Host Matrix Modulation by Tumor Exosomal CD44v6 and Proteases Promotes Motility and Invasiveness

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“All truths are easy to understand once they are discovered; the point is to discover them.”

Galileo Galilei

To my loving parents

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DECLARATION

I hereby declare that this thesis is the result of my own work, that I used no other than the indicated references and resources, that all the information that has been taken directly or indirectly from other sources is indicated as such, and that I have regarded the statute of the Karlsruhe Institute of Technology on securing good scientific practice in its currently applicable version.

Wei Mu

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List of Abbreviations

ADAM: A Disintegrin And a Metalloproteinase Domain
ADAMST: A Disintegrin And a Thrombospondin Motifs
Alix: ESCRT-II Adaptor Protein ALG-2 Interacting Protein X
APC: Allophycocyanin
ASML: Bsp73ASML, Pancreatic Carcinoma Line
ASML-CD44v Kd: Stable Knockdown Of CD44v4-v7 In ASML Wildtype Cell
BSA: Bovine Serum Albumin
˚C: Degree Celcius
CD: Cluster Of Differentiation
CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A
CXCR4: C-X-C Chemokine Receptor Type 4
d: Day
DHPE: Lissamine™ Rhodamine B 1,2-Dihexadecanoyl-Sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt
DIC: Disseminated Intravascular Coagulation
DMSO: Dimethyl Sulfoxide
ECM: Extracellular Matrix
ECL: Enhanced Chemiluminescence
EDTA**:** Ethylene Diamine Tetraacetic Acid
EGF: Epidermal Growth Factor
EGFP: Enhanced Green Fluorescent Protein
EGFR: Epidermal Growth Factor Receptor
ERM: Ezrin, Radixin, Moesin
ESCRT: Endosomal Sorting Complex Required For Transport
EMT: Epithelial-Mesenchymal Transition
EMMPRIN: Extracellular Matrix Metalloproteinase Inducer
FACS: Fluorescence-Activated Cell Sorting
FCS: Fetal Calf Serum
FGF: Fibroblast Growth Factor
FITC: Fluoresceinisothiocyanate
FN: Fibronectin
h: Hour
HA: Hyaluronic Acid
HB: Heparin-Binding
Hcl: Hydrochloric Acid

HE: Hematoxilin-Eosin
HEPES: 4-(2-Hydroxyethyl)-1-Piperazine Ethane Sulfonic Acid
HDGF: Hepatoma-Derived Growth Factor (HDGF)
HIF: Hypoxia-Inducible Factor
HRE: Hypoxia Response Element
HRP: Horse Radish Peroxidase
HGF: Hepatocyte Growth Factor
HGFR: Hepatocyte Growth Factor Receptor
Igg: Immunoglobulin G
IP: Immunoprecipitation
Ifp: Intrafoodpad
Ip: Intraperitoneal
GAG: Glycosaminoglycan
LAMP: Lysosomal-Associated Membrane Proteins
LN: Laminin
LN1: Laminin111
LN5: Laminin332
LRP-1: Lipoprotein Receptor-Related Protein 1
Min: Minute
Mrna: Messenger Ribonucleic Acid
Mirna: Micro Ribonucleic Acid
MAPK: Mitogen-Activated Protein Kinase
MHC: Major Histocompatibility Complex
MMP: Matrix Metalloproteinase
Mvbs: Micro Vesicular Bodies
MT1-MMP: Membrane-Type1-MMP; MMP14
OD: Optical Density
PAGE: Polyacrylamide Gel Electrophoresis
PBS: Phosphate Buffered Saline
PDAC: Pancreatic Ductal Adenocarcinoma
PDGF: Platelet-Derived Growth Factor
PDGFR: Platelet-Derived Growth Factor Receptor
PE: R-Phycoerythrin
PHD: Prolyl-Hydroxylase Domain
PI: Propidium Iodine
PI3K: Phosphatidylinositol 3-Kinase
Plgf: Placental Growth Factor

PMA: Phorbol 12-Myristate 13-Acetate
PMSF: Phenyl Methyl Sulphonyl Fluoride
Prog: Progressor Cells
RAS: Proto-Oncogene
RAEC: Rat Aorta Endothelial Cells
Rpm: Revolutions Per Minute
RT: Room Temperature
Ptks: Protein-Tyrosine Kinases
RPMI: Roswell Park Memorial Institute
SD: Standard Deviation
SDS: Sodium Dodecyl Sulphate
Sirna: Small Interfering RNA
STP: Serine Threonine Proline
TACE: TNF-Alpha Converting Enzyme, ADAM17
TAE: Tris Acetate EDTA
TAPI: TACE Inhibitor
Taq: *Thermus Aquaticus*
TEMED: N,N,N´N´-Tetramethylene Diamine
Tems: Tetraspanin Enriched Microdomains
TEN: Tris EDTA Nacl
TGF: Tumor Growth Factor
TIMP: Tissue Inhibitor of Metalloprotease
TLC: Thin Layer Chromatography
TNF: Tumor Necrosis Factor
U: Unit
Upa: Urokinase-Type Plasminogen Activator
Upar: Urokinase Receptor
VCAM-1: Vascular Cell Adhesion Molecule 1
VEGF: Vascular Endothelial Growth Factor
VEGFR1: Vascular Endothelial Growth Factor Receptor 1-Positive
V/V: Volume/Volume
WB: Western Blot
Wk: Week
W/V: Weight/Volume
µm: microMolar

ZUSAMMENFASSUNG

Exosome sind kleine Vesikel, die von den meisten Zellen eines Organismus produziert werden und als das wichtigste interzelluläre Kommunikationsmittel diskutiert werden. Tumorzellen sezernieren besonders viele Exosomen. Da sich Exosomen im gesamten Organismus verteilen, können Tumorexosomen sowohl mit benachbarten Tumorzellen, tumorassoziierten Wirtszellen und mit entfernten Zellen, z.B. hämatopoetischen Zellen, vaskulären Endothelzellen und verschiedenen Stromazellen, kommunizieren. Es ist bekannt, dass die Kommunikation zwischen Zellen und Exosomen hauptsächlich über rezeptorvermittelte Signaltransduktion nach Bindung der Exosomen und durch Reprogrammierung der Zielzelle nach Übergabe des exosomalen Kargos in die Zielzelle, erfolgt. Hingegen gibt es kaum Informationen über den Einfluss von Tumorexosomen auf die extrazelluläre Matrix, die aber einen wichtigen Bestandteil des Tumormikromilieus darstellt. Daher fragte ich, ob Tumorexosomen auch die Organisation der extrazellulären Matrix beeinflussen und ob diese Veränderungen auf Stromazellen und Tumorzellen rückwirken.

Als Modellsystem verwendete ich Exosomen der metastasierende Pankreastumorzelllinie ASML der Ratte und eine schwach metastasierende ASML-CD44v6 knockdown Linie. Ich konnte zeigen, dass

I) Tumorexosomen im gesamten Organismus an Komponenten der extrazellulären Matrix binden.

Die bevorzugten Bindungspartner hängen vom Expressionsprofil an Adhäsionsmolekülen der Tumorexosomen ab. ASML Exosomen, die eine hohe CD44- und α4β6-Expression aufweisen, binden bevorzugt an Hyaluronsäure und Laminin 332.

II) Tumorexosomen die extrazelluläre Matrix verändern.

Kollagen, Laminin, Fibronektin und native Matrizes werden durch exosomale Proteasen degradiert. Die höhere Effizienz des Matrix Abbaus durch ASML im Vergleich zu ASML-CD44v6 knockdown Exosomen beruht auf der durch CD44v6 unterstützten Transkription von uPAR und der Assoziation von CD44v6 mit MMP-9, MMP-14 und TACE.

III) die durch Tumorexosomen modulierte Matrix Stromazellen und hämatopoetischen Zellen beeinflusst.

Der Matrixabbau durch Tumorexosomen beeinflusst Adhäsion, Migration und Invasivität, sowie Proliferation und Apoptoseresistenz von Stromazellen. Dies ist zumindest teilweise auf die Generierung bioaktive Abbauprodukte der Matrix und die Freisetzung von Wachstumsfaktoren und Chemokinen aus der Matrix zurückzuführen.

Tumorexosomen binden und modulieren sowohl die Tumormatrix als auch die extrazellulären Matrix entfernt liegender Organe. Die durch Tumorexosomen modulierte extrazelluläre Matrix ist ein kritischer Faktor in der Kommunikation zwischen Tumorzellen und dem Wirt. Dies schließt die Rekrutierung hämatopoetischer Zellen und die Vorbereitung der prämetastatischen Nische ein. Unter physiologischen Bedingungen könnte die Modulation der extrazellulären Matrix durch Exosomen zu Organogenese, Vaskulogenese und Angiogenese beitragen und bei Gewebereparatur wie Wundheilung und Koagulation nach Verletzung von Blutgefäßen beteiligt sein.

1. INTRODUCTION

Cancer remains a leading cause of death. This is mainly due to the capacity of tumor cells to spread through the body and to settle in distant organs, which process is called metastasis (Alberts *et al.*, 2008; Jemal, *et al*., 2011). Metastasis formation is tumor cells show additional genetic alterations that allow primary tumor cell detachment, invasion and growth at distant site. Despite extensive studies on tumor cell biology that provide many helpful suggestions on early cancer detection and new treatment strategies, there are still many open questions on tumor progression, drug resistance and settlement at distant sites which are the major causes of the clinical failure in surgery or chemo- and radiotherapy.

1.1 Pancreatic adenocarcinoma

Pancreatic cancer is characterized as an aggressive malignancy, with the poorest diagnosis in gastrointestinal cancers. (Keleg *et al*., 2010). The cause of pancreatic cancer so far remains unknown, but most common known risk factors are smoking, high-calorie diet, chronic pancreatitis and diabetes (Jones *et al*., 2008; Dumitrascu *et al.,* 2009; Carney *et al*., 2003).

Pancreatic ductal adenocarcinoma (PDAC) could account for the large majority (92%) of all pancreatic neoplasms. The estimated 5 years survival rate is less than 6% (Michaud *et al*., 2007). PDAC originates in ductal epithelium and evolves from pancreatic intraepithelial neoplasias (PanINs). This progression from minimal grade PanIN-1 (low-grade) to more severe grades including PanIN-2 (intermediate-grade) or -3 (high-grade), and finally to invasive carcinoma, results from the successive accumulation of genetic alterations. The lower grade PanIN-1 or -2 harbors genetic alterations in the GTPase KRas (KRAS) (Dumitrascu *et al.,* 2003; Alberts *et al.*, 2008), and cyclin-dependent kinase inhibitor 2A (p16/CDKN2A) (Brabletz *et al.,* 2003), whereas the higher grade PanIN-3 and invasive adenocarcinomas not only contain the genetic

alterations in KRAS and p16/CDKN2A genes, but also specially harbor several mutations in tumor protein p53 (TP53) and SMAD4 (Jones. *et al*., 2008).
Although great achievements have been made in pancreatic cancer research, it remains the deadliest form of solid malignancy, and after metastatic spread there are no effective therapeutics. As a matter of fact, lack of clinical symptoms still results in poor early detection in PDAC patients. The high lethality of PDAC is mainly due to the fact that most patients have advanced disease stages when diagnosed, with the tumor rapidly spreading and being resistant to chemotherapy and radiotherapy (Krautz *et al.*, 2011). Thus, tumor metastasis will be the main therapeutic target for patients with PADC when removal by surgery failed.

1.2 Tumor metastasis

Tumor metastasis is the spreading of cancer cells through lymphatic and blood vessels from the organ where the cancer originates to other organs like bone, lung and brain, which are not directly connected with the primary tumor organ (Birchmeier et al., 2003; Brabletz *et al.,* 2010). Tumor metastasis is responsible for over 90% of the mortality rate in pancreatic cancer patients and is the most life-threatening aspect of cancer (Fidler, 2003). Metastasis formation requires cancer cells to escape apoptotic signals and to survive despite the host immune responses (Hunter et *al*. 2008). Briefly, during cancer metastasis, cancer cells first detach from the surrounding tumor cells and escape from the primary site, migrate through the basement membrane, invade and penetrate through the blood vessels or lymphatic channels, arrest in the circulation, extravasate into secondary sites, finally adapt and outgrow in a new microenvironment (Figure 1).

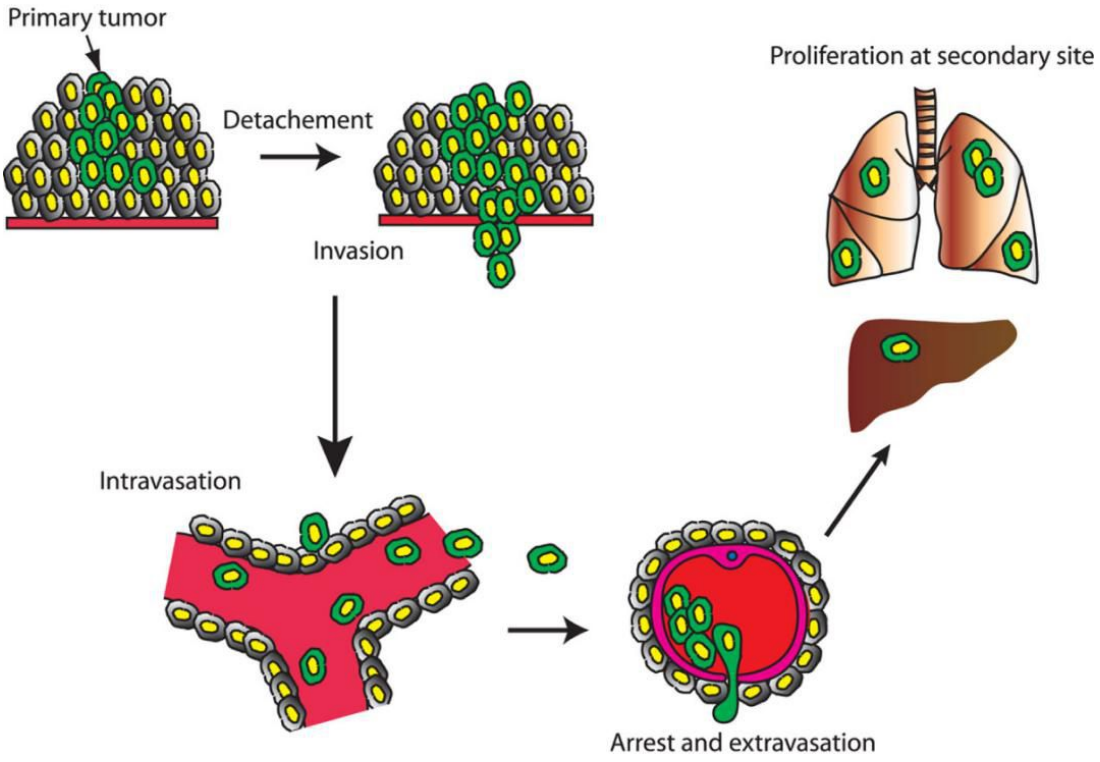


Figure 1. The development of malignant tumor cells including detachment, invasion, extravasation and proliferation in a second organ. (Hunter *et al.* 2008)

Many theories already proposed to explain the process of tumor metastasis. Among them, the 'seed and soil' hypothesis offers a great insight into the tumor development and metastasis (Paget, 1889; Fidler, 2003). This hypothesis supposes that the tumor cells (seeds) are disseminated systemically, but they can only grow in specific organ microenvironments (soils).

Since then, the concept of the pre-metastatic niche has been proposed, which states that the primary tumor cells can transmit signals to create an environment prior to the arrival of metastatic cells (Alderton *et al.,* 2012; Birchmeier et al., 2003). It means that the 'soil' is prepared much earlier than expected in the period of tumor carcinogenesis. In fact, before the migrating tumor cells approach distant sites, the target organs already have been colonized by myelomonocytic cells originating from the bone marrow (BM) (Erler *et al.,* 2009). These myelomonocytic cells form an inflammatory-like microenvironment, which is similar to the cancer microenvironment that is produced by the primary tumor, and promotes tumor cell settlement and survival

in a new hostile environment (John *et al.,* 2003; Castells *et al.,* 2012). Thus, elaborating the mechanisms underlying metastatic niche establishment can substantially improve the early therapeutic targeting of disseminated tumor cells, as well as the prognosis for patients with advanced malignancy.

To date, it is widely accepted that the cancer microenvironment is crucial for the metastatic process including pre-metastatic niche formation. (Epifano *et al.,* 2004; Hunter et *al*., 2008). The cancer microenvironment is created by the cancer cells and dominated by tumor stroma, blood vessels, tissue-associated cells and infiltrating inflammatory cells (Stewart *et al.,* 2011;Keleg *et al.,* 2003). Apart from the effect on tumor metastasis, there are emerging evidences showing that the cancer microenvironment also promotes tumor initiation and proliferation.

1.3 Cell-Extracellular matrix interactions

The extracellular matrix (ECM) is a mixture of extracellular molecules secreted by cells, which provides biochemical and structural support to the surroundings (Hynes *et al*., 2009; Kopfstein *et al*., 2006). The interaction between cells and extracellular matrix components plays significant roles in regulating many different cellular functions like cell motility, proliferation, differentiation and drug resistance (Brizzi *et al*., 2012; Shields *et al*., 2012). In the context of cancer, the extracellular matrix is the main component of the cancer microenvironment. During cancer development, the extracellular matrix helps cancer cells to build a niche and to establish a microenvironment that favor the growth of the primary tumor and tumor metastasis. As the extracellular matrix has such a critical role in tumor metastasis, clarifying cell-ECM interactions will contribute to the understanding of cancer microenvironment and cancer progression.

1.3.1 Integrins

Integrins present on the cell surface are the main receptors responsible for cell-ECM interactions (Hakulinen *et al.,* 2003). They are transmembrane proteins containing transmembrane alpha and beta subunits, large extracellular domains for the interaction with the ligands in the extracellular matrix and an intracellular domain interacting with cytoskeleton proteins (Kawakami *et al*., 1999; Epifano *et al*., 2012). In mammals, 18 alpha subunits and 8 beta subunits have been characterized, and these subunits can combine in various ways to form 24 integrin receptors (Hynes, 2002). Integrins function as the mediator of the extracellular signal when binding ligands to transmit chemical and mechanical signals into the cytoplasm, which leads to the rearrangements of the cytoskeleton and activation of various signaling pathways (Brizzi *et al*., 2012).

During the main process of tumor carcinogenesis, the expression of integrins significantly changes, and different types of tumors show distinct integrin expression profiles (Rabinovitz *et al.*, 1996; Bertotti *et al.*, 2002). Importantly, integrins can directly promote cell adhesion by serving as ligands of the extracellular matrix, or indirectly activate growth factors and intracellular signaling pathways to regulate extracellular matrix remodeling. For instance, integrins interact with receptor tyrosine kinases (RTKs) on cell surface and activate cellular signaling pathways that are critical for tumor metastasis (Baass *et al.*, 1995). In particular, integrin α6β4, which associates with the epidermal growth factor receptor (EGFR), is abundant in pancreatic carcinoma. Integrin αVβ3 in cooperation with the EGFR and the platelet-derived growth factor (PDGF) dominates in glioblastomas and melanomas (Hammond, 2001; Recio *et al.*, 2002). Furthermore, most integrins activate focal adhesion kinase (FAK), which causes the phosphorylation of downstream signaling protein (Yang *et al*., 2011; Reilly *et al*., 2001). Taken together, the crosstalk between integrins and cell surface receptors is important for the transduction of inter- and extracellular signals.

1.3.2 Proteases influence extracellular matrix remodeling

A balance between cells and surrounding extracellular matrix is crucial for promoting cell development. However, during cancer progression, this balance is broken mainly due to the remodeling of the ECM by proteases secreted by tumor cells (Samnegard *et al.,* 2005). Proteases are molecules of relatively small size and are compact, spherical structures that can catalyze the cleavage of peptide bonds or hydrolyze large proteins to smaller molecules (Apte *et al.,* 2004; Fayard *et al.,* 2009). To date, proteases are broadly identified as metalloproteases, serine proteases, threonine proteases, cysteine proteases and glutamic acid proteases.

Cleavage and degradation of extracellular matrix components by different families of proteases are the main processes during extracellular matrix remodeling and are important for regulating extracellular matrix abundance and structure (Fayard *et al.,* 2009), as well as for releasing biologically active molecules, such as growth factors (Colige *et al.*, 2005). During metastatic dissemination, two essential families of proteases, metalloproteinases and serine proteases, are the main enzymes involved in extracellular matrix remodeling.

1.3.2.1 Metalloproteinase

Matrix metalloproteinases (MMPs)

The family of metalloproteinases contains matrix metalloproteinases (MMPs) and adamalysins (ADAMs). MMPs, a family of zinc-dependent endopeptidases, are the main enzymes involved in extracellular matrix degradation. MMPs were originally categorized based on their specificity for extracellular matrix components, including gelatinase A (MMP-2), gelatinase B (MMP-9), stromelysins, collagenases and matrilysin. However, as a growing list of MMP substrates have been identified. MMPs are currently divided into secreted MMPs (Escrevente *et al.,* 2011) and membrane-type MMPs (MT-MMPs)

(Hakulinen *et al.,* 2008). According to the structural blueprint of MMPs, most MMPs display three domains, including the propeptide (Fayard *et al.,* 2009; Ngora *et al.,* 2012), a catalytic domain, as well as the hemopexin-like C-terminal domain that is linked to the catalytic domain. Interestingly, the activity of MMPs is low in normal conditions but increase during repair or remodeling processes as well as in pathological or inflammatory tissue (Sanderson *et al.*, 2005). In fact, all the MMPs are synthesized and secreted in the latent form. They are activated by sequential proteolysis of the propeptide that binds and blocks the active site cleft (Yu *et al.,* 2012; Samnegard *et al.,* 2005).

Adamalysins

The adamalysin family includes ADAMs, which is a disintegrin and metalloproteinases, and ADAMTS, the ADAMs contain a thrombospondin motif. ADAMs contains a disintegrin and a metalloproteinase domain, whereas ADAMTS are ADAM proteases with a thrombospondin motif (Colige *et al.*, 2005; Apte *et al.,* 2004). Although all ADAMs contain a metalloproteinase domain, only less than half of them show proteolytic activity. ADAMs are membrane-bound enzymes which can cleave transmembrane protein ectodomains adjacent to the cell surface, thereby releasing the complete ectodomain of cytokines, adhesion molecules, receptors, and growth factors (Escrevente *et al.,* 2011).

Clinically, the adamalysin family is implicated in various pathological events including tumor metastasis and fibrosis through shedding of growth factors, cytokines and apoptosis-inducing ligands (Fayard *et al.,* 2009; Ngora *et al.,* 2012).

Metalloproteinase inhibitors

Since there is a clear connection between extracellular matrix degradation through proteases and tumor invasion, numerous studies have linked inhibition

of proteases by synthetic inhibitors with a corresponding suppression of tumor metastasis. Furthermore, the tissue inhibitors of metalloproteinases (eg., TIMPs) are natural metalloproteinases inhibitors and commonly expressed at tumor sites. They are the most essential physiological inhibitors of metalloproteinases (Kawaguchi *et al.,* 1997). The TIMP family is comprised of four members (TIMP1 to TIMP4), which form 1:1 stoichiometric complexes with active MMPs, ADAMs and ADAMTS, leading to the inhibition of their proteolytic activity.

In the cancer microenvironment, different sets of metalloproteinases and TIMPs are mostly generated by tumor and non-transform cells (Zoller 2009), and released into the outside extracellular space, where they regulate the matrix around tumor cells (Khokha, 1994; Kawaguchi *et al.,* 1997). The function of proteases in tumor progression depends on a delicate local balance between metalloproteinases and their physiological inhibitors (Fayard *et al.,* 2009). Thus, during the invasive process, TIMPs are presented primarily by the cancer cells and serve as a regulatory mechanism for controlling the activity of the MMPs.

1.3.2.2 Serine proteases

The serine protease family occupied above one-third of all the identified proteases. Their distinctive feature is the nucleophilic serine in their active site (Kriegbaum *et al.*, 2011). The urokinase plasminogen activator (uPA) is the most essential serine protease, and has been recognized as a prognostic marker in many human malignancies. uPA always interacts with its receptor uPAR (CD87) on the cell surface, and facilitates tumor cell motility and extravasation via regulating local proteolysis and these separating cells from the extracellular matrix components (Xu *et al.*, 2010; Kawaguchi *et al.,* 1997).

The inhibitors interaction like uPA/uPAR are important suppressors for tumor cell invasion, angiogenesis and metastasis. Plasminogen activator inhibitor-1

(PAI-1), which located in the uPA/uPAR system is able to prevent the cleavage of plasminogen as well as excludes plasmin from their surrounding microenvironment.

1.3.2.3 Proteases affect extracellular matrix modulation and tumorigenesis

During the first step of tumor migration and invasion, cancer cells require to detach from adjacent cells and surrounding matrix through the cleavage by protease (Kessenbrock *et al.,* 2010). In this process, the extracellular matrix is remodeled and components of the extracellular matrix are degraded by the proteases to facilitate cancer progression. For instance, cleavage of laminin-5 γ2 chains by MMP-2 and MMP-14 produces a fragment containing epidermal growth factor (EGF)-like motifs that engages EGFR (EGF receptor) and integrin signaling on cell surface, leading to cell migration (Hakulinen *et al*., 2008; Ngora *et al.*, 2012). Several types of the collagen family including collagen III, IV, IX and X, as well as elastin, and fibronectin are degraded by MMP-3. This matrix protein degradation affects cell adhesion and migration (Samnegard *et al.,* 2005). Furthermore, the various interactions between MMPs promote focal degradation of the extracellular matrix during invasion. (John *et al*., 2001; Egeblad *et al*., 2002).

Beyond the cleavage of extracellular matrix components, proteases also take an important part in interfering with the interaction during tumor cell of progression (Figure 2). For example, E-cadherin is a critical molecule for cell-to-cell adhesion. The deregulation of E-cadherin by MMP-3 and MMP-7 can induce epithelial-mesenchymal transition (EMT), thereby promote tumor metastasis. In addition, MMP-14 can degrade the tyrosine-protein kinase-like 7 (PTK7), a key inhibitor of tumor invasion, which leads to cancer cell motility (Castells *et al.*, 2012). CD44, a main surface receptor for hyaluronic acid (HA), is cleaved by MMP-9 and MMP-14. Studies showed that the disruption of the

CD44-MMP-9 complex at the cell surface through overexpression of the CD44 extracellular domain could inhibit tumor migration and invasion (Zöller, 2011).

Besides being involved in cell adhesion and motility, emerging evidences indicate that proteases are engaged in the formation of a metastatic niche (van Hinsbergh *et al.,* 2008; Yu *et al.,* 2000). As mentioned, the formation of a premetastatic niche mainly requires soluble factors (e.g. VEGF) secreted by primary tumor cells and the fibronectin that accumulates at surrounding stroma cells. MMP-2 and MMP-9 take important roles on releasing and activating the Kit ligand, and other molecules like vascular endothelial growth factor, which are involved in creating a tumor metastasis supporting microenvironment (Figure 2). Furthermore, MMPs also display multiple functions during tumor angiogenesis (John *et al.,* 2001). Taken together, several genes or matrix proteins are regulated by proteases that trigger extracellular matrix remodeling and thereby the switch towards metastasis and neovascularization at distant sites.

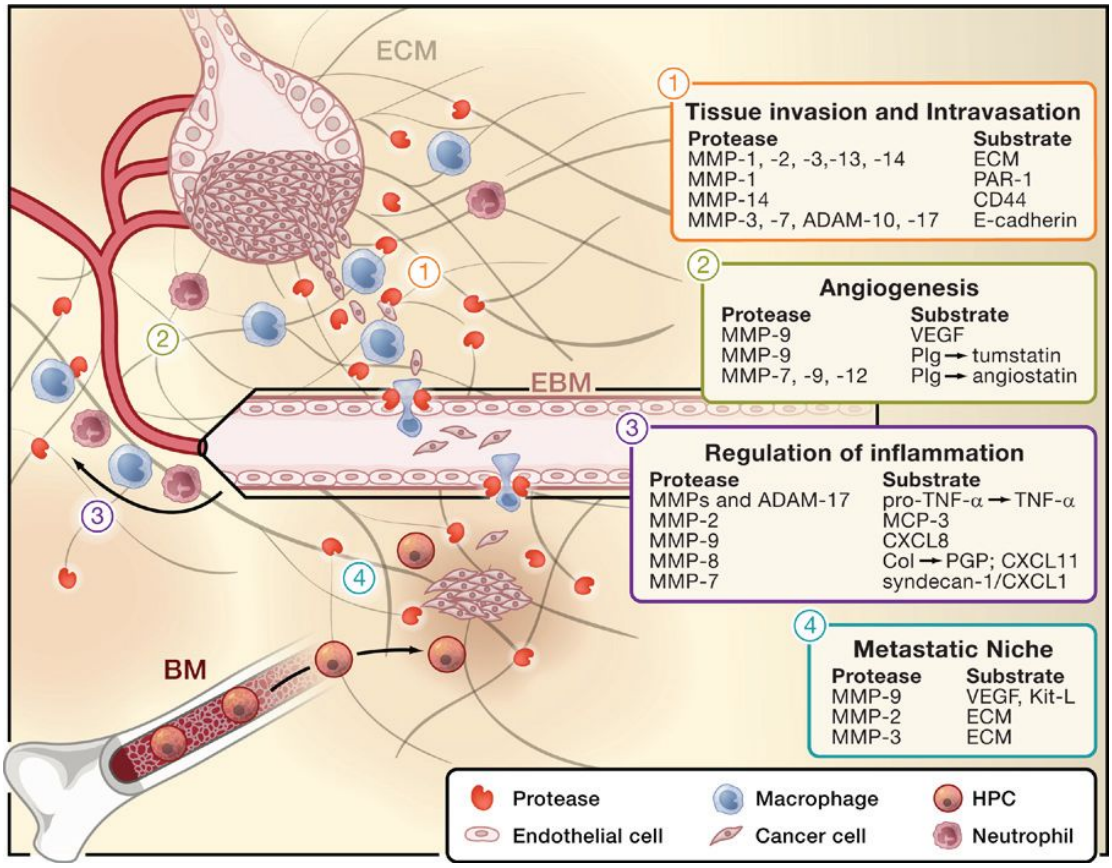


Figure 2. Functions of MMPs in the tumor microenvironment. Proteinases and their substrates in each of the tumor progression steps are as follows, 1) Tissue invasion and cancer cell intravasation. 2) Highly vascularized tissues and new blood vessel generation (angiogenesis). 3) Recruitment of inflammatory cells to the tumor microenvironment; 4) Metastatic niche formation. MMP-2, MMP3 and MMP-9 are involved in this process by releasing factors from the bone marrow (BM). (Kessenbrock *et al.,* 2010)

1.4 Role of CD44 in tumor metastasis

1.4.1 Structure and expression of CD44

The glycoprotein CD44 is encoded by a single gene, however the corresponding mRNA is composed of 20 exons (Bennett *et al.,* 1992; Arch *et al.,* 1992). As shown in figure 3, the first, as well as the last five (exon 1-5 or exon 6- 20) exons are all constant exons, whereas the intermediate No. 6-15 exons are combinations due to alternative splicing of the CD44 pre-mRNA. Exons 6-15 combination leads to the generation of many variable regions and different isoforms, which named as CD44 variant v1-v10 (Banerji *et al.,* 1998).

Up to now, at least 20 different isoforms of CD44, with a molecular weight of 85-230 kDa, have been identified in many cell types (Günthert *et al.*, 1997). The smallest CD44 molecule (85-95 kDa) is the standard isoform (CD44s), which does not harbor any variant exon and is ubiquitously expressed in vertebrate cells. The extracellular domain of CD44 contains a hyaluronic acid (HA) and other glycosaminoglycan (GAG) binding sites (Zöller, 2011; Christ *et al.,* 2002). The cytoplasmic domain could be encoded by exons 9 or 10, which is essential in cell migration as well as cellular signal transduction via binding cellular molecules (Hyman *et al.,*1998; Günthert *et al.*, 1997).

CD44 variants are closely related with tumor progression. For instance, expression of variant isoforms including CD44v6 correlates with poor prognosis in many cancer types such as cervical cancer, colorectal carcinoma (Kuhn *et al.,* 2007; Berg *et al.,* 2012), acute myeloid leukemia (Legras *et al.*, 1995; Hirata *et al.,* 1998), and pancreatic carcinoma (Marhaba *et al.*, 2004; Megaptche *et al.,*

2010). Moreover, high expression of CD44v6 correlates with lymph node metastasis formation (Kunishi *et al.,* 1997; Wang *et al.,* 2013).

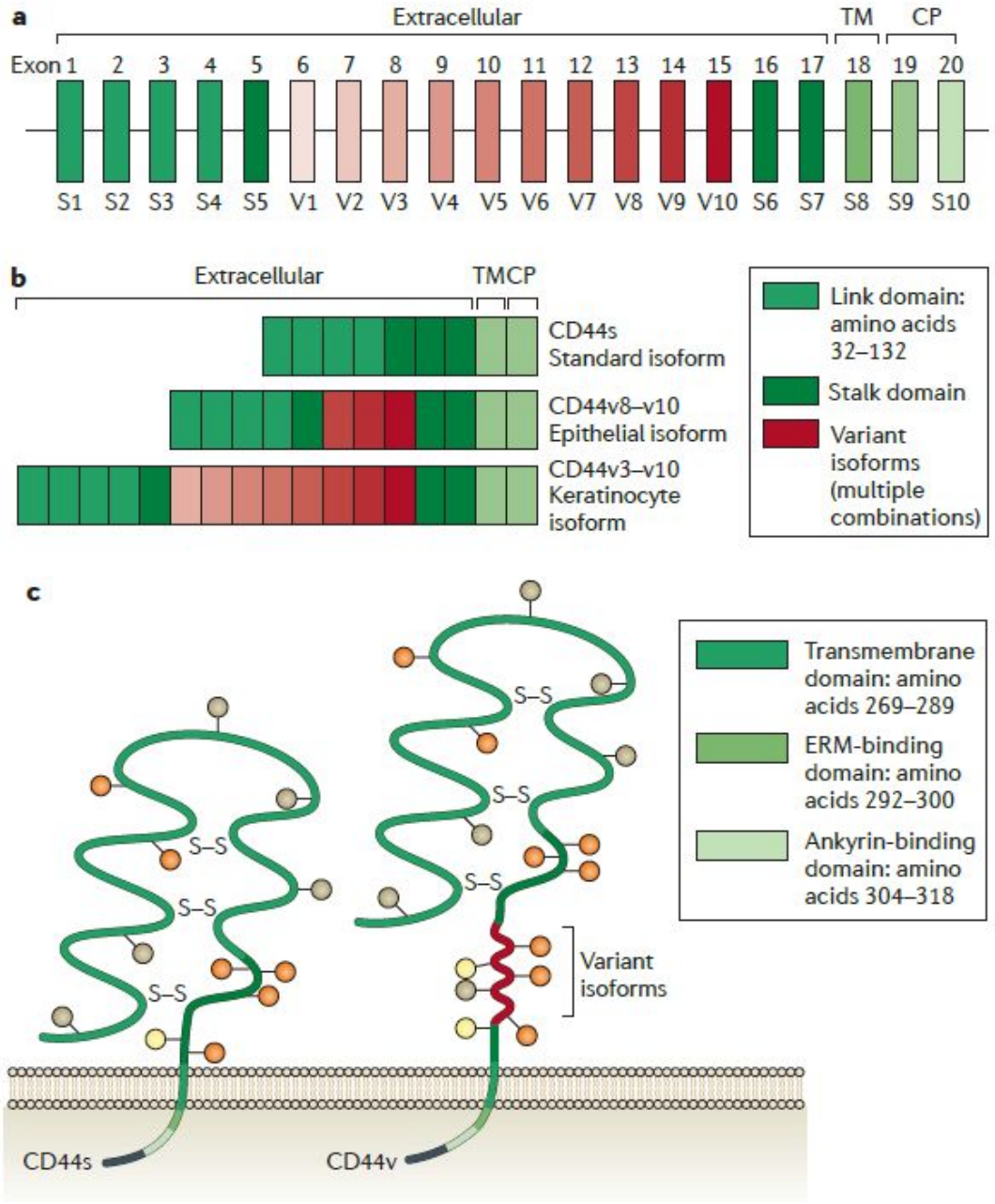


Figure 3. The CD44 gene molecule and CD44 protein structure is shown. a) Exons of CD44. b) Examples of alternatively spliced CD44 proteins. c) The CD44 molecule comprises many extracellular domains, a stalk-like region close to the transmembrane region where the variant exons products can be inserted. There are multiple sites for N- and O-glycosylation and two active glycosaminoglycan (GAG) binding sites (Zöller, 2011).

In a clinical study, it has been indicated that CD44v5, CD44v6, and CD44v7 are present in non-small cell lung carcinomas (NSCLCs), whereas there is low expression level of these variants v5 to v7 in lung tissues (Legras *et al.*, 1995; Miyoshi *et al.,* 1997). Another case of in vivo experiments showed lung metastasis formation of breast cancer cells occurred after orthotopic transplantation of CD44 variant-expressing breast cancer cells 4T1 (Yae *et al.*, 2012). However, the high expression of CD44 isoforms does not always have a positive correlation with tumor metastasis. For instance, in oral squamous cell carcinomas, the low expression of CD44v6 promotes lymph node metastasis formation (Kunishi *et al.,* 1997). Overall, the physiologic and pathologic behavior of various cell types is associated with different expression of CD44 variant isoforms.

1.4.2 CD44 associated molecules in cancer

CD44 also serves as receptors for many extracellular matrix components such as laminins, fibronectin, and other ligands (Bourguignon *et al.,* 2002). Hyaluronic acid (HA) is the well-known ligand for both CD44s and CD44 variants. The interaction between CD44 and HA is characterized as a principal mediator of cell proliferation in breast carcinoma, glioma and malignant mesothelioma (Bloor *et al.,* 2001, Tienthai *et al.,* 2007).

CD44 could act as a important co-receptor for receptor tyrosine kinases system (RTKs). CD44 influence the activation of RTKs such as the hepatocyte growth factor receptor (HGFR/c-Met). In fact, the variant isoform CD44v6 plays a prominent role as a co-receptor for RTKs (Orian-Rousseau *et al.,* 2002). The activation of c-Met through theCD44v6 bind its ligand, like hepatocyte growth factor (Tremmel *et al.,* 2009).

Beyond this, CD44 also interacts with MMPs and it serves as an important platform where MMPs can associate with their substrates, which contributes to cancer progression. For instance, the activation of transforming growth factors-β

(TGF-β) is strongly dependent on MMP-9 binding to the cell surface via CD44v6 (Schmidt *et al.,* 2004; Yu & Stamenkovic, 2000). The recruitment of MMP-9 to the cell surface supported by CD44 not only contributes to matrix proteins degradation, but also causes the proteolytic activation of the TGF-β precursor to induce tumor angiogenesis and metastasis (Ge *et al.*, 2012).

1.4.3 CD44 is involved in extracellular matrix assembly

CD44 serves as a transmembrane receptor of hyaluronic acid, thus the interaction of CD44-HA does not only promote tumor cell arrest and extravasation (Yae *et al.,* 2012), but also modify the surrounding matrix to support cell colonization.(Yae *et al.,* 2012). For instance, tumor cells support the increase of hyaluronic acid expression that facilitate the formation of the pre-metastatic niche (Hirata *et al.,* 1998; Bourguignon *et al.,* 2000).

Some previous researches provide some evidences that CD44 is directly involved in extracellular matrix (Bennett *et al.,* 1995; Jung *et al.,* 2009; Bourguignon *et al.,* 2012)remodeling and cancer progression. Klingbeil *et al.* established CD44v4-v7 (CD44v) stable knockdown cell lines from a highly metastatic tumor line (Klingbeil *et al.,* 2009). These stable knockdown cell lines showed a significant reduction in metastatic capacity, which was mostly due to an altered tumor extracellular matrix (Jung *et al.,* 2009). This altered matrix secrete by CD44v knockdown tumor cells does not support adhesion of neither CD44v knockdown nor wildtype cell lines. CD44v4-v7 associates with matrix proteins to regulate cell adhesion and to form a supporting microenvironment for the pre-metastatic niche might be in a CD44v6-dependent manner (Jung *et al.,* 2012). In fact, the absence of CD44v is accompanied by a strikingly reduced expression of hyaluronan synthases type 3 (Jung *et al.,* 2009; Jung *et al.,* 2012).

1.5 Exosomes

Exosomes are endocytic origin, small (30-100nm) vesicles that are released from various cell types in vitro and in vivo (Lakkaraju *et al*., 2008, Peinado *et al.,* 2012). Exosomes are derived from multivesicular bodies (MVB), which either fuse with lysosomes or fuse with the plasma membrane (PM) and secrete intraluminal vesicles (ILVs). Thus, exosomes have similar biochemical characteristics to the internal vesicles of MVB (Théry *et al.,* 2002, Zöller*,* 2009). Notably, exosomes contain many lipids (Mittelbrunn *et al*., 2011; Zöller*,* 2009), proteins, mRNA and miRNA (Alderton *et al.,* 2012; Théry *et al.,* 2002), these components which are crucial in intercellular communication.

1.5.1 Exosome generation

The generation of exosomes starts with the endocytosis of membrane domains from the cell surface, with the endocytosed membrane being called early endosomes. The early endosome formation occurs either in a clathrin-dependent, or in a clathrin-independent manner, which is generated by lipid-rafts. The formation of MVB is initiated in the late endosomes through sorting of ILVs via inward budding of limiting membranes (Alderton *et al.,* 2012; Théry *et al.,* 2002). As figure 4 shows, MVBs can be processed via two routes. MVBs either traffic to lysosomes, leading to the degradation in the proteasome, which are named degradative MVBs, or fuse with the plasma membrane and release their cargo ILVs into the extracellular space, which are named exocytic MVBs, and the released ILVs are named exosomes (Mittelbrunn *et al*., 2011; Zöller*,* 2009).

Inward budding and subsequent fission of early endosomes and their transport to MVBs requires several groups of complexes (Bache *et al*., 2003). In general, ESCRTs are involved in the mechanisms governing the degradation of MVBs in an ubiquitinylation-dependent manner (Hegmans *et al*., 2008). The ESCRT are composed of four different protein complexes (ESCRT 0-III), which are

essential for ILV construction. The ESCRT-0 complexes recognize and recruit ubiquitylated proteins onto the endosomal membrane that initiates MVB biogenesis. ESCRT-I and ESCRT-II complexes serve for membrane deformation into buds with sequestered proteins, and the ESCRT-III protein complex could subsequently drive vesicle scission (Sheldon *et al*., 2010; Niel *et al*., 2011).

However, it has been suggested that MVBs and ILVs can also be formed through ESCRT-independent (ubiquitinylation-independent) pathways. Indeed, the MVBs synthesis is not abolished in the absence of ESCRT. Cells can employ another alternative manner to sorting proteins into MVB, which is regulated by lipid membrane constituents of the MVB. In this sorting mechanism, the proteins are assigned into the ILVs based on their intrinsic physical properties, and then segregated into raft-like microdomains (de Gassart *et al.,* 2003).

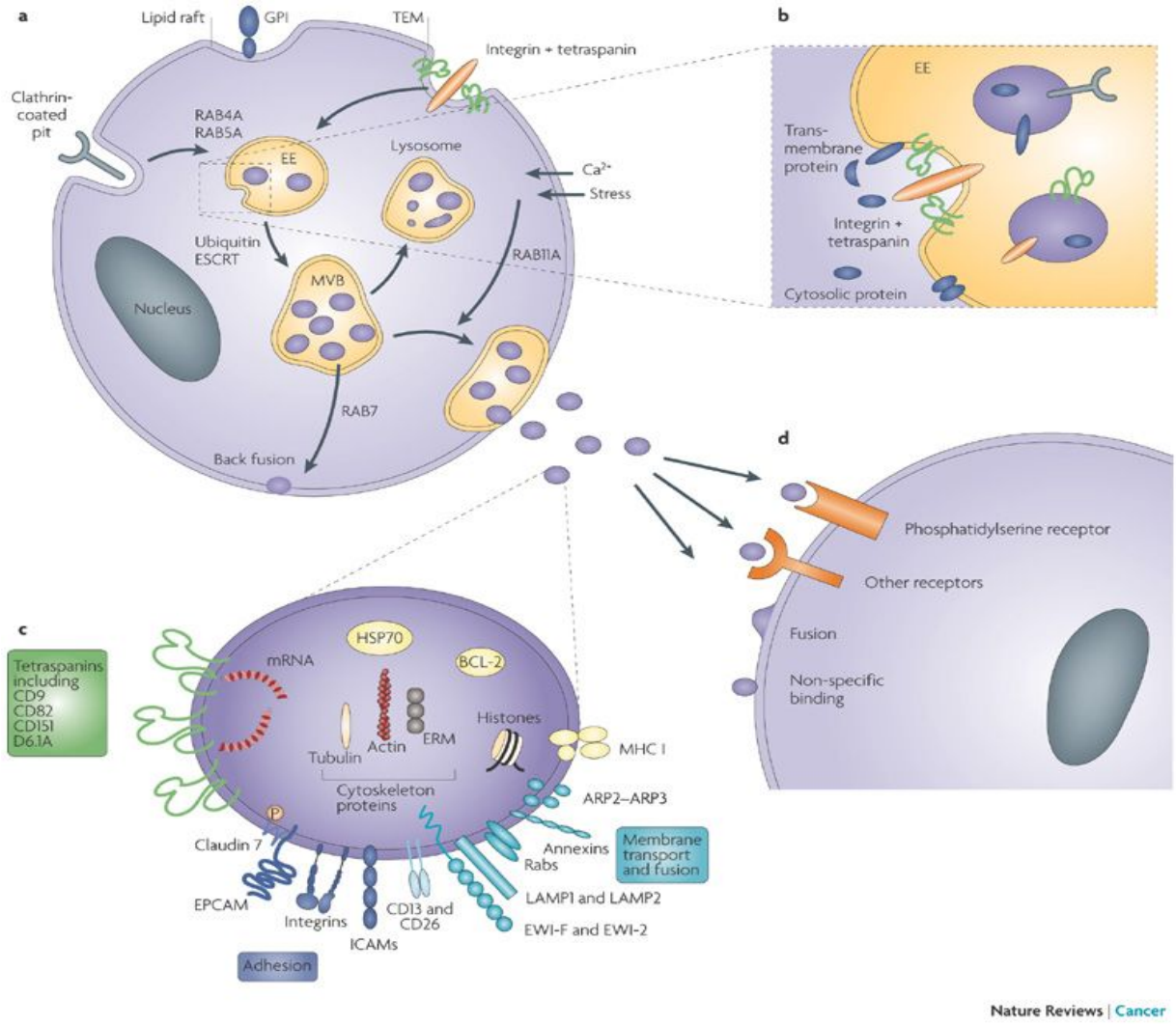


Figure 4. Exosome composition and generation. a) and b) Exosomes are derived from multivesicular bodies (MVB), the fusion of MVB with the plasma membrane (PM) and then secrete the intraluminal vesicles. c) The common components of exosomes. d) Exosomes uptake follows several routes such as specific receptors binding, phosphatidylserine receptor binding, or fusion with the target cell membrane. EE: early endosome; TEM: tetraspanin-enriched microdomain. (Zöller, 2009)

1.5.2 Exosome composition

The component of exosomes can be identified by various techniques including western blotting (WB), enzyme linked immunosorbent assay (ELISA), flow cytometry, and genomic technologies such as miRNA or mRNA microarrays. Analysis of exosomes by different technologies expands our insight into the exosome-microenvironment crosstalk.

As Figure 4c shows, exosomes carry a large number of common proteins, mRNA and miRNA (Zöller, 2009). One major class of exosome cargo proteins

is adhesion molecules. These proteins include integrins, CD146, CD18, CD11a, CD11-b, CD11-c and milk-fat-globule EGF-factor VIII (MFG-E8) (Théry, 2011; Mittelbrunn *et al*., 2011; Valadi *et al*., 2007). Another widely identified protein group is tetraspanins, a family of transmembrane proteins abundantly expressed on the exosome surface. Tetraspanins, such as Tspan8 (Nazarenko *et al.,* 2010), CD9 (Murayama *et al,* 2008), CD81, CD63 (Berditchevski *et al,* 2007) and CD151 (Yue *et al,* 2013), modulate biological functions via tetraspanin-enriched microdomains (TEMs) that are critical in tumor metastasis and angiogenesis (Rana *et al*. 2011; Wang *et al*. 2012). In addition, exosomes are also rich in metabolic enzymes, heat-shock proteins, tubulin, proteases, antigen presentation proteins like the major histocompatibility complex (MHC) class I and II, signal transduction molecules, and the lysosomal-associated membrane proteins, LAMP1 and LAMP2 (Thery *et al.,* 2001; Silverman *et al.,* 2010; Simons & Raposo *et al.,* 2009; Hegmans *et al.,* 2004). Notably, tumor derived exosomes contain large numbers of tumor specific proteins, mRNA and miRNA, and thereby transfer these cargoes to their target cells. These tumor exosomal molecules such as cadherin-17, Tspan8 and epithelial cell adhesion molecule (EpCAM) are tumor cell-specific, which can be clinically used as diagnostic markers (Zöller, 2009).

1.5.3 Target modulation by exosomes

The extracellular or intercellular communication between tumor and stroma cells was for a long time considered to require cell-cell or cell-ECM interactions to regulate cancer progression. It is well accepted that exosomes fulfill at least part of these activities. Thus, despite serving as potential diagnostic marker (Zöller, 2013; Madhavan *et al.,* 2015), exosomes are also recognized as critical mediators of the intercellular crosstalk with the tumor microenvironment.

Exosomes can transfer messages to target sites in the microenvironment basically through three manners (Figure 4d). First, exosomal membrane proteins

can bind to the surface of target cells, acting as ligands for receptors (e.g. phosphatidylserine receptors), thereby activating or regulating intracellular signaling. Second, exosomes, can fuse with the membrane of target cells so that exosomal membrane proteins and lipids become integrated into the host cell membrane and modify the plasma membrane through the addition of new membrane receptors and different lipid components. Third, liberation of the exosome contents after uptake can strongly affect the targets and lead to target cells reprogramming. Thus exosomes can modulate target cell composition as well as activate signaling pathways to regulate physiological and pathological processes (Stoeck *et al.,* 2006; Simons & Raposo *et al.,* 2009).

The tumor cell derived exosomes contribute to tumor metastasis through modulating their targets and breaking anti-tumor immune reactions (Ge *et al.,* 2012). These processes depend on activation of ligands, as well as extracellular matrix degradation by exosomal proteases (Egeblad *et al.,* 2002). Furthermore, tumor exosomes also release many regulators of proteases, which enhance the activity of proteases and thereby stimulate the pro-invasive factors (e.g. HGF) in recipient cells (Cho *et al.,* 2012).

It is also well known that tumor cells create their own matrix and further affect host cells to generate a microenvironment that support tumor cells survival and motility (Garnier *et al.,* 2010; Jung *et al.,* 2009), but how tumor exosomes participate and influence this progress is still poorly understood. Interestingly, current studies indicate that the interaction between exosomal proteases and target cells also requires the cooperation with CD44 (Simpson*et al.,* 2009; Stoeck *et al.,* 2006). Since CD44 is abundantly expressed in exosomes secreted by many types of cancer cells, and is an essential receptor for matrix protein, understanding how the interaction between exosomal proteases and CD44 contributes to targets modulation becomes essential and important.

1.6 The BSp73 tumor model

The BSp73 tumor is a pancreatic adenocarcinoma that spontaneously arose in the BDX rat strain (Matzku *et al*., 1983). The primary BSp73 tumor harbors two different sublines, with a local tumor in the pancreatic organ and an ascitic metastasic tumor. The subcutaneously implanted local tumor maintained local growth and generated a solid tumor at the implantation site (AS cell line), whereas tumor cells derived from the ascites, instead of locally growing, rapidly spread via the lymphatic system to the lung, where they form excessive miliary metastases (ASML cell line). The highly metastatic variant ASML has a spherical-like morphology, while the non-metastatic variant AS shows fibroid tube morphology. To further characterize the different metastatic properties between AS and ASML, monoclonal antibodies were generated and selected for specific binding to the metastasizing ASML (Seiter *et al.,* 1993; Matzku *et al*., 1989). Through these studies, many proteins were identified as metastasis-supporting molecules. It is very interesting to find that, among them, CD44v6, acting as a tumor invasion inducer in pancreatic cancer is the most prominent one. Thus, the BSp73 tumor provides a good model to study the function of CD44v6 in cancer metastasis.

1.7 Aim of the thesis

The essential hallmark of multicellular organisms is intercellular communication that can be mediated by molecule transportation or direct cell-cell contact. However, in the last decades, exosomes, particularly tumor-derived exosomes, have been demonstrated as the third mechanism of intercellular communication. Tumor exosomes can communicate with neighboring tumor cells, stroma cells, hematopoietic cells as well as the host matrix.

To date, information has been collected mostly for the impact of tumor exosomes on stroma and hematopoietic cells, whereas the communication with the host matrix has been neglected. In my thesis, I focused on the latter point and attempted to answer the following questions:

1. Do tumor exosomes communicate with the host matrix and if yes, what are the exosomal molecules that are engaged in this communication?
2. What are the consequences of the interaction between tumor exosomes and the extracellular matrix components?
3. How do tumor exosome-induced alterations of the matrix cross talk with stroma host cells?
4. What is the special role of exosomal CD44v6 in the communication between exosomes and the host matrix?

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

Name	Company
Camera system Spot CCD	Diagnostic Instruments, Sterling Heights,
Carl Zeiss LSM710 confocal	Carl Zeiss, Hamburg, Germany
Charge-coupled device (CCD)	Hamamatsu, Herrsching,Germany
Cell chamber Neubauer improved	Brand, Wertheim
Centrifuge Sorvall RC5B Plus	Kendro, USA
Centrifuge Biofuge fresco	Heraeus, Hanau, Hanau
DNA-agarose gel electrophoresis	Bio-Rad, Munich
FACS Calibur	Becton-Dickinson, Heidelberg
Fuji Film Imaging Plate BAS-MS	Fuji Photo Film, Düsseldorf
Hyper processor (for processing	Amersham, Freiburg
Incubator for molecule biology	Melag, Berlin
Incubator for cell culture	Labotec, Goettingen
Master cycler (PCR cycler)	Eppendorf, Hamburg,
Magnetic stirrer 3000	Heidolph, Keilheim
Microscope DMBRE	Leica, Bensheim
Microwave	Phillips, Wiesbaden
Photocassette	Amersham, Freiburg
Ph-Meter-761 Calimatic	Knick, Berlin
Photometer Ultraspec III	Amersham, Freiburg
Pipettus-Akku	Hirschmann, Eberstadt
Pipettes	Eppendorf, Hamburg
Power supply PS 9009	GIBCO, Darmstadt
Rotor GSA	Kendro, USA
Rotor SW34	Kendro, USA
Rotor SW41 Ti	Beckman Coulter, Krefeld
Sterile hood	Heraeus, Hanau
Sonicator Sonoplus	Bandelin, Berlin

Table top centrifuge	Heraeus, Hanau
Thermo-mixer	Eppendorf, Hamburg
Ultrasound homogenizer	Bandelin Electronik
Water-bath	Julabo, Seelbach
Weighing scale RC210 D	Sartorius, Goettingen
Whirlmixer Vortumor exosome	Si Inc., New York, USA

2.1.2 Miscellanoeus materials

Name	**Company**
Cell culture flasks 25cm^2, 75cm^2	Greiner, Frickenhausen
Cell culture 96-well, 24-well, 6-well	Greiner, Frickenhausen
Centrifugal concentrators Vivaspin	Vivascience, Hannover
Cryovials	Greiner, Frickenhausen
Coverglass	R. Langenbrinck, Emmendingen
Dako pen	DakoCytomat., Glostrup, Denmark
Falcon tubes 15ml, 50ml	Greiner, Frickenhausen
Glass slides	R. Langenbrinck, Emmendingen
Hyperfilm ECL	Amersham, Freiburg
Needles	BD Biosciences, Heidelberg,
Nitrocellulose membrane Hybond	Amersham, Freiburg
Parafilm	Greiner, Frickenhausen
Petridishes	Greiner, Frickenhausen
Pipette tips	Sarstedt, Numbrecht
Sterile filter 0,2µm	Renner, Darmstadt
Syringes	BD Biosciences, Heidelberg
Transwell migration (Boyden)	Neuroprobe, New York, USA
WhatmanTM 3MM paper	Scleicher & Schüll, Dassel

2.1.3 Chemicals and reagents

Name	Company
Acetic acid	Riedel-de Haen, Seelze
Acetone	Fluka, Buchs, Switzerland
Agarose	Sigma, Steinheim
Ammonium persulphate (APS)	GIBCO, Darmstadt
Bio-Rad, Munich Bradford reagent	Bio-Rad, Munich
Bovine Serum Albumin (BSA)	PAA, Pasching, Austria
Brij 96	Fluka, Buchs, Switzerland
Calcium chloride	Merck, Darmstadt
Chloroform	Riedel-de Haen, Seelze
Carboxyfluorescein-succinimidylester	Invitrogen, Darmstadt
cis-Diaminplatinum(II)dichlorid	Sigma-Aldrich, München
Crystal violet	Sigma, Steinheim
Dimethyl formamide	Merck, Darmstadt
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Ethanol	Riedel-de Haen, Seelze
Ethidium bromide	Merck, Darmstadt
Ethylenediamine tetraacitic acid (EDTA)	Sigma, Steinheim
Foetal Calf Serum (FCS)	PAA, Pasching, Austria
Formaldehyde (37%)	Merck, Darmstadt
G418 sulphate	PAA, Pasching, Austria
Gelatine (cold water fish skin)	Merck, Darmstadt
Glucose	Merck, Darmstadt
L-Glutamine	AppliChem, Darmstadt
Glycerine	Roth, Karlsruhe
Glycine	GERBU, Gaiberg
HEPES	GERBU, Gaiberg
HiPerfect-Reagent for transfection	Quiagen, Hilden
Hydrochloric acid (HCl)	Riedel-de Haen, Seelze
Hygromycin	PAA, Pasching, Austria

Immersion oil	Zeiss, Goettingen
Magnesium carbonate	Merck, Darmstadt
Magnesium chloride	Merck, Darmstadt
Magnesium sulphate	Merck, Darmstadt
Milk powder	Roth, Karlsruhe
Methanol	Riedel-de Haen, Seelze
N,N,N´N´-Tetramethylenediamine	Sigma, Steinheim
Paraformaldehyde	Sigma, Steinheim
Penicillin	Sigma, Steinheim
Phenylmethylsulphonylfluoride (PMSF)	Sigma, Steinheim
PMA	Sigma, Munich, Germany
Potassium chloride	Merck, Darmstadt
Potassium dihydrogenphosphate	Merck, Darmstadt
Potassium tetrathionate	Merck, Darmstadt
Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim
Protein G Sepharose 4 Fast Flow	Amersham Biosciences, Freiburg
RPMI 1640	GIBCO, Darmstadt cell culture
Sodium acetate	Merck, Darmstadt
Sodium azide	AppliChem, Darmstadt
Sodium carbonate	AppliChem, Darmstadt
Sodium chloride	Fluka, Buchs, Switzerland
Sodium hydrogen phosphate	Merck, Darmstadt
Sodium dodecyl sulphate (SDS)	GERBU, Gaiberg
Sodium hydrogen carbonate	AppliChem, Darmstadt
SP-Dio18(3) dye for exosome labeling	Invitrogen, Darmstadt
Tris	Roth, Karlsruhe
Triton-X-100, Triton-X-114	Sigma, Steinheim
Trypan bue	Serva, Heidelberg
Trypsin	Sigma, Steinheim
Tween 20	Serva, Heidelberg

2.1.4 Buffers and solutions

Bicarbonate buffer	15mM Na2CO3, 35mM NaHCO3, pH 9.6
Blot buffer	25 mM Tris, 192mM Glycine,0.1% SDS, 20% Methanol
Ethidium Bromide	0.01% (w/v) in water. Store in dark.
Freezing medium	10% DMSO in FCS
HEPES buffer	25mM HEPES, 150 mM NaCl, 5mM MgCl2, 1 mM PMSF, Protease inhibitors
6x Laemmli-buffer	350mM Tris, pH6.8, 10% (w/v) SDS, 36% (w/v) Glycerine, 0.01% (w/v) Bromophenol blue
LB medium	10g peptone, 5g yeast extract, 10g NaCl. Make volume to 1l. Add 15g agar for LB plates
Running buffer for SDS-PAGE (10X)	1%SDS (w/v), 144g Glycine, 30g Tris. Make volume to 1l with bidestilled water
PBS	137 mM NaCl, 8.1mM Na2HPO4, 2.7 mM KCl, 1.5mM KH2PO4, pH 7.4
Stripping buffer	62.5 mM Tris-HCl (pH 6.8), 2% SDS ,0.1 M, 2-Mercaptoethanol
TAE buffer	242g Tris base, 57.1ml Glacial acetic acid, 100ml 0.5M EDTA pH 8.0. Make volume to 1l and adjust pH to 8.5
TEN buffer	40mM Tris/Cl pH 7.5, 1 mM EDTA pH 8.0, 150 mMNaCl

2.1.5 Kits

Name	**Company**
Quiaquick gel extraction kit	QUIAGEN, Hilden, G.
Quiaquik Midiprep kit	QUIAGEN, Hilden, G.
ECL Western Blotting Detecting	Amersham, Freiburg, G.

2.1.6 Markers

Name	Company
GeneRulerTM 100bp and 1Kb DNA-Ladder Plus	MBI Fermentas, St. Leon-Rot
PagerulerTM Prestained Protein Ladder	MBI Fermentas, St. Leon-Rot

2.1.7 Antibodies

2.1.7.1 Primary antibodies

Antibody	Supplier
α6β4 (clone B5.5)	Matzku et al., 1989
actin	BD, Heidelberg, G.
ADAM10	Santa Cruz, Heidelberg, G.
ADAM17	Santa Cruz, Heidelberg, G.
ADAMTS1	Santa Cruz, Heidelberg, G.
ADAMTS5	Santa Cruz, Heidelberg, G.
ADAMTS8	Santa Cruz, Heidelberg, G.
bFGF	Oncogene, Boston, USA
CD9 (clone B2C11)	BD Biosciences, Heidelberg; Developmental studies Hybridoma bank
CD11a	BD, Heidelberg, G.
CD11b (clone Ox42)	European Association of Animal Cell Cultures
CD11c (clone Ox41)	European Association of Animal Cell Cultures
CD13	Chang et al, 2005

Antibody	Supplier
CD18	BD Biosciences, Heidelberg, G.
CD29; (clone FW4.10.1)	BD Biosciences, Heidelberg, Developmental studies Hybridoma bank
CD44s (clone Ox50)	European Association of Animal Cell Cultures
CD44v6 (clone A2.6)	Matzku et al., 1989
CD49c (clone Ralph3.1)	BD Biosciences, Heidelberg, Developmental studies Hybridoma bank
CD49b	BD Biosciences, Heidelberg, G.
CD49c	BD Biosciences, Heidelberg, G.
CD54	BD Biosciences, Heidelberg, G.
CD104	BD Biosciences, Heidelberg, G.
CD151	Claas et al., 2005
CD106	Biozol, Eching, G.
CD151	Matzku et al., 1998
claudin-6	Santa Cruz, Heidelberg, G.
collagen I	Rockland, Gilbertsville, USA.
collagen II	LabVision, Frem., CA, USA.
collagen III	ARB, Golden, CO, USA.
collagen IV	Rockland, Gilbertsville, USA.
CXCR4	Santa Cruz, Heidelberg, G.
D6.1A (clone D6.1)	Matzku et al., 1998

Antibody	Supplier
EpCAM (clone D5.7)	Matzku et al., 1998
EGFR	Santa Cruz, Heidelberg, G.
ERK1/2	BD, Heidelberg, G.
FGFG	BD, Heidelberg, G.
Fibronectin	BD, Heidelberg, G.
HAdase	Santa Cruz, Heidelberg, G.
Hyaluronan	Rockland, Gilbertsville, USA.
HGF	Santa Cruz, Heidelberg, G.
JNK	BD, Heidelberg, G.
Jun	BD, Heidelberg, G.
Laminin1	Rockland, Gilbertsville, USA.
Laminin5	BD, Heidelberg, G.
MMP2	Dianova, Hamburg, G.
MMP3	Santa Cruz, Heidelberg, G.
MMP9	Dianova, Hamburg, G.
MMP13	Dianova, Hamburg, G.
MMP14	Santa Cruz, Heidelberg, G.
Metallothio.	Santa Cruz, Heidelberg, G.
Palladin	Santa Cruz, Heidelberg, G.

Antibody	Supplier
PDGF	BD, Heidelberg, G.
PDGFR	BD, Heidelberg, G.
pJNK	BD, Heidelberg, G.
pJun	BD, Heidelberg, G.
pRas	BD, Heidelberg, G.
Ras	Santa Cruz, Heidelberg, G.
SDF1	Abcam, Cambridge, UK.
TF	Santa Cruz, Heidelberg, G.
TGF	Santa Cruz, Heidelberg, G.
Tenascin	LabVision, Fremont, CA.
uPA	Calbiochem, Darmstadt, G.
uPAR	Calbiochem, Darmstadt, G.
VEGF	Biotrend, Köln, G.
VEGFR1	Biotrend, Köln, G.
Vitronectin	Biotrend, Köln, G.
vWF	Abcam, Cambridge, UK.

2.1.7.2 Secondary antibodies

Antibody	Company
anti-mouse IgG-APC	BD, Heidelberg, Germany
anti-mouse IgG-HRP	Dianova, Hamburg, Germany
anti-mouse IgG-PE	Dianova, Hamburg, Germany
anti-rabbit IgG-HRP	Dianova, Hamburg, Germany
anti-hamster IgG	Dianova, Hamburg, Germany
Streptavidin-HRP	Sigma, Steinheim, Germany

2.1.8 Matrix proteins

Protein	Supplier	Concentration
Collagen I	Sigma, Munich, G	10 µg/ml
Collagen II	Sigma, Munich, G	10 µg/ml
Collagen IV	Sigma, Munich, G.	10 µg/ml
Hyaluronan	Sigma, Munich, G.	
Fibronectin	Sigma, Munich, G.	2 µg/ml
rat LN111	Sigma, Munich, G.	2 µg/ml
rat LN332 (LN5)	804G exosome-depleted culture supernatant w/o FCS	50 µg/ml
Tenascin	SKmel exosome-depleted culture supernatant w/o FCS	50 µg/ml
Vitronectin	Sigma, Munich, G.	2 µg/ml
Matrigel	BD, Heidelberg, G.	4.5 mg/ml (1:1 dilution)

2.1.9 Inhibitors

Inhibitor	Supplier	Concentration
MMP9/ MMP13 inhibitor	Merck, Darmstadt, G.	10 µg/ml
TIMP-2 inhibitor	Merck, Darmstadt, G.	10 µg/ml
TAPI (TACE Inhibitor)	Merck, Darmstadt, G.	10 µg/ml

2.1.10 Cell lines

BSp73ASML (ASML-wt)	metastatisizing rat pancreatic adenocarcinoma line (Matzku *et al.,* 1983)
BSp73ASMLCD44v4-v7 knockdown (ASML-CD44vkd)	Stable knockdown of CD44v4-v7 in ASML-wt cell (Klingbeil *et al.,* 2009)
804G	rat bladder carcinoma line, LN5 secreting (Homma *et al.,* 1985)
Rat lymph node stroma cells (Lnstr)	Isolated from rat lymph nodes (LeBedis *et al.,* 2002)
Rat lung fibroblasts (LuFb)	Isolated from lungs of BDX rats with NiSO4
Rat aorta endothelial cells (RAEC)	Isolated from Wistar rats, Cell lining, Berlin
Lymph node cells (LNC)	Isolated from lymph nodes of BDX rats
Bone marrow cells (BMC)	Isolated from femur and tibia by flushing the bones of BDX rats

2.1.11 Rat strain

BDX rats between 8-12 weeks were used for cell preparation and in vivo experiments. The BDX rats were bred in the animal facility of the University of Heidelberg (IBF), under pathogen-free conditions and provided steile food and water *ad libitum*.

2.2 Methods

2.2.1 Cell biology

2.2.1.1 Cell culture

Cells were grown and maintained in RPMI 1640 medium containing 5%-10% heat inactivated the FCS (also named as fetal calf serum) and antibiotics (penicillin and streptomycin 100U/ml). The cells were cultured in flasks or plates at 37°C, 5% CO_2 and 95% humidity. For passage, confluent cells were detached by trypsin, EDTA or strong pipetting, and were divided into new flasks.

Long term cell storage was performed by washing cells once with sterile PBS, resuspending the cells in pre-cold FCS containing 10% DMSO. After step-cooling, cells were kept in a -80°C freezer overnight and then vials were transferred into liquid nitrogen.

For recovery, vials were put into a 37°C water bath, and instantly transferred into pre-warmed medium, washed once at 4°C, 400rpm centrifugation to remove the DMSO. The cell pellet was resuspended in fresh medium and cells were transferred in culture flasks.

For functional experiments, the viability of cells was assessed by light microscopy after trypan blue staining. Viability of over 95% was considered sufficient.

2.2.1.2 Recloning of transfected cells through limiting dilution

To establish stable knockdown clones, the shRNA transfected cells were cloned at 1-3 cells/well in F-bottom 96-well plates. Clones were evaluated by flow cytometry, to select for clones with significant downregulation. However, the shRNA transfected into tumor ASML cells were maintained in selection medium containing 750 μg/ml geneticin (G418), and surviving cells were regularly examined by Western blot or flow cytometry analysis for the selection of stable knockdown clones.

2.2.1.3 Exosome preparation

Confluent cell cultures were cultured in serum free medium and supernatants were collected after 48 to 72h of culture. Exosome isolation proceeded through a series of centrifugation steps: 500g for 10min, 2000g for 10min, 5000g for 20 min, 10,000g for 30min. After this, exosomes were isolated by 2.5h of ultra-centrifugation at 100,000g using a SW41Ti rotor in a Beckman Coulter ultracentrifuge. The supernatant was collected as CM^{-exo}.

Exosomes were further purified via a ucrose cushion centrifugation or a discontinuous sucrose gradient centrifugation. Briefly, exosomes float at

densities harbor from 1.15 to 1.19 g/ml on layer of sucrose gradients. The exosome pellet was resupended in 800µl of buffer HEPES and totally mixed with 80% sucrose, the mixture could be placed at the bottom of the 4ml ultracentrifuge tube. Over this, 1.6ml of 30% sucrose in HEPES were carefully layered, and followed by a next layer of 5% sucrose on top. The tubes were carried to the SW41Ti rotor and centrifuged at 100,000g for 16h. Thereafter, 12 equal fractions were collected, the fractions 3 to 7 contain the purified exosomes.

For sucrose cushion, the exosome pellet generated by ultracentrifugation was resupended in 20ml PBS, and the mixture of exosome-PBS was layered on 20ml of 40% sucrose, and was ultracentrifuged at 100,000g for 90min. The top layer was removed after centrifugation, and the lower sucrose layer, which contains the exosomes was diluted with PBS and washed by ultracentrifugation (90 min, 100,000g). The method of sucrose cushion centrifugation was used for separating dye-labeled exosomes from free-dye.

2.2.1.4 Fluorescent dye-labeling of ASML wt or kd exosomes

For exosmes labeling, 1mg of exosomes was labeled in 200µl of PBS containing DHPE or SP-DioC18 $_{(3)}$ at 1:10,000 dilution in PBS for 25min. After this, the labeled exosomes were washed twice and incubated with exosome-depleted FCS in the dark for 30min so that the free dye binds to proteins in FCS. Dye-labeled exosomes were centrifuged down after the wash with PBS. The labeled exosome-pellets were purified by 40% sucrose cushion as described. The dye-labeled exosomes were stored at -80˚C till further use.

2.2.1.5 Tumor/stroma matrix generation

Tumor ASML wildtype cells (ASML-wt) and ASML CD44v4-v7 knockdown cells (ASML-CD44vkd), as well as rat lymph nodes stroma cells (Lnstr), lung fibroblasts (LuFb) and aorta-derived endothelial cells (RAEC) were cultured as described. The culture supernatants of tumor and stroma cells are referred as tumor or stroma matrix, also named as conditioned medium (CM^{+exo}). The

CM^{+exo} was further washed by centrifugation (90 min, 100,000g) to deplete exosomes (CM^{-exo}).

2.2.1.6 Adhesion assay

For measuring cell adhesion, F-bottom 96-well plates were pre-coated with matrix proteins or cell culture conditional medium (CM^{-exo}) at 4°C overnight, and then blocked with 2% PBS/BSA. Adhesion of cells was checked by CFSE fluorescence or by crystal-violet staining.

CFSE fluorescence assay was performed as follows: cells were labeled with CFSE (5μM) for 25min in serum free medium until they obtained viewable yellow color. $2x10^4$ cells of these co-incubated w/wo exosomes were seeded on pre-coated 96-well plates for 2 hours at 37°C. The plates were washed with PBS and adhesion of cells was determined in a Fluoroskan Ascent multiplate reader (excitation/emission: 490 nm/518 nm).

The crystal-violet staining assay was performed as follows: $2x10^4$ cells co-incubated w/wo exosomes were seeded on pre-coated 96-well plates for 2 hours at 37°C. Cells were harshly washed by PBS twice, the adherent cells were fixed by cold methanol and then stained with fresh 0.1% crystal violet solution for 30min. After staining, cells were dissolved in 10% acetic acid and staining intensity was evaluated by a fluorescence ELISA reader (OD: 595 nm).

2.2.1.7 Migration assay

Cell migration was evaluated by wound healing, transwell migration measurement and video microscopy. Firstly, the confluent monolayers were cultured in a 24-well plate and scratched with a pipette tip. Wound healing was observed by light microscopy after 12h-72h. Transwell migration was assessed by the Boyden chamber assay. Cells ($5x10^3$) were seeded in the upper part of the chamber in 40μl of RPMI/0.1% BSA. The lower part of the chamber, separated by a 8-μm pore size polycarbonate membrane, contained 30μl of CM^{-exo} w/wo ASML or -CD44v knockdown exosomes (10μg/ml). Migrated stroma cells

(Lnstr, LuFb and RAEC) were evaluated after 16 hours by staining the lower surface of the membrane side with 0.1% crystal violet, measuring OD595 nm after acetic acid lysis. Migration rate is presented as percentage of all input cells.

For video microscopy, cells were pre-treated w/wo different stroma CM-exo or exosomes. Cell migrating routes were evaluated for 24 hours via an Olympus IX81 inverse microscope, equipped with an incubation chamber (37°C, 5%CO_2), a charge-coupled device (CCD) camera and a ScanR acquisition software. The photograph system took pictures every 20 minutes for each well and created videos or albums of migrating cells. Migration routes were quantified according to a software ImageJ, manual_tracking plugin (F.P. Cordeliére, Centre de Recherche). The mean value of cell migration length per 1 hour is presented.

2.2.1.8 Invasion assay

For cell invasion, matrigel was thawed at 4˚C and diluted 1:1 with serum free RPMI 1640 w/wo exosomes. 100µl of this mixture was seeded carefully into an insert transwell chamber and incubated overnight at 37°C. The following day, $2x10^4$ stroma cells (Lnstr, LuFb and RAEC) in serum free medium were layered on the matrigel mixture and kept in a cell culture incubator for 24 hours. Cells which stayed in culture medium or at the surface of the matrigel were removed, and invading cells within the matrigel or penetrated through the transwell chamber were documented by light microscope and were counted via ImageJ, cell counting display.

2.2.1.9 Immunofluorescence

$3x10^5$ cells were seeded and grown on cover slides for 48h. Prior to staining, cells were fixed, and then permeabilized with 0.2% of Tween in PBS and 1%BSA. Thereafter, cells were blocked with 0.2% gelatin (derived from freshwater goldfish) for 15min and were incubated with primary antibodies (2-10µg/ml) for 1h, 4˚C. Cells were incubated with fluorochrome-conjugated secondary antibody. Finally, cover slides were washed and mounted in elvanol.

Digitized images were generated by a Carl Zeiss LSM710 confocal microscope and analyzed by software Carl Zeiss Axioview Rel. 4.6

2.2.1.10 Flow cytometry for cells and exosomes

The flow cytometry is a technique used to measure fluorescence labeling of individual cells or exosomes. Cells ($2x10^5$ per sample), detached with trypsin or EDTA, were seeded into U-bottom 96-well plates. Cells were incubated with the primary antibody (1-5µg/ml). The fluorochrome-conjugated secondary antibody (0.3-0.5µg/ml) was added and cells were incubated in the dark for 30min, at 4˚C. For intracellular staining, cells were fixed in 1% formalin for 20min, and permeabilized with 0.2% Tween in FACS buffer before incubation with the primary and secondary antibodies. Samples were acquired and analyzed with the FACS Calibur.

FACS was also used to characterize exosomes. Dye-labeled exosomes were incubated with 4-µm aldehyde-sulfate latumor exosome beads. Where indicated, latumor exosome beads (1µl) were coated with antibody or matrix proteins (10µg/ml), blocking free aldehyde groups by incubating with 100mM glycine for 20 min at room temperature before incubation with exosomes.

2.2.1.11 Apoptosis assay

For evaluating cell apoptosis, Lnstr, LuFb and RAEC ($1x10^5$) were co-cultured with autologous CM^{-exo} pretreated w/wo ASML-wt or -kd exo and were seeded onto a 96-well plate in the presence of 5µg of cisplatin for 24h or 48h. Thereafter, cells were washed with PBS/1% FCS. To monitor survival, cells were incubated with Annexin-FITC/PI according to the manufacturer's conditions, afterthen, it could be evaluated by flow cytometry with two channels, channel FL-1 for annexin-FITC, however, and channel FL-3 for PI.

2.2.1.12 Cell proliferation

Cells ($3x10^3$) pretreated with stroma CM^{-exo} w/wo ASML or -CD44v knockdown exosomes were seeded on F-bottom 96-well plates along with ^3H-

thymidine (10μCi/ml) and were cultured for 24 to 72h. The ^{3}H-thymidine incorporation was evaluated in a β-counter.

Alternatively, cells were labeled by CFSE and cell division was determined. Briefly, stroma cells ($3x10^{3}$) were collected and washed twice by sterile PBS, and were co-incubated with 5μM CFSE for 20min at 37˚C. The CFSE-labeled cells were co-cultured with CM^{-exo} pretreated with exosomes for 24h, 48h and 72h. Thereafter, dilution of the CFSE-labeled cells was evaluated by flow cytometry.

2.2.2 Protein Biochemistry

2.2.2.1 Immunoprecipitation (IP)

Cells were lysed in lysis buffer (HEPES buffer containing 1% Brij96, protease inhibitor cocktail) for 60min at 4°C with shaking. 1 mg of cell lysates or 100 μg of exosomal lysates were immunoprecipitated with corresponding antibody (2μg/ml or 200μl hybridoma supernatant) overnight at 4°C. Thereafter, samples were precipitated with 5% ProteinG Sepharose for 1h at 4°C with rotation. The complexes were washed three times with lysis buffer. All liquid was removed through a 35g-needle attached to a vacuum pump to ensure minimal background. Complexes were dissolved in Laemmli buffer, boiled at 95°C for 5min and centrifuged shortly to separate sepharose beads from proteins.

2.2.2.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples

(the SDS-PAGE running gel)

SDS-PAGE is a technique used to separate proteins according to their size. The PAGE allows different size proteins to move at different rates. Two gel layers were used to separate proteins, however the stacking gel (375mM Tris pH6.8, 0.1%SDS, also with 4% acrylamide-bisacrylamide, 0.1%TEMED (v/v), 0.1% (w/v) APS allows them to enter the resolving gel. At the bottom, 5ml of separating gel (375mM Tris pH8.8, 0.1%SDS, 10% acrylamide-bisacrylamide,

0.1%TEMED (v/v), 0.1% (w/v) ammonium persulphate) allows the separation of proteins. After complete polymerization, gels were loaded and run in SDS running buffer at a constant voltage of 90V. Gels were either stained with coomassie blue or subjected to Western blot analysis.

2.2.2.3 Western blotting

After SDS-PAGE, the gel was equilibrated for 10min in transfer buffer: This transfer set was placed into an apparatus with black side facing black, and the protein transfer was performed at 30V, overnight, at 4°C. After transfer had been completed, the membrane was quickly checked with ponceau solution and blocked with 5% (w/v) fat-free milk for 1h. The membrane was blotted with the primary antibody, overnight at 4°C on a shaking platform. After three times washing with PBST (PBS+ 0.1%Tween 20), the membrane was probed with horse radish peroxidase (HRP)-conjugated secondary antibody (diluted 1:10000 in PBST) for 1h at room temperature, which was followed by additional three washing steps. For biotinylated proteins, the membrane was detected with Streptavidin-peroxidase. Detection was done by the Enhanced Chemiluminescence system (ECL, Amersham Biosciences) and also these X-ray films.

2.2.2.4 Zymography

Zymography is an electrophoretic technique for hydrolytic enzyme detection. ASML and -CD44v knockdown CM^{-exo} were collected from serum-free cell cultures. The CM^{-exo} and ASML and -CD44v knockdown exosomes (30µg /sample) were mixed with Laemmli buffer and incubated for 15min at 37°C. Samples were separated in a 10% acrylamide gel containing 1mg/ml gelatin. Thereafter, the gelatin gel was washed three times by 2.5% Triton for 10min to remove SDS, and was incubated in developing buffer (50mM Tris, 10mM $CaCl_2$, 150mM NaCl, 2µM $ZnCl_2$, pH 7.5) for 48h, 37°C. After 2 days, the gel was stained with Coomassie-blue 250 and washed by destaining solution.

2.2.2.5 Protease Activity

To investigate the protease activity of exosomes, 50µg ASML or -CD44v knockdown exosomes were incubated with matrix proteins (1µg) or stroma CM^{-exo} (50µg) overnight, 37°C. The matrix protein degradation was evaluated by SDS-PAGE gel running and Western blot (WB) or zymography.

For determining the protease inhibitor effect on indicated proteases, the ASML and -CD44v knockdown exosomes were pre-treated with 10µg/ml inhibitor (TAPI, TIMP-2 and MMP-9/13 inhibitor) for 5h, 37°C, and the altered exosomes were co-incubated with matrix protein overnight as described. After then, the matrix protein degradation was evaluated by WB.

2.2.3 Animal experiments

2.2.3.1 In vivo exosome targeting

Dye-labeled ASML and -CD44v knockdown exosomes (400 µg) were i.v. injected into rats. BDX rats (three per group) were sacrificed after 48 hours and various organs were excised and shock frozen in liquid nitrogen. Tissue sections were counterstained by hematoxylin and eosin (H&E), and the recovery of exosomes was detected by Zeiss confocal microscopy.

2.2.3.2 In vivo exosome modulation of ECM

Matrigel was mixed (1:1) with tumor exosomes (400 µg) in PBS and incubated overnight at 37°C. The matrix-tumor exosome was subcutaneously (s.c.). injected into BDX rats (five per group). The plug was removed after 5 days and thereafter shock frozen. Plug sections were stained with the indicated primary and secondary antibodies. After drying, samples were stained with H&E. The sections of the exosome-modulated stroma matrix were evaluated by Zeiss confocal microscopy.

2.2.4 Statistical analysis

All assays were statistically evaluated, values represent the mean (±SD) of triplicates and/or 3 repetitions. P-values<0.05 (Student's t-test) were considered statistically significant.

3. RESULTS

There are emerging researches of the crosstalk between tumor exosomes and host cells (e.g. stroma, endothelial and hematopoietic cells). However, the impact of tumor exosomes on the extracellular matrix is largely unknown. As extracellular matrix is an essential component of the organism, and contributes to tumor cell expansion and spread, the first aim of my thesis was to clarify whether and how tumor exosomes interact and influence host matrix.

3.1 Tumor derived exosomes bind to matrix proteins

To explore the hypothesis that tumor exosomes can influence host matrix, exosomes from the highly metastatic rat pancreatic adenocarcinoma ASML and the poorly metastatic ASML-CD44v4-v7 (ASML-CD44v) knockdown cells were used. The ASML wild type exosomes and ASML-CD44v knockdown exosomes were isolated by ultracentrifugation and further purified by a sucrose gradient centrifugation.

3.1.1 Matrix proteins delivered by tumor and stroma cells

The conditioned medium (CM) from serum-free cell culture supernatant is enriched in many different types of matrix proteins, which could be used as a model of the extracellular matrix in vitro. To avoid interference of autologous exosomes, the conditioned medium was depleted exosomes (CM^{-exo}) by ultracentrifugation.

My study started with characterizing matrix protein in ASML and ASML-CD44v knockdown lines. As shown in figure 5A, collagen I, tenascin and vitronectin were expressed at a similarly level in both cells, whereas the expression level of collagen II, collagen IV, fibronectin and laminin111 in ASML-CD44v knockdown cells was 40% less than in ASML cells.

The secreted matrix proteins also displayed distinct expression profiles in ASML-wt and ASML-CD44v knockdown CM^{-exo}. Despite the same expression level of vitronectin and laminin332, secretion of these matrix proteins was

significantly decreased in ASML-CD44v knockdown CM^{-exo} compared to ASML CM^{-exo}. Furthermore, secreted collagen I, -IV and fibronectin in ASML CM^{-exo} were around five times more than in ASML-CD44v knockdown CM^{-exo}. Finally, collagen II was neither delivered by ASML or -CD44v knockdown cells.

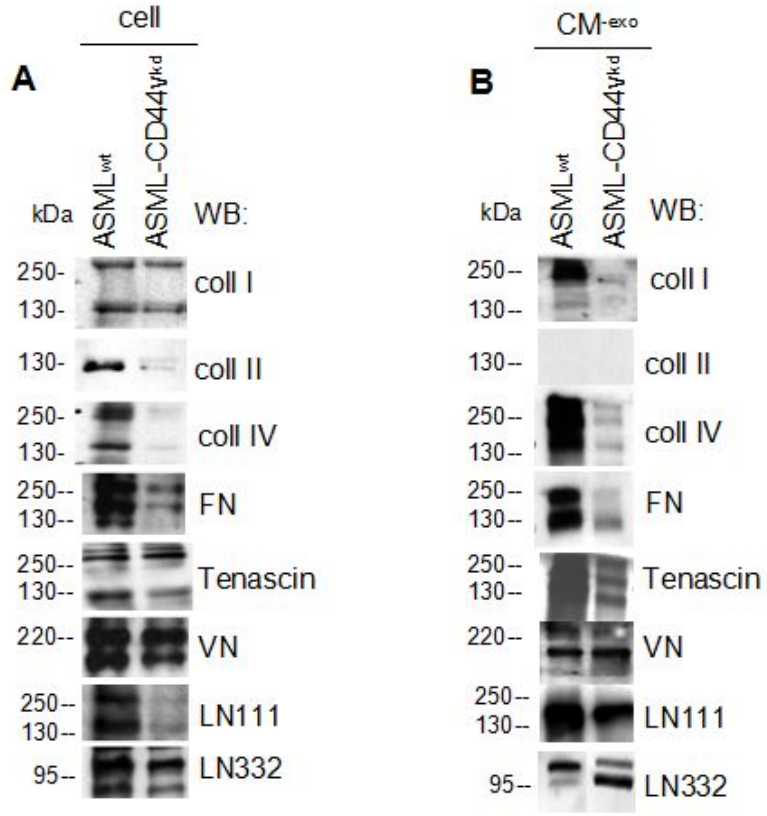


Figure 5. Recovery of matrix proteins in tumor cells and CM^{-exo}. (A, B) WB analysis of collagen I , collagen III, collagen IV and fibronectin , Tenascin, vitronectin (VN), laminin111 and laminin332 expression in ASML and ASML-CD44v knockdown cells (A) and CM^{-exo} (B).

Lymph node stroma cells (LnStr), as well as lung fibroblasts (LuFb) are major targets of ASML exosomes. Both stroma cells abundantly expressed matrix proteins (Figure 6A), and the expression of secreted matrix proteins in CM^{-exo} showed only slight differences compared to the cellular expression. As shown in figure 6, the expression of collagen I and II was higher in LnStr than LuFb cells and CM^{-exo}. However, collagen IV, which is enriched in LuFb CM^{-exo}, was not recovered in LnStr CM^{-exo}. Other matrix molecules, including fibronectin, vitronectin, tenascin and laminin were highly expressed in both stroma CM^{-exo}. With the above information, the stroma CM^{-exo} as a host matrix model to verify

and elucidate the interaction between tumor exosomes and extracellular matrix components.

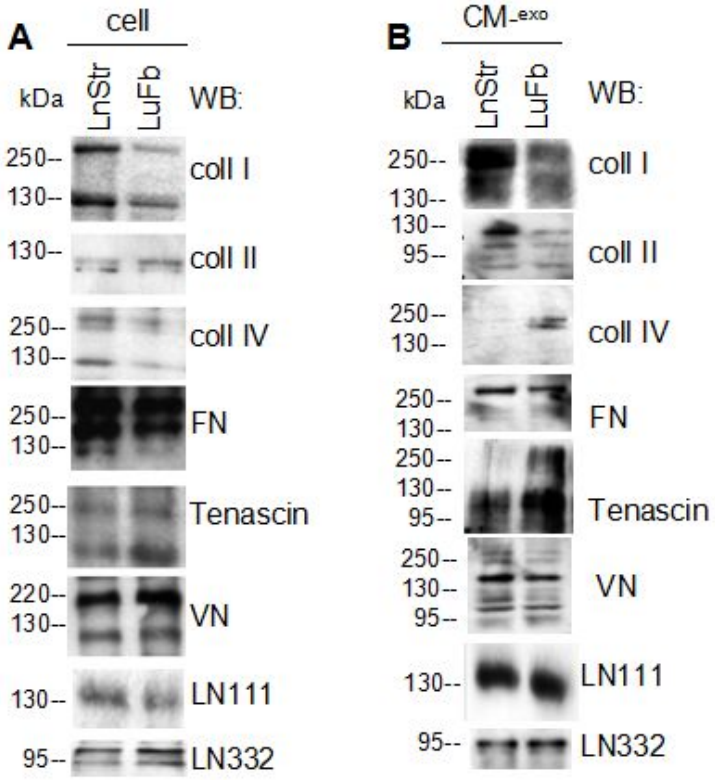


Figure 6. Recovery of matrix proteins in stroma cells and CM^{-exo}. (A, B) WB analysis of collagen I (coll I), collagen II (coll II), collagen IV (coll IV), fibronectin (FN), Tenascin, vitronectin (VN), laminin111 (LN111) and laminin332 (LN332) expression in LnStr- and LuFb cells (A) and CM^{-exo} (B).

3.1.2 Tumor exosomes bind to matrix proteins in vitro

To determine the binding of tumor exosomes to purified matrix proteins as well as host matrix, ASML and ASML-CD44v knockdown exosomes were purified as described and labeled with the fluorescent dye SP-Dio$_{18(3)}$. The latex beads were coated with distinct matrix proteins overnight, and dye-labeled exosomes were incubated with the matrix protein-coated beads for 2 h. The binding capacity of tumor exosomes was evaluated by measuring the fluorescence intensity of the labeled exosomes that bound to the beads. Flow cytometry analysis revealed that ASML and ASML-CD44v knockdown exosomes showed comparable binding capacities for laminin 111, laminin 332 and vitronectin. However, ASML-CD44v knockdown exosomes strikingly lost binding capacity for hyaluronic acid. ASML exosomes also exerted 20% stronger binding

capacity for collagen I, -III, -IV and fibronectin than ASML-CD44v knockdown exosomes (Figure 7A).

Tumor exosomes also bound to tumor and host matrix. CM^{-exo} from ASML and -CD44v knockdown lines, as well as LnStr-, LuFb- and rat aorta endothelial (RAEC) lines were seeded on 96-well plates (F-bottom, ELISA plate) overnight so that the components of matrix could attach to the plates. Dye-labeled ASML or -CD44vkd exosomes were added and co-incubated with the matrix for 2h. The binding of dye-labeled exosomes was analyzed by the fluorescence microplate reader (Figure 7B). ASML exosomes strongly bind to ASML autologous-, Lnstr-, LuFb- and RAEC CM^{-exo}. Compared with ASML exosomes, ASML-CD44v knockdown exosomes showed 30% less binding capacity for Lnstr- and RAEC CM^{-exo}, as well as 50% less binding capacity for ASML CM^{-exo} respectively. Importantly, ASML and -CD44v knockdown exosomes hardly bound to ASML-CD44v knockdown CM^{-exo}, which probably due to the reduced level of matrix protein expression and distinct matrix organization in the absence of CD44v6. To further confirm this finding, I seeded tumor and stroma CM^{-exo} onto glass cover-slides, and co-incubated the cover-slides with dye-labeled ASML or -CD44v knockdown exosomes for 2h. The cover-slides were observed by confocal microscopy. I found that tumor exosomes (red fluorescent spots) could bind to LnStr-, LuFb-, RAEC- and ASML CM^{-exo}, whereas the binding of CD44v knockdown exosomes to matrix was much weaker. Furthermore, Quantification of the exosome-covered area confirmed the stronger binding capacity of ASML exosomes (Figure 7C).

Taken together, tumor exosomes can directly bind to matrix proteins and stroma matrix in vitro. The binding capacity of ASML-CD44v knockdown exosomes was significant reduced. In addition, the CD44v knockdown matrix possibly is disorganized so that even ASML exosomes showed decreased binding.

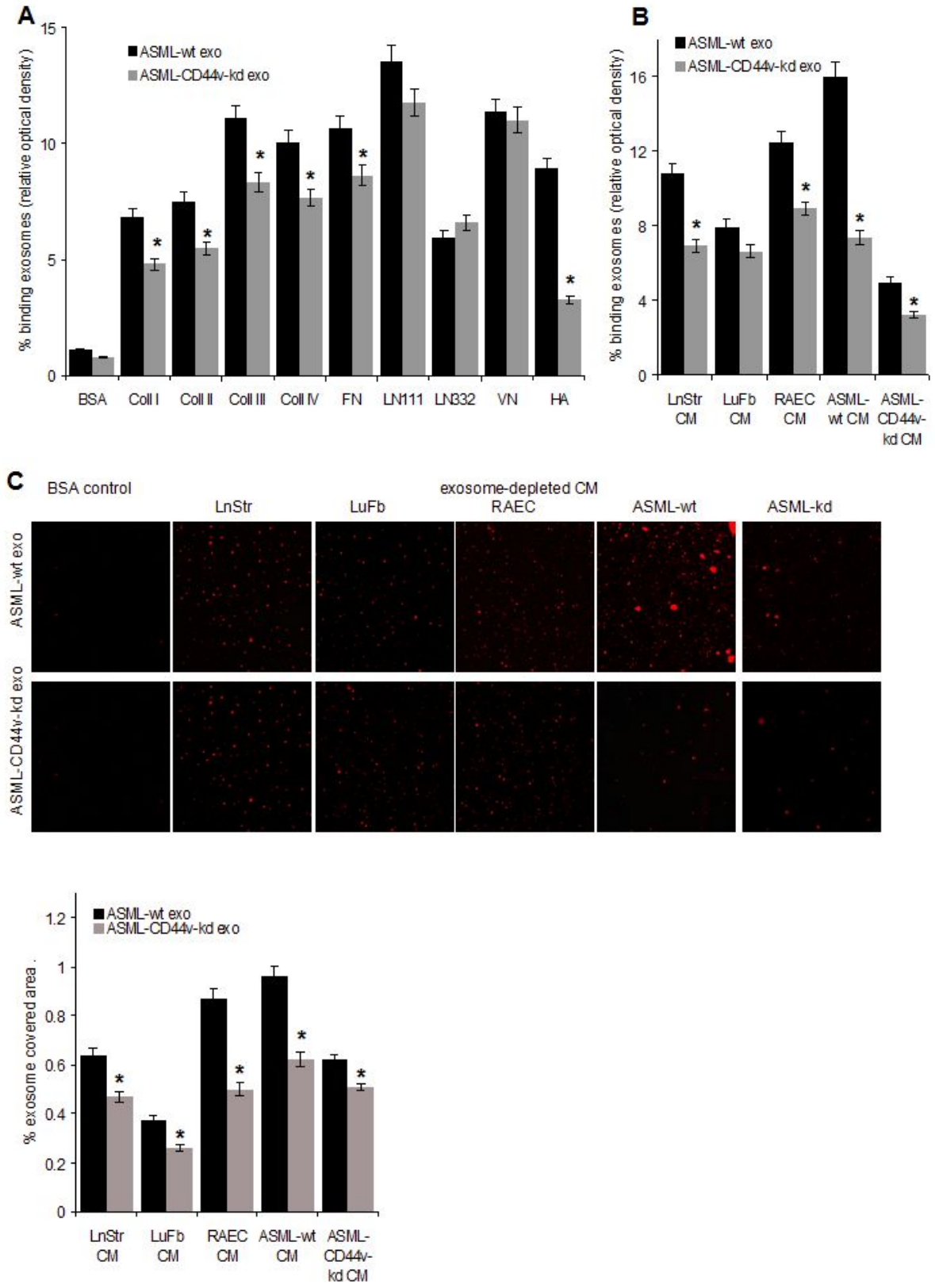


Figure 7. Tumor exosomes bind to matrix proteins in vitro. (A–C) ASML-wildtype (wt) and ASML-CD44v knockdown (kd) exosomes were labeled with SP-Dio$_{18}$, and free dye was removed by 2 times washing and ultracentrifugation. (A) Dye-labeled exosomes were co-incubated with matrix protein–coated latex beads and then wash off to remove the un-bound exosomes, the matrix protein

binding was measured by flow cytometry. (B) Tumor and stroma cell CM^{-exo} were collected and seeded. The co-incubation with dye-labeled exosomes for 2h. (C) Confocal microscopy was used to evaluate the binding of exosomes to tumor or stroma CM^{-exo} (scale bar, 10μm), and the exosome covered area was quantified by imageJ. (A, B and C)

3.1.3 Adhesion molecule receptors are engaged in matrix protein binding

As the binding capacity of ASML wildtype and -CD44v knockdown exosomes displayed significant differences, it became important to figure out the contribution of exosomal adhesion molecules in matrix protein binding.

I first evaluated the adhesion molecule expression in ASML and -CD44v knockdown exosomes by flow cytometry and WB. As shown in figure 8A, the ASML exosomes expressed CD29, CD44 and CD104 (β4) at a significantly higher level than ASML-CD44vkd exo, whereas expression levels of CD11b, CD11c, CD18, CD49b, CD49c and CD54 were similar in both exosomes.

To determine whether highly expressed adhesion molecules in ASML exosomes contribute to stronger matrix binding, dye-labeled exosomes were incubated with anti-CD44, anti-CD49c and anti-CD104 to block the exosomal adhesion molecule. These “antibody-blocked” exosomes were seeded on matrix protein coated 96-well plates for 2h. Thereafter, the plates were washed to remove the non-bound exosomes and the binding was measured by ELISA. The antibody blocking of exosomal adhesion molecules significantly inhibited the exosome binding. As shown in figure 9B, ASML exosome blocked with anti-CD44 hardly bind to collagen IV, laminin 111 and fibronectin , and particularly not to hyaluronic acid, where the exosome binding decreased to one fifth compared to non-blocked exosomes. ASML exosomes blocked with anti-CD49c showed significantly weaker binding to fibronectin and laminin111. Furthermore, when blocked with anti-CD104, binding of exosomes to laminin111 was most strongly reduced (Figure 8B). Thus, I concluded that ASML exosomes selectively bind to matrix proteins mainly through adhesion receptors. CD44 predominantly binds

to hyaluronic acid and laminin111, CD49c to collagen IV, laminin111 and fibronectin, and CD104 to laminin111.

Taken together, tumor-derived exosomes bind to various matrix proteins in a non-random process, which relies on the composition of the stroma matrix as well as the expression of exosomal adhesion molecules.

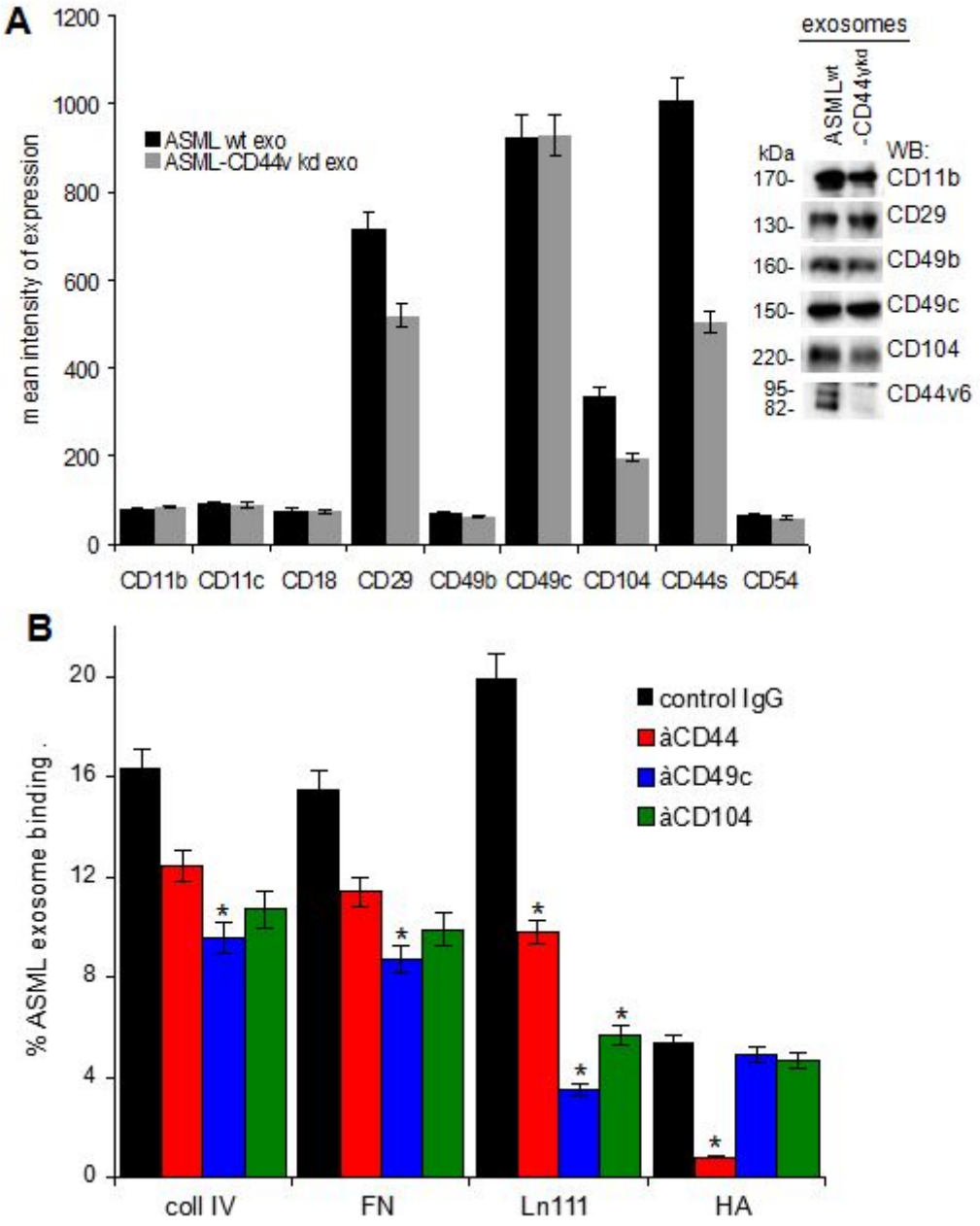


Figure 8. Adhesion molecule . (A) FACS and WB analysis of adhesion molecule expression in ASML-wildtype (wt) and ASML-CD44v knockdown (kd) exosomes (B) Dye-labeled ASML exosomes were pre-incubated with the indicated antibodies. Non-bound staining antibodies were removed via centrifugation. The pellets were then collected and co-incubated with different pure

matrix proteins that coated on ELISA plates for 2 hours at 4°C, and fluorescence intensity of binding (% of total exosomes) was evaluated.

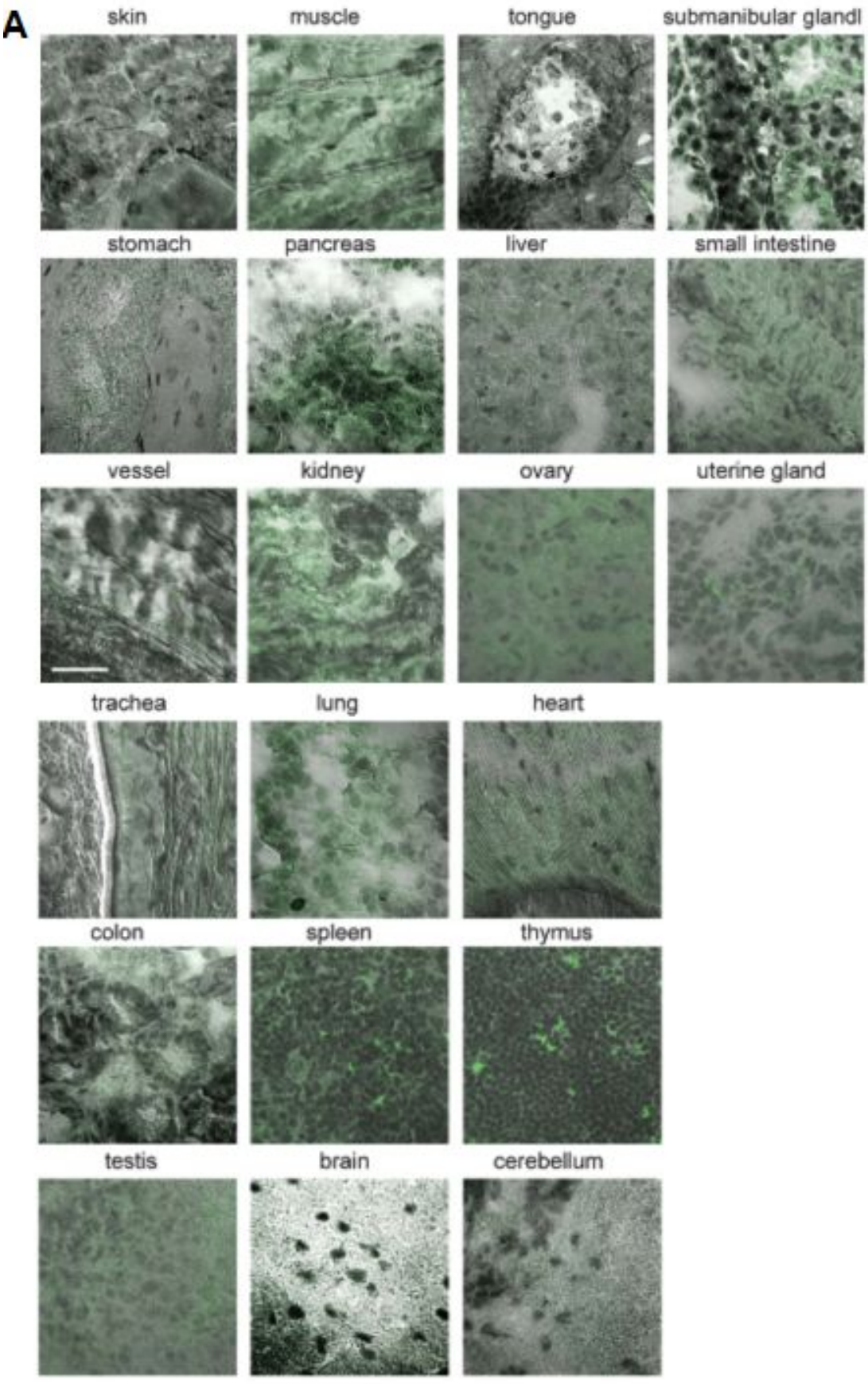
3.1.4 Exosomes bind to matrix proteins in vivo

Tumor exosomes binding and uptake is a non-random process. Thus, it became interesting to investigate whether the ASML exosome also selectively bind to specific tissue matrix.

To evaluate binding of exosomes to native tissue matrix in vivo, rats received an i.v. injection of 400μg florescence dye-labeled ASML or ASML-CD44v knockdown exosomes and were sacrificed after 48 h. Tissues were excised and shock frozen. As shown in figure 9A, it is striking to find that the ASML exosomes were present in many organs, such as tongue, colon, lung, and muscle, and particularly in some tissue organ like skin and heart. Therefore, recovery of exosomes implied ASML exosomes selectively bind to extracellular matrix in vivo. This hypothesis was further explored by immunohistochemistry staining.
To determine whether ASML exosomes could bind to selective matrix proteins, sections of shock frozen tissue samples were immuno-stained with matrix protein specific antibodies and subjected to hematoxylin and eosin staining (H&E). Confocal microscopy was performed with two different channels. One fluorescence channel (FL-G) was used for evaluating the present of green fluorescence dye-labeled exosomes, the other bright-field channel (BL-T) for detecting expression of the indicated matrix proteins in different rat organs. The digital overlays revealed that ASML exosomes (green) were specifically enriched in the regions stained for defined matrix proteins (red parts). As shown in figure 9B, ASML exosomes significantly bound to collagen IV in skeletal muscle, to laminin 111 in heart muscle, and to vitronectin in the skin. In contrast, ASML-CD44v knockdown exosomes were poorly discovered in these matrix. These findings confirmed that ASML exosomes have a significant advantage in binding to host matrix in vivo.

Finally, to determine whether metastasizing tumor cells also take advantage of exosome-matrix binding. The tumor preferentially metastasizes through the lymphatic system to the lung, without invading the epidermis or forming a local tumor nodule. However, occasionally muscle metastasis are observed, where an example is shown in figure 9C. It is notable that ASML cells grew along the basement membrane of the muscles without destroying the muscle. This fits to basement membrane surrounding skeletal myofibers being strong attractants for ASML exosomes.

Taken together, tumor exosome binding to selected matrix proteins appears to attract tumor cells and promote their migration along with the tumor exosome-decorated matrix. Thus, I proceeded to explore the impact and consequence of exosome-binding to host matrix.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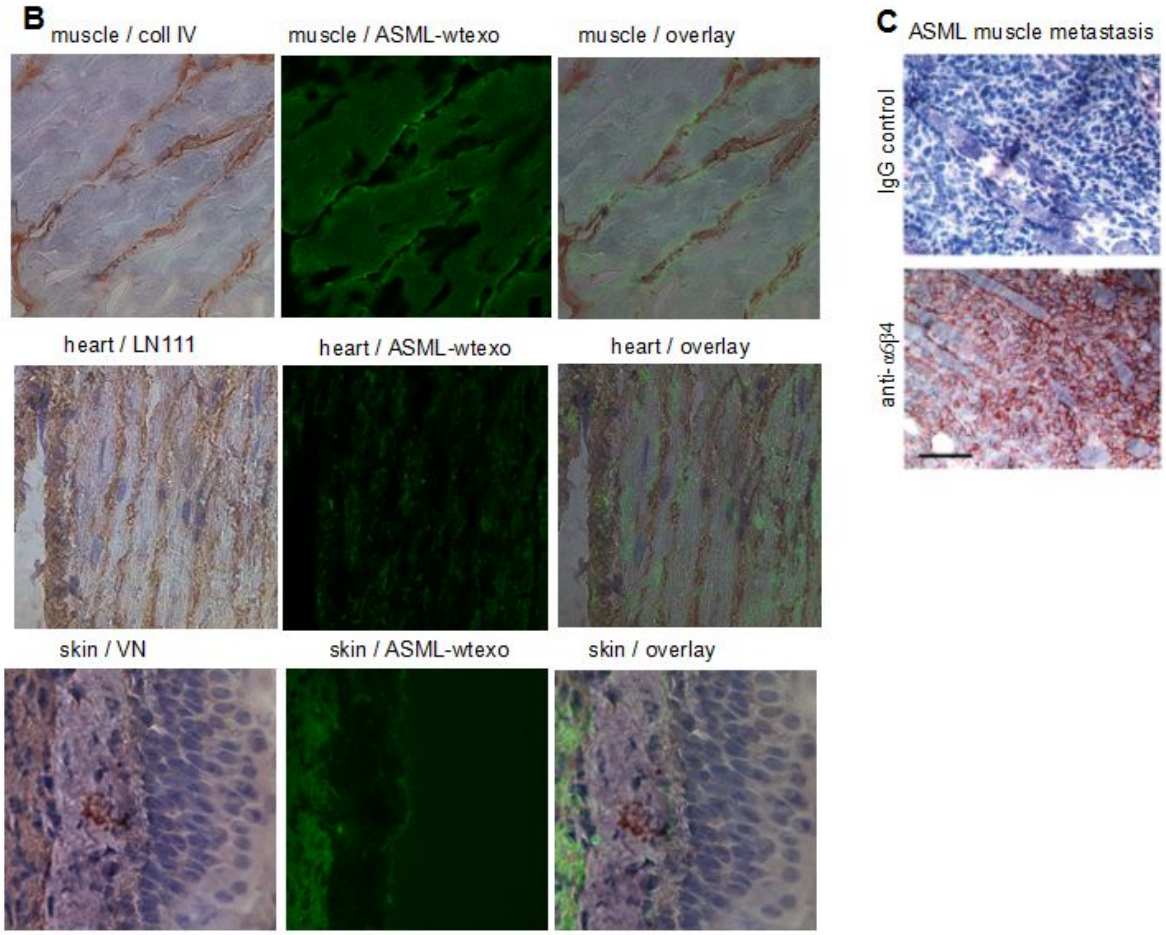


Figure 9. Tumor exosome bind to matrix proteins in vivo. (A and B) Dye-labeled ASML-wildtype (wt) and ASML-CD44v knockdown (kd) exosomes (400 μg) were i.v. injected into rats, and rats were sacrificed after 48 hours. Their organs were excised and then shock frozen and stained. These tissue sections were counterstained with the hematoxylin and eosin (H&E). (A) The recovery of exosomes was evaluated by Zeiss confocal microscopy. (B) The bright-field photo of confocal microscopy presents immunohistochemistry of matrix proteins. The overlay of H&E staining and fluorescence by image J demonstrates the distribution of dye-labeled exosomes and the co localization with the indicated matrix proteins (scale bar, 10 μm). (C) ASML cells were intrafootpad injected into rats. Tissue sections of the abdominal wall muscle were stained with control IgG or B5.5 (anti-α6β4; scale bar, 20 μm).

3.2 CD44v6 cooperates with proteases in exosomes

Tumor derived exosomes are enriched in several proteases. The expression profiles of proteases in ASML and -CD44v knockdown exosomes were evaluated by WB and flow cytometry. ASML exosomes were abundant in uPAR, MMP-2, MMP-3, MMP-9, MMP-13, MMP-14 (MT1-MMP) as well as ADAM17 (TACE), ADAMTS1 and ADAMTS8. Similar to ASML exosomes, ASML-CD44v knockdown exosomes also highly expressed MMP-2, MMP-14,

ADAMTS1 and ADAMTS8. However, the expression level of uPAR, MMP-9 and TACE were around 35% less in -CD44v knockdown exosome than ASML exosomes. Notably, MMP-13 had the most striking reduction, with only less than 40% protein presented in -CD44v knockdown compare with ASML exosomes (Figure 10, A and B).

The enrichment of proteases in exosomes may contribute to extracellular matrix degradation. To support this hypothesis, the enzyme activities of MMPs were evaluated by zymography. As CM^{-exo} and exosomes are both secreted from cells, I used CM^{-exo} as a control to characterize the protease activity. As shown in figure 10C, compared to CM^{-exo}, the activity of MMPs was remarkably higher in exosomes. ASML exosomes exerted a high expression level of pro-MMP-9 (97kDa) and active-MMP-9 (92kDa), as well as the MMP-2 (72kDa) level. On the contrary, the activity of MMPs in ASML-CD44v knockdown exosomes was strikingly reduced. This finding suggested that protease recovery in tumor exosomes may correlated with the presence and association with CD44v6. Thus, it became interesting to investigate whether exosomal proteases cleave and degrade matrix proteins, at least partly, in a CD44v6 dependent manner.

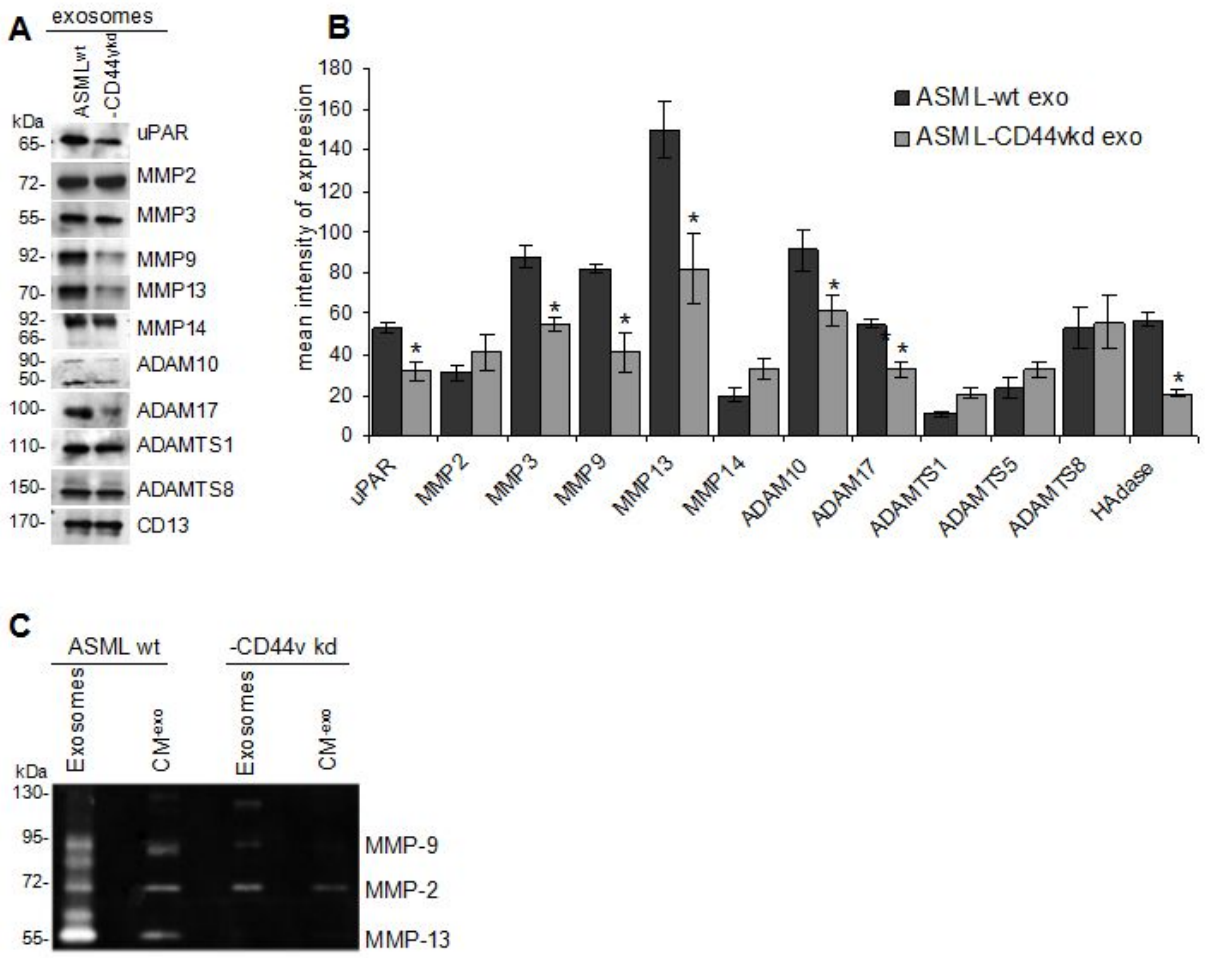


Figure 10. The recovery of exosomal proteases in dependence on the presence of CD44v6. The protease expression in ASML-wt or -CD44v knockdown exosome was evaluated by (A) WB and (B) flow cytometry. Significant differences between ASML-wt and -CD44v kd exosomes are indicated by asterisk. (C) Zymography for evaluating MMP enzyme activities in ASML and ASML-CD44vkd exosomes and CM^{-exo}.

3.3 Matrix degradation by exosomal proteases

To determine the impact of exosomal proteases on the extracellular matrix, purified matrix proteins and native host CM^{-exo} were co-incubated with tumor exosomes, and the degradation of matrix proteins was evaluated by WB.

Collagen I, collagen IV, fibronectin and laminin111 were significantly degraded by ASML exosomes, while ASML-CD44v knockdown exosomes strikingly reduce the capacity of matrix degradation. The degradation of collagen II

displayed a similar level in both exosomes. However, vitronectin did not show any degradation after co-incubation with ASML exosomes (Figure 11A).

I next estimated the degrading capacity of tumor exosomes on host matrix. The CM^{-exo} from LnStr, LuFb and RAEC lines served as targets for the exosomes. As shown in figure 11B, the degradation patterns of the matrix proteins in the CM^{-exo} by tumor exosomes were distinct, which may be due to the different compositions of the extracellular matrix delivered by LnStr, LuFb and RAEC cells. As shown in figure 11B, laminin111 and laminin332 in LuFb CM^{-exo}, as well as collagen I in RAEC CM^{-exo} were significantly degraded by ASML exosomes, respectively, while these matrix proteins were barely degraded by ASML-CD44v knockdown exosomes.

To confirm the contribution of exosomal proteases to matrix protein modulation, the ASML exosomes were treated with MMP-2, MMP9/13 and TACE inhibitors, respectively, to abolish the activity of exosomal proteases (Figure 11C). The degradation of collagen I and collagen IV, particularly laminin332 were significantly impaired after MMP-9/13 inhibitor and TACE inhibitor treatment, but were only slightly affected after treatment with the MMP-2 inhibitor, it means the modulation of these matrix proteins required MMP-9/13 and TACE. Moreover, the cleavage of fibronectin specifically relied on TACE. Thus, differences in exosome-mediate matrix modulation were strongly associated with the interactions between CD44v6 and MMP-9, MMP-13 and TACE.

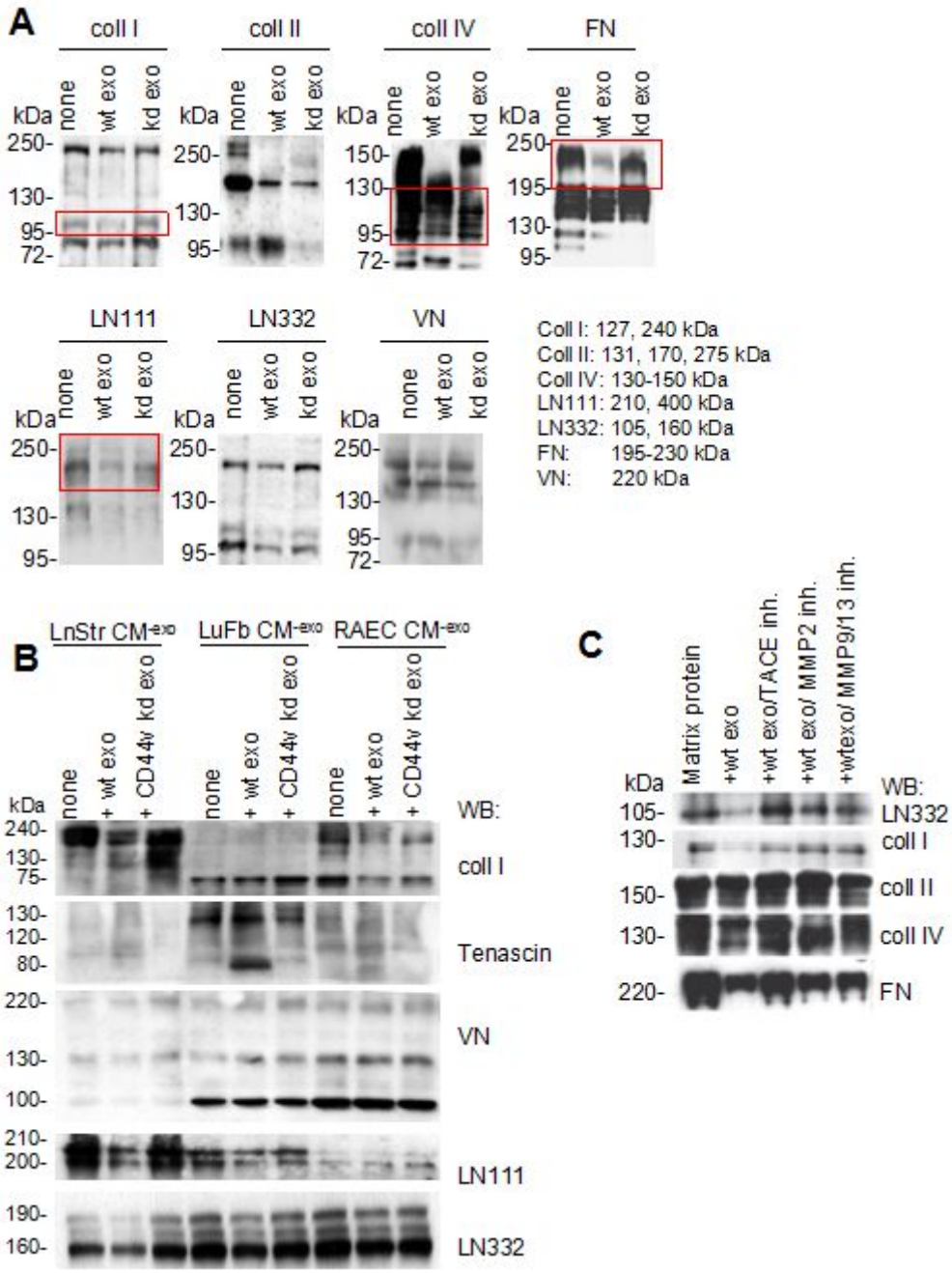


Figure 11. Exosomal proteases contribute to matrix degradation. (A and B) Purified matrix proteins and LnStr-, LuFb- and RAEC CM^{-exo} were co-cultured with ASML-wildtype (wt) and ASML-CD44v knockdown (kd) exosomes for 12h. Matrix degradation was evaluated for distinct matrix proteins by blotting with antibodies to collagen I (coll I), collagen II (coll II), collagen IV (coll IV), fibronectin (FN), vitronectin (VN), laminin111 (LN111) and laminin332 (LN332) (C) The matrix protein degradation by tumor exosomes was inhibited by TACE, MMP2 and MMP9/MMP13 inhibitors. ASML-wt exosomes were co-incubated with protease inhibitors for 5h and co-cultured with different matrix proteins for 12h. Matrix degradation was evaluated by WB.

3.4 The engagement of exosomal CD44v6 in stroma cell adhesion

Adhesion of cells is regulated by their interaction with various components of the extracellular matrix. Thus, it became important to determine whether the tumor exosome-modulated host matrix affects stroma cell adhesion. First, the protrusive activity of cells under different treatments was observed by confocal microscopy. Stroma CM^{-exo}, ASML exosomes and CM^{-exo} modulated by ASML-wt exosomes were co-cultured with LnStr, LuFb and RAEC cells respectively. Figure 12A showed that the spreading of stroma cells increased significantly when co-cultured with stroma CM^{-exo} modulated by ASML exosomes as there were more focal adhesion points. However, when co-cultured with ASML exosomes, most stroma cells displayed less sprouts, particularly LuFb.

In addition, as shown in figure 12B, stroma cell adhesion increased to a comparable level after treatment with autologous matrix or the ASML-CD44v knockdown exosomes-modulated matrix, whereas stroma cells adhered more significantly in the presence of the ASML exosomes-modulated matrix, The results indicated that CM^{-exo} modulated by tumor exosomes promoted cell adhesion.

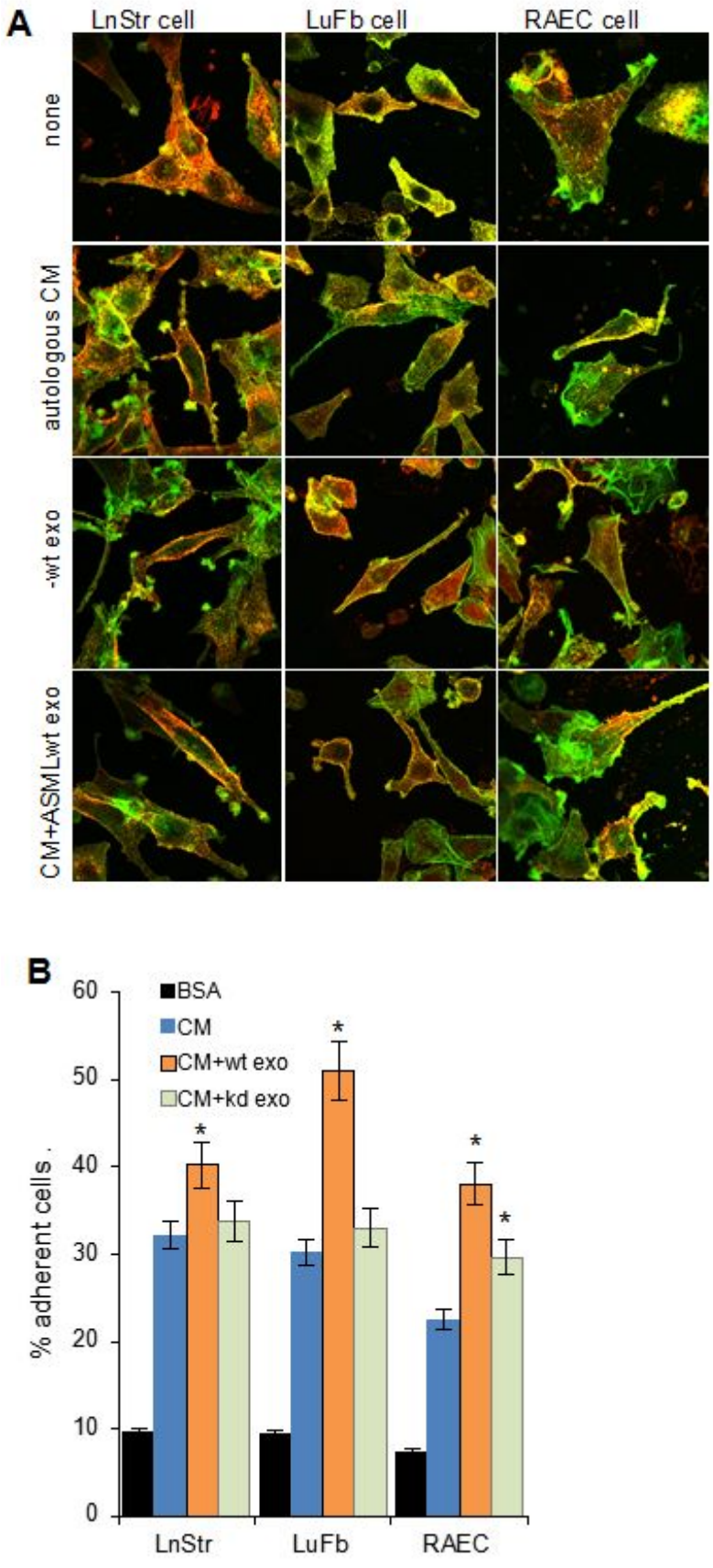


Figure 12. The tumor exosome-modulated matrix and host cell adhesion. (A) LnStr, LuFb, and RAEC cells were seeded on cover slides coated with BSA, autologous CM^{-exo}, ASML-wt exosomes and exosome-modulated CM^{-exo}, and were stained with anti–CD44 and phalloidin

fluorescein isothiocyanate. Confocal microscopy of representative examples (scale bar, 100μm) (B) BSA, CM^{-exo}, or ASML-wt or ASML-CD44vkd exosome-modulated CM^{-exo} were pre-coated in 96-well plates overnight. LnStr, LuFb, and RAEC lines were seeded in these plates for 2h, and washed 2 times to remove the non-bound cells. Adhesion was evaluated by crystal violet staining of adherent cells. The mean± SD of the percent adherent cells is shown. Significant differences between cells adherent to stroma matrix and the exosome-modulated matrix are indicated by asterisk.

3.5 Tumor exosome-modulated matrix contribute to stroma cell motility

Extracellular matrix modulation could facilitate tumor invasion and migration so as to promote tumor metastasis. As I found that tumor exosomes signficantly modulated the matrix components, I wondered whether the stroma matrix modulated by the tumor exosomes could better serve the demands of metastasizing cancer cells.

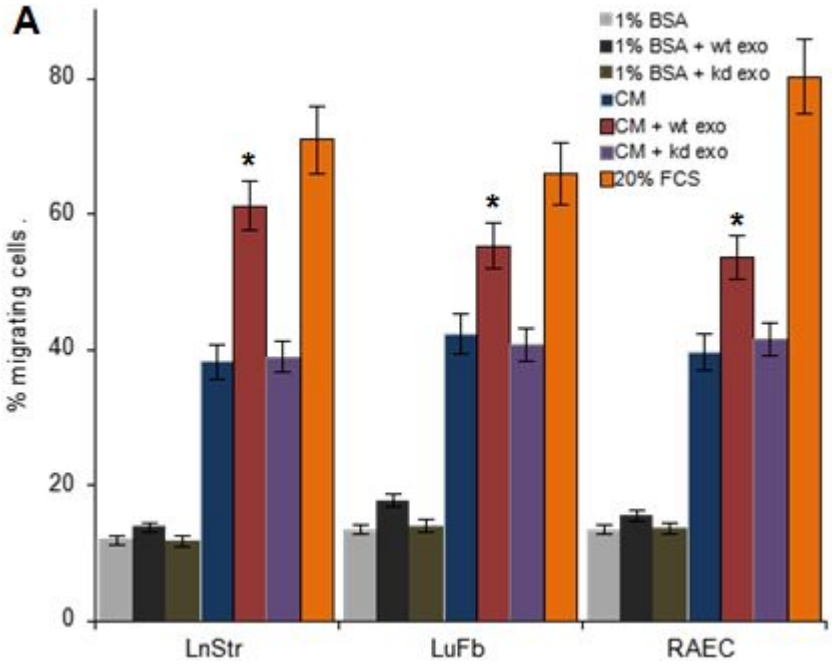
3.5.1 Migration

The transwell chamber assay was used to evaluate the impact of tumor the exosome-modulated matrix on cell migration. LnStr, LuFb and RAEC cells were seeded in the upper part of Boyden chambers, with the lower parts containing 2% BSA as a negative control, 20% FCS as the positive control, stroma cell autologous CM^{-exo}, exosomes from the ASML or -CD44v knockdown cells and tumor exosome-modulated CM^{-exo}. The numbers of migrating cells through the membrane were counted after 16 h. As shown in figure 13A, the transwell assay revealed that stroma cells co-cultured with ASML or -CD44v knockdown exosomes hardly strengthened the migration capacity. However, more than 50% of stroma cells migrated, when their autologous CM^{-exo} was modulated by ASML exosomes while stroma cells co-cultured with autologous matrix or -CD44v knockdown exosome-modulated CM^{-exo} showed a lower migration rate. To confirm this finding, a wound healing assay was performed. stroma cell monolayers were scratched and co-cultured with ASML or -CD44v knockdown exosomes. Images were captured at the starting point and at regular intervals during cell migration. As in this setting, the host cell matrix was continuously

exposed to tumor exosomes during the observation time, the impact of tumor exosomes on stroma CM^{-exo} modulation was directly evaluated. As shown in figure 13B, stroma cell migration was strongly accelerated in the presence of ASML exosomes, particularly for LnStr and RAEC. Thus, migration of LnStr, LuFb and RAEC was strengthened only when their autologous CM^{-exo} modulated by ASML exosomes.

Finally, the migration route of single cell was evaluated by live-cell video microscopy for LnStr and LuFb lines lasting 12h. As described, the stroma CM^{-exo} was treated with ASML or -CD44v knockdown exosomes, and stroma cells were co-cultured either with these altered CM^{-exo} or directly with tumor exosomes (Figure 13C). Stroma CM^{-exo} treated with ASML exosomes significantly promoted LnStr and LuFb migration, whereas stroma cells treated with exosomes or stroma CM^{-exo} treated by ASML-CD44v knockdown exosomes showed unaltered migration.

Taken together, tumor exosomes can modulate the stroma matrix to promote cell migration. This obviously depends on the exosomal CD44v6 as ASML-CD44v knockdown exosomes exerted very weak effects, which might be due to the weaker binding and the lower amount of proteases for matrix degradation.


A
1% BSA
1% BSA + wt exo
1% BSA + kd exo
CM
CM + wt exo
CM + kd exo
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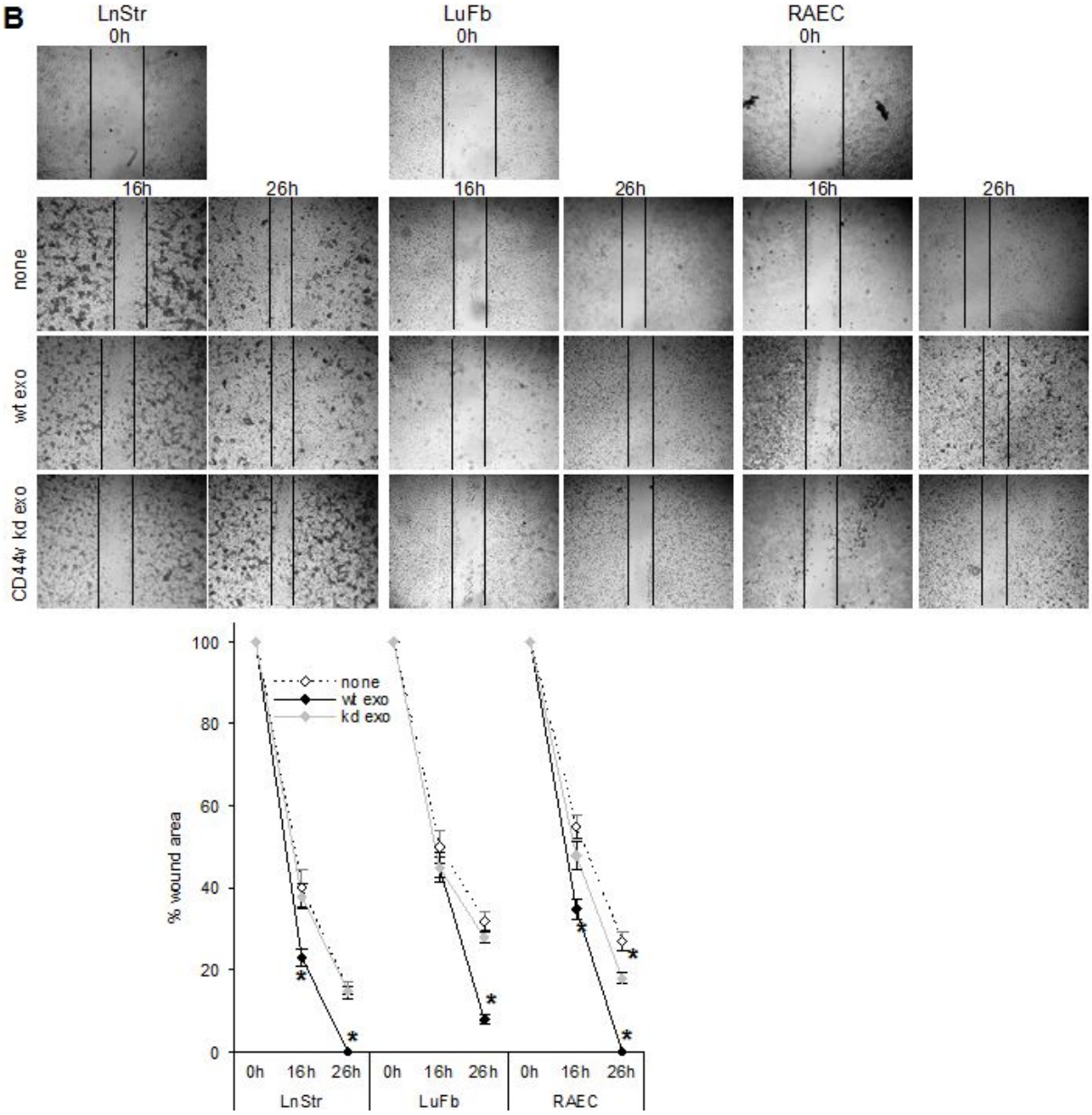
40
60
80
*
*
*
LnStr
LuFb
RAEC


B
LnStr
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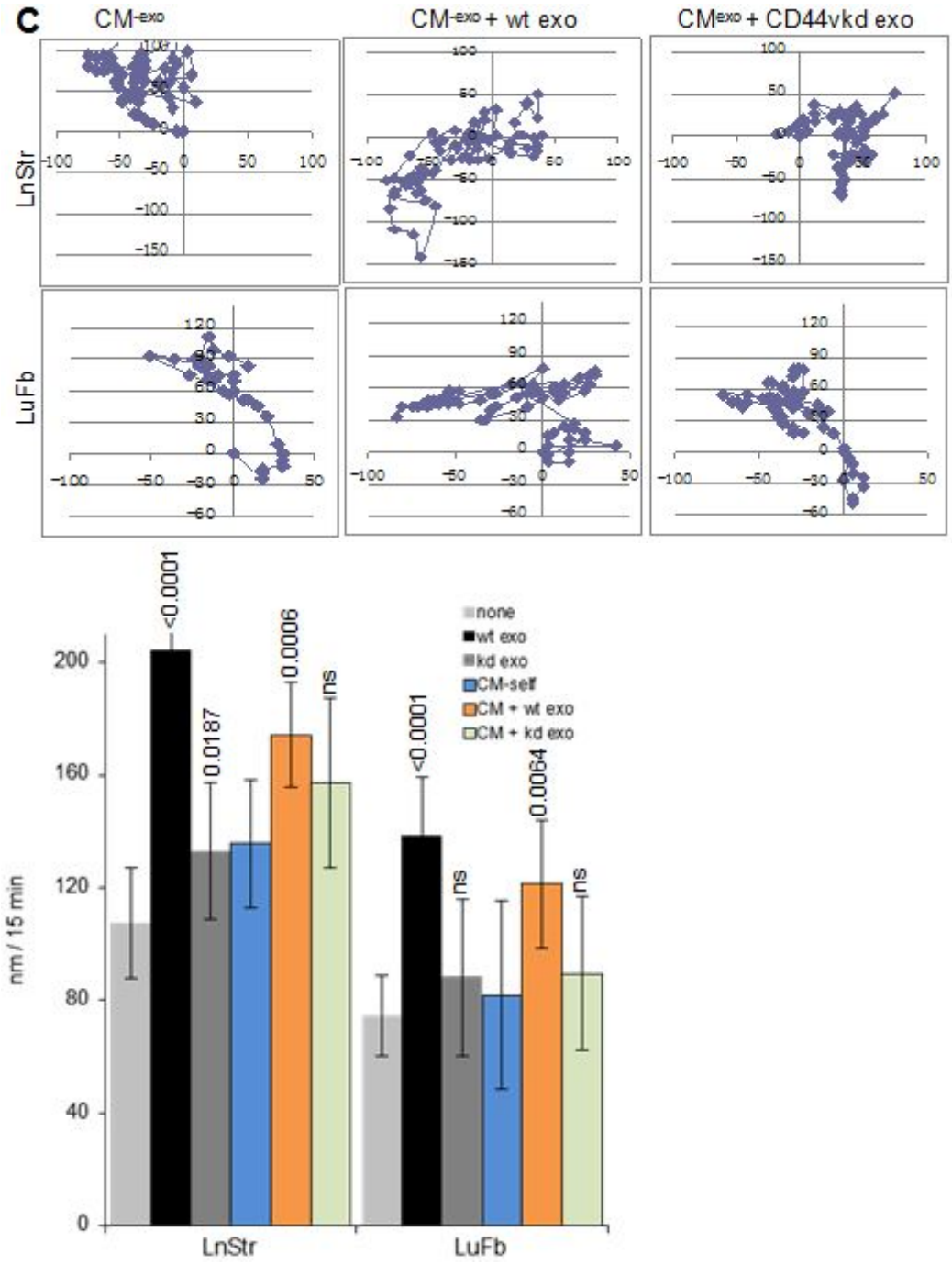
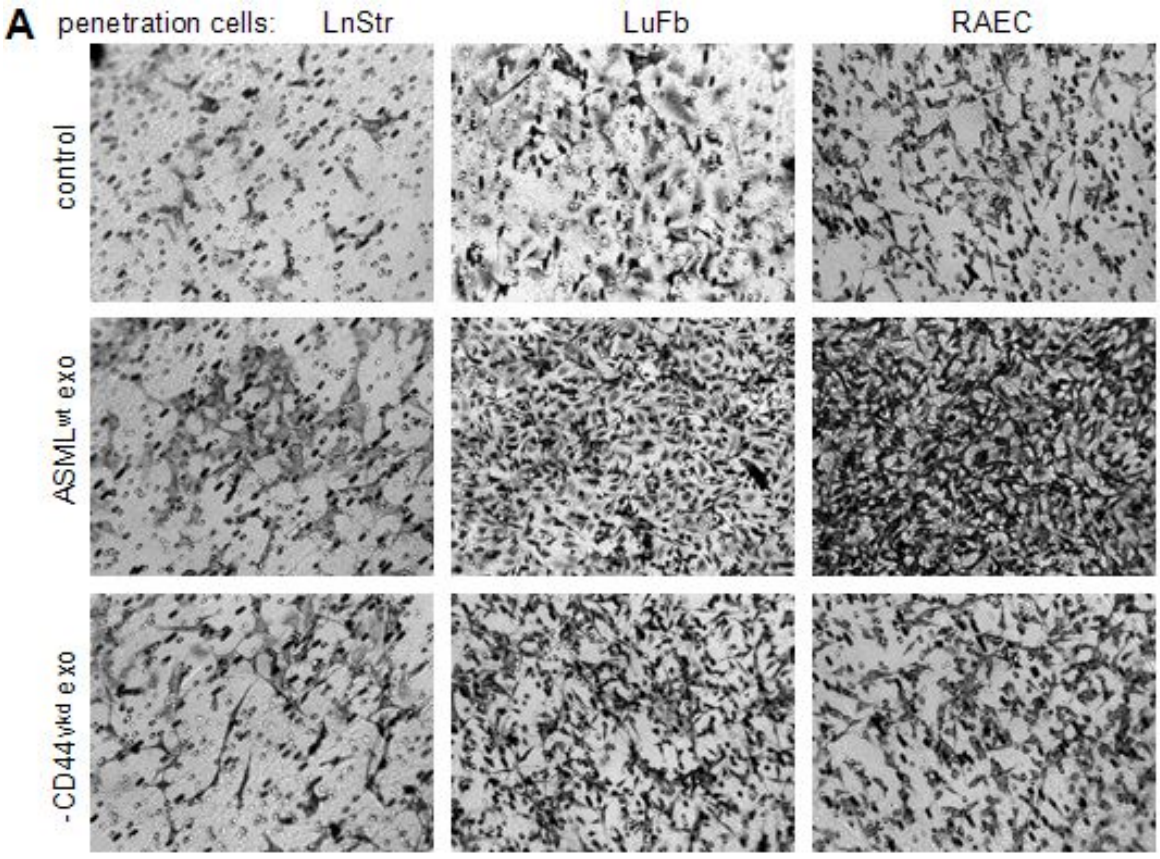


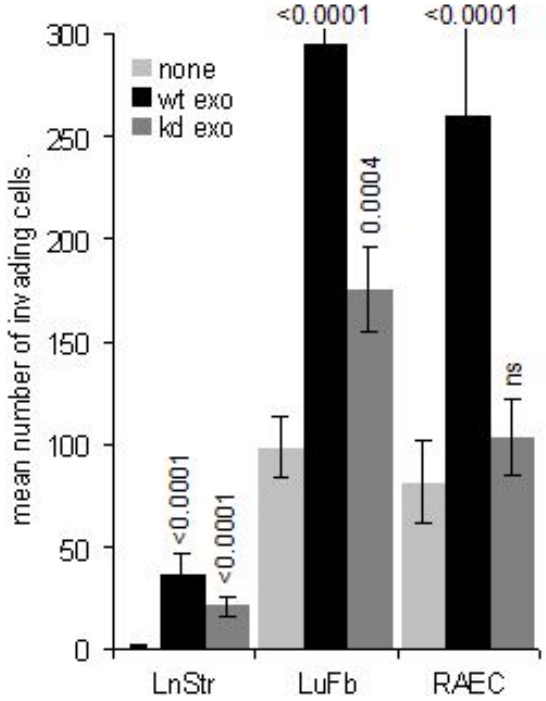
Figure 13. The tumor exosome-modulated matrix supports host cell migration. (A) stroma cells were seeded in the upper part of Boyden chambers. The lower parts contained BSA, 20% FCS, or CM^{-exo} pretreated with ASML-wt or –CD44v knockdown exosomes. Migration was evaluated after 16 hours by crystal violet stain onto the lower membrane site. (B) Cells were seeded on plates coated with CM^{-exo} or tumor exosome-modulated CM^{-exo}. Subconfluent monolayers were scratched by pipette tips and observation followed for 26 hours. Representative examples (scale bar, 250 µm) and the mean ± SD (three wells) are shown. (C) Cells were seeded on CM^{-exo} or exosome-modulated CM^{-exo}. Cell migration was observed for 12 hours. Representative examples and the mean value ± SD track of 15 cells per 20 minutes are shown. (A-C) Significant differences in stroma cell migration by exosomes treatment are shown or indicated by asterisk.

3.5.2 Invasiveness

Cell invasiveness is related to cell migration. However, it requires a cell to migrate through a barrier of extracellular matrix by enzymatic degradation. To evaluate whether the invasiveness of stroma cell could be influenced by tumor exosomes, I performed the matrigel transwell assay. Matrigel was co-incubated with ASML or ASML-CD44v knockdown exosomes overnight for degradation and remodeling. LnStr, LuFb, and RAEC were seeded on untreated or exosomes-modulated matrigel for 24h. The penetration of stroma cells was shown by crystal-violet staining and the number of invading cells was counted by image J. As shown in figure 14A, the invasion capacities of LuFb and RAEC were significantly increased when seeded on ASML exosome-modulated matrigel. However, ASML-CD44v knockdown exosomes exerted a much weaker effect. Furthermore, LnStr hardly invaded the matrigel spontaneously, but passed through it when the matrigel was degraded by ASML exosomes. The matrigel modulated by ASML-CD44v knockdown exosomes only slightly supported LnStr invasiveness.

These in vitro findings were strongly supported by an in vivo matrigel plug assay (Figure 14B). BDX rats were s.c. injected with matrigel mixed with ASML or -CD44v knockdown exosomes. After 5 days, the plug was removed and shock frozen. Plug sections were stained with antibodies for the indicated matrix proteins, integrin (anti-CD49c) as identify marker, or fibroblasts (vimentin) and endothelial cells (CD31). CD49c+, vimentin+ and CD31+ cells as well as the indicated matrix proteins were only abundantly recovered in ASML exosome-treated plugs, while collagen I and vimentin were also recovered at a low level in ASML-CD44v knockdown-treated plugs. Thus, ASML exosomes exerted a more pronounced effect on matrix than -CD44v knockdown exosomes. To sum up, exosomal CD44-protease complexes can facilitate host cell migration and invasiveness evidently through remodeling and degradation of the target matrix.


A
penetration cells:
LnStr
LuFb
RAEC
control
ASML wt exo
- CD44 vkd exo


mean number of invading cells
300
250
200
150
100
50
0
none
wt exo
kd exo
<0.0001
<0.0001
<0.0001
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ns
LnStr
LuFb
RAEC

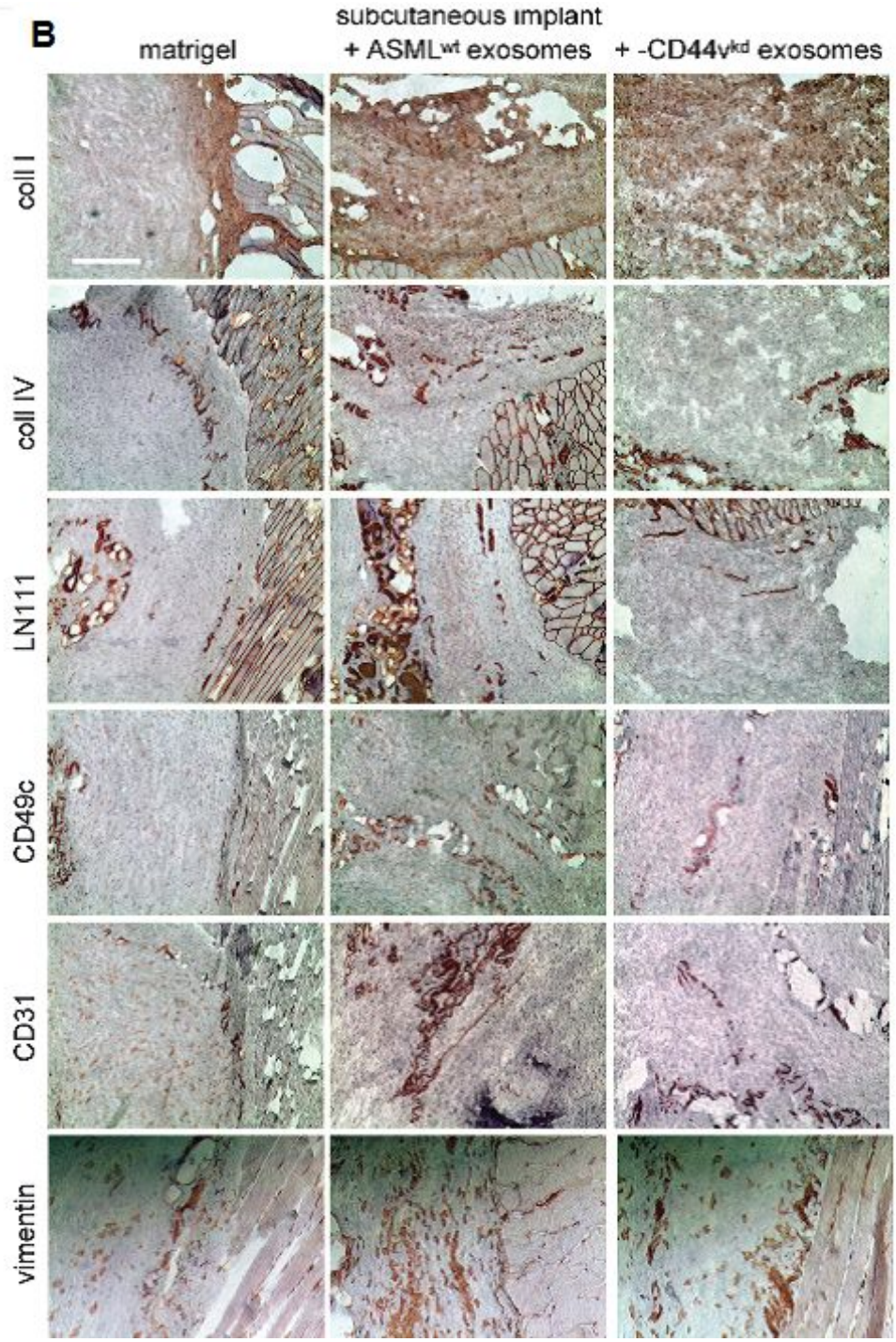

Figure 14. Tumor exosome-modulated matrix promotes invasiveness. (A) Matrigel mixed with the RPMI medium or tumor exosome. Representative different examples (scale bar, 200 µm) and the value mean numbers (triplicates) ± SD of penetrating cells are indicated. Significant differences between matrigel and tumor exosome-pretreated matrigel are indicated. (B) Matrigel was mixed (1:1) with PBS, which contained ASML or ASML-CD44v knockdown exosomes. The plug was removed after 5 days and was shock frozen. (scale bar, 200 µm).

3.6 The tumor exosome-modulated matrix facilitates cell proliferation

The tumor matrix, inherently modulated by autologous exosomes, exerted a feedback on the tumor cells by increasing tumor cell proliferation and apoptosis

resistance (Jung et al, 2011). Thus, it became essential to explore whether besides promoting cell motility, the tumor exosome-modulated host matrix also activates non-transformed cell proliferation.

First, the stroma cell lines LnStr, LuFb and RAEC, as well as lymph node cells (LNC) and bone marrow cells (BMC) were co-culture with ASML or -CD44v knockdown CM^{-exo}, and the proliferation of these cells was evaluated by ^{3}H-thymidine incorporation. As shown in figure 15A, ASML CM^{-exo} significantly promoted LuFb proliferation, while it had only a mild effect on LnStr, LNC and BMC, and did not influence RAEC proliferation. Compared with ASML CM^{-exo}, ASML-CD44v knockdown CM^{-exo} hardly affected LnStr, LuFb and LNC cell proliferation.

Surprisingly, in contrast to tumor CM^{-exo}, ASML exosomes displayed an even stronger effect on LnStr, LuFb, and RAEC proliferation (Figure 15B). For instance, the proliferation of LnStr increased 3-fold after co-incubation with autologous CM-exo modulated by ASML exosomes.

Accelerated cell proliferation might be accompanied by up-regulation of growth factor receptors and activation of downstream signaling pathways. As shown in figure 15C, when co-cultured with ASML exosome-modulated CM^{-exo}, LnStr and LuFb expressed a higher level of epidermal growth factor receptor (EGFR) and chemokine receptor 4 (CXCR-4), particularly the vascular endothelial growth factor receptor 1 (VEGFR1). However, CM^{-exo} modulated by ASML-CD44v knockdown exosomes only promoted the slightly fibroblast growth factor receptor present in LnStr, and VEGFR1 expression in LnStr and LuFb. Furthermore, the PDGFR also changed, which was particularly associated with LuFb growth, was much higher when the cells were co-cultured with ASML exosome modulated LuFb CM^{-exo}.

In addition, the autologous CM^{-exo}, several growth promoting signaling cascades were activated by the ASML exosome-modulated CM^{-exo} particularly

extracellular signal-regulated kinase 1/2(ERK1/2), as well as other signaling like c-jun (Figure 15D). In LuFb the effect was much stronger than in LnStr. The distinct impact on these two cell lines might be due to the different amount of growth factor delivered by these cells into the extracellular matrix. Thus, activation of signaling cascades by tumor exosome-modulated matrix may be initiated by liberation of growth factors in the extracellular matrix. To sum up, the effect of the ASML exosome-modulated matrix differs for individual target cell lines likely due to the accessibility of matrix-deposited growth factors and forced liberation by the tumor exosome-modulated matrix.

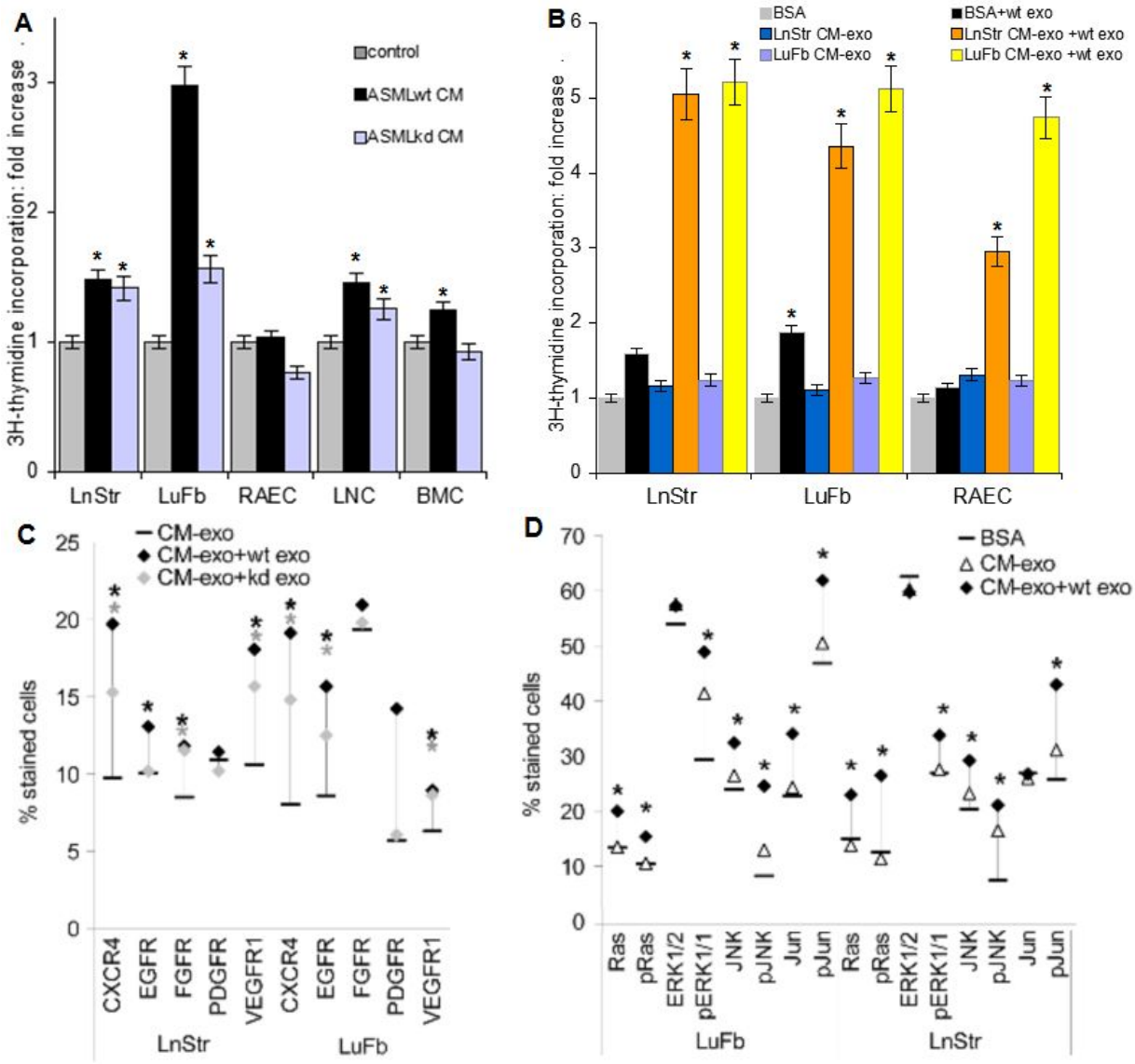

Figure 15. The tumor exosome-modulated matrix promotes cell proliferation. (A and B) Cells were treated with CM, CM^{-exo}, or tumor exosomes pretreated CM^{-exo}. Cell proliferative activity was

evaluated by the assay of ^{3}H-thymidine incorporation after 3 days of culture. (C) Flow cytometry analysis of LnStr and LuFb that were treated with CM^{-exo} w/wo ASML-wt or -CD44v knockdown exosomes. Mean values (three assays) of stained cells are shown. (D) LnStr and LuFb expression check by flow cytometry. (E) WB analysis of cytokines and chemokines in LnStr, LuFb, and RAEC CM^{-exo}. (A-D) Significant differences in the presence of tumor exosomes or tumor exosome-modulated CM^{-exo} are indicated by asterisk.

3.7 The tumor exosome-modulated matrix affects apoptosis

To clarify whether the tumor exosome-modulated host matrix can also procet target cells from apoptosis, stroma cells were co-cultured with tumor matrix or tumor exosome-modulated host matrix and stained with AnnV/PI and analyzed by flow cytometry after 3 days of culture in the presence of cisplatin. As shown in Figure 16A, ASML wild type CM^{-exo} displayed a mild apoptosis-protective effect on LnStr, LuFb and RAEC, but not on LNC or BMC. The ASML-CD44v knockdown CM^{-exo} exerted no protective effect on host cells. However, when LnStr and LuFb CM^{-exo} were pre-treated with ASML exosomes and then co-cultured with their originating cells, AnnV/PI staining revealed that the ASML exosome-modulated stroma CM^{-exo} exerted a stronger protective effect than the autologous matrix. Particularly, for LnStr, the apoptotic rate decreased 40% compare with control cells (Figure 16B).

Although the underlying mechanism is still poorly elucidated, we figured out that the ASML exosome-modulated LnStr CM^{-exo} provided signals to enhance the phosphatidylinositol 3-kinase (PI3K)/Akt pathway activation along with the liberation of apoptosis regulator Bcl-2 family. As shown in figure 16C, after induction of cell apoptosis by cisplatin, B-cell lymphoma 2 (Bcl2), B-cell lymphoma-extra large isoform1 (BclXl) as well as Bcl-2-associated death promoter (BAD) phosphorylation were upregulated in LnStr cells co-cultured with ASML exosome modulated CM^{-exo}. On the other hand, the expression of other pro-apoptotic molecules like Bcl-2-associated X protein (Bax) and Bcl-2 homologous killer (Bak), cleaved caspase-9, and activated caspase-3 were decreased. In contrast, LnStr cells which were co-cultured with autologous CM^{-exo} as well as the ASML-CD44v knockdown exosome-modulated CM^{-exo} only

induced sightly changes in PI3K, Bcl2 and BclXl expression, which resulted in the reduced apoptosis resistance. Further studies should focus on the initial trigger which was delivered by tumor exosome-modulated matrix that support the activation of the PI3K/Akt pathway.

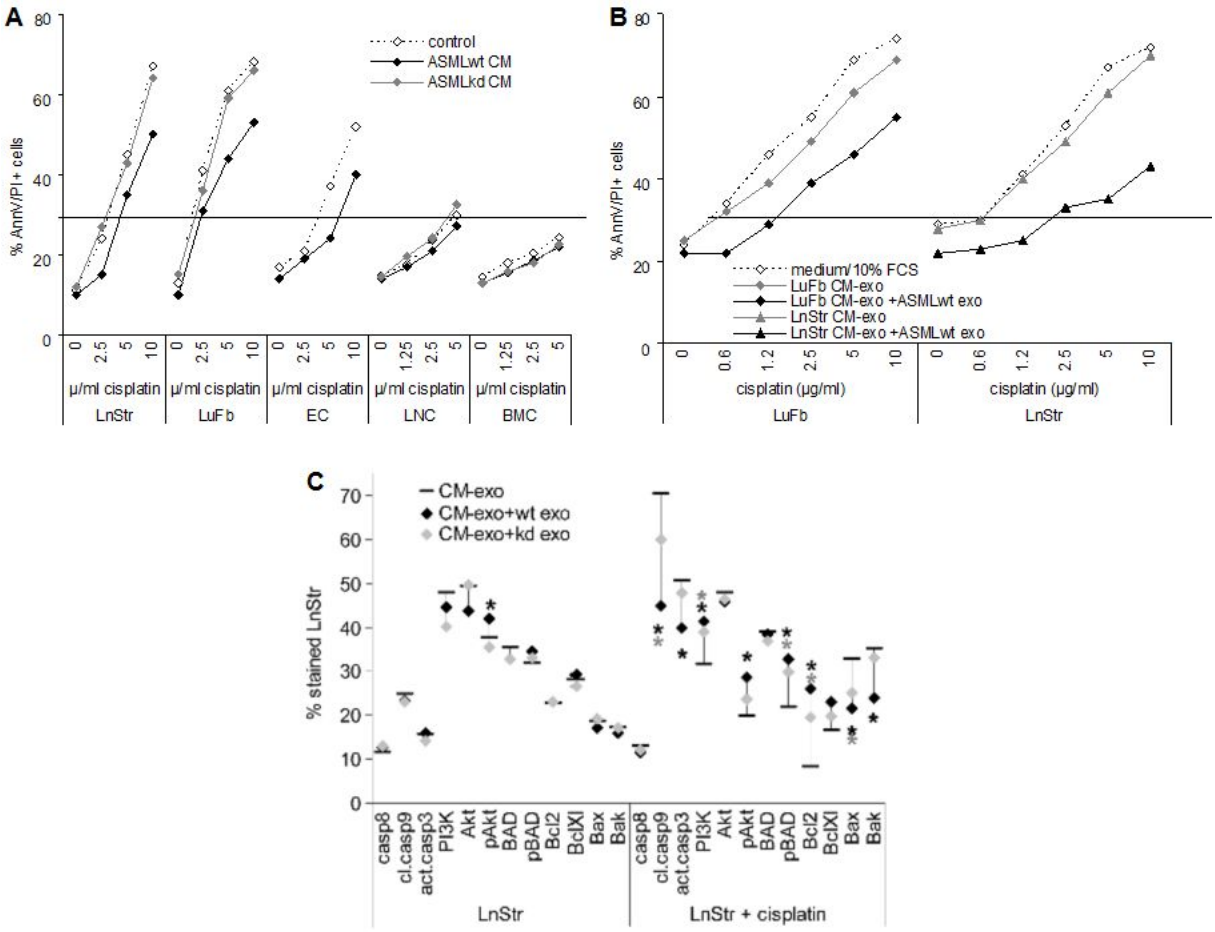

Figure 16. The tumor exosome-modulated matrix can support drug resistance. (A and B) stroma cells were co-incubated with ASML CM or tumor exosome-pretreated autologous CM^{-exo}. Apoptosis resistance was evaluated by AnnV/PI staining after 3 days of culture. (C) Cells were incubated for 24 hours with CM^{-exo} or tumor exosome-pretreated host CM^{-exo}, where indicated cultures contained 10 µg/ml cisplatin. Cells were fixed and permeabilized, and evaluated by FACS flow cytometry, and mean values (three assays) are shown. (C) Significant differences between host CM^{-exo} and tumor exosome-pretreated CM^{-exo} are indicated by asterisk.

4. DISCUSSION

Exosomes mediate intercellular communication through transportation of their bioactive molecules including enzymes, receptor proteins, mRNA and microRNA. For tumor exosomes, it has been demonstrated that their crosstalk with the host considerably contributes to tumor progression. This was elaborated particularly for the influence of tumor exosomes crosstalk with stroma cells in (pre) metastatic organs. I report here that, apart from the crosstalk with host cells, tumor exosomes also affect the extracellular matrix and that the exosome-modulated host matrix also has great impact on tumor progression. Using a highly metastatic rat pancreatic cancer line, ASML, I demonstrated that tumor exosomes directly bound to and degraded host matrix via exosomal adhesion molecules and proteases. Notably, I also show for the first time that the ASML exosome-modulated matrix strongly supports stroma cell motility and facilitates proliferation and apoptosis resistance. Last, but not least, I could elaborate a major role of exosomal CD44v6 in host matrix modulation.

4.1 The choice of tumor exosomes

BSp73 is a pancreatic adencarcinoma that consists of two sublines, the non-metastatic BSp73 AS line and the highly metastatic BSp73 ASML line (Matzku *et al.,* 1983). The latter differs from the non-metastatic line by expression of CD44 variant v4-v7 (CD44v). CD44v expression suffices to initiate the metastatic phenotype (Günthert *et al.,* 1991). The role of the CD44v in metastasis was confirmed by the loss of the capacity to metastasize of ASML cells with a CD44v knockdown (Klingbeil *et al.,* 2009). Our previous study also demonstrated that CD44v6 is sufficient for assembling a soluble matrix, which allows tumor exosomes to modulate the pre-metastatic organ to facilitating tumor cell embedding and growth (Jung *et al.,* 2009).

The availability of a metastatic line and a non-metastatic line that differed only in the expression level of CD44v6 together with their corresponding exosomes

provided a good tool to evaluate the impact of metastatic tumor cell derived exosomes on the host matrix and the contribution of CD44 variant isoforms in tumor metastasis.

4.2 Tumor exosomes bind to the host matrix

Exosomes constitutively express tetraspanin proteins and several membrane proteins (e.g. CD44), which are responsible for the binding selectivity of exosomes (Rana *et al.,* 2012; Rana and Zöller 2011). Apart from binding to the target cells, ASML exosomes could also bind to extracellular matrix proteins. To study the molecular function of exosomal CD44v6 in the crosstalk between tumor exosomes and native host matrix (including extracellular matrix proteins), I evaluated and compared the different binding efficiency of ASML and ASML-CD44v knockdown exosomes.

Notably, the binding capacity of ASML exosomes to hyaluronic acid, collagen I, -IV, fibronectin and laminin 111 was stronger than that of ASML-CD44v knockdown exosomes. As CD44 could bind to via associated integrins, I performed antibody blocking assay. These experiments revealed that tumor exosomes bound to laminin 111 mostly through integrin α3 and α6β4, to collagens predominantly through α3, and to hyaluronic acid through CD44. Thus, the lower binding efficiency of ASML-CD44v knockdown exosomes to the extracellular matrix components is probably due to the decreased expression of exosomal CD44v6 and its associated integrin α6β4.

Further more, in vivo experiments indicated that ASML exosomes preferentially bound to selected matrices. ASML exosomes preferential bound to muscles, the perivascular region, the submucosa of the gastrointestinal tract, and the basal lamina of the skin. Tissue staining for matrix proteins revealed that preferred targets of ASML tumor exosomes were collagen IV, fibronectin and laminins, which are also preferred targets of CD44v6 and its associated integrins. A first hint toward the biological significance of tumor exosomes binding to selective

tissue matrix came from the finding that ASML cells, which metastasize preferentially via lymph nodes to the lung, occasionally grow along tissue matrix (e.g. muscle and skin), without destructing or invading the adjacent tissue.

Take together, ASML exosomes bind to selective matrix components via adhesion molecules which are engaged in exosomal CD44v6 protein complexes. The reduced expression of α6β4 and CD44v6 in ASML-CD44v knockdown exosomes mainly accounts for the weak binding.

4.3 Exosomal proteases and CD44v6 contribute to matrix degradation

Exosomes contain large amount of proteases, some of which are known to associate with CD44 or are regulated by CD44 (Hakulinen *et al.,* 2008; Jung *et al.,* 2009; Alderton *et al.,* 2012). CD44 not only directly binds to several proteases (Fayard *et al.,* 2009), but also serves as a platform where proteases are brought together with their substrates (Hakulinen *et al.,* 2008; Samnegard *et al.,* 2002; Xu *et al.,* 2010). It is well known that MMP-9 is recruited to the cell surface by docking to CD44 (Yu *et al.,* 2002). In addition, the stimulation of CD44 by its ligand hyaluronic acid could up-regulate uPA and uPAR expression (Bourguignon *et al.,* 2012). Then, the question arose, whether tumor exosomes degrade extracellular matrix proteins or the host matrix, and whether exosomal CD44v6 contributes to matrix protein degradation.

Indeed, the expression level of uPAR, MMP-9, MMP-13, and TACE are higher in ASML exosomes than in ASML-CD44v knockdown exosomes. Zymography confirmed the high level of MMP-9 activity in ASML exosomes, and strongly impaired enzyme activity in CD44v6 deficient exosomes. Differences in exosomal protease expression were accompanied by pronounced difference in the degradation of collagen I, collagen IV, fibronectin and laminin111. The contribution of individual proteases was verified by the use of protease inhibitors. It is found that MMP-9 expression accounted predominantly for collagen I, collagen IV and laminin332 degradation, and TACE expression

particularly for fibronectin degradation. Importantly, these two proteases are associated with CD44v6 and are hardly expressed in the ASML-CD44v knockdown exosomes. Thus, poor matrix degradation correlates with the lack of CD44v6 and its associated proteases.

One additional point should be mentioned, CD44v6 also associates with integrins α3β1 and α6β4, which are adhesion ligands for matrix proteins. Thus, the exosomal proteases evidently profit from focalization of proteases at adherent points, which facilitates matrix degradation. I found that exosomal MMP-9, MMP-13, uPAR and TACE, which are regulated by CD44v6, contribute to the modulation of the host matrix.

4.4 Host matrix modulation by tumor exosomes supports cell motility

Controlled degradation of extracellular matrix components is essential for regulating biological effects on host cells. For instance, after cleavage and degradation, some laminin fragments promote cell motility (Malinda *et al.,* 2008; Kim *et al.,* 2011), and small hyaluronic acid fragments facilitate induction of an inflammatory milieu (Tienthai *et al.,* 2003). Thus, tumor exosomal proteases may not only be responsible for matrix protein degradation, but also initiate new activities by releasing biologically active molecules from the extracellular matrix.

Indeed, the host matrix that was modulated by ASML exosomes could promote stroma cell migration, whereas ASML-CD44v knockdown exosome-modulated matrix did not. Beyond migration, ASML exosomes also allowed host stroma cells to invade this modulated matrix. In line with the lower efficiency of ASML-CD44v knockdown exosomes in degrading matrix proteins, host cell invasiveness was hardly affected by the ASML-CD44vkd exosome-modulated host matrix.

The findings in my study provide strong evidence that tumor exosomes facilitate host cell motility and invasiveness not only by directly affecting host cells, but

also by modulating the host matrix. These findings expand the range of known tumor exosomes activities and explain some confusing phenomena, such as the tumor-induced stroma reaction, which is common in pancreatic cancer (Epifano *et al.,* 2002;Erkan *et al.,* 2012), the recruitment of endothelial cells (Umezu *et al.,* 2013), and the recruitment of hematopoietic progenitors towards the tumor cells (Vogel *et al.,* 2012). Additional work is required to elaborate whether this is only due to the biologically active matrix degradation products, or whether the liberation of matrix protein-deposited proteases, growth factors and cytokine/chemokine also contributes to host cell motility.

4.5 Host matrix modulation by tumor exosomes affects cell proliferation and apoptosis resistance

Extracellular matrix also sequesters and locally releases growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and other signaling molecules (Hynes *et al.,* 2009; Ge *et al.,* 2012). In addition, CD44 interacts with some of the molecules deposited in the extracellular matrix, which triggers activation of signal transduction and subsequent cell proliferation (Jung *et al.,* 2009; Thuma and Zöller *et al.,* 2014; Orian-Rousseau, 2010). Therefore, I wondered whether the tumor exosome-modulated host matrix could affect target cell proliferation.

The up-regulation of these growth factor receptors could support the activation of the mitogen-activated protein kinases (MAPK) (Hynes *et al.,* 2009), as well as the phosphoinositide 3-kinase/Akt pathways (Bryant *et al.,* 2005). Another up-regulated molecule, tumor growth factor-β (TGFβ) activates the c-Jun N-terminal kinases (JNK), MAPK and the nuclear factor-κ of activated B-cells (NFκB) pathways (Holmes *et al.,* 2007;Dey *et al.,* 2010). These signaling pathways, when activated, could promote target cell proliferation. Indeed, I found that ASML exosomes modulated host matrix promoted stroma cell proliferation.

Interestingly, I found that the pronounced PI3K and Akt phosphorylation, Bad phosphorylation and Bcl-2 expression were also up-regulated in the stroma cells after incubation with ASML exosome-modulated matrix. In line with this observation, expression of the caspase-3 became only mildly up-regulated by cisplatin treatment, and the cleavage of caspase-9 was significantly reduced when stroma cells were co-cultured with the ASML exosome-modulated matrix.

It is important to note that the bioavailability of growth factor is regulated by the extracellular matrix stiffness (Hynes 2009; Brizzi *et al.,*2012), which is regulated and modulated by proteases (Marhaba *et al.,* 2005; Bryant *et al.,* 2005). For instance, activation of EGFR signaling requires TACE to expose the ectodomain of EGFR to its ligands (Urban *et al.,* 2002; Tsruya *et al.,* 2002). As the exosomal proteases (e.g. MMP-9 and TACE) are regulated by CD44v6, it is reasonable to make the conclusion that the contribution of exosomal CD44v6 in modulating the extracellular matrix may rely on the CD44v6-associated proteases that facilitate liberation of ligands for stroma cells (Zoller 2009).

It already be well accepted that the binding and uptake of tumor exosomes can stimulate and reprogram host cells (Hakulinen *et al.,* 2008; Park *et al.,* 2010). I here present evidence that exosomes affect the host matrix and that the exosome modulated host matrix in a feedback loop contributed to stroma cell activation. ASML exosomes, which highly express CD44v6, α6β4 and MMP-9 and TACE, supported an intense crosstalk between the pancreatic cancer cells and the pancreatic tissue stroma. These tumor exosomal molecules also directly or indirectly modulate stroma matrix. Finally, the modulated stroma matrix and the liberated growth factors generate a milieu that favors stroma cell and hematopoietic cell recruitment, motility, activation and apoptosis resistance. As exosomes impact on matrix modulation can equally affect the local tumor microenvironment, the hematopoietic system and pre-metastatic organs.

5. Conclusion and Outlook

Conclusion

Tumor exosomes communicate with their originating tumor cells as well as host stroma cells, endothelial cells and hematopoietic progenitor cells. Up to date, researches have proved that tumor exosomes bind to and are uptake by target cells, which significantly alter target cells. I here certify that tumor exosome also modulate the host extracellular matrix and thereby promote host cell motility, proliferation and apoptosis resistance. From my research, I could make several conclusions about the functions of tumor exosomes.

First, tumor exosomes bind to selected matrix proteins in a non-random manner in vitro and in vivo. Tumor exosomes further modulate the host matrix through exosomal proteases. The extracellular matrix is important for bioactive compound storage and tissue repair, as well as for the cross-talk between tumor and stroma cells (Sangaletti *et al.,* 2008). Taking this into account, the host matrix modulated by tumor exosomes supports creation of space, liberation of cytokines/chemokines and proteases and generation of cleavage products that promote stroma cell activation.

Second, besides understanding the effect of tumor exosomes on the host extracellular matrix modulation, I focused on the special contribution of exosomal CD44v6. CD44v6 is involved in a multitude of functions of the exosomes, which is evidenced by the fact that tumor exosomes derived from ASML-CD44v knockdown cells exerted only a very weak capacity to modulate host matrix and stroma cells. I confirmed that CD44v6 in complexes with MMP-9, uPAR and TACE are particularly important for matrix protein degradation. Taken together, exosomal CD44v6 binds extracellular matrix components and interacts with several proteases that are pivotal in matrix modulation.

Outlook

Although I started to characterize potential functions of exosomes and exosomal CD44v6 in the modulation of the extracellular matrix, there remains several questions that need to be addressed.

First, I reported the impact of CD44v6-competent tumor exosomes on the host stroma matrix or cells. It might still requires a detailed proteome analysis of stroma modulation and a correlation of the modulated individual stroma components with the stroma cell response.

Second, exosomes can directly activate stroma cells and can be uptaken by stroma cells. The impact of CD44v6-competent tumor exosomes on stroma cell activation remains to be explored. The uptake of CD44v6-competent tumor exosomes by stroma cells has a significant impact on stroma cell reprogramming, which is at least partly due to the transfer of miRNA (Rana *et al.,* 2013). However, reprogramming of host cells by CD44v6-competent tumor exosomes remains to be comprehensively analyzed. Notably, there is evidence that CD44v6 is also engaged in the recruitment of miRNA into exosomes. Further experiments are required to define the mode of miRNA recruitment by CD44v6. Also, reprogramming of host cells by CD44v6-competent tumor exosomes has not been comprehensively analyzed. Finally, activation of signaling cascades in stroma cells by CD44v6-competent exosomes remains to be explored.

Third, CD44v6 is a cancer-initiating cell marker. There is evidence that CIC exosomes transfer CIC features, particularly the capacity for EMT towards non-CIC. An engagement of CD44v6 was postulated, but needs confirmation.

Taken together, the function of tumor exosomes in inducing metastasis and the strong engagement of CD44v6 in this process demand further exploration in hope for therapeutic intervention via a blockage of CD44v6-competent tumor exosome activities.

6. Summary

Tumor-derived exosomes, recognized as important intercellular communicators in the cancer microenvironment, facilitate tumor development and metastasis. Several groups have reported on the crosstalk between exosomes and their target cells in tumorigenesis and tumor progression. However, the question whether exosomes affect the extracellular matrix was largely neglected. Previous researches suggested that CD44v6 interacts with matrix proteins and that CD44v6 might be responsible for functional activities of tumor exosomes. Thus I hypothesized that tumor exosomes modulate the host matrix, such that it facilitates tumor progression in a CD44v6-dependent manner.

To approach this topic, I used exosomes from the highly metastatic rat pancreatic adenocarcinoma ASML and the poorly metastatic ASML CD44v6 knockdown cell lines. The binding of ASML exosomes to individual extracellular matrix components was higher than ASML-CD44v6 knockdown exosomes in vitro and in vivo. Preferential targets of tumor exosomes vary with the expression profile of exosomal adhesion molecules. In ASML exosomes, high CD44v6 expression is essential for hyaluronic acid binding, and high integrin α6β4 expression accounts for laminin 332 binding.

Tumor exosomes binding to matrix is accompanied by significant modulation of the extracellular matrix through exosomal proteases. The matrix proteins including collagens, laminins, fibronectin and hyaluronic acid were degraded after the binding of ASML exosomes. The particular contribution of CD44v6 relies on affecting the transcription of uPAR and the association of proteases including MMP-9, MMP-14 and TACE. Degradation of the extracellular matrix via these proteases was confirmed by using protease inhibitors.

The host extracellular matrix modulated by ASML exosomes promoted stroma cell adhesion, migration and invasiveness. These effects were not or weakly induced by host matrix modulated by ASML-CD44v6 knockdown exosomes.

Degradation of the matrix proteins is also accompanied by liberation of growth factors and chemokines.

Taken together, modulation of the extracellular matrix by tumor exosomes is a critical factor in the cross-talk between a tumor and the host. Thus, I suggest that modulation of the extracellular matrix by exosomes may also be important in organogenesis, vasculogenesis, angiogenesis, and tissue repair including wound healing and clotting after vessel disruption.

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