STUDIES ON SYNDIECAN-1 IN MESENCHYMAL TUMORS

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To my parents

献给我的父母
ABSTRACT

The syndecans are a four-member family of type I transmembrane proteoglycans. Their core proteins have a short cytoplasmic domain, a transmembrane domain and a large N-terminal extracellular domain possessing glycosaminoglycan chains. All the protein domains and the glycosaminoglycan chains can contribute to the functions of syndecans. Syndecans are involved in many cellular processes including cell differentiation, proliferation, adhesion and migration. Syndecan-1 is the prototype and a hybrid proteoglycan containing both heparan sulfate and chondroitin sulfate chains.

Human malignant mesothelioma and fibrosarcoma are aggressive mesenchymal tumors; their low endogenous expression of syndecan-1 may correlate to a more malignant phenotype. In this thesis, we aimed to better understand the role of syndecan-1 in mesenchymal tumors, focusing on its nuclear translocation and structure-function relationship.

We stably transfected mesenchymal tumor cells with a full-length syndecan-1 construct and three truncated variants, namely 78 lacking the extracellular domain with the exception of the juxtamembrane DRKE sequence proposed to be essential for oligomerization; 77 lacking the whole extracellular domain; and RMKKK being a potential nuclear localization signal within the cytoplasmic domain.

Syndecan-1 and basic fibroblast growth factor share the tubulin-mediated transport route and co-localize with heparanase in the nucleus. The cytoplasmic RMKKK sequence of syndecan-1 is sufficient for its nuclear translocation and thus serves as a nuclear localization signal, with the arginine residue being vital for this function.

Overexpression of syndecan-1 influences the expression profile of the other syndecan family members, in particular downregulating syndecan-2. Both full-length and truncated syndecan-1 constructs decrease the proliferation of mesenchymal tumor cells in two ways: the full-length syndecan-1 prolongs the S phase of the cell cycle, whereas the extracellular truncated variants 77 and RMKKK prolong the G0/G1 phase.

Overexpression of syndecan-1 decreases mesenchymal tumor cell migration and motility, but enhances cell adhesion. Distinct protein domains have differential effects; the extracellular domain is more important for promoting cell adhesion, while the transmembrane and cytoplasmic domains are sufficient for inhibition of cell migration.

Reduction of endogenous chondroitin sulfate levels inhibits fibrosarcoma cell adhesion, motility and migration, whereas addition of exogenous chondroitin sulfate chains increases cell motility and migration through JNK and tyrosine kinase signaling pathways, but decreases cell adhesion.

Taken together, these results address the importance of nuclear translocation, and the functional protein domains and glycosaminoglycan chains, thereby providing new insights into the role of syndecan-1 in tumor progression.
LIST OF PUBLICATIONS

Syndecan-1 and FGF-2, but not FGF receptor-1, share a common transport route and co-localize with heparanase in the nuclei of mesenchymal tumor cells.

II. **Fang Zong**, Eleni Fthenou, Juan Castro, Bálint Péterfia, Ilona Kovalszky, László Szilák, Georgios Tzanakakis and Katalin Dobra.
Effect of syndecan-1 overexpression on mesenchymal tumour cell proliferation with focus on different functional domains.
*Cell Proliferation*, 2009, 00, 1–12.

III. **Fang Zong**, Eleni Fthenou, Filip Mundt, Ilona Kovalszky, László Szilák, David Brodin, Georgios Tzanakakis, Katalin Dobra and Anders Hjerpe.
Specific syndecan-1 domains regulate mesenchymal tumor cell adhesion, motility and migration.
*Manuscript.*

IV. Eleni Fthenou, **Fang Zong**, Alexandros Zafiropoulos, Katalin Dobra and Anders Hjerpe and Georgios Tzanakakis.
Chondroitin sulfate A regulates fibrosarcoma cell adhesion, motility and migration through JNK and tyrosine kinase signaling pathways.
LIST OF ABBREVIATIONS

BrdU  Bromodeoxyuridine
CS    Chondroitin Sulfate
DS    Dermatan Sulfate
ECM   Extracellular Matrix
EGFP  Enhanced Green Fluorescent Protein
FACS  Fluorescence-Activated Cell Sorting
FGF   Fibroblast Growth Factor
FGFR  Fibroblast Growth Factor Receptor
GAG   Glycosaminoglycan
GalNAc N-Acetyl-Galactosamine
GlcA  Glucuronic Acid
GlcNAc N-Acetyl-Glucosamine
GlcNS N-Sulfo-Glucosamine
HGF   Hepatocyte Growth Factor
HS    Heparan Sulfate
IdoA  Ido A Acid
JNK   c-Jun N-terminal Kinase
MAPK  Mitogen-Activated Protein Kinase
MM    Malignant Mesothelioma
NLS   Nuclear Localization Signal
PDGF  Platelet-Derived Growth Factor
PG    Proteoglycan
SDS   Sodium Dodecyl Sulfate
TGF-β Transforming Growth Factor-β
VEGF  Vascular Endothelial Growth Factor
WST-1 Water-Soluble Tetrazolium-1
1 INTRODUCTION

1.1 SYNDECANS

Proteoglycans (PGs) are macromolecules composed of a core protein to which one or more glycosaminoglycan (GAG) chains of variable structure are covalently attached. This heterogeneous group of complex molecules is generally present on the cell surface and in the extracellular matrix (ECM), though there are also intracellular and circulating forms [1].

Syndecans are a major family of transmembrane proteoglycans. The first molecule identified as an integral membrane protein with substitution of GAGs [2] was named syndecan, from the Greek word synedēin meaning “to bind together”, because it was thought that this molecule linked ECM components to the actin-containing cytoskeleton [2, 3]. So far, four members of this family have been characterized, namely syndecan-1 (syndecan) [2], syndecan-2 (fibroglycan) [4], syndecan-3 (N-syndecan) [5, 6] and syndecan-4 (amphiglycan or ryudocan) [7, 8].

The four syndecan family members occur in mammals and probably all vertebrates, whereas only one occurs in invertebrates [9]. The gene family for these syndecan core proteins may have arisen by gene duplication and divergent evolution from a single ancestral gene, resulting in four distinct genes in mammals. Syndecan-1 and -3 and syndecan-2 and -4, belong to two subfamilies, based on the similarities in their core protein sequences [10].

The characteristic structure of the syndecans endows them with multiple cellular functions. Syndecans interact with a wide variety of cellular ligands and mediate cellular signal transduction. Consequently, they are involved in the control of many cellular processes, including cell differentiation, proliferation, adhesion and migration. Syndecans are key regulators in the interplay between cells and their microenvironment, earning them the name of “tuners of transmembrane signalling” [11].

1.1.1 Structural organization

The four syndecans share a general structural organization (Figure 1). Their core proteins are typical type I transmembrane proteins. All four syndecans have a short C-terminal cytoplasmic domain, a single-pass transmembrane domain and a large N-terminal extracellular domain, containing several consensus sequences for GAG attachment. All mature syndecans are produced by cleavage of an 18-22 amino acid signal peptide present in their precursors [10, 12].

1.1.1.1 Core proteins

Cytoplasmic domain

The cytoplasmic domains contain two highly conserved regions, denoted as C1 and C2, which are membrane proximal and distal, respectively (Figure 2). These subdomains
are conserved both within a specific syndecan across all species, as well as between all four syndecan members [13], with the exception of a conservative R for K substitution in the C1 of human syndecan-2. A central variable region (V), between C1 and C2, is unique for each family member (Figure 2), but shows sequence conservation across species [12].

**Transmembrane domain**

The single-pass transmembrane domain is highly conserved among the four syndecan family members (Figure 2).

**Extracellular domain (Ectodomain)**

The ectodomains are variable in length and sequence between syndecan members, but contain conserved motifs for GAG attachment, cell interaction, proteolytic cleavage and oligomerization. This overall variation is even apparent within a specific syndecan type across species.

GAG attachment occurs to serine residues in Ser-Gly sequences of the ectodomain. Syndecan-1 and-3 contain two regions for GAG attachment, one is close to the N-terminus, and the other near the cell membrane. Within each mammalian syndecan ectodomain there is at least one repeating Ser-Gly motif besides single Ser-Gly motifs [10].

**Figure 1.** Schematic illustration of human syndecans.

- Signal peptide.
- Double dotted lines represent cell membrane.
- Black dashed lines indicate GAG attachment sites.
Figure 2. Some conserved amino acid sequences of the four human syndecan family members. The potential nuclear localization signal is shown in bold. Membrane-adjacent tetrapeptides (shown in italics) are supposed to be important for syndecan oligomerization and shedding.

1.1.1.2 GAG chains

GAGs are linear polysaccharides that consist of repeating disaccharides units composed of a uronic acid and a N-substituted hexosamine. GAGs substituted in syndecans are heparan sulfate (HS) and chondroitin sulfate (CS)/dermatan sulfate (DS).

HS is the principal GAG present in all four syndecans; in addition, syndecan-1 [14], syndecan-4 [15], and possibly syndecan-3 [10] are also substituted by CS. In syndecan-1 there are three consecutive Ser-Gly sequences in the N-terminal region, flanked by hydrophobic and acidic residues, as the canonical motif for HS attachment [16, 17]. Two singular Ser-Gly sequences in the region near the cell membrane are attachment sites for CS. Similarly, this site preference for specific GAG addition may also occur in syndecan-3 [18]. In contrast, each of the three GAG attachment sites near the N-terminus of syndecan-4 is capable of bearing either HS or CS, without obvious preference [15]. These GAG differences may relate to the nature of the wider peptide environments around the individual Ser-Gly attachment sites.

The biosynthesis of GAGs commences with the covalent addition of a xylose to the serine residue of a defined Ser-Gly sequence of the core protein (Figure 3). Then three more sugar molecules are sequentially added to form a common tetrasaccharide linker (xylose-galactose-galactose-glucuronic acid), as the basis for subsequent specific GAG chain elongation [19]. Chain elongation proceeds with sequential addition of N-acetyl hexosamine, either N-acetylglucosamine (GlcNAc) in HS or N-acetylgalactosamine (GalNAc) in CS, alternating with glucuronic acid (GlcA). As the polysaccharides are assembled, some of the individual monosaccharides are subjected to a number of stepwise modification reactions. In HS, the first step is deacetylation/N-sulfation of
GlcNAc to yield N-sulfoglucosamine (GlcNS). This can be followed by C5 epimerization of adjacent GlcA to iduronic acid (IdoA). A series of O-sulfations can also occur at various sites, principally at the 6-O-position of GlcNAc/GlcNS and the 2-O-position of IdoA, and rarely at the 3-O-position of GlcNS [3] (Figure 4).

CS is also modified by various patterns of O-sulfation [20]; each disaccharide may be non-sulfated or sulfated to varying degrees (Figure 4). The hydroxyls at C4 or C6, or both positions, of GalNAc can become sulfated, designated as CS-A, -C, or -E, respectively, though the latter is relatively rare. Sulfation at C2 of the GlcA, besides that at C6 of GalNAc, gives the rare CS-D. Sometimes the GlcA is epimerized to IdoA, as in HS/heparin, giving CS-B, better known as DS; the IdoA can then also be 2-O-sulfated [21].

These GAG modifications generate complex sulfation patterns that are diverse in degree, positions and combinations of sulfation within a disaccharide, as well as distribution along the GAG chain, giving rise to what is referred to as the GAG “fine structure”. Incomplete modifications, and their typical clustering, results in formation of domain structures within the polymer chains. Typical HS chains contain highly-modified domains, flanked by short, lesser-modified “transitional domains”, interspersed among larger unmodified domains [22, 23].

In addition, at the cell surface, GAG chains may also undergo further modification: sulfatase selectively removes 6-O-sulfates from HS and heparanase cleaves HS chains at a few specific sites into fragments of 10-20 sugar units (Figure 4). The generated fragments are biologically more active than the native HS chains in basic fibroblast growth factor (FGF-2) signaling [24].
1.1.2 Functional interactions

The specific structure of syndecans determines their many varied functions. Syndecans participate in several important cellular processes including cell proliferation, adhesion and migration. Syndecans exert these functions via interactions with a large variety of ligands by their GAG chains and/or core proteins. Regulation of syndecan expression affects all these cellular functions.
1.1.2.1 GAG chain interactions

The natural occurrence of mature syndecans without GAGs has not been described. The addition of GAG chains to syndecan protein cores is critical for syndecan function, as the attached GAGs provide most of the binding sites for extracellular ligands. The binding of proteins to GAGs is to a large extent electrostatic, due to the high content of negatively-charged carboxyl and sulfate groups in GAGs interacting with positively-charged amino acid side chains (i.e. lysine and arginine), although other types of interaction such as hydrogen bonding also contribute (Kjellén L. and Lindahl U., 1991). Consequently, sulfation is critically important for GAG binding; the highly sulfated and IdoA-rich domains are the main regions for the recognition of growth factors and other proteins by HS [25]. The sugar ring of IdoA is particularly flexible in its conformation which may allow the best presentation of sulfate groups to take place for optimal physical interaction with different proteins [26].

Syndecan GAGs exhibit great structural diversity including the type, number, length and fine structure [3]. It has been shown that HS binds a large number of ligands including ECM components and cell-surface adhesion molecules [27, 28], chemokines [29], growth factors [30, 31], and growth factor receptors [32] (Table 1). It is well documented that HS interacts with both FGF2 and FGF receptor-1 (FGFR1) thereby forming a ternary complex to exert efficient cell growth signaling [33].

The role of CS in syndecans is less clear but, by analogy with other PG interactions, it could also modify their protein binding [34]. A study has suggested that CS chains on syndecan-1 and syndecan-4 can cooperate with HS chains in ligating the growth factors midkine and pleiotrophin-2 [35]. Another study showed that the ECM protein laminin induces adhesion of normal human keratinocytes and fibrosarcoma cells through binding to both HS and CS chains of syndecan-1 [36].

Table 1. Selected HS-binding ligands

<table>
<thead>
<tr>
<th>Growth factors and Growth factor receptors</th>
<th>FGF family (except FGF19), FGFR-1, HGF, HB-EGF, VEGF, PDGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM proteins</td>
<td>Fibronectin, Vitronectin, Laminins, some Collagens, Fibrillin-1, Tenascin-C, Thrombospondin</td>
</tr>
<tr>
<td>Cell-cell adhesion molecules</td>
<td>L-selectin, N-CAM, PECAM</td>
</tr>
</tbody>
</table>

Footnote: this list is selected and summarized based on reviews of Carey et al. [3]; and Xian et al. [12]). Abbreviation used: HB-EGF, heparin-binding epidermal growth factor; N-CAM, neural-cell adhesion molecule; PECAM, platelet-endothelial cell adhesion molecule.
1.1.2.2 Protein core interactions

The syndecan protein core is the structural base for GAG attachment. Moreover, discrete domains of the core proteins can themselves bind or associate with ECM components, cell surface and cytoplasmic ligands, thus modulating various cellular processes (Figure 5).

The first “cell-binding domain” described for syndecans was localized within the region of amino acids 56-109 of the mouse syndecan-4 ectodomain, and was shown to mediate cell adhesion [37]. A conserved NXIP motif of syndecan-4 ectodomain is now known to be required for its cell adhesion property [38]. The ectodomains of syndecan-2 and -4 promote the adhesion of mesenchymal cells via an indirect interaction with β1 integrin [39]. A similar property of a stretch of 5 hydrophobic amino acids AVAAV, within the ectodomain close to the plasma membrane, was identified as being critical for syndecan-1-mediated inhibition of cell invasion [40]. Recently, a site in syndecan-1 for the direct binding and activation of integrins αVβ3 and αVβ5 has been mapped to a region of the ectodomain (amino acids 88-121) where the GAG chains are apparently not involved [41].

The transmembrane domain is important for syndecan self-oligomerization (also refer to section 1.1.4). Oligomerization could enhance the proximity between syndecan core proteins, thereby enlarging their interaction surfaces and increasing the probability of binding to other membrane proteins [42]. This could be the mechanism by which syndecans associate with the actin cytoskeleton after antibody-induced, or insoluble ligand-induced, clustering [3, 10, 13]. This oligomerization is essential for the activation of the cytoplasmic domain for downstream signaling [43, 44]. All four syndecans have been shown to bind neurofibromin, the tumor suppressor protein encoded by the neurofibromatosis type I gene. Neurofibromin interaction requires the transmembrane domain and a membrane-proximal region of the cytoplasmic domain of syndecan [45].

The cytoplasmic domain of syndecan-1 interacts with the small GTPase Rab5, and this interaction regulates syndecan-1 ectodomain shedding [46]. This domain binds cytoskeletal and PDZ-domain proteins through the C1 and C2 regions, respectively, and thus regulates the dynamics of the actin cytoskeleton and membrane trafficking. These interactions control syndecan recycling through endosomal compartments, promote internalization of accompanying protein cargo, and regulate cell adhesion and various signaling systems [43, 44, 47]. One of the PDZ-domain proteins that interact with the C2 domain is syntenin, which co-localizes with syndecan-1 at cell adhesion sites [48]. The V region of syndecan-1 has also been ascribed essential roles in lamellopodial spreading, actin bundling and cell migration [49].
ED: ectodomain
TM: transmembrane domain

**Figure 5.** Schematic representation of human syndecan-1, indicating the positions of specific peptide sequences or domains that have corresponding known ligand interactions or functions

1.1.3 Cellular distribution

Syndecans are integrated in the cell membrane and can be localized to specific cell membrane compartments. Immunocytochemical staining reveals that syndecan-1 is expressed by most epithelial cells. Its distribution on the cell membrane in different epithelial cell types ranges from over the entire cell surface to only on the basolateral surface [50]. In mammary epithelial cells, it has been observed that, in newly seeded cultures, syndecan-1 is present initially over the entire cell surface, but becomes restricted to the basolateral surface as the cells polarize [51]. In polarized myeloma cells, syndecan-1 is concentrated on the uropod, a discrete membrane domain located at the trailing edge of the cell, where syndecan-1 promotes adhesion and sequesters HS-binding growth factors [52].
Traditionally, syndecans have been thought to exert their functions at the cell surface due to their membrane localizations. However, emerging evidence show that syndecans can also be present in the cytoplasm and nucleus. We previously investigated the subcellular distribution and tubulin-dependent rearrangement of syndecan-1 in malignant mesothelioma (MM) cells, by using immunocytochemical staining and confocal laser microscopy. Syndecan-1 is initially present in the cytoplasm, and localizes to the cell membrane later, usually at the cell-cell contact sites. Syndecan-1 also shows prominent nuclear immunoreactivity, and this nuclear translocation occurs in a time- and tubulin-dependent manner. This pattern of reactivity to syndecan-1 was revealed using a panel of antibodies to both the endo- and ecto-domains of the protein, as well as antibodies to HS, indicating that the entire molecule is present. The nuclear localization of syndecan-1, and its co-localization with tubulin in the mitotic spindle, has also been shown in various carcinomas, in neuroblastoma and in benign mesothelial and endothelial cells [53].

There are reports that other PGs can also be present in the nucleus of different cell types, including a nuclear HSPG in corneal stromal fibroblasts [54], syndecan-2 in the injured adult rat cerebral cortex [55] and in central chondrosarcoma [56], glypican and biglycan in the nuclei of neurons and glioma cells [57].

Interestingly, overexpression or addition of heparanase decreases the level of syndecan-1 in the nucleus of myeloma cells in a concentration-dependent manner, suggesting that HS chains are important for the nuclear translocation of syndecan-1 [58].

The role of nuclear PGs and GAGs has been linked to the control of cell division [59], the shuttling of FGF2 into the nucleus [60] and inhibition of DNA topoisomerase I activity [61]. The latter inhibitory effect suggests that the presence of HS in the nucleus may inhibit gene transcription [61]. Intriguingly, nuclear syndecan-1 seems to influence cell proliferation; TGF-β2 delays the nuclear translocation of syndecan-1 concomitantly with an antiproliferative effect on MM cells [62].

Observations of nuclear PGs, and the implication of their functional importance, have triggered investigations on the underlying mechanisms of their nuclear translocation. The nuclear import of large proteins depends on the presence of a nuclear localization signal (NLS) corresponding to a short peptide. The typical peptide NLS consists of one or more short sequences of positively charged lysines and/or arginines. The first NLS discovered was PKKKRK [63], and the sequence K-K/R-X-K/R is proposed as a consensus monopartite NLS [64]. A bipartite NLS consists of two clusters of basic amino acids, separated by a spacer of variable length; the prototype is KR[PAATKKAGQA]KKKK [65].

Functional NLSs have been identified in the core proteins of glypican and biglycan [57]. The conserved, membrane-proximal RMKKK sequence in the cytoplasmic domains of human syndecan-1, -3 and -4 (but conservatively altered to RMRKKK in syndecan-2) may be a potential NLS.
The presence of PGs/GAGs in the nucleus raises interesting questions: how do PGs get to the nuclear destination, and what are their molecular partners along the route? What are their functions in the nucleus?

1.1.4 Core protein-mediated oligomerization

Upon extracellular ligand binding, cell surface receptors activate intracellular signaling pathways. This signal transduction often initiates from a process known as receptor dimerization or oligomerization [33, 66]. Syndecan transmembrane core proteins exhibit a propensity to form tight, non-covalent dimers or oligomers [3]. Syndecans exist as dimers in vivo, regardless of clustering by extracellular ligands. These dimers, or even oligomers, are SDS-resistant and can only be separated into monomers by a single freeze-thaw process [67].

The structural requirement for this oligomerization of syndecan core proteins was investigated initially using rat syndecan-3 as a model. Recombinant syndecan-3 core protein forms SDS-resistant dimers, tetramers and higher-order oligomers. Analysis of recombinant protein domains, and the effects of site-directed mutations, revealed that the transmembrane domain is required, but is not sufficient for oligomerization of syndecan-3. An adjacent extracellular tetrapeptide (ERKE) is also needed, and the two basic R and K residues are essential to confer this oligomerization [68]. Studies in syndecan-2 and-4 showed that transmembrane domains are sufficient for inducing syndecan self-oligomerization, and transmembrane domain-induced oligomerization is crucial for the function of these syndecan receptors [69]. A more recent study showed that the transmembrane domains of the syndecan family display a hierarchy of homotypic and heterotypic interactions; the strengths of transmembrane self-dimerization are in the order of syndecan-2 > syndecan-4/-3 >> syndecan-1. Each syndecan transmembrane domain can also form heteromeric complexes with the other paralogs, and these interactions exhibit selectivity, e.g. syndecan-1 shows stronger interactions with syndecan-2 and -3, but a weaker self-interaction, and no interaction with syndecan-4 [70]. A conserved GXXXG sequence is present within the transmembrane domains of all four syndecans. This has been identified as a dimerization motif, allowing the formation of both homo- and hetero-dimers. Mutation of either of these glycine residues hampers such oligomerization [68-70].

The weak self-oligomerization of syndecan-1 transmembrane domains suggests that oligomerization of the full-length protein would require additional interaction sequence and/or partners. For example, coupling to the cytoskeleton via the cytoplasmic domains may further stabilize the oligomers [71, 72]; Selectivity of syndecan-1 heteromeric interactions implies that specific residues outside the conserved GxxxG motif must contribute to this specificity [70]. The additional sequence, besides the transmembrane domain, that is compulsory for syndecan-3 oligomerization, is the adjacent tetrapeptide ERKE within the ectodomain. Within this tetrapeptide there is only one amino acid difference between syndecan-1 and syndecan-3. It is intriguing to know whether the corresponding DRKE sequence in syndecan-1 has an equivalent function.
1.1.5 Ectodomain shedding

Syndecan ectodomains can be released from the cell surface by endogenous proteolytic cleavage in a process known as shedding [73]. Ectodomain shedding converts syndecans from membrane-associated receptors to soluble HSPG effectors that can compete in binding the same ligands as their cell surface counterparts [74].

The intact ectodomains of each mammalian syndecan are constitutively shed from cultured cells [75] as part of normal cell surface HSPG turnover [76]. Syndecan shedding is often accelerated in response to pathophysiological cues. Shed syndecan-1 and -4 are usually found in the wound fluids that accumulate following tissue injury and inflammation [77, 78]. Elevated levels of soluble syndecan-1 ectodomain have been demonstrated in sera from patients with lung cancer [79], multiple myeloma [80] and Hodgkin’s lymphoma [81].

Shedding can be accelerated by a variety of physiological stimuli, such as growth factors, chemokines, bacteria, and cellular stress [24]. Recent studies showed that heparanase enhances syndecan-1 shedding by stimulation of protease expression. This suggests a novel mechanism for the stimulation of tumor growth and metastasis [82, 83]. How extracellular stimuli influence proteases to mediate syndecan cleavage still needs to be elucidated.

The release of syndecan ectodomains from the cell surface by shedding has functional consequences. Membrane-bound and soluble forms of syndecan-1 have different roles in breast cancer cells. Overexpression of membrane-bound syndecan-1 stimulates proliferation, but inhibits invasiveness, of adenocarcinoma cells, whereas overexpression of a constitutively shed syndecan-1 decreases the proliferation, but promotes invasion, of the same cells [84]. Soluble syndecan-1 promotes growth of myeloma tumors in vivo. This is demonstrated by transfection of myeloma cells with a syndecan-1 construct lacking its transmembrane and cytoplasmic domains, which structurally and functionally mimics the shed syndecan-1 ectodomain in vivo. When injected into the marrow of human bones implanted in severe combined immunodeficient mice, the cells expressing this truncated form of syndecan-1 grow much faster than the cells transfected with full-length syndecan-1 [85].

Syndecan ectodomain shedding is carried out by a variety of proteases of the matrix metalloproteinase family, via cleavage of the core protein at specific sites close to the cell membrane. One identified cleavage site is G245-L246, about 7 amino acids from the cell membrane in human syndecan-1 [86], while another cleavage site is supposed to be the dibasic RK within the DRKE motif adjacent to the cell membrane [77, 87]. One of our truncated syndecan-1 constructs, which lacks the ectodomain with the exception of this DRKE motif, mimics the remaining portion of the core protein after ectodomain shedding.

1.1.6 Expression in normal tissues

So far, all cells studied except B-cells express at least one syndecan member; most cells express multiple syndecans, and a few may express all four. Syndecan-1 is the major
Syndecan on epithelial cells, syndecan-2 is present mainly on cells of mesenchymal origin, syndecan-3 is primarily in neuronal tissue and cartilage, while syndecan-4 is more ubiquitously expressed. However, each syndecan is expressed in highly regulated cell-, tissue- and development-specific patterns [12, 75].

In the adult individual, syndecan-1 is expressed predominantly in epithelial tissues, especially in normal squamous and transitional epithelia, where it localizes to the basolateral surfaces of simple epithelial cells, and the cell-cell contacts of stratified epithelial cells. It is generally absent in most mesenchymal cells, although it is expressed on Leydig cells and some plasma cells [88]. However, during embryonic development, syndecan-1 is transiently expressed in the condensing mesenchymal cells in some tissues, especially during epithelial-mesenchymal interactions [3]. In addition, a small amount of uniformly-distributed syndecan-1 has been seen in cultured fibroblasts [89]. It is also expressed in pre-B cells in the bone marrow, but lost immediately before the mature B-cells are released into the circulation [90].

Syndecan-1 expression is induced in response to wound healing; its expression is increased in the proliferating keratinocytes at the wound margins [91], and in the endothelial cells within the granulation tissue [92]. This expression can be modified in cultured cells by treatment with various growth factors and cytokines, and the responses are cell type-dependent. For example, syndecan-1 expression is induced in 3T3 fibroblasts by FGF-2 [93], and in vascular smooth muscle cells by platelet-derived growth factor (PDGF) [94]. In contrast, syndecan-1 expression is decreased in endothelial cells by tumor necrosis factor-α [95], and in MM cells by epidermal growth factor (EGF), insulin-like growth factor (IGF) and transforming growth factor-β2 (TGFβ2) [62]. On the other hand, PDGF and TGF-β increase its expression in human periodontal fibroblasts and osteoblasts [96].

### 1.1.7 Syndecan-1 in cancers

Syndecan-1 expression is altered in pathological states [89]. Decreased levels have been observed in cell transformation models. For example, inhibition of syndecan-1 in epithelial cells causes them to transform to a mesenchymal morphology [97]; an epitheliod phenotype can be restored when the cells re-express syndecan-1 by transfection with syndecan-1 cDNA [98]. The cell surface syndecan-1 in mouse epidermis is decreased upon the malignant transformation induced by ultra-violet irradiation [99].

Syndecan-1 is expressed in various human cancers (see review [100]). The presence of syndecan-1 in myeloma cells [101] is used as a diagnostic marker for this tumor. Up-regulation has also been reported in breast cancer, and in some cases this correlated to higher histological tumor grading, tumor size and poor prognosis [102]. As described earlier (section 1.1.5), syndecan-1 has also been associated with lung cancer and Hodgkin’s lymphoma. However, other studies of lung, colon, gastric, cervical, esophageal and oral cancers have demonstrated that its expression is down-regulated [103-111]. In squamous cell carcinomas of the head and neck, and also uterine cervix, loss of syndecan-1 correlates with less differentiation and worse clinical outcome [112,
13]. Down-regulation has also been reported in some mesenchymal tumors including human fibrosarcoma [114]. A correlation between syndecan-1 expression and cell differentiation has been shown in MM [115, 116].

The expression level of syndecan-1 can differ within a particular type of cancer. For example, several reports demonstrate that syndecan-1 is up-regulated in prostate cancer and elevated levels are associated with a higher tumor grade and recurrence [117-119]. However, another report shows that syndecan-1 is almost absent in high grade prostate carcinoma samples [120]. Also, syndecan-1 is up-regulated in pancreatic cancer tissue samples [121], however, more than 70% of pancreatic carcinoma specimens have weak or negative immunoreactivity for both epithelial and stromal compartments [122]. These findings are contradictory in an as yet unexplained way.

1.1.8 Syndecan expression affects tumor cell behavior

Experimental studies on the role of syndecan-1 in malignancy have shown that syndecan-1 expression associates with the maintenance of epithelial morphology and inhibition of tumor cell growth and invasiveness. Several studies have also shown that modulation of syndecan-1 expression can affect tumor cell behavior.

1.1.8.1 Cell Proliferation

Syndecan-1 overexpression influences tumor cell proliferation in a cell type-dependent manner. In mouse squamous cell carcinoma [109], fibrosarcoma cells [123] and human endometrial cancer cells [124] such overexpression increased cell proliferation. On the other hand, decreased cell growth has been seen when overexpressing full-length syndecan-1 in mouse mammary tumor cells [98].

A limited number of studies have so far dissected the roles of distinct syndecan-1 domains in tumor cell proliferation, and mainly focused on the ectodomain. Overexpression of various truncated syndecan-1 constructs that lack the C-terminal cytoplasmic tail induce hepatocyte [125] and myeloma proliferation [85]. However, inhibitory effects have been described as well. For example, the entire mouse syndecan-1 ectodomain suppresses the growth of mouse mammary tumor cells [126]. A similar effect is obtained by transfection of these cells with minican, a shorter segment of the syndecan-1 ectodomain containing the distal GAG attachment sites [127]. This duality in the role of syndecan-1 in cell proliferation indicates a complex regulatory mechanism, which is tissue and/or tumor type-specific, and at least partly dependent upon serum conditions [128].

1.1.8.2 Cell adhesion, motility and migration

Tumor cell invasion of surrounding tissue is the key factor in malignancy. It is dependent on a complex interplay of tumor cell adhesion, motility and migration. Tumor cells must adhere to ECM and cell surface molecules as they invade. Cell motility and migration are dynamic processes that require continuous assembly and disassembly of cell-cell and cell-matrix adhesions [129]. These cell behaviors are mediated by the interactions between cell membrane receptors and the surrounding...
microenvironment as well as the internal cytoskeleton. Among the many interacting cellular molecules, syndecans are emerging as important regulators for these processes and thus crucial for tumor invasion.

Expression of syndecan-1 inhibits myeloma cell invasion into type I collagen gel [130]. An invasion regulatory domain within the ectodomain of syndecan-1 has been identified and proved to inhibit the invasion of myeloma cells [40]. Overexpression of full-length syndecan-1, or a truncated variant which lacks the ectodomain, promotes metastasis of fibrosarcoma in a mouse model [123]. Down-regulation of syndecan-1 expression by siRNA disrupts αVβ3 integrin-dependent cell spreading and migration of human mammary carcinoma cells [71]. It has also recently been reported that overexpression of syndecan-2 enhances the migration and invasion of melanoma cells [131].

Interestingly, a recent study shows differential roles for membrane-bound and soluble syndecan-1 in breast cancer progression [84]. Overexpression of full-length syndecan-1 increases cell proliferation, whereas shed syndecan-1 decreases it. Constitutive membrane-bound syndecan-1 inhibits invasiveness, whereas the soluble form promotes invasion of cells into Matrigel. Thus the proteolytic conversion of syndecan-1 from a membrane-bound form into a soluble molecule marks a switch from a proliferative to an invasive phenotype.

So far, however, the molecular mechanisms behind syndecan regulation of the cellular behavior of mesenchymal tumors are still poorly understood. It is challenging to address the role of syndecan-1 in mesenchymal tumors and the importance of its different functional domains.

1.2 MESENCHYMAL TUMORS

MM and fibrosarcoma are two highly malignant tumors of mesenchymal origin. Their differentiation patterns vary, with MM cells showing either epithelioid or sarcomatoid (fibroblast-like) morphology, while fibrosarcoma tissue mainly has the latter kind of phenotype. In culture, however, fibrosarcoma cells may also grow in an epithelioid fashion. The growth patterns acquired can in vitro undergo a reversible morphological transition between these two phenotypes. This property of these two mesenchymal tumor cell types allows the study of how cell differentiation and tumor progression are regulated. So far, however, there are only limited studies on the role of syndecan-1 in these two mesenchymal tumors.

1.2.1 Malignant mesothelioma

MM is the primary malignant tumor of serous membranes, originating from mesothelial cells. The mesothelium is a monolayer of flattened epithelial-like cells resting on a thin basement membrane supported by connective tissue. Their mesenchymal nature is reflected by production of connective tissue ECM components like type-I and −III collagens [132-135].
Mesotheliomas most frequently develop in the pleura, but also in the peritoneum and occasionally in the pericardium and in tunica vaginalis testis. They start as a localized tumor, but spread rapidly along mesothelial surfaces [136]. MM invades the surrounding tissues and cause distant metastases in up to 80% of patients [137, 138]. The most frequent sites of metastases for pleural MM are through lymphatics to bronchopulmonary, hilar, and mediastinal lymph nodes; occasionally, lymphangitic spread is observed [139]. This highly aggressive tumor allows a median survival of about one year, and the best available therapy will, so far, only improve survival by a few months [140].

MM is almost always caused by exposure to fibrous minerals such as asbestos [141, 142]. Asbestos fibers can interact with the microtubules of the mitotic spindle [143], and can interfere with normal chromosome segregation [144], leading to aneuploidy. Asbestos can also cause DNA damage by direct physical interaction [145], or indirectly by formation of hydroxyl free radicals. Hydroxyl radicals are generated by the iron precipitates that accumulate to form the asbestos bodies [146]. The final consequences of such DNA/chromosome damage are the loss/inactivation of tumor suppressor genes and activation of proto-oncogenes [147]. Indeed, MM cells express a plethora of growth factors and growth factor receptors, including EGF receptor [148], TGF-α and-β [149], PDGF [150], vascular endothelial growth factor (VEGF) [151], FGF [152], hepatocyte growth factor (HGF) and c-Met [153]. In particular, expression levels of FGF-1 and -2 correlate with a poor survival of MM patients [152]. FGF-2 stimulates synthesis of PGs in MM cells via an autocrine mechanism [154].

The production of PGs and GAGs in MM reflects the mesenchymal nature of the tumor; it produces a variety of macromolecules commonly found in the ECM of soft connective tissues, e.g. versicans, biglycan, decorin, perlecan and hyaluronan [116]. MM cells also synthesize a number of cell-associated PGs including syndecan-1, -2 and -4. Among them, syndecan-2 and -4 seem to dominate especially in the epithelioid subtype [116]. In these cells the expression of syndecan-1 was relatively low in both phenotypes, while in another study, syndecan-1 expression was higher in the epithelioid cells, but reduced or lost in the sarcomatoid subtype [115].

### 1.2.2 Fibrosarcoma

Fibrosarcoma is a rare tumor derived from fibroblasts that can develop as a result of exposure to radiation. This sarcoma is predominantly found around bones or in soft tissues. It usually originates in the fibrous tissue of bone and invades long and flat bones. It also involves periosteum and overlying muscle. The tumor may present different degrees of differentiation. Poorly differentiated tumors contain pleomorphic, giant and multinucleated cells with numerous atypical mitoses. These tumors are associated with abnormal deposition of collagen and other ECM components. The presence of immature blood vessels favors metastasis through the bloodstream. The lungs are the primary sites of metastasis for fibrosarcomas that develop in the extremities. Once metastasis to the lungs has occurred, the chances of survival are significantly decreased [155].
Fibrosarcomas have been found to be rich in GAGs and PGs [156]. The content, cellular disposition and turnover of the ECM components are variable between individual cell lines of fibrosarcoma origin [157]. Although immunohistochemical staining of syndecan-1 in fibrosarcoma sections was negative [114], some fibrosarcoma cell lines express syndecan-1 in culture. Fibrosarcoma cells migrate spontaneously on ECM components [158], and shedding of syndecan-1 stimulates fibrosarcoma cell migration [86]. Overexpression of syndecan-2 results in increased migration/invasion and anchorage-independent growth [159]. Fibrosarcoma cells overexpressing syndecan-1 have higher growth rates in vitro and in vivo, and develop more lung metastases [123].

The above studies were all performed with the HT1080 fibrosarcoma cell line that has an epithelioid morphology. The importance of syndecan-1 has, so far, not been thoroughly studied in the B6FS cell line with a fibroblast-like morphology. One exception is a study demonstrating that CS-A chains enhance PDGF-mediated proliferation [155].
2 AIM OF THE STUDY

The overall aim of this thesis was to study the role of syndecan-1 in regulating various aspects of mesenchymal tumor cell behavior, such as proliferation, adhesion, motility and migration, with a focus on the subcellular distribution of syndecan-1 and its structure-function relationship. The thesis work has been divided into four sub-projects, the specific aims of which were:

1) To clarify the cellular translocation routes of syndecan-1, FGF-2 and FGFR-1, and to identify the minimal structural requirement for the nuclear transport of syndecan-1 (Paper I).

2) To investigate the role of syndecan-1 in mesenchymal tumor cell proliferation, focusing on the importance of distinct core protein domains (Paper II).

3) To investigate the role of syndecan-1 in adhesion, motility and migration of mesenchymal tumor cells, focusing on the contribution of the specific protein domains (Paper III).

4) To examine the effects of both endogenous and added CS on fibrosarcoma cell adhesion, motility and migration, and the main downstream pathways involved in mediating these effects (Paper IV).
3 COMMENTS ON METHODOLOGY

3.1 SYNDECAN-1 CONSTRUCTS

**Figure 6.** Schematic illustration of syndecan-1/EGFP constructs.

Human syndecan-1 constructs were designed based on potentially functional domains (Figure 6). The constructs were cloned into pEGFP-N1 or pEGFP-C3 vectors (Clontech, USA). Apart from the human full-length syndecan-1/EGFP construct (FL), we also studied the truncated variants 78, 77 and RMK. The 78 construct lacks the extracellular domain with the exception of the DRKE motif, which supposedly is important for oligomerization and is also a protease cleavage site. The 77 construct lacks the entire extracellular domain, mimicking the part remaining after shedding, and the RMK transcript is a potential NLS.

We selected these EGFP vectors because the expressed EGFP retains the fluorescent properties of the native protein, allowing the visualization and localization of the fusion protein. A neomycin-resistant cassette allows stably transfected eukaryotic cells to be selected using geneticin (G418).

The RMK/EGFP construct was further subjected to point mutations to confirm the NLS property of the transcript. For this purpose the basic amino acids arginine (R) and lysine (K) were replaced by the neutral amino acids alanine (A) and leucine (L), respectively. For comparison with the wild type RMK, three mutated constructs were tested: AMLK, AMKL and RMLK.
3.2 MESENCHYMAL TUMOR CELL LINES

We used two MM cell lines, with different in vitro growth patterns, established from the pleural fluid of a patient with malignant pleural mesothelioma. The cell line STAV-AB, established in human AB serum-supplemented medium, has an epithelioid morphology, while the cell line STAV-FCS established in fetal calf serum-supplemented medium grows with a fibroblast-like morphology.

The cell lines derived from fibrosarcomas can also differentiate into either an epithelioid or a fibroblast-like phenotype. The human fibrosarcoma cell line HT1080 grows as rounded cells, sometimes in multiple layers to form microtumors [160]. On the other hand, the human fibrosarcoma cell line B6FS is predominately represented by small, blunt fibroblast-like cells [161]. While previous studies on syndecans in fibrosarcoma have mainly addressed the HT1080 cell line, our present study focuses on the B6FS cell line, because of its low basal synthesis of syndecan-1.

These mesenchymal tumor cell lines with low expression levels of endogenous syndecan-1 were used for transfection of the different syndecan-1 constructs. Transient transfection showed that transfection efficiency was generally low in both MM cell lines, whereas it was much higher in the fibrosarcoma cell line. Stable transfections were achieved in both STAV-AB and B6FS cell lines by geneticin selection; the B6FS transfectants were further selected by fluorescence-activated cell sorting (FACS) to isolate the stronger expressing cells. These stable transfectants were then used for the functional studies.

3.3 BIOASSAYS

The effects on tumor cell behavior of syndecan-1 overexpression were evaluated by various bioassays.

3.3.1 Cell proliferation

Cell proliferation was assessed by using the water-soluble tetrazolium-1 (WST-1) assay and the bromodeoxyuridine (BrdU) ELISA. The WST-1 assay is based on the cleavage of the tetrazolium salt WST-1 by metabolically active cells. The BrdU ELISA measures DNA synthesis by quantification of BrdU incorporated during a labeling pulse.

3.3.2 Cell adhesion, motility and migration

Cell migration is a process depending on both the adhesion and motility properties of the cells.

CyQUANT GR® Dye was used to measure cell numbers in cell adhesion and migration assays. The adherent or migrated cells are lysed and stained by the dye, which binds to DNA. The resulting fluorescence is thus proportional to the number of cells and is measured directly using a fluorometer.
Cell migration was assayed by using a Transwell plate with 8 µm pore polycarbonate membrane inserts, either with (chemotaxis assay) or without a chemoattractant. The cells migrated through the membrane were quantified as above.

Cell motility was also assessed using the “Random Movement” and “Wound Healing” assays. In “Random Movement”, cell movements are recorded in non-confluent cultures, using time-lapse microscopy, and the moving paths of individual cells are tracked using ImageJ software. Total distances and final displacements of moving cells are calculated based on their path tracking data. Total distance represents general motility of the cell, whereas the final displacement also involves directional movement.

In the “Wound Healing” assay, an artificial wound is made in a confluent cell monolayer and the subsequent closure of the wound is monitored using a digital image processor connected to a microscope. Wound width is measured using ImageJ software and converted to cell migration speed. The measure obtained by the “Wound Healing” assay reflects not only cell motility, but also depends on cell proliferation.
4 SUMMARY OF PAPERS

4.1 PAPER I

Syndecan-1 and FGF-2, but not FGF receptor-1, share a common transport route and co-localize with heparanase in the nuclei of mesenchymal tumor cells.

Syndecan-1, as a co-receptor, binds both FGF-2 and its cognate receptor FGFR-1, to form and therefore stabilize the signaling ligand/receptor complex at the cell surface. We previously found a tubulin-mediated nuclear translocation of syndecan-1. In this study, we further explored the transport route and functional significance of nuclear syndecan-1 in mesenchymal tumor cells.

First, we examined the subcellular distribution of syndecan-1, heparanase, FGF-2 and FGFR-1 by immunocytochemistry and subsequent confocal laser microscopy. We found that FGF-2 and heparanase co-localized with syndecan-1 in the nucleus, whereas FGFR-1 was enriched mainly in the perinuclear area.

Secondly, we examined the effects of drugs that interfere with microtubule assembly on the nuclear translocation of these molecules. Vinblastine treatment resulted in depolymerization of tubulin. Double-labeling experiments showed that both FGF-2 and syndecan-1 co-precipitated with depolymerized tubulin, and the tubulin depolymerization completely hampered the nuclear transport of both syndecan-1 and FGF-2. In contrast, FGFR-1 was not associated with tubulin, and its distribution was not affected by vinblastine treatment. Doxorubicin, known to arrest cells in the G2 phase of the cell cycle, almost completely inhibited the nuclear transport of syndecan-1.

Thirdly, we performed immunoprecipitation of syndecan-1 and FGF-2, and found that they both existed in crude nuclear extracts. Importantly, overexpression of syndecan-1 leads to an enhancement of nuclear FGF-2, suggesting an important role of syndecan-1 for this nuclear transport.

Finally, to elucidate the minimal structural requirement for the nuclear transport, we transfected the cells with a full-length syndecan-1/EGFP construct or a truncated RMKKK/EGFP construct, in which the RMKKK motif may serve as a nuclear localization signal. The subcellular distributions of the fusion proteins were monitored by fluorescence microscopy. Distinct nuclear localization was observed in the RMKKK/EGFP transfected cells, while full-length syndecan-1/EGFP fusion protein revealed nuclear, cytoplasmic and focal cell membrane reactivity. EGFP transfected control cells revealed only faint cytoplasmic reactivity. The RMKKK sequence was then subjected to site directed mutagenesis. All three mutants, mutant-1 (AMLKK), mutant-2 (AMKLK) and mutant-3 (RMLLK) hampered nuclear translocation. When quantified as the proportion of cells with nuclear localization, mutant-1 and -2 showed a significant decrease, whereas mutant-3 did not, suggesting that the arginine (R) residue is essential for this translocation.
The results demonstrate that syndecan-1 and FGF-2 share the tubulin-mediated transport route and co-localize with heparanase in the nucleus, whereas FGFR-1 is enriched mainly in the perinuclear area suggesting a different route. The cytoplasmic RMKKK sequence of syndecan-1 is sufficient for its nuclear translocation and may therefore act as a NLS.

4.2 PAPER II
Effect of syndecan-1 overexpression on mesenchymal tumor cell proliferation with focus on different functional domains.

Human syndecan-1 was overexpressed in two malignant mesenchymal tumor cell lines: the mesothelioma STAV-AB line, which has a low expression level of syndecan-1, and the fibrosarcoma B6FS line, in which endogenous syndecan-1 is virtually absent. The cells were transfected with a human full-length syndecan-1/EGFP construct or different truncated variants: construct 78 lacking the extracellular domain with the exception of the DRKE sequence adjacent to the cell membrane (possibly important for oligomerization and shedding), construct 77 lacking the whole extracellular domain, and the RMKKK within the cytoplasmic domain, supposed to be a NLS.

Stable overexpression of the full-length syndecan-1 caused a 5-10 fold increase in syndecan-1 mRNA levels, as evaluated by quantitative real time-PCR, and a 2-3 fold increase in protein level, as analyzed by flow cytometry. Overexpression of syndecan-1 profoundly influenced the expression of the other syndecans. In the MM cells native syndecan-1 was upregulated by all constructs except for the RMKKK, while syndecan-2 was simultaneously downregulated. Syndecan-4 was upregulated by all constructs except for the 78. For B6FS cells overexpression of the full-length syndecan-1 downregulated syndecan-4. Construct 78 upregulated both syndecan-2 and 4, whereas construct 77 upregulated only syndecan-2. None of these constructs influenced significantly the expression of syndecan-3.

The proliferation rates were calculated as doubling times based on the growth curves using the WST-1 cell proliferation assay. In parallel, DNA synthesis in proliferating cells was monitored using the BrdU Cell Proliferation ELISA. Most transfectants showed longer doubling times compared with the EGFP vector control. The growth inhibition was seen in both cell lines with either the full-length syndecan-1 or its truncated constructs. To further investigate the underlying mechanism for this decrease in cell proliferation, cell cycle analysis was performed based on cellular DNA contents determined by FACS analysis. Though the length of G2/M phase was similar for the different transfectants, an increased G0/G1 phase was most evident in the RMKKK and 77 transfectants, whereas a prolonged S phase was more obvious in the full-length transfectants. This could be verified with the DNA incorporation assay, which showed a small decrease only in the full-length transfectants. Moreover, when the morphology of the stably transfected fibrosarcoma cells was monitored, all syndecan-1 constructs
resulted in a significant decrease of the cellular length/width ratio, indicating a transition to a more epithelioid phenotype, in parallel with the decreased proliferation activity.

Overall, the results show that the syndecan-1 constructs decrease the cell growth rate. The reduction seems mainly to depend on the cytoplasmic/transmembrane domains, although the effect differs when the extracellular portion is present.

### 4.3 PAPER III

**Specific syndecan-1 domains regulate mesenchymal tumor cell adhesion, motility and migration.**

We aimed to investigate the function of syndecan-1 in tumor cell adhesion and migration, with special focus on the importance of its distinct protein domains, to better understand the structure-function relationship of syndecan-1 in tumor progression.

We utilized the same two mesenchymal tumor cell lines which stably overexpress the full-length syndecan-1 or truncated variants. Various bioassays for cell adhesion, chemotaxis, random movement and wound healing were used. Furthermore, we performed gene expression microarray to analyze the global gene expression pattern influenced by overexpression of syndecan-1. We found that full-length syndecan-1 enhanced cell adhesion in a dose-dependent manner, while the truncated constructs only affected adhesion marginally, or not at all. All constructs inhibited the serum-induced chemotactic cell migration and wound closure. The full-length syndecan-1 and the 78 constructs specifically reduced cell motility/migration in the random movement assay. These results indicate that adhesion mainly depends on the extracellular domain with some minor contribution from the juxtamembrane DRKE motif. Effects of syndecan-1 on migration are more complex; the pro-adhesive effect of the extracellular domain is one factor hampering migration, but the transmembrane/cytoplasmic portions have more impact on cell motility. Gene expression microarray analysis identified a number of candidate genes coding for proteins associated with cell adhesion and migration that were differentially regulated in syndecan-1 overexpressing cells.

### 4.4 PAPER IV

**Chondroitin sulfate A regulates fibrosarcoma cell adhesion, motility and migration through JNK and tyrosine kinase signaling pathways.**

CS is abundant in connective tissue ECM and is also a constituent GAG chain of syndecan-1. Recent studies have shown that CS chains stimulate fibrosarcoma cell proliferation through a tyrosine kinase pathway, whereas they inhibit normal fibroblast proliferation, motility and migration. In this study, we examined the effect of both
endogenous and exogenous CS on fibrosarcoma cell behavior, as well as investigated their main cellular effectors.

First, the effects of endogenously synthesized GAGs were examined. Sodium chlorate was used to reduce sulfation of all PGs. This reduction inhibited the basal level of all tested functions, i.e., cell adhesion, motility and migration. Furthermore, in order to investigate the specific role of CSPGs on these functions, a β-D-xyloside was utilized. P-nitrophenyl-β-D-xyloside competitively and selectively disabled the addition of CS chains to the core protein thus specifically inhibiting CSPG biosynthesis. Our results demonstrated that the reduction in CSPG levels strongly inhibited the basal level of all tested fibrosarcoma cell functions. The effect of p-nitrophenyl-β-D-xyloside treatment was similar to that caused by the reduction in total GAG sulfation. To further investigate the possible participation of cell-associated CS chains in these functions, we used chondroitinase AC to remove cell surface-bound CS chains, and found a severely inhibitory effect on all these fibrosarcoma cell functions. The results indicate that CSPGs constitute a major class of PGs that regulate fibrosarcoma behaviors.

Secondly, in order to evaluate the role of exogenous CS chains on these fibrosarcoma cell functions, we added 10-100 µg/ml of purified CS to the culture medium. Free CS-A chains altered these fibrosarcoma cell functions at high concentrations (30 and 100 µg/ml). Cell motility and migration were enhanced, whereas cell adhesion was inhibited.

Thirdly, to elucidate the signaling pathways that mediate the CS-induced fibrosarcoma cell motility and migration, inhibitors of the main cellular signaling pathways, which regulate actin cytoskeleton rearrangements, were utilized; these inhibitors target mitogen activated-protein kinases (MAPK), including c-Jun N-terminal kinase (JNK), MEK kinase and p38. Moreover, a general tyrosine kinase inhibitor (genistein) was also used. The results showed that CS chains increased cell motility through the MAPK pathway, mainly through JNK but not MEK1/2, whereas CS-induced cell migration required a tyrosine kinase-dependent pathway.

This study indicates a crucial role for CS in mesenchymal tumor cell adhesion, motility and migration.
5 CONCLUSIONS

* Syndecan-1 and FGF-2 share the tubulin-mediated transport route and co-localize with heparanase in the nucleus. The minimal sequence requirement for the nuclear translocation of syndecan-1 is the RMKKK motif within the C1 region of core protein, serving as the NLS.

* Syndecan-1 influences the expression profile of other syndecan family members. Overexpression of syndecan-1 downregulates syndecan-2 in MM cells.

* Syndecan-1 decreases the proliferation of mesenchymal tumor cells in two ways. With the extracellular domain present, syndecan-1 leads to a longer S-phase; whereas the transmembrane/cytoplasmic domains result in a prolonged G0/G1 phase.

* Syndecan-1 decreases migration and motility, and enhances adhesion of mesenchymal tumor cells in an expression level-dependent manner. Distinct protein domains have differential effects; the extracellular domain is more important for promoting cell adhesion, while the transmembrane and cytoplasmic domains are sufficient for inhibition of cell migration.

* Reduction of endogenous CS levels inhibits fibrosarcoma cell adhesion, motility and migration, whereas exogenously added CS chains increase cell motility and migration through JNK and tyrosine kinase signaling pathways, but decrease cell adhesion.
6 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

This thesis work provides some new insights into the role of syndecan-1 in tumor progression, which merits further investigations.

Our group was the first to demonstrate that the entire syndecan-1 molecule translocates to the nucleus in several malignant and benign cell types. This thesis has identified the RMKKK sequence in the C1 region as a NLS. Another recent report has shown that heparanase decreases the level of syndecan-1 in the nucleus [58]. There are now an increasing number of publications about nuclear PGs, including syndecans [54-57]. The mechanisms behind the nuclear localization of PGs are still incompletely understood and await further studies. Indirect evidence points to possible functions of the nuclear PGs in cell proliferation, but the precise role(s) remains to be clarified.

Transfections with the present full-length syndecan-1 construct results in reduced nuclear translocation of the fusion protein compared to the native syndecan-1. This could be explained by the C-terminal attachment of the EGFP, which might hamper binding to the tubulin transporting structures. An alternative construct with N-terminal EGFP should therefore be tested. The RMKKK sequence should then be deleted from this construct, to further confirm the NLS role of this motif. Further investigation on the partner molecules of nuclear syndecans will give a better insight into their possible functions.

Another interesting finding is that syndecan-1 influences expression of other syndecan members, seemingly in a compensatory manner. Overexpression of syndecan-1 downregulated syndecan-2, and upregulated syndecan-4, in MM cells. There might be some overlapping functions between syndecan members, which must be taken into consideration, when evaluating the effects on tumor cell behavior. Simultaneous downregulation of syndecan-2 and -4, which both have higher endogenous expressions than that of syndecan-1 in MM, might be an approach to elucidate which effects are directly associated with syndecan-1, and which are secondary to the altered expressions of the other syndecan family members.

The core protein and the GAG chains are both involved in biological processes important for tumor progression, and the functional domains participate in different ways. The different domains of the protein core are also involved in different ways in the regulation of migration and proliferation of these tumor cells. The different effects obtained with 77 and 78 constructs point towards importance of the short extracellular DRKE motif close to the cell membrane. This indicates that the formation of homo- or hetero-oligomers also participates in the regulation of these processes.

Better knowledge about the precise mechanisms for these structure-function relationships, including specific protein and oligosaccharide sequences, may provide a basis for subsequent targeted therapy in these tumors. One interesting concept concerns possible interactions with specific GAG sequences, where specific interactions may be
interfered with using soluble HS oligosaccharides to perturb the function of cell surface HSPGs. We have established a library of heparin fragments, and initial results, using capillary zone electrophoresis, indicate that specific fragments have distinct binding capacities for different growth factors. The results are, however, preliminary and need to be further investigated.

Gene expression microarray allows a quick screening for genes whose expression levels are influenced by the manipulation of the target gene. This helps to find partner molecules involved in specific functions. Furthermore, an analysis of the pathway network may further explain the underlying mechanisms.

Taken together, the results in this thesis show that syndecan-1 is involved in regulation of the proliferation and dissemination of tumor cells. A better understanding of the mechanisms behind these functions could make this family of PGs a potential target for future therapy.
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8 REFERENCES


