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Collagen of the extracellular matrix in carcinoma

Functional and structural studies

PER OLOF OLSSON
DEPARTMENT OF LABORATORY MEDICINE | LUND UNIVERSITY 2017

Collagen of the extracellular matrix in carcinoma
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Per Olof Olsson

LUND
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DOCTORAL DISSERTATION

By due permission of the Facultry of Medicine, Lund University, Sweden.
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Functional and structural studies

Abstract

The cancer microenvironment has been attracting increasing attention as the realization of its importance for both disease treatment and malignant progression. Here the aim was to elucidate some of the effects and mechanisms of the tumor microenvironment related to the extracellular matrix (ECM). We hypothesized that collagen fibril assembly is an instrumental component in the formation of the barriers for transport of small molecules into carcinoma that have been seen in the treatment of carcinoma. To investigate this hypothesis we have utilized human xenograft and syngeneic generated tumors in mice, cellular and biochemical analyses. We found that fluid flow through carcinoma interstitium can be increased through STI571 treatment, and that this flow corresponds with a decreasing collagen fibril diameter. Additionally STI571 was found to increase blood flow and tumor oxygenation in a manner that correlates to matrix composition. Collagen fibril diameter was decreased via inhibition of the TGF-β activating integrin αvβ6 which corresponded to a clear trend toward a decreased interstitial fluid pressure, in all but the most fibrotic tumors. This illustrates the diversity of actable agents involved in stromal change. It points to a central role of collagen in the maintenance and regulation of fluid and solute exchange in tumors. Based on these results it may be possible to beneficially augment tumor treatment by altering one or more of the regulatory elements in the stroma of carcinoma.

Key words. Collagen, Extra cellular matrix. TGF-beta, integrin $\alpha_v\beta_6$

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Collagen of the extracellular matrix in carcinoma

Functional and structural studies

Per Olof Olsson

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A special thanks to:
My family. For their sacrifice and encouragement through time and distance

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Psalm 139:14
I will praise thee; for I am fearfully and wonderfully made:
marvellous are thy works; and that my soul knoweth right well.

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Overview

Scientific Question

My thesis work concerns the collagenous backbone of the extracellular matrix (ECM) in carcinoma, specifically the role of transforming growth factor-beta (TGF-β) for and the effects of the small tyrosine kinase inhibitor STI571 on the modulation of a dense collagenous matrix. Our approach at investigating the effects of the ECM in tumors has been to utilize transplanted carcinoma models in mice. Focused studies using pharmacological treatments, as well as genetic modification have been used experimentally. Gene expression, collagen and glycosaminoglycan content together with Magnetic Resonance Imaging (MRI), Electron microscopy (EM), and immunohistochemical staining in *in vivo* xenograft and syngeneic system tumor studies as well as *in vitro* models of TGF-β activation and of gene expression.

Introduction

Collagens are the most abundant proteins in the body and form the backbone of all tissues outside of the central nervous system. STI571 is a low molecule weight tyrosine kinase inhibitor, which is used clinically for the treatment of specific malignancies, developed for the treatment of chronic myeloid leukemia [1, 2]. It has been tested for potential use in atherosclerosis and even Alzheimers [3, 4]. It is also known under its trade names Imatinib and Glivec. Its previous reported effects, in solid experimental carcinoma, include: increasing radiation and chemotherapeutic treatment efficiency, the reduction of interstitial fluid pressure and increased extracellular volume, but does not affect growth in experimental carcinoma [5]. It is a selective inhibitor of intrinsic receptor tyrosine kinases and known to inhibit Abl, PDGF receptors, c-Kit, and DDR1, a collagen receptor [2, 6]. It has been shown to lower interstitial fluid pressure,. The mechanism behind these effects is investigated here.

Integrin $\alpha_V\beta_6$ expression, in carcinoma, has been correlated to a poorer patient prognosis, and shown to bind to and activate the latent TGF-β complex. A

functional inhibitory antibody to $\alpha_V\beta_6$ became available, and it became a target of interest as previous work in the group had focused on the effects of direct TGF-β inhibition. The investigation as to the viability of $\alpha_V\beta_6$ as a potential target to modulate carcinoma stroma, and thereby improve treatment efficacy was performed with the intent of inhibiting an upstream effector of the known ECM modulator TGF-β.

Fibromodulin (FMOD) is a small leucine rich proteoglycan (SLRP). FMOD and other SLPRs affect the collagen matrix assembly. Depletion of FMOD reduces the deposition of collagen in models of fibrosis and experimental carcinoma.

Hypotheses

The mechanism of the chemotherapeutic STI571 (Imantinib/Gleevec) to increase treatment efficacy in tumors involves modulation of tumor stromal matrix and blood flow.

The integrin $\alpha_V\beta_6$ is a modus involved in tumor pathology as relates to modulation/activation of latent TGF-β.

FMOD is the effector of TGF-β on collagen morphology and against the background that it affects the collagen architecture and fluid transport parameters; we further hypothesized that it may affect glycosaminoglycan in carcinoma.

List of Papers

Paper I

The tyrosine kinase inhibitor Imatinib augments extracellular fluid exchange and reduces average collagen fibril diameter in experimental carcinoma
P. Olof Olsson, Renata Gustafsson, René in 't Zandt, Tomas Friman, Marco Maccarana, Emil Tykesson, Åke Oldberg, Kristofer Rubin and Sebastian Kalamajski
Mol Cancer Ther. 2016 Oct;15(10):2455-2464. PMID: 27474147

Paper II

Imatinib increases oxygen delivery in extracellular matrix-rich but not in matrix-poor experimental carcinoma
Mikhail Burmakin, Tijs van Wieringen, P. Olof Olsson, Linda Stuhr, Aive Åhgren, Carl-Henrik Heldin, Rolf K. Reed, Kristofer Rubin and Carina Hellberg
Manuscript submitted

Paper III

Inhibition of integrin $\alpha_V\beta_6$ changes fibril thickness of stromal collagen in experimental carcinomas.
P. Olof Olsson, Renata Gustafsson, Alexei V. Salnikov, Maria Göthe, Kathrin Zeller, Tomas Friman, Bo Baldetorp, Louise A. Koopman, Paul H. Weinreb, Shelia M. Violette, Nils-Erik Heldin and Kristofer Rubin
Manuscript

Paper IV

Fibromodulin deficiency reduces collagen architecture but not glycosaminoglycan content.
P. Olof Olsson, Sebastian Kalamajski, Marco Maccarana , Åke Oldberg, Kristofer, Rubin.
Manuscript submitted

Paper V

Mice lacking NCF1 exhibit reduced growth of implanted melanoma and carcinoma tumors.
Tiina Kelkka, Angela Pizzolla, Juha Petteri Laurila, Tomas Friman, Renata Gustafsson, Eva Källberg, Olof Olsson, Tomas Leandersson, Kristofer Rubin, Marko Salmi, Sirpa Jalkanen, Rikard Holmdahl
PLoS One. 2013 Dec 16;8(12):e84148. doi: 10.1371/journal.pone.0084148. eCollection 2013. PMID: 24358335

Abstract

The cancer microenvironment has been attracting increasing attention as the realization of its importance for both disease treatment and malignant progression. Here the aim was to elucidate some of the effects and mechanisms of the tumor microenvironment related to the extracellular matrix (ECM). We hypothesized that collagen fibril assembly is an instrumental component in the formation of the barriers for transport of small molecules into carcinoma that have been seen in the treatment of carcinoma. To investigate this hypothesis we have utilized human xenograft and syngeneic generated tumors in mice, cellular and biochemical analyses. We found that fluid flow through carcinoma interstitium can be increased through STI571 treatment, and that this flow corresponds with a decreasing collagen fibril diameter. Additionally STI571 was found to increase blood flow and tumor oxygenation in a manner that correlates to matrix composition. Collagen fibril diameter was decreased via inhibition of the TGF-β activating integrin $\alpha_v\beta_6$ which corresponded to a clear trend toward a decreased interstitial fluid pressure, in all but the most fibrotic tumors. This illustrates the diversity of actable agents involved in stromal change. It points to a central role of collagen in the maintenance and regulation of fluid and solute exchange in tumors. Based on these results it may be possible to beneficially augment tumor treatment by altering one or more of the regulatory elements in the stroma of carcinoma.

Terms and Abbreviations:

Angiogenesis	the formation of capillaries from preexisting blood vessels, occurs in a variety of physiological and pathological settings, including embryonic development, wound healing and tumor growth.
3G9	Short for the 6.3G9 anti-integrin $\alpha_v\beta_6$ monoclonal mouse antibody
$\alpha_v\beta_6$	Arg-Gly-Asp binding integrin involved in LAP activation
BSA/FBS	Bovine Serum Albumin / Fetal Bovine Serum
CAF	Cancer Associated Fibroblast
C38	Murine colon adeno carcinoma syngeneic to C57Bl6 mice
CD31	A monoclonal antibody directed to "Platelet endothelial cell adhesion molecule" (PCAM-1) that can be used to detect tissue endothelial cells, which also express PCAM CD31
ECM	Extra Cellular Matrix
ECV	Extra Cellular Volume
EMT	Epithelial-Mesenchymal Transition
FMOD	Fibromodulin - murine fibromodulin denoted Fmod. A small leucine-rich proteoglycan
KAT-4	Human colon cancer cell line misidentified from result of thyroid cell cross contamination, also known as HT-29
LTGFB	Latent TGF-β
LAP	Latency Associated Peptide
LLC	Large Latent Complex, a member of the TGF-β complex
MAF	Myofibroblast
NG2	Neural/glial antigen 2, used here as marker for Pericytes

OOC38	A cell line derived, for this work, from C38 for more consistent tumor growth.
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PIF IFP	Interstitial Fluid Pressure, may also be referred to as
Proteoglycans	Proteins substituted with glycosaminoglycan chains
Starling Equation	$Jv=K[(Pc-PIF)- \sigma(\pi p-\pi if)]$, describes fluid flow across membranes (capillaries)
STI571	A tyrosine kinase inhibitor, also known as: Gleevec or Imatinib
SLRP	Small Leucine Rich Proteoglycans
TAMs	Tumor Associated Macrophages
TGF-β	Transforming Growth Factor β
TMLC /MLEC	Transformed Mink Lung carcinoma cell line (also known as C32 or MLEC) with luciferase dependent production via PAI-1 under membrane bound TGF-βR

I Introduction

Cancer is a major medical problem and the incidence of cancer is increasing globally. Worldwide cancer accounts for approximately 15% or 8.2 million of the 56 million deaths in 2012, the total number of cases are projected to increase by 70% in the next 20 years[7] Research into new treatment regimens is thus highly warranted.

Stroma refers to the part of an organ that has a supportive, e.g. blood vessels, structural and connective role, whereas the functional parts of the organ are termed parenchyma. The pathogenic role of the stroma and stromal cells in carcinoma has acquired an increased interest. It is evident that they play a physiologic role where stromal cells and ECM components collaborate with malignant cells relating to development, as well as suppression and restraint or promotion of growth and metastasis of carcinoma [8-11].

Carcinoma stroma mostly resembles loose connective tissue. Loose connective tissues make up approximately 15% of the body by weight, and are present at varying degrees in all organs outside the central nervous system. The extra-cellular compartment of this tissue is composed of many different classes of macromolecules, which together make up an extra-cellular matrix (ECM) [12-14]. Evidence suggests that cells and their behavior, including both malignant and stromal cells, are in part regulated by the ECM [15-17].

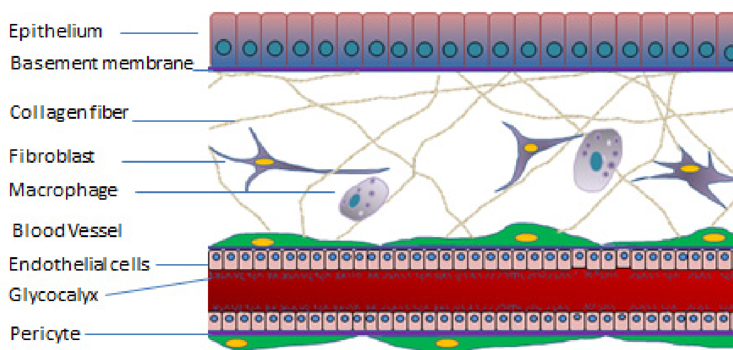

Figure 1
Loose connective tissue

II Stroma

Tissue stroma consists of those parts which do not possess the specific function of a tissue, the functional part being the parenchyma. The stroma harbors blood vessels, nerves and connective tissue cells. Inflammatory reaction, tumor invasion and transport from blood to parenchyma occur in the stroma. The core ECM is argued to be composed of 200 human genes and 195 in mice. ECM associated genes, which make up the matrisome, has been said to number 278 genes in humans and 274 in mice [13].This thesis will address only a fraction of the total components of the matrisome and its effectors.

i. Loose Connective Tissue

Also referred to as areolar tissue, is the space where the majority of interactions, transport and the barrier functions (see Barrier function) are generated. It is named as such as it fills the space between, connects and supports nerve and vascular tissues, as well as provides a supportive and connective function of the parenchyma [18]. It also plays a pivotal role in immune surveillance [19, 20].

There exist a number of cell types within loose connective tissues, which are in turn surrounded by a moderately viscous ground substance, these may include: mast cells, fibroblasts, macrophages, some adipocytes and infiltrating leukocytes to varying degrees. Nerve fibers are also present but rarely nerve cell bodies. Nutrients and oxygen are conveyed through the ground substance to avascular cells (tissue cells) [18]. The interstitial space, occupied by the ground substance, is primarily composed of glycosaminoglycan (GAG) chains, apart from which the free GAG hyaluronan (HA) are attached to a protein core. GAGs are negatively charged which helps maintain the physiologically important under-hydrated state of the ECM during homeostasis in loose connective tissues. GAGs are enclosed within a matrix which is primarily composed of collagenous fibers and is maintained by the forces exerted by contractile cells on collagen and other fibers of the ECM [13, 21, 22].

Connective tissue is characterized by its primary constituent, the ECM, which apart from the ground substance, discussed above, is composed of protein fibers:

collagens, elastic and micro fibers(see section II ii) . This tissue has a high ECM to cell ratio. Reticular and elastic fibers may also be involved and the formation of a provisional matrix where proteins like fibronectin (FN), also present during homeostasis, vitronectin (VN) and fibrin may provide vital structural elements, being actively regulated by contractile cells, *e.g.,* fibroblasts [23, 24].

Integrins are common transmembrane proteins which are the primary component cells utilize to bind to and adhere to the ECM, forming and unifying the ECM components into interconnected complexes, which together with the cells within, form loose connective tissue. Integrins couple the ECM to exert cellular attachment and contractile forces, internal cellular attachments are primarily with actin filaments, where they bind indirectly via complexes containing, *e.g.*, kindlin alpha-actinin and talin. They also can activate intracellular signaling pathways [25-27].

ii. Vessels

Vessels are in essence tubes in which fluid is transferred between tissues. Normal vasculature is well organized and has distinct vessel types, from veins to capillaries. Vessels have an endothelium which in normal tissues is tight and contain a series of proteoglycans at their apical surface which compose a glycocalyx on the interior of the vessel which aids in regulation of transfer across the vessel wall addition to the basement membrane and cellular barriers. The glycocalyx is composed of glycoproteins and proteoglycans, attached via the transmembrane section of the proteoglycan core proteins [28]. Tumor vessels are aberrant and their structure, function and distribution chaotic, even exhibiting cross directional flow, where normal vasculature have distinct veins, venules and arteries, arterioles[29].

a. Blood vessels

There are three major types of blood vessels. Major carries, such as arteries, carrying blood from the heard, veins, which bring blood to the heart and capillaries, micro-vessels which enable the transfer of water, oxygen, and nutrients. Arteries and veins are further categorized by their smaller components, arterioles and venules. These make up a branching network with capillary beds in between. Arterioles are thought to be the main controllers of the microcirculation within the capillaries[30]. Blood in vessels is the means by which oxygen nutrients and waste are transported, without which tumors, or any tissue, are incapable of growing. Oxygen diffuses through tissue very slowly, with a coefficient of 1.04 ×

10^{-5} cm^2/s, which corresponds to only 200 µm total, and so a network of vessels carrying oxygen rich blood must be present to provide the oxygen needed for cellular metabolism [31, 32]. This is the primary reason for treatment aimed at inhibiting tumor angiogenesis, the development of new vessels. Angiogenesis occurs when new blood vessels are formed. This is different from vasculogenesis, in which endothelial cells differentiate from the embryonic mesoderm [32, 33]. One major angiogenic player is vascular endothelial growth factor (VEGF) (see section IX). Apart from the development of new vessels, some tumors are capable of co-opting host vessels [34]. The composition of blood is approximately 45% cellular and 55 % plasma, of which nearly 50% is albumin, which accounts for a large proportion of the generation of oncotic pressure in the capillaries (see section II iv).

b. Lymph

Lymph vessels, like blood vessels are composed of endothelial cells, and stain positive with anti CD31 antibodies. Other lymph endothelial cell markers are tissue specific, expressing varied markers in different tissues. Lymph is important for the drainage and maintenance of homeostasis in most tissues. Tumors however, generally do not possess lymphatic vessels within themselves, but instead have peritumoral lymphatic tissue [35].Lymphatic vessels, which have a discontinuous basement membrane, take up protein rich fluid from the interstitium. They require specific VEGF isoforms to form, this process generally occurs in the periphery and not intertumorally leaving most carcinoma with poor tissue lymphatic drainage [36].

iii. Basement membrane

Basement membranes are sheet like ECM structures which separate endothelium and epithelia, muscles and peripheral nerves from the underlying connective tissues. They function in providing support and separation of tissue compartments. They are composed of collagen IV, laminin, nidogen, fibrillin and among others proteoglycans, notably perlecan. They are anchored to cells by collagen VII fibrils. Pericytes together with endothelial cells produce the components, *e.g.*, collagen IV, laminin, fibronectin, nidogen, and perlecan and together form vascular basement membranes in development and in wound healing [37-39].

a. Collagen IV

Collagen IV are collagens that are formed by six separate polypeptide chains, α1-α6 that form sheets composed of heterotrimers, differentially expressed in specific tissues. It acts as a scaffold interacting with laminin, nidogen and perlecan forming the basement membrane. It is exclusively found in basement membranes. A number of disorders are associated with collagen IV such as Goodpasture´s syndrome, which is associated with autoimmunity against collagen IV α3 chain resulting in bleeding of the lungs and renal failure and Alport´s syndrome, characterized by renal dysfunction, due to mutations affecting production or assembly of collagen IV, specifically the α-3, -4, and -5 chains. Collagen IV interacts with cellular and ECM components, providing stromal and cellular support [38, 40].

b. Laminin

Laminin is a component of the basement membrane. It is composed of a several isoforms (16) which consist of three chains, α, β, and γ chains, all are glycosylated and some have been argued to have GAG chains [41]. They are built in cross shapes with long and short components which adhere to cells, via their G domain, present primarily on their α chain via integrins or glycoproteins. They interact with collagen IV and other ECM proteins which influence cellular processes from adhesion and migration to apoptosis, through membrane receptors including integrins. Laminins play an active in their cellular modulation behavior[42]. They mediate forces between cells and ECM through their connection to both integrin and their various ECM components [24, 43].

iv. Exchange between blood and interstitium

The Starling´s equation, $Jv=K[(Pc-PIF)- \sigma(\pi p-\pi if)]$. Jv is fluid flow in mL per minute. K represents the constant of proportional permeability of water to the capillary, the higher the permeability the larger the value K. Pc is capillary hydrostatic pressure; PIF represents interstitial fluid pressure; σ represents the reflection coefficient which describes the correction for the impermeability of the capillaries to proteins. $\pi p-\pi if$ is the difference between the oncotic pressure and the interstitial oncotic pressure, meaning the osmotic pressure exerted by proteins in the capillary minus the oncotic pressure in the interstitium.

J_v is flow of fluid in mL per minute
P_c is capillary hydrostatic pressure
PIF represents interstitial fluid pressure
σ represents the reflection coefficient
π_p is the capillaries oncotic pressure
π_{if} is the oncotic pressure of the interstitium.

The Starling equation was first described in 1914, describes the fluid flow in mL per minute from blood vessels. The question to whether or not interstitial fluid pressure is actively controlled or not will change the view and approach to understanding the fluid, and molecular, transport within tumors. We believe that this pressure is regulated, and its effectors also relate to plasma protein leakage. Capillary pressure (hydrostatic pressure) designated here Pc, generated by the heart, is generally maintained around 10 mmHg. 50% of the protein in blood, and capillaries is albumin, at about 45mg/mL and as low as about 5 mg/mL in the interstitium, which affects the interstitial fluid pressure (PIF). Albumin is relatively small (55kd unreduced and 67kd reduced) and has a high affinity for water and the total colloid osmotic pressure is generated by protein concentration in the plasma of blood vessels. . As the vascular membranes are not normally permeable to protein leakage, fluid is drawn from the interstitium into the capillaries[44]. This can be seen by the reflection constant, which is normally 0.95 but in cases of edema can be reduced to 0.3. The presence of the endothelial glycocalyx has also been suggested to have a regulatory role in the fluid exchange between capillaries and the interstitium[45].

v. Cellular components in stroma

Fibroblasts are a cell type, found throughout the body, which synthesize ECM and collagen. They have other roles than the production of ECM, including maintaining homeostasis and wound repair. They are the most common cell type of connective tissues and are generally dormant in most tissues and can be activated in the presence of wound healing or in tumors. Activation may occur through substrate stiffness or through growth factor activation. In response to activation, fibroblasts become more contractile and produce greater amounts of ECM, which can lead to fibrosis. These activated fibroblast may be referred to as myofibroblasts (MFs) [46-49]. It is generally understood that fibroblasts are the main ECM producers depositing proteoglycans and the free GAG HA, the fibrous proteins*, i.e.,* collagen, elastin and reticular fibers, as well as glycoproteins. Within the ECM is the “ground substance” which is primarily water, held in place by GAGs, most notably HA [21].

Vascular cells in loose connective tissue are primarily endothelial cells and pericytes, in larger vessels smooth muscle cells are also present. Endothelial, cells can be identified with the CD31 (anti-PECAM marker, pericytes, however, are a much more difficult cell type to describe and were originally defined as intra-mural cells (meaning within the vascular wall), encompassed in the basement membrane of capillaries [37]. Whether pericytes are a single or multiple cell population remains to be determined, this as they are known to express various cellular markers, especially when activated. These markers include NG2 originally described in human tissues as the high molecular weight melanoma associated antigen (in activated tissues), tropomyosin, aSMA, and desmin among others[50, 51]. They have are also been reported as producers of ECM including collagen and most likely participate in the production of a basement membrane type of ECM [52]. There exist also a population of resident and infiltrating immune cells, active in immune surveillance and in maintaining homeostasis. Immune cells within loose connective tissue have been established as being effectors of both stromal activation as well as immune response and can be seen as a means for stromal cell activation and thus change in the ECM [19].

III ECM components

i. Collagen and Collagen Assembly

Collagens are heavily modified post-translationally and the fiber assembly proceeds in a step-wise fashion. Collagen synthesis starts in the rough endoplasmic reticulum in the cells and synthesized as large molecules known as pre-procollagen. Three alpha chains folds into triple helices, at which point disulfide bonds occur at the terminal ends resulting in, the then named, procollagen. Newly synthesized chains are hydroxylated on proline residues by the enzyme prolyl-4-hydroxylase, which is dependent on Fe^{2+} for its function. Ascorbic acid (vitamin C) is needed to reduce Fe^{3+}, which is generated in each hydroxylation cycle, back to Fe^{2+}. The hydroxylated proline (Hyp) residues are instrumental to stabilize the triple helical conformation. Hyp composes ~38% of the proline and ~9% of the total amino acids within collagen[53]. Hyp compared to proline has the addition of a hydroxyl group, which is electronegative due to the oxygen, giving the stabilizing effects which provide collagen its rigid structure [53, 54]. The collagen triple helices are transported through the Golgi where, in the case of collagen type I, a limited number glucose and glucose-galactose residues are added. Collagen containing transport vesicles are fused with the plasma membrane where it is believed that initial fibril formation takes place. In the case of tendon collagen-producing cells this process occurs at specialized membrane invaginations called fibropositors [55]. The C-terminal and N-terminal ends are cleaved off leaving tropocollagen, and free collagen pro-peptides. Fibrils are assembled in a complex process involving fibronectin and integrins [54, 56]. Further modifications during fibril/fiber maturation involve cross-link formation at short non-helical stretches at the collagen termini denoted telopeptides. Lysyl residues are deaminated and oxidized to lysine aldehydes by lysyl oxidases (LOX and LOX-like) and the aldehyde groups spontaneously form cross-links with unmodified lysyl or histidyl-residues in an adjacent collagen α1chain. In addition, some lysyl residues are hydroxylated by lysyl-hydroxylases and, after oxidation by LOX to hydroxylysine aldehydes can form cross-links. It has also been reported that collagen cross-links can form through the action of trans-glutaminase (TGM2). The cross-links greatly affect the stability and strength of the fibril [57]. These crosslinks are thought to be tissue specific, accrue with age, infer a more

stiff matrix and reduce the overall turnover rate of collagen which is important in the understanding of both the physiological and pathologic function of collagen[58, 59].

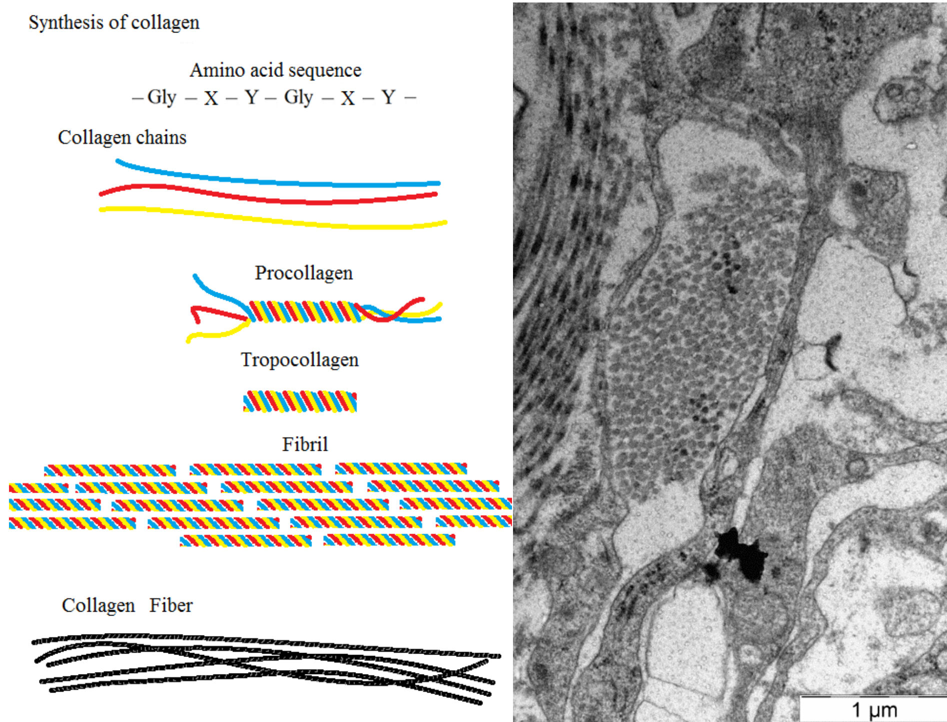

Figure 2
An illustration of the process of collagen synthesis (left). Transmission electron microscopy of collagen fibers and their fibrils in an OOC38 derived experimental carcinoma (right).

In the case where Vitamin C (Ascorbic acid) is not present, P4H is not capable of adding hydroxyl groups as both Ascorbic acid and Iron are necessary cofactors for the enzyme. Ascorbic acid is kept in its reduced state by NADPH or Glutathion. Reduced or non-oxidized Ascorbic acid plays its role in returning the Fe3+ to Iron2 Oxide (Fe2+Oxide) after conversion in the hydroxylation process. Conditions like scurvy derive from the reduced amount of hydroxylated proline and lysine residues in the pro-a chain, preventing the formation of stable triple helices, which in turn prevents the formation of normal fibrils, resulting in weaker collagen structures leading to the some of the symptoms associated with scurvy. Hydroxylases are vital for a number of other physiological process as well, which long known underscores the importance, of adequate Vitamin C in the diet.

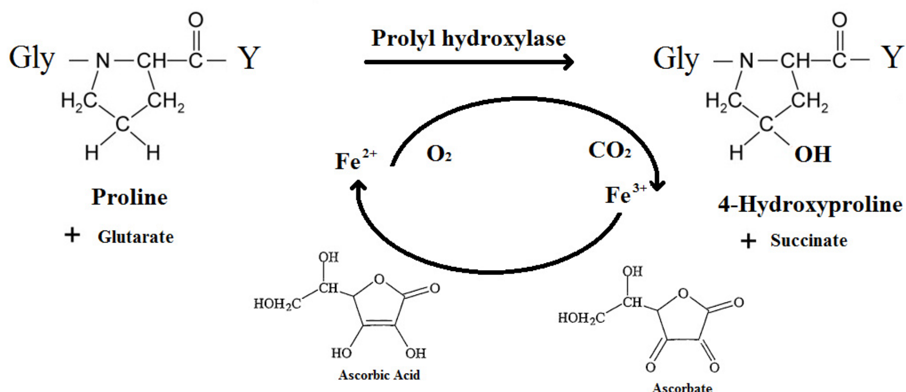

Figure 3
The role of ascorbic acid in the hydroxylation of proline

ii. Non collagenous components of the ECM

a. Elastin

Collagen and elastin are two of the greatest expressed components of the ECM in tissues requiring strength and elasticity like the aorta [60, 61]. Elastin, like collagen, is associates to and is crosslinked by LOX. Elastin forms heterofiber complexes with fibulins and microfibril-associated protein 4 (MFAP4), which in turn also binds collagen [62]. Elastin gives an elastic component compared to the strength provided by collagen fibers [60]. Generally elastic fibers are produced during development, and their synthesis is ceased with the maturation of the associated tissue[61]. The lack of elastin in development is embryonically lethal [24, 60]. Elastin turnover may be one critical aspect to understanding the effects of TGF-β in tumors, as it binds LTGB, and has been shown to be expressed at greater concentrations in patients with cancer as has been indicated through turnover measured indirectly in urine [63].

b. Fibronectin

Fibronectin (FN) is a dimeric disulfide-inked glycoprotein with multiple domains and is a component of most extracellular matrices. Its domains allow it to interact with multiple ECM components including collagen [64]. The FN domains control

self-assembly, while it binds various ECM components, *i.e.*, integrins and collagen via separate ligand binding. FN may be activated by integrins, where it undergoes a conformational change and becomes insoluble precipitating into stable fibrils [65]. These fibrils once deposited aid in the organization of other ECM proteins. The role in organization of the ECM is possibly due to its multi-domain nature, allowing for the binding of multiple components concurrently. These components include major ECM contributors such as collagen I and III, as well as microfibrils. Fibronectins is inhibited by tenascin-C, although this does not affect its adhesive properties, and fibronectin dependent cell movement continues *in* vitro where the two are combined. It has also been implicated in cell determination and differentiation in development [24, 64, 66, 67].

c. Fibrinogen

Fibrinogen is a large protein, most commonly associated with the coagulation of blood. It is composed of terminal globular domains connected by a rod like structure. Fibrinogen is known to bind activated $\alpha_{IIb}\beta_3$ integrin on the platelet surfaces and termed fibrin when deposited in insoluble fibrils. It is produced by the liver and is primarily a provisional matrix protein associated with wound healing, and substrate over which cells can migrate Fibrin has also been shown to act as an intermediary connector between cells and the ECM as it binds collagen and connects cells via integrin $\alpha_v\beta_3$ binding and fibronectin. [12, 23, 68, 69]. Fibrinogen fibers are termed fibrin when fibrinogen fibrinopeptides, located at the center of the molecule, are cleaved off by thrombin rendering the protein insoluble, as it is self-adhesive to other fibrin molecules, which have also been relieved of their fibrinopeptides, thus becoming fibrin molecules. These fibrin monomers self-assemble forming fibers. Fibrin fibers branch and form a mesh in three dimensions. Factor XIIIa acts on glutamine and lysine between fibers, forming direct cross-links providing the strength and stability necessary for clot formation. Fibrin is degraded by plasmin, which is activated by fibrin and tissue plasminogen activator interactions. In addition to providing a provisional matrix, fibrin has affinity to several factors which are important in the ECM including: fibulin, von Willebrand factor, FGF-2, VEGF and IL-1 [24, 70, 71].

d. Fibrillins

Fibrillins are an order of ECM proteins which contain a high degree of cysteine containing TGF-β binding regions. They interact with tropoelastin and integrins and form a significant component of the microfibrils in the ECM. They have a high affinity for calcium ions; contain three described isoforms, fibrillin 1-3. They

are described as being essential for the formation of elastic fibers. They are described as needing fibronectin for proper assembly and bind collagen in a complex binding fibronectin as multimers but not monomers. They have been described as a vital component in the activation of TGF-β and functional compromising mutations are diseases, referred to as fibrillinopathies as they affect the formation and function of microfibrills. One such associated disorder is Marfans syndrome, which is thought to be driven in part by pathologies in TGF-β sequestration or activation [64, 72, 73].

e. Fibulins

Fibulins are group of seven (FBLN1-7) glycoproteins which incorporate into fibrilllar ECM components. They have several basement membrane binding sites and are known to interact with various ECM proteins including fibronectin, laminin, tropoelastin and various proteoglycans. They are known to bind endothelial integrins and are thought to regulate crosslinking as they have been shown to interact with members of the lysyl oxidase-like family. Additionally they are implicated in various cell functions and tumor growth and or inhibition [74-76]. One example of the effect of fibulin is that like FBLN, when mutated its sequestration properties of TGF-β are reduced, resulting in the overabundance of the growth factor and the associated Marfans syndrome [77].

f. Tenascins

Tenacins are a heterogeneous group of glycosylated ECM proteins which similar to fibrinogen have FN binding and C-terminal globular domains. They are known to interfere with integrin mediated cell attachment to fibronectin and laminins and are differentially expressed in different tissue types. Tenascin-C is and –W are those which have thought to be most involved in tumor progression [67]. Although Tenascin –C interferes with fibronectin it does not have the same adhesive properties and reduces cell spreading. Integrin signaling via FAK and RhoA are also impaired. Their seemingly anti-adhesive properties have been proposed as a mechanism for the re-modulation of tissues and as a mechanism for the progression of cancer [78, 79]. Tenascin regulation is reportedly perturbed in many cancers and connective tissue diseases. It is associated with the pathological collagen deposition in Dupuytren's disease, but likely functions in conjunction with other ECM components such as the laminins, fibronectin, and collagen [24, 80, 81]. Mutations in Tenacin –X has been found in Ehlers-Danlos Syndrome, which was previously thought to be a group of diseases associated only with mutations in collagen types I, III, or V[82].

g. Thrombospondins

Thrombospondins (TSPs) are a group of ECM glycoproteins (TSP1-5) which are known to bind various ECM molecules as well as cell receptors. TSP-5 is also known as Cartilage Oligomeric Matrix Protein (COMP), which is known to have a modulatory effect on collagen in cartilage. Unlike many of the other ECM components discussed, TSPs are not known to form large structures or complexes, but rather modulate cell function from cell surface and ECM interaction [83, 84]. TSP-2 has been described as a modulator of collagen [85].

TSP-1, which is known to be stored in platelets, has been shown to activate latent TGF-β, thought to occur by interaction with the latent complex preventing the LAP from inhibiting active TGF-β binding to its cognate receptors. TSP-1 has elevated expression levels in many fibroproliferative pathologies, including tumor development [86, 87]. TSPs have been associated with both positive and negative prognosis in disease. [17, 88-91].

iv. Glycosaminoglycan and Proteoglycans

GAGs apart from the only known free GAG, Hyaluronan (HA), are attached to proteins thus forming the proteoglycans. These proteoglycans have one or several GAG chains which are covalently attached to the protein core. The GAGs are built from unbranched repeating disaccharide units consisting of, in the case of chondroitin sulfate (CS) glucuronic acid –N-acetylgalactosamine. Dermatan sulfate consists of the same basic disaccharide unit but also possess some uronic acid-residues epimerized to iduronic acid. Heparan sulfate consists of uronic/iduronic acid and N-acetlyglucosamine and keratin sulfate (KS) glactose and N-acetylglucosamine. These GAGs are sulfated to various degrees. HA is built form repeating glucuronic acid –N-acetylglucosamine disaccharides that are not sulfated. CS/DS and HS are linked through serine residues in the core protein via a tetrasaccharide bridge [21, 92, 93].

GAGs by area are main components of the ECM and act as fillers and are the primary component of viscous “ground substance”, their negative charge allows them to bind cations as well as water regulating fluid flow through the ECM. They have also been attributed as having a stabilization effect on the ECM and ECM related proteins. Proteoglycans are synthesized in the endoplasmic reticulum and glycosylated in the Golgi. They can be found as ECM, or transmembrane proteins. Their function varies and is reported to include wound repair [21]. Additionally, some proteoglycans are also known to function as co-receptors with transmembrane domains which transmit signals from the ECM[25]. Proteoglycan

cores have specific GAG chain binding, for instance CS/DS is associated with versican, biglycan and decorin and HS/CS associates to perlecan, CS to aggrecan, brevican, and KS to fibromodulin and lumican KS [94].

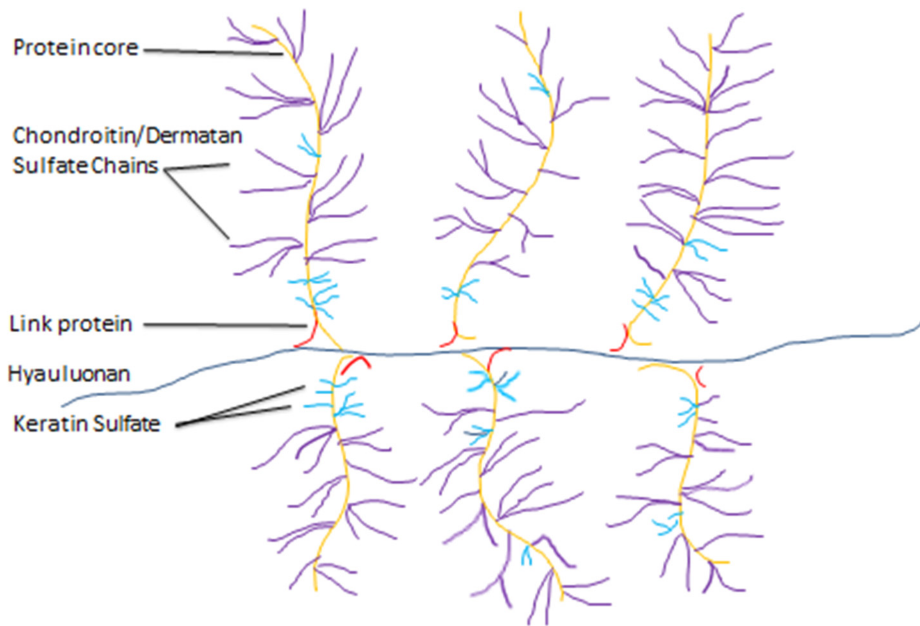

Figure 4
An illustration of a proteoglycan (aggregan like).

a. Role of the fibromodulin

The research groups around professor Åke Oldberg and the late professor Dick Heinegård pioneered the work on the small leucine-rich protein fibromodulin, originally isolated from cartilage [95-97]. Later our groups established a difference between the collagen architecture in tumors grown in wild type compared to Fmod deficient mice [98]. The latter tumors had a less dense collagenous matrix and more extra cellular fluid volume (ECV). Fmod belongs to the small leucine rich proteoglycan family (SLRPs). It along with other SLRPs are of interest with respect to the ECM some are known modulators of collagen, affecting both assembly and maintenance of collagen fibrils [99, 100]. Additionally tendon tensile strength corresponds to Fmod deficiency [101]. Fmod is also known to play a role in TGF-β modulation [102].

IV Stroma and the Microenvironment

Loose connective tissue stroma is composed of a number of ECM proteins (see section II). ECM components are obviously tissue specific; many however are expressed in multiple tissue types and have a common function. Tumors with the same cellular origin which express different ECM components have been shown to metastasis differently. Differences in basement membrane proteins were higher in non-metastatic tumors, compared to more metastatic tumors. Elastin and elastin binding emilins were also increased in tumors with increased metastasis, although the presence of most of the ECM was unchanged [13]. In a study on primary colorectal adenocarcinoma, FMOD is one such ECM protein which is observed in primary tumors when compared to adjacent healthy colon submucosa [103]. The question to the role of the ECM as being permissive or driving in tumorigenesis is thus relevant.

It has long been known that malignant tumors often develop where chronic inflammation is not stemmed. Colon and liver cancers are examples where fibrosis due to a chronic inflammation is either determinant or determined by cancer formation. This leads to a question to the origin, relevance and causal elements of the microenvironment related to cancer[104]. It has also been demonstrated that an aberrant tumor stoma (mesenchyme) can lead to the selective loss of tumor-suppressor genes via increased selective pressures, evident by the experimental loss of p53[105]. The properties of cancer, were famously delineated in 2000 by Hanahan and Weinberg in an article titled “The Hallmarks of Cancer” are as follows: Self-sufficient in growth signals, insensitive to anti-growth signals, evade apoptosis (programmed cell death), immortal (limitless replicative potential), angiogenic (develop blood vessels) and invade tissue and metastasize[106].

Years later Hanahan and Weinberg wrote a follow up to their article “Hallmarks of cancer” titled “Hallmarks of cancer: the next generation”, there the discussion of inflammation and the tumor microenvironment where the issue of inflammation and cancer development was emphasized. This line of thinking dates to 1863 with Virchow’s observation of leucocytes in cancer tissue. Two other hallmarks, primarily evading the immune system and the reprograming of metabolism[107].

Cancer metabolism and the use of glucose, known as the Warburg effect, have been well known since described by Otto Warburg in 1924.

This discussion about tumorigenesis may not be directly linear. Changes in the microenvironment may be due to the growth of a tumor creating its own microenvironment favorable for continued growth, or due to other factors, *i.e.*, chronic inflammation, which leads to an environment permissive to tumor development. The Warburg effect has recently been cited discussed as one such example, where the use of glucose may be a tumor initiator, rather than a symptom of tumor growth[108]. Additionally changes in the stromal microenvironment, as in the presence of long term fibrosis, correlates to an increase incidence of cancer. Likewise certain patients have been known to develop metastatic tumors at points of injury, or surgery at wound margins. This follows the same line of thinking that tumors require specific microenvironments to become established [109] . The microenvironment of wounds and healing tissue may correlate with carcinoma physiology and pathology. Moreover, that the conditions present in a wound, are favorable for the establishment, or progression of tumors[110]. The concept of cancer as a non-healing would has been around since the 1980s [111] The effects of scaring, or chronic irritation by a foreign body, *i.e.*, an ulcer, fistula or other element has been known to proceed the formation of cancerous lesions for over 40 years [110, 112].

Wound repair, can be broken down into three basic components, an inflammatory stage, a regenerative stage and a remodeling stage. Tumors seem to exhibit the first two aspects, but fail when it comes to the third, remodeling; instead inducing inflammatory markers and proliferating without the regulation seen in normal wound healing. Several correlative examples between tumor development and wounds have been shown, first with the Rous sarcoma virus in chickens, tumors grew anywhere wounds were present, or initiated [113], the connection was further established to inflammation when anti-inflammatory agents prevented the formation of tumors at these wounding sites[114].

Cancer has been thought to be driven by a number of mutations in either driver, so called oncogenes, or tumor suppressor genes. This view is being challenged in some contexts as it has been shown that certain tumors can be maintained by a subpopulation, which induces alterations in the microenvironment, specifically in collagen and in drafting vascularization in tumors [115]. To this extent it is even argued that tumors can be classified as organs in their own right, containing a degree of functionality between themselves and their microenvironment[116]. Stromal conditions change during tumor progression and can promote metastasis. A major stromal change in reactive tumor stroma is the presence of CAFs, though to precede tumor progression, induced by growth factors and cytokines. The crosstalk between stromal and malignant cells may induce the priming for the

generations of CAFs, in essence seeding the stroma to a pro-malignant microenvironment phenotype. This is referred to as the seed-soil hypothesis[117].The ability of malignant cells to take advantage of and manipulate their microenvironment, may make the stroma more permissive to metastatic invasion, compared to the tumor suppressive characteristics associated with healthy stroma [8, 16].These stromal microenviornmental effects are linked to immune related inflammation, (see section VII). Due to the established ECM interactions, as effectors of the stromal microenvironment and cancer progression, many have been and are being considered as targets for treatment [118].

The origins of the proteins in the ECM of tumors have been confirmed in some models, using human xenographed tumors in mice. It has been shown that, although to a differential degree, both tumor and stromal cells contributed to the ECM. These studies have shown near complete production by the host (mouse), some proteins however, such as fibrillin-1, fibronectin, collagens IV and XVIII, perlecan and nidogen are produced by both tumor and host cells. [13, 14].

V Epithelial-to-mesenchymal transition (EMT).

EMT may be induced by a number of factors often associated with carcinoma, such as TGF-β and tissue necrosis factor (TNF)[119]. It is a process of transition by epithelial cells to mesenchymal sells, here cells gain a level of mulipotency, and are able to differentiate into a variety of cell types. It is characterized by loss of cellular polarity and has a number of associated markers and drivers including: TWIST, Snail and slug, PI3K and AKT, activated by a number of pathways, including activation by matrix stiffness via TGF-β, FGF, NFKB, hedgehog signaling. Its role in metastasis is controversial [120-122].

It is also characterized by repression of intermediate filaments like cytokeratins in favor or more mesenchymal intermediate filaments like vimentin (VIM), desmin and a change from E- to N-cadherin expression, thus VIM and a change in cadherin expression, suggest a move towards EMT. EMT has also been suggested as the cause or source of so called cancer stem cells, defined as a non-metabolic, dormant cell population which gives rise to secondary cells responsible for tumor formation[123]. Mesenchymal cells are those responsible for the deposition of the primary ECM components which appear to be linked with the negative association with EMT and patient prognosis.

As we consider tumors to be non-healing wounds, the cell types which are involved in the response to injury are key players. Fibroblasts produce a number of key growth factors, and deposit the extracellular matrix which both provides the support for the stroma and a large portion of its compartment. They secrete chemokines and are involved in angiogenesis and are associated with cancer and its progression at all stages. Fibroblasts are the primary collagen producers and modifiers of the ECM, they utilize integrins to interact with the microenvironment and are characterized by their actin and vimentin intermediate filaments, the marker FSP1 (fibroblast specific protein 1, in the S100 calcium binding protein family). In healthy tissue, fibroblasts have minimal ECM producing activity, they can be activated by a number of growth factors, including TGF-β, proteases and chemokines among others[124]. Activated fibroblasts in carcinoma, also referred to as CAFs or cancer associated fibroblast , are known to be associated with all stages of tumor progress and together with the cancer cells, the ECM, immune cells, vessels and the basement membrane, comprise the tumor stroma[124, 125].

VI Physiology of carcinoma

i. Loose connective tissue in carcinoma.

In carcinoma the loose connective tissue and stroma differs in many ways from normal tissue. The presence of microfibrils, and stromal components are not only different but their structures may be dysregulated, when compared to healthy tissues. The types and number of cells also differs from normal loose connective tissue. Macrophages and cancer associated fibroblasts are often associated with carcinoma (see sections VII and VI ii). Much of the regulation of the matrix is controlled by auto and paracrine signaling from factors such as TGF-β and PDGF (see section IX).

The major lethal cancers lung, breast or prostate and colorectal cancers are solid in nature and generally have a pervasive extracellular matrix. Cellular forces and various other factors maintain the structure of the ECM. These matrices are generally rich in collagen and fibronectin, among others, including SLRPs and GAGs. Fibrillar components like collagen, elastin and provisional fibers like fibrin, VN and FN are often overexpressed. Fibrillar collagens may be present as homotrimeric collage type I ($\alpha1(I)_3$, which may be more resistant to remodeling, instead of the normal $\alpha1(I)_2\ \alpha2(I)$) . Provisional matrix deposition, such as fibrin deposition, is one of the reasons carcinoma can be compared with healing wounds [13, 22, 126].

ii. Producers of stromal ECM in Carcinoma

Cancer associated fibroblasts (CAFs) are often one of the most prominent cell types within the stroma of many cancer types. It has been reported that CAFs can originate from local fibroblasts, where present locally. Fibroblasts may potentially be derived from pericytes then continue to become MFs and CAFs [52, 127, 128]. CAFs are often identified by their expression of the myofibroblast (MF) marker α-SMA [129] Fibroblasts ubiquitously express PDGF receptors *in vitro* and are seen, as previously discussed, as main depositors of ECM proteins, especially collagen[128]. PDGF receptors are reported to be upregulated in carcinoma,

inflammation and in healing wounds, PDGF inhibition; has been reported to inhibit the differentiation capacity of pericytes[130]. Pericytes are also considered to be mesenchymal stem cells and capable of myogenic, osteogenic, chondrogenic and adipogenic differentiation, i.e. they are capable of becoming muscle, bone, cartilage and fat related cell types [130, 131].

The difference in the types of contraction generated by smooth muscle cells (SMC) and MFs is temporal acute and transient in SMC versus long and sustained, the latter being from MFs. *In vitro* the cytoskeleton exerts a tension on the ECM via integrins at cellular focal adhesions. A cell mediated contraction can, additionally activate latent TGF-β which in turn drives the formation/differentiation and contraction of MFs or CAFs [49]. The source of the ECM in tumors is primarily of stromal origin, of which cells are argued to be derived from pericytes , circulating fibrocytes, or infiltrating fibroblasts from the surrounding tissue, *i.e.*, those cells capable of becoming mesenchymal ECM producers[13] [52, 132-134]. It is also well known that stromal cells have an important role in tumor progression, through paracrine factors including TGF-β and PDGF, but also many others including: IGF, HGF, FGF and MMPs, which are expressed at increased levels when associated with carcinoma and are known ECM regulators [135].

iii. Blood vessels and the exchange of fluid and solutes in carcinoma.

Tumors have an aberrant vascular structure, which when normalized, can both increase treatment efficacy, decrease tumor growth, and sensitivity to radiation. Radiation sensitivity occurs due to an increase of oxygenation, of which radiation produces free radicals which in turn extirpate their surroundings. It is believed by many that the hypoxia commonly seen in carcinoma explains the relative radiation-insensitivity; oxygen is a radiation-sensitizer. In hypoxia, the concentration of oxygen radicals that are generated by radiation is lowered. These radicals are DNA-damaging and responsible for a large part of the radiation-toxicity. This occurs as a result of increasing the blood flow to the tumor resulting in an increased transfer of solutes and greater oxygenation[136]. Tumor progression requires angiogenesis and has long been seen as a target for cancer therapy[137]. Oxygenation through the interstitium occurs via diffusion. Oxygen can only diffuse through tissue to a distance of about 200 μm, the diffusion coefficient for oxygen through the interstitium has been experimentally established at a rate of 1.04×10^{-5} cm^2/s[31], whether or not this is true in tumors is unknown. The requirement for oxygen necessitates the complex vascular

network, which itself is aberrant in carcinoma [138]. The fact that carcinoma can grow at accelerated rates also limits their oxygen availability and along with the associated hypoxia comes a number of inflammatory cytokines and angiogenic factors.

Tumors may possess capillaries and small blood vessels, and even some larger arteries and veins which feed and drain the tumor. Capillaries contain two basics cell types, endothelial cells and their supporting pericytes. In healthy capillaries, the endothelial cells are well-ordered and supported by a basement membrane. They have tight junctions which allow them to regulate the flow of fluids across the barrier and maintain a degree of internal polarity. In addition to tight junctions there ae various channel proteins, such as aquaporins, which transport water across the endothelial plasma membrane, these seem to increase in cancer[139]. The endothelial glycocalyx is an additional regulator of fluid flow[45]. In tumors there may be large gaps in the capillary walls, pericytes may be absent and the vessel shapes and organization are not ordered [29]. It has been shown that when vessels are normalized in tumors, the probability of metastasis decreases, and tumors themselves become less aggressive and more responsive to treatment [140].

The exchange between capillaries and the interstitium within carcinoma, which are often leaky for plasma proteins, due in part to that VEGF/VPF and inflammatory factors directly affects the barrier to fluid exchange[12, 141]. Cellular regulators of blood vessels include endothelial cells, SMCs and pericytes [52].

Still it has been shown by Levick in 1987 when applying Darcy's Law, for the flow through porous beds, together with the Carman-Kozeny equation from 1937, that it is not the cellular compartment of the interstitium which is primarily responsible for the drag effect, reducing interstitial conductivity, but instead as he termed it "…the drag created by innumerable fixed fibrous obstacles spanning the intercellular spaces."[142].

Plasma volume in in the experimental carcinomas that we have studied was approximately 0.12mL/g dry weight and total tissue water was around 4 - 5 mL per g dry weight, plasma volume in the skin is approximately 0.008mL/g dry weight compared to total tissue water equal to 1.99mL/g. This shows how little water is present in the plasma compartment by volume [143-145].

VII Carcinoma and the Immune system

i. Inflammation profiles

Chronic inflammation has been linked to tumorigenesis (see above). Inflammation is a normally a response to activation of the immune system.

Inflammation allows for the increased passage through the interstitium of fluid, protein and cells in response to infection or injury. Among these are immune cells, infiltrating leukocytes, of which include monocytes, that receive different signals, from the interstitial environment, to become macrophages. are further broken down into two types, M1 and M2 [146, 147]. M1 have a phagocytotic pro-inflanmmatory phenotype, whereas M2 are considered to be rebuilding phenotype. IL-4 is known to stimulate differentiation into M2 macrophages. This was seen to be upregulated in tumors generated in NCF1 deficient mice (Paper V). Other immune cell types are also present including T-cells, neutrophils (granulocytes) and NK cells, many of which are modulated by TGF-β and other growth factors [148, 149]. Additionally overexpression of these growth factors stimulate may lead to the recruitment of macrophages in the tissue [150]. Mast cells are another infiltrating immune cell which has also been shown to have a role in the upregulation of angiogenesis and promotion of tumor progression [9].

Immune response and immune avoidance are also prerequisites for the survival and progression of tumors. A number of oncogene as well as tumors pressor genes are associated with immune system recognition or avoidance. Macrophages are also involved in immune surveillance and activity. High macrophage content in carcinoma however, is associated with a negative patient prognosis[146] . They and other immune cells are capable of generating reactive oxygen species (ROS) as a mechanism to destroy fungus, bacteria, and other invading cells, which would include carcinoma. These ROS are produced by NOX complexes. The innate immune response is also active, and modulated within carcinoma. NFκB, antigen suppression and surveyor cell avoidance are key to tumor survival, anti-tumor T and NK and monocytes cells are known to be dependent on NOX family oxidases for their function, and require the NcF1 gene to form a functional complex[151-

153]. We have shown that where ROS are limited a greater number of tumors develop in models for experimental carcinoma (Paper V) [154].

ii. Neutrophil cytosolic factor 1 (Ncf-1) a down-regulation of autoimmunity.

Macrophage-derived oxygen radicals, reactive oxygen species (ROS), have been described as modulators of the autoimmune response against cancer. Downregulation of *p47phox* known as the phagocyte NADPH oxidase/NOX2 regulator, (NCF1). NADPH oxidases are enzymes which are involved in the productions of ROS[155]. The NOX2 complex is protective against autoimmunity and is implemental in immunity against pathogens. Certain mutations in *Ncf-1* decrease the ability of antigen presenting cells to produce ROS that represent important negative modulators of T-cell activity thereby a mutated non-functional Ncf-1 protein increases the probability of autoimmunity [152]. The idea of mutating NCF1 was that tumors may not be as able to avoid destruction by the immune system. On the other hand a decreased T-cell activity could allow malignant tumors with subtle antigenic differences from the host tissue that otherwise would be killed by immunosurveillance. It has been reported that, in the liver, NOXes regulate TGF-β via ROS, and that this interaction is involved in the processes surrounding fibrosis [156].

VIII Barriers to transport

Interstitial conductivity for fluid and thus access to tissue, distal to vasculature, is perturbed in tumors [157]. Many factors influence interstitial conductivity and the flow of fluid through loose connective tissue and the ECM, these components very by tissue and tumor type. The heterogeneous nature of tumors also results in a varied resistance to fluid flow or transport between regions within individual tumors. Facets include cellularity, as cell surfaces restrict fluid flow due to their low hydraulic conductivity. The amount and concentration of GAGs and collagen also effects this flow and conductivity[142]. Collagen fibers, by number, are more prevalent that GAGs , although GAGs take up a greater area (see section III iv) in tissue and their effect on transport has not been completely understood. They can affect intratumoral flow and hydraulic conductivity of fluid by GAG and volumetric exclusion as discussed by Levick [142, 158]. The negligible flow through collagen fibrils themselves, argues against them as being conduits of flow. GAGs interact with the fibrous ECM and provide resistance to tissue conductance, this affects the interstitial hydraulic permeability [158].The mechanism behind the observed effects of collagen, is likely due to the basic obstructive effects of GAGs on fluid flow through the ECM as they are prevented from expanding by the collagen network. This in effect increases the length and resistance to flow, through both physical obstruction as well as the charged disaccharides addition of an electrokinetic resistance. Fluid flow may also be hindered by additional elements other than collagen, cells and GAGs including proteoglycan protein cores[142] (see section III iii). Aukland and Reed have proposed that it is the Starling forces (see section VI iii) which are perturbed preventing normal transfer between blood and carcinoma interstitium [159].

A stiffer ECM in associated with carcinoma, gives rise to some of their barrier functions and is also associated with EMT as well as tumor progression [120]. In addition, even when accessible, treatment is further hindered by the complex interplay between various ECM regulatory systems.

i. Interstitial Fluid Pressure (PIF)

PIF has been described as a correlating factor to barriers for cancer therapy, which when lowered significantly improves treatment [157, 160]. PIF has been proposed

to result from mechanic and tensile-forces exerted by cellular interactions on components of loose connective tissue [157]. Interstitial fluid volume seems to be determined by collagen fibril diameter more than the modulation of GAGs, as reported in 2003, that PIF does not correlated to HA content alone[161]. Fibrillar ECM components provide the main skeleton for which cells can exert these forces, generating PIF[162]. The intolerant or impaired penetrability of cytostatics in carcinoma has been shown to be reduced by lowering PIF [149, 157]. Our group has previously reported that STI571 reduces PIF in KAT-4 experimental carcinoma. Tumor perfusability was also increased in patients treated with STI571 and observed with PET in a study conducted in Uppsala [163]. The relatively low increased 5-year survival rate elucidated by Morgan, Ward and Barton with curative and adjuvant chemotherapy treatments may reflect the fact that reported by Heldin, that cytostatics penetration into tumors is poor or impaired [157, 164].

It has now been shown that PIF can be reduced in tumors treated by not only STI571, but with Anti-VEGF, Prostaglandin E_1, Anti-PDGF, Anti-TGF-β1 and –β3 , Anti-IL-1 and in Fmod negative mice[145, 160, 165]. We additionally indicate that tumor PIF can be reduced via anti- $\alpha_v\beta_6$ (paper III).

Changes of this collagen fibril network by either effecting density or fibril structure are known to correlate with changes in PIF [98, 144, 166-168]. This may be correlated with collagen fibril diameter, with a decrease in collagen diameter has been repeatedly seen to correlate with a reduction of PIF. The opposite is also potentially true, a recent study where collagen fibril diameter was increased, so to was PIF [162].It has been shown that a reduction in GAGs reduces PIF, which is known to be associated with negative treatment efficacy [157, 169]. It has further been shown that the characteristically elevated PIF in pancreatic ductal carcinoma is dependent on the GAG HA, and that when removed enzymatically in a model system, PIF is decreased and microvascular constriction is removed[170]. When prostaglandin E_1 is administered, there is an immediate reduction in PIF in tissue; PDGF and TGF-β inhibition have a similar reduction but over time [160, 165, 171, 172]. The question to where fibroblasts or other stromal cells provide the tension, or is it the collagen seems to imply that there is both a cellular regulated immediate tension and a structural component which is maintained by the structure/morphology of the matrix, which both are involved in the regulation of fluid transport.

Cells act as barriers to the flow of fluids, due to their membranes, and restrict fluid movement to narrower regions and interspace. This interspace is filled with ground substance, the ground substance is primarily composed of GAGs, fibroblasts act on collagen and other fibrillar ECM components containing the ground substance, resulting in an increased PIF[173].

IX ECM and growth factor signaling

In addition EMT transition of carcinoma cells[174], there exist several mediators produced by inflammatory or malignant cells which can drive the formation of CAFs [124, 125]. CAFs are major producers of stromal ECM, TGF-β is well known for its modulatory effects on the formation and regulation of the stromal and the development of CAFs in carcinoma[175].

PDGF is present in most cancer and its receptors are ubiquitously expressed in fibroblasts and pericytes *in vitro* and in activated tissues. It has been implicated in EMT and the differentiation of cell types. PDGF inhibition has been shown to increase tumor treatment efficiency and overcome some of the barriers to transport [5]. PDGF is a stimulator of growth and survival, but also effects homeostasis and cell movement in tissue and mesenchymal cells. It is a known effector of pericyte differentiation and reported to effect angiogenesis. Auto and paracrine PDGF signaling in cancer has been reported as an ECM promotor and of tumor progression [130, 176, 177].

Vascular endothelial growth factor (VEGF) is important for tumor progression and metastasis. Its general role is in that of vessel formation, primarily in embryonic development. The VEGF family proteins bind their cognate tyrosine kinase receptors (VEGFR) with interspecific specificity. VEGF is also involved in maintaining vascular integrity, effecting vascular permeability [178, 179].

IL-1 affects VEGF expression and effects. VEGF expression has been shown to be activated by IL-1β via the P38, MAPK, and the JNK signaling axis [180]. Il-1α was shown in 2005 to increase VEGF levels as well [181]. This further illustrates the same signaling mechanism of both the IL-1 isoforms, downstream of the IL-1 receptors, which has been known for some time. IL-1 is best described as the main workhorse of inflammation, wound healing and immune response. It is associated with a number of diseases, and the lack of IL-1Ra in animal models results in spontaneous arthritis [182] . The importance of codependent processes of IL-1, VEGF and TGF-β underscore the complexity of the extracellular matrix.

TGF-β is also heavily involved in angiogenesis and works together in interplay with VEGF, leading to the production of new vessels[183]These factors have at first glance opposing roles, TGF-β induces endothelial apoptosis where VEGF exerts a protective effect on the cells. It was, however, shown that TGF-β

upregulates VEGF synthesis through the intermediate promotion of FGF-2 expression, and that the inhibition of flk-1, aka VEGF receptor 2, terminates TGF-β induced p38 (MAPK) activation and apoptosis [184].

Both IL-1 and TGF-β, in addition to the deposition and remodeling of the ECM, are involved in cell migration and cell signaling. They affect angiogenesis independent of VEGF, showing a further complex role in the alteration of the tumor microenvironment and a more convoluted than previously understood means of tumor progression and intercellular regulatory mechanisms [185]. This cross-talk illustrates multifactorial signal mediation, where any factor alone may have distinct and even opposite effects.

i TGF-β: TGF-β activation and biological significance.

TGF-β is central in this study. It is described as a modulator of a large number of biological processes. The exact functions are not binary but it plays a role in and affects a number of processes, it is known to be an important factor for tumor growth as it has been shown to affect the ECM. It is active in the development of fibrosis and is a potent stimulator of collagen production in cultured cells [186, 187].

TGF-β plays a vital role in a wide variety of processes from embryology to immune regulation to cancer, and when it or its activators are not present, it leads to embryonic lethality [175, 188]. Most if not all of the secreted TGF-β is done so in its inactive form, bound to a latency associated peptide, which in turn is covalently bound to the latent TGF-β binding proteins on secretion [189]. The contradictory nature of its effects can be seen in early versus later tumor development. It has been indicated to reduce tumor growth by more than 90 percent in some tumor models but is also described as an early inhibitor of tumor progression [190, 191].

TGF-β is generated in a non-active form, which together with the co-secreted latency-associated peptide (LAP) form the Latent TGF-β complex also known as the Large Latent Complex (LLC). LAP originates from the cleavage of the C-terminal pro-region of the transcribed protein. The remainder, the N-terminal portion, being the active element, most commonly referred to simply as TGF-β. The association with the cleaved LAP and TGF-β prevent biological interaction unless activated, as such it is termed latent TGF-β (LTGFB)[192]. TGF-β may additionally be secreted in a small latent complex (SLC), however it is not secreted by most cells, and is generally only common in bone [193]. The LAP associates with LTBP via disulfide bonds between Cys repeats and can bind

proteins during secretion [194]. The LAP contains the arginine-glycine-aspartic acid (RGD) motif sequence which is recognized by a series of integrins, most notably $\alpha_v\beta_6$ and $\alpha_v\beta_8$ [195-197]. The LTBP proteins are members of fibrillin-like ECM proteins and are known to associate with several other ECM proteins including: elastin, fibronectin, fibrillin and vitronectin [24, 198, 199]. The LTBP is covalently affixed to the ECM proteins via transglutamination, and ECM interactions may be cleaved with plasmin [199, 200].

Other than integrin mediated tension latent TGF-β is activatable by proteolytic cleavage (MMPs(MT-1[201]-2,-9[202], proteases cathepsin and plasmin[203, 204], elastase and chymase[205], thrombin and conformational change and release of the active form after interacting with the TSP-1[87]; exposure to low pH or reactive oxygen species [202, 206-209].

TGF-β receptors are nearly ubiquitously expressed, which suggests in itself a complex activation requirement. TGF-β signaling acts by receptor phosphorylation leading to phosphorylation of Smad2 and 3. Smad2 and 3 dissociate from the receptor and form complexes with Smad4 that are translocated to the nucleus where they regulate the transcription of a large number of target genes. Non Smad signaling occurs as well, a main example, associated with the apoptotic effects of TGF-β is the activation of the p38 MAP kinase pathway [210, 211]. TβRII forms complexes with TβRI after first binding TGF-β. TGF-β isoforms all bind TβRII. TGF-β isoforms –β1 and –β3 have high affinity to TβRII and low TβRI affinity. TGF-β2 has a low affinity for TβRII and requires a co-receptor for activation[212]. TGF-β receptors, type I (TβRI) which is also known as ALK-5, and II (TβRII) are serine/threonine kinases receptors. The two receptor types occur in various states but are stabilized as heterotetramers when bound to a ligand [213, 214].

X Aims of the thesis

i. Ambition of the studies.

The general aim for the works conducted during my doctoral studies, culminating in this thesis, has been to elucidate structure and functional relationships of stroma ECM in experimental carcinoma. The ambitions during this time have been to study the role of integrin-mediated activation of latent TGF-β in carcinoma; to study the functional consequences and mechanisms of STI571 treatment in experimental carcinoma; and to investigate the potential role of Fmod in the organization of the stromal ECM in experimental carcinoma.

An ambition, stemming from work previously showed by this relating to the barriers of cancer therapy has been to lower interstitial fluid pressure in carcinoma facilitating greater anticancer efficacy of cytostatic treatment[5, 157, 160].

The AIM of paper I

"***The tyrosine kinase inhibitor Imatinib augments extracellular fluid exchange and reduces average collagen fibril diameter in experimental carcinoma",*** was to determine which aspects of the ECM were changed after the admission of STI-571 to tumor bearing animals, and its effect on fluid flow through the matrix. This was built on the fact that PIF decreases in tumors treated with STI571 as well as an increased response to chemotherapeutics and a study showing increased oxygen perfusion in patients metastatic tumors with PET after being treated with the same[5, 163, 215].

The AIM of paper II

"Imatinib increases oxygen delivery in extracellular matrix-rich but not in matrix-poor experimental carcinoma", was to determine if the mechanism behind the effects seen in Paper I [216], were due to vascular effects and if the effect was linked to the observed matrix effects.

The AIM of paper III

"***Inhibition of integrin $\alpha_V\beta_6$ changes fibril thickness of stromal collagen in experimental carcinoma"***, was to help elucidate the effects of the $\alpha_V\beta_6$ integrin on TGF-β activation in carcinoma, and if the previously observed TGF-β effects could be recapitulated by solely inhibiting the $\alpha_V\beta_6$ integrin. Additionally the mechanisms of TGF-β activation were investigated. Previously TGF-β inhibition has been shown to have similar effects in carcinoma as treatment with STI571, as such potential ECM and cellular contributors were also included in the investigation[172].

The AIM of paper IV

"**Fibromodulin deficiency reduces collagen architecture but not glycosaminoglycan content."**, was formulated to investigate whether the effects seen in papers I,II and III were due to the observed reduction of Fmod seen after treatment with either STI571 or anti TGF-β treatments.

The AIM of paper V

"***Mice lacking NCF1 exhibit reduced growth of implanted melanoma and carcinoma tumors"***, was based on the premise that a change in ROS production could affect the implantation and growth of tumors. This can be connected to the other four papers via the role of reduced or inhibited macrophages and other immune cells in carcinoma tissue.

XI Methods and Models

The following is meant to discuss, in part, the potential benefits and drawback of some of the lesser known models and methods used to complete the work done for this thesis.

i. Model System

Here we have employed models to investigate several properties which chronic inflammatory lesions shared with carcinoma. Additional carcinoma characteristics such as aberrant blood vessels, high interstitial fluid pressure, and various matrix components have been investigated. Our work has primarily used human cell lines xenografted into immuneincompetent mice. We have also utilized syngeneic tumor cell lines in mice, to avoid potential effects of a missing immune system. Although these methods may not best represent the human condition, they afford easier access to tumor material, growth and more consistency in a notoriously heterogeneous system.

ii. Cell and Mouse lines

The KAT-4 cell line, which is used in several studies in the thesis (papers I-III) have been characterized extensively by our research groups , was thought to have its origins in a human thyroid cancer[217], but was shown to most likely be the colorectal carcinoma HT-29 [218] which we later confirmed. Our Capan-2 and KAT-4 cell lines were verified by Identicell of Aarhus Denmark.

Lewis lung carcinoma and B16 melanoma cell lines were used in paper V and these cell lines are syngeneic to the C57Bl6 mouse line. CT26 is a cell line syngeneic to the BALB/c mouse line. Further studies used OOC38, a clone derived from C38 as it was deemed more desirable to use the C57 Bl6 mouse line with a more human like collagen composition. For this purpose the C38 cell line was chosen.

The C38 cell line was originally chemically derived and has been used extensively in carcinoma and metastasis studies and is characteristically insensitive to therapies [219]. From the original C38 several other lines have arisen through selection such as the SL4 and MC38 [220]. After *in vivo* passage a clone was selected and designated OOC38. OOC38 exhibited a higher degree of TGF-β activation and more pronounced colonial like growth, while being negative for markers of EMT.

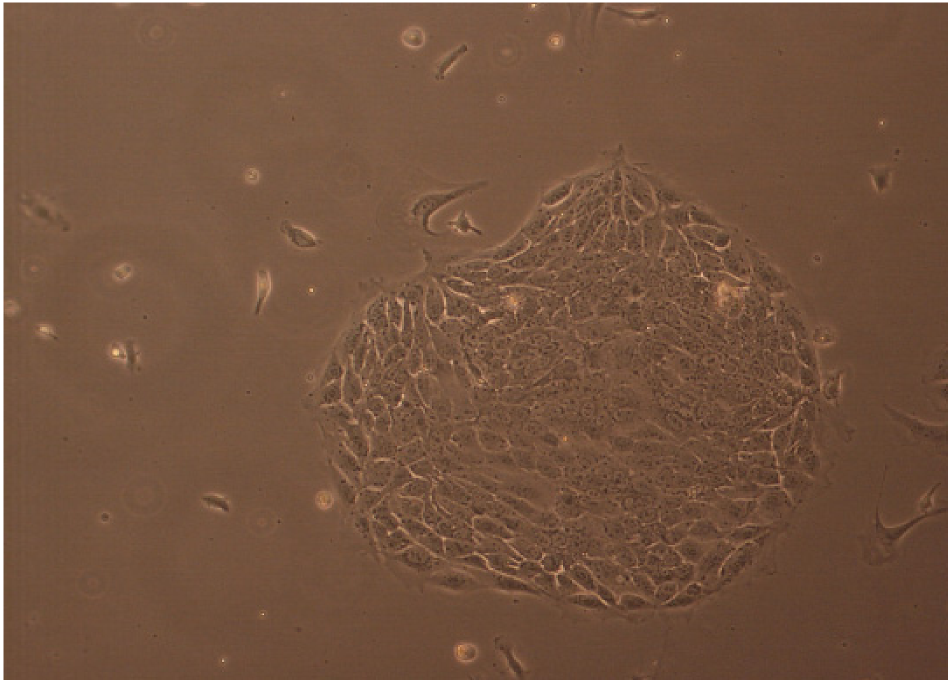

Figure 5
OOC38 line

The C57 Bl6 inbred mouse line from Jackson was used for the syngeneic mouse tumor growth with the C38 cell line as well as an isogenic Fmod -/- line maintained in Lund. The strain was chosen for its extensive use, being widely used and best characterized strain of laboratory mouse [221]. Its originates from breeding done by C.C. Little in 1921 at the Bussey Institute for Research in Applied Biology. The C57Bl6 line used in paper IV was backcrossed to C57Bl6 mice purchased from the Jackson laboratory to avoid potential subpopulation divergence which may have occurred over the years . As described by Hughes 2007 much diversity can persist even in heavily inbred strains, *i.e.,* C57Bl6 [222].

All animal handling was performed in accordance with ethical permission and licensing. Anesthetization of all mice was performed though inhalation of

Isoflurane. Respiration and reflexes were monitored in all cases, and heat was provided in the form of a thermal pad at 37°C for pressure measurements. Mice were sacrificed by cervical dislocation when anesthetized, or through isoflurane overdose.

iii. Hexosaminidase Assay to determine cell numbers

Cell numbers were determined by hexosaminidase reaction as described by Landegren in 1984[223]. This method works well for the identification of cells in various conditions, where direct cell counting is not possible. It is, however, limited as hexosaminidase may be released from dying cells and accumulated over time in the media. To overcome these potential drawbacks, relative differences between conditions instead of absolute values were used, often to verify the lack of treatment toxicity, or cell proliferation and survival in various assays.

iv. TGF-β Luciferase Assay

This assay measures luminesces from the presence of luciferase when given the luciferin substrate. As luciferase is expressed in MLEC under the PAI-1 promotor, which is downstream of TGF-β receptor activation, it is used as a measurement of TGF-β. There is a question of consistency in the measurements as a degree of variability within and between assays is common. To overcome this biological variation in the method, many multiple independent experiments were performed, positive and negative controls were used and results normalized to known activators, or to the MLEC baseline when necessary. Additionally anything which may interfere in PAI-1 activation other than TGF-β may confound the results, resulting in a potential false negative or reduced signal.

a. $\alpha_v\beta_6$ 3G9 antibody inactivation

This is central to paper III. The 3G9 monoclonal antibody against $\alpha_v\beta_6$ was developed in Biogen Idec, Inc. in 2004. As there are a number of integrins which bind the RGD site of the TGF-B LAP including $\alpha_{IIb}\beta_3$, β_1 integrins, and all of the α_v integrins. The $\alpha_v\beta_6$ integrin is the only one specific to epithelial cells. There are a total of five β subunits which are capable of pairing with the α_v integrin subunit these are: β_1, β_3, β_5, β_6, and β_8 [197]. The integrin $\alpha_v\beta_6$ is upregulated in healing

wounds and in tumors [224], consistent with the non-healing wound paradigm and is a suggested target for various cancer therapies [225-227].

The $\alpha_V\beta_6$ integrin has been thoroughly described as a primary activator of the LLC [209, 228]. Mouse studies utilizing genetic modification have shown that integrin mediated TGF-β activation are vital for TGF-β dependent processes investigated by knock downs of $\alpha_V\beta_6$ and $\alpha_V\beta_8$ resulted in embryonic lethality [188, 228]. The integrin $\alpha_V\beta_6$ has been implicated in development [188] and cancer [190]. $\alpha_V\beta_6$ expression is restricted to epithelial cells and is a negative prognostic factor upregulated in a number of cancers [229]. $\alpha_V\beta_6$ has been implicated in the degradation of elastin [230].

$A v\beta_6$ is also known to bind proteins other than LAP, including tenascin, fibronectin and vitronectin [231, 232]. The RGD sequence which is associated with the integrin binding on the LAP is present in both LAPβ1 and LAPβ3. LAPβ2 contains instead an SGD sequence, which the integrins do not bind.

The 3G9 antibody was shown to inhibit LAP binding with an IC50 of 2.7ng/ml. more than four times less than the ED_{50} for $\alpha_v\beta_5$ activation of latent TGF-β[233]. The other potential integrin associations, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, were confirmed to be negative for antibody binding being tested with cell-expressed and purified integrin. No significant cross-reactivity of the $\alpha_v\beta_6$ antibodies with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins was observed. [234] (see also section IX i).

v. PIF measurement

Tumor interstitial fluid pressure was measured, *in vivo,* using capillary displacement across a membrane, with a wicked needle inserted into tumors. In addition to the large degree of variation between tumors, there exist many issues with mearing PIF in this way. The instability of measurements, and the large degree of variation in the stromal compartment, relating to ECM composition, confounds population based measurements of such small scale. The difficulty and time required also limits the number of tumors which can be investigated. There exist a number of other challenges and disadvantages with measuring PIF. The potential issue with the size of the needle changing the stroma which it comes in contact with or puncturing vessels is a concern. The wick in the needle method is still however preferred to micro-puncture as micro-puncture will not work in the tumors of living mice as the rise and fall of the tissue from their respiration prevent the use of glass capillary needles. Micro-puncture will however work on immobilized tissue, *i.e.*, mouse or rat skin or foot pads. Measuring PIF in a living animal is necessary, due to need for circulation as PIF is directly correlated to Pc.

When circulation is abrupted, PIF is reduced, but not to zero, due to the persisting oncotic pressure [234].

vii. Hydroxyproline determination

Carcinomas tissues were hydrolyzed in 6M HCl for 4 hours at 120°C at a pressure of 2 atmospheres. Hydroxyproline content in the hydrolysates was determined essentially as described [235]. Collagen standards of known concentration were also hydrolyzed to verify complete hydrolyzation of the samples, and for an additional verification of the hydroxyproline standards.

When hydroxyproline measurements were compared to image analysis of traditional collagen staining, Sirus Red/Tricrome, the consideration of the types of collagen may be vital, as these staining methods work on intact fibers, whereas hydroxyproline assay represents total collagen, including all forms present, i.e. non-fibrillar and degraded/unformed collagen.

vii. Illumina

mRNA expression analysis was performed on RNA extracted from free floating 3 dimensional collagen gels containing tumor cells or co-cultures of tumor cells with fibroblast. Cells were allowed to normalized and contract their gels for 48 hours before being harvested for RNA extraction. One major shortcoming here is that the 3D culture systems are known to differ from their 2-dimensional counterparts. When cells are grown on stiff plastic substrates, they become generally activated, fibroblast grown in relaxed (free floating) collagen gels are known to revert to a more physiological inactivated state [236, 237]. This can potentially lead to differences when comparing, for instance, TGF-β activation in 2D verses 3D systems. It is however beneficial to be able to use a single cell type, to better understand activation in and between stromal and malignant cells, which is not possible in an *in vivo* situation. One limitation of this study was the lack of a fibroblast expression base line; all differences must be understood from the context of relative expression between the RNA expressed in malignant or co-cultured gels. This also points to a shortcoming of the platform as batch effects prevent the direct comparison between multiple chips. Determining the functional background of the assay can also lead to the detection of signals which should otherwise be removed from analysis.

Further drawbacks to the analyses, all samples with a q value above five, to minimize the potential for false discovery, and as well as any genes which did not differ significantly between the malignant cell types by more than five times were removed. A shortcoming of this type of analysis includes not having a fibroblast gene expression baseline. The media type is also something which should be considered, cells were conditioned to the same media before use, and however the presence of serum in their expansion and the lack of serum during the experiment itself may both pose as factors which introduce variation into the system. Many of the genes which are not caught by the analysis may in fact have a biologically significant effect. Any findings should therefore be verified with multiple other methods, preferably from in vivo material as well. The differences can be seen in the supplemental tables of paper III.

XIII Results of papers I-V

Results of paper I

The tyrosine kinase inhibitor Imatinib augments extracellular fluid exchange and reduces average collagen fibril diameter in experimental carcinoma.

STI571: In paper I we show that collagen structure, specifically fibril diameter, is affected by treatment with STI571, which leads to an increased tumor permeability. We show a reduction of collagen fibril diameter, interstitial fluid pressure, and an increases extracellular volume in connection with STI571 treatment.

Results of paper II

Imatinib increases oxygen delivery in extracellular matrix-rich but not in matrix-poor experimental carcinoma

In paper II we show that STI571, in various tumor models of colonic carcinoma, has an effect on the PIF and pO2, relative to collagen content as measured by hydroxyproline. Where it varied from 1 .21 ± 0.21 (n = 4) and 0.725 ± 0.496 (n = 4) mg/g by wet weight in KAT-4 and CT26 derived carcinoma tissue, respectively. No collagen was detectable in B16BB derived tissue. We also noted an effect on the number of vessels per area, defined by CD31 staining in KAT-4 tumors but not CT26, nor an effect on other vascular parameters. These data suggest, given that STI571 does not affect oxygen consumption, that the blood flow is increased in tissues rich in ECM compared to ECM poor models. This further suggests that tumor physiological changes induced by STI571 are dependent on the modulation of the ECM, specifically the collagen network.

Results of paper III

Inhibition of integrin $\alpha_V\beta_6$ changes fibril thickness of stromal collagen in experimental carcinomas.

TGF-β latent protein complex activation, with binding via $\alpha_V\beta_6$ and possibly other matrix components, is inhibited by preventing the action of $\alpha_V\beta_6$. In paper II we show that not all cells which express $\alpha_V\beta_6$ are capable of activating latent TGF-β. We see that like TGF-β itself, the inhibited of this integrin reduces desmoplasmic response *in vivo*. Although it is known that latent TGF-β can be activated by other means, we see the same basic response after inhibition of TGF-β, as with inhibition of $\alpha_V\beta_6$ in our experimental carcinoma models. Investigation of the role of the integrin $\alpha_V\beta_5$ is underway to potentially explain differences observed between models.

We further show that the $\alpha_V\beta_6$ integrin has an indirect role in establishing the structure of stromal collagen in carcinomas. This structural difference is strikingly similar to other work shown in paper I. This may implicate a link between the activation and/or signaling in a hypothetical $\alpha_V\beta_6$, TGF-β, PDGF axis.

Results of paper IV

Fibromodulin deficiency reduces collagen architecture but not glycosaminoglycan content.

Here we show a similar collagen structural phenotype to tumors with inhibited TGF-β and $\alpha_V\beta_6$ as well as those treated with STI571. No differences in the type or concentrations of GAGs were observed in response to Fmod deficiency.

Results of paper V

Mice lacking NCF1 exhibit reduced growth of implanted melanoma and carcinoma tumors.

NCF1 removal resulted in an overall decrease in the tumorigenesis of transplanted cells. In spontaneously formed tumors no difference was observed. The tumors which did developed in NCF1 deficient mice did not differ significantly from their wild type counterparts in the areas tested except in the expression of IL-4.

VI Discussion

i. On Carcinoma

The importance of a steady concentration of GAGs as well as the increased tumor volume after treatments of STI571, together with the reduced fibril diameter suggests that the regulatory mechanism for the fluid exchange between the intistium and blood is most likely collagen. Collagen architecture is thus suggested to be a contributor to the barrier function in carcinoma. We, together with others argue that PIF, believed to be a measurement of this barrier, is generated by stromal characteristics, which are causal in the interference with fluid and solute uptake in carcinoma tissue. The correlation between collagen fibril density, fibril structure, network architecture, and PIF in experimental carcinomas can be considered as established [98, 166-168]. Here we have shown that the fibril diameter of collagen fibers is affected by treatment with STI571, TGF-β and integrin αvβ6 inhibition as well as Fmod deficiency.

The interconnection between Fmod deficiency, TGF-β inhibition and treatment with STI571 remain to be fully characterized. It appears as though TGF-B may be central in all three models. This may provide a means of affecting elements of the ECM without the overarching effects of total TGF-β inhibition, *i.e.*, direct FMOD targeting or a combination therapy utilizing the matrix altering effects of STI571 to enhance the delivery of therapeutic compounds.

ii. Summary of the interplay

Inflammation: Inflammatory cytokines, PDGF, TGF-β among others.

The question of an interconnected PDGF and TGF-β interplay in matrix production and modification is a possibility with the knowledge that both have downstream ABL activation. ABL is a known target of STI571, and both TGF-β and PDGF pathways share ABL activation down-stream of their cognate receptors [238, 239]. ABL has been shown to act upstream of early growth response factor 1 (Egr-1) which is a known matrix regulator and is reported to have an active role in

fibrotic processes [240, 241]. Integrin mediated forces; cellular signaling and the maintenance of ECM interactions may additionally influence already complex interactions. Similar effects on PIF have been seen in both treatments with STI571 and with direct PDGF inhibition, using an aptamer [5], when compared to TGF-β inhibition. This begs the question to whether or not PDGF and TGF-β share a common activation profile. TGF-β may have a circular relationship with PDGF each stimulating the production of the other in a positive feedback loop. It was suggested by work done at the Ludwig Institute in Uppsala that PDGF can activate the TGF-β signaling, it has also been reported that TGF-β increases a PDGF response[242]. Further questions of the effects of VEGF, IL-1 and other growth factors and cytokines, seen to be expressed by either tumors or malignant cells remain. One example is fibroblast growth factor binding protein (FGFBP) as found in paper III to be a potential candidates for further investigation. FGFBP plays a role in ECM regulation through the release of sequestered FGF-1 and -2 activating fibroblasts [243]. We have also observed, in unpublished work, that malignant cells were able counter some of the effects of IL-1 in fibroblast mediated 3-dimensional collagen gel contraction.

iii. On TGF-B activation

We focused on the TGF -β1 and –β3 isoforms as they the –β2 isoform is not integrin mediated. TGF- β2 interacts only weakly with TβRII. Additionally Fc:TβRII, the recombinant extracellular domain of TβRII fused to an IgG Fc carrier, specific to TGF-β1 and -β3, was sufficient to effect collagen fibril formation, and abrogated PAI-1 activation, indicating no evidence for the role of TGF-β2 in the systems studied.

We have also seen that the mRNA expression of the different LTBP proteins differs within but not between the tumor cell types. One potential line of inquiry would be to establish if the different LTBP isoforms are present in different concentrations in tumors, and if there would be a difference in the substrate proteins to which they are known to be incorporated.

One observation from the mRNA expression results to be an INFγ like response signature in naïve Capan-2 cells. Along with TGF-β, INFγ are known activators of macrophages, which are in turn are known to activate TGF-β. This may present a reasonable explanation for the delayed response to $\alpha_v\beta_6$ inhibition in Capan-2 derived tumors, compared to direct TGF-β receptor inhibition, as macrophages can enzymatically activate latent TGF-β [148].

TGF-β associated gene table								
Gene name	Gene symbol	T-test (p)	Fold Change	q-value (%)	**KAT-4**	*SD*	**Capan-2**	*SD*
	GCG	0,28	0,97	27,70	**6,61**	*0,02*	**6,57**	*0,05*
Latent Transforming Growth Factor Beta Binding Proteins 1 – 4								
	LTBP1	0,07	1,13	11,08	**6,54**	*0,09*	**6,71**	*0,07*
	LTBP2	0,18	1,10	18,59	**6,68**	*0,05*	**6,82**	*0,12*
	LTBP3	0,68	1,21	54,19	**8,73**	*0,51*	**8,96**	*0,72*
	LTBP4	0,52	1,08	46,32	**7,56**	*0,03*	**7,66**	*0,22*

log_2 mRNA expression of LTBP isoforms with glucagon (GCG) illustrating background expression level

iv. On STI571 effects

One of our main findings and goals is the modification of matrix components to effectively increase, or open, blood flow in tumors. This was best illustrated in our paper (Paper I) which delineated the effects of STI571 on collagen superstructure and tumor fluid perfusion in spite of the lack of apparent vascular effects. This also indicated an increase in blood flow as oxygen levels were increased after treatment (paper II) [143, 216].

High PIF may itself affect blood flow to tumors, via constriction of the vessels, or through increased tension in the tissue. We suspect that the blood vessels are constricted in these tumor models. We see no increase in vascular leakage, or number of vascular structures after treatment with STI571.

The number of non-vascular associated NG2 cells, was changed after treatment with STI571. Whether this is the affecting element on the observed collagen fibril diameter decrease or a result of decreased migration or pericyte differentiation, is unknown. In paper II we show that the effects seen on fibrous tumors, such as KAT-4 and CT26 were not mirrored in tumors that did not possess as detectable collagen matrix. This change in the collagen structure, did not affect PIF in the more collagen rich Capan-2 derived tumors (see paper III). This may indicate that there is a threshold or a window of fibrosity, in which at certain levels a decrease will affect tumor perfusability. This may be similar to reinforcement in building, or other structures, if the diameter of a reinforcement is decreased its reinforcement capacity is also decreased, but when the total number of

reinforcements are such that the total structural effect is greater than the difference observable by the decrease in the diameter of any number of individual supports, no difference will be observed.

PDGF is a well-known inducer of cellular contraction. Contraction is vital for the integrin mediated activation of the LTGFB complex which is anchored to ECM protiens, *i.e.* vitronectin, fibronectin and fibrillin [24]. This raises the question whether PDGF may be indirectly responsible for the activation of TGF-β.

There is a possibility that STI571 has its effect on the TGF-β pathway, not only on contraction but via direct inhibition as we see that when administered *in vitro* TGF-β signaling, measured by PAI-1, is completely ablated (see XIV).

v. On FMOD

One potential point of discrepancy in comparing the KAT-4 and OOC38 tumor models is the noted difference in in the amount of GAGs. OOC38 tumors have less than ½ the amount of GAGs than do KAT-4 derived tumors , from around 1000 in KAT-4 derived tumors to about 350 ng/mg by dry weight in OOC38 tumors. This may explain limited pressure differences observed in various OOC38 derived tumors, in unpublished pilot studies, when compared to other tumor models.

TGF-β is generally thought to associate with the matrix via transglutamination LTBP to fibulin or fibrillin. It has also been reported to be sequestered by proteoglycans binding tighter with higher sulfation to HA, HS, CS/DS and the proteoglycan core proteins, greater [244-246]. Previous studies have shown that TGF-β bind decorin in the nM range independent of GAGs. How FMOD affects TGF-β concentration and availability is not clear. FMOD regulation of the ECM by TGF-β sequestration may be a potential mechanism additionally linking the processes investigated in this thesis.

vi. On NCF-1

NCF1 deficiency decreased acceptance towards tumor establishment, but did not otherwise effect progression of established tumors [154].

NCF1 deficiencies in the B16 melanoma cell lead to an increase in the cytokine IL-4, but not the other measured inflammatory cytokines, *e.g.,* IL-6. IL-4 is a cytokine which is suggested regulate various immune cells including T-cells and macrophages. An overexpression is also associated with an overactive immune

response. IL-4 is also reported to be an inducer of cellular proliferation and ECM production. It has even been suggested to be listed as a "fibrogenic cytokine" as Fibroblasts have been shown to increase ECM gene expression in response to IL-4 stimulation [247-250]. Additionally it has been reported that ROS are involved in the regulation of TGF-β [156]. This may warrant additional investigation into the effects of NCF-1 deficiency on ECM composition.

XIV Future perspectives

The observed increase of IL-4 in NCF1 deficient tumors and the potential for IL-4 induced ECM production may be a link between the various observed results in papers I to IV. Further study to determine the IL-4 expression levels in treated and untreated tumors may be warranted. Additionally the effect on infiltrating immune cells may be helpful in understanding and developing potential therapeutic methods for the treatment of carcinoma.

One observation from the various tumor studies, done in this thesis, is that the tumors where we see the greatest effects on the ECM are often the same tumors which have been observed the largest other effects . A confounding factor in determining effects has been the variation within groups and the statistical power of the group sizes. One way to overcome these issues would be to expand our methodology, *i.e.*, the use of KO animals and to increase group sizes.

The Barrier function remains an issue to current and novel treatments. Using a combination therapy may prove to increase efficacy in various treatment modalities. In addition to the published and submitted works it has been observed that there appears to be a large deposition of fibrin in tumors, which correlates spatially to the collagen ECM in imunohistochemical stainings of tumors. Interestingly when treated with STI571 and increase in fibrin was observed in tumor sections. Some preliminary studies were done to establish if this fibrin could be removed *in vivo* and indeed with the intravenous administration of tissue plasminogen activator (TPA) it could. The question remains however to fibrin´s role in and its potential effect on the barrier function tumors.

One interesting, and unpublished findings was that STI571, completely abolished the PAI-1 response in the TGF-β assay, indicating either an effect on the receptor or on the signaling cascade. We know that the Rho/ROCK are important for actin turnover, and that when inhibited integrins forces cannot be mediated, It is uncertain how we are able to have a PAI-1 signal when directly adding TGF-β to our assay as it has been reported that TGF-β has been is incapable of activating its primary promotor, PAI-1, when the Rho/ROCK pathway is absent [251]. ROCK inhibition did not however make a significant difference, this may indicate that ABL is not the effected element in the STI571 signal inhibition, as ABL is reportedly dependent on the Rho/ROCk pathway [252].

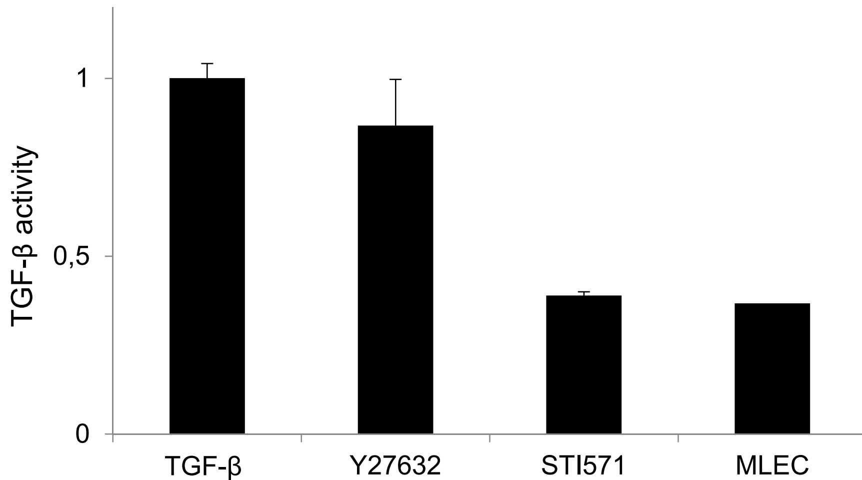

Figure 6
TGF-β activity measured by luciferase production in the MLEC assay under the PAI-1 promotor after administration and treatment with 10µM STI571 and 10µM ROCK inhibitor (Y27632) relative to TGF-β treated and untreated MLEC controls. TGF-β TGF-β n=6, STI571 $p<0.001$(n=3), Y27632 $p=0.06$ (n=5), MLEC (n=5).

The opportunity to increase the effectivity of these substances with the modulation of matrix structure, allowing for better treatment efficacy and shorter treatment times may be paramount in the treatment of especially highly fibrotic tumors.

XV Popular scientific summary

Cancer is the second leading cause of death in the world. Nearly one in every two individuals will be diagnosed with cancer in their lifetimes. The majority of cancer types are carcinomas affecting the breast, lung, prostate and colon. One common problem to the treatment of many tumors is that it is difficult to reach throughout the tumors with current treatments. This problem is in part due to the high fibrosity, and interstitial fluid pressure which are present in many tumors. This pathological environment arises from both cancer malignant and stromal cells which make up the tumors. The barrier to treatment is affected by the matrix and supports the structure of the tumors. Collagen is a main structural component of this extracellular matrix (ECM). Here we strive to affect and understand the components of the matrix which are incurring the pathological environment.

To best study tumor ECM its effects and modulators we have utilized mice as a model for the human disease. These studies have allowed us to show that a decrease in the diameter of collagen fibers is associated with a decrease in the interstitial fluid pressure. This type of change has previously been shown to have a positive effect on treatment. We have used a conventionally available drug and shown that the effects are related to a change in the structure of collagen. Additionally collagen structural changes can be induced through the inhibition or removal of factors involved in the process of matrix formation and maintenance.

Tumors, like all tissue require oxygen to survive and grow. The network of blood vessels in tumors however is not normal. Normalizing blood flow and vessels, has been shown in some instances to decrease the pathological nature of tumors and affect treatment outcome. Our studies have also indicated that through modulation of the ECM we can increase blood flow within the tumors, which may both provide better drug penetration and potential for treatment.

Just as important as the effectors of the ECM we show that certain components in tumors are unchanged, yielding functional information about the interplay of ECM components. There currently exist a number of substances which could prove effective for treating cancer if they were able to reach their targets. In better understanding the mechanism of the formation and control of the ECM and the associated barrier to treatment seen in tumors, we hope to be able create a more targeted means of therapy. This research helps to do just that.

There is a limit to knowledge and understanding, no one person is capable of possessing the whole of human knowledge. Much of what we believe to be true will eventually be shown to be false.

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