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TRANSLATIONAL STUDIES OF SYNDECAN-1 AS ANGIOGENESIS INHIBITOR - FROM BASIC RESEARCH TO CLINICAL APPLICATIONS

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Cover illustration: Front cover is design by Iman Shahbandeh and illustrates the main concept of the thesis combined with a poem by Hussein Panahi that says:
Behind this window, science is talking about sun while holding an umbrella of doubt.

پشت این پنجره، علم
چتر شک دستشه و از افتاب حرف میزنه.

TRANSLATIONAL STUDIES OF SYNDECAN-1 AS ANGIOGENESIS INHIBITOR – FROM BASIC RESEARCH TO CLINICAL APPLICATIONS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Joman Javadi

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To my beloved Sam

میراث من!
نه به قید قرعه،
نه به حکم عرف،
یکجا سند زدم همه را به حرمت چشمانت،
به نام تو!

Do not go with the flow. Be the flow.

Elif Shafak

POPULAR SCIENCE SUMMARY OF THE THESIS

Asbestos is a group of minerals that are found in soil and rocks in many parts of the world. Asbestos fibers are strong, resistant to heat and do not conduct electricity. Because of these characteristics, they are useful in the industry. Asbestos fibers are in the air and when they are inhaled, they stick to mucus in the throat and end up in the lungs and the mesothelial surface. These fibers injure the mesothelial cells and cause chronic inflammation, DNA damage and finally malignant mesothelioma.

Malignant Mesothelioma (MM) is an aggressive and uncurable type of cancer that is up to 80% caused by asbestos. People who worked with asbestos or have been exposed to it (male are involved more than female) have the highest risk of getting mesothelioma cancer.

It usually takes 20-50 years after asbestos exposure for the tumor to appear, and once diagnosed most patients has a short survival time (in the order of one year or less). There are four different locations of MM within the body, the most common involves the pleural. Shortness of breath, chest pain, loss of weight, vomiting, coughing blood and increasing of the pleural fluid are first symptoms for pleural malignant mesothelioma.

Attempts to treat the tumor can involve surgery, radiation, or chemotherapy. Surgery can in some countries be an option during early stages of the disease, although the benefits of this option are still questioned. During more advanced stages treatment is confined to chemotherapy, sometimes supported by radiotherapy. Although today's therapeutic options will not cure the patient, therapy of mesotheliomas will have better effects n early stages and when initiated soon after diagnosis. It is therefore urgent to learn more about the progression of mesothelioma progression, in order to detect the tumor earlier and, if possible, tailor treatment options for this particular kind of tumor.

The aim of this thesis was to study the role of Syndecan-1 (a cell membrane protein) in malignant mesothelioma angiogenesis, the ability of the tumor to disseminate, the survival of patients, and to find useful biomarkers to be analyzed in parallel. For this purpose, in **paper I** we identified angiogenesis-related genes which were affected by Syndecan-1. Moreover, we showed that higher level of syndecan-1 inhibits proliferation, tube formation and wound closure in endothelial cells. In **paper II** we studied the effect of Syndecan-1 on epithelial-mesenchymal transition of lung cancer (A549) and fibrosarcoma (B6FS) cell lines. We showed that syndecan-1 overexpression associated with less invasive fibrosarcoma cells.

In addition to this, in **paper III** we focused on finding novel biomarkers for earlier diagnosis of the disease. We found nine diagnostic biomarkers for malignant mesothelioma. Additionally, we showed that four out of nine of these biomarkers can be used for distinguishing malignant mesothelioma from lung carcinoma. In **paper IV,** we characterized extracellular vesicles, isolated from pleural fluid. We showed that these small vesicles carry biological molecules which can affect other tumor and non-tumor cells behavior in the extracellular environment.

More studies to better understand the mechanisms involved in malignant mesothelioma progression are required. Additionally, combining several biomarkers can help us promote earlier diagnosis and longer patients' survival.

ABSTRACT

Malignant mesothelioma (MM) is a highly aggressive primary tumor of the pleura, associated with poor prognosis. MM is mainly related to exposure to mineral fibers such as asbestos. The diagnosis of MM, despite multiple diagnostic tools, is challenging and treatment options are limited. Previous studies have shown that angiogenesis plays an important role in MM progression thus, anti-angiogenic agents show promising use in MM therapy. In addition to that, detection of new soluble diagnostic or prognostic biomarkers may improve patients' outcomes and therapeutic options.

Syndecan-1 (SDC-1) is a membrane proteoglycan which regulates various biological processes in tumor cells by acting as a co-receptor for growth factors. SDC-1 is also a significant mediator of cell-cell and cell-matrix interactions. Cell membrane SDC-1 can be shed by sheddases and its Heparan Sulfate (HS) degraded by heparanase-1 (HPA-1). Additionally, syndecan-1 can translocated to the nucleus through a tubulin-dependent mechanism. Loss of cell membrane SDC-1 is associated with epithelial-mesenchymal transition (EMT) and worse prognosis, while nuclear translocation of SDC-1 seems to have the opposite effect.

The purpose of this thesis work was to investigate the role and potential value of syndecan-1 as an angiogenic factor and as a diagnostic biomarker for MM.

We showed the inhibitory effect of SDC-1 overexpressing mesothelioma cells on migration, proliferation, and tube formation capacity in endothelial cells. We also found that silencing of SDC-1in MM cells promoted experimental wound closure but had no effect on tube formation in endothelial cells (**paper I**). These effects were mediated by angiogenic factors comprising Angiopoietin-1, FGF-4, HGF, TGF-β1, TIMP-1, TSP-1, and TRG1-β1 which were significantly up- or down regulated by SDC-1 overexpression, as well as IL8 which was significantly up-regulated by SDC-1 silencing. In the same study, we evaluated the expression level of SDC-1 and VEGF in pleural effusion and showed the prognostic value of VEGF in malignant mesothelioma patients.

We furthermore studied the potential value of Angiopoietin-1, HGF, and TIMP-1 among other angiogenic-related proteins as diagnostic and prognostic biomarkers in patient material. We found that Galectin-1, Mesothelin, Osteopontin, VEGF, HGF, shed SDC-1, MMP-7, NRG1-β1, and TIMP-1 were significantly higher in malignant pleural mesothelioma patients. Additionally, we showed that shed SDC-1, MMP-7, Mesothelin, and Galectin-1 significantly discriminated MM from metastatic adenocarcinoma patients (**paper III**). We further verified our result in **paper I** and showed that shed SDC-1 and VEGF were prognostic in MM patients.

The role of nuclear SDC-1 on epithelial-mesenchymal plasticity of tumor cells – a key event in tumor spread and metastasis – was studied in **paper II.** Using tumor cell lines, we found that loss of nuclear SDC-1 was associated with cellular elongation and induced EMT in

human lung adenocarcinoma cells. Further investigation revealed that nuclear SDC-1 reduce mesenchymal properties and invasiveness of fibrosarcoma cells.

Extracellular vesicles have recently gained much interest for their ability to mediate cell-to-cell communication. In **paper IV** we characterized extracellular vesicles including, apoptotic bodies, microvesicles, and exosomes, derived from pleural effusion from malignant pleural mesothelioma, metastatic lung adenocarcinoma, and benign patients. We found that the size of exosomes was in accordance with previous studies, but their concentration varied between individuals in the same patients group. Additional characterization showed that the presence of CD2, CD8, CD9, CD81, CD24, CD44, CD105, CD146, CD133, MCSP, and ROR1 were higher in the exosome fraction compared with microvesicles and apoptotic bodies whereas, CD40 and CD45 were lower. Furthermore, we demonstrated the presence of the angiogenesis -related proteins which we studied in **paper III** in extracellular vesicles.

LIST OF SCIENTIFIC PAPERS

I. **JOMAN JAVADI****, Ghazal Heidari-Hamedani**, Angelika Schmalzl, Tünde Szatmári, Muzaffer Metintas, Pontus Aspenström, Anders Hjerpe and Katalin Dobra*
Syndecan-1 overexpressing mesothelioma cells inhibit proliferation, migration and tube formation of endothelial cells by synchronized action of angiogenesis related factors. cancers, 2021, 13 (4), 655

II. Ashish Kumar-Singh, Malgorzata Parniewska, Nikolina Giotopoulou, **JOMAN JAVADI**, Wenwen Sun, Katalin Dobra, * Anders Hjerpe and Jonas Fuxe, *
Nuclear syndecan-1 regulates epithelial-mesenchymal plasticity in tumor cells. Manuscript/submitted.

III. **JOMAN JAVADI** *, Katalin Dobra, Anders Hjerpe
Multiplex Soluble Biomarker Analysis from Pleural Effusion. Biomolecules, 2020, 10 (8), 1113

IV. **JOMAN JAVADI**, Andre Görgens , Hanna Hjerpe Vanky, Dhanu Gupta, Anders Hjerpe, Daniel Hagey, Katalin Dobra
Characterization of different extra-cellular vesicles populations derived from pleural effusion. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AD	Adenocarcinoma
ADAMs	A-disintegrin-and-matrix metalloproteinases
AP-1	Activator protein 1
ARRDC-1	Arrestin domain-containing protein-1
AUC	Area under the curve
BAP-1	BRCA 1- associated protein-1
BE	Benign
CD	Cluster of differentiation
CDKN2A	Cyclin dependent kinase inhibitor 2A
CEA	Carcinoembryonic antigen
CRS	Cytoreductive surgery
CT	Computerized tomography
cDNA	Complementary DNA
dsDNA	Double-strand DNA
ECM	Extra-cellular matrix
ED	Ectodomain
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMA	Epithelial membrane antigen
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinases
EpCAM	Epithelial cell adhesion molecule
ESCRT	Endosomal sorting complexes required for transport
EVs	Extracellular vesicles
Exts	Glycosyl-transferases
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridization
GAG	Glycosaminoglycan

GalNAc	N-acetylgalactosamine
GlcA	Glucuronic acid
GlcNAc	N-acetylglucosamine
GLUT-1	Glucose transporter 1
HA	Hyaluronan
HEG-1	HEG homolog 1
HGF	Hepatocyte growth factor
HUVEC	Human umbilical vein endothelial cells
ICC	Immunocytochemisry
IdoA	Iduronic acid
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IL	Interleukin
ILVs	Intraluminal vesicles
LDH	Lactate dehydrogenase
LOH	Loss of heterozygosity
MCSP	Melanoma chondroitin sulphate proteoglycan
MM	Malignant mesothelioma
MMPs	Matrix metalloproteinases
MPF	Megakaryocyte-potentiating factor
MPM	Malignant pleural mesothelioma
MSLN	Mesothelin
MTAP	Methyladenosine phosphorylase
MT-MMPs	Membrane-tethered matrix metalloproteinases
mRNA	Messenger RNA
Ndsts	Deacetylase-N-sulfotransferases
NF2	Neurofibromatosis 2
NRG1-β1	Neuroglin-1 beta-1
OPN	Osteopontin
PAR	Partitioning defective
PDGF	Platelet drived growth factor

PE	Pleural effusion
PI3K	Phosphatidylinositol-3-kinase
PLD	Phospholipase D
RNS	Reactive nitrogen species
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RTKI	Receptor tyrosine kinase inhibitor
SCRIB	Scribble
SDC-1	Syndecan-1
siRNA	Small interfering RNA
SMRP	Soluble mesothelin-related protein
SV-40	Simian virus 40
TIMP-1	Tissue inhibitor of metalloproteinases
TF	Transcription factor
TGF-β1	Transforming growth factor beta 1
TJ	Tight junction
TMD	Transmembrane domain
TNF-α	Tumor necrosis factor α
TSGs	Tumor suppressor genes
TSP-1	Thromboaspondin-1
TTF-1	Thyroid transcription factor 1
VEGF	Vascular endothelial growth factor
WST-1	Water soluble tetrazolium salt 1
WT-1	Wilms tumor protein 1
ZEB	Zinc Finger E-Box Binding
ZO-1	Zonula occludens
α-SMA	α smooth muscle actin

1 INTRODUCTION

The key to growth is the introduction of higher dimensions of consciousness into our awareness.

Lao Tzu

1.1 PLEURAL EFFUSION

1.1.1 Structure of the normal pleura

The lungs are enclosed by the pleura, which consists of mesothelial cells. The flat superficial mesothelial cells rest on a basement membrane under which fibroblast-like mesothelial cells are found. The pleura also covers the thoracic wall, thus the inner visceral layer and the outer parietal layer form together the pleural cavity. This cavity is one out of four serous cavities in the body (the others are pericardium, peritoneum, and tunica vaginalis testis in male). In adults, the pleural cavity contains physiologically a tiny amount (0.1 – 0.3 ml/kg) of pleural fluid which is secreted from the vasculature of parietal and absorbed into the lymphatic circulation. Under normal conditions the pleural fluid has mainly two functions which are, lubricating the pleural surface to allow frictionless movements during breathing, and generating surface tension between inner and outer layer of the pleura. The second function allows the thoracic cavity to expand during inhalation [1-3].

1.1.2 Etiology and pathogenesis

Oncotic and hydrostatic pressure gradient between parietal and visceral blood capillaries regulate secretion, volume, and movement of pleural fluid. Lymphatic vessels in the parietal pleura drains pleural fluid into lymphatic capillaries. In normal conditions, there is a balance between production and resorption of the fluid [4]. Accumulation of pleural fluid is due to a wide variety of pathologic conditions and diseases such as heart failure, pleural infections, and malignancies, either because of excessive production or decreased resorption of the fluid or both [5, 6]. Pleural fluids caused by altered hydrostatic and/or oncotic pressure are classified as transudate. Exudates, on the other hand, is caused by increased vessel permeability, this fluid showing higher concentration of proteins. Heart failure, liver cirrhosis, nephrotic syndrome and other causes of hypoalbuminemia are common causes of transudate whereas, exudates are seen in inflammatory processes and in malignancies [6, 7].

1.1.3 Management and treatment

Congestive heart failure, pleuro-pneumonia, and cancer are the most common causes of pleural effusions (PE). If a pleural effusion is suspected, physical examinations, chest x-ray, computerized tomography (CT) scan, chest ultrasound, and patient's clinical history are required to reveal the etiological causes of effusion. From clinical point of view, chest imaging and measuring PE/serum ratio of total protein and LDH, and PH are useful to diagnose and distinguishing transudate from exudate [7-9]. Patients with PE might present

symptoms such as dyspnea, pleuritic chest pain, and dry cough or be asymptomatic depending on the volume of pleural fluid. Thoracentesis and thoracoscopy are therapeutic options for treatment and relief of dyspnea and chest pain of the patients [10].

1.2 MALIGNANT MESOTHELIOMA

1.2.1 Etiology and pathogenesis

Malignant mesothelioma (MM) is a highly aggressive, and so far, incurable primary tumor of mesothelial cells. MM is mainly caused by exposure to mineral fibers such as asbestos [11, 12]. Asbestos consists of crystalline silicates fibers that cause oxidative stress and chronic inflammation. These processes lead to alteration of gene expression and intracellular signaling pathways through generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Up-regulation of nitric oxide radical and peroxynitrite ion by asbestos lead to DNA damage and modification of DNA bases from G to T. Previous studies showed that phosphorylation of epidermal growth factor receptor (EGFR) by reactive oxygen species in rat mesothelial cells, leads to activation of extracellular-regulated kinases (ERKs). Activation of ERKs is linked to cellular processes such as cell proliferation, differentiation, and apoptosis. The duration of exposure and type of asbestos correlate with the risk of developing malignant mesothelioma [13-16]. Erionite, radiation, simian virus 40 (SV-40) and genetic predispositions such as BRCA1-associated protein 1 (BAP1) gene are less common risk factors for MM. Malignant mesothelioma has a high mortality rate and occurs after a long (20 to 50 years) latency period between commencement of asbestos exposure and diagnosis. The survival time after diagnosis is less than one year [17, 18].

1.2.2 Localization of malignant mesothelioma

Malignant pleural mesothelioma (MPM) is the most common (70%), arising of mesothelial cells of the pleura. Most cases of malignant pleural mesothelioma start by inhalation of asbestos fibers that later are captured in lungs and mesothelial cells. MPM has a poor prognosis, short survival time and no effective treatment options [19, 20]. Dyspnea and pleural effusion are common first symptoms of MPM [21].

Less than 30% of malignant mesothelioma cases originate from peritoneum. Compared to MPM, it seems to be less associated to asbestos exposure and more often develops in younger individuals and in women. Median survival time is less than eight months, but cytoreductive surgery (CRS) in early-diagnosed patients may increase this time to 30-90 months [22, 23].

Pericardial mesothelioma is rare (< 1%) and aggressive with a shorter survival time of 5-7 months compering with pleural and peritoneum mesothelioma. Chest pain, shortness of breath, cardiac tamponade, heart failure, constrictive pericarditis and pericardial effusion are pericardial mesothelioma’s symptoms [24].

Malignant pleural mesothelioma is the subject of this thesis and from now on I will focus on this type of malignant mesothelioma.

1.3 MALIGNANT PLEURAL MESOTHELIOMA

1.3.1 Epidemiology of malignant pleural mesothelioma

Asbestos exposure is the main risk factor in 90% of the cases. Although asbestos is banned in most developed countries, in Russia, India, Brazil, and China it is still in use. Consequently, the incidence of MPM will increase in the next few decades due to earlier asbestos exposure and a long latency period until the disease develops [25, 26]. Hence, more diagnostic biomarkers for earlier detection of MPM will be needed.

1.3.2 Diagnosis of malignant pleural mesothelioma

The diagnosis of MPM can be obtained in different ways. It can be based on histological biopsy material or the cytological evaluation of cells in an effusion. Although not considered sufficient for a definitive diagnosis, the analysis of soluble biomarkers in the pleural effusion may offer strong support to effusion cytology.

Malignant pleural mesothelioma is often diagnosed at a late stage when the tumor cells are less responsive to current therapies. The survival time of the patients are only in the order of one year. Over the past decade, improving diagnostic and prognostic tools has been subject of considerable interest.

1.3.2.1 Histological and cytological diagnosis

Histological diagnosis is based on both morphology and positive immunohistochemical markers for mesothelial cells such as calretinin and Wilms tumor 1 (WT-1) and excluding markers such as BerEp4 and CEA.

Histologically, malignant pleural mesothelioma is classified into three subtypes: epithelioid (60-80%), sarcomatoid (<10%) and biphasic (10-15%) [27]. These features can be identified by routinely stained tissue. The epithelioid subtype contains polygonal, oval, or cuboidal cells is less aggressive with longer survival time, comparing with the sarcomatoid subtype which contains spindle cells. The biphasic subtype of MPM contains both epithelioid and sarcomatoid cells in the same tumor [17, 27, 28] and the proportion of epithelioid or sarcomatoid component determines the survival time.

Pleural effusion is a common symptom of MPM and often the first available material for cytological diagnosis. Sensitivity of the cytological diagnosis varies in different laboratories between 30%-75%. The difficulty of the diagnosis lies in overlapping of the atypical features and immunoreactivity among benign and malignant cells [28]. Cytological examination of an effusion often reveals abundant mesothelial cells with various degree of atypia arranged frequently in 3D papillary structures. Some MPM effusions are, however, devoid of such typical cytomorphology, and the cytological diagnosis must always be based on typical immunocytochemical reactivities. When the cytomorphological diagnosis obtained, combining morphology with immunocytochemistry, and also supported by ploidy analyses, using fluorescence in-situ hybridization (FISH); a definitive diagnosis of MPM can be obtained up to 73% of all cases. Like in histopathology these adjuvant methods are compulsory for a reliable diagnosis [27, 29].

1.3.2.2 FISH and IHC/ICC diagnostic markers

The pathological diagnosis and distinguishing benign from malignant conditions are challenging. Hence, additional methods are used to further support diagnosis and differentiate MPM from benign and other types of cancer. A variety of IHC markers such as the BRCA1 associated protein 1 (BAP1), HEG homolog 1 (HEG-1), methyladenosine phosphorylase (MTAP), glucose transporter 1 (GLUT-1), epithelial membrane antigen (EMA), desmin, CD44 molecule (CD44), and FISH analysis of cyclin dependent kinase inhibitor 2A (CDKN2A) gene, better known as p16, are useful for diagnosis of MM. Among these markers, evaluation of BAP1 by immunohistochemistry and homozygous deletion of p16 by FISH are considered specific for defining a malignant condition, i.e., distinguishing MM from reactive mesothelial proliferations [30, 31].

BRCA1-associated protein1 (BAP1) is a tumor suppressor which regulates cell cycle, cell proliferation and cell death. Mesothelioma cells often show somatic mutation of BAP1 gene which correlates with loss of nuclear immunohistochemical staining while benign and inflammatory cells retain the IHC staining even with one wild-type copy of BAP1. Desmin is a muscle marker which in a similar way is not expressed in malignant mesothelial cells. BAP1 and desmin are markers commonly used to distinguish benign and malignant mesothelial populations by immunocytochemistry [30, 32].

A common genetic alteration in MM is the homozygous deletion of cyclin-dependent kinase inhibitor 2A (CDKN2A), also known as p16. Loss of p16 gene in tissue sections or pleural effusion cytology specimens can be detected by fluorescence in situ hybridization (FISH). The two major benefits of p16 FISH analysis are that no benign samples show homozygous loss of p16 and it give prognostic information. Deletion of p16 gene correlates with more aggressive tumor [30, 33].

1.3.2.3 Soluble biomarkers

Earlier diagnosis may provide better chances for successful chemotherapy. Generally, a biomarker is a measurable distinctive indicator of a certain biological process that can help with the diagnosis, hopefully leading to better patient prognosis. So far, several diagnostic and prognostic biomarkers have been investigated for MM including mesothelin, hyaluronan, osteopontin, and miRNAs [34, 35].

Mesothelin

Mesothelin is a 71-KDa blood-based precursor protein which expressed at low level in normal mesothelial cells of pleura, peritoneum and pericardium [36]. Mesothelin can be cleaved by endoprotease furin into two proteins: a shed form (N-terminal megakaryocyte-potentiating factor (MPF)), which is secreted into the blood, and a membrane- bound mesothelin (C-terminal glycosylated phosphatidylinositol-linked glycoprotein) [17, 37]. Soluble mesothelin can be released from the cell to the blood and pleural effusion. Several studies have shown that, this shed form of mesothelin is elevated in many cancers including

MPM, ovarian cancer and pancreatic cancer. There is a correlation between expression level of soluble mesothelin and tumor size and survival time. Therefore, mesothelin can be used as diagnostic and prognostic marker for MPM [38-40].

Hyaluronan

Hyaluronan (Hyaluronic acid/HA), a member of glycosaminoglycan family, is a negatively charged high-molecular-weight (HMW) polysaccharide (4–800 KDa) which consists of the repeating disaccharide (glucuronic acid and N-acetylglucosamine). HA is a widely distributed component of ECM in normal connective tissues. Different cellular processes including cell migration, differentiation, cell growth and adhesion are influenced by this glycosaminoglycan. Hyaluronic acid is often highly expressed in pleural effusion and serves as a diagnostic biomarker for MPM [41-44].

Osteopontin

Osteopontin (OPN) is a calcium-binding glycol-phosphoprotein which is secreted by osteoclasts, cardiac fibroblasts, macrophages and activated T cells. Binding of osteopontin to its receptors (αvβ1, αvβ3, αvβ5 and CD44) play roles in tumor development, cell migration and cell matrix interaction. Higher levels of osteopontin in serum, plasma and pleural effusion have been found in MPM patients compared with benign subjects. Hence, osteopontin can be used as another diagnostic marker for MPM [45-47].

Micro-RNAs

Micro-RNAs (miRNAs) are short (~22 nucleotides) single strand non-coding RNAs that are involved in differentiation, proliferation, and apoptosis by regulating the gene expression at the post-transcriptional levels. It is important to note that alterations in the expression pattern and level of miRNAs associates to carcinogenesis. The expression of several miRNAs is altered in MPM (miR-30b*, miR-195*, miR-32*, miR-584, miR-483-3p, miR-9, miR-7-1* etc.). In addition, miR-143, miR-210 and miR-200c distinguishing MPM from primary and metastatic lung adenocarcinoma and benign diseases. Data suggest that miRNAs can be used as diagnostic biomarkers for MPM [48-50].

Exosomes

Exosomes are small membrane nanovesicles (30-150 nm) of endosomal origin that are secreted by different cell types including epithelial and tumor cells. Exosomes contain nucleic acids (including DNA, mRNA, noncoding RNA, and microRNA), and signaling proteins (transmembrane and cytoskeletal proteins) which are involved in various functionalities of the tumor cells including, the establishment of tumor environment, tumor growth and progression. Exosomes are released in abundance from tumor cells into different body fluids and can be used as biomarkers and cell-cell or cell-matrix interactions mediators [51-55].

1.3.3 Molecular processes in malignant pleural mesothelioma

Alterations in gene expression and loos of protein function are frequent in MPM. Loss of heterozygosity (LOH) leads to alteration of some tumor suppressor genes (TSGs) such as *p14*, *p15*, *p16* at 9p21, NF2 at 22q12, and BAP-1 at 3p21 in MPM [56, 57]. Homozygous deletion of 9p21 (CDKN2A gene), results in loss of p16INK4a and p14ARF, which are cell cycle regulators. p53 and pRb are indirectly inactivated in MPM. p14ARF stabilizes p53 through inactivation of MDM2 and p16INK4a stabilizes pRb by inhibiting CDK4/6 [58].

In addition to inactivation of tumor suppressor genes, activation of transcription factors such as activator protein-1 (AP-1) and β-catenin also regulate a variety of cellular processes including proliferation and apoptosis [59]. Moreover, many growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF) A and B, and endothelial growth factor (EGF) are involved in the tumor development and pathogenesis of MPM (Figure 1) [60-65]. VEGF plays an important role in regulating angiogenesis of mesothelioma cells which has a prognostic value in MPM.

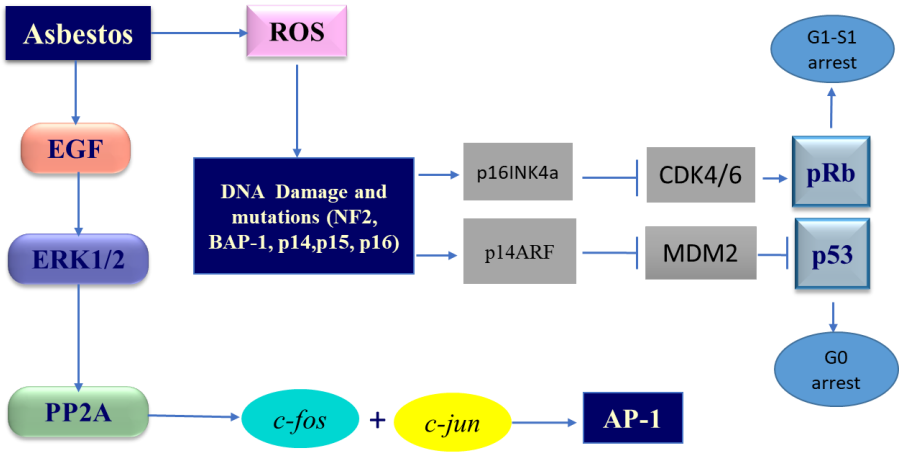

Figure 1. A schematic diagram of the main players in pathogenesis of malignant mesothelioma. Several receptors and growth factors are activated by asbestos which leads to phosphorylation of RTKs and other transcription factors which promote cell proliferation, and inflammation. Genetic changes including mutations caused by ROS.

1.3.4 Epithelial to mesenchymal transition in malignant pleural mesothelioma

Epithelial-to-mesenchymal transition (EMT) is a physiological and pathological process that occurs during cancer progression. It confers motility to the cancer cells necessary for invasiveness and subsequent metastasis. During EMT cells lose their cell-cell contacts (through disassembly of adherens-, tight-, and gap- junctions) and apical-basal polarity (through disruption of polarity complexes such as scribble (SCRIB) and partitioning defective (PAR)) [66, 67]. Additionally, cells change their transcriptional programs which leads to down-regulation of epithelial markers (E-cadherin, zonula occludens (ZO-1) and β-catenin) and up-regulation of mesenchymal markers (vimentin, fibronectin, and smooth muscle actin (α-SMA). EMT is regulated by a variety of transcription factors including ZEB-1, Snail, Twist (Figure 2) which down-regulate E-cadherin gene [68-70].

The differentiation of MPM has a major impact on patient survival. The epithelioid subtype of MPM has a better prognosis compared to the sarcomatoid and biphasic subtypes, hence suggesting a role of EMT in malignant pleural mesothelioma [71, 72]. The EMT master regulator master genes, ZEB-1, Snail, and Twist are up-regulated in MPM. Among other growth factors including, HGF, TNF-α, FGF-2, and EGF induce EMT in MPM. TGF-β on the other hand has a crucial role in down-regulating E-cadherin [71, 73].

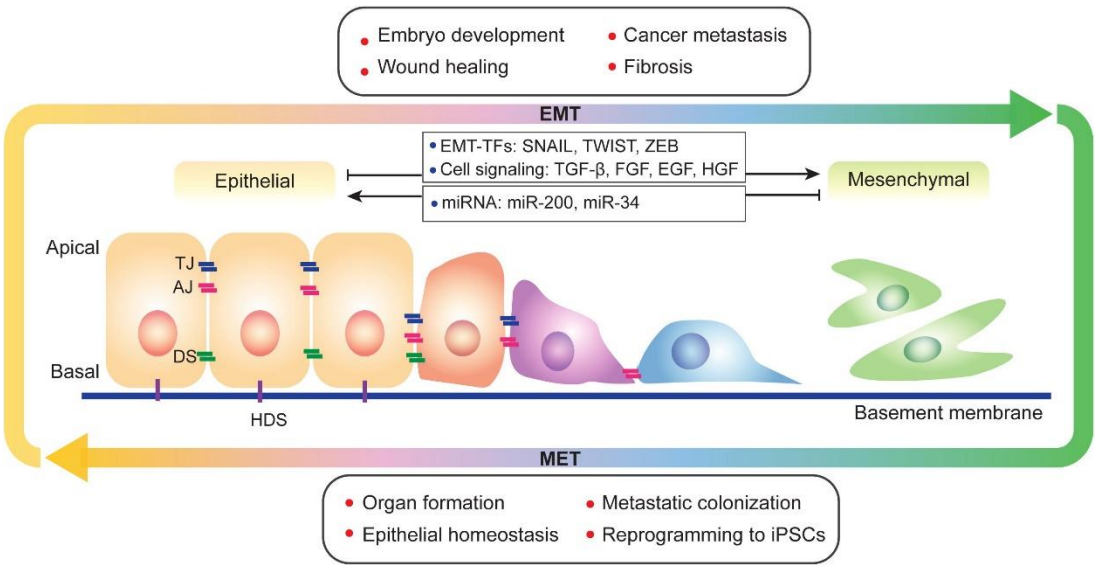

Figure 2. The basic of epithelial-mesenchymal transition. Epithelial cells lose their polarity and cell-cell contact and become mobile mesenchymal cells. Epithelial and mesenchymal markers are listed. MPM has an intermediate phenotype, co-expressing both epithelial and mesenchymal markers, but retained ability to shift from one phenotype to the other [67].

1.3.5 Angiogenesis in malignant pleural mesothelioma

Forming of new blood vessels or angiogenesis is a general prerequisite required for tumor growth and metastasis. Angiogenesis has an important role in the progression and invasiveness of cancer and its inhibition has become the target for intense development of malignant diseases treatment [74].

Pro-angiogenic factors, secreted from tumor cells, are stimulating angiogenesis in solid tumors. Secretion of pro-angiogenic factors from tumor cells leads to creation of immature, and permeable blood vessels. There are several growth factors, including Vascular endothelial growth factor (VEGF), Transforming growth factors (TGF-β) families, and platelet-derived growth factor (PDGF), involved in the formation of defective new blood vessels. Additionally, a tumor angiogenetic phenotype is depending on the balance between pro-angiogenic and anti-angiogenic factors activity [75-78].

In MPM, VEGF, matrix metalloproteinases (MMPs), hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), PDGF, and interleukin-8 (IL-8) are positive regulators of angiogenesis. Among these angiogenic factors, VEGF is the most potent factor which significantly increases vascular permeability and tumor aggressiveness [79, 80]. VEGFs act via their receptors and this interaction leads to receptor dimerization and phosphorylation which furthermore leads to activation of signaling pathways. Bevacizumab (anti-VEGF antibody), Vendetanib (triple tyrosine kinase inhibitor) and E7080 (multi-tyrosine kinase inhibitor) are antibodies which neutralize VEGF activities. VEGF inhibitors in combination with chemotherapeutic agents such as pemetrexed will inhibit hyper-permeability and angiogenesis of endothelial cells which can be used as a therapeutic strategy against MPM [79]. In addition, Nintedanib, another receptor tyrosine kinase inhibitor (RTKI) that inhibits VEGF/FGF receptors 1-3, PDGF receptors α/β, and Src family [81, 82] has been proved to be effective in MPM.

1.3.6 Treatment of malignant pleural mesothelioma

Despite greater understanding of MPM tumorigenesis and advanced technologies, malignant pleural mesothelioma is still an often treatment resistant malignancy and its treatment remains a challenge. Depending on the tumor subtype, epithelioid, sarcomatoid and biphasic, median overall survival is 12-27, 7-18 and 8-12 months respectively [83, 84]. The main therapeutic strategies for MPM are, chemotherapy and surgery in combination with chemotherapy or radiotherapy (RT) or both, although an optimal treatment has not been established. Multimodality therapy of MPM including surgery followed by chemotherapy and radiotherapy may prolongs patient's survival time [85, 86].

Cancer immunotherapy is a new promising treatment option for MPM patients. The expression of immune-checkpoint proteins such as PD-L1 is altered in tumor cells. It has been shown that PD-L1 is overexpressed in sarcomatoid subtype of MPM. PD-L1/PD-1 is a negative regulator of active T-cells proliferation. Immune-check point inhibitors such as

pembrolizumab and nivolumab have shown promising development in many tumors including MM [87, 88].

1.4 SYNDECANS

1.4.1 General features

Proteoglycans (PGs) are biologically active macro-molecules, produced by most eukaryotic cells. PGs consist of a core protein with covalently attached glycosaminoglycan (GAG) chain. Proteoglycans, based on their protein core, composition of their GAG chain, localization, and size, are classified into three groups. Syndecans and glypicans are cell membrane proteoglycans [89, 90].

The syndecan (SDC) family in vertebrates consists of four members (SDC-1, SDC-2, SDC-3, and SDC-4) and belongs to the type I transmembrane heparin sulfate proteoglycans family [91, 92]. In adult tissues, syndecan-1, which is the focus of this thesis, expressed mostly in the epithelial cells, syndecan-2 in connective tissues, syndecan-3 in neural tissues and cartilage while syndecan-4 is ubiquitously expressed [93]. The synthesis of the syndecan core proteins occurs in ribosomes whereas subsequent modification and binding of glycosaminoglycans occurs in Golgi. Later, Syndecans translocate to the cell membrane by exocytosis [94].

1.4.2 Syndecans structure

Syndecan core proteins have a similar structure, consisting of an extracellular domain, a highly conserved transmembrane domain, an intracellular domain of about 30 amino acid length [95, 96].

The extracellular domain (ED) of Syndecans carries covalently attached GAG chains that are regulating many of syndecans's cellular functions [97]. The biosynthesis of the GAG side chains is performed by enzymes, such as exostosin glycosyl-transferases (Exts) and deacetylase-N-sulfotransferases (Ndsts), activity. Exts enzymes are required for HS chain elongation, by adding repeating disaccharide units, while Ndsts are required for HS chain modification, by N-deacetylation of N-acetylglucosamine residues and N-sulfation of same amino group [98-100].

The transmembrane domain (TMD) of Syndecans plays role in dimerization and interaction of these proteoglycans with other receptors and proteins respectively [101-103]. This domain is highly homologous and contains GXXXG motif which modifies homo- and hetero-dimerization of Syndecans. Additionally, the TMD transmits signals to the cytoplasmic domain which regulates cell signaling [104].

The cytoplasmic domain (CD) of Syndecans consists of two highly conserved regions (C1 and C2) which flank a variable region (V1) [105, 106]. This domain of Syndecans mediates interaction of Syndecans with other cytoplasmic proteins. For example, binding of C1 region of syndecan-2 to ezrin/radixin/moesin (ERM) proteins links syndecan-2 to actin cytoskeleton.

Moreover, C region of syndecan-1 contains MKKK motif which mediates endocytosis through activation of ERK and Src kinases. In addition to this, we previously reported that RMKKK motif av syndecan-1 mediates nuclear translocation of syndecan-1 [107-109]. The variable region is differing between the four different Syndecans. Syndecan-4 V region contains KKXXXKK motif which bind to phosphatidylinositol 4/5-biphosphate and stabilizes SDC-4 dimers. Additionally, interaction of this motif with protein kinase C-alpha (PKCα), and Rac-1 regulates focal adhesion and migration, respectively [110, 111].

Heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS) are linear sulfated polysaccharides which are attached covalently to the ED of syndecans via serine residues [89, 112]. SDC-1/3 carry HS and CS whereas SDC-2/4 carry only HS (Figure 3). GAG's biosynthesis begins with the binding of xylose to serine residues on core protein followed by the addition of two galactose (Gal) and a glucuronic acid (GlcA) units. This tetrasaccharide is common between HS and CS GAGs [113]. HS synthesizes by adding of a repeating disaccharides regions consist of D-glucuronic acid (GlcA) and N-acetyl-glucosamine (GlcNAc) to the tetrasaccharide. Whereas in CS addition of N-acetylgalactosamine (GalNAc) and D-glucuronic acid (GlcA) is subsequent step [114]. Following synthesis, modification of HS initiates by N-deacetylation/N-sulfation of GlcNAc by N-deacetylase/N-sulfotransferase (NDST) enzyme. C5-epimerization of GlcA into iduronic acid (IdoA), 2-O-sulfation in the C2 position of IdoA and GlcA, and 6-O-sulfation of GlcNAc/GlcNS are other modifications of HS [115]. Sulfation of the CS polymer occurs at C4/C6 of GalNAc residues by sulfotransferase enzymes [113]. Highly sulfated GAGs regulate different cellular processes by interacting with a wide range of proteins [116].

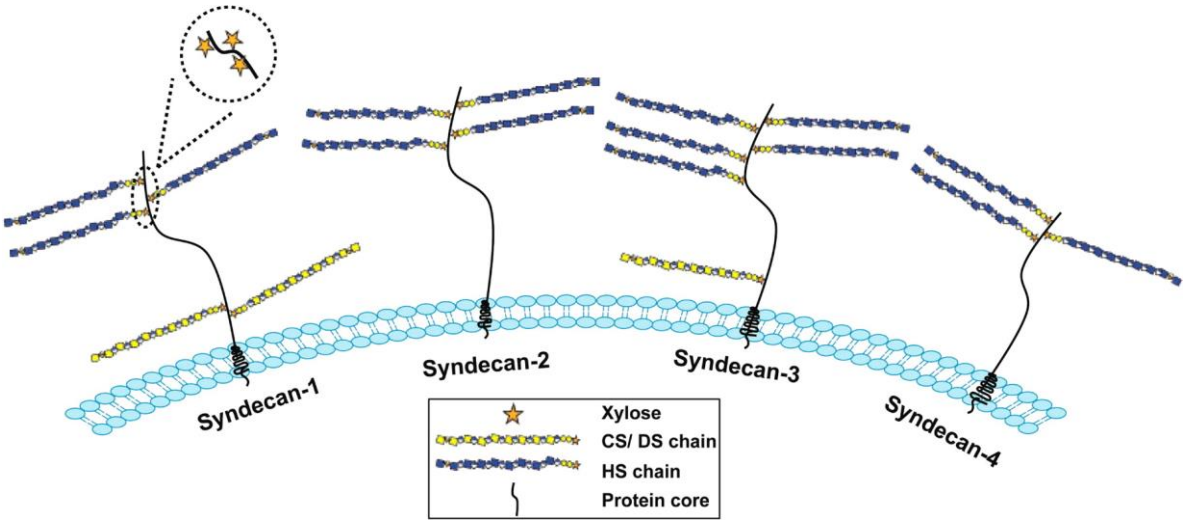

Figure 3. Schematic representation of syndecans structure. Syndecans are carrying GAG side chain. Syndecan-1 and 3 are carrying HS and CS, while syndecan-2 and 4 are carrying only HS [117].

1.4.3 Shed Syndecans

Syndecans interact via their HS side chains with many proteins and link the cytoskeleton to the ECM. Additionally, it has been shown that this interaction plays role in cell adhesion, growth, migration, proliferation, and morphogenesis [97, 102, 118]. Syndecans are bound to the cell-membrane, but their ectodomain can be released to the extracellular matrix [119] by the action of different.

Sheddases such as matrix metalloproteinases (MMPs), disintegrin and metalloproteinase (ADAMs), at cleavage site that are located at a juxtamembrane site and are common between the four SDCs. Matrilysin (MMP-7), MMP-2, MMP-9, MT1-MMP, and MT3-MMP are known to cleave SDC-1. Syndecan-2 and Syndecan-4 can be cleaved by MMP-2 and MMP-9 [120-123]. Syndecans can be cleaved at different site depending on the shaddases. Thrombin, and plasmin cleave human SDC-4 at Lys114-Arg115 and Lys129-Val130, respectively [124]. The ectodomain Syndecan-1 and 4 can be cleaved by ADAM-7 [125].

This process is regulated by growth factors, heparanase, trypsin, and cytokines. For example, activation of ERK/MAPK signaling pathway by EGF- and thrombin-receptor induces Syndecan-1 and 4 shedding [119, 120, 126-130]. Intracellular mechanism such as phosphorylation of tyrosine residue on cytoplasmic domain of Syndecans by tyrosine kinases, regulates SDC-1 shedding [119, 131].

The shed extracellular domain of SDC carries its GAG chains as it becomes soluble in the extracellular fluid. Thus, the released Syndecans can participate in the same interactions as on the cell membrane, and in this way be competitive to their parent molecule. Such solubilized SDC-1 can bind to VEGF receptor2 and FGF-2, having a stimulatory effect on growth and invasion of tumor cells [132, 133].

1.4.4 Syndecans in cancer

In addition to Syndecans important role in normal homeostasis, they play an important role during cancer development and progression. Syndecans act as a co-receptor, at the cell surface and as soluble variants in various body fluids, and bind to molecules such as cytokines, chemokines, growth factors and extracellular matrix (ECM) components via their GAG chains and regulate many cellular processes. In breast carcinomas expression of SDC-1 correlates with unfavorable prognosis. In addition, SDC-1 regulates tumorigenesis in mouse mammary epithelial cells by activating Wnt-1 signaling, and tumor-growth in breast carcinoma by activation of fibroblast [134-137]. Furthermore, loss of SDC-1 expression increases cell migration and invasion, and differentiation in breast, liver, gastric, lung adenocarcinoma, and mesothelioma carcinomas which is in correlates to with unfavorable patient's outcome [138-142].

Although there were not so many studies regarding the role of SDC-2 in cancer, recent studies showed that, overexpression of SDC-2 activates K-ras signaling pathway to induce invasive phenotype in pancreatic adenocarcinoma [143]. The same effect was observed in breast cancer patients, where the level SDC-2 promoting cells invasion by regulating

RhoGTPase [144]. Additionally, SDC-2 is a prognostic factor for head and neck cancer [145].

The loss of receptor protein tyrosine phosphate β/ζ (RPTPβ/ζ) initiate EMT and promoting cell migration and invasion in prostate cancer. Loss of RPTPβ/ζ activates Src, Pten, and ERK1/2 through activation of pleiotrophin/syndecan-3 activity [146]. In addition, higher expression level of SDC-3 is correlated with worse prognosis in pancreatic cancer [147]. SDC-4, like other SDCs, interacts with growth factors and regulates several processes including cell adhesion, migration, and progression in cancer. Higher expression of SDC-4 is correlated with favorable prognosis in patients with breast cancer [135, 148].

1.5 EXTRACELLULAR VESICLES

Extracellular vesicles (EVs), comprising apoptotic bodies, microvesicles, and exosomes are heterogeneous cell-derived membranous nanoparticles ranging from 30-4000 nm. EVs are released from all cells types and can be found in all biological fluids. They have been demonstrated to regulate diverse pathological and physiological processes as well as intercellular communication. In the beginning, EVs were considered to be cell waste for eliminating cell's unneeded compounds. However, now we know that EVs carry and deliver various functional factors such as proteins, lipids, and nucleic acids to specific target cells and that this can affect the recipient cell's activity [149-151].

1.5.1 Biogenesis and secretion of extracellular vesicles

Extracellular vesicles classes have different biogenesis mechanisms although all are involved in membrane-trafficking processes.

1.5.1.1 Apoptotic bodies

Unlike exosomes and microvesicles which are produced by healthy cells, apoptotic bodies are released during apoptosis which is a regulated mechanism of cell death. Apoptosis is initiated by pyknosis, i.e., irreversible condensation of chromatin, and cell shrinkage. Later, plasma membrane blebbing regulated by actin-myosin interactions occurs, followed by disintegration of the cellular content into membrane enclosed small vesicles form apoptotic bodies. Apoptotic bodies are large (500-4000 nm), contain specific organelles, and nuclear fragments [152-154].

1.5.1.2 Microvesicles

Microvesicles are formed by outward vesiculation and fission of vesicles from the plasma membrane into the extracellular space. These vesicles are smaller than apoptotic bodies and range from 50 to 1000nm in diameter [155, 156]. Microvesicle formation does utilize the endosomal machinery and is thus a distinct process from exosome formation. This process is depending on the Ras-related GTPase ADP ribosylation factor 6 (ARF 6) and components of ESCRT system [157]. Microvesicle budding is initiated with the activation of phospholipase D (PLD) by ARF6 which leads to phosphorylation of myosin light-chain kinase (MLCK) by ERK. Activation of MLCK triggers the release of microvesicles [155, 158].

In addition to the above, endosomal sorting complex required for transport (ESCRT) machinery also plays a role in secretion of microvesicles. Interaction of ARRDC1 with TSG101 (components of ESCRT system) delocalizes TSG101 to the plasma membrane and subsequently results in the secretion of microvesicles which contain these two proteins [157].

1.5.1.3 Exosomes

Exosomes are formed during maturation of early endosomes into late endosomes or multi-vesicular bodies (MVBs). During maturation of early endosome to late endosome, contents are sorted into 30-100 nm vesicles via inward budding of the membrane. Late endosomes or

MVBs are identifiable by the presence of multiple intraluminal vesicles (ILVs) [159, 160]. Protein sorting of ILVs is a highly regulated ESCRT dependent or independent process.

The first process involves four multi protein complexes of the ESCRT machinery which are involved in the ubiquitination of proteins on the inward budding part of the endosomal membrane. In the beginning of the process, ESCRT-0 recognizes mono ubiquitinated proteins. In the next step, ESCRT-I and ESCRT-II join ESCRT-0 and drive membrane budding. Later, ESCRT-III pinch off the membrane to complete the budding process. ESCRT-III is recruited to ESCRT-I and II via Alix which can carry and deliver un-ubiquitinated proteins such as Syndecans and tetraspanin CD63 into the ILVs. Now, ILVs are ready to fuse with plasma membrane or be destroyed by the lysosome. Rab27A/B leads ILVs toward the cell membrane where the SNARE complex helps them to fuse with membrane. Such vesicles released to the extracellular space are called exosomes (Figure 3) [161-165]. The second process involves endosome membrane proteins called tetraspanins. Tetraspanins consist of four transmembrane domains that are required for protein-protein interaction. ILVs form through these protein-protein interaction [166, 167]. Alix, CD9, CD63 and CD81 serve as exosome markers.

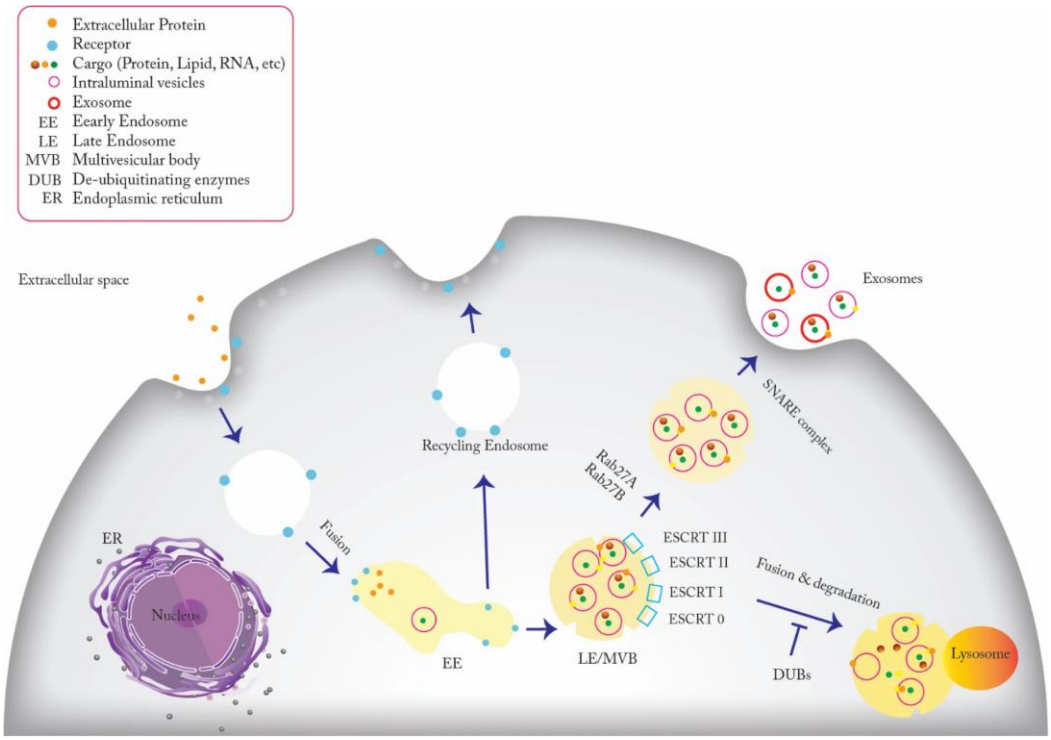

Figure 3. Exosome biogenesis and secretion. Early endosome (EE) can undergo two pathways either transforming to late endosome (LE/MVB) or recycling endosome. Early endosome converts to late endosome by packing their proteins content into small intraluminal vesicles (ILVs). Proteins sorting and packing can be depending on the ESCRT system. Later, targeted MVBs are ready to fuse their membrane with plasma membrane and release the intraluminal vesicles to the extracellular space. These vesicles are called exosome now [168].

1.5.2 Targeting of recipient cells

Recently, EVs have gained notable attention as mediators of intercellular communication. The released EVs can allow intercellular communication by reaching recipient cells and delivering their contents to them. Up-take of the EVs by recipient cells requires docking at the cell membrane, activation of cell membrane receptors, and internalization of their cargo. The mechanism of these processes is not completely understood and is dependent on the origin of the extracellular vesicles and the target cell [169, 170].

Interaction of EVs with target cells depends on the surface receptors and adhesion molecules such as tetraspanins, lipids, extracellular matrix components, integrins, and heparan sulfate proteoglycans on both target cells and EVs [171-176].

After binding to the recipient cell, which depends on the composition and cell membrane structure, EVs can either be internalized by endocytosis or remain at the cell membrane. For example, the presence of lipid rafts in the cell membrane increase uptake of EVs [177, 178]. EVs which remain bound on the cell membrane can be involved in antigen presentation and cell signaling. For example, B cell-derived exosomes induce MHC II antigen transfer to T-cells and stimulates their proliferation [179]. In addition, embryonic stem cells-derived microvesicles stimulate trophoblast migration by activation of JNK and FAK signaling kinases [180]. On the other hand, EVs can deliver their cargo in the recipient cell after internalization which occurs by two mechanisms: via the endocytic pathway or direct fusion with the cell membranes. EVs can transport various proteins, lipids, or nucleic acids to recipient cells and thus regulate their gene expression [52, 181-183].

1.5.3 Extracellular vesicles in cancer

Extracellular vesicles derived from tumor cells play an important role in tumor progression and metastasis by mediating intercellular communication between tumor cells as well as between tumor and stromal cells in the niche or at distance. Primary tumor cells require active communication with surrounding cells to grow and metastasize. For example, Epidermal growth factor receptor variant III (EGFRvIII)-positive glioma cells, secrete vesicles bearing EGFRvIII and deliver it to EGFRvIII negative glioma cells in the same tumor. EGFRvIII activates MAPK and Akt signaling pathways which leads to recipient cell growth [184, 185]. Presence of tetraspanin 8 on the surface of pancreatic cancer-derived exosomes induce VEGF-independent angiogenesis in endothelial cell by recruiting other proteins such as CD49d and CD106 [172].

In the last decade, the effect of EV-borne mRNA and miRNA on recipient cell gene expression and fate has attracted attention. Astrocyte-derived EVs containing PTEN-targeting miRNA induce invasion and metastasis in brain tumor [181, 186].

Remodeling of the extracellular matrix during cancer progression induces cancer invasion and cell motility. Presence of MMPs in EVs leads to proteolytic degradation of ECM

components which in turn promote tumor invasion and angiogenesis [187, 188]. Additionally, EVs can also induce EMT in endothelial cells [189].

1.5.4 EV's potential as biomarkers and therapeutic targets

Tumor-derived EVs contain tumor specific molecules such as RNAs and proteins, which can serve as diagnostic biomarkers or therapeutic targets. Thus, EV-associated proteins and RNAs can provide information about disease status.

Epithelial cell adhesion molecule (EpCAM) and CD24 are two tumor derived EVs biomarkers. EV-borne CD24 and EpCAM levels were significantly higher in patients with ovarian cancer when compared with benign and healthy controls [190, 191]. Additionally, exosomal integrins can predict organ-specific metastasis. For example, exosomal integrins αvβ5 and α6β4/α6β1 were associated with liver and lung cancer, respectively [176].

Exosomal genetic material, including DNA, RNA, mRNA, and miRNA, can be used as diagnostic and/or prognostic biomarkers. Exosomal miRNA-17-92a cluster and miRNA141/miRNA375 are significantly higher in colorectal cancer and prostate cancer, respectively [192-194].

Due to tumor derived EVs capacity to transform healthy cells to cancer cells, tumor derived EVs have been considered as a new target for cancer treatment. EVs biogenesis, content, and surface protein composition can serve as a target in cancer therapy. Rab27, which is involved in exosomes production, knockdown reduce exosome production, tumor growth and metastasis in metastatic melanoma [195-197].

2 THESIS BACKGROUND

Syndecan-1 is a differentiation marker structurally corresponding to a single type I transmembrane proteoglycans (PGs) with an N-terminal signal peptide, an extracellular ectodomain carrying glycosaminoglycan (GAG) side chains, a single hydrophobic transmembrane domain, and a short C-terminal cytoplasmic domain [198, 199]. Syndecan-1 exhibits a characteristic pattern of cell- and development-specific expression and a complex regulation both on transcriptional and post-translational level. It is proposed as "fine tuner of cellular signaling", as it plays critical roles for differentiation, proliferation, wound healing, migration, and angiogenesis in various pathological conditions [103, 200, 201]. The HS chains of syndecan-1 contain defined short sequences that specifically bind growth factors to their respective receptors, necessary for the receptor function. On the cell membrane the Syndecans are turned over within a few hours through proteolytic cleavage and shedding of most of the ectodomain as an intact fragment which instantly converts the membrane-bound ectodomain into soluble effector, which in turn may act as agonists or antagonists that regulate the effect of the cognate growth factor receptor [199]. This shedding might constitute one important contributing factor to decreased cell-surface syndecan-1 level, adverse prognosis, and increased therapy resistance of tum ours such as malignant mesothelioma.

Syndecan-1 is expressed in epithelial cells in a temporo-spatial manner [202] and it is down-regulated in dedifferentiated tumor components and mesenchymal tumors [203]. Very few previous studies have addressed the regulation of syndecan-1 in mesenchymal cells, or the importance of the respective functional domains of this PG in angiogenesis. Syndecan-1 may serve as a prognostic marker probably depending on whether syndecan-1 is cell bound or shed to the stroma. We aim to further characterize the functional domains of syndecan-1 and evaluate the role of this protein in pleural effusions.

Traditionally syndecan-1 is associated with functions at the level of the cell membrane, where it binds and activates several different growth factors (GF) – growth factor receptor (GFR) complexes, thus acting as a co-receptor in signalling [199, 200]. We have, however, previously shown a substantial proportion of syndecan-1 at intracellular locations and preliminary data point toward involvement in regulating angiogenesis [204].

Syndecan-1 acts as a co-receptor for a wide range of extracellular ligands, including VEGF, a key signaling molecule in angiogenesis both during embryogenesis and tumor growth [205-211]. SDC-1 regulates angiogenesis by interacting with integrins and insulin-like growth factor-1(IGF-1R) receptor. Upon activation a ternary receptor complex is formed on tumor cells and activated endothelial cells undergoing angiogenesis, that activates intracellular integrin signaling. In addition, there is a unique sequence on the Syndecan-1 extracellular domain, known as synstatin, which inactivates the integrin/IGF-1R/ Syndecan-1 complex and consequently the signaling pathways involved in angiogenesis. This angiogenesis modulating potential of syndecan-1 may very well serve as a therapeutic approach [212].

The mesenchymal cells we use as model display an inherited biphasic phenotype. Presence of syndecan-1 on the surface of malignant mesothelioma cells is associated with favourable prognosis, whereas the decrease of syndecan-1 deteriorates the prognosis, suggesting a role as prognostic factor. This effect, however, seems to depend on whether syndecan-1 is tumor

cell derived, cell-surface associated, shed or synthesized by the tumor stroma. Furthermore syndecan-1 might sequester soluble factors regulating angiogenesis and thereby modulate their local concentration, leading to altered angiogenesis. Experimental data also suggest that the shed syndecan-1 might be involved in therapy resistance through an EGFR regulated mechanism.

Our recent studies show that restoration of syndecan-1 hampers the proliferation [213] cell cycle progression and migration of mesenchymal cells. Subsequent pathway analysis indicates the involvement of many possible mediators including a critical role for several tyrosine kinase receptor mediated pathways and TGB-β [210].

The sulphation pattern of HS chains is regulated by several enzymes and has a crucial role in their interactions with the signaling molecules. One of these enzymes acting at the cell surface is sulfatase-1 (SULF1), an endosulfatase, catalyzing selective removal of 6-O sulfate groups from S domains of heparan sulfate chains, highly regulating the GF binding and by this, a multitude of signaling pathways. Data suggest that SULF1 could be a TGFβ responsive gene [214, 215]. Syndecan-1 on the other hand can function as a negative regulator of TGFβ signaling. Our data suggest that syndecan-1 has complex interactions with TGFβ mediated signaling, and that deregulation of SULF-1 could potentiate the effects of syndecan-1. The interaction between Syndecan-1, TGFβ and SULF-1 may constitute an interesting loop of co-regulation, which warrants further investigations.

3 RESEARCH AIMS

There are two major aims of this thesis. The first general aim was to investigate the role of SDC-1 in the angiogenesis of mesenchymal cells and epithelial to mesenchymal transition and the second was to study the diagnostic and prognostic utility of syndecan-1 in combination with novel and established biomarkers. The specific aims of this thesis work were as follows:

Specific aims

Paper I: To investigate the role of syndecan-1 in angiogenesis of mesenchymal cells. How does syndecan-1 influence angiogenesis?

Paper II: Does modulation of syndecan-1 expression influence the TGF-β induced epithelial-mesenchymal transition.

Paper III: To evaluate soluble (shed) syndecan-1 and angiogenic-related factors as possible diagnostic and prognostic biomarkers.

Paper IV: To characterize the different extracellular vesicle populations in pleural effusions.

4 MATERIALS AND METHODS

4.1 CELL LINES

In this thesis work we used A human malignant mesothelioma cell line, STAV-AB with epithelial-like morphology (paper I), and a fibrosarcoma cell line, B6FS with fibroblast-like morphology (paper II), one human lung adenocarcinoma cell line, A549 with epithelial-like morphology (paper II), and the human umbilical vein endothelial cells (HUVECs, paper I).

STAV-AB cells were established from pleural effusion while B6FS cells were from poorly differentiated fibrosarcoma. A549 and HUVEC cells were obtained from ATCC.

STAV-AB and B6FS cells have originally low endogenous SDC-1 expression level. SDC-1 was overexpressed in these cells by stable transfection with a plasmid vector carrying the human full-length SDC-1 gene (FL) or with the same vector lacking SDC-1 gene (EV) as a negative control [213]. Cells overexpressing SDC-1 were selected by Geneticin. STAV-AB overexpressing SDC-1 cells have been used to study the effect of SDC-1 on angiogenesis of HUVEC cells.

B6FS cells (no SDC-1 expression) were transfected with the same vectors carrying human full-length SDC-1 gene (FL) and (EV), as well as a truncated variant lacking RMKKK peptide responsible for nuclear translocation of SDC-1 (RMKKKdel). B6FS cells transfected with the above constructs have been used to study the effect of nuclear translocation of SDC-1 on EMT.

4.2 PLEURAL EFFUSIONS

Pleural effusions from patient with malignant pleural mesothelioma, metastatic lung adenocarcinoma and benign conditions were collected and diagnosed at the Department of Pathology and Cytology, Karolinska University Hospital at Huddinge, Sweden and at the Department of Chest Disease, Medical Faculty of Eskisehir, Turkey (paper I, III, and IV). Pleural effusions from Sweden were further verified by extensive immunocytochemistry and biomarker analyses including Hyaluronan and Mesothelin. All samples from both Sweden and Turkey were collected before any treatment was given. The study was approved by the ethical review board of Stockholm, Sweden (2009/1138-31/3 and 2007/1089-32) and the ethical review board of Eskisehir University, Turkey. Pleural effusion was used to study diagnostic/prognostic biomarkers and extracellular vesicles characterization (paper III and IV).

4.2.1 Extracellular vesicles fractionation

All pleural effusions (paper IV) were collected and diagnosed at the Department of Pathology and Cytology, Karolinska University Hospital. Samples were centrifuged at 300g for 10 minutes to discard cell debris (C), 2000g for 10 minutes to isolate apoptotic bodies (AB), 10000g for 10 minutes to isolate microvesicles (MV), 100 000g for 90 minutes to isolate exosomes (EX). Additionally, dissolved free proteins in the supernatant (SP) remained from last centrifugation step (100 000g), were concentrated.

4.2.2 Nanoparticle tracking analysis (NTA)

To determined exosomes size and concentration, nanoparticle tracking analysis (NTA) (NanoSight Techniques) was used (paper IV). Nanoparticle analysis is based on the Brownian motion and characterizing nanoparticles from 10 nm – 1000 nm in solution. The system introduces a laser beam to the samples through a glass prism which illuminates the particles. Particles are visualized by optical microscope fitted with video camera. NTA software identifies particles size and concentration.

4.3 FLUORESCENCE ACTIVATED CELL SORTING (FACS)

To measure SDC-1 expression level in SDC-1 overexpressed cells (STAV-AB and B6FS) prior each experiment (paper I and II), we used FACS. Cells were stained with primary antibody against SDC-1 (1:20 dilution, MCA2459, Bio-Rad Laboratories, Solna, Sweden) for 30 min on ice, and secondary antibody Alexa 488-conjucated goat anti mouse (A-110017; Invitrogen/Thermo Fisher Scientific) for 15 min in dark. Becton Dickinson Flow cytometry (Mountain View, CA, USA) was used to identify fluorescence intensity.

4.4 BEAD-BASED MULTIPLEX EXOSOME FLOW CYTOMETRY ASSAY

To characterize surface markers of different fractions derived from pleural effusion, we used MACSPlex assay coupled by flow cytometry (paper IV). We used commercial kit (MACSPlex Exosome kit, human, Miltenyi Biotec) which comprises a cocktail of various fluorescently labelled bead populations which coated with a specific antibody, and detection reagents. The kit allows detection of 37 exosomal surface epitopes plus two isotype controls. Samples surface epitope bind to the antibody and be detected by different fluorescence intensities by flow cytometry.

4.5 PROTEOME PROFILER ARRAY

To determine expression level of multiple angiogenesis-related proteins (paper I), we used Proteome Profiller Human Aniogenesis Antibody Array (R&D Systems, Inc., Minneapolis, MN, USA) kit. Proteome profiler array is a membrane-based immunoassay which the membrane is coated with capture antibodies against specific proteins. A mixture of sample with a cocktail of biotinylated detection antibodies was added to the membrane. Chemiluminescent detection reagents were used to visualized targeted proteins.

4.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

To measure SDC-1, VEGF and Mesothelin level in cell lysate, supernatant (SDC-1) and pleural effusion (SDC-1, VEGF and Mesothelin), ELISA was used (paper I and III). We used commercial kits (human CD138/SDC-1; Gen-Probe Diaclone, France, cat. No. 950.640.192, human VEGF; Quantikine ELISA, R&D systems, Minneapolis, MN, USA, cat. No. DVE00, and human N-ERC/mesothelin Assay kit, IBL) for all three antigens of interest. ELISA is a sensitive assay and is based on a capture antibody coated onto a plate surface. The antibody

binds to the relevant antigen in the sample, can be detected by labelled secondary antibody, and quantified by reading absorption in a spectrophotometer.

4.7 LUMINEX ASSAY

To assess the level of different biomarkers simultaneously, Luminex bead-based immunoassay was used (paper I, III, and IV). We measured SDC-1 level in SDC-1 silenced-cells (paper I), and 10 different biomarkers, including angiopoietin-1, galectin-1, osteopontin, mesothelin, HGF, VEGF, NRG1-β1, TIMP-1, MMP-7, and SDC-1, level in pleural effusion (paper III) and exosomes isolated from pleural effusion (paper IV) by using Human premixed multi-analyte Luminex kit (cat: LXSAHM).

Analytes specific antibodies, coupled to bead region with fluorophores at set ratio for each unique bead region, were incubated with sample. Subsequently, samples were incubated with a mixture of biotinylated detection and streptavidin-phycoerythrin (PE) reporter antibodies. Beads were illuminated by two spectrally distinct light emitting diodes (LEDs), one to determine beads region and the second to determine the PE-derived signal which is proportional to the amount of analyte bound. The median fluorescence intensities were determined on a Luminx® 100/200 analyzer.

4.8 IMMUNOFLUORESCENCE ANALYSIS

To measure the subcellular localization of SDC-1, epithelial cell markers (E-cadherin and CAR), and mesenchymal cell markers (vimentin and ZEB-1) in lung adenocarcinoma cell line (A549), immunofluorescence (IF) staining, was used (paper II). Primary antibody staining was performed overnight at 4°c with following antibodies: anti-SDC 1 (Abcam, ab128936, rabbit) 1:500, anti-Ecad (Cell Signaling, 3195S, rabbit) 1:500, anti-CXADR (SIV), rabbit, 1:10, anti-Vimentin (HPA027524, Sigma-Aldrich, Stockholm, Sweden, rabbit) 1:50, and anti-Zeb1 (HPA027524, Sigma, Aldrich, rabbit) 1:50. Secondary antibody staining was performed for 1 hour at room temperature using Alexa Fluor 488 goat anti-rabbit (A32731, Thermo Fisher Scientific). immunofluorescent staining was localized to cell surfaces (E-cadherin, CAR, vimentin, and ZEB-1) and to the nucleus (SDC-1).

4.9 IMMUNOBLOT ANALYSIS

To determine levels of E-cadherin, N-cadherin, vimentin, and SDC-1 (paper II) and identify differential centrifugation fractions specific proteins (paper IV), western blot was used. Protein concentration were determined with BCA assay (Bio-Rad) using bovine serum albumin as standard. The following primary antibodies were used: rabbit polyclonal antibody against E-cadherin (Cell Signaling, Leiden, The Netherlands, 1:1000), N-cadherin (Cell Signaling, 1:1000), vimentin (Cell Signaling, 1:1000), SDC-1 (Abcam, Cambridge, UK, 1:1000) in the second paper, and CD9 (Abcam ab-92726, 1:2000), cleaved caspase 9 (Cell Signaling 9505S, 1:1000), CD81 (Santa Cruz sc-9158, 1:200) in the fourth paper. Freshly prepared secondary antibodies (Donkey Anti-Rabbit or Sheep Anti-Mouse IgG F(ab´)2 Fragment Specific, Peroxidase conjugated, Thermo Fisher) and (Li-Cor 926-68020, 926-152

68071, 926-68079, 1:15000) were used in paper II and paper IV, respectively. In this technique proteins are separated based on their weight through gel electrophoresis and detected by anti-body against to the protein of interest.

4.10 GENE SILENCING

To reduce the expression level of SDC-1 (paper I and II) and MMP-7 (paper I) genes, transient knockdown by small interfering RNA (siRNA) was used. A cocktail of three siRNA (SDC-1 silencer pre-designed siRNA, s12632, s12633, and s12634, Ambion/Thermo Fisher Scientific) constructs was used to silence SDC-1 and a scrambled siRNA, with no target mRNA, was used as a negative control. SDC-1 expression level was validated on mRNA level by qPCR. We used High Pure RNA Isolation kit (Roche, Mannheim, Germany) for total RNA isolation and cDNA synthesis was performed using First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England).

The qPCR was performed with Platinum® SybrGreen qPCR SuperMix-UDG kit (Invitrogen) with the following set of sense/antisense primer (CyberGene AB, Sweden) sequences: SDC-1 fwd: TCT GAC AAC TTC TCC GGC TC and SDC-1 rev: CCA CTT CTG GCA GGA CTA CA and GAPDH fwd: ACA TCA TCC CTG CCT CTA CTG G and GAPDH rev: AGT GGG TGT CGC TGT TGA AGT C.

Same procedure was used to reduce the expression level of MMP-7 gene with the difference that we used one siRNA construct (Cat. No.sc-41553, Santa Cruz Biotechnology, Inc, Dallas, TX, USA). Scrambled siRNA with nonsense sequence was used as a negative control. qPCR was performed as it described above with the following set of sense/antisense primers which were designed by us: MMP-7 fwd: GAG TGC CAG ATG TTG CAG AA and MMP-7 rev: AAA TGC AGG GGG ATC TCT TT, and GAPDH fwd: ACA TCA TCC CTG CCT CTA CTG G and GAPDH rev: AGT GGG TGT CGC TGT TGA AGT C.

4.11 CELL PROLIFERATION ASSAY

To study the effect of SDC-1 overexpression on the proliferation of HUVEC cells (paper I), the WST-1 assay was used. HUVEC cells proliferation was assessed by Cell Proliferation Reagent WST-1 (Roche Diagnostics Scandinavia AB, Bromma, Sweden). The HUVEC cells were treated with conditioned medium from SDC-1 overexpressing mesothelioma cells. Their proliferation was assessed by the WST-1 reagent. Anionic tetrazolium salt (WST-1) requires an electron coupling reagent to be cleaved (formazan) and permeates into the cytosol of metabolically active cells. This process is depending on dehydrogenase enzyme activity. Presence of active dehydrogenase enzyme reduces this process. Thus, the amount of formazan dye formed correlates to the number of metabolically active cells. Optical densities were quantitated with a spectrophotometer.

4.12 CHEMOTOAXIS ASSAY

To investigate the effect of SDC-1 on the chemotactic capability of HUVEC cells, Transwell migration assay with a pore size of 3µm (Transwell chamber, Corning, NY, USA) was used

(paper I). HUVEC cells were seeded in the upper chamber and conditioned medium from SDC-1 overexpressing and control cells were added to the lower chamber. To initiate cell movement across the membrane, recombinant VEGF (10 ng/ml) was added to the conditioned medium. After 24 hours incubation time, the membrane between the two compartments was fixed in 4% formaldehyde and cells were stained with 0.2% crystal violet. The percentage of area covered by migrated cells to the underside of the membrane was determined.

4.13 INVASION ASSAY

To study the effect of nuclear SDC-1 in SDC-1 overexpressing cells on the invasive behavior of B6FS cells, Invasion assay was used (paper II). The assay was performed by using 8µm pore cell culture inserts (Merk Chemicals and Life Science, Stockholm) and 3mm cylinder. This assay is based on the cell migration capacity towards chemoattractants. B6FS cells overexpressing SDC-1 (FL, RMKKK deleted, and control) were seeded on top of growth factor-reduced Matrigel (3mg/ml, AH Diagnostics) into the cell culture insert. The insert was suspended over a larger well which contained medium (control), or medium + TGF-β (10ng/ml). Migrated cells to the lower insert were counted using Alamar blue and Countess (Invitrogen/Thermo Fisher Scientific).

4.14 WOUND HEALING ASSAY

To further study the effect of SDC-1 overexpression on the HUVEC cells migration and proliferation rate, wound healing assay was used (paper I). We used CELL BIOLABS CytoSelect 24-well wound healing assay kit. The wound field (0.9 mm) was created by a standardized plastic insert and the HUVEC cells were cultured till they formed a confluent monolayer. At this point, the insert was removed, and HUVEC cells culture medium replaced was with the conditioned medium from SDC-1 overexpressing and SDC-1 silenced mesothelioma cells. Relevant controls were used for each condition. The closure of the wound was measured at different time points (0, 4, 8, and 24 hours) and the area was measured with ImageJ software.

4.15 TUBE FORMATION ASSAY

Tube formation assay is a widely used in vitro assay to study angiogenesis. It is based on the reassembling and establishing new cell-cell contacts to form new vessel lumen. To study the effect of SDC-1 overexpression on the tubulogenesis of the HUVEC cells, we performed this tube formation assay (paper I). HUVEC cells were seeded on extracellular matrix gel (ECM gel, Cell Biolabs, San Diego, CA, USA), and treated with the conditioned medium from SDC-1 overexpressing cells, SDC-1 silenced cells, and their relevant controls with a ratio of 1:2 conditioned medium/HUVEC complete medium (vol/vol). Cells were incubated for 6 hours in conditioned medium and then tube formation was monitored. Quantification performed using image processing software to measure the tube area, the length and/or number of branch points, and total number of tubes.

4.16 STATISTICAL METHODS

All statistical analysis in were performed using GraphPad software. The level of significance was less than 0.05 ($p<0.05$).

4.16.1 Kaplan-Meier Survival analysis

To investigate the correlation of different biomarker levels with the survival of malignant mesothelioma patients, Kaplan-Meier survival curves were constructed (paper I and III). Cutoff Finder was used to determine a cut-off value for each biomarker based on the most significant and highest hazard ratio.

4.16.2 Receiver Operating characteristic (ROC) analysis

To assess the diagnostic efficiency of individual biomarkers (paper III), ROC analysis was used. In general, ROC curve shows the trade-off between the sensitivity and the specificity of a classifier. The area under the ROC curve (AUC), which ranges between 0.5-1, shows how good a classifier is. AUC values exceeding 0.7 indicates possible diagnostic value of the classifier.

4.16.3 Logistic Regression

To develop a model for identifying and combine diagnostic biomarkers for diagnosis (paper III), logistic regression was used. Logistic regression tests the potential association between multiple exposure variables (X) with an outcome variable (Y) which is obtained by Odds ratio (OR). Odds ratio shows the association between an exposure and an outcome. Odds ratio ranges between zero and infinity where 1 shows that there is no difference between studied variants.

5 RESULTS

A little progress each day, adds up to big results.

Paper I: Syndecan-1 overexpressing mesothelioma cells inhibit proliferation, wound healing, and tube formation of endothelial cells

Angiogenesis is an important process in aggressive tumors like MM. In this paper we studied the role of syndecan-1 (SDC-1) overexpression on angiogenesis. The first hypothesis was to study the effect of SDC-1 overexpression on the secretion of angiogenesis-related proteins. The result of this analysis showed that, several angiogenesis-related proteins were significantly altered by SDC-1 overexpression and silencing including, FGF-4, NRG1-β1, TGF-β1, HGF (were significantly up-regulated) angiopoietin-1, TIMP-1, and TSP-1 (were significantly down-regulated) upon syndecan-1 overexpression, and IL-8 (was significantly up-regulated) upon SDC-1 silencing. After profiling the angiogenesis-related proteins affected by overexpression, we studied the effect of these proteins on the proliferation, migration, and tube formation of endothelial cells.

The conditioned medium from SDC-1 overexpressing MM cells, acted inhibitory on all these function, while conditioned medium from SDC-1 silenced MM cells, increased the ability of endothelial cells to migrate while there was no effect on tubulogenesis. The inhibitory effect of SDC-1 seemed to be regulated through angiogenic proteins.

Furthermore, there was a significant positive correlation between shed SDC-1 and VEGF in pleural effusion from MM patients. In addition to this, VEGF turned out to be of prognostic value in MM patients.

Paper II: Nuclear syndecan-1 regulates epithelial-mesenchymal plasticity in tumor cells

One main cause of death in cancer is related to the formation of metastases. Epithelial-mesenchymal transition (EMT) give the malignant cells invasive and metastatic properties. SDC-1 is mainly expressed in epithelial cells and is deregulated during EMT. In this study, we investigated the possible role of nuclear SDC-1 in EMT in tumor cells.

Nuclear SDC-1 was knocked down by interfering RNA in A549 cells, which gave more mesenchymal morphology to these cells. Simultaneously, the E-cadherin level was significantly reduced compared to the scrambled control cells. This loss was complete after exposure to TGF-β. In addition to this, the N-cadherin level was unaffected by SDC-1 knockdown but significantly increased after TGF-β treatment.

When validated by immunofluorescence staining, E-cadherin and CAR were lost in both SDC-1 knockdown A549 cells and control cells after TGF-β treatment. Moreover, vimentin and Zeb1 were more expressed in SDC-1 knockdown compared to the control cells after TGF-β treatment.

Paper III: Multiplex soluble biomarker analysis from pleural effusion

The diagnosis of MM can be based on the examination of cytological or histological materials. Whatever the diagnostic material used, the diagnosis is a challenge. Analyses of soluble biomarkers in the effusion can in some cases provide help. A diagnostic battery would, however, gain if additional markers could be added. SDC-1 plays dynamic roles in different cancers and in the pleura, it is shed into the effusion together with angiogenesis-related proteins studied in paper I. Using the Luminex assay several of these compounds seemed to give diagnostic information, distinguishing MPM, metastatic adenocarcinoma and benign mesothelial proliferations.

Thus galectin-1, mesothelin, osteopontin, shed SDC-1, VEGF, MMP-7, HGF, and TIMP-1 had significantly higher level, and NRG1-β1 had significantly lower level in MPM patients compared with the benign conditions. As indicated by ROC curve analyses galectin-1, mesothelin, osteopontin, NRG1-β1, and shed SDC-1 all were of high diagnostic value in MPM patients.

In addition, patients with MPM and metastatic adenocarcinoma could be distinguished using galectin-1, mesothelin, MMP-7, and shed SDC-1. Galectin-1 and mesothelin had significantly higher level whereas MMP-7 and shed SDC-1 had significantly lower level in MPM effusion compared with metastatic adenocarcinoma patients. Moreover, stepwise logistic regression analysis showed that MMP-7, mesothelin, and osteopontin had higher predictive value for distinguishing MPM from adenocarcinoma.

We could also demonstrate that the level of shed SDC-1 in MPM patients correlated positively with the levels of HGF and NRG1-β1.

Furthermore, the levels of shed SDC-1 and VEGF have prognostic value in MPM patients which was in accordance with our previous result (paper I). High level of these two compounds had significantly worse prognosis.

Measuring the concentration of these nine compounds can be useful in useful adjuncts in effusion cytology, more often allowing a definite diagnosis based on the first diagnostic material available.

Paper IV: Characterization of different extracellular vesicle populations present in the pleural effusion

Tumor micro-environment is a dynamic regulator of tumor development, progression, and metastases. Extracellular vesicles including, apoptotic bodies, microvesicles, and exosomes, are nanoparticles released from cells specially cancer cells. These particles bear functional molecules and can be taken-up by target cells which highlight their importance as cell-to-cell mediators. In this study we isolated and characterized extracellular vesicles derived from pleural effusion from patients with malignant pleural mesothelioma (MPM) or metastatic adenocarcinoma (AD), and from patients with benign (BE) conditions.

Extracellular vesicles (apoptotic bodies, microvesicles, and exosomes) were isolated from pleural effusions using differential ultra-centrifugation. To validate differential the ultra-centrifugation fractions, we detected CD81 in the microvesicles and exosomes, and cleaved caspase 9 in the protein lysate of the apoptotic bodies.

The concentration and size distribution of the exosomes was in accordance with previous studies and the concentration of exosomes varied between individuals. The surface markers CD2, CD8, CD9, CD24, CD44, CD81, CD105, CD133, CD146, MCSP, and ROR1 were elevated while CD40, CD45, and CD49e had lower levels on the exosomes compared to apoptotic bodies and microvesicles.

All studied angiogenesis-related proteins were present in the extracellular vesicles derived from pleural effusion of MPM, AD, and benign conditions. In addition to this, the level of galectin-1, mesothelin, osteopontin, VEGF, MMP-7, HGF and NRG1-β1 were significantly lower in exosomes compared with supernatant whereas, there were no significant differences in the level of angiopoietin-1, TIMP-1, and shed SDC-1.

Angiopoietin-1 and TIMP-1 seemed to be preferably transported in exosomes while the other factors mainly presented in supernatant as soluble molecules. Most importantly galectin- 1, osteopontin, mesothelin, and VEGF had higher level whereas SDC-1 and HGF had lower level in exosomes derived from MPM patients compared to patients with AD and benign mesothelial proliferations.

6 DISCUSSION

The aim of argument, or of discussion, should not be victory, but progress.

Joseph Joubert

Angiogenesis, neoformation of new vascular networks, is an important feature of solid tumors since it is required for tumor growth and metastasis. Pro- and anti-angiogenic proteins are produced during angiogenesis. The phenotypic switch in tumor cells is the result of a balance between pro- and anti-angiogenic regulators. Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF-β) are important angiogenesis regulators in tumors [78, 205, 216]. Importantly, increased vascularity is associated with poor prognosis and advanced tumor stage [207, 217]. Syndecan-1, a transmembrane proteoglycan, acts as a co-receptor for several extracellular ligands and regulates various cellular processes including angiogenesis [218-220]. Membrane-bound SDC-1 is released to ECM by shaddases resulting of a free SDC-1 ectodomain which contain HS chain and is active [221]. Hence, we aim to study the role of SDC-1 in malignant pleural mesothelioma angiogenesis.

In this study, we showed that angiopoietin-1, thrombospondin-1 (TSP-1), and tissue inhibitor of metalloproteinases-1 (TIMP-1), among other angiogenesis-related proteins, were significantly down-regulated upon SDC-1 overexpression, whereas IL-8 was significantly up-regulated upon SDC-1 silencing. In addition to this, TIMP-1, and TSP-1, among other proteins which were altered by both SDC-1 overexpressing and silencing, were significantly up-regulated upon SDC-1 silencing.

Previous studies showed that activation of Tie-2 receptor tyrosine kinase by angiopoietin-1 play key roles in endothelial cell vascularization by increasing level of TJ-associated proteins (ZO-1, occludin, and claudin-5) [222, 223]. TSP-1 is known as both anti-angiogenic and immunomodulatory factor which regulates various cellular processes including migration, proliferation, cell adhesion, extracellular matrix organization, and growth factor activity [224-226]. Additionally, TSP-1 can activates TGF-β which is an important regulator of angiogenesis [227]. Furthermore, TIMP-1 acts as an anti-angiogenic factor through downregulation of MMP-1 and inhibition of endothelial cells migration [228].

Moreover, up-regulation of pro-angiogenic factors including, FGF-4 [229, 230], HGF [231, 232], NRG1-β1 [233], had no significant effect on HUVEC cells proliferation, migration, and tube formation in our system. This might be due to a stronger effect of anti-angiogenic factors.

VEGF is a key regulator of angiogenesis. It has been shown that several growth factors, including FGF, TGF-β, EGF, and TNF, and hypoxia induce VEGF expression [234, 235]. In addition, VEGF level is significantly correlated with tumor angiogenesis and metastasis in many cancers including MPM [236-240]. Shed SDC-1 level is associated with cancer chemotherapy resistance, progression, and prognosis [221, 241]. We previously showed that

mesothelioma patients with lower level of shed SDC-1 had a longer survival [242]. In this study we showed that there is positive correlation between shed SDC-1 and VEGF.

Taken together, our data demonstrate an anti-angiogenic effect of SDC-1 on HUVEC cells regulated by an array of anti- and pro- angiogenesis-related factors. Moreover, shed SDC-1 could be a prognostic indicator in MPM.

Another feature of importance for a malignant tumor is the epithelial-mesenchymal transition (EMT). This is a process in which epithelial cells increase their motility and invasiveness by acquiring more mesenchymal phenotypes. Previous studies have shown that this process can be induced in different ways by extracellular signals, involving factors such as hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) plays an important role in this process.

SDC-1 knockdown repressed E-cadherin, induced N-cadherin, and increased nuclear Zeb1 in human lung adenocarcinoma cell lines (A549). In addition, SDC-1 overexpression decreased the level of N-cadherin and vimentin in fibrosarcoma (B6FS) cells. We previously showed that overexpression of SDC-1 enhanced cell adhesion and inhibit cell migration in fibrosarcoma cells (B6FS) [243]. Furthermore, we showed that overexpression of SDC-1 down-regulates TGF-β in MM cells [210]. Taken together, these data suggest the role of SDC-1 in EMT.

The diagnosis of MPM and distinguishing it from metastatic adenocarcinoma and benign conditions is challenging. The search for new diagnostic biomarkers is therefore essential. While hyaluronan and mesothelin can be used in clinical practice [43], previous studies has indicated that other factors such as osteopontin and fibulin-3 can be valuable [17, 35, 244-246].

In addition, TGF-β level is higher in MPM effusions compared to those in lung adenocarcinoma patients [247, 248]. In our multi-parameter test it could be shown that galectin-1, NRG1-β1, shed SDC-1, VEGF, and TIMP-1 also had good diagnostic efficiency in MPM patients. The shed SDC-1 level positively correlated with VEGF level in MM (paper I). These two factors correlated in this material to shorter overall survival, and these factors may thus also be of prognostic value.

None of these new possible factors have sufficient specificity to motivate their use as single biomarkers in diagnostic routine. A diagnostic battery must therefore be based on many biomarkers. Multivariate tests like the Luminex used here could be the basis for such analysis. The evaluation of such multiparameter test, however, needs a logistic model, which can be deduced using larger patient materials.

The diagnosis of a malignant condition in the pleura – primary MPM or metastatic adenocarcinoma – is a diagnosis if an advanced condition. Earlier diagnosis of MPM may improve the therapeutic options, but this must then be obtained before the effusion is established. One possibility could be pre-symptomatic analysis of serum from persons at risk

(asbestos or erionite exposed individuals). For such a future option the appearance of these factors in blood must be clarified.

Release EVs into the extracellular space are important mediators of cell-to-cell communication and a way for the malignant cell to interact with its surroundings. The tumor cells can induce physiological and pathological changes in recipient cells by delivering the cargo of active proteins and RNA. When EVs are released from pleural tissues, they will also end up in the effusion. The size of vesicles in the exosome fraction was in accordance with previous studies [249]. Their concentration in the effusion depends on several factors such as their rate of biogenesis (tumor burden, number of inflammatory cells) and the volume depending on rate by which the fluid is accumulated. It is therefore natural that their amounts varied between the effusions, also when thoracocentesis had been repeated.

The exosomes present in the effusion may have been produced by any of the cells present. Characterization of EVs surface markers is a way to mirror their cellular origin [250]. Such factors are CD2, CD8 (T-cells), CD9, CD81 (tetraspanin family), CD24 (B-lymphocytes), CD44 (large number of mammalian cells), CD133 (malignant cells), CD105, CD146 (endothelial cells), MCSP (epidermal stem cells) and ROR1 (cancer cells). These factors were all more abundant in the exosome fraction compared to microvesicles and apoptotic bodies. These results indicate that the origin of exosomes present varied. Specific analyses of tumor exosomes should be possible using immunoprecipitation with factors such as CD133.

Still the content of exosomes was different in the malignant effusions, indicating origin in tumor cells. The exosomes isolated from MPM effusions had higher level of mesothelin, osteopontin, galectin 1, and VEGF compared to those in AD and benign effusions. Due to the ability of EVs in mediating cell-to-cell communication, these biologically active proteins can be delivered to other cells, a way for the MM cells to regulate the recipient cells signaling pathways [161, 251]. For example, activation of PI3K/Akt and MAPK/ERK signaling pathways by mesothelin inhibit apoptosis. In addition, mesothelin can increase cell proliferation and invasion by activating STAT3 signaling pathway and interacting with CA125 proteoglycan, respectively [252, 253].

Similarly, the pleural effusions from AD patients had higher exosome levels of SDC-1 and HGF compared to MPM and benign effusions. Previous studies have shown that EVs cargos are cell-type-specific and regulated by some proteins and enzymatic activities. For example, up-regulation of heparanase influences exosomes secretion and their protein content [175].

Taking together, this study suggests that exosomes actively participate in cell-to-cell communication. Since the exosomes also enter the pleural cavity, it is possible that their regulatory role involves pleural tissue at distance. Further dissemination with even more distant cells is then also possible when the effusion is drained through the stomata directly into lymphatic vesicles. The better understanding of the exosome function in malignant conditions is one component of importance to possible future designing of targeted therapies.

7 CONCLUSIONS

A theory that explains everything, explains nothing.

Karl Popper

In this thesis work, we investigate the role of SDC-1 overexpression in angiogenesis and epithelial to mesenchymal transition. Further, we studied the potential value of ten angiogenesis-related factors, including SDC-1, as diagnostic and prognostic biomarkers for MPM. Lastly, we characterized pleural effusion derived EVs for a better understanding of MM tumor progression and metastasis. The major conclusions of this thesis work are:

- SDC-1 over-expression effects growth factors secretion of mesothelioma cells.
- SDC-1 overexpression inhibits endothelial cells proliferation, migration, and tube formation.
- Loss of nuclear SDC-1 induce epithelial-mesenchymal transition in A549 lung adenocarcinoma cells.
- Loss of nuclear SDC-1 increase B6FS fibrosarcoma cells invasion.
- Galectin-1, mesothelin, osteopontin, shed SDC-1, VEGF, MMP-7, HGF, TIMP-1, and NRG1-β1 can be diagnostic biomarkers for distinguishing malignant pleural mesothelioma and metastatic adenocarcinoma from benign condition.
- Galectin-1, mesothelin, MMP-7, and shed SDC-1 can be diagnostic biomarkers for distinguishing malignant pleural mesothelioma from metastatic lung adenocarcinoma patients.
- VEGF and shed SDC-1 are prognostic biomarkers for malignant pleural mesothelioma.
- Pleural effusion derived exosomes have higher level of osteopontin, galectin, mesothelin, and VEGF in malignant pleural mesothelioma patients compared with adenocarcinoma and benign conditions.
- SDC-1 and HGF are present at higher level in exosomes isolated from pleural effusion from adenocarcinoma patients.

8 FUTURE PERSPECTIVE

The future is always beginning now.

Mark Strand

The aim of this thesis was to investigate the role of SDC-1 in angiogenesis of mesenchymal cells and its impact on epithelial to mesenchymal transition along with evaluation of the efficiency of SDC-1 in combination with other stablished biomarkers. Although we covered the aims of this thesis, there are more findings which merit further investigation.

Our results showed that SDC-1 overexpression inhibits endothelial cell migration, proliferation, and tube formation. Additionally, we showed that nuclear SDC-1 reduces EMT in cancer cells. It will be interesting to study the mechanisms and pathways which are involved in these processes.

Our results showed the presence of biological active proteins in exosomes. Further analysis to study the effect of tumor cell-derived exosomes on the neighbor cells by transferring their cargo is needed.

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Osho

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