures. This work was performed and completed entirely in my laboratory under my supervision. Should you have any further requests, please do not hesitate to contact me.

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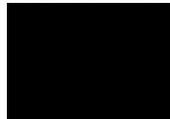
Matrix Biol. 2017 Nov 7. pii: S0945-053X(17)30326-8. doi: 10.1016/j.matbio.2017.10.005.

**Decorin is a devouring proteoglycan: Remodeling of intracellular catabolism via autophagy and mitophagy.**

Buraschi S, Neill T, Iozzo RV.

Simone actively contributed to the conceptualization, and writing of the paper and design of the figures. This work was performed and completed entirely in my laboratory under my supervision. Should you have any further requests, please do not hesitate to contact me.

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Annabel Torres, PhD  
703 Downing CT  
Westampton, NJ 08060

March 12, 2020

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Coventry, UK

**RE: STATEMENT OF AUTHORSHIP OF PUBLICATION**

To the Chair of the Board of Graduate Studies,

This letter confirms that Simone Buraschi contributed to published manuscripts that were part of my Doctoral Thesis at Thomas Jefferson University. I worked with Simone while I was a graduate student in the laboratory of Dr. Renato Iozzo. Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the following papers, which were subsequently inserted into my Doctoral Thesis:

Neill T, Torres A, Buraschi S, Owens RT, Hoek J, Baffa R, Iozzo RV. Decorin induces mitophagy in breast carcinoma cells via peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) and mitostatin. *J.Biol.Chem.* 2014;289:4952-68

Buraschi S, Neill T, Goyal A, Poluzzi C, Smythies J, Owens RT, Schaefer L, Torres A, Iozzo RV. Decorin causes autophagy in endothelial cells via Peg3. *Proc.Natl.Acad.Sci.U.S.A.* 2013;110:E2582-E2591

Moreover, he contributed to the following review, which was written after the findings published in his PNAS paper (Buraschi et al., 2013):

Neill T, Torres AT, Buraschi S, Iozzo RV. Decorin has an appetite for endothelial cell autophagy. *Autophagy* 2013;9:1626-8

Should you have any further requests, please do not hesitate to contact me.

Best personal regards,

Annabel Torres



Thomas Neill, Ph.D.  
Assistant Professor  
Department of Pathology, Anatomy, and Cell Biology  
1020 Locust Street  
Jefferson Alumni Hall Suite 336G  
Philadelphia PA, 19107



March 13, 2020

University of Warwick  
Coventry, UK

**RE: STATEMENT OF AUTHORSHIP OF PUBLICATION**

To the Chair of the Board of Graduate Studies,

This letter confirms that Simone Buraschi contributed to published manuscripts that were part of my Doctoral Thesis at Thomas Jefferson University. I have been working with Simone since 2009 and have worked together on many projects focused on elucidating the cellular and molecular mechanisms decorin bioactivity. Specifically, Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the following papers, which were subsequently inserted into my Doctoral Thesis:

1. Neill T, Torres A, Buraschi S, Owens RT, Hoek J, Baffa R, Iozzo RV. Decorin induces mitophagy in breast carcinoma cells via peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) and mitostatin. *J.Biol.Chem.* 2014;289:4952-68
2. Neill T, Painter H, Buraschi S, Owens RT, Lisanti MP, Schaefer L, Iozzo RV. Decorin antagonizes the angiogenic network. Concurrent inhibition of Met, hypoxia inducible factor-1 $\alpha$  and vascular endothelial growth factor A and induction of thrombospondin-1 and TIMP3. *J.Biol.Chem.* 2012;287:5492-506

Moreover, Simone and I actively collaborated on the execution and writing of the following manuscripts which are part of Simone's application:

1. Buraschi S, Neill T, Iozzo RV. Decorin is a devouring proteoglycan: Remodeling of intracellular catabolism via autophagy and mitophagy. *Matrix Biol.* 2019 Jan;75-76:260-70
2. Neill T, Torres AT, Buraschi S, Iozzo RV. Decorin has an appetite for endothelial cell autophagy. *Autophagy.* 2013;9:1626-8
3. Buraschi S, Neill T, Goyal A, Poluzzi C, Smythies J, Owens RT, Schaefer L, Torres A, Iozzo RV. Decorin causes autophagy in endothelial cells via Peg3. *Proc.Natl.Acad.Sci.U.S.A.* 2013;110:E2582-E2591

4. Buraschi S, Neill T, Owens RT, Iniguez LA, Purkins G, Vadigepalli R, Evans B, Schaefer L, Peiper SC, Wang Z, et al. Decorin protein core affects the global gene expression profile of the tumor microenvironment in a triple-negative orthotopic breast carcinoma xenograft model. *PLoS ONE* 2012;7:e45559
5. Buraschi S, Pal N, Tyler-Rubinstein N, Owens RT, Neill T, Iozzo RV. Decorin antagonizes Met receptor activity and downregulates  $\beta$ -catenin and Myc levels. *J.Biol.Chem.* 2010;285:42075-85

Should you have any further requests, please do not hesitate to contact me.

Best personal regards,



Thomas Neill, Ph.D.

Rick T. Owens, Ph.D.

March 26, 2020

University of Warwick  
Coventry, UK

**RE: STATEMENT OF AUTHORSHIP OF PUBLICATION**

To the Chair of the Board of Graduate Studies,

This letter confirms that Simone Buraschi contributed to several published manuscripts in which I am a co-author. Simone studies the biology of decorin, and I have provided, throughout the years, recombinant human decorin for his *in-vitro* and *in-vivo* experiments in the laboratory of Dr. Renato V. Iozzo. I, therefore, certify that Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the following papers:

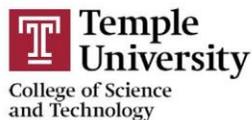
1. Buraschi, S., Pal, N., Tyler-Rubinstein, N., Owens, R. T., Neill, T., and Iozzo, R. V. (2010) *J. Biol. Chem.* **285**, 42075-42085
2. Iozzo, R. V., Buraschi, S., Genua, M., Xu, S.-Q., Solomides, C. C., Peiper, S. C., Gomella, L. G., Owens, R. T., and Morrione, A. (2011) *J. Biol. Chem.* **286**, 34712-34721
3. Neill, T., Painter, H., Buraschi, S., Owens, R. T., Lisanti, M. P., Schaefer, L., and Iozzo, R. V. (2012) *J. Biol. Chem.* **287**, 5492-5506
4. Buraschi, S., Neill, T., Owens, R. T., Iniguez, L. A., Purkins, G., Vadigepalli, R., Evans, B., Schaefer, L., Peiper, S. C., Wang, Z., and Iozzo, R. V. (2012) *PLoS ONE* **7**, e45559
5. Buraschi, S., Neill, T., Goyal, A., Poluzzi, C., Smythies, J., Owens, R. T., Schaefer, L., Torres, A., and Iozzo, R. V. (2013) *Proc. Natl. Acad. Sci. U. S. A.* **110**, E2582-E2591
6. Neill, T., Torres, A., Buraschi, S., Owens, R. T., Hoek, J., Baffa, R., and Iozzo, R. V. (2014) *J. Biol. Chem.* **289**, 4952-4968

Should you have any further requests, please do not hesitate to contact me.

Best personal regards,



Rick T. Owens, Ph.D.



Department of Biology  
Sbarro Institute for Cancer  
Research and Molecular Medicine  
and Center for Biotechnology

Andrea Morrione, Ph. D.  
1900 North 12th Street Room 429B  
Philadelphia, PA 19122



March 27, 2020

University of Warwick  
Coventry, UK

RE: STATEMENT OF AUTHORSHIP OF PUBLICATION

To the Chair of the Board of Graduate Studies,

With this letter I would like to confirm that Simone Buraschi contributed to the JBC 2011 article listed below, where I am the corresponding author. In this manuscript, we provide a novel mechanism for decorin in negatively modulating the IGF system by acting on ligands and membrane receptors. The experiments were part of an ongoing collaboration between my laboratory and the laboratory of Dr. Iozzo at Jefferson. I confirm that Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the manuscript.

Iozzo, R. V., Buraschi, S., Genua, M., Xu, S.-Q., Solomides, C. C., Peiper, S. C., Gomella, L. G., Owens, R. T., and Morrione, A. (2011) J. Biol. Chem. 286, 34712-34721

Should you have any further requests, please do not hesitate to contact me.

Best personal regards,



Andrea Morrione, Ph.D.

**DawangFu, Inc**

**120 Schor Ave, 2<sup>nd</sup> Floor, Leonia, NJ 07605-0760, U.S.A.**

---

Shi-Qiong Xu, MD, MPH, PHD,  
VP, Global Sourcing, Senior Health Consultant  
Dawangfu, Inc.  
120 Schor Ave, 2nd Floor,  
Leonia, NJ 07605-0760  
Email: [REDACTED]  
Mobile: [REDACTED]

March 30, 2020

**University of Warwick  
Coventry, UK**

**RE: STATEMENT OF AUTHORSHIP OF PUBLICATION**

To the Chair of the Board of Graduate Studies,

This letter confirms that Simone Buraschi contributed to the manuscript listed below in which I am a co-author. Simone studies the biology of decorin, and I have collaborated with him on this paper while working in the laboratory of Dr. Andrea Morrione. I certify that Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the following article:

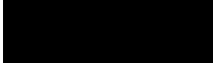
Iozzo, R. V., Buraschi, S., Genua, M., Xu, S.-Q., Solomides, C. C., Peiper, S. C., Gomella, L. G., Owens, R. T., and Morrione, A. (2011), Decorin antagonizes IGF Receptor I (IGF-IR) function by interfering with IGF-IR activity and attenuating downstream signaling. *J. Biol. Chem.* **286(40)**: 34712-34721

Should you have any further requests, please do not hesitate to contact me.

With best personal regards,

[REDACTED]

**Chiara Poluzzi, Ph.D.**  
Senior Post-Doctoral Fellow  
Pediatric Hematology/Oncology  
Laboratory - Stem cell transplant  
and Gene therapy – Prof. Dr. Biffi A.  
Fondazione Istituto di Ricerca  
Pediatrica - Città della Speranza  
Corso Stati Uniti, 4  
35127 Padova  
PD, Italy



March 31, 2020

University of Warwick  
Coventry, UK

**RE: STATEMENT OF AUTHORSHIP OF PUBLICATION**

To the Chair of the Board of Graduate Studies,

This letter confirms that Simone Buraschi contributed to the PNAS manuscript of which I am a co-author. In this manuscript, we have described a new function for decorin in evoking endothelial cell autophagy. Simone actively contributed to the conceptualization, experimental design and execution, validation, and writing of the manuscript, which is inserted into his application:

Buraschi S, Neill T, Goyal A, Poluzzi C, Smythies J, Owens RT, Schaefer L,  
Torres A, Iozzo RV. Decorin causes autophagy in endothelial cells via Peg3.  
*Proc.Natl.Acad.Sci.U.S.A.* 2013;110:E2582-E2591

Should you have any further requests, please do not hesitate to contact me.

Best personal regards,

Chiara Poluzzi, Ph.D.





European Patent and  
Trade Mark Attorneys,  
Chartered Patent Attorneys and  
Chartered Trade Mark Attorneys

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2 April 2020

**For: STATEMENT OF AUTHORSHIP OF PUBLICATION**  
**Applicant: SIMONE BURASCHI**

To the Chair of the Board of Graduate Studies,

In order to satisfy the requirements for an award of a PhD by Published Work from the University of Warwick, I confirm that Simone Buraschi contributed significantly to the below-referenced publication. I worked with Simone from June 2009 until August 2010 in the laboratory of Dr Renato V. Iozzo, MD. as a pre-doctoral fellow. I confirm that Simone was the lead researcher on the project and was involved in the experimental design and execution at all stages, including the writing of the paper.

Buraschi S, Pal N, Tyler-Rubinstein N, Owens RT, Neill T, Iozzo RV. Decorin antagonizes Met receptor activity and down-regulates {beta}-catenin and Myc levels. *J Biol Chem.* 2010;285(53):42075–42085. doi:10.1074/jbc.M110.172841

I declare that the information given above is true to the best of my knowledge. If you require any further information, please do not hesitate to contact me.

Kind regards,



**Nadia Tyler-Rubinstein Ph.D**  
Patent Attorney

**Boulton Wade Tennant LLP**  
Salisbury Square House, 8 Salisbury Square, London EC4Y 8AP

European Patent and Trade Mark Attorneys, Chartered Patent Attorneys and Chartered Trade Mark Attorneys  
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Raffaele Baffa, MD, PhD

University of Warwick  
Coventry, CV4 7AL  
UK

3 April 2020

**For: STATEMENT OF AUTHORSHIP OF PUBLICATION**  
**Applicant: SIMONE BURASCHI**

To the Chair of the Board of Graduate Studies,

In order to satisfy the requirements for an award of a PhD by Published Work from the University of Warwick, I confirm that Simone Buraschi contributed significantly to the below-referenced publication. I confirm that Simone was involved in the experimental design and execution at all stages, including the writing of the paper.

J Biol Chem. 2014 Feb 21;289(8):4952-68. doi: 10.1074/jbc.M113.512566. Epub 2014 Jan 8.  
**Decorin induces mitophagy in breast carcinoma cells via peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) and mitostatin.**  
Neill T, Torres A, Buraschi S, Owens RT, Hoek JB, Baffa R, Iozzo RV.

I declare that the information given above is true to the best of my knowledge. If you require any further information, please do not hesitate to contact me.





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University of Warwick  
Coventry, CV4 7AL  
UK

Bearbeiter

Datum 03. April, 2020

**For: STATEMENT OF AUTHORSHIP OF PUBLICATION**  
**Applicant: SIMONE BURASCHI**

To the Chair of the Board of Graduate Studies,

In order to satisfy the requirements for an award of a PhD by Published Work from the University of Warwick, I confirm that Simone Buraschi contributed significantly to the below-referenced publications. I confirm that Simone was involved in the experimental design and execution at all stages, including the writing of the papers.

J Biol Chem. 2012 Feb 17;287(8):5492-506. doi: 10.1074/jbc.M111.283499. Epub 2011 Dec 22. **Decorin antagonizes the angiogenic network: concurrent inhibition of Met, hypoxia inducible factor 1 $\alpha$ , vascular endothelial growth factor A, and induction of thrombospondin-1 and TIMP3.**

Neill T, Painter H, Buraschi S, Owens RT, Lisanti MP, Schaefer L, Iozzo RV.

PLoS One. 2012;7(9): e45559. doi: 10.1371/journal.pone.0045559. Epub 2012 Sep 19. **Decorin protein core affects the global gene expression profile of the tumor microenvironment in a triple-negative orthotopic breast carcinoma xenograft model.**

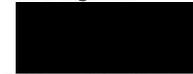
Buraschi S, Neill T, Owens RT, Iniguez LA, Purkins G, Vadigepalli R, Evans B, Schaefer L, Peiper SC, Wang ZX, Iozzo RV.

Proc Natl Acad Sci U S A. 2013 Jul 9;110(28): E2582-91. doi: 10.1073/pnas.1305732110. Epub 2013 Jun 24. **Decorin causes autophagy in endothelial cells via Peg3.**

Buraschi S, Neill T, Goyal A, Poluzzi C, Smythies J, Owens RT, Schaefer L, Torres A, Iozzo RV.

I declare that the information given above is true to the best of my knowledge. If you require any further information, please do not hesitate to contact me.

Kind regards,



Liliana Schaefer, MD

Universitätsklinikum  
Institut für Allgemeine Pharmakologie  
und Toxikologie  
Theodor-Stern-Kai 7  
Haus 74 / 3 OG  
60590 Frankfurt

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**Prof. Dr. L. Schaefer**  
Institut für Allgemeine  
Pharmakologie und Toxikologie  
Stellvertretende Direktorin

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**Institut für Allgemeine  
Pharmakologie und Toxikologie**  
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Direktor: Prof. Dr. Dr. G. Geißlinger

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Telefax: +49-69-6301-7636

Atul Goyal, PhD

University of Warwick  
Coventry, CV4 7AL  
UK

3 April 2020

**For: STATEMENT OF AUTHORSHIP OF PUBLICATION**  
**Applicant: SIMONE BURASCHI**

To the Chair of the Board of Graduate Studies,

In order to satisfy the requirements for an award of a PhD by Published Work from the University of Warwick, I confirm that Simone Buraschi contributed significantly to the below-referenced publication. I confirm that Simone was the lead researcher on the project and was involved in the experimental design and execution at all stages, including the writing of the paper.

Proc Natl Acad Sci U S A. 2013 Jul 9;110(28):E2582-91. doi: 10.1073/pnas.1305732110. Epub  
2013 Jun 24.

**Decorin causes autophagy in endothelial cells via Peg3.**

Buraschi S, Neill T, Goyal A, Poluzzi C, Smythies J, Owens RT, Schaefer L, Torres A, Iozzo RV.

I declare that the information given above is true to the best of my knowledge. If you require any further information, please do not hesitate to contact me.

Kind regards,



Atul Goyal, PhD

Email: 

## **Appendix B**

Work published by the author and submitted for this thesis

## **Appendix C**

Full bibliography of all the works published by the author

1. Buraschi S, Pal N, Tyler-Rubinstein N, Owens RT, Neill T, Iozzo RV. Decorin antagonizes Met receptor activity and downregulates  $\beta$ -catenin and Myc levels. *J.Biol.Chem.* 2010;285:42075-85
2. Iozzo RV, Buraschi S, Genua M, Xu S-Q, Solomides CC, Peiper SC, Gomella LG, Owens RT, Morrione A. Decorin antagonizes IGF receptor I (IGF-IR) function by interfering with IGF-IR activity and attenuating downstream signaling. *J.Biol.Chem.* 2011;286:34712-21
3. Neill T, Painter H, Buraschi S, Owens RT, Lisanti MP, Schaefer L, Iozzo RV. Decorin antagonizes the angiogenic network. Concurrent inhibition of Met, hypoxia inducible factor-1 $\alpha$  and vascular endothelial growth factor A and induction of thrombospondin-1 and TIMP3. *J.Biol.Chem.* 2012;287:5492-506
4. Tobia C, Chioldelli P, Nicoli S, Dell'Era P, Buraschi S, Mitola S, Foglia E, van Loenen PB, Alewijnse AE, Presta M. Sphingosine-1-phosphate receptor-1 controls venous endothelial barrier integrity in zebrafish. *Arterioscler.Thromb.Vasc.Biol.* 2012 Sep;32:e104-e116
5. Genua M, Xu S-Q, Buraschi S, Peiper SC, Gomella LG, Belfiore A, Iozzo RV, Morrione A. Prolyne-rich tyrosine kinase 2 (Pyk2) regulates IGF-I-induced cell motility and invasion of urothelial carcinoma cells. *PLoS ONE* 2012;7:e40148
6. Buraschi S, Neill T, Owens RT, Iniguez LA, Purkins G, Vadigepalli R, Evans B, Schaefer L, Peiper SC, Wang Z, et al. Decorin protein core affects the global gene expression profile of the tumor microenvironment in a triple-negative orthotopic breast carcinoma xenograft model. *PLoS ONE* 2012;7:e45559
7. Buraschi S, Neill T, Goyal A, Poluzzi C, Smythies J, Owens RT, Schaefer L, Torres A, Iozzo RV. Decorin causes autophagy in endothelial cells via Peg3. *Proc.Natl.Acad.Sci.U.S.A.* 2013;110:E2582-E2591
8. Neill T, Torres AT, Buraschi S, Iozzo RV. Decorin has an appetite for endothelial cell autophagy. *Autophagy* 2013;9:1626-8
9. Morcavallo A, Buraschi S, Xu S-Q, Belfiore A, Schaefer L, Iozzo RV, Morrione A. Decorin differentially modulates the activity of insulin receptor isoform A ligands. *Matrix Biol.* 2014;35:82-90
10. Neill T, Torres A, Buraschi S, Owens RT, Hoek J, Baffa R, Iozzo RV. Decorin induces mitophagy in breast carcinoma cells via peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) and mitostatin. *J.Biol.Chem.* 2014;289:4952-68
11. Tanimoto R, Morcavallo A, Terracciano M, Xu SQ, Stefanello M, Buraschi S, Lu KG, Bagley DH, Gomella LG, Scotlandi K, et al. Sortilin regulates progranulin action in castration-resistant prostate cancer cells. *Endocrinology* 2015 Jan;1:58-70

12. Xu SQ, Buraschi S, Morcavallo A, Genua M, Shirao T, Peiper SC, Gomella LG, Birbe R, Belfiore A, Iozzo RV, et al. A novel role for drebrin in regulating progranulin bioactivity in bladder cancer. *Oncotarget*. 2015 Mar 12;10:10825-39
13. Tanimoto R, Lu KG, Xu SQ, Buraschi S, Belfiore A, Iozzo RV, Morrione A. Mechanisms of Progranulin Action and Regulation in Genitourinary Cancers. *Front Endocrinol.(Lausanne)* 2016;7:100
14. Buraschi S, Xu SQ, Stefanello M, Moskalev I, Morcavallo A, Genua M, Tanimoto R, Birbe R, Peiper SC, Gomella LG, et al. Suppression of progranulin expression inhibits bladder cancer growth and sensitizes cancer cells to cisplatin. *Oncotarget*. 2016 May 23;28:39980-95
15. Neill T, Buraschi S, Goyal A, Sharpe C, Natkanski E, Schaefer L, Morrione A, Iozzo RV. EphA2 is a functional receptor for the growth factor progranulin. *J.Cell Biol* 2016 Dec 5;215:687-703
16. Tanimoto R, Palladino C, Xu SQ, Buraschi S, Neill T, Gomella LG, Peiper SC, Belfiore A, Iozzo RV, Morrione A. The perlecan-interacting growth factor progranulin regulates ubiquitination, sorting, and lysosomal degradation of sortilin. *Matrix Biol*. 2017 Apr 20;64:27-39
17. Buraschi S, Neill T, Iozzo RV. Decorin is a devouring proteoglycan: Remodeling of intracellular catabolism via autophagy and mitophagy. *Matrix Biol* 2019 Jan;75-76:260-70
18. Karalis TT, Heldin P, Vynios DH, Neill T, Buraschi S, Iozzo RV, Karamanos NK, Skandalis SS. Tumor-suppressive functions of 4-MU on breast cancer cells of different ER status: Regulation of hyaluronan/HAS2/CD44 and specific matrix effectors. *Matrix Biol* 2018 Apr 16
19. Xu SQ, Buraschi S, Tanimoto R, Stefanello M, Belfiore A, Iozzo RV, Morrione A. Analysis of Progranulin-Mediated Akt and MAPK Activation. *Methods Mol.Biol*. 2018;1806:121-30
20. Passi A, Vigetti D, Buraschi S, Iozzo RV. Dissecting the role of hyaluronan synthases in the tumor microenvironment. *FEBS J*. 2019 Apr 11;286:2937-49
21. Gubbiotti MA, Buraschi S, Kapoor A, Iozzo RV. Proteoglycan signaling in tumor angiogenesis and endothelial cell autophagy. *Semin.Cancer Biol*, In Press 2019 May 9
22. Tobia C, Chiodelli P, Barbieri A, Buraschi S, Ferrari E, Mitola S, Borsani G, Guerra J, Presta M. Atypical Chemokine Receptor 3 Generates Guidance Cues for CXCL12-Mediated Endothelial Cell Migration. *Front Immunol*. 2019;10:1092
23. Mongiat M, Buraschi S, Andreuzzi E, Neill T, Iozzo RV. Extracellular matrix: the gatekeeper of tumor angiogenesis. *Biochem.Soc.Trans*. 2019 Oct 31;47:1543-55

24. Neill T, Chen CG, Buraschi S, Iozzo RV. Catabolic degradation of endothelial VEGFA via autophagy. *J.Biol Chem.* 2020 Mar 24;295:6064-79
25. Buraschi S, Neill T, Xu SQ, Palladino C, Belfiore A, Iozzo RV, Morrione A. Progranulin/EphA2 axis: A novel oncogenic mechanism in bladder cancer. *Matrix Biol* 2020 May 15
26. Neill T, Buraschi S, Kapoor A, Iozzo RV. Proteoglycan-driven Autophagy: A Nutrient-independent Mechanism to Control Intracellular Catabolism. *J.Histochem.Cytochem.* 2020 Jul 6;In Press