Function of Surface-Associated Protein and DNA on Extracellular Vesicles

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Cover illustration:
"Yin-Yang: Relation between known and unknown"
Designed by Ganesh Shelke.
Yin: Mast cell-derived extracellular vesicle (green: EV Lipid) uptake by lung epithelial cells (blue: Nucleus). **Yang**: Transmission electron micrograph of human mast cell-derived extracellular vesicles (courtesy: Cecilia Lässer).

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To my family for everything

“One thing leads to another, everything is connected”

-Art on the London Underground

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Abstract

Extracellular vesicles (EVs), including exosomes, are nano-sized, lipid bilayer-enclosed vesicles that are released into the extracellular environment from by almost all cells. EVs contain biomolecules, such as proteins, lipids, and nucleic acids, and they are suggested to play vital roles in cellular communication. In addition, they are used as biomarkers and have therapeutic applications. The goal of this Ph.D. thesis was to define the localization of EV-associated cargo (particularly proteins and DNA) and to determine the role of EVs in regulating biological processes. We addressed these questions by using mast cell-derived EVs (exosomes) and determining their effects on signaling pathways in primary human mesenchymal stem cells, epithelial cells, and monocytes.

We have made three important discoveries.

First, we showed that the protein cargo, TGFβ-1, present on the surface of EVs derived from mast cells, activated a migratory phenotype in primary human MSCs. The major form of TGFβ-1 was inactive and was associated with heparan sulfate proteoglycans. Moreover, these EVs enhanced the immunosuppressive phenotype of MSCs in a mouse model of allergic airway inflammation. EVs activated prolonged and efficient TGFβ-signaling and were retained in the endosomal compartments of MSCs during this period. Furthermore, based on the protein expression and the morphological features that were induced in lung epithelial cells, we also concluded that the epithelial-to-mesenchymal transition could be induced by these EVs. Additionally, we found that these EVs could activate the phosphorylation of proteins that are involved in EMT.

Second, we showed that the surface of EVs is associated with extracellular-DNA that induced the aggregation of EVs. Additionally, DNA was also present on the inside of EVs. The DNA on both the inside and outside of the EVs consisted of both mitochondrial and nuclear DNA. In this study, we were able to separate the EVs based on their density, followed by detection of the DNA that was associated with the EVs. The EV-associated DNA was able to initiate the activation of innate immune signaling by phosphorylation of interferon regulatory factor-3 in monocytes.

Third, we evaluated and found that 18 hour is more efficient than 1.5 hours of ultracentrifugation in depleting EV-associated RNA (as well as DNA) from fetal bovine serum prior to its use in cell culture media.

We conclude that mast cell-derived EVs harbors bioactive molecules (e.g., TGFβ-1 and DNA) on their surfaces. These EVs can affect MSCs by regulating the immune environment of the lung during inflammation. Some portion of the secreted TGFβ-1 is inactive and is attached to the surface of EVs. This might target the EVs to the acidifying compartment of early/late endosomes and lead to the activation of TGFβ-1 along with the uptake of the EVs. Additionally, EVs also carry DNA. Most of the DNA molecules were present on the surface of the EVs and were able to activate the DNA sensors in recipient cells. Thus, EVs assist in the uptake of DNA into the cytoplasm of the recipient cell, and this mechanism has implications in autoimmune disease and in the maintenance of inflammation.

Keywords: Extracellular Vesicles, Exosomes, Mast cell, Mesenchymal stem cells, TGFβ-1, Endosomes, Epithelia-to-mesenchymal transition, Extracellular DNA, IRF-3

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Sammanfattning på svenska

Extracellulära vesiklar (EV), inklusive exosomer, är vesiklar i nanostorlek inneslutna i ett dubbelskikt bestående av lipider. Dessa vesiklar frisätts till omgivningen av nästan alla typer av celler. EV innehåller biomolekyler som proteiner, lipider och nukleinsyror som troligen spelar en viktig roll i kommunikationen mellan celler. Dessutom kan de användas som biomarkörer och har en roll inom terapeutiska användningsområden. Målet med denna avhandling var att främst definiera var EV-associerade proteiner och deoxyribonukleinsyra (DNA) var lokaliserade och bestämma deras roll i hur EV reglerar biologiska processer. För att besvara dessa frågor använde vi oss av EV (exosomer) från mast celler och studerade deras effekt på signaleringsvägar i primära humana mesenkymala stamceller (MSC), epitelceller och monocyter.
I denna avhandling gjordes tre viktiga upptäckter.

I. Vi kunde visa att proteinet TGFβ-1, bundet till ytan på EV, kunde stimulera en migrerande fenotyp hos primära humana MSCs. TGFβ-1 är ett protein som uppträder i olika former med olika aktivitet. Den huvudsakliga formen här var inaktiv och bunden till heparinsulfatproteoglykaner. Dessa är allmänt förekommande makromolekyler som är viktiga kofaktorer i vidhäftningsprocesser. För övrigt kunde dessa EV förstärka en immunosuppressiv fenotyp hos MSCs i en experimentell modell för allergisk luftvägsinflammation. EV stimulerade en effektiv och förlängd TGFβ signalering och behölls endosomalt i MSCs under denna tid. Vidare, baserat på protein uttryck och morfologiska karaktäristiska egenskaper som var inducerade i lungepitelceller, kunde vi konkludera att omvandlingen av epitelceller till mesenkymala celler (Epithelial-Mesenchymal Transition, EMT) kunde induceras av EV. Dessutom fann vi att dessa EV kunde stimulera fosforylering av proteiner involverade i EMT.

II. Vi har även visat att ytan på EV band extracellulärt DNA som kunde inducera aggregering av EV. Vi fann även DNA inuti EV. Detta DNA samt DNA bundet till ytan på EV bestod av både mitokondriellt DNA och kärn DNA. I denna studie, kunde vi separera EV baserat på deras densitet, följt av detektion av det DNA som var associerat med EV. Detta DNA kunde initiera

aktivering av den medfödda immunsignaleringen i monocyter genom fosforylering av interferon-reglerande faktor-3 (IRF3).

III. Dessutom utvärderades ett protokoll för effektiv reducering av EV-associerad ribonukleinsyra (RNA) och DNA från fetalt bovint serum för användning i cell kultur media.

Sammanfattningsvis drar vi slutsatsen att EV från mast celler bär med sig bioaktiva molekyler (t ex TGFβ-1 och DNA) på sin yta. Dessa EV kan påverka MSC genom att reglera den immunologiska mikromiljön i lungan vid inflammation. En del av det frisatta TGFβ-1 är inaktivt och är bundet till ytan på EVs. Troligen är detta inriktat mot EV i den surgörande delen av tidiga/sena endosomer och leder till aktivering av TGFβ-1. Dessutom kan EV bära med sig DNA. Den övervägande delen av DNA molekylerna bundna på ytan kunde aktivera DNA sensorer i mottagarceller. Därmed visade vi att EV hjälper till med upptag av DNA in i mottagarcellens cytoplasma. Denna mekanism kan ha betydelse inom autoimmuna sjukdomar samt i upprätthållandet av inflammation.

List of publications

This thesis is based on the following studies, referred to in the text by their roman numerals.

I. **Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum**.
Ganesh Vilas Shelke, Cecilia Lässer, Yong Song Gho and Jan Lötvall.
Journal of Extracellular Vesicles (2014) (PMID: 25317276)

II. **Regulation of mesenchymal stem cell function by mast cell exosome surface TGFβ-1-role of endosomal retention.**
Ganesh Vilas Shelke**#**, Yanan Yin**#**, Su Chul Jang, Cecilia Lässer, Stefan Wennmalm, Hans Jürgen Hoffmann, Jonas Nilsson, Li Li, Yong Song Gho, Jan Lötvall. (**#** Equal Contribution)
(*Submitted*)

III. **Epithelial-mesenchymal transition induction in respiratory epithelial cells by mast cell extracellular vesicles.**
Ganesh Vilas Shelke, Yanan Yin, Hjalmar Brismar, Cecilia Lässer, Jan Lötvall
(*In Manuscript*)

IV. **Human mast cells release extracellular vesicle-associated DNA.**
Ganesh Vilas Shelke[#], Su-Chul Jang, Yanan Yin, Cecilia Lässer, Jan Lötvall (# Corresponding Author)
Matters (2016), (doi: 10.19185/matters.201602000034)

V. **Extracellular vesicle-associated DNA is present on both the inside and the surface of vesicles and has a possible role in the activation of STING-associated pathways in recipient cells.**
Elisa Lázaro-Ibáñez, Ganesh Vilas Shelke, Rossella Crescitelli, Su Chul Jang, Anaís Garcia, Cecilia Lässer, Jan Lötvall
(*In Manuscript*)

Publications not included in the thesis

VI. **Two distinct extracellular RNA signatures released by a single cell type identified by microarray and next-generation sequencing.**
Cecilia Lässer, **Ganesh Vilas Shelke**, Ashish Yeri, Dae-Kyum Kim, Rossella Crescitelli, Stefania Raimondo, Margareta Sjöstrand, Yong Song Gho, Kendall Van Keuren Jensen and Jan Lötvall.
RNA Biology (2016) (PMID: 27791479)

VII. **Mast cell exosomes promote lung adenocarcinoma cell proliferation – role of KIT-stem cell factor signalling**.
Hui Xiao, Cecilia Lässer, **Ganesh Vilas Shelke**, Juan Wang, Madeleine Rådinger, Taral Lunavat, Carina Malmhäll, Li Hui Lin, Jia Li, Li Li, Jan Lötvall
Cell Communication and Signaling (2014) (PMID: 25311367).

VIII. **Dual-Wavelength Surface Plasmon Resonance for Determining the Size and Concentration of Sub-Populations of Extracellular Vesicles.**
Deborah Rupert, **Ganesh Vilas Shelke**, Gustav Emilsson, Virginia Claudio, Stephan Block, Cecilia Lässer, Andreas Dahlin, Jan Lötvall, Marta Bally, Vladimir Zhdanov, Fredrik Höök.
Analytical Chemistry (2016) (PMID: 27644331)

IX. **Exosomes in the nose induce immune cell trafficking and harbour an altered protein cargo in chronic airway inflammation**
Cecilia Lässer, Sarina E O' Neil, **Ganesh Vilas Shelke**, Carina Sihlbom, Sara Folkesson Hansson, Yong Song Gho, Bö Lundbäck, Jan Lötvall
Journal of Translational Medicine (2016) (PMID: 27320496)

X. **Escherichia coli outer membrane vesicles can contribute to sepsis induced cardiac dysfunction**.
Kristina Svennerholm, Kyong-su Park, Johannes Wikström, Cecilia Lässer, Rossella Crescitelli, **Ganesh Vilas Shelke**, Su Chul Jang, Shintaro Suzuki, Elga Bandeira, Charlotta Olofsson, Jan Lötvall
Scientific Reports (2017) (Pubmed: 29234030)

XI. Apoptosis induced by combination of TNF-α and IFN-γ is associated with upregulation of Par-4 and decreased NF-kB and Akt in human neuroblastoma cells.

Ganesh Vilas Shelke#, Jayashree Jagtap#, Richa Shah, Gowry Das, Mruthyunjaya.S, Radha Pujari, Padma Shastry. (# Equal contribution) *Biomedicines* (2018) (Pubmed: 29278364)

XII. Optical density and lipid content of extracellular vesicles revealed using optical waveguide scattering and fluorescence microscopy.

Déborah L. M. Rupert#, Mokhtar Mapar#, **Ganesh Vilas Shelke**, Matthias Elmeskog , Karin Norling, Stephan Block, Björn Agnarsson, Jan O. Lötvall, Marta Bally, Fredrik Höök (# Equal Contribution) (*Submitted*)

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ABBREVIATIONS

ABs	Apoptotic bodies
ADP	Adenosine diphosphate
AKT	A protein-serine/threonine kinase
ANXA1	Annexin A1
APCs	Antigen presenting cells
BMMCs	Bone marrow mast cells
BMP7	Bone morphogenetic protein-7
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CRISPR	Clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ECAD	E-cadherin
EGFR2	Epidermal growth factor receptor
ELISA	Enzyme linked immuno-sorbent assay
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal-regulated kinase
EVs	Extracellular vesicles
FBS	Fetal bovine serum
GFP	Green Fluorescent Protein
HEK	Human embryonic kidney
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
HSP	Heat shock proteins
HSPG	Heparan sulfate proteoglycan
ICAM	Intercellular Adhesion Molecule
IFN-γ	Interferon gamma
Ig	Immuno globulin
ILVs	Intra lumenal vesicles
IRF3	Interferon regulatory factor-3
ISEV	International Society of Extracellular Vesicles
LAMP1	Lysosomal-associated membrane protein 1
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex

MMP	Matrix metallo proteinases
MSCs	Mesenchymal stem cells
MVBs	Multi-vesicular bodies
MVs	Micro-vesicles
NCAD	N-cadherin
NK	Natural killer
NKG2D	Natural killer (NK) group 2 member D
NSF	N-ethylmaleimide-sensitive factor
RAB	Ras-related protein Rab-27A
RAC1	Ras-related C3 botulinum toxin substrate 1
RNA	Ribo nucleic acid
shRNA	Short hairpin RNA
STAM-1	Signal transducing adaptor molecule-1
SUMO	Small ubiquitin-like modifier
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TRP-1	Transient receptor potential-1
TSG	Tumor susceptibility gene
VAMP	Vesicle-associated membrane protein
WNT5A	Human Wnt family member 5A
ZEB	Zinc-finger E-box-binding
ZO-1	Zonula occluden-1

1 INTRODUCTION

Cells depend on their extracellular surroundings for their survival. Various biochemical meditators (ions, cyclic adenosine monophosphate, nucleotides, and metabolites) can interact with cells and signal the availability of nutrients or the presence of danger. This process has been well studied in lower organisms, e.g., a group of behavior responses in bacteria called “quorum sensing” [1]. In eukaryotes, cell-to-cell communication was first observed in the late 1950s and early 1960s, but the transfer of biochemical meditators was not well defined, and it was thought that the mode of mediator transfer was restricted to neighboring cells. The transfer of mediators was shown to be limited to the size of the mediator because the observed transfer was mediated through the gap junctions between cells [2, 3]. Subsequent research revealed that hormones, secreted proteins, and other mediators could regulate cellular functions at distant sites far away from the origin of the signaling molecules [4].

The past decades has witnessed the discovery of a new mode of cell-to-cell communication called extracellular vesicles (EVs). EVs have emergent properties that are conserved from bacteria to higher eukaryotes. They are broadly termed “EVs” and have broad implications for our basic understanding of cellular communication, for biomarker discovery, and for therapeutic applications.

In this part of thesis, I will discuss the relevant background information on EVs, including the history of their discovery, their cellular origins, and descriptions of their associated cargo. I will also provide the relevant background information on the biological messages that are delivered by EVs. It is not possible to cover all of the literature, but I will attempt to highlight the relevant and essential findings in this growing field.

1.1 Extracellular vesicles

1.1.1 Historical background

Membrane-enclosed structures released from cells are broadly termed EVs. Early work on EVs dates back to the 1940s when components of clotting factors were found in platelet-free plasma that was pelleted/removed by high-speed centrifugation [5]. It was not until 1964, however, that Peter Wolf and colleagues coined the term "platelet dust" to describe this pelleted material [6]. The initial understanding of membrane shedding came from studies in reticulocytes, which shed about a third of their membranes during maturation, and the mechanisms behind this were thought to contribute to intracellular budding of vesicles to form multi-vesicular endosomes [7]. The most critical finding came in 1983 when the research groups of Philip Stahl and Rose Johnstone independently showed the exocytosis of vesicles with transferrin receptor recycling (**Figure 1**) [8, 9]. Subsequently, in 1987 Johnstone used the term "exosomes", but only with regards to the vesicle structures that are released into the extracellular milieu [10]. In the current literature, "exosomes" refers to all intraluminal vesicles (ILVs) that are derived from multi-vesicular bodies (MVBs).

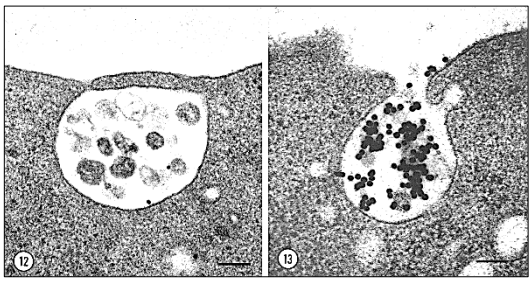

Figure 1. *Exocytosis of a multivesicular endosome releasing exosomes containing transferrin receptor. Clifford V. Harding et al. J Cell Biol 2013; 200:367-371).Reprint permission kindly given by JBC.*

Exosome research was dormant for a decade because they were initially considered to be nothing more than a "waste disposal system" or "trash can" and were a system for protein/membrane component removal in differentiating cells, primarily reticulocytes. However, in 1996 a similar process of exosome release was discovered in immune cells, and it was shown that this exosomes release was associated with a biological function. Electron microscopy of B-cells revealed the presence of exosomes that were presenting antigen and that

induced a major histocompatibility complex (MHC) class II-restricted T-cell response [11]. This idea of exosomes with antigen presentation was studied in a pre-clinical mouse model. Tumor growth was reduced using an exosomes-primed T-cell response, where the exosomes were obtained from tumor peptide-pulsed B-cells/dendritic cells [12]. Within a short time, human clinical trials were set up in 2005 following the same line of thinking, and patients with metastatic melanoma and non-small cell lung cancer were administered EVs derived from autologous dendritic cells [13].

Up to this point, EV and associated protein studies were predominating. However, proteomics studies of EVs were gradually accumulating and indicated the presence of RNA translation machinery in many different EVs. These observations led Jan Lötvall and his team to popularize the hypothesis that EVs mediate the transfer of RNA, especially micro-RNA (miRNA) and messenger-RNA (mRNA), from one cell to another [14]. In addition, the use of EV-derived RNA with biomarker potential boosted the field of exosome research exponentially [15, 16].

1.1.2 Classification and nomenclature

EV is an umbrella term used for all lipid bilayer structures released from cells and ranging in size from 30 nm to 2,000 nm. The common denominator that defines EVs is having a lipid bilayer, while EV components such as proteins, RNA, and DNA are still debated as to whether they should be considered defining features of EVs [17, 18]. The EV field is still developing, and numerous methods are being used to differentiate them, and they are often given interchangeable names such as exosomes, macrovesicles, micro-particles, and large-oncosomes [19]. For example, vesicles are called dexosomes, platelet dust, or proteasomes if they are derived from dendritic cells (DCs), blood platelets, or prostate epithelial cells, respectively. Currently the naming of EVs is based on the size of the vesicles, their floating density, and their cell of origin. The most widely used method of sequential ultracentrifugation yields EV subsets (apoptotic bodies (ABs), micro-vesicles (MVs), and exosomes) based on their sedimentation rate [20, 21].

Currently the term “exosomes” is used for vesicles that are released after the fusion of MVBs carrying ILVs to the plasma membrane, and the formation of MVs is defined by membrane budding [22]. However, it is difficult to separate exosomes from the pool of EVs. Also, EVs that bud from cell membranes can vary in size and can be as small as exosomes (<150 nm) or as large as ABs (>2,000 nm). In this thesis, I will be referring to the preparations as EVs, and these are an exosome-enriched vesicle preparation.

The heterogeneity of EVs is reflected in the isolation method to separate ABs, MVs, and exosomes when using differential centrifugation. It has been shown that all of these subsets share common exosome markers [21] , and this was further supported when inhibition of the small GTPase Rab27a at the protein level led to the reduction of CD63 on exosomes while other markers like CD9 remained unchanged [23]. We and others have observed certain subsets of EVs that are derived from mitochondria, and although we do not know the mechanism behind the origin of these EV subsets, they certainly seem to be biologically relevant because these mitochondria-derived subsets are increased in melanoma [24-26]. Immuno-affinity-based capturing of EV subsets has been used to isolate and characterize sub-populations of EVs and has been used to produce cleaner EV samples [23, 27]. Significant structural heterogeneity in terms of shape and size can be seen in EV preparations with cryo-electron microscopy in which samples are unprocessed and can be observed without any damage from the preparation [28]. Some studies have attempted to tackle the question of heterogeneity of EVs that come from different cell types in the same culture condition by detecting them one by one and thus establishing their cellular origin [29, 30]. Certainly the heterogeneity that is discussed in the literature is based on proteins, but in the near future EVs will likely be classified based on other cargo such as lipids, RNAs, and DNAs. The EV field is just beginning to address EV heterogeneity, and it will requires time and robust approaches to address this question.

1.1.3 Biogenesis of extracellular vesicles

Cells release EVs by shedding lipid bilayer structures that are formed from a series of interactions between protein complexes and lipids on the cell surface and the endomembrane. Cell surface membranes are rapidly recycled and

replaced by new membranes, and the rate at which this happens remains unclear but has been reported to be between 10 and 20 minutes [31, 32]. Cell membranes are constantly shuffling in order to carry out important functional actions, including antigen processing, nutrient uptake, cholesterol/lipid efflux, and receptor regulation [31, 33]. Even though lipids are one of the defining features of EVs, only a few studies have been performed to identify these different lipids [34-37]. Thus, most of the information on EV-biogenesis is based on EV protein composition. In order to determine the biogenesis of EVs (mostly exosomes and MVs), several methods are used such as the detection of specific marker proteins (CD63, CD81, Alix, and TSG101) and measuring the particle number in the secreted supernatant [38]. With these techniques, we have gained some key insights into the biogenesis pathways that involve MVBs (i.e., exosomes) and membrane budding (i.e., MVs) (**Figure 2**). Even though EVs (MVs and exosomes) have separate sites of origin, they share similar cargo clustering mechanisms. Cargo clustering at the site of origin and clustering of various proteins induces exclusion/budding and the ultimate release of vesicles from the cell membrane. The budding process is essential for the release of MVs and exosomes, and different protein clusters are involved in their biogenesis.

Cargo clustering

The primary signal for the loading of any cargo in EVs is the cargo itself. For example, simply overexpressing any cargo (such as MHC class II or melanocyte-specific glycoprotein Pmel17) can trigger MVB formation and thus become part of the released EVs [39, 40]. The membrane cargoes usually come from internalized plasma membrane or Golgi compartments and become part of the ILVs that then become part of MVBs [41]. In order to become part of the ILV membrane, there has to be an alteration in the regulators of endosome recycling or retrograde endosome-to-Golgi transport. For example, syntenin is one such protein that regulates the sorting of syndecan into exosomes [42, 43].

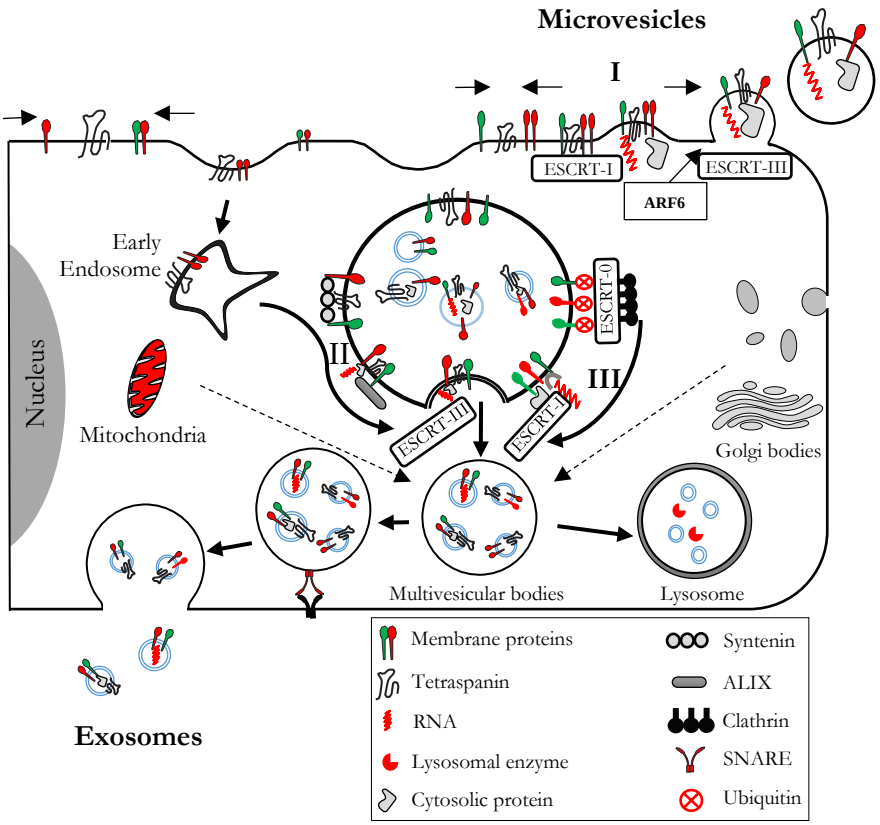

***Figure 2. Extracellular vesicles biogenesis**: **Microvesicles: 1**. Formation of microdomain by clustering of membrane protein and lipids on the cell surface. Cytoplasmic side of microdomain engage and bring cytoplasmic cargo and undergoes outward budding. **Exosomes: 2. ESCRT-independent pathway**: Clustering of lipid and membrane proteins in limiting membrane of endosomes with Syntenin and ALIX, followed by recruitment of cytoplasmic cargoes (RNA, protein), finally leading to membrane invagination generating ILVs in MVBs**. 3. ESCRT-dependent pathway**: Targeted/ubiquitinated protein assemble with ESCRT-0/Clathrin, followed by recruitment of cytoplasmic cargo and ESCRT-I complex. ESCRT-III component act is scission that generate ILV from MVB. **ESCRT**: Endosomal Sorting Complex Required for Transport, **ILV**: Intraluminal vesicles, **MVB**: Multivesicular body.*

Microvesicles biogenesis

Unlike exosomes, biogenesis of MVs involves outward budding of the membrane followed by fission of plasma membrane (**Figure.2. I**). Initiation of MVs starts with redistribution of phospholipid-like phosphatidylserine causing shrinking of actin-myosin assemblies [44]. This process involves the ADP-ribosylation factor 6 (ARF6)-initiated signaling cascade leading to myosin light chain (MLCK) based release of MVs.ARF6 is also required for targeting Integrin β-1, MHC class-I, and MMP14 to the MVs [45]. Recently, TSG101, a component ESCRT-I, was shown to be associated with a tetrapeptide protein within the Arrestin 1 domain–containing protein 1 (ARRDC1) that facilitates its recruitment to MVs [46]. Various cues like calcium influx and hypoxia can also trigger MV release with different cargo content [47, 48].

ESCRT-dependent exosome biogenesis

ESCRTs are a set of protein complexes that act on the membrane and its cargo to induce invagination to form ILVs and MVBs, and this occurs concomitant with cargo clustering [49]. In a step-wise manner, ESCRT-0 clusters ubiqutinated cargo with the help of ESCRT-I protein complexes on the cytoplasmic side of the endosomal membrane (**Figure 2. III**).This is followed by recruitment of ESCRT-II and ESCRT-III that help in budding and the generation of ILVs that will become exosomes [50]. This process occurs in parallel with other essential events that act as regulators of EV formation. For example, accessory proteins like VPSD4 ATPase facilitate invagination and recycling of endosome [45].

Components of ESCRT-0, including HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM-1 (signal transducing adaptor molecule-1), are known to recognize ubiqutinated proteins and play central roles in many cells types [50-52]. Interestingly, even though components of the ESCRT-II protein machinery are found in exosome proteomes, their elimination does not have a significant effect on EV secretion. Other key members of the ESCRT family, like ALIX, are involved in associating cargo to the ESCRT III complex [43]. ALIX interacts with another protein called syntenin (an adaptor protein for heparan sulfate proteoglycan receptor) to regulate the production of MHC

class II exosomes in HeLa cells [43]. However, the same machinery in DCs reduces the production of EVs containing CD63, CD81, and MHC class II molecules [50]. VPS4, a protein that is needed for ESCRT III disassembly, has been the subject of much debate in terms of its role in EV biogenesis [43, 50, 53]. In some cells like Oli-neu cells, ESCRT-independent exosome secretion is proposed [54].

ESCRT-independent exosomes biogenesis

Lipids in EVs are a defining feature of EVs along with their associated proteins, and any changes in lipid availability can alter the EV number and composition (**II. Figure.2**). Proteolipid protein-positive exosomes are produced independently of the ESCRT proteins. Inhibition of sphingomyelinase impairs ceramide synthesis and thus impairs the secretion of exosomes [54, 55]. Inhibitor studies have indicated that it is not exosomes, but other membrane-derived compartments, including membrane-budded MVs, that are affected in their secretion. However, inhibitors might have some nonspecific effects, and thus it is difficult to draw firm conclusions from such studies. Altering the level of cholesterol can have an effect on EV secretion, and in an experiment in which cholesterol was accumulated in MVBs, EVs were secreted that contained ALIX, CD63, and flotiline-1 [56]. Enzymes like phospholipase D2 that catalyzes the synthesis of phosphatidic acid from phosphatidylcholine can promote the invagination of ILVs in MVBs [57]. Additionally, tetraspanin, which plays a role in bringing together different cargos, is essential for the recruitment and biogenesis of vesicles [58, 59].

Trafficking and fusion of MVBs

Control of the movement of MVBs (as described above) is essential so that they can fuse to the plasma membrane and release the sorted vesicles. The RAB family of proteins are key proteins that regulate multiple events in vesicular trafficking, including budding, transport, docking, and finally fusion to the plasma membrane. RAB11 and RAB35 have been implicated in regulating the transferrin receptor and proteolipid protein-containing EVs, respectively [60, 61]. A RAB-depletion experiment of RAB5A, RAB9A, RAB2B, RAB27A, and RAB27B using shRNA showed reduced exosome secretion [39].The role of RAB27B has been shown in a number of cancer studies [62]. Different RAB

proteins are associated with different organelles; for example, RAB11 and RAB35 are associated with recycling endosomes, whereas RAB27A/B is associated with MVB docking to the plasma membrane [39, 63]. After the docking of the limiting membrane to the site of fusion, a protein called soluble NSF-attachment protein receptor (SNARE) forms complexes with the VAMP7 and YKT6 proteins, and these complexes are required for the release of EVs along with their cargo (**Figure 2**) [52, 64]. Our understanding of EV secretion is restricted to certain subsets of EVs because of the difficulty in determining the cargoes and subpopulations of EVs. Unexpected membrane proteins from other organelles in EV preparations have not yet been defined, so all of the experiments described above will be revisited in the future.

1.1.4 Methods for EV isolation

Various isolation, purification, and detection methods are used to obtain EVs (exosomes, MVs, and ABs). An ideal EV preparation would have high recovery yield, no contamination with free proteins, no cross contamination with other EV subsets, and little physical or chemical damage following the isolation procedure. However, there are currently no perfect methods for obtaining EV preparations, but some strategies such as coupling existing methods or developing new methods are under way to attain the "ideal EV preparation". The classical method of differential ultracentrifugation can be used to enrich the EV population, and then an iodixanol/sucrose-based density gradient can be used to obtain purified EVs, and this method remains the preferred choice of isolation for many labs and can be found at www.evtrack.org [65, 66]. The combination of ultracentrifugation followed by density gradient centrifugation uses the sedimentation rate and density of EVs to yield relatively pure preparations. However, many studies have used polyethylene glycol-based EV precipitation, an approach that yields EVs but can also co-precipitate free proteins [67]. Body fluids like serum and plasma require additional steps to obtain pure EVs because density-based flotation alone results in the co-flotation of lipoprotein complexes along with EVs. The combination of a density cushion along with size-exclusion chromatography yields relatively pure EV preparations [68], and size-exclusion chromatography has also been shown to be associated with improved EV integrity over ultracentrifugation-based EV isolation [69]. Another strategy is

ultracentrifugation followed by density gradients and then immune-affinity capture, and this can achieve a purer EV preparation with known cargo [70, 71]. Another combination that is often used for high-volume cell culture is ultrafiltration (or tangential flow filtration) coupled with size exclusion chromatography and the use of an affinity tag to further select and improve EV preparations [72]. This type of approach can easily be adapted for and mixed with the other methods listed in **Table 1** to generate EVs. Along with the expansion of EV isolation techniques, guidelines have been established by the International Society of Extracellular Vesicles (ISEV) to ensure the uniformity of methods for future comparative studies [73].

Table 1. *Various methods of EV isolation.*

Method		Principle	Ref.
Ultracentrifugation	Ultracentrifugation	Sedimentation	[14]
	Ultracentrifugation with sucrose	Sedimentation + Density	[74, 75],
	Ultracentrifugation with iodixanol		[76]
Ultrafiltration		Size	[77]
Size-exclusion chromatography			[78, 79],
Dialysis			[80]
Protein-based capture	Capture antibody	Immune affinity	[70, 71],
	Tim-4 protein	Non-covalent interaction	[81]
	Annexin A5		[82]
	Vn peptide		[83]
Sugar-based capture	Heparin	Affinity binding to sugar	[84]
	Lectin		[85, 86]
Precipitation-based	Polyethylene glycol	Insoluble ionic precipitation	[87]
	Sodium acetate	Salting out	[88]
	Protamine	Charge-based precipitation	[89]
	Organic solvent	Organic solvent precipitation	[90, 91]
Two-phase isolation		Phase separation	[92]

1.1.5 Extracellular vesicle cargoes

EVs are almost ubiquitously present and are commonly found in all body fluids as well as in the extracellular matrix. The contents of EVs reflect their source cells, and this property of EVs has made them a valuable source of biomarkers. EVs consist of a lipid bilayer structure that carries lipids, proteins, nucleic acids, and metabolites, and this cargo can be present on the surface as well as protected inside the lumen of the EVs. Studies on the composition of EVs using various omics approaches are listed in databases such as EVpedia, Vesiclepedia, and Exocarta (http://student4.postech.ac.kr/evpedia2_xe/xe/, www.exocarta.org/, and http://www.microvesicles.org/).

Lipids

Lipids are a fundamental feature of EVs and provide a common site where all other cargoes cluster, and they are released as part of the EVs. Different types of lipids are present on EV subsets (e.g. exosomes and MVs), but interestingly their lipid compositions also differ from the membrane of the cell of origin [34, 35]. The type and packaging of lipids in the exosomes are dependent on the conditions under which the source cells are grown, e.g. pH [93]. EVs are usually enriched in phosphatidylserine, cholesterol, and sphingomyelin [94]. A detailed lipidomics study on glioblastoma cells, hepatocellular carcinoma cells, and human mesenchymal stem cells (MSCs) showed a clear enrichment of glycolipids and free fatty acids in exosomes, whereas ceramides and sphingomyelins were enriched in MVs [34]. The lipids in EVs can act as "autacoids" by transporting eicosanoids, prostaglandins, and leukotrienes [94-96], while other lipid components like cholesterol and ceramide are involved in exosome biogenesis, and lysobisphosphatidic acid has been shown to play role in ILVs [97]. Despite being the common denominator among EVs, the different lipids are still not well studied and will be the focus of future research.

Proteins

The first work on EVs involved transferrin receptor proteins, and since then proteins have been the most studied components of EVs [98]. Proteins such as cargo proteins and proteins required for biosynthesis are well catalogued in the

literature [22]. The majority of information in the literature comes from studies on stem cells, immune cells, and cancer cells, and the most commonly identified proteins in all of these studies include junctional, chaperone, cytoskeletal, membrane trafficking, structural, and transmembrane receptor/regulatory adaptor proteins [99, 100]. These proteins can be categorized based on where they are located, e.g., whether they are surface proteins, transmembrane proteins, membrane-anchored proteins, or luminal proteins. Some of the membrane proteins such as tetraspanins, MHC class II, and luminal proteins (TSG101 and ALIX) are used as EV markers. EVs are also known to contain adhesion molecules such as integrins, glycoproteins, and selectins [101, 102]. Some subdomains of EVs have lipid rafts that tend to cluster tetraspanins and integrin [59, 103]. Annexins are also frequently identified in EVs and aid in intracellular fusion events through their interactions with phospholipids [104, 105]. Some members of the RAB protein family assist in membrane transport and EV biogenesis [39, 106]. Interestingly, EVs also contain other various metabolic enzymes such as peroxidases, pyruvates dehydrogenase, enolases, and certain kinases. Exosomes from different body fluids were shown to contain cell surface peptidases such as dipeptidylpeptidase-IV and aminopeptidase [107], and the presence of a matrix metalloproteinase (MMP) in EV preparations was shown to have matrix-degrading properties that aid in migration of cells in the matrix [108, 109].

Post-translational modification of EV proteins and their detection with sensitive methods have led to a new understanding of EV physiology because these modifications can influence the structure and function of EVs [110]. For example, phosphorylation of the MET protein has been reported, and the protein was found to be transferred via exosomes released from aggressive melanoma that makes metastatic niche at distance organ [62]. In Alzheimer's disease, phosphorylated tau protein has been suggested to spread via its association with EVs [111]. Component of phosphorylation machinery (kinases and phosphatases) can be found in EV preparations suggesting the ability of EVs to activate phosphorylation in recipient cells [112-114]. Phosphorylation of epidermal growth factor receptor (EGFR) and other receptor tyrosine kinases in EVs can modulate the phospho-proteome of recipient cells [115].

In addition to phosphorylation, the functionality of many secreted proteins and membrane proteins is regulated by glycosylation. In glycosylation, a glycan moiety is added to a protein in an enzymatic (glycosidase and glycosyltransferase) reaction that occurs in the endoplasmic reticulum and Golgi apparatus. Studies on glycan identified a common glycome between MVs and the HIV virion suggesting their common origin and uptake pathway [116]. The modification of EV cargo by glycosylation alters exosome uptake, as seen in the case of galectin-3 binding protein [117]. Tetraspanins found in EVs are known to undergo variable glycosylation. Other modifications such as ubiquitination are seen in MVs derived from plasma [118]. The addition of a SUMO group to the hnRNPA2B1 protein drives the miRNA sorting into EVs [119]. The covalent attachment of interferon-stimulated gene-15 to a protein triggered by type I IFNs, also called ISGYlation, is involved in the innate immune response and cancer [120], and this modification drives EV secretion and lysosomal degradation [121].

Nucleic acids

The presence of nucleic acids in EVs has been shown to be a novel method of cell-to-cell communication. Since the presence of RNA in EVs was reported a decade ago, a large number of studies on EVs have been performed [14, 16, 122]. Over the past few years, work on EV-associated DNA has helped bring the research community together by resolving certain debates such as the amount of miRNA per EV and whether or not DNA associated with EVs is an isolation artifact, and this work has allowed the field of nucleic acid research to move forward [123]. In the following sections I will highlight the analysis of the RNA and DNA contents of EVs.

RNA

The presence of RNA in ABs was described the 1990s, but it was only after 2007 that its functional role began to be explored [14]. The most commonly enriched RNAs in EVs are small RNAs, transfer-RNAs (tRNA), and 18S and 28S rRNAs. With the ease of next-generation sequencing, various RNAs such as micro-RNAs, short and long non-coding RNAs, tRNA fragments, piwi-RNA, vault RNA, and Y RNA have been reported in EVs [21, 87, 124-128]. Moreover, circular RNAs have been found to be stable in EVs [129]. The

selective packaging of RNA in EVs has been reported, and miRNA overexpression studies in cells and analyses of 3' untranslated region-specific RNA in EVs have revealed a tendency for RNA loading in EVs [130, 131]. Mechanistic studies have been proposed to clarify the loading of RNA in EVs. The GGAG motif in certain miRNAs interacts with a ribonucleic protein complex (hnRNPA2B1) to assist in their loading in MVBs that contain future exosomes [119]. A recent study showed the enrichment of miRNA in CD-63-positive EVs and showed the importance of the RNA-binding Y-box protein for the sorting of miR-223 in a cell-free reaction [71]. Certain post-translation modifications such as the SUMOylation of ribonucleoproteins, uridylation, and the adenylation of RNA have been reported to dictate the loading of RNA in EVs and hence the abundance of RNA in EVs [132, 133]. Enhancing cholesterol biosynthesis by nSMase2 biosynthesis has been shown to increase the amount of miRNA in EVs by enhancing exosome production [55], and a component of the RNA silencing machinery (AGO2) has been shown to be involved in the loading of miRNA into EVs [134, 135]. However, the localization of the AGO2 protein with secreted EVs needs to be confirmed in order to clarify its role in miRNA-associated EV biogenesis [136]. Counter intuitively, the debate over the amount of miRNA loaded inside EVs has added to the discussion on the methods for measuring miRNA in isolated EVs and the location of RNA cargo in EV [123, 137]. Most of these finding are restricted to simple observations on the pool of EVs and will certainly be revisited following the development of clearer definitions of EVs and their subpopulations [70, 71].

DNA

Unlike RNA, EV-associated extracellular DNA (ex-DNA) has been surprisingly little studied in terms of its origin, and it is often argued to be produced from dying cells [138]. It is well known that ABs, the largest EV subset in terms of size, carry DNA [138, 139], but information on ex-DNA in other EV subsets (exosomes and MVs) is scarce. Recent studies on EVs from serum and cell culture supernatants have identified the presence of ex-DNA [140-143], and the data usually indicate both nuclear (presentation of chromosomes) and mitochondrial origins [144-147]. Interestingly, there is more in the literature on DNase-resistant luminal ex-DNA with much less focus on the non-protected ex-DNA that is present and that is considered a potential

contaminant. Indeed, a few studies found no DNase-sensitive DNA in human plasma or smooth muscle cell EVs [141]. However, a study on floated EVs treated with DNase found that ex-DNA that floated along with EVs was DNase sensitive and was present on the EV surface, as supported by other findings [148-150]. The use of EV-associated ex-DNA independent of its location could have a huge impact on the disease biomarker field, including early cancer detection [144, 151].

1.2 Extracellular-vesicles in cell-to-cell communication

With all the cargoes described above, EVs present a potent information delivery system from one cell to another. They can influence local sites close to their site of production or they can be directed to distant sites. To enable communication, it is essential for the EVs to dock on the plasma membrane and engage with surface receptors, which in turn activate other molecules on the surface and trigger rapid downstream signaling, uptake, or fusion with the recipient cell membrane. Available studies to date have described the surface interactions and intercellular fates of EVs. Common ways of evaluating the uptake of EVs is to use a membrane-specific lipid dye (PKH and Di-dyes), a membrane-anchoring tag with a fluorescent protein, or an EV-associated protein fused with green fluorescent protein (GFP) or m-cherry. This section describe how EVs engage with recipient cells and transfer their cargo.

1.2.1 Surface-to-surface interactions of EVs

Proteins present on the plasma membrane of recipient cells are key determinants that engage with the surface components of EVs. Various tetraspanins, proteoglycans, integrins, lipids, and matrix components all play a role in mediating EV–cell interactions. Several features of cell-to-cell interactions are phenocopied in the EV-to-cell interaction, e.g., integrins from EVs interact with Intercellular Adhesion Molecule (ICAMs) on the cell surface [152, 153]. Alternatively, integrins can engage with surface matrix components such as fibronectin and laminin for exosome and MVs binding [154, 155]. In recipient cells, the dimerization of integrin can provide organ specificity for EV uptake, as shown in a mouse model [101]. These integrins also bind to EV

tetraspanins to assist in the docking and uptake of the EVs [156]. One of the most interesting classes of proteins in terms of EV binding is the heparan sulfate proteoglycans, which are multi-modular proteins that can be found on both EVs and the cell surface and are essential for the attachment of cellular CD44 and act as multiple sources of ligands (e.g. TGFβ-1) [157-159]. As discussed earlier, these receptors induce the engagement and clustering of lipid rafts, which initiates membrane reorganization and drives the remaining processes in the signaling cascade.

1.2.2 Uptake of EVs

EV uptake is mediated through various processes such as direct membrane fusion and endocytosis. Endocytosis is further classified into clathrin-mediated endocytosis, caveolin-mediated endocytosis, macro-pinocytosis, and phagocytosis. Most the uptake is not very exclusive and depends on the cell type and cellular state. EV endocytosis is an active process and requires appropriate physiological conditions. Reducing the temperature to 4°C, fixing the cells with paraformaldehyde, and inhibiting actin polymerization with cytochalasin D in the recipient cells result in low or almost no EV uptake. Studies using various chemical inhibitors and blocking peptides have revealed some of the key pathways for EV uptake, which are listed in **Table 2.**

Table 2. *Route of EV uptake by recipient cells with inhibitors and their target.*

Drugs	Targeted molecules	Cell types
Endocytosis		
Heparin	Heparan sulfate proteoglycans	Glioblastoma multiforme tumor [160] SW-780 bladder cancer cells [161]
Di-fluoromethyl-ornithine	Heparan sulfate proteoglycans	Glioblastoma multiforme tumor [160]
Asialofetuin	Galectin-5	Macrophages [162]
Human receptor- associated protein	CD91	Dendritic cells [163]
RGD (Arg-Gly-Asp) peptide	Fibronectin	Macrophages [164] Dendritic cells [152]
Ethylene diamine Tetra acetic acid	Calcium	Dendritic cells [152, 165] Macrophages [162]

Cytochalasin D	Actin	Epithelial A549 cells [166], Microglia [167] Dendritic cells [152], Macrophages [164]
Cytochalasin B		Macrophages [162]
Latrunculin A		HUVEC [168]
Latrunculin B		RAW264.7 macrophages [169]
Clathrin dependent Endocytosis		
NSC23766	Dynamin	Microglia [167]
Dynasore	Dynamin-2	Macrophages[162], Microglia [167]
Chlorpromazine	Receptors for neurotransmitters	SKOV-3 ovarian cancer cells [170], Microglia [167]
Macropinocytosis		
5-(N-Ethyl-N-isopropyl)amiloride (EIPA)	Sodium/proton exchanger	SKOV-3 ovarian cancer cells [170], RAW-264.7 macrophages [169]
Bafilomycin A, Monensin, Chloroquine	Sodium/proton exchanger H(+)-ATPase activity	Microglia [167]
Phagocytosis and Macropinocytosis		
Annexin-V	Phosphatidylserine	Neuro-2A mouse neuroblastoma cells [171], Microglia [167]
Phagocytosis		
Wortmannin	Phosphoinositide 3-kinases (PI3Ks)	RAW-264.7 macrophages [169]
LY294002		
Lipid raft mediated Endocytosis		
Methyl-β-cyclodextrin	Cholesterol	SKOV-3 ovarian cancer cells [172], RAW-264.7 macrophages [169], BT-549 breast cancer cells [173], HUVEC and U87-MG glioblastoma cells[168]
Filipin		BMDC [174], Melanoma cells [93], HUVEC [168]
Simvastatin		HUVEC [168]
Fumonisin B1	Glycosphingolipid	Jurkat cells and HEK-293T kidney cells[175]
N-butyldeoxynojirimycin hydrochloride		
U0126	ERK1/2	HUVEC, HeLa, Mouse embryonic fibroblasts[168]
Membrane fusion		
Proton pump inhibitor	Sodium reabsorption	Melanoma cells [93]

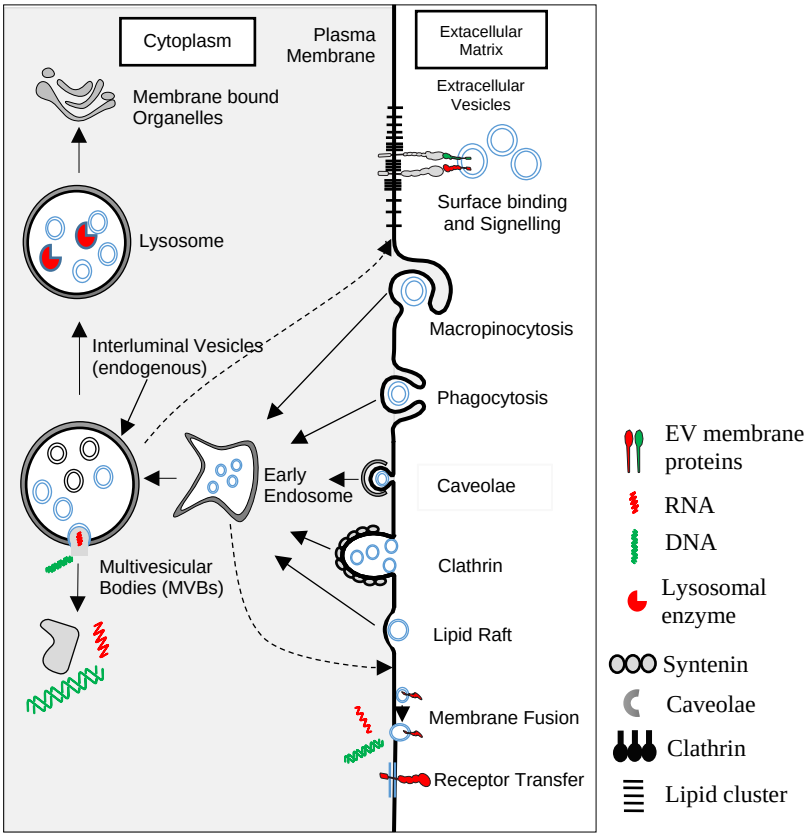

Figure 3: Uptake of extracellular vesicles: *Cellular uptake of EVs is done by endocytosis or direct membrane fusion. To deliver a function the cargo present on EV surface can bind with surface protein to activate cell surface receptor or fuse with surface to deliver its content. Majority of uptake is mediated by multiple endocytic pathways (⟶) and arrives in early endosomes where they fuse with endogenous multivesicular bodies (MVBs) or can recycle back to the cell surface (⇢). The fate of MVBs can be release of EV-cargo in cytoplasm or fusion with lysosomes or other organelles.*

1.2.3 Functions of the delivered EV cargo

EVs have multiple cargo proteins (growth factors, chemokines, enzymes), RNA (miRNA, mRNA), DNA (genomic and mitochondrial), and lipids in various locations with regards to the EV surface [176]. As the properties of EVs have been determined, EVs have been implicated in a wide variety of physiological and pathological functions. They have been shown to influence processes such as coagulation, angiogenesis, inflammation, injury repair, and adaptive and innate immunity. However, knowledge on EVs with regard to their role in the delivery of associated cargo is mostly restricted to single cargos (**Table 3**). Depending on the cargo they are carrying, EVs can be more or less effective in inducing a phenotype change. For example, if the cargo is an enzyme that catalyzes a reaction, then large amounts of enzyme will be needed because most of it will be exhausted. Similar half-life can be seen for mRNA cargo where only a few recipient cells are effectively primed (RNA transfer). On the other hand, cargoes such as signaling proteins have the capacity to amplify signals or miRNA can target multiple mRNA and can be more effective in influencing a phenotype. EVs can contain various growth factors, and thus they can act as reservoirs of various cytokines and chemokines. The surfaces of EVs are decorated with various proteoglycans that are known to carry various factors. These EVs activate recipient cells by acting as a ligand, or can fuse with cells to transfer the bioactive receptor to cellular receptors and initiate the downstream signaling that decides cellular fate. In summary, cells seem to release EV-associated cargos that have varying degrees of effector function and can function for shorter or longer times or can have permanent phenotypic effects.

Table 3. *List of biological functions delivered by various cargos in recipient cells. Cargo are classified based on their topological location on EV.*

	Cargo	Source of EV / Recipient cell	Function	Ref.
Surface Cargo				
Membr	cKIT	Mast cells /Epithelial cells	c-Kit transfer activation of PI3K signaling, Proliferation	[177]
		Gastrointestinal stromal tumor / smooth muscle cells	Invasive phenotype and MMP-1 secretion	[178]

	Proteoglycan, Syndecan, Glypican	Hepatic Stellate Cells (HSC) / hepatocytes or HSC	Growth factor binding, fibronectin binding, and enhance uptake of EV	[179]
		GBM cell line / GBM cell line	EV uptake mediated by surface HSPG, GBM development	[160]
		Myeloma Cell/Myeloma Cell	Binding of fibronectin via HSPG assist uptake and cross talk	[155]
	TβRII	Stromal fibroblast / breast cancer cells	TGFβ-signaling and therapy resistance	[180]
	MHC-II	Epithelial cell line EVs / mesenteric lymph nodes	Humoral immune response	[181]
	Tetraspanin-8	Adenocarcinoma / endothelial cells (EC)	Proliferation, migration, sprouting, maturation of EC progenitors	[182]
Secreted factors	TGFβ-1	Thymus tumor/T-cell	Induction of regulatory T cell	[183]
		OvCa cell lines, Squamous cells /T cells	Induction of regulatory T cell	[184]
		Mesothelioma cell line / peripheral blood lymphocytes	Impair lymphocyte responses to Interleukin-2, T cells	[185]
		TS/A or 4T-1 tumor cells and T cell / tumor	MDSC-induced immunosuppression	[186]
	IL-1β	THP-1 monocytes/HeLa cell	IL-1 receptors phosphorylates signaling	[187]
		Murine macrophage with ATP stimulation	Cytokine production	[188]
		Dendritic cells stimulated with nucleotide	Activating T lymphocytes	[189]
	TNF	Synovial fibroblasts / CD3-activated T-cells	Delay T-cell apoptosis	[190]
	LFA1	Dendritic cells /Dendritic cells	Ag-specific naive T cells	[191]
	VEGF	Mesenchymal stromal cells/renal tubular epithelial cells	Pro-angiogenesis for tissue regeneration	[192]
		Platelet micro particles / Angiogenic outgrowth cells	Proliferation, migration, and capillary tube formation by mature endothelial cells	[193]
	TRAIL	K562 cells / KMS11 multiple myeloma.	Growth inhibition and Necrosis in KMS11. Cell death in all cell lines but not in primary	[194]
		MSC cells/cancer cell line	Bronchial epithelia cells	[195]
	Wnt –PCP	Fibroblast cell/Breast cancer cell	Protrusive activity and motility	[196]
	Wnt4	Human mesenchymal stem cells / skin HaCAT cells	β-catenin activation, proliferation, and migration	[197]
Luminal Cargo				
RNA	mRNA	Human Mast cells /Mouse mast cells	Human protein synthesis in mouse cells	[14]
	GFP mRNA	Endothelial progenitor cell/ fibroblasts	Neo-angiogenesis and GFP expression in microvascular ECs	[198]
	Oct 4 mRNA	Embryonic stem cell / bone marrow cells	Survival, increase pluripotency marker Oct4 Nanog and Rex-1	[122]

	cre mRNA	Hematopoietic cell /Neuronal cells in the mouse brain	More Cre mRNA based recombination during inflammation	[199]
		Myeloid-derived suppressor-cell /mouse carcinoma and glioma	Induce recombination and enhanced immunosuppressive phenotype and an altered miRNA	[200]
	Hepatitis C viral RNA	Hepatitis C virus-infected plasmacytoid dendritic cell (pDC) / non-infected pDC	EVs induce secretion of type I interferon	[201, 202]
	miRNA-223	Human embryonic kidney cells 293T / NA	NA	[71]
DNA	Genomic DNA	Damages human diploid fibroblasts/ normal human diploid fibroblasts	Innate immune signaling by the STING pathway and regulation reactive oxygen species	[203]
		Plasmodium falciparum-infected red blood cells/ Mosquitoes	DNA-dependent transfer of drug resistance and fluorescence between malaria-infected red blood cells	[204]
	Mitochondrial DNA	Breast cancer cells / C2C12 myoblasts	No direct function but transfer machinery for signal transduction	[147]
Proteins	Angiotensin-converting enzyme 2	Human Urine (chronic Kidney disease with transplanted kidney during Diabetic and non-diabetic	ACE activity and protein	[205]
	Leukotriene synthesis enzyme	Human macrophages and dendritic cells / granulocytes	Promote granulocyte migration	[206]
	Mitochondrial ATP Synthase	MSCs/ macrophages	Active enzyme providing enhanced bioenergetics, innate immunomodulatory activity of MSCs	[24]
	Casein kinase I	Bladder carcinoma cell / breast cancer	Metastasis and differential expression of EMT factors	[207]
	Annexin A2	Bladder carcinoma cell / breast cancer	Microenvironment for metastasis	[208]
Lipids	Lysophosphatidy-lcholine	Dendritic cells or RBL mast cells/NA	Maturation of DCs and lymphocyte chemotaxis via the G protein-coupled receptor	[36]
	Endocannabinoids	Microglia /Neuron	Synaptic function, inhibit presynaptic transmission	[209]

1.2.4 EVs in inflammation

EVs can modulate cellular functions and can activate cells in the local microenvironment by moving through the circulation. Under normal “healthy” physiological conditions, the status of EVs and their compositions are difficult to assess. Instead, EVs derived from altered cells have been linked with certain functions in the disease state of cells, mostly inflammatory diseases such as

asthma, allergies, cancer, and infections. Thus EVs have been implicated in the pathophysiology of a large number of diseases. The following section highlights some of the key features of EVs that dictate various inflammatory conditions.

EVs in the circulation are released from various types of cells (including pathogens and damaged cells) and are rapidly engulfed by phagocytic cells that are present in various organs, e.g., Kupffer cells (liver), DCs , and macrophages (lungs) [210, 211]. These cells either release cytokines or, produce immunomodulatory EVs loaded with antigens. Such EVs modulate the immune profile by interacting with other immune cells; for example, DCs and B-cells show enhanced EV production following interactions with T-cells [212, 213]. EV secretion from T-cells can be amplified because a single cell can release a large number of EVs with the T-cell receptor on their surface [214]. The cells that participate in both innate and adaptive immune regulation can release EVs, e.g., mast cells with the Fc receptors crosslinking with IgE or activation via calcium ionophores that induces EV release [215, 216]. The upregulation of EVs in the circulation or in body fluids is seen in hypoxia, cancer, and infection [217-219]. This suggest that EVs are capable of regulating immunological functions.

1.2.5 Antigen presentation via EVs

DCs, B-cells, and macrophages are antigen presenting cells (APCs) that modulate immunological functions by directly interacting with CD4+ and CD8+ T-cells and with natural killer (NK) cells and NK T-cells [220]. This interaction occurs through their membrane proteins such as MHC class I and II complexes and co-stimulatory molecules (B7, CD80, CD86, Programmed cell death protein 1, and ICAM). EVs derived from such cells harbor these molecules, giving them similar properties as the cells they originate from. B-lymphocytes secrete antigen-presenting vesicles with the MHC class II molecule and other co-stimulatory signals, making them suitable for use in vaccines, and this suggests that these EVs can maintain a disease-free state by potentially targeting tumor antigen [11]. Tumors in mice were suppressed when EVs derived from DCs (exposed with a tumor peptide) were injected into mice due to a cytotoxic T-cell response [12]. EVs derived from an array of APCs carry MHC class I and MHC class II complexes, and these can

potentially activate corresponding CD8+ or CD4+ T-cells [11, 221, 222]. A similar CD8+ T-cell response was observed in EVs derived from cells exposed to a viral peptide [223].

DCs (professional APCs) participate in “cross dressing”, which is the transfer of whole peptides associated with MHC class I complexes or MHC class II complexes from a peptide source cell to the cell surface of an acceptor APC or another type of recipient cell [224, 225]. The transferred peptide-MHC complexes are recognized by T-cells without the need for further antigen processing. Here, EVs from different sources that carry antigenic peptide–MHC complexes are presented to T-cells in the following ways. (i) Some of these EVs attached to the DC surface can be directly presented to T-cells with co-stimulation from the DCs, (ii) DCs can take up these EVs, process them, and present the antigen to recipient APCs, or (iii) autologous EVs are created that carry the antigen that is then presented to the T-cells. As an example this process is clearly demonstrated in allograft transplantation where cross-dressing occurs between host APCs and EVs containing donor MHC class II [226, 227].

EV-associated tumor antigens, including HER-2/neu, EGFR2, CEA, MART-1, gp100, TRP-1, mesothelin, and HSP 70 and HSP 90, are seen in many body fluids [228-231]. Melanoma patient-derived EVs contain the MART-1 antigen, and when these EVs are presented to DCs they generate cytotoxic T lymphocytes specific to the MART-1 antigen [228]. Additionally, the status of tumor cells under stress or damaging conditions is often reflected in EVs with a high immunogenic potential [232]. The immunomodulation of these tumor-derived EVs can be regulated by the reduction of the cytotoxic capacity of NK cells by regulating NKG2D, which is required for DC generation [233].
During infection, pathogens not only damage cells at the site of infection, but also produce EVs that carry antigens. This also could be a way by which the infected cell relays the danger signal to the immune cells. This has been demonstrated with *Plasmodium yoelii* in reticulocytes, *Mycobacterium bovis bacillus* in macrophages, *Toxoplasma gondii* in DCs, human immunodeficiency virus in T-cells, and cytomegalovirus in endothelial cells [234-237]. However, certain infections, especially parasitic infections, generate an EV dependent response that dampens or activates the immune system in a stage-specific manner [238, 239]. Macrophages

infected/phagocytized with microbes generate EVs with antigen with pathogen-associated molecular pattern with the capacity to activate Toll-like receptor in inflammation [240]. Overall, this indicates the capacity of immune cell-derived EVs to present antigen and produce immunomodulatory effects.

1.2.6 Regulation of immune response by mast cells and mast cell-derived EVs

Mast cells derive from hematopoietic stem cells that circulate as immature progenitor cells and mature after they target a site [241]. They are located throughout the vascular system, specifically near the lining of the epithelium in the airway, gut, and skin [241]. Because of their proximity to the external environment, mast cells can respond to pathogens through their constant surveillance of any danger signal released from damaged cells (e.g., alarmins or metabolites) or by the pathogen itself. As a response, they release an array of cytokines, chemokines, and lipid mediators that recruit immune effector cells. To perform this function, mast cells have a large repertoire of receptors that allows them to respond to a wide range of stimuli [242]. Activation of mast cells can occur in an IgE-dependent or IgE-independent manner. IgE-dependent activation requires the binding of IgE antibodies from B cells to the FcεRI receptor on mast cells [242]. However, they can also be activated by other receptors for IgA, IgG, adenosine, C3a, chemokines, cytokines, and pathogen-associated molecular patterns, as well as toll-like receptors. Activation of mast cells is typically related to the release of the contents of secretory granules into the extracellular space (degranulation). However, it is important to highlight that not all activation signals cause degranulation, e.g., toll-like receptor (TLR-1/4/5/6) activation does not induce degranulation, but their activation results in the secretion of cytokines, chemokines, cysteinyl-leukotrienes, and other eicosanoids [243, 244]. As an example, non-IgE-dependent activation has been reported to cause bronchoconstriction in asthma, which is associated with vascular permeability, inflammation, and tissue remodeling [245, 246]. It is important to note that along with growth factors and cytokines, mast cells also synthesize and release negatively charged heparin or chondroitin sulfate proteoglycans [247-249]. These proteoglycans have a high affinity for growth factors and act as sponges or reservoirs [250, 251].

Mast cells and their secretory components interact with different cells involved in innate and adaptive immunity. As a part of the innate immune system, they interact with DCs, NK-cells, eosinophils, neutrophils, and myeloid-derived suppressor cells [252-256], and as part of the adaptive immune system they interact with T-cells, inducible regulatory T-cells, and B-cells [257-260]. Not all interactions between mast cells and cells of the adaptive immune system require physical interaction. Tkaczyk et al. showed for the first time that unstimulated mouse bone marrow mast cells (BMMCs) were able to induce the proliferation of resting B-cells and to stimulate IgM production [261]. This indicated that some factor from mast cells was released and transferred a signal in a contact-independent manner [261, 262]. Studies have also shown that mast cells release exosomes that drive these functions because they are decorated with co-stimulatory molecules such as MHC-II, CD86, CD40, CD40L, LFA-1, and ICAM-1 [263]. BMMC-derived exosomes have also been shown to enhance the differentiation of naive $CD4^+$ T-cells into Th2 cells [264]. Mast cells produce EVs with different compositions depending on whether the mast cells are in their activated or non-activated states [263, 264]. A recent study showed that BMMC-derived exosomes bind to IgE via FcεRI and inhibit the activation of mast cells by quenching away free IgE [265]. Thus exosomes act as suppressors of IgE mediated immune activation. The studies discussed above clarified two important aspects of mast cells in the regulation of immune function. i) Mast cell degranulation is not essential for their adaptive immune function, and ii) mast cells release EVs in the resting/non-stimulated state. These findings are central to the methodologies that we have used in this thesis where we have used EV that were derived from non-stimulated mast cells.

1.2.7 Epithelial-to-mesenchymal transition

Many features of EVs from various cells are involved in inducing cellular migration and the invasiveness of various cells [266, 267]. EV-associated cargoes such as proteins, [178, 196, 268, 269] and RNA [270, 271] are known to induce EMT. The following section will describe the general steps involved in EMT.

EMT occurs through a series of cellular events that result in the trans-differentiation of epithelial cells into mesenchymal cells with migratory features. EMT is a fundamental feature of cellular behavior and is essential for

development, wound healing, and stem cell function [272, 273]. EMT also contributes to epithelial damage, fibrosis, and cancer progression [273-275]. Cells that undergo EMT lose their cell-to-cell junctions (by reduction of claudin and occludin) and apical-basal polarity and reorganize their cytoskeleton, and this causes cells to detach from their neighboring cells [276-279]. Increasing expression of MMPs (MMP-2, MMP-3, and MMP-9) alters the cellular attachment to the ECM [280]. Additionally, protease activity in the ECM can release stored growth factors like TGFβ-1 [281] .

EMT induces signaling that affects the gene expression of EMT-regulating proteins. The hallmark of EMT is characterized by the "cadherin switch" where downregulation of E-cadherin (E-CAD) and the upregulation of mesenchymal neural cadherin (N-cadherin) is frequently observed [272, 282, 283]. Activation of mesenchymal regulatory transcription factors like SNAIL, TWIST, and ZEB have been shown to be critical for the generation of proteins that drive EMT [284-287].

Among EMT regulators, the TGFβ family is well described in the literature. TGFβ-1 is involved in fibrosis of the airway, liver, and heart [288-290]. TGFβ-1 can activate the TGF-pathway via SMAD and non-SMAD signaling. In SMAD-dependent signaling, the TGFβ family of proteins interacts with type-II TGFβ receptors that phosphorylate and thereby activate type I transmembrane kinases, and these kinases in turn phosphorylate effector SMADs (SMAD2/SMAD3/SMAD4) [291]. Another member, bone morphogenetic proteins (BMP), can activate different SMADs (SMAD1, SMAD5, and SMAD4) [291-293]. Non-SMAD pathways that are also initiated by TGFβ-1 include, PI3K, and MAPK pathway proteins [293, 294] which also contribute to EMT [295, 296]. Taken together, it is clear that TGFβ-1 can engage in activating multiple signaling cascades that regulate the events in the EMT process. The presence of EVs in the matrix presents an opportunity to dissect the regulation induced by the growth factors (e.g. TGFβ-1) present on the EV surface. The evidence of for EV-associated TGFβ-1 supports the EMT regulation seen in cancer cells.

2 AIM

Broadly, the aim of this work was to determine the function of mast cell-derived EVs in transferring protein and DNA cargo to recipient cells and to determine the topological orientation of the cargo in the EVs as well as their biological effects on recipient cells.

Specific Aims:

- To establish a protocol for eliminating EVs from fetal bovine serum (**Paper I**).
- To determine the protein cargo and the protein topology in human mast cell-derived EVs (**Paper II**).
- To determine the biological functions of the protein cargo in EVs on human MSCs (**Paper II**) and epithelial cells (**Paper III**).
- To determine the location of the DNA cargo in EVs (**Paper IV, V**).
- To determine the characteristics of the EV-associated DNA cargo and its capability to induce signaling in innate immune cells (**Paper V**).

3 EXPERIMENTAL STRATEGIES AND CONSIDERATIONS

This section provides a more general description of the experimental approaches that were taken and discusses the possible limitations of the studies included in the thesis. A detailed step-by-step description of the methods can be found in the "Materials and Methods" sections of each of the attached papers listed in the Appendix.

3.1 Isolation strategy for EVs (Papers I–V)

EVs released by cells into the extracellular milieu are a heterogeneous population of membrane-bound structures. EV heterogeneity can occur for multiple reasons. First, multiple cell types can release EVs into the microenvironment or the circulating body fluids and thus contribute to "systemic heterogeneity". Second, EV heterogeneity occurs because the sources of membranes from which EVs are derived can be "inherently heterogeneous". This heterogeneity gives rise to "exosomes" that are formed from the fusion of MVBs to the cytoplasmic side of the cell surface, while those that are formed from the outward membrane budding of the cell surface are referred to as MVs.

In order to eliminate "systemic heterogeneity," we used primary cells and cell lines based on in vitro cell culture systems to obtain EVs from mono-cultures. "Inherent heterogeneity" was eliminated by the use of differential low-speed centrifugation based on the pelleting of larger structures such as ABs, MVs, and other cell debris. The remaining supernatant was then used to isolate exosomes enriched in EVs pelleted by ultracentrifugation.

3.2 EV purification strategies (Papers II, IV, V)

Following ultracentrifugation, the exosome-enriched EV pellet was enriched in not only exosomes, but also in free proteins because of the similar sedimentation rates of some free proteins and exosomes. In order to remove the co-isolated proteins, we used an iodixanol-sucrose density gradient centrifugation method to float exosome-enriched EVs at their flotation density.

The isolation of EVs by this method was possible because of the lipids in the membrane of EVs, which are a defining feature of EVs but are absent in free proteins.

3.3 Tracking EV uptake in recipient cells by physical separation of endosomes and lysosomes (Paper II)

The cellular uptake of EVs is an active process and can occur either by direct membrane-to-membrane fusion on the cell surface or by the endocytosis of EVs, which are then targeted to early or late endosomal or lysosomal compartments. To determine the physical location of EVs within the cells, we first labeled the surface proteins of EVs with biotin and incubated them with recipient cells (MSCs and HEK-293 cells). These recipient cells were then lysed, and the cytoplasmic contents, including various organelles, were separated during the first round of ultracentrifugation followed by iodixanol-sucrose density gradient fractionation. The crude fraction enriched in lysosomes and endosomes were further purified during a second round of iodixanol-sucrose density gradient fractionation. With these sequential centrifugation steps, we enriched the lysosomal and early endosomal compartments. We finally probed the EVs using the streptavidin-based detection of biotinylated proteins. This approach helped us probe the EVs and to determine which organelles they targeted after they are taken up by recipient cells. To our knowledge, this is the first physical method developed to isolate organelles to trace EV in recipient cells.

3.4 Determining whether EVs contain the active or inactive form of TGFβ-1 (Paper II)

Most immune cells release TGFβ-1 into the extracellular milieu. However, the majority of this secreted form is inactive TGFβ-1. The physiological activation of inactive TGFβ-1 requires proteolysis or acid hydrolysis that converts inactive TGFβ-1 into its active form, which is then able to bind to and activate surface TGFβ receptors for downstream signaling. In order to determine how much TGFβ-1 is active or inactive in our EV preparations, we performed a

sandwich enzyme linked immune-sorbent assay (ELISA) using hydrochloric acid-based activation of inactive/latent TGFβ-1 to detect total TGFβ-1, and the sample without treatment will yield active TGFβ-1.Using these values we could calculate the proportion of active and inactive TGFβ-1 in the EV preparation.

3.5 EV surface-associated TGFβ-1 (Paper II)

The isolation of EVs by differential ultracentrifugation-based methods is a commonly used approach to concentrate EVs. Interestingly, EVs that are enriched in these preparations often include some free proteins that are also co-pelleted. Because we were interested in identifying the true protein cargo on the surface of EVs, we used different strategies to determine the validity of our findings. We used the following approaches to avoid possible contaminating protein in our EV preparation:

3.5.1 Membrane proteomics to determine true EV membrane proteins

The common feature of EVs is the presence of a lipid membrane. Therefore, proteins that are anchored on or are associated with lipid membranes would most likely be true EV proteins. We used a stringent membrane proteomics method to identify true EV proteins. In these steps, EVs isolated after pelleting were treated with high salt and low-pH conditions to remove weakly associated non-EV proteins. This approach allowed us to identify the true EV-associated proteins that would otherwise have been masked by contaminating proteins.

3.5.2 Pellet-free EVs isolation to reduce damage induced by ultracentrifugation

Centrifugation-based pelleting of EVs on the wall of an ultracentrifuge tube can damage the EVs and release or exposed the luminal TGFβ-1. In order to rule out the damage caused by centrifugation, we employed an iodixanol-sucrose density cushion method directly on the precleared-supernatant to collect EVs directly at the interphase of the 10% and 30% layers. These interphase samples were then loaded onto the bottom of an iodixanol-sucrose density gradient and floated to obtain pellet-free EVs. These samples were then

analyzed for their active and total TGFβ-1 contents. Preclearing of the supernatant prior to use was performed by low speed centrifugation that removed ABs and MVs.

3.5.3 Enzymatic treatments: Trypsin and Heparinase-ii

The presence of proteins on the surface of EVs can easily be demonstrated by the enzymatic digestion of EVs with either trypsin or proteinase K. We digested EVs with heparinase-II to remove the heparan sulfate proteoglycan (HSPG)-based association of TGFβ-1 on the surface and subsequently determined the interaction of TGFβ-1 with the sugars present on EV-associated proteins.

3.6 High-resolution separation of EVs from free DNA (Papers IV and V)

EVs can have a variable density (high density and low density) that causes them to co-float with other free DNAs in the iodixanol-sucrose density gradient. We, therefore used a strategy to separate them on a gradient by multiple layering (1 ml) of various percentages of iodixanol-sucrose (20, 22, 22, 24, 26, 28, 30, 35, 50%).

4 RESULTS AND DISCUSSION

4.1 Fetal bovine serum contains biologically active EVs (Paper I)

To understand the biology of EV-mediated cellular communication, in vitro cell-derived conditioned medium is often used. This medium is regularly supplemented with 10–20% fetal bovine serum (FBS) for cellular maintenance. However, FBS contains large amounts of EVs and micro particles. Larger-size particles, including vesicles larger than 100 nm, are removed by filtration during the commercial preparation of FBS, but many vesicles smaller than 100 nm are retained in the FBS. To determine the function of cell-derived EVs, careful removal of FBS-derived EVs and their associated cargo is essential to avoid false-positive signals.

To remove EVs, we ultracentrifuged the FBS for 18 h, and the supernatant was then filtered (0.2 μm pore size) before using it as a supplement for the culture medium. We observed a significant decrease in the migration of epithelial cells in an in vitro cell migration assay in medium where the FBS was EV-depleted (**Paper I**, Fig 1). This indicated that ultracentrifugation of the FBS removed the components that are essential for cell migration, which includes EVs. To evaluate the EVs that are present in FBS, we washed the FBS-EV pellets with PBS and used electron microscopy for structural evaluation and western blotting analysis for protein determination, e.g., TSG101, CD81, and CD63. Interestingly the antibodies had cross-reactivity with bovine TSG101, CD81, and CD63 that have approximately 99%, 95%, and 89% sequence similarity with humans, respectively (**Paper I**, Fig 2a, 2b). We did not want to highlight the cross-reactivity, but the fact is that the similarity in proteins can have biological effects that will remain unnoticed if not removed prior to any functional assays. Moreover, FBS-EVs were readily taken up by epithelial cells and induced a dose-dependent migratory response, similar to what is seen for many cell-derived EVs.

4.2 FBS contains EV-associated RNA (Paper I)

EV-associated RNA is one of the most studied areas in EV research, and RNAs such as lncRNA, miRNA, and mRNA have been shown to affect cellular functions [200, 297]. When we started this study, many researchers were using an FBS EV-depletion protocol with 1.5 hours of ultracentrifugation. Therefore, we based our evaluation by measuring the removal of RNA from FBS after the different protocols used for EV-depletion in the research community. The traces of nucleotides found in FBS-EV preparations were resistant to RNase and proteinase-K. However, treatment with Triton-X100 made the EV-RNA partially sensitive to RNase activity (**Unpublished data.** Fig 4a). This indicates that the RNA present in FBS-derived EVs is protected. Recently, cell culture-based studies have reported the presence of RNA in cell-derived EVs could be contributed by EV-associated RNA from FBS, indicating the need for

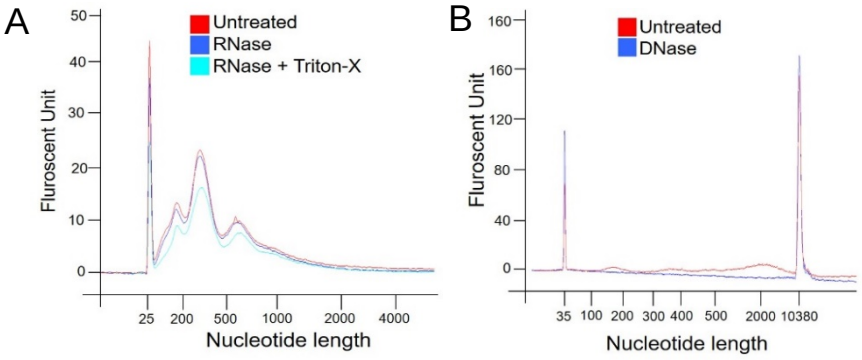

Figure 4. *Nucleotide profiling of FBS-derived EVs. A) Presence of RNA determined by using a pico-gram sensitivity RNA chip using and a Bioanalyzer system. B) Presence of DNA determined by using a high sensitivity DNA chip and using a Bioanalyzer system.*

EV- RNA depletion [298]. In our analysis, we found that prolonged (18 hours) ultracentrifugation of FBS reduces the amount of RNase-insensitive nucleotides from FBS (**Paper I,** Fig 4a, 4b). Surprisingly, this does not guarantee that all EVs are removed because we observed small EVs (30–60 nm) in the FBS subjected to EV depletion (**Paper I,** Fig 4c).

When this work was published, very little was known about EV-associated DNA. However, our recent data suggest the presence of longer segments of bovine DNA in FBS-EV preparations that were obtained by ultracentrifugation followed by density-based flotation (**unpublished data.** Fig 4a). However, EV-associated DNA was not protected and was completely degraded with DNase, suggesting that it is associated with the surface of EVs or associated proteins (**unpublished data.** Fig 4a).

In summary, caution must be exercised while working with cell culture systems in which FBS is used as a growth supplement. Apart from various factors in FBS, the EVs and their associated cargo that are found in FBS can regulate cellular function [299, 300]. Conserved proteins and RNA (and possibly DNA) share sequence similarity between humans and bovines and this must be considered when studying biological functions or when performing sequencing or proteomics studies. Currently, there are few techniques for depleting EVs from FBS. Methods like the use of ultrafiltration [301], activated charcoal, or adapting cells to serum-free conditions can help to reduce FBS-EV contamination. However, these methods are either expensive or cannot be used for primary cell cultures. For now, prolonged ultracentrifugation appears to be the optimal protocol for removing EV-associated RNA from FBS.

4.3 Mast cell-derived EVs carry TGFβ-1 associated with heparan sulfate proteoglycans (Paper II)

EVs from immune cells, including mast cells, participate in the regulation of immune responses [302, 303]. Mast cells can act as a component of both the innate and the adaptive arms of the immune system [304], and they harbor characteristic secretory granules along with a large number of ILVs in MVBs [305]. **Paper II** demonstrated the presence of EVs secreted from both primary human mast cells as well as a human mast cell line, and electron microscopy showed nano-sized vesicles (**unpublished data,** Fig. 5) and FACS analysis for the presence of tetraspanins such as CD81, CD63, and CD9 (**Paper II, supplementary** Fig. 1).

EVs harbor multiple proteins with potentially multiple functions, including immunomodulatory functions. Interestingly, if we look at the membrane protein composition of mast cell-derived EVs, we can see, in addition to exosome proteins, the contribution of proteins from other organelles such as the mitochondria and Golgi bodies (**unpublished data** Fig. 6) [306, 307]. Indeed, membrane protein contributions can either be due to membranes from dying cells or from the exchange of intercellular membrane compartments [308-310]. Many organelle-associated proteins have been reported in studies on EVs, and some of these include CAV1 and mitochondrial proteins [26, 311, 312]. EV proteomics databases are enriched in organelle-derived proteins, which are often overlooked because they could be isolation artifacts of in vitro cell cultures. They cannot be explained by conventional MVB-derived exosomes

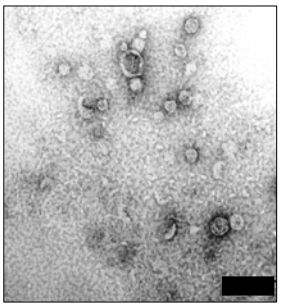

Figure 5*: Electron micrograph showing EVs derived from primary human mast cells. Bar = 0.2 μm*

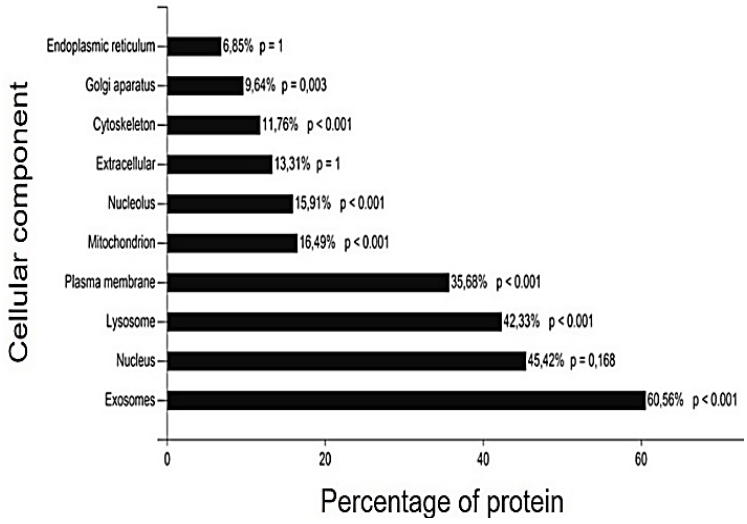

Figure 6*: Analysis of the Gene Ontology terms under cellular component that is most associated with the mast cell-derived EV membrane proteome. Chart represent the top 10 organelles associated with the proteins found in the EVs. Analysis was made by Funrich software.*

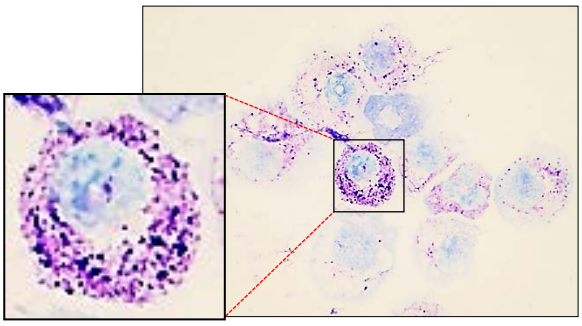

Figure 7: *Primary mast cells from mouse bone marrow stained with toluidine blue staining and image in 40x resolution.*

or surface membrane budding. Other studies from our group have identified traces of mitochondrial inner-leaflet protein (MTCO2) along with exosome markers [26]. This strongly suggests a process of membrane exchange between MVBs and other membrane compartments. EV-associated proteins commonly include various HSPG and HSPG-like proteins (e.g., syndecan and glypican) that play an important role in EV biogenesis [158, 313]. Interestingly, in humans a large proportion of secretory granules are produced by mast cells [314]. Indeed, while staining the cytospin slides of mast cells with toluidine blue dye, we observed purple-stained vesicles, which are typical of negatively charged heparan and chondroitin sulfate proteoglycans (**unpublished data** Fig. 7). Additionally, when we investigated the core membrane proteins of EVs, approximately 33% of the total membrane proteins had glycan binding sites (**unpublished data** Fig. 8). HSPGs are known to associate with various growth factors such as TGFβ-1, EGF, and VEGF. TGFβ family is a growth factor known to regulate multiple immune and wound healing functions in both disease states and in cellular homeostasis [315, 316]. In **Paper II**, EVs derived from human mast cells were validated for the presence of TGFβ-1 (**Paper II** Fig 3). We found an association between TGFβ-1 and EV markers (CD63, flotillin-1) using various approaches such as fluorescent correlation spectroscopy, ELISA, and immune-capturing. Interestingly, the TGFβ-1 identified on EVs was in both the active and inactive form. The co-localization of EVs and TGFβ-1 might be a non-specific interaction due to high-speed centrifugation. To rule out this possible interaction, we used a method of EV isolation that did not involve pellet formation (**Paper II, supplementary** Fig. 5). Instead, the cell culture media was directly loaded onto a density gradient. The EVs isolated in this way still showed the presence of TGFβ-1, indicating that the presence of TGF-β in/on EVs was not an artifact of the sample preparation.

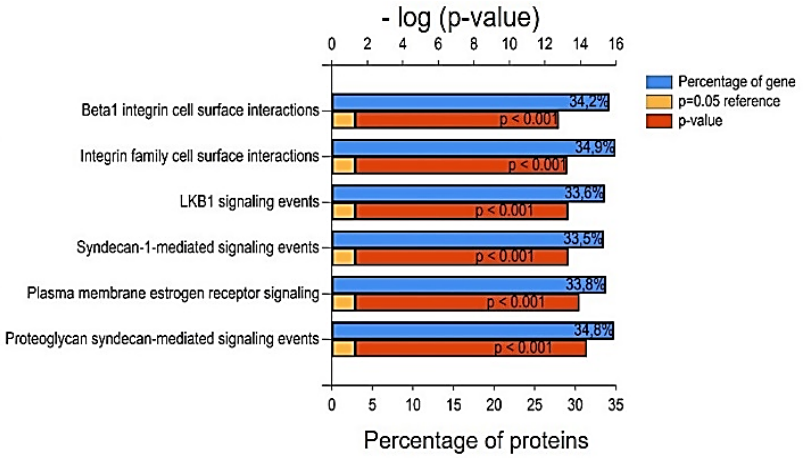

***Figure 8**: Analysis of the Gene Ontology terms under biological processes that is most associated with the mast cell-derived EV membrane proteomes. The top six biological processes and percentage of genes involved in those biological processes are shown. Analysis was done using Funrich software.*

Binding of TGFβ-1 to HSPG is well studied in the literature [317]. Interestingly, we observed that the presence of TGFβ-1 on the surface of exosomes was bound via surface HSPG. When we blocked the biosynthesis of glycan chains using p-Nitrophenyl-beta-D-xylopyranoside, an inhibitor of the enzymes essential for the addition of glycans to core glycoproteins, we observed a significant decrease in the association of the latent/inactive form of TGFβ-1 (**Paper II,** Fig. 6C-E). However, we do not know whether the association of TGFβ-1 with the glycan chain of HSPG occurs inside the cell during organelle fusion (MVBs and Golgi vesicles) or after the EVs are released into the extracellular milieu. Moreover, Golgi vesicles in cells contain inactive forms of TGFβ-1 that we also found in EV preparation [306, 307]. It is documented that a constant exchange of membranes occurs between MVBs and other vesicles compartment; however, in our case further studies are required to elucidate the exact site of TGFβ-1 loading to EV.

4.4 Mast cell-derived EVs induce migratory and immunosuppressive phenotypes in human MSCs (Paper II)

During inflammation, human MSCs have been reported to provide tissue repair and immunosuppressive functions; therefore, they have been adopted for various therapeutic applications [318]. However, pre-conditioning methods are often used to enhance MSC function, e.g., pretreatment with cytokines, small molecule drugs, and various growth factors [319]. The properties of mast cell-derived EVs with regards to their membrane composition and their role in immune regulation led us to hypothesize that the EVs from mast cells can enhance the immunomodulatory functions of MSCs. To test this, we utilized well-established OVA-induced allergic inflammatory models that are characterized by increased numbers of eosinophils and reduction in their number would be sign of immunosuppression [320]. In vivo imaging of MSCs (expressing Luciferase–GFP) injected into the tail vein showed a higher degree of MSC retention in the lungs when the MSCs had been pre-conditioned with EVs compared to untreated MSCs (**Paper II,** Fig. 1B). After 72 hours, traces of GFP in the lung parenchyma were seen along with reduced numbers of eosinophils in the broncho-alveolar lavage, indicating reduced inflammation (**Paper II,** Fig.1C-D).

4.5 Mast cell-derived EVs co-localize with the endosomal compartment during TGF signaling (Paper II)

Mast cell-derived EVs have multiple cargoes that can simultaneously regulate an array of pathways in recipient MSCs and provide features like immunosuppression and retention in the lung. However, we focused on EV-associated TGFβ-1 for the following reasons: (i) TGFβ-1 has been identified in our mast cells-derived EV-proteomics, (ii) it is known to be associated with HSPG, and (iii) it plays a role in immunomodulation and regulation of cell migration (**Paper II**, Fig 1D, 2). We found that the majority (>80%) of the TGFβ-1 isolated in the EV preparation (from HMC-1, mast cell line) was found in the floating fractions, indicating that the majority of the TGFβ-1 in the

ultracentrifugation pellet was EV-associated (**Paper II**, Fig 3A), and thus we used the EV pellet for further functional studies. In vitro evaluation of the migratory phenotype of MSCs treated with primary mast cell-derived EVs showed enhanced migration and wound healing response towards a pan-chemo attractant (i.e. FBS). TGFβ-1 in its active form binds to the TGFβ-receptor and rapidly activates the downstream signaling pathway. Accordingly, EVs were able to activate phosphorylation of SMAD in MSCs, which is a surrogate of TGFβ-signaling (**Paper II**, Fig 4). Blocking experiments, either with a TGFβ-1 blocking antibody, CRISPR-cas9 knockout of TGFβ-1 in EV-producing mast cells, or TGFβ-receptor 1 blocking (with LY215799), led to downregulation of the activation of pSMAD and to reduced migration of recipient MSCs. This clearly suggested the participation of TGFβ-1–induced signaling. However, this signal activation by EVs was initiated after 60 min of exposure (**Paper II**, Fig 5A), which was in contrast to free TGFβ-1 where the signal activation was seen already at before 30 min. During this time we traced biotinylated-EVs, and found the presence of EVs in the endosomal-enriched fraction (EEA1 positive) after 60 min of EV-exposure as compared to the lysosomal compartment (LAMP1 positive) (**Paper II**, Fig 5C,5D, Supplementary Fig 10).

The pathway of TGFβ receptor internalization is rapid and is not always dependent on its attachment to the ligand (TGFβ-1) [321]. In addition, this pathway is shared by EV uptake [322, 323] and is targeted to acidifying compartments. Moreover, the presence of TGFβ receptor in endosomes is well documented [324]. Previously, we found that the TGFβ-1 that is associated with EVs exists in both active and latent/inactive forms. The latent form of TGFβ-1 needs acid or proteases from the microenvironment for its activation [325-327]. Presently, it appears that this latent form of TGFβ-1 on the surface of EVs might be activated in the acidifying microenvironments of the endosomal compartments, where TGFβ-1 bind to ligand-free TGFBR followed by phosphorylation of SMAD. However, more experiments are needed to test this hypothesis.

We then compared the differences in signaling efficiency between free TGFβ-1 and EV-associated TGFβ-1. Surprisingly, EV-associated TGFβ-1 after 60 min was able to induce more phosphorylation of SMAD2 than a similar concentration of free TGFβ-1 (**Paper II**, Fig 5A). This suggested that EV-associated TGFβ-1 acts differently compared with free TGFβ-1. EVs are

known to be taken up by cells in their endosomal compartment that gradually undergoes acidification. In our organelle-based EV-tracking experiments, at 60 min we saw the presence EVs in the EEA1-positive compartment (endosomes). The presence of inactive forms of TGFβ-1 on EVs after their uptake into acidifying endosomal compartments suggests a possible way of activating of TGFβ-1–induced signaling.

In summary, TGFβ-1 is associated with the surface of mast cell-derived EVs and activates human MSCs by regulating signaling events that determine the migratory phenotype. The association of the inactive form of TGFβ-1 with the EV surface via HSPG presents a potential new layer of TGFβ-1 regulation that could be mediated by endosomal acidification and signaling. However, more studies are needed to confirm these findings.

4.6 Mast cell-derived EVs enhance EMT in epithelial cells (Paper III)

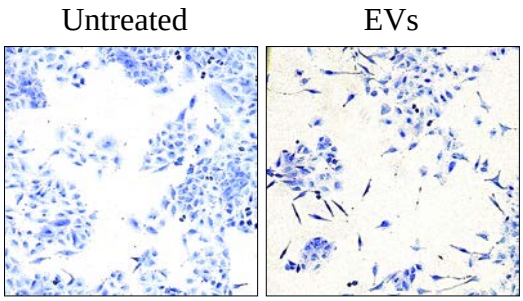

Figure 9: *Morphology of epithelial cells (A549) after EV treatment visualized by using Coomassie Brilliant Blue staining of cellular protein.*

Based on our current understanding of mast cell-derived EVs (and their associated cargo), it is clear that EVs can affect various downstream events that decide the fate of cells. Physiologically, the highest density of mast cells reported to date is found in the lungs and airways, and these cells can affect other cells in close proximity, such as epithelial cells, through secretory factors, including EVs. In **Paper III**, we sought to determine the effect of mast cell-derived EVs on airway epithelial cells (A549 cells). In our study, we observed the uptake of mast cell-derived EVs by airway epithelial cells (**Paper III**, Fig 1A), which induced morphological changes such as an elongated cell morphology (**Unpublished data**, Fig 9). EVs from various cell types have been shown to induce EMT, with characteristics

including morphological changes and a migratory/invasive phenotype. The EMT process is upregulated in various inflammatory conditions such as chronic obstructive pulmonary disease, lung repair, fibrosis, and cancers [266, 267]. To determine this we performed transcript analysis of genes that are commonly reported during EMT in epithelial cells upon EV exposure. A significant change in the transcript level was seen for TWIST1, MMP9, WNT5A, TGFB1, and BMP7 (**Paper III**, Fig 2). Activation of EMT was further confirmed by the differential expression of protein markers such as MMP, Snail, NCAD, and ECAD (**Paper III**, Fig 3A). Upon EV exposure, the epithelial cells secreted active matrix metalloproteinases that are required to degrade the matrix and facilitate migration (**Paper III**, Fig 3C-D) [109]. Currently, we do not know the mechanism underlying EMT; however, we speculate on the involvement of multiple signaling components present in EVs. Taken together, mast cell-derived EVs activate signaling events that induce the EMT phenotype.

4.7 Mast cell-derived EVs induce rapid phosphorylation of multiple EMT-regulating proteins (Paper III)

The steps involved in EMT are complex and can be initiated by multiple regulators in the cellular microenvironment. As described in **Paper II**, the protein cargo within mast cell-derived EVs can be used to study the activation of signaling in recipient epithelial cells. When a ligand binds to the corresponding receptor on the cell surface, it activates the receptor and associated proteins by phosphorylation. Protein phosphorylation at serines and threonines is the most common post-translational (PTM) modification that modulates the signaling events that eventually decide cellular fate. We cataloged the protein phosphorylation in epithelial cells after 30 min of exposure to EVs using an antibody microarray (**Paper III**, supplementary Table 1). The analysis of the data revealed differentially regulated phosphoproteins, and a literature review of these proteins revealed that the EV-treated group were directly or indirectly implicated in EMT (e.g., TGM2, MMP9, CADH1 and VCAM-1) (**Paper III**, Fig 4B). Interestingly, the phosphoprotein component of the PI3K-AKT pathway was highly activated in response to EV exposure (**Paper III**, supplementary Fig 2). This result was in

line with our previous findings regarding the phosphorylation of AKT in epithelial cells [177]. Taken together we found that epithelial cells responds rapidly to EV but inducing protein phosphorylation.
In **Paper II**, TGFβ-1 was found to be an essential component of EVs. In **Paper III** we shown that EV was able to induce EMT in epithelial A549 cells. Therefore, when we looked at the activation status of TGFβ-signaling in epithelial A549 cells, as well as bronchial epithelial BEAS-2B cells (data not shown), after EV stimulation, we observed the rapid activation of SMAD2, which is an indicator of TGFβ-signaling (**Paper III**, Fig 5A). Additionally, EV-treated A549 cells produced more TGFβ-1 in the supernatant (**Paper III**, Fig 5C). Therefore, EV stimulation can activate the cell to increase the production and release of endogenous TGFβ-1. Large numbers of studies have clearly demonstrated that free TGFβ-1 activates the EMT cascade [328, 329]. However, at this stage it is not clear whether EV-associated TGFβ-1 is in any way involved directly in activating the EMT cascade or it can also be regulated by other EV-associated cargo.

In summary, this study highlights the capacity of mast cell-derived EVs to induce EMT in epithelial cells, and protein phosphorylation data suggest the involvement of multiple regulatory pathways. Collectively, this information on EV-associated cargo and the signaling events in recipient cells will be valuable in identifying the mechanism underlying EMT in epithelial cells.

4.8 EVs carry DNA on their surface (Papers IV, V)

This section focuses on ex-DNA, an EV cargo that is not well described in the literature. DNA is reportedly the most stable biomolecule, and its presence was reported in ABs [138]. However, recent studies have suggested that ex-DNA is also produced by non-apoptotic cells, and thus the characterization of these molecules is necessary in order to understand their biological roles and diagnostic potential [143]. Nevertheless, the possibility of the association of DNA on the surface of EVs was not considered until recently.

In **Paper IV,** we describe, for the first time, the presence of DNA on the surface of mast cell-derived EVs. We collected EVs by ultracentrifugation followed by an iodixanol-sucrose density gradient and found the presence of

DNA in floating layers. The phosphate backbone of the DNA imparts a net negative charge to the EVs (**Paper IV**, Fig B). Treating the EV samples with DNase reduced the aggregation of the EVs and we observed an increase in particle number. This observation was common in other EV samples obtained from cell-lines and FBS (**unpublished data,** Fig10). It is well known that liposomes mixed with DNA are effective agents for transfection and are readily taken up by cells [330]. Similarly, upon incubation, EVs (with associated DNA) were taken up by recipient MSCs, and were found in the cytoplasm as DNA-foci (**Paper IV**, Fig D, E). Currently, it is unclear whether the mechanism of DNA uptake is dependent on EVs. However, this study suggested a novel mechanism for EV-assisted DNA uptake and presented a possible mechanism by which cell-free DNA can move within body fluids.

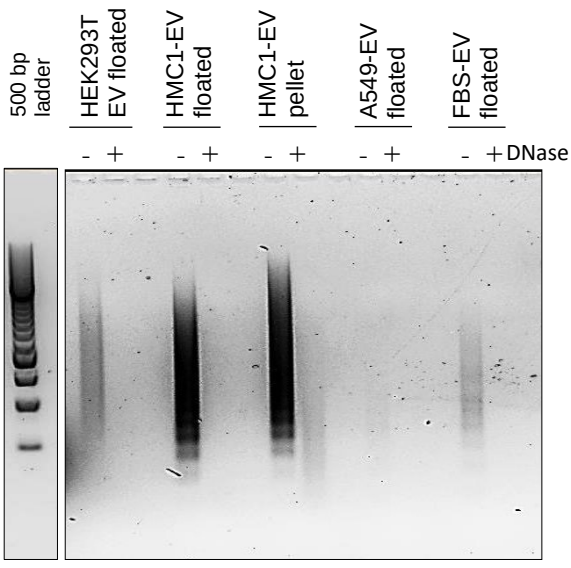

Figure 10: *DNA isolated from different EV sources was separated using agarose gel electrophoresis and visualized using GelStar nucleic acid stain. EVs obtained by ultracentrifugation are referred to as the EV pellet, and upon purification using an iodixanol density gradient are referred to as EV-floatation.*

To understand the distribution of EV-associated DNA in regards to whether it is present on the inside or outside of EVs, we analyzed the DNA as well as RNA present in all fractions of the density gradient (**Paper IV**, 2). In **Paper V**, we described a high-resolution iodixanol-sucrose density gradient used to separate EVs (**Paper IV**, Fig 1A). We extended this work to include EVs derived from erythroid-leukemia cells (TF-1 cells) in addition to the mast cell

line (HMC-1 cells) used previously. The distribution of two protein markers (Flotillin-1 and CD81) across the gradient revealed the presence of EVs in two fractions (fractions 1–3: low-density EVs, and fractions 5–7: high-density EVs) (**Paper V**, Fig 1B). In both fractions, the majority of the DNA was found to be associated with the EV surface (~85%) with little DNA being protected on the inside of the EVs (**Paper V**, Fig 4). To determine the source of the DNA, we performed a DNA sequencing analysis. Both mitochondrial and genomic DNA were detected in the EVs (**Paper V**, Fig 5A-B). Interestingly, the sequencing reads suggested that the DNA molecules were found both inside and outside of EVs (**Paper V**, Fig 5C). We obtained higher sequence coverage of DNA in the DNase-treated EVs (the DNA was protected on the inside of EVs). Coverage of the whole genome has been reported in EV-associated DNA [143], and deep sequencing only on DNase-treated EVs was used in previous analysis [144]. Some researchers used DNase-treated EVs followed by genomic amplification and PCR-based methods to detect EV-associated DNA [142]. Nevertheless, with these approaches, the identity of surface-associated DNA remained undetermined. Our study highlighted this issue and described the localization of extracellular DNA by using multilayer iodixanol density gradients and DNase treatment with deep sequencing analysis of both the protected DNA and the total DNA.

4.9 Surface-associated DNA on EVs induces innate immune signaling (Paper V)

Although the source of the DNA remains unclear, a few recent studies have indicated that the presence of DNA is reflected by its source and by the cell state [149, 150]. Studies from Edit Buzas's group indicated that the association of DNA with EVs was due to the presence of fibronectin on the surface of the EVs [149]. Interestingly, fibronectin is known to interact with HSPG, and in **Paper II** we showed that mast cell-derived EVs harbor many proteoglycans on their surface [149]. Although not confirmed, this could to be a potential mechanism of DNA-EV interaction. Alternatively, this association might be explained by the presence of histones and other DNA-binding proteins on EVs, as identified by proteomic analysis for both high and low-density EV fractions (**Paper V**, Fig 3).

The presence of DNA on EVs and the uptake of EVs by cells into the cytoplasm makes this DNA available as a danger-associated molecular pattern [331, 332]. DNA segments in the cytoplasm activate a set of DNA sensors (e.g., STING) and trigger an innate immune response [333]. We set out to determine if the EV can activate DNA sensors by determining the activation of interferon regulatory factor-3 (IRF3) and the release of innate cytokines such as IL-6 and IL-1β. In an experiment with human peripheral blood mononuclear cells as the recipient cells, EV treatment showed an increase in IL-6 and IL-1β at 24 hours after EV treatment (**Paper V**, Fig 7B). We went further and determined if surface DNA on EVs can influence the release of innate cytokine in monocyte cells. Upon removal of DNA from the surface of the EVs, the increased level of cytokine showed a reduction back to basal level of secretion (**Paper V**, Fig 7B). Additionally, we observed that activation of IRF3 was partially dependent on the DNA present on the EV surface (**Paper V**, Fig 7C-D). Our preliminary data for EV-associated DNA uptake experiments revealed that DNA uptake was strictly dependent on its association with EV (**unpublished data,** data not shown) [334]. This suggested that both DNA and EVs might share (at least partly) a common uptake pathway. At this point, we do not know how EV-associated DNA is transferred to the cytoplasm and activates the DNA sensors after EVs are taken up by the cells.

Taken together, **Papers IV** and **V** provided evidence that the association between DNA and EVs might act as a regulator of the innate immune response. Efficient uptake of DNA after EV association presents a novel method to generate sterile immunity, and a better understanding of EV-associated DNA will help establish better diagnostic approaches and achieve the effective generation of immune responses.

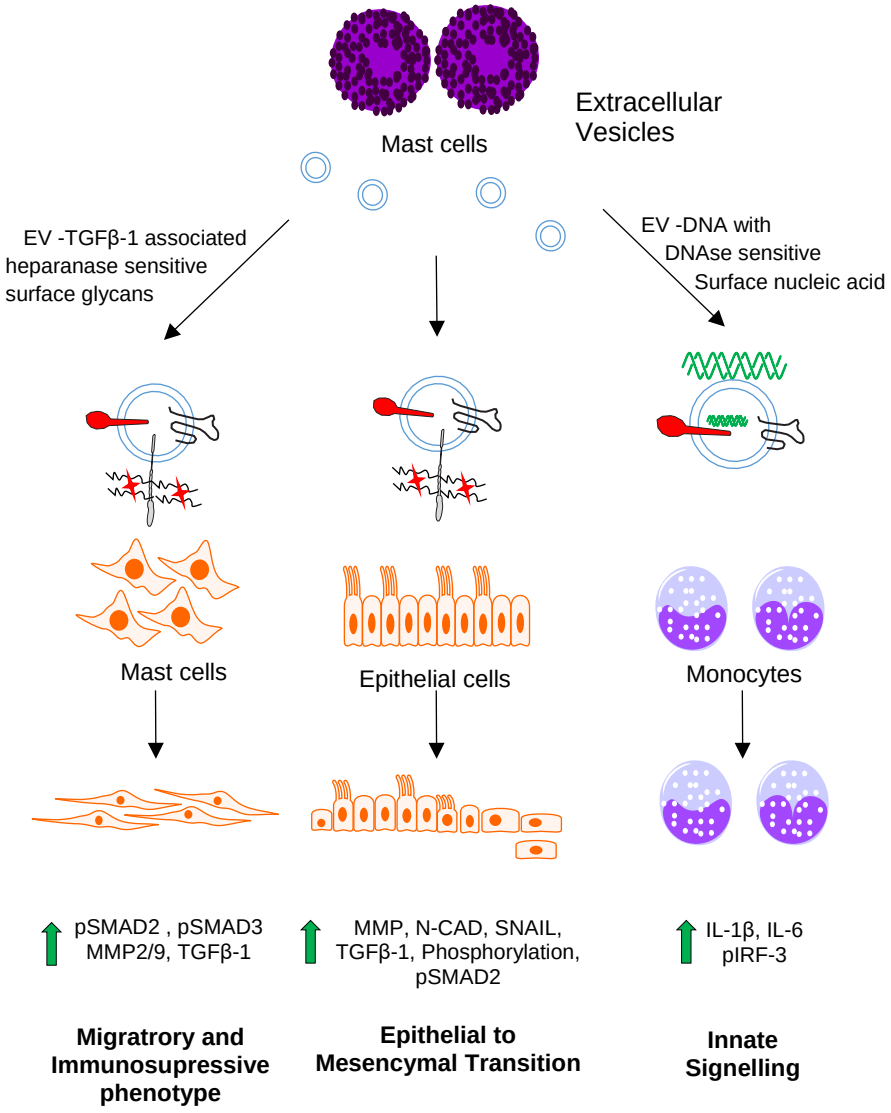

Figure: 11 *Role of mast cell derived extracellular vesicles in cell to cell communication. Mast cells release EVs that carries TGFβ-1 on their surface via Heparan sulfate proteoglycan and majority of DNA on surface. EVs influence cellular phenotype of primary human mesenchymal stem cells (MSCs), epithelial (A549) cells and monocyte (THP-1) cells.*

5 CONCLUSION

Data published in the articles and attached manuscripts have established the following and also summaries in figure 11.

Paper I

EVs derived from FBS contribute to the migratory phenotype of recipient cells, and a significant amount of the RNA component of EVs can be removed by prolonged ultracentrifugation of FBS. However, this does not guarantee the complete removal of EVs. Dilution of FBS prior to prolonged ultracentrifugation does not result in any additional EV depletion.

Paper II

The surface of mast cell-derived EVs is associated with soluble proteins, including TGFβ-1. Both active and inactive forms of TGFβ-1 are associated with EVs, with the inactive form being associated with HSPGs that are present on the surface of EVs. The EV-associated TGFβ-1 is able to activate TGFβ/SMAD signaling in MSCs. Furthermore, mast cell-derived EVs induce migratory and immunosuppressive phenotypes in MSCs by reducing eosinophil recruitment. EVs are taken up and are targeted to endosomal compartments, and this appears to induce the activation of TGFβ-1 because these compartments provide an acidic environment.

Paper III

Mast cell-derived EVs can activate EMT in epithelial cells with upregulation of mesenchymal markers and downregulation of epithelial markers. EMT changes are accompanied by the activation of differential regulation of phosphorylated proteins upon EV exposure.

Paper IV

A portion of the extracellular DNA is associated with mast cell-derived-EVs, and it induces aggregation and gives a negative charge on the EVs. This association assists in the uptake of DNA by recipient cells.

Paper V

The EV-associated DNA, both on the inside and on the outside of the EVs, comes from both mitochondrial and genomic DNA. The majority of EV-associated DNA is DNase sensitive, and only a small fraction is protected. Because most of the DNA is associated with the surface of the EVs, the EV-associated DNA can be sensed by cytoplasmic DNA sensors that induce the activation of interferon regulatory factor-3, a component of the innate immune signaling pathway.

6 FUTURE PERSPECTIVES

Taken together, the studies undertaken in this thesis have shown how mast cell-derived EV cargo is associated with the surface of the EVs. In addition, we also proposed how these cargoes influence signaling within recipient cells and thus influence the biological functions of these cells. In this section, I will put my work in a broader context and reflect on the gaps that should be addressed in future EV research.

The past decade has seen a huge interest in the EV field with increased interest in EV-based biomarkers and therapeutics. This has in part been due to the availability of "omics technology" to generate a large amount of data along with highly sensitive detection assays. However, the methods for working with EVs are still evolving, and the field still faces significant challenges regarding the purity of samples after isolation, the complexity of biological samples, and the wide variety of subpopulations of EV.

Inside cells, there is the constant generation and exchange of membranes between various cellular compartments, and this suggests that at least some of the EV subpopulations can come from the fusion of various membrane compartments. Our understanding of EVs is still limited to a handful of proteins, such as tetraspanins, but it is known that numerous other membrane proteins are found in different EV preparations. Lipid-based isolation of EVs is likely to be a way forward in our understanding of EV function, and isolating EV proteins from captured lipids will likely transform our understanding of EV cargo. Once we know all of the proteins associated with EVs, we can possibly dissect the biogenesis of the numerous EV subpopulations that currently have unknown origin.

Currently, information about EV biogenesis comes from MVs generation via surface membrane budding and from exosomes derived from MVBs. Our work started with the finding that TGFβ-1 on the surface of EVs is associated with HSPG (**Paper II**). However, the standing question remains as to how this association is achieved mechanistically, and it is not clear whether this process is specific for TGFβ-1 or if this occurs regularly with other growth factors. This interaction could most likely happen either during the fusion of MVBs with Golgi-vesicles or after release of EVs and TGFβ-1 outside the cell in the

extracellular matrix. Interestingly, mast cells are the major contributor of HSPG, thus we can speculate that this interaction might have a larger impact on diseases. Cells could release EVs with HSPG that can bind to growth factors and render them capable of activating to activate nearby cells. Validation of this finding in a disease model would be an interesting avenue for future research.

The DNA cargo in EVs is considered to be a valuable source of disease biomarkers, and to have potential to use for gene or mutation-specific detection in different body fluids in a non-invasive manner. Identification of tumor cell-specific DNA methylation on EV-associated DNA would be a way forward to accelerate improving diagnostic accuracy. The literature suggests the presence of DNA inside the lumen of exosomes (an EV subpopulation), but our findings in mast cell-derived EVs (**Paper IV** and **V**) argue against this because we found that the majority of DNA was not protected inside the EVs but was either in free form or was in small amounts sticking to the surface of EVs. We obtained this finding by purifying EVs with density-based gradient ultracentrifugation. For diagnostic purposes, it would be of interest to know if this association occurs actively inside the cell of origin or outside of the cell as a passive interaction. Eosinophils and granulocytic cells are known to release "DNA traps" outside the cells that can capture pathogens, but at this time we do not know if the DNA from mast cells is released as such a DNA trap. We have observed the presence of nucleosome complexes (histone protein) in EVs suggesting that the observed DNA might be at least partially associated with DNA-binding proteins and thus that there might be some interactions between histones and the EV surface. An additional hypothesis for DNA–EV interaction could the binding ability of DNA with fibronectin, as well as the ability of fibronectin to interact with HSPG present in EVs. Taken together, the surface of EVs derived from mast cells presents a foundation that might be genetically engineered with different proteoglycans that will specifically conjugate with different growth factors and DNA molecules.

EV-cargo can interact with the cell surface, fuse with the membrane, or be taken up within a cell via various endocytic pathways. However, the release of EV cargo into the cytoplasm presents a challenge, where cargo inside the EV lumen has to cross two different bilayers to reach the cytoplasm. This part of the delivery process is still not well understood. Most of the EV cargo is

targeted to the lysosomal compartment, but this degradation is avoided by certain EVs. Certain EV cargo can either spend more time in early endosomes or can be recycled back to the cell surface or fused with MVBs. We have found the presence of EVs in endosomes at the time when we observed EV-associated TGFβ-1 signaling, which suggests a possible engagement of TGFβ-1 with the TGFβ receptor present in the endosomes. However, we need more evidence in order to confirm such spatial regulation of signaling. At this point, we do not know if the signaling that is observed is because of activation of latent TGFβ-1 in the endosomes or because of active TGFβ-1 on the cell surface engaging with the TGFβ-receptor to initiate signaling. Further studies are needed to discriminate between cell surfaces vs. endosomal TGFβ-1 signaling and to determine which form of EV-associated TGFβ-1 (active vs. latent) is involved in endosomal TGFβ-1 signaling. On a similar line, the fate of EV-associated DNA inside the recipient cells is poorly understood. It has been shown that DNA complexed with EVs is much more effectively delivered to the cells, but more work needs to be done to dissect the mechanism of DNA transport from endosomes to the cytoplasm or any other uptake machinery of the recipient cell.

EVs derived from non-activated mast cells are bioactive, and they induce an immunosuppressive phenotype in MSCs and can initiate the EMT cascade in epithelial cells. These properties of secreted EVs are not a surprise because the secretome of non-activated mast cells has immunomodulatory properties. But how these EVs with multiple cargoes possibly initiate parallel signaling pathways is not known. It is possible that the cargos can act as ligands that rapidly activate receptors through phosphorylation and drive the downstream EMT signaling cascade. Analysis of these pathways and their crosstalk remains to be investigated.

To summarize, we need to obtain more information about EV surface-associated cargo using carefully evaluated methods to isolate EVs and to determine their true cargo. The understanding of the topology of EV cargo will enhance the usefulness of EVs in both diagnostic and therapeutic applications. The function of these surface cargoes can have many parallel effects that regulate biological function in both health and disease conditions.

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