Regulation of Hyaluronan Synthesis and Signaling via CD44 in Cancer

MERIMA MEHIC
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Abstract

Hyaluronan is a ubiquitous glycosaminoglycan which is an important constituent of the extracellular matrix (ECM). In addition to organizing the extracellular matrix and regulating tissue homeostasis, hyaluronan, by binding to its main cell surface receptor CD44, is involved in intracellular signaling pathways regulating major cellular processes during development, wound healing, inflammation and cancer. Accumulation of hyaluronan in cancer promotes progression of the disease and correlates with poor prognosis. This thesis focuses on the regulation of hyaluronan synthesis and its signaling in normal and cancer cells.

Cancer cells in solid tumors are surrounded by stroma, which has an essential role in the growth and metastasis of tumors. Prominent members of the tumor stroma are fibroblasts, which synthesize ECM components, such as hyaluronan, and secrete growth factors, and activate intracellular signaling pathways. We demonstrate a cross-talk between the receptors for platelet-derived growth factor BB (PDGF-BB), transforming growth factor β (TGFβ) and CD44 in dermal fibroblasts. We found that PDGF-BB can activate the Smad signaling pathway downstream of the TGFβ receptor I (TβRI), and that PDGF-BB-induced migration depends on TβRI. CD44 forms a ternary complex with the receptors for PDGF-BB and TGFβ, and negatively regulates their signaling. Furthermore, we demonstrate that TGFβ stimulation of mammary epithelial cells transcriptionally upregulates hyaluronan synthase 2 (HAS2), which is essential for TGFβ-induced epithelial-mesenchymal transition (EMT); in this process, polarized epithelial cells adapt a mesenchymal phenotype which facilitates migration and invasion.

HAS2 protein activity and stability is regulated by posttranslational modifications, including ubiquitination. We investigated the ubiquitination of HAS2 in aggressive breast cancer cells, whose metastasizing capability depends on HAS2-synthesized hyaluronan. We identified two deubiquitinating enzymes, USP4 and USP17, which target HAS2 and affect its activity and stability.

In summary, these studies increase the knowledge about the regulation of hyaluronan production and its role in cancer progression.

Keywords: Hyaluronan, CD44, TGFβ, PDGF-BB, cancer, signaling, hyaluronan synthase, epithelial-mesenchymal transition

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Za tatu
(To my father)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


* Indicates that the authors contributed equally to the work

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ARF-BP1</td>
<td>ARF-binding protein 1</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>CSC</td>
<td>cancer stem cell</td>
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<td>DUB</td>
<td>deubiquitinating enzyme</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
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<tr>
<td>ERM</td>
<td>ezrin, radixin, moesin</td>
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<tr>
<td>FANCD2</td>
<td>Fanconi anemia group D2</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>GlcNAc</td>
<td>N-Acetylglucosamine</td>
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<tr>
<td>GlcUA</td>
<td>D-glucuronic acid</td>
</tr>
<tr>
<td>HAS</td>
<td>hyaluronan synthase</td>
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<tr>
<td>HBP</td>
<td>hexosamine biosynthetic pathway</td>
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<tr>
<td>HMG1A2</td>
<td>high-mobility group AT-hook 2</td>
</tr>
<tr>
<td>IKKγ</td>
<td>inhibitor of nuclear factor kappa-B kinase subunit γ</td>
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<tr>
<td>LAP</td>
<td>latency associated protein</td>
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<tr>
<td>LTBP</td>
<td>latent TGFβ binding protein</td>
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<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
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<tr>
<td>MDM2</td>
<td>mouse double minute 2 homolog</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>mono-Ub</td>
<td>monoubiquitination</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating nuclear cell antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PDGFRβ</td>
<td>platelet-derived growth factor receptor β</td>
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<tr>
<td>PI3-kinase</td>
<td>phosphatidylinositol 3’kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol-12 myristate 13-acetate</td>
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<tr>
<td>poly-Ub</td>
<td>polyubiquitination</td>
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<tr>
<td>RCE1</td>
<td>ras converting enzyme 1</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>R-Smad</td>
<td>receptor Smad</td>
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<td>Abbreviation</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<tr>
<td>SARA</td>
<td>smad anchor for receptor activation</td>
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<tr>
<td>SH2</td>
<td>Src homology 2</td>
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<tr>
<td>STAMBP</td>
<td>STAM-binding protein</td>
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<tr>
<td>TAF</td>
<td>tumor associated fibroblast</td>
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<tr>
<td>TAM</td>
<td>tumor associated macrophage</td>
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<tr>
<td>TF</td>
<td>transcription factor</td>
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<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
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<tr>
<td>TβRI</td>
<td>transforming growth factor β receptor I</td>
</tr>
<tr>
<td>UNP</td>
<td>ubiquitous nuclear protein</td>
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<tr>
<td>USP</td>
<td>ubiquitin-specific protease</td>
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Introduction

The human body is made up of cells, which are small compartments filled with proteins, and a nucleus that contains all the information needed to make a human being. Cells are surrounded by a complex network of proteins and sugars, called the extracellular matrix (ECM), to which they attach and depend upon for many processes. A prominent member of the ECM is hyaluronan to which cells bind through a cell surface receptor called CD44.

Like ourselves, the cells can grow, reproduce, process information, talk to each other and carry out an amazing number of chemical reactions. Several cells organize into collaborative compartments to form tissues that organize into bigger units and make organs. Like cities, these compartments are well structured so that every cell is specialized to carry out its duties and collaborates with other cells to build a coordinated organism more capable than its single units. To manage this, cells communicate with neighboring cells and with distant cells by sending out proteins, such as growth factors, and ECM components. For this society to function, every cell needs to know when to divide and propagate, when to specialize and do its duties, how to respect the territorial boundaries, and when to die for the sake of the whole organism. There is information about these rules in the center of each cell, the nucleus, and during cell division, the information, in the form of deoxyribonucleic acid (DNA), is copied and every daughter cell inherits the same information. A mistake introduced during the replication of the information is called a mutation. In a complex multicellular organism, cell division occurs about $10^{16}$ times during a life span; this can give rise to many mutations. Most mutations are repaired by the elaborate control systems our cells posses, and not all mutations are bad. In essence, evolution is brought forward through mutations that rendered upon the organism new and more favorable traits, which make it more successful to survive and propagate.

Sometimes, a set of mutations occur in a single cell which endows upon the cell the possibility to propagate despite the control mechanisms, an advantage which is then favored by natural selection. This is what gives rise to cancer; an individual cell begins to propagate without control which destroys the cellular society. This is reinforced when cancer cells spread to distant sites by metastasis. But cancer cells cannot do it alone; their neighboring cells and environment, the soil, help them in the process. This thesis will
discuss components of the tumor microenvironment, the soil, which aid in tumor progression. To begin with, we will describe a cross-talk between signaling proteins, which cooperate and deliver an altered cell behavior. Then we will elucidate how signaling proteins lead to changes in the micro-environment, which ultimately aid in metastasis and tumor progression. Finally, we will decipher how cancer cells themselves produce and maintain components in the tumor microenvironment.

Cancer

Cancer is a disease characterized by uncontrolled cell growth, with the potential of invasion of surrounding tissue and spreading to distant sites in the body. 8.2 million people die each year of cancer, which is an estimated 13% of all deaths worldwide (World Health Organization (WHO) http://www.who.int/cancer/en/). Solid tumors derived from epithelial cells, called carcinomas, are the most prevalent. The five most common cancers in men are (in order of appearance): lung, prostate, colorectal, stomach and liver; the six most common cancers in women are: breast, colorectal, lung, uterine, cervix and stomach. Breast cancer is the second most common cancer in the world, and ranks as the fifth cause of death from cancer overall (WHO).

Tissue homeostasis is maintained by intricate genetic control systems that govern the release of growth promoting and growth inhibiting signals. Cancer results from uncontrolled cell growth due to failure of the mechanisms that normally control the growth, survival and proliferation of cells. As normal cells evolve progressively to become tumor cells, they acquire a set of capabilities, which enable them to escape the control mechanisms that govern normal tissue homeostasis [1]. The most fundamental capabilities are sustained proliferative signaling and escaping growth inhibitory signaling; furthermore, cancer cells are resistant to cell death signals, can replicate indefinitely, and stimulate the outgrowth of blood vessels which fuel the growing tumor. Two emerging hallmarks, which have gained much attention recently, are deregulated energy metabolism and evading immune destruction [2]. In the presence of oxygen, normal cells process glucose first by glycolysis to pyruvate, which then generates a lot of ATP in the mitochondria through oxidative phosphorylation. Paradoxically, cancer cells switch their energy metabolism towards only glycolysis even in an oxygen-rich environment, generating relatively little ATP compared to oxidative phosphorylation. The increased glycolysis allows the glycolytic intermediates to enter alternative biosynthetic pathways, such as the hexose biosynthetic pathway (HBP), and generate building blocks, such as amino acids and nucleosides, needed for cell growth [2]. Enabling capabilities which further
enhance the tumorigenic process are genome instability and inflammation [2]. Finally, the one trait which makes a tumor malignant and ultimately leads to death is the ability to invade surrounding tissue, enter the bloodstream and spread to distant sites to form new tumors, a process called metastasis [1, 3].

The malfunction of cellular regulation mechanisms that give rise to cancer is due to genetic damage, which results in mutations of proto-oncogenes, tumor suppressor genes and caretaker genes. The genetic changes that underlie oncogenesis are a result of errors in the replication and repair of genes and exposure to environmental insults, so called carcinogens. Proto-oncogenes normally promote cell growth, and are changed into oncogenes by mutations that make them overactive, thus promoting excess growth. Tumor-suppressor genes lead to growth inhibiting signals, and are inactivated by mutations in cancer, thus allowing uncontrolled cell division. Caretaker genes are genes that protect the integrity of the genome, such as DNA repair mechanisms. Mutations in these genes result in a higher rate of mutations, a genome instability which is a characteristic of cancer cells.

Tumors are clones of a single cell, which by accumulating enough mutations over time, progressively become dangerous. Thus cancer takes time to develop and usually arises late in life. It occurs most frequently after the age of reproduction and is thus not evolutionary selected against; together with an increasing life time, this makes cancer more and more common. Cancer-causing mutations occur in the somatic cells, cells of the body, and are not passed on to the next generation. However, occasionally mutations in the germ-line can be inherited and cause increased susceptibility to cancer.

All solid tumors are composed of two components: the cancer cells and the stroma or microenvironment which they produce, and which is essential for their growth [4]. The stroma is composed of blood vessels, cells of the immune system and fibroblasts, and the ECM.

The extracellular matrix and its role in cancer

“The best work in the pathology of cancer is done by those studying the nature of the ‘seed’. They are like scientific botanists; and he who turns over the records of cases of cancer is only a ploughman, but his observations of the properties of the ‘soil’ may also be useful” [5, 6].

The extracellular matrix is a complex three-dimensional network of proteins and polysaccharides secreted by cells into the space between them, regulating tissue integrity and homeostasis. The ECM holds tissues together and is
tailored by combinations of different components to the specific function of
the tissue: e.g. strength in a tendon or tooth, or cushioning in cartilage. Cells
attach to components of the ECM, such as collagen, laminin, fibronectin and
hyaluronan, through the binding of cell adhesion receptors in the cell mem-
brane, thus connecting the interior and exterior of cells. The ECM controls
the availability of cytokines and growth factors, such as platelet derived
growth factor (PDGF) and transforming growth factor β (TGFβ), which can
bind to cell surface receptors and alter cell fate. In addition, the matrix pro-
vides a surface through or on which cells can move, for example during em-
bryonic development or tissue assembly. This bidirectional flow of infor-
mation is essential to many biological processes such as proliferation, migra-
differentiation and survival. Prominent members of the microenviron-
ment are fibroblasts, which form the structural foundation supporting normal
epithelial tissues. The physiological roles of fibroblasts include secretion of
ECM components and growth factors, regulation of tissue homeostasis and
differentiation of surrounding cells, and wound healing [7].

Changes in the ECM, which is constantly remodeled, degraded and rebuilt,
can provide environmental cues and modulate the behavior and interactions
of cells. Thus defects that alter the interactions and flow of information be-
tween cells and their ECM inevitably lead to human disease. Tumors are
characterized by a stiff ECM, as a result of increased deposition and altered
organization of the ECM components [8].

Recent research has revealed that the biology of tumors can no longer be
understood simply by enumerating the traits of the cancer cells, but instead
must include the contributions of the “tumor microenvironment” to
tumorigenesis [2]. One must take into account the individual specialized cell
types within a tumor, the signaling pathways controlling their function and
the tumor microenvironment that they build. The signals which instruct the
entry of cells into and their progression through the cell cycle are most often
conveyed by growth factors. Cell cycle progression requires the adhesion of
cells to the ECM, which permits growth factor signaling. Epithelial cancer
cells acquire the ability to secrete their own ECM, which helps them to
evade growth suppression and survive in hostile environments. Cancer cells
also secrete growth factors such as PDGF and TGFβ into their microenvi-
ronment which stimulates the recruitment and activation of fibroblasts [7].
Activated fibroblasts respond by secreting more growth factors, cytokines
and ECM components, such as hyaluronan, that act back on cancer cells,
promoting their invasive properties. Adhesion receptors on cancer cells, such
as CD44, interact with ECM molecules and fine-tune growth factor signal-
ing, altering their malignant phenotype [9, 10]. Thus, elucidating the cross-
talk between growth factor receptors and adhesion molecules is crucial for
understanding the complex and diverse biology of cancer.
Growth factors

Growth factors are extracellular signaling molecules, peptides or hormones, which by binding to specific cell surface receptors initiate a signaling cascade that results in different cellular responses such as proliferation, migration or apoptosis. Important members of the growth factor family are epidermal growth factor (EGF), TGFβ and PDGF. These peptides act by binding to the extracellular part of a kinase receptor which leads to activation and phosphorylation of the cytosolic part of the receptor. This recruits and activates cytosolic signaling proteins which translocate to the nucleus and determine the precise pattern of gene expression.

A typical mammalian cell expresses more than 100 different types of cell-surface receptors, many of which activate the same or similar signaling pathways; this requires an extensive regulatory mechanism and tight control over the signals’ effect on cell behavior. The response of a signaling molecule does not just depend upon the receptor to which it binds, but also upon the cellular context. There is an extensive cross-talk between different growth factor receptors (GFRs) and cell adhesion receptors bound to ECM components [11]. The outcome that determines cell fate depends upon the cell’s repertoire of receptors and the signals it receives from the microenvironment.

Platelet-derived growth factor and its receptors

PDGF isoforms constitute a family of growth factors which can potently stimulate proliferation, migration and survival of connective tissue cells, such as fibroblasts and smooth muscle cells. They also have important functions during embryogenesis and wound healing [12]. Originally identified as a constituent of whole blood serum, PDGF was purified from human platelets in 1979 [13, 14]. The PDGF family consists of four related, cationic polypeptide chains: A, B, C and D, encoded by four different genes [15]. The active molecules are disulfide-linked homo- or heterodimers.

The PDGF isoforms exert their effects on cells by binding to and dimerizing two structurally related protein tyrosine kinase receptors, the α- (170 kDa) and β- (180 kDa) receptors (PDGFRα and PDGFRβ, respectively) [12, 16, 17]. Fibroblasts and smooth muscle cells express higher levels of β- than α-receptors. Some cells, like human platelets, express only the α-receptor, whereas others, such as mouse capillary endothelial cells, express only β-receptors [12]. PDGF isoforms interact with the receptors with different specificities causing the formation of homo- or heterodimeric receptor complexes. PDGF-AA and PDGF-CC isoforms bind the α-receptor, forming α-
receptor homodimers whereas PDGF-DD binds the β-receptor forming β-receptor homodimers. PDGF-AB forms α-receptor homodimers or αβ – receptor heterodimers. PDGF-BB can bind both α- and β-receptors, thus forming all combinations of homo- and heterodimers [12, 18, 19]. There is genetic evidence in mice only for a few interactions: PDGF-AA and PDGF-CC via PDGFRα and PDGF-BB via PDGFRβ [18].

Upon PDGF binding, dimerization of receptors juxtaposes the intracellular parts, leading to autophosphorylation of tyrosine residues in trans between the two receptors in the complex (Fig. 1). Phosphorylation of conserved tyrosine residues in the kinase domain of the α-receptor (Tyr-849) and the β-receptor (Tyr-857) is crucial and increases the catalytic activity of the kinases. Autophosphorylation of tyrosine residues outside the kinase domain causes additional conformational changes, as well as creates docking sites for signal transduction molecules containing Src homology 2 (SH2) domains, such as phosphatidylinositol 3′kinase (PI3-kinase), phospholipase C (PLC)-γ, the Src family of tyrosine kinases, and Grb2 and other adaptors [12, 19, 20].

**PDGF signaling**

Signaling pathways activated by PDGF lead to cell growth, chemotaxis, actin reorganization and prevention of apoptosis, in a cell type- and context-dependent manner. The PI3-kinase pathway induces actin reorganization and migration through the Rho family of GTPases, and promotes survival through activation of Akt/Protein kinase B (PKB). The Erk 1/2 mitogen activated protein (MAP) kinase pathway is activated by Sos1-mediated activation of Ras and promotes cell growth, differentiation and migration. The PDGFR-activated PLC-γ generates the second messenger inositol 1,4,5-trisphosphate which mobilizes intracellular Ca^{2+} levels and diacylglycerol; together these molecules activate protein kinase C (PKC), leading to directed cell motility and proliferation [12, 19, 21, 22].

PDGF receptor signaling is negatively regulated by tyrosine phosphatases, such as SH2-containing protein tyrosine phosphatase 2 (SH-PTP2) [23] and T-cell protein tyrosine phosphatase (TC-PTP) [24]. Loss of TC-PTP leads to increased PDGFRβ phosphorylation by inducing recycling of the receptor [25]. PDGF receptor signaling can also be terminated by internalization and degradation of ligand-receptor complexes in lysosomes. The ubiquitin ligase Cbl ubiquitinates activated PDGF α- and β- receptors [26] and promotes their degradation via the proteasome pathway [12, 27, 28].

PDGF-mediated intracellular signaling is tightly linked with and regulated by the extracellular matrix (ECM). PDGF stimulates the production of ma-
Matrix molecules and is often retained in the ECM by interaction with matrix molecules such as heparan sulfate proteoglycans [12]. In particular, PDGF-BB has a potent stimulatory effect on the synthesis of the glycosaminoglycan hyaluronan, which through interaction with its cell surface receptor CD44 can modulate the response of cells to PDGF-BB signaling [9, 29-31].

Figure 1. Overview of PDGF-BB signaling pathway. P, phosphorylation; TFs, transcription factors
PDGF signaling in cancer

Many human tumors express PDGF and PDGF receptors, and autocrine PDGF signaling loops contribute to tumorigenesis by providing self-sufficiency for growth and survival signals and thus promote proliferation. In many cases, genetic aberrations of the corresponding genes, such as overexpression or gene translocation, cause uncontrolled PDGF signaling. In human glioma, the expression of PDGFRα is increased as the tumor progresses to a high grade malignant tumor [12]. In some epithelial tumors such as breast carcinoma, autocrine PDGF signaling promotes invasion and metastasis by promoting and maintaining epithelial-mesenchymal transitions (EMT) in cooperation with TGFβ [18, 32].

Paracrine PDGF signaling derived from tumor cells is involved in the recruitment and proliferation of tumor stromal cells, such as fibroblasts and pericytes. This contributes to tumor angiogenesis, which is crucial for growth and survival of tumor cells. By affecting the tumor stroma, PDGF signaling also contributes to increased interstitial tissue fluid pressure in the tumor, which limits cancer drug delivery and thus contributes to drug resistance [18].

Transforming growth factor-β and its receptors

The TGFβ family consists of 33 members, most of which are dimeric, secreted polypeptides, including bone morphogenetic proteins (BMPs), activins, growth and differentiation factors (GDFs) and Müllerian inhibiting substance (MIS) [33]. TGFβ was discovered in the 1980s as a polypeptide secreted from transformed fibroblasts with the ability to elicit transformation of normal rat kidney fibroblasts in soft agar assays [34]. TGFβ was later demonstrated to control a diverse set of cellular processes. During embryonic development, this family contributes to tissue patterning and regulation of stem cell self-renewal and differentiation [35]. The effects of TGFβ family members depend on the state of responsiveness of the target cell; TGFβ potently inhibits epithelial, endothelial and hematopoietic cell proliferation and induces their apoptosis, regulates differentiation and proliferation of mesenchymal cells and increases motility of epithelial cells [33, 36]. Three TGFβ isoforms (TGFβ1, 2 and 3) have been described in mammals and are secreted from cells as a latent complex composed of the mature TGFβ-dimer and the TGFβ propeptide (called latency associated protein, LAP); in addition the LAP molecule is often covalently bound to latent TGFβ binding protein (LTBP) [37, 38]. Latent TGFβ is inactive and linked to the extracellular matrix, creating a storage place, from where it can be liberated through proteolytic cleavage by proteases, such as elastase or ma-
trix metalloproteinases, or by interaction with integrins and matrix proteins [35, 39].

TGFβ ligands transmit signals by binding to type I and type II serine/threonine kinase receptors, that form heterotetrameric complexes in the presence of the dimeric ligand. Upon binding of the active TGFβ dimer to the type II receptor (TβRII), the type I receptor (TβRI) is recruited to the complex and phosphorylated on serine and threonine residues in the glycine-serine rich (GS) domain by TβRII. Once activated, TβRI initiates the intracellular signaling cascade by phosphorylating a family of transcription factors, the Smads. The receptor Smads (R-Smads), Smad2 and 3, upon phosphorylation by TβRI, bind to Smad 4, a common mediator Smad (co-Smad). Then the Smad-complexes translocate to the nucleus and interact with the DNA at Smad binding elements (SBE), recruiting co-activators and co-repressors of TGFβ responsive genes, regulating transcription in a cell-type- and context-dependent manner (Figure 2) [33, 35]. TGFβ can also induce signaling pathways in a Smad-independent manner. For example, TβRI can be autophosphorylated on its tyrosine residues, leading to binding and activation of the adaptor protein ShcA, which recruits Sos1/Grb2 complex and activates the Erk1/2 MAP kinase pathway [40]. Furthermore, TGFβ stimulation leads to phosphorylation of p38 MAP kinase in a TRAF6-dependent manner, independently of TβRI kinase activity [41].

The activated TGFβ receptors can be internalized via two distinct routes that determine if the outcome is signaling or receptor degradation. Internalization via clathrin-coated vesicles promotes signaling by guiding the receptors to early endosomes, which are enriched for the Smad Anchor for Receptor Activation (SARA). SARA binds and recruits Smad2 and 3 to the TβRI, facilitating their phosphorylation [42]. Internalization of TGFβ receptors via lipid rafts and caveolin-positive vesicles targets the receptor for polyubiquitination and degradation [43].

Inhibitory Smads, Smad 6 and 7, participate in negative feedback loops that may regulate the intensity or duration of TGFβ responses. Smad7 expression is increased upon TGFβ stimulation and its binding to the TβRI competitively inhibits R-Smad phosphorylation, and recruits Smurf ubiquitin ligases and phosphatases to downregulate receptor levels and function [44].
Figure 2. Overview of TGFβ signaling pathway. P, phosphorylation

TGFβ in cancer

TGFβ plays a dual role in cancer, acting as a tumor suppressor at early stages of carcinogenesis while it promotes invasiveness and metastasis at later stages. The components of the TGFβ pathway are commonly inactivated in many epithelial-derived tumors, such as Smad4 in pancreatic cancer or TβRII in hereditary colon cancer [36, 45]. However, many human cancers retain a functional signaling pathway and find ways to evade the growth inhibitory effects of TGFβ. Tumor cells often show increased expression or
production of TGFβ which correlates with poor prognosis. Through its effect on the microenvironment and the induction of epithelial-mesenchymal (EMT) transition, TGFβ promotes invasiveness and metastasis [46]. Interestingly, during TGFβ-induced EMT an autocrine PDGF signaling loop in breast epithelial cells and hepatocytes is up-regulated, which is crucial for EMT, and promotes breast and liver cancer metastasis [32, 47]. These findings indicate a close link between the signaling pathways of PDGF and TGFβ.

**TGFβ-induced EMT**

During embryonic development, cells of epithelial character can transit to a mesenchymal, migratory state and travel to new sites to form tissues. This shift towards the mesenchymal state, which modifies the adhesion molecules expressed by the cell giving it a more invasive and migratory phenotype, is called epithelial-mesenchymal transition (EMT). The reverse process, mesenchymal-epithelial transition (MET), allows the mesenchymal cells to transform back to epithelial cells at a new embryonic site, restoring apicobasal polarity and cell-cell adhesions. This process is also important in adults during wound healing, and is implicated in the process of metastasis and invasion of tumors [48].

Cellular changes associated with EMT include loss of adherens- and tight-junction markers, such as E-cadherin, ZO-1 and occludin, loss of apical-basal polarity, and induction of mesenchymal markers N-cadherin, vimentin and fibronectin (Figure 3) [49]. These changes are accompanied by an induction of ECM components which favors a migratory and invasive phenotype.

Figure 3. Epithelial cells progressively lose epithelial characteristics and go through intermediate EMT phenotypes before adopting a full mesenchymal phenotype.
TGFβ is a potent inducer of EMT due to its ability to induce secretion of many other cytokines, growth factors and chemokines, as well as to ECM components. The different signaling pathways cross-talk and cooperate in establishing and maintaining a set of transcription factors (TFs) that drive the EMT gene expression program, leading to the transdifferentiation [50]. TGFβ-signaling, through both Smad-dependent and Smad-independent pathways, leads to the upregulation of EMT transcription factors (EMT-TFs), such as the Snail family of zinc finger proteins (Snail1, 2, 3), the ZEB family of zinc and homeodomain proteins (ZEB1, 2) and the Twist family of basic helix-loop-helix proteins. Additionally, TGFβ upregulates other TFs, such as c-Jun and c-Fos, chromatin remodeling factors, such as HMG2, micro-RNAs (miRNAs) of the miR-200 family, and long non-coding RNAs (lncRNAs). This transcriptional and translational re-programming leads to the repression of epithelial genes and induction of mesenchymal genes [50].

TGFβ is a potent inducer of EMT both during normal conditions, such as development and tissue repair, and in pathologies such as cancer. For example, during heart valve formation, TGFβ2 activates TGFβRIII to initiate EMT, and TGFβ3-activated TGFβRII promotes invasion into the cardiac cushion [48]. TGFβ also drives EMT in normal as well as transformed cells in culture, such as the normal mouse mammary epithelial cells NMuMG and the aggressive breast cancer cells MDA-MB-231 [51-53].

The dissemination of carcinoma cells from the primary tumor to distant organs and seeding of a secondary tumor have been suggested to rely on EMT (Figure 4). Epithelial cancer cells need to lose their cell-cell attachments, degrade the local microenvironment and the basement membrane, in order to reach the blood vessels and intravasate. They are then carried by the blood stream to distant sites, where they extravasate and colonize the new tissue to form macrometastases. It is proposed that a small population of cancer cells in the invasive front of a growing tumor show an EMT profile, while the remaining bulk of tumor cells remain epithelial. This demonstrates the heterogeneous nature of tumors, with tumor cells eliciting a spectrum of phenotypes from epithelial, to intermediate and full EMT states [54]. Circulating cancer cells show an intermediate EMT phenotype, expressing both epithelial and mesenchymal markers; upon extravasation from the blood stream, mesenchymal cancer cells need to revert back to an epithelial phenotype to colonize a new site.
Figure 4. Invasion and metastasis of cancer cells relies on EMT. The multi-step process of tumor invasion and metastasis begins with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels. At distant sites, cancer cells escape from the lumina of vessels and extravasate into the parenchyma, undergo MET, and form secondary tumors. Tumor-associated macrophages (TAMs) and tumor-associated fibroblasts (TAFs) in the stroma facilitate tumor growth, invasion and migration by secreting growth factors, proteolytic enzymes, angiogenic factors, and immune-suppressive factors.

The EMT process has been implicated in the acquisition of cells with stem-like properties, called cancer stem cells (CSCs). These cells are characterized by pluripotency and self-renewal capabilities, and are suggested to drive tumor progression by generation of heterogeneous cancer cell lineages. Furthermore, EMT confers resistance to cell death and senescence as well as to cancer therapy [48].
Hyaluronan

Since the discovery of hyaluronan in 1934 by Meyer and colleagues [55], the knowledge, applications and medical significance of this glycosaminoglycan has never ceased to grow. The name was derived from hyaloid, vitreous humor from which it was isolated. Hyaluronan is an unbranched, negatively charged and linear glycosaminoglycan (GAG), composed of repeating disaccharide units of \((\beta1-3)\) D-glucuronic acid (GlcUA) and \((\beta1-4)\) N-acetyl-D-glucosamine (GlcNAc) (Figure 5) [56]. The molecular mass of hyaluronan at normal physiological conditions is \(\geq 0.5 \times 10^6\) Da. It has remarkable hydrodynamic characteristics, being able to bind large amounts of water and provide a template for interactions with proteoglycans and other extracellular macromolecules [57]. Creating a viscous biological meshwork, hyaluronan organizes the extracellular and pericellular matrices around cells.

Figure 5. Chemical structure of hyaluronan. \(n\) is about 10 000.

Hyaluronan is widely expressed in the human body and most abundant in soft connective tissue and skin [58]. It is localized in the extracellular matrix, at the cell surface and inside cells. It is crucial in many morphogenetic processes during vertebrate embryogenesis, when its expression is spatially and temporally regulated. Hyaluronan tissue distribution is ubiquitous, and hyaluronan is particularly concentrated in pericellular matrices surrounding proliferating and migrating cells [59, 60]. Hyaluronan synthesis is increased during mitosis and is required for cell detachment during mitosis and migration [61, 62]. Concomitantly, there is an increase in intracellular hyaluronan, both cytoplasmic and nuclear, with hyaluronan surrounding the chromosomes at metaphase and filling the space between separating chromosomes during anaphase [62]. Thus hyaluronan has a functional role both
intracellularly and in the pericellular matrix of a proliferating and migrating cell.

Underlying the role of hyaluronan in major cellular processes such as proliferation, migration, differentiation and angiogenesis, is its ability to bind a set of extracellular and cell surface associated hyaluronan binding proteins (HABPs), termed hyaladherins [63]. Two well known hyaladherins which mediate intracellular and cell surface hyaluronan signaling are CD44 and RHAMM (receptor for hyaluronan mediated motility)[64].

The biological outcome of hyaluronan signaling is largely dependent on its size. Intermediate size hyaluronan fragments are pro-angiogenic, inducing endothelial cell proliferation, migration and tube formation [65]. In addition, intermediate hyaluronan fragments induce inflammatory cytokines and transcription of matrix metalloproteinases (MMPs). Oligomers of 6-12 kDa size induce inflammatory gene expression in dendritic cells, while the smallest oligosaccharides are anti-apoptotic [66]. Thus, while high molecular weight hyaluronan is anti-inflammatory, immunosuppressive and protects endothelial cell barriers, fragmented hyaluronan is highly angiogenic, inflammatory and immune-stimulatory.

Hyaluronan is synthesized by glycosyltransferases named hyaluronan synthases (HASes) at the plasma membrane. There are three genes in the human genome encoding HASes, namely \textit{HAS1}, \textit{HAS2} and \textit{HAS3}. Hyaluronan is degraded by hydrolase enzymes called hyaluronidases; Hyal-1 and Hyal-2 are the major hyaluronidases responsible for degradation of hyaluronan in somatic tissues [66]. Hyal-1 is a lysosomal enzyme active at low pH and Hyal-2 is anchored to the membrane by a glycosylphosphatidylinositol (GPI) link. Hyaluronan is taken up by cells for degradation through binding to CD44, which together with Hyal-2, tether hyaluronan to the cell surface. This complex is enriched in plasma membrane invaginations called lipid rafts, where the \textit{Na}^{+}-\textit{H}^{+} exchanger creates an acidic environment and Hyal-2 degrades hyaluronan to a 20 kDa product, corresponding to about 50 disaccharides; these fragments are then internalized to lysosomes where Hyal-1 further degrades them to tetrasaccharides [66]. During inflammation, hyaluronan can be fragmented by reactive oxygen species (ROS), producing low molecular weight hyaluronan, which is then involved in the inflammatory response.

The balanced biosynthesis, uptake and degradation of hyaluronan are prerequisites for normal tissue homeostasis. There is accumulation of hyaluronan whenever there is rapid tissue turnover and repair, such as during inflammation, wound healing and neoplasia. Extensive research has demonstrated a correlation between levels of hyaluronan production and malignan-
cy in several tumor types, both in animal models and in human patients. For example, normal breast tissue has low levels of hyaluronan while in 56% of breast cancer, the hyaluronan levels are elevated in the malignant epithelium and peritumoral stroma; moreover, increased hyaluronan content promotes the progression of the disease and correlates to bad prognosis [67]. Most interestingly, accumulation of a very high molecular mass hyaluronan (6-12 MDa) most likely mediates the cancer resistance of the naked mole rat through involvement in an anticancer mechanism called early contact inhibition [68].

Thus hyaluronan, as a microenvironmental cue that regulates cell behavior, has a fundamental and profound role during embryonic development, wound repair, inflammation and tumor progression. Understanding of its biosynthesis and regulation is crucial for gaining insight into its role in these processes.

**Hyaluronan in cancer**

Hyaluronan is a prominent member of the tumor microenvironment with an established role in tumor progression [57, 67, 69]. Besides influencing tumor cell migration and cancer invasion by modulating the hydration, structural integrity, and osmotic balance in the tumor environment, hyaluronan also triggers intracellular signaling, which promotes the malignant phenotype [57, 58, 67, 70, 71].

Most likely, hyaluronan is involved in almost all steps in the process through which cells evolve and become invasive cancers. Experimentally increased synthesis of hyaluronan in tumor cells via overexpression of hyaluronan synthase enzymes leads to an enhanced malignant phenotype, including anchorage-independent growth and metastasis of tumors in animal models [70-74]. Hyaluronan promotes anchorage-independent, but not normal, anchorage dependent, growth by binding to CD44 and induction of the PI3-kinase-Akt pathway, which activates survival and anti-apoptosis signals [75, 76]. Highly hydrated hyaluronan matrices promote invasiveness and hyaluronan-CD44 interactions recruit and activate matrix metalloproteinases (MMPs) necessary for ECM degradation and invasion [77, 78]. Hyaluronan oligosaccharides promote angiogenesis and overproduction of hyaluronan by ectopic expression of HAS2 promotes EMT in normal mammary epithelium and mesothelioma cells [76, 79-81].

In addition to the tumor cell-derived hyaluronan, there is a prominent role for the stroma-derived hyaluronan in cancer progression. Overexpression of HAS2 in spontaneous Neu-induced mammary tumors induces a hyaluronan-rich intratumoral stroma, which promotes angiogenesis through recruitment
of stromal cells (mesenchymal stem cells, myofibroblasts and endothelial cells) and release of stroma-derived angiogenic signals [82].

There is a preference of breast cancer to metastasise to bone, suggesting that this “soil” is somehow attractive and permissive for the breast cancer “seed”. Hyaluronan might be one of the factors that guide the breast cancer cells to bone. Namely, highly metastatic breast cancer cells are characterized by elevated hyaluronan production (by the means of high HAS2 expression) [77] and enrichment in a cancer stem-like cell (CSC) population [83]. CSCs from aggressive breast cancer cells that have high hyaluronan production have a greater ability to adhere to bone microvessel endothelium and transmigrate compared to low hyaluronan-producing breast cancer cells. Furthermore, hyaluronan on CSCs promotes interactions with tumor associated macrophages (TAMs), which by stimulating stromal cells in bone promote proliferation of the CSCs, and thus tumor invasion and growth in bone [83].

The hyaluronan synthases

A single bacterial protein, termed Has, was found to be necessary and sufficient for hyaluronan biosynthesis [84, 85]. This enzyme, residing at the plasma membrane [86], differs from most enzymes in glycobiology since it catalyzes the addition of both GlcUA and GlcNAc onto the growing high molecular weight hyaluronan chain, simultaneously extruding it into the extracellular space [85].

The first gene encoding a hyaluronan synthase was identified in *Xenopus laevis* and named “developmentally expressed during gastrulation clone 42 (DG42, or xHAS1); subsequently a second Has gene was molecularly cloned from group A streptococci [59, 87]. This finally lead to the discovery of three mammalian HAS genes, *HAS1*, *HAS2* and *HAS3* which encode distinct but related HAS enzymes, all capable of hyaluronan biosynthesis [88-90]. The promoter regions of the three HAS genes contain regulatory elements, and transcriptional regulation of the genes has been demonstrated in response to growth factors, cytokines, hormones and prostaglandins [30, 91, 92].

Each mammalian HAS protein has a molecular mass of about 63 kDa and is predicted to be an integral membrane protein with six transmembrane regions, 2 membrane-associated regions and a highly conserved cytoplasmic region which probably contains the catalytic domain (Figure 6) [59, 85]. The crystal structure and thus mechanism of enzymatic activity for the three HAS proteins remains elusive and represents a major challenge in the field.
During mouse embryogenesis, the three Has genes are expressed in distinct spatial and temporal manners, with Has2 being the dominant hyaluronan synthase both during embryogenesis and in adult tissues [59]. Has2-deficient mice die during embryogenesis due to severe cardiac and vascular abnormalities, while Has1- and Has3-null mice are viable. In Has2-null embryos, the cardiac endothelium fails to undergo epithelial-mesenchyme transition (EMT), with subsequent loss of endocardial cushions and cardiac jelly formation [93].

Due to the elevated levels of hyaluronan in many cancers and correlation to bad disease outcome, the expression of the three HAS enzymes has been studied. In breast cancer, high HAS protein levels in both stromal and carcinoma cells are associated with poor differentiation of the tumor, HER-2 positivity and poor patient outcome [94].

HAS2, among the three HASes, has been implicated in breast cancer invasiveness, progression of mesothelioma and colon carcinoma; aggressive breast cancer cell lines are characterized by HAS2-overactivity, and HAS2 (but not exogenous hyaluronan or CD44) is crucial for TGFβ-induced EMT in normal mammary epithelial cells [67, 77, 79, 80]. Furthermore, overexpression of HAS2, but not exogenous hyaluronan, induces the EMT phenotype and acquisition of cancer stem cell properties in breast carcinoma cells.
by induction of Twist, TGFβ, TNFα and ultimately Snail, through TβRI- and p38-dependent pathways [95].

In many epithelial cancers, such as endometrial carcinoma, the mRNA levels of the HASes did not correlate with protein expression, or the hyaluronan accumulation [96, 97]; this suggested alternative regulatory mechanisms of the hyaluronan synthases. Thus, increased knowledge about the regulation of HAS enzymes, in particular HAS2, is important for understanding the impairment and aberrant activity which leads to hyaluronan accumulation and drives tumorigenesis.

**Regulation of hyaluronan synthases**

Due to the obvious importance of HAS2 both during embryogenesis and in adulthood, the promoter of the HAS2 gene has been extensively studied, revealing response elements for binding of transcription factors, such as CREB, STAT and RAR [98]. Increased hyaluronan synthesis in response to growth factors, such as PDGF-BB and TGFβ, is often due to the induction of the HAS2 gene in normal mammary epithelial cells, mesothelial cells and fibroblasts [31, 80, 91]. In addition, the HAS2 mRNA possesses a natural antisense transcript, called HAS2-AS1, which is simultaneously induced with the HAS2 mRNA and acts to stabilize and promote the expression of the HAS2 mRNA by modulating chromatin accessibility [99, 100].

Recent data has shed light on the post-translational modifications that control the HASes. Stimulation of fibroblasts with phorbol-12 myristate 13-acetate (PMA), which activates PKC, increased hyaluronan synthesis in fibroblasts and this was not due to increased mRNA levels [92]. Additional studies have implicated phosphorylation and glycosylation in the regulation of HAS activity [101-103]. While phosphorylation increased the activity of HASes in these studies, the phosphorylation of HAS2 on threonine 110 by AMPK reduced its activity [104]. This indicates that the energy status of the cell affects the activity of HAS2, and when the ATP:AMP ratio is low, AMPK is activated and inhibits the energy demanding process of hyaluronan synthesis by phosphorylation of HAS2. Furthermore, O-GlcNAcylation of HAS2 on serine 221 increases its activity and stability [105]. Namely, UDP-GlcNAc, which is an efficient nutrient sensor in the cell, is also used for O-GlcNAcylation of proteins, and can thus regulate HAS2 activity both as a substrate for hyaluronan and by posttranslational modifications. Similarly, HAS3 plasma membrane residence and recycling was increased, while lysosomal degradation was decreased by a surplus of UDP-GlcNAc and O-GlcNAcylation [106].
HAS2 is also regulated by dimerization and ubiquitination. Importantly, HAS2 is monoubiquitinated on lysine 190, and mutation of this residue, which resides in the catalytic domain and is conserved amongst the three HASes, abolishes the hyaluronan-synthesizing activity of HAS2 [107]. The ubiquitination of HAS2 and its regulation is further studied and described in this thesis. HAS2 is also polyubiquitinated with both lysine 48- and lysine 63-linked chains, which are efficiently removed by a deubiquitinase called USP17 (Figure 7) [108].

Furthermore, all three HAS enzymes can form homo- and heteromeric complexes, with the HAS2-HAS3 heteromeric complex being the most active [107, 109]. Formation of dimers, or multimers, could explain the intriguing fact that a 63-kDa protein can form a membrane pore for the bulky hyaluronan chain, in addition to having two UDP-transferase activities, and ability to retain the growing chain [110].

Figure 7. HAS2 is active as a dimer and modified by Lys48- and Lys63-linked ubiquitin chains which are removed by USP17. Monoubiquitination is removed by USP4.
CD44

The lymphocyte homing receptor CD44 is a widely distributed transmembrane glycoprotein first described in 1983 [111], and later found to be the principal cell surface receptor for hyaluronan [112]. CD44 is implicated in multiple physiological functions, such as cell migration during morphogenesis and angiogenesis, differentiation, adhesion and lymphocyte homing and activation; binding of CD44 to hyaluronan affects cell adhesion to the extracellular matrix and is implicated in the stimulation of aggregation, proliferation and migration. Under pathological conditions, CD44 is involved in inflammation and various steps of tumorigenesis [113-115].

The CD44 gene has 20 exons; extensive alternative splicing of this gene results in multiple variants of CD44 [116] (Figure 8A). The structural variability is further enhanced by post-translation modifications, such as O-linked and N-linked glycosylation, as well as glycosaminoglycan attachments [117]. The most widely expressed isoform of CD44, standard CD44 (CD44s), contains exons 1-5 and 16-20. The extracellular domain is the site of alternative splicing, yielding CD44 variant isoforms (CD44v1-10). The variant isoforms have a more restricted pattern of expression, and are preferably expressed in epithelial cells and tumors. All isoforms contain the hyaluronan-binding domain [114]. The transmembrane domain is highly conserved, and is thought to be responsible for CD44 clustering, which is required for efficient activation by hyaluronan [118]. The cytoplasmic domain (exon 20) contains 6 potential serine phosphorylation sites, and interacts with cytoskeletal proteins and signaling molecules [113, 117] (Figure 8B). For example, the ERM (ezrin, radixin and moesin) proteins, thought to be important for cell migration and cell shape, interact with a basic amino-acid motif in the cytoplasmic tail of CD44 and crosslink it to the actin cytoskeleton [115].

The CD44-ERM complex is regulated by phosphorylation; in cultured cells, CD44 is constitutively phosphorylated at Ser325, and this form binds active ERM protein that couples CD44 to the actin cytoskeleton. Activation of PKC results in dephosphorylation of Ser325 and concomitant phosphorylation of Ser291, which leads to disengagement of the ERM protein and loss of cytoskeleton association [119]. Furthermore, the c-Src kinase binds to the cytoplasmic tail of CD44 and hyaluronan-CD44 interactions stimulate the c-Src kinase activity in lipid rafts, inducing cytoskeleton-mediated tumor cell migration [64]. Similarly, TGFβRI kinase activated by hyaluronan can phosphorylate CD44, which enhances its binding to the cytoskeletal protein ankyrin in metastatic breast tumor cells, leading to hyaluronan-mediated breast tumor cell migration [120].
Figure 8. A) CD44 gene with 10 standard exons (s1-10) and 10 variant exons (v1-10). TM, encoding the transmembrane part; CP, encoding the cytoplasmic part. B) CD44s protein with the extracellular hyaluronan-binding region (link domain), the stem (variant) region where the variant exon products are inserted, and the cytoplasmic region which binds intracellular partners. The extracellular region contains many sites for N-glycosylation (orange circles) and O-glycosylation (yellow circles) and disulfide bridges (S-S).

In addition to engaging the cytoskeleton, CD44 can transduce intracellular signaling events leading to proliferation, migration and differentiation. CD44 has no intrinsic kinase activity, but modulates signaling by interaction with other molecules, acting as a co-receptor for many receptor tyrosine kinases (RTKs), including the fibroblast growth factor receptor family, epidermal growth factor receptor family (EGFR), the c-MET RTK, and others [11]. Furthermore, hyaluronan-engaged CD44 was found to inhibit PDGF-BB-induced fibroblast migration [9] and hyaluronan–engaged CD44 was shown to promote TGFβ-dependent fibroblast proliferation through promoting the interaction between CD44 and EGFR in lipid rafts [121, 122]. In this thesis,
a cross-talk is described between CD44, PDGFRβ and TβRI, where CD44 has a negative modulatory effect on the signaling pathways of these receptors [10]. These opposing effects of CD44 are mediated by two modes of CD44, namely the growth-promoting and growth-inhibitory mode, which depend on the biological conditions of the cells. This dual mode of CD44 is mediated through merlin, an ERM-related tumor suppressor encoded by the neurofibromatosis-2 (NF2) gene. At high cell density, merlin is hypophosphorylated and binds to a c-terminal ERM binding site in CD44, thus inhibiting ERM binding and proliferation of cells. At low cell density, merlin is hyperphosphorylated and occurs in a complex with the ERM proteins [123]. Proliferation is permitted and CD44 is activated by other ligands, presumably growth factors binding to their GFRs, to which CD44 acts as a co-receptors. This molecular switch consisting of CD44 and merlin, that instructs normal cells when cell-cell or cell-matrix contact has occurred, is mediated by hyaluronan in the ECM binding to CD44 and intracellularly presumably by a protein phosphatase [9, 123].

In addition, CD44 acts as a docking station for matrix molecules and enzymes, such as for MMP9 which by docking to CD44 cleaves and activates latent TGFβ [39]. Finally, the extracellular stem region of CD44 is proteolytically cleaved by MT1-and MT3-MMPs, and this step is necessary for further processing of the CD44 c-terminal fragment in the transmembrane region by presenilin-1/γ-secretase [119, 124]. This cleavage results in a CD44 intracellular domain fragment (CD44-ICD), which was demonstrated to translocate to the nucleus and act as a transcription factor, up-regulating CD44 mRNA amongst others [125].

**Ubiquitination and its function**

Ubiquitination is a post-translational modification of proteins with ubiquitin, which governs a variety of biological processes ranging from proteolysis to cell signaling. Ubiquitin is a highly conserved 76-amino acid protein which can be covalently attached via its carboxyl terminus to primarily lysine residues of a substrate protein, creating an isopeptide bond [126-128]. This reaction requires three types of enzymes: an activating enzyme (E1), a conjugating enzyme (E2), and an ubiquitin ligase (E3) (Figure 9) [129]. Monoubiquitination (mono-Ub) is an attachment of a single ubiquitin molecule, whereas polyubiquitination (poly-Ub) is the formation of a ubiquitin chain on the target protein; the linkage between the ubiquitin molecules in a chain encodes information about the substrate’s fate in the cell. Ubiquitin has seven lysine residues (K6, K11, K29, K33, K48 and K63), all of which can be conjugated to form a polyubiquitin chain. Lys48-linked poly-Ub chains are generally believed to target proteins to the proteasome for degra-
dation, while Lys63 linkages have been shown to regulate processes such as endocytosis, DNA repair and kinase activation through proteasome-independent mechanisms [130, 131].

Mono-Ub controls important cellular functions, such as histone regulation, endocytosis of plasma membrane proteins, protein signaling, DNA damage response, and protein localization. Mono-Ub is sufficient as an endocytic internalization signal [132]; furthermore, it regulates the endocytic machinery itself [133, 134]. Mono-Ub and endocytosis are required for Notch receptor signaling, a regulator of cell development and fate. Activation of the nuclear factor κB (NFκB) pathway, involved in apoptosis, innate and adaptive immune responses, proliferation and migration, is regulated through post-translational modifications, including ubiquitination, of IKKγ. Specifically during DNA damage and stress, a mono-Ub signal on IKKγ is required for NFκB activation [131, 135]. DNA damage repair by Fanconi anemia group D2 protein (FANCD2) requires its mono-Ub [136], and the same holds for recruitment of Y family DNA polymerases to proliferating nuclear cell antigen (PCNA) upon stalled DNA replication. Furthermore, ubiquitination serves an important function in the p53 pathway, which is important for cell cycle arrest, apoptosis and tumor suppression. Mono-Ub of p53 by MDM2, a RING-type E3 ligase, leads to nuclear export but not proteasomal degradation of p53 [127, 135].

Figure 9. Ubiquitin is activated in an ATP-dependent manner by a ubiquitin-activating enzyme (E1), transferred to a ubiquitin conjugating enzyme (E2), which interacts with a ubiquitin ligase (E3). The E3 recruits specific substrates and cooperates with the E2 to transfer ubiquitin to the substrate.
Deubiquitinating enzymes (DUBs)

DUBs are proteases which mediate the removal and processing of ubiquitin. The functions of DUBs, other than to reverse ubiquitination of proteins modified with either mono-Ub or poly-Ub, can be generation of free ubiquitin from the ubiquitin precursor, recycling of ubiquitin, disassembly of ubiquitin chains so ubiquitin monomers can re-enter the free-ubiquitin pool, and finally, DUBs can modify ubiquitin chains on proteins and thereby exchange one signal for another [137].

The human genome encodes approximately 95 putative DUBs, divided into five families based on their ubiquitin-protease domains: 58 ubiquitin-specific proteases (USP), 4 ubiquitin C-terminal hydrolases (UCH), 14 ovarian tumor proteases (OTU), 5 Machado-Joseph disease proteases (MJD) and 14 JAMM domain-containing proteases (JAMM). The USPs, UCHs, OTUs and MJD are cysteine proteases whose enzymatic activities rely on a thiol group of a cysteine residue in the active site. The JAMMs are metalloproteases which use a Zn$^{2+}$-bound polarized water molecule to cleave the peptide bond [138].

As ubiquitination is a key regulatory post-translational modification, the DUBs, having the power to reverse it, control many important cellular processes. DUBs are involved in control of endocytosis, cell signaling, proteasomal degradation, transcription and RNA processing [139]. The TGFβ signaling pathway is highly regulated by ubiquitination, and several DUBs have been discovered as critical modulatory components in this pathway. USP15 was identified as a deubiquitinating enzyme for R-Smads [140], as well as for the TβRI [141]; silencing of USP15 lead to impaired TGFβ signaling. OTUB1 is another interesting DUB which negatively affects the TGFβ pathway by inhibiting Smad2/3 polyubiquitination independent of its catalytic activity; additionally, this DUB also controls Ras signaling [142, 143].

DUBs are emerging as important regulators of cell cycle control, chromatin remodeling, epigenetic control of gene expression and DNA damage repair processes. This has lead to the development of small molecule inhibitors of DUBs, which are promising as cancer drugs and may provide new therapy options in the future [144].

DUB specificity can be directed either against the target protein, called target specificity, or it can be towards the ubiquitin moiety itself, called substrate specificity [138]. Some DUBs, as in the case of STAMBP (STAM-binding protein), prefer Lys63-linked chains, whereas others have specific targets and can deubiquitinate both mono- and polyubiquitinated proteins, as for example USP4 and USP17 [145].
Two DUBs, USP17 and USP4, were found to specifically act on HAS2 and affect its stability and activity, and will be further described in this thesis.

Ubiquitin-specific protease 17

The USP17 family of DUBs is a set of cytokine-inducible early-response genes encoded by the highly polymorphic human tandem repetitive DNA sequence RS447 on chromosome 4, and by additional sequences on chromosome 8 [146, 147]. The multiple family members are highly similar (>95%) and conserved throughout mammals. They will be collectively called USP17 due to the high similarity and difficulties to distinguish by molecular methods so far.

USP17 was originally identified as cytokine-inducible in murine hematopoietic cells [148], and subsequently in humans by IL-4 and IL-6 stimulation [149]. Constitutive overexpression of USP17 caused cell cycle arrest in G1/S and G2/M phases of the cell cycle, as well as apoptosis [148, 149]. It was then showed to block proliferation by regulating Ras membrane localization, and thereby MAP kinase activation, through deubiquitination of Ras Converting Enzyme 1 (RCE1) [150]. Interestingly and somewhat surprising, a set of studies then followed showing that USP17 promotes oncogenic transformation and is highly expressed in human tumors [151-155]. Additionally, USP17 was also induced by chemokines and essential for both mesenchymal and ameboid migration, and cytoskeleton rearrangements [156]. It then became clear that USP17 expression is tightly regulated in the cell cycle and balanced levels of USP17 are crucial for G1-S and G2-M transition. The mechanism whereby USP17 controls S-phase entry is again by regulating the proper membrane localization and activation of GTPases Ras and Rho, whose proper activation is crucial for ERK MAP kinase signaling and degradation of cyclin-CDK inhibitors p21cip1 and p27kip1 [151].

Sequence analysis of USP17 family members revealed that they contain the conserved hyaluronan binding motif (R/K)X7(R/K), where R is arginine, K is lysine and X is any non-acidic amino acid. It was shown to bind hyaluronan, and the hyaluronan-binding motifs are important for the apoptosis-inducing ability and inhibition of anchorage-independent growth of USP17 when overexpressed in cancer cells [157, 158].

Ubiquitin-specific protease 4

Prior to the standardization of the nomenclature, USP4 was known as UNP. Human UNP (ubiquitous nuclear protein), originally identified in the mouse genome, is located on chromosome band 3p21.3; the mRNA was found elevated in small cell tumors and adenocarcinomas of the lung [159]. Subse-
sequently, two tissue selective isoforms were identified, with the longer iso-
form being predominant in brain and the shorter in testis. The protein was
found to be mainly cytoplasmic and reduced in small cell lung cancer, in
contrast to previous studies [160].

Since its discovery, the knowledge about the important targets of USP4, such
as members of the NF-κB pathway [161-164], β-catenin [165], p53 [166],
and TβRI [167] has increased. AKT phosphorylation of USP4 results in its
re-localization to the membrane and deubiquitination of the TβRI, reinforce-
ing the pro-tumorigenic effects of TGFβ signaling in breast cancer cells
[167]. Furthermore, USP4 has an inhibitory effect on p53 signaling by
deubiquitinating and stabilizing the E3 ubiquitin ligase ARF-binding protein
1 (ARF-BP1) [166].

The role of USP4 in cancer is controversial; USP4 is overexpressed in vari-
ous cancers and plays a role in invasiveness and metastasis [165-168], but
has also been shown to inhibit breast cancer cell growth [169] and lung can-
cer cell migration [161, 163].
Present investigations

The mechanisms driving cancer initiation and progression involve a complex interplay between cancer epithelial cells and stromal cells, such as fibroblasts. Hyaluronan has a prominent role as a microenvironmental cue that can influence the malignant process. We have studied the regulation of hyaluronan synthesis by HAS2 in normal and breast cancer cells, and its role in EMT, migration and cell cycle progression. We have also investigated the signaling pathways of PDGF-BB, TGFβ and CD44 in fibroblasts, and how their integrated message can alter cell behavior.

Our specific aims have been:

Paper I: to understand the crosstalk between the signaling pathways of PDGF-BB, TGFβ and CD44 in dermal fibroblasts

Paper II: to elucidate the role of HAS2-produced hyaluronan and CD44 in TGFβ-induced EMT and migration of breast epithelial cells

Paper III: to identify deubiquitinating enzymes that target HAS2 and affect its activity in breast cancer cells

Paper I

PDGF β-receptor, TGFβ type I receptor and CD44 modulate each other´s signaling and stability.

Growth factor signaling is modulated by context-dependent cross-talk between different signaling pathways, which results in the complex regulation of proliferation, migration and survival of cells. We found that the receptors for PDGF-BB and TGFβ form a complex which is ligand-independent and mediated through the extracellular part of PDGFRβ. Interestingly, PDGF-BB stimulation led to phosphorylation of Smad2, the main downstream effector of the canonical TGFβ pathway, as well as to clearance of TβRI from the cell surface. The PDGF-BB-mediated phosphorylation of Smad2 was dependent on the kinase activities of TβRI and PDGFRβ, the Src kinase and TGFβ. Importantly, silencing of PDGFRβ suppressed the total levels of TβRI, and
the cell surface associated fraction. Furthermore, CD44 formed a ternary complex with PDGFRβ and TβRI and depletion of CD44 by siRNA increased the signaling via PDGFRβ and TβRI by stabilizing the receptors and Smad proteins. Finally, PDGF-BB-induced fibroblast migration was dependent on the TβRI kinase activity. Our data indicate that a cross-talk between PDGFRβ and TβRI occurs in dermal fibroblasts, and that CD44 negatively modulates the signaling via these receptors.

Paper II
Efficient TGFβ-induced epithelial-mesenchymal transition depends on hyaluronan synthase 2.

Epithelial-mesenchymal transition (EMT) is a developmental program whereby polarized epithelial cells change their morphology into motile mesenchymal cells. EMT contributes to tumor progression by allowing cancer cells to invade the surrounding tissue, and migrate towards blood vessels. TGFβ is a potent inducer of EMT and has a well established role in cancer invasion and metastasis. We found that TGFβ stimulation of NMuMG normal breast epithelial cells leads to upregulation of HAS2 and hyaluronan production. Upon TGFβ stimulation these cells are known to acquire mesenchymal traits through EMT, and knockdown of HAS2 lead to a partial inhibition of EMT, demonstrated by real time PCR and microscopy analysis of major EMT markers such as Snail, Zeb1 and ZO-1. Silencing of HAS2 also inhibited TGFβ-, but not EGF-mediated migration. Silencing of CD44, blocking CD44-hyaluronan interactions with CD44-blocking antibodies, or removal of extracellular hyaluronan with Streptomyces hyaluronidase, did not affect TGFβ-induced EMT; furthermore, overexpression of a dominant negative catalytic mutant of HAS2 also did not inhibit TGFβ-induced EMT. These results indicate that HAS2 expression, but not extracellular hyaluronan, is important for TGFβ-induced EMT.

Paper III
The deubiquitinating enzymes USP4 and USP17 target hyaluronan synthase 2 and differentially affect its function.

Altered hyaluronan synthesis, due to deregulated HAS2 gene expression and/or activity, is implicated in the progression and metastasis of carcinomas, and hyaluronan accumulation in the tumor stroma correlates with bad prognosis of many cancers, including breast cancer. The activity of HAS2 is regulated by post-translational modifications, including ubiquitination. In order to identify the deubiquitinating enzyme(s) (DUBs) that are involved in
deubiquitination of HAS2, a retroviral expression library of 69 Flag-HA-tagged DUBs [170] was screened in HEK293T cells for the ability to deubiquitinate myc-tagged HAS2. The most efficient DUBs found to target HAS2 were USP4 and USP17. USP4 preferentially decreased monoubiquitination of HAS2, previously found to be crucial for activity [107], while USP17 efficiently removed polyubiquitination and significantly stabilized HAS2 protein levels. Importantly, silencing of USP4 potentiated, while silencing of USP17 decreased hyaluronan production in aggressive breast cancer cells. These results indicate that the activity of HAS2 is regulated by ubiquitination, and that the DUBs USP4 and USP17 target HAS2 to modulate its activity.
Future perspectives

Paper I

There is evidence that autocrine PDGF-BB signaling is important for progression of TGFβ-induced EMT in many cancers [32]. Our observation that PDGF-BB induces phosphorylation of Smad2 and that silencing of PDGFRβ interfered with TGFβ signaling might be one of the mechanisms by which PDGF-BB signaling pathway affects TGFβ-induced EMT. It would be of interest to investigate the cross-talk between the receptors for PDGF-BB and TGFβ in a model of breast cancer and to see whether it is of importance for progression of the disease. The role of CD44 for this complex would also be interesting to study in such a model since CD44 can act both as a tumor suppressor and as a promoter.

The mechanism of PDGF-BB-induced Smad2 phosphorylation remains to be elucidated, and our data indicate that it involves both the TGFβ ligand and receptor; thus it might involve activation of latent TGFβ in a protease-independent manner, since our results indicate that proteases are not involved. A possible mechanism to explore would be fibroblast contraction mediated by integrins, which has been shown to activate latent TGFβ [171].

Paper II

Although the role of HAS2 in EMT has gained much attention, and it is well known that HAS2 is important for EMT and metastasis of breast cancer, the mechanism remains unknown. The role of extracellular hyaluronan in EMT is still somewhat unclear; our studies and those of others indicate that extracellular hyaluronan and CD44 are not important [80, 95], but this needs further investigation. For example, the role of polydispersed or low molecular weight hyaluronan has not been investigated. Ongoing studies in our laboratory aim at investigating the role of the HAS2 antisense transcript (HAS2-AS) in EMT. Another interesting observation by Chanmee and coworkers [95] is that hyaluronan production by cancer cells may act to shift cellular metabolism towards the hexosamine biosynthetic pathway (HBP) and glycolysis because of the huge consumption of UDP-substrates; this established a positive feedback loop with HIF-1α signaling and hyaluronan production.
which maintains the CSC conversion of breast cancer cells. Hyaluronan production may have additional roles except for acting as a fuel for the HBP pathway since hyaluronan might activate signaling pathways that induce glucose uptake, such as the PI3K-Akt pathway, which would further enhance the metabolic shift of cancer cells. This line of research is just emerging and holds promise for the future.

Paper III

While there is substantial knowledge about the transcriptional regulation of HAS2, the posttranslational regulation has been staggered due to lack of specific, reliable and sensitive antibodies. Most studies about the protein modifications of HAS2 are therefore done in cells overexpressing HAS2, which may include some pitfalls and difficulties. This area of research needs more attention and knowledge about the extensive modifications of HAS2 might aid in developing better antibodies or ways to detect endogenous HAS2. We have demonstrated that HAS2 is modified by both Lys48- and Lys63-linked chains, and further investigations will aim at elucidating the specific roles of these modifications, as well as for the possible role of monoubiquitination in the control of HAS2 intracellular trafficking, localization, stability and function. Furthermore, we aim to investigate the effect of growth factor stimulation on the ubiquitination of HAS2, which might give further insight into the aberrant accumulation of hyaluronan in cancer.

We identified USP17 as a very potent DUB for deubiquitination of HAS2; this enzyme has two hyaluronan binding motifs (HABPs), and is also found in the nucleus. Many intracellular proteins with HABMs, such as CDC37 and RHAMM, are important for cell cycle progression and proliferation [172], indicating that intracellular hyaluronan is important for these processes. Interestingly, we found that the complex between HAS2 and USP17 is regulated through the cell cycle, and complex formation is increased during the G2/M transition, concomitant with increased hyaluronan secretion. It would be of interest to explore this further; preliminary results indicate that USP17 stabilizes HAS2 specifically during the G2/M phase and further experiments are ongoing to resolve whether this is the case. Furthermore, immunofluorescence staining and proximity ligation assay indicate that HAS2 and the HAS2/USP17 complexes are found in the nucleus; this finding could shed light on the question of the origin of intracellular and nuclear hyaluronan, but further investigations are needed.
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I remember my first day at BMC, the registration day for students about to study the *Biomedicine program* at Uppsala University. Afraid that I would get lost in this huge building, I followed the signs directing me to the registration room, and on my way I encountered the sign “Ludwig Institute for Cancer Research”. I stopped for a second, and wished I could work there one day.

Now, almost ten years later, I look back at my adventure and I am truly grateful that my paths lead me to the **Ludwig Institute for Cancer Research** in Uppsala. My years spent here doing my PhD have been fun and at times very challenging, which has made me a stronger person. I learned a lot and made friends from all over the world; I will try to mention a few but the list of past and present **Ludwigos** is long and I thank you all for your encouragement and friendship during these years.

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References


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