Extracellular Vesicles and RNA Interference in Tumors

Taral R Lunavat

Department of Internal Medicine and Clinical Nutrition
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: Confocal microscopy image of exosomes (green) and microvesicles (red) taken up by A549 cells. The image was captured at the Centre for Cellular Imaging (CCI), University of Gothenburg. Image by Taral R Lunavat.

ॐ भूर्भुवः स्वः तत्सवितुर्वरेण्यं ।

भर्गो देवस्य धीमहि धियो यो नः प्रचोदयात् ॥

Oh Creator of the Universe!

We meditate upon thy supreme splendour.

May thy radiant power illuminate our intellects,

destroy our sins, and guide us

in the right direction!

To my beloved Family and Friends

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Taral R Lunavat

Department of Internal Medicine and Clinical Nutrition, Institute of Medicine
Sahlgrenska Academy at University of Gothenburg, Göteborg, Sweden

ABSTRACT

Extracellular vesicles (EVs) including apoptotic bodies (ABs), microvesicles (MVs), exosomes (EXOs) and cell derived artificial nanovesicles (NVs) are important mediators of cell-to-cell communication, in part by transferring bioactive molecules such as DNA, mRNA, miRNA, siRNA, proteins, and lipids. These EVs are released by many cell types, including melanoma cells, and are found in many body fluids. EVs derived from various cell types differ in their molecular composition making them as important diagnostic and prognostic markers. The overall aim of this thesis was to use small-RNA sequencing techniques to define the molecular RNA cargo in the EV subsets described above as well as to examine the functional relevance of the EV-associated miRNA and siRNA on recipient cells.

Characterization of EVs showed distinct RNA profiles in ABs, MVs, and EXOs, and there were significantly greater amounts of total RNA in EXOs compared to ABs and MVs. Small RNA sequencing revealed distinct repertoires of noncoding RNAs in the EV subsets. EXOs contained unique sets of miRNAs, which were shown to be differentially expressed in melanoma tumors compared with benign naevi in previously published studies, thus making them potentially useful as carriers of therapeutic agents. This study demonstrates that distinct sets of RNA molecules are present in subsets of EVs, and this provides unique insights into the contribution of extracellular RNA in cancer development and progression.

The BRAF^{V600E} inhibitor vemurafenib inhibited the growth of *in vitro* melanoma cell cultures, and EVs isolated from the treated cells had significantly higher RNA and protein contents compared to EVs from non-treated cells. Small RNA sequencing revealed distinct non-coding RNA species with significant alterations in miRNA between treated and non-treated cell-derived EVs. Moreover, treated cells and the EVs derived from them showed significant upregulation of miR-211 *in vitro* and *in vivo*. Furthermore, when vemurafenib-treated cell-derived EXOs were transferred to BRAF^{WT} cells, KCNMA1 and IGF2R, genes that are known to play roles in tumor progression, were down-regulated and this resulted in growth

attenuation. Overall, miR-211 could be used as a biomarker of response in patients diagnosed with BRAF-mutant melanoma. This study also provides the framework for further investigations into the function of miR-211 in melanoma cells and EVs as well as in cells that might receive miRNA from EVs.

Artificial EXO-mimetic NVs were developed by serial extrusion, and they showed similar characteristics as EXOs. Exogenous loading of GFP-siRNA in NVs led to down-regulation of GFP in endothelial cells. Cell-derived NVs carrying endogenously expressed Myc-siRNA showed significant down-regulation of human cMyc both transcriptionally as well as translationally in lymphoma cells. These NVs were efficiently loaded with siRNA and were taken up by recipient cells resulting in the reduction of target gene expression. In conclusion, this study suggests that EXO-mimetic NVs can be a platform for delivering siRNA to cells.

Taken together, EVs have significant therapeutic potential. EVs have emerged as a novel and functionally important vehicle of cell-cell communication that can mediate multiple biological effects. In addition, these vesicles might provide unique signatures that can be used as biomarkers of response to drug treatment.

SAMMANFATTNING PÅ SVENSKA

Extracellulära vesiklar (EV), så som apoptotiska kroppar (AB), mikrovesiklar (MV) och exosomer (EXO) som frisätts från celler, och artificiella nanovesiklar (NV) som tillverkas från celler, har de senaste åren visat sig ha en viktig funktion vid cellkommunikation genom att överföra aktiva molekyler så som DNA, mRNA, miRNA, siRNA, proteiner och lipider mellan celler. EVs frisätts av de flesta celler, inklusive melanomceller, och återfinns i flertalet kroppsvätskor. Det molekylära innehållet i dessa vesiklar skiljer sig från det i cellerna som producerar dem och de lämpar sig därför väl som diagnostik- och prognosmarkörer. Det övergripande målet med denna avhandling har varit att bestämma RNA-innehållet med sekvensering i de ovan nämnda EV subpopulationerna, samt att förstå de vesikelassocierade miRNA- och siRNA-effekterna i mottagarceller.

Karaktäriseringen av subpopulationerna av EVs visade tydligt olika RNA profiler mellan AB, MV och EXO, med signifikant mer RNA i EXO jämfört med de andra två subpopulationerna. Sekvenseringen av små RNA visade att de olika subpopulationerna av vesiklar innehöll distinkt olika typer av icke-kodande RNA. EXO innehöll en unik uppsättning av miRNA, som visade sig ha förändrat uttryck i melanomtumörer jämfört med födelsemärken i tidigare publicerade studier, vilket därmed gör dem till unika bärare med terapeutisk potential. Denna studie visar därmed att subpopulationer av EV innehåller unika uppsättningar av RNA molekyler vilket ger en förståelse för hur dessa kan bidra till extracellulärt RNA i cancer.

Vemurafenib är en BRAFv600E inhibitor som användes för att behandla melanomceller i in vitro-kulturer vilket resulterade i blockering av celltillväxt. EV isolerade från behandlade celler hade signifikant mer RNA och proteininnehåll jämfört med icke-behandlade celler. Sekvensering av små RNA påvisade tydligt att vesiklar från behandlade och icke-behandlade celler innehöll olika icke-kodande RNA-molekyler, med signifikant skillnad i miRNA-innehållet. Behandlade celler och deras vesiklar hade en signifikant uppreglering av miR-211 in vitro och in vivo. Vidare så visade det sig att när EXO från behandlade celler gavs till BRAFWT-celler avstannade tillväxten vid 48 timmar genom nedreglering av generna KCNMA1 och IGF2R, gener som tidigare har visat sig spela en viktig roll vid tumörprogression. Sammanfattningsvis kan miR-211 användas som en biomarkör för respons i patienter som bär på melanom med BRAF-mutationer. Denna studie ligger även till grund för fortsatta undersökningar av vilken funktion miRNA-211 har i melanomceller och EV samt i potentiella mottagarceller.

Artificiella exosom-liknande NV skapades genom att sekventiellt pressa celler genom filter. NV som laddades exogent med GFP-siRNA, d.v.s. efter att de hade skapats, kunde sedan när det gavs till endotelceller nedreglera GFP i dessa mottagarceller. NV som laddades endogent med Myc-siRNA, d.v.s. cellerna innehöll redan siRNA när vesiklarna skapades, kunde signifikant nedreglera humant cMyc både transkriptionellt och translationellt i lymfomceller som odladats tillsammans med dessa vesiklar. Sammanfattningsvis så kunde dessa NV laddas effektivt med siRNA som togs upp av mottagarceller vilket resulterade i en nedreglering av målgenen. Denna studie föreslår att exosom-liknade NV kan användas för att leverera siRNA till mottagarceller.

Sammanfattningsvis kan EV vara terapeutiskt viktiga. De framträder som en tidigare okänd och funktionellt viktig förmedlare av cellkommunikation och kan dessutom erbjuda en unik plattform i deras användning som biomarkör för behandlingsrepsons.

LIST OF PAPERS

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

I. Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells – evidence of unique microRNA cargos.
Lunavat TR*, Cheng L*, Kim DK, Bhadury J, Jang SC, Lässer C, Sharples RA, López MD, Nilsson JA, Gho YS, Hill AF, Lötvall J.
RNA Biology 2015 July; 12:8, 810-823. (PMID: 26176991)
*These authors contributed equally

II. BRAF-inhibition alters melanoma vesicular secretome microRNA cargo.
Lunavat TR, Cheng L, Einarsdottir B, Olofsson R, Sharples RA, Lässer C, Gho YS, Hill AF, Nilsson JA, Lötvall J.
Manuscript.

III. siRNA Delivery by Exosome-Mimetic Nanovesicles – Implications for Targeting c-Myc in Cancer.
Lunavat TR*, Jang SC*, Nilsson L, Park HT, Repiska G, Lässer C, Nilsson JA, Gho YS*, Lötvall J*.
Submitted.
*These authors contributed equally

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Additional publications not included in this thesis:

Xiao H, Lässer C, Shelke GV, Wang J, Rådinger M, Lunavat TR, Malmhäll C, Lin LH, Li J, Li L*, Lötvall J*. *Mast cell exosomes promote lung adenocarcinoma cell proliferation – role of KIT-stem cell factor signaling.* Cell Communication and Signaling 2014 Apr 4; 12:64.

Mudduluru G*, Abba M*, Batliner J*, Patil N, Scharp M, Lunavat TR, Leupold JH, Oleksiuk O, Juraeva D, Thiele W, Rothley M, Benner A, Neriah YB, Sleeman J, Allgayer H. *A Systematic Approach to Defining the microRNA Landscape in Metastasis.* Cancer Research 2015 Aug 1; 75:3010. DOI: 10.1158/0008-5472.CAN-15-0997

CONTENTS

ABBREVIATIONS

ABs	Apoptotic bodies
ALIX	Apoptosis-linked gene 2-interacting protein X
ARRDC-1	Arrestin domain containing 1
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CME	Clathrin-mediated endocytosis
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
EGFRvIII	Epidermal growth factor receptor variant III
EpCAM	Epithelial cell adhesion molecule
ESCRT	Endosomal sorting complexes required for transport
EVs	Extracellular vesicles
EXOs	Exosomes
FBS	Fetal bovine serum
GM-CSF	Granulocyte macrophage colony-stimulating factor
Hsp90	Heat shock protein 90
IGF2R	Insulin growth factor 2 receptor
ILVs	Intraluminal Vesicles
INF-γ	Interferon gamma
JAK	Janus kinase
LAMP	Lysosomal-associated membrane protein
LKB1	Polarization-related protein
MAPK	Mitogen activated protein kinase
MART	Melanoma-associated antigen recognized by T cells
miRNA	Micro ribonucleic acid
MITF	Microphthalmia-associated transcription factor
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MVBs	Multivesicular bodies
MVs	Microvesicles
NKs	Natural killer cells
NKG2D	Natural-killer group 2, member D
NTA	Nanoparticle tracking analysis

NVs	Nanovesicles
PBS	Phosphate buffer saline
PDGFRβ	Platelet-derived growth factor receptor beta
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PTEN	Phosphatase and tensin homolog
PTX	Paclitaxel
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROCK1	Rho-associated protein kinase 1
SCID	Severe combined immunodeficiency
siRNA	Short interfering ribonucleic acid
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
STAT	Signal transducer and activator of transcription
TEM	Transmission electron microscope
TNFα	Tumor necrosis factor alpha
TSAP6	Tumor suppressor activated pathway-6
VAMP	Vesicle-associated membrane protein
VEGFR1	Vascular endothelial growth factor receptor 1
VPS	Vacuolar protein-sorting-associated protein

1 INTRODUCTION

1.1 Extracellular vesicles

Extracellular vesicles (EVs) including apoptotic bodies (ABs), microvesicles (MVs), exosomes (EXOs), membrane-like particles, ectosomes, and EXO-like vesicles are released by many cell types in an evolutionarily conserved manner from prokaryotes to eukaryotes [1-3]. These EVs have different characteristics and properties and are formed in a variety of ways. According to their cellular origin, these vesicles can be classified as ABs (which are derived from cells undergoing apoptosis), MVs (which are shed from the plasma membrane), or EXOs (which are derived from multivesicular bodies (MVBs)) [4-7].

The importance of these EVs lies in their ability to transfer genetic information to neighboring cells thereby influencing their function [8-10]. The transfer of signals between cells can occur through a variety of different biomolecules, including proteins, lipids, nucleic acids, sugars, etc. The unique cargos carried by EVs allow cells to adapt to changing environments and allow cells to communicate with each other even if they are located some distance away from each other within the body.

EXOs were initially described nearly 35 years ago when two groups observed that MVBs in reticulocytes released such vesicles into the extracellular space [11-13]. Before that, EXOs had been observed as pro-coagulant platelet-derived particles in plasma [14], and these were referred to as "platelet dust" by Wolf in 1967 [15]. Later, these vesicles were observed by Raposo and colleagues, and they demonstrated that these vesicles from transformed B-lymphocytes were antigen presenting and were able to induce T-cell responses [16]. In 2007, EXOs received substantial interest as important mediators of cell-cell communication with the discovery that these EVs contain mRNA and miRNA [8, 9]. These vesicles have since been isolated from most cell types and have been shown to be present in most biological fluids, including breast milk, amniotic fluid, saliva, urine, nasal and bronchial lavage, plasma, serum, and seminal fluid [17-23].

Many groups have coined the nomenclature of these EVs subgroups and have proposed specific characteristics for them, but there is still a lack of specific markers for defining these subpopulations [24, 25]. EVs are generally isolated by differential centrifugation by pelleting the ABs at $2000 \times g$, the

MVs at $16,500 \times g$, and the EXOs at $120,000 \times g$ [26, 27]. In addition, EV subsets can also be fractionated by affinity chromatography, antibodies against the known EV markers [28, 29], size exclusion chromatography [30], charge separation, or isoelectric focusing [31-33]. The contents of the EV subsets usually differ in their molecular composition depending on their source and the enrichment or isolation technique.

1.1.1 Biogenesis

Endocytosis is the process by which cells internalize solutes, fluids, plasma membrane components, and macromolecules through the invagination of the plasma membrane to form vesicles and vacuoles through membrane fission [34]. This pathway consists of highly dynamic membrane compartments that are involved in the internalization of the cellular components or extracellular ligands, recycling to the plasma membrane, and subsequent degradation [35, 36]. As the early endosomes mature into late endosomes, they become characterized by the formation of intraluminal vesicles (ILVs) within the lumen of the endosome. Because of their characteristic features, these late endosomes are referred to as MVBs or multivesicular endosomes [37]. ILVs are formed by inward budding of an early endosomal membrane, thus randomly or specifically engulfing cytosolic contents and incorporating peripheral and transmembrane proteins into invaginating membranes [38].

In most cells, the biogenesis of EVs, and especially EXOs occurs by inward budding of the limiting membrane to form the MVBs. The fate of the MVBs is to fuse to either lysosomes or the plasma membrane of the cell. When they fuse with lysosomes, the contents of the vesicles are degraded by acid hydrolysis inside the lysosomes. It has been hypothesized that the contents of the MVBs are sorted and that proteins that are destined for degradation by the hydrolases are ultimately found inside the ILVs. On the other hand, proteins that have functions are usually found on the outside of the ILVs of the MVBs. However the exact process by which the sorting of cargo occurs is not yet understood [39, 40].

The other way in which MVBs release their contents is by fusion with the plasma membrane of the cell in an ion-dependent manner, thereby releasing ILVs via the exocytosis pathway into the extracellular environment [41]. These vesicles, now referred to as EXOs, bear the tetraspanin CD63, the lysosomal membrane proteins LAMP1 and LAMP2, and other molecules that are generally thought to be present in late endosomes [42]. Interestingly, in reticulocytes, MVBs only bear early endosomal markers as compared to the

late endosomal markers such as RAB4 or RAB5 [43]. Altogether, these findings propose that subpopulations of MVBs are destined for the degradation pathway or are fated for exocytosis.
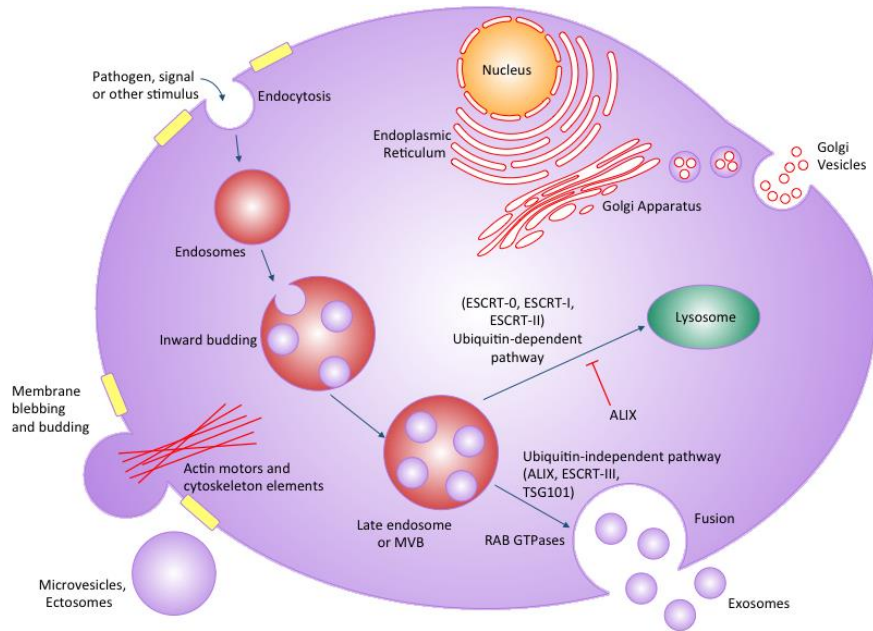
Figure 1. Biogenesis of extracellular vesicles. Intraluminal vesicles are formed by endocytosis in response to ligands, pathogens, or other stimuli. These endocytic vesicles mature from early endosomes into late endosomes, which leads to the formation of multivesicular bodies (MVBs). Rab GTPase regulates MVBs maturation and fusion with the plasma membrane by ubiquitin-independent pathways to release exosomes. ALIX inhibits the late endosomes from undergoing lysosomal degradation and thus favors exosomal release. Microvesicles bud directly from the plasma membrane lipid micro-domains, and is controlled by cytoskeleton elements and regulatory proteins thus promoting membrane curvature and budding into the extracellular environment. Microvesicles shedding also requires specific factors such as ARF-6, VPS4, and the plasma membrane protein ARRDC1. Golgi vesicles are also released after synthesis in the endoplasmic reticulum, where the protein cargo is transported to the Golgi apparatus, modified and packaged into small vesicles. Adapted from [44].

The biogenesis of EXOs also depends on the ESCRT (endosomal sorting complex required for transport) machinery to form the MVBs thus following a mechanism that parallels the endolysosomal degradation pathway (Figure 1) [41]. Indeed, RNA interference (RNAi) of the key ESCRT proteins tumor susceptibility gene 101 (TSG101, ESCRT-I), vacuolar protein sorting 22 (VPS22, ESCRT-II), charged multivesicular body complex protein (CHMP 2A, CHMP 4A/B/C, ESCRT-III), or ALIX (or VPS4A/B) (ESCRT-III

interacting protein) have been shown to significantly reduce EXO production in breast cancer cells [45]. In addition, another report has shown decreased EXO secretion after knockdown of the ESCRT-0 protein (Hrs; hepatocyte growth factor-regulated tyrosine kinase substrate) in antigen-presenting cells [46]. These four ESCRT protein complexes transport ubiquitinated proteins along the endosomal pathway [47]. The ubiquitin is removed from the cargo protein just prior to delivery into the ILVs suggesting that the mono-ubiquitination of proteins targets them into the ILVs for sorting in MVBs [48, 49]. However, it is not yet known whether the ESCRT machinery sorts the molecules into ILVs of MVBs for lysosomal degradation or for secretion.

Another mechanism of EXO biogenesis is ceramide enrichment in the endosomal membranes [50]. Due to its small, cone-like shape, ceramide directly influences the inward budding of the plasma membrane to form endosomes. The transfer of the cargo, which is enriched in distinct subdomains, into the lumen of the endosomes is not dependent on the ESCRT machinery but on the sphingolipid ceramide. However, it has not been directly confirmed whether ceramide functions to sort specific cargo molecules into the membranes of EXOs.

Another set of proteins involved in the biogenesis of MVBs are the Rab proteins, a small family of GTPases, including Rab5, Rab27, and Rab35, that regulate the transport, docking, and fusion of vesicles and thus play critical roles in the endosomal pathway [51]. Rab5 regulates early endosomal fusion and trafficking and is mostly found on clathrin-coated endocytic vesicles and early endosomes [51-53]. It has been suggested that both Rab4 and Rab5 regulate protein movement in and out of the early endosomes [51], and Rab7 has been suggested to function in the transition from early endosomes to late endosomes [52]. Several other Rabs, including Rab11, Rab27, and Rab35, are involved in EXO secretion via exocytosis [52]. It has been further shown that inhibition, depletion, or mutation of these Rabs affects the biogenesis of MVBs [54-56].

The biogenesis of microvesicles or ectosomes occurs by direct budding from the plasma membrane, but very little else is known about MV formation [4]. Despite this mechanistic distinction, it has been proposed that the elements required for EXO biogenesis are common for MV production and MV budding. For instance, it has been suggested that the topology of outward budding is similar to the budding into the lumen of MVBs [57]. Recent studies have shown that MV shedding from the plasma membrane occurs as a result of the interaction between arrestin-domain containing 1 (ARRDC-1) and the ESCRT component TSG101 [58]. In glial cells, the production of

ceramide promotes membrane curvature during MV formation [59]. Another example of ectosomes or MV formation involves the actin-based motors in the context of the enterocyte brush border. The membrane binding motor myosin-1a exerts direct force on the apical membrane that distributes along the length of the microvillus [60]. This results in the accumulation of membrane at the microvillar tips that in turn initiates the formation and release of MVs into the gut lumen. Further, it has been shown that depletion of myosin-1a in mice results in the production of MVs that lack the characteristic enrichment of cargo molecules [61]. These studies suggest that the mechanism of plasma membrane manipulation is of prime importance to the formation and release of MVs.

ABs are generally formed when normal or cancerous cells undergo apoptosis [62, 63]. A dying cell undergoes several stages, starting with nuclear chromatin condensation followed by membrane blebbing and the distribution of cellular contents into distinct membrane vesicles called ABs or apoptosomes [62]. ABs are generally formed during programmed cell death whereas EXOs and MVs are secreted during normal cellular processes. It has been suggested that the membrane blebbing is mediated by actin-myosin interactions [64]. The Rho effector protein ROCK1, which phosphorylates myosin light chains, contributes to this interaction in the plasma membrane. During apoptosis, Rho effector protein ROCK1, is cleaved to generate the active form, and the active form is sufficient to form the membrane blebs and to re-localize the fragmented DNA into these blebs and ABs [64, 65].

The complexity of the endosomal pathway shows that many proteins are involved into the biogenesis of EVs from the formation of ILVs to MVBs to the transport and fusion of these vesicles and the budding of membranes. These processes might also be important in the release of different cargo molecules into the EVs.

1.1.2 Molecular composition

EVs released by most cell types have heterogeneous molecular contents, and several methods, including immunoblotting, protein staining, and liquid chromatography-mass spectrometry are required to fully characterize the protein content of EVs. EVs that are purified after washing with phosphate buffered saline (PBS) or density gradient centrifugation are devoid of contaminants such as serum proteins and soluble proteins as well as some intracellular compartments like the mitochondria and the endoplasmic reticulum. EXOs contain proteins that are involved in the biogenesis of

MVBs, such as the annexins, SNARE proteins, flotillin, Rab GTPase, etc. EVs are also enriched in membrane proteins that cluster into microdomains at the plasma membrane, e.g. tetraspanins. The tetraspanins are a family of more than 30 proteins that contain four transmembrane domains [66]. Tetraspanins were first identified in B-cell EXOs and were found to be >100 fold enriched in EXOs relative to the transferrin receptor, which is considered to be a genuine marker for the plasma membrane and early endosomes [67]. Several studies have confirmed that numerous tetraspanins such as CD81, CD63, CD82, CD9, CD53, and CD37 are enriched in the exosomal fraction (Figure 2) [67-71].
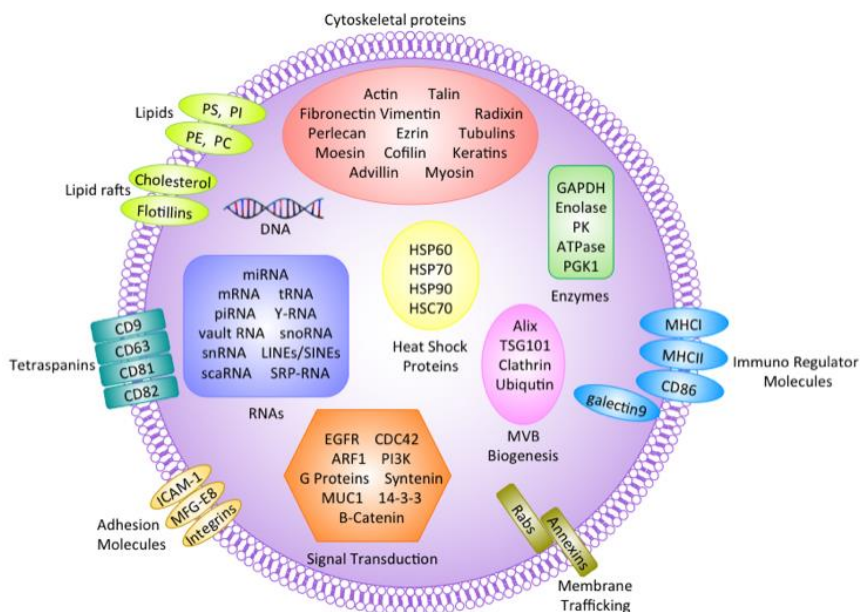
Figure 2. Molecular composition of extracellular vesicles. The luminal cargo of extracellular vesicles mainly consists of DNA, lipids, RNA such as miRNA and mRNA, and a myriad of different proteins depending on cellular origin. mRNA-messenger RNA; miRNA- microRNA; piRNA- piwi-interacting RNA; snoRNA- small nucleolar RNA; snRNA- small nuclear RNA; LINE/SINE- long/short interspersed nuclear element; scaRNA- small Cajal body RNA; tRNA- transfer RNA; SRP-RNA-signal recognition particle RNA; MHCI- Major histocompatibility complex I; ICAM-1- intercellular adhesion molecule 1; MFG-E8- milk fat globulin-EGF factor 8 protein; CD- cluster of differentiation; MVBs- multivesicular bodies; HSP70- heat shock protein 70; HSC70- heat shock cognate 70; PK- protein kinase; PS-phosphatidylserines; PI- phosphatidylinositol; PC- phosphatidylcholines; PE-phosphatidylethanolamines; TSG101- tumor susceptibility gene 101; EGFR-epithelial growth factor receptor. Adapted from [72-74].

EVs have also shown to be enriched in proteins that are associated with lipid rafts, including flotillin and glycosylphosphatidylinositol-anchored proteins [75, 76]. In addition, EXOs isolated from many cell types are highly enriched in sphingomyelin, cholesterol, and hexosylceramides [76-79]. Little is known about the molecular content of MVs and whether particular components are enriched in MVs in relation to the plasma membranes from which they originate, but recent studies have shown the presence of tetraspanins and other exosomal markers such as CD81, CD63, and flotillin-1 in MVs [27, 80].

A major breakthrough came a decade ago with the discovery that miRNA and mRNA are found in EXOs and that these vesicular mRNAs can be translated in the recipient cells, and since then much effort has been put into identifying other RNA cargos in the EV subsets [8, 81]. Many other studies have confirmed the presence of RNA in EVs from cell cultures [9, 10] and body fluids [23, 82-84]. EVs also contain various non-coding RNA in addition to miRNA, including Y RNA, vault RNA, long non-coding RNA, transfer RNA, mitochondrial RNA, small interfering RNA, RNA transcripts from protein-coding mRNA, etc., and these have been analyzed and confirmed by deep sequencing methods [85-88]. In addition to these sequencing strategies, studies have also confirmed the enrichment of RNA by validating them in the EVs [27, 86]. Although the mRNA content appears to differ slightly in these EVs from the parent cells [8, 9, 89], studies have shown that the miRNAs that are found in these EV subsets are more similar to those of the parent cell [29, 82, 90]. There is evidence suggesting that the miRNA contents in EXOs and their parent cells differ much more than the miRNA contents in EXOs from different cell types, and this is indicative of the heterogeneous nature of EV cargo [91]. The extensive characterization of RNA and proteins in EVs has generated a lot of data that have been compiled into online database like EVpedia, ExoCarta, and Vesiclepedia [92-96].

1.1.3 Release and uptake of EVs

Release

The release or secretion of EVs, especially EXOs, is regulated by the fusion of MVBs with the plasma membrane. As described earlier in the biogenesis of EXOs, many proteins are involved in the release of these vesicles. The underlying mechanism of MVBs fusion with the plasma membrane is not yet understood, but some recent studies have shed light on this topic. Changes in the intracellular calcium levels in melanoma cells [97], cultured cortical

neurons [98], human erythroleukemia cells [99], and oligodendrocytes [100] have been shown to induce the release of EVs. Studies have also shown that depolarization of cells induced by potassium can also increase the release of EXOs [98]. It has also been shown that the secretion of vesicles is modulated by the glutaminergic activity in cultured cortical neurons [101] and that crosslinking of receptors by cellular activation in mast cells or T-cells can induce the secretion of EXOs [102-104]. Furthermore, it has also been shown that activation of the P2X7 receptor triggers ATP-mediated release of vesicles in leukocytes and epithelial cells [105].

The role of Rab proteins in intracellular trafficking and the fusion of cellular compartments have also been investigated. In human erythroleukemia cells, the secretion of EXOs was triggered when cells were transfected with wild-type Rab11 whereas the release was inhibited when cells were transfected with mutant Rab11 [54, 106]. In contrast, different short hairpin RNA (shRNA) has been used to silence different Rab proteins, and it has been shown that EXO secretion is not decreased when using shRNA against Rab11A [56]. Instead, silencing of Rab27 effectors such as Slp4 and Slac2b has been shown to decrease EXO secretion thus phenocoping Rab27a and 27b [56]. The regulation of EXO secretion by Rab27 was also determined by nanoparticle tracking analysis (NTA) [107]. In addition to Rab11 and Rab27a and 27b, Rab35 is also involved in the release of proteo-lipid protein enriched EXOs [55]. However, it has not been shown how these Rab proteins control the different steps of EXO biogenesis and EXO release through MVBs fusion with plasma membranes in different cell types. It has been shown that different lipids are involved in regulating EXO release, and stimulating cells with ionomycin triggers phospholipase D2 translocation from the plasma membrane into intracellular compartments where it induces EXO secretion [108]. Also, diacylglycerol kinase α regulates the secretion of EXOs [109].

The last step of EXO secretion requires the fusion of the MVBs with the plasma membrane. This process usually requires soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). Vesicular SNAREs (v-SNAREs) are localized on the MVBs, which interact with target SNAREs (t-SNAREs) that further localize to the intracellular side of the plasma membrane thus forming a membrane bridging SNARE complex that is responsible for the membrane fusion [110]. Fader *et al* demonstrated that the v-SNARE complex TI-VAMP/VAMP7 is involved in the fusion of MVBs with the plasma membrane [111].

Many studies have demonstrated that EXO secretion is controlled by stress conditions. Activation of p53 regulates the transcription of the tumor

suppression activated pathway 6 (TSAP6) gene, which has been implicated in EXO secretion [112, 113]. Thus the stress response of p53 not only produces secreted protein mediators but also releases EXOs that communicate with neighboring recipient cells to influence their phenotype. In addition, it has also been shown that melanoma cells release more EXOs when cultured in acidic conditions that mimic the tumor microenvironment [114].

Only a few studies have looked at MV secretion or the shedding of vesicles (ectosomes) in different cell types. Most of the stimulating agents used in these studies have tended to enhance the production of MVs that usually bud from the plasma membrane, and it has been suggested that a process of local disassembly of the cytoskeleton is necessary for the membrane abscission that occurs during the shedding of MVs [115]. It is also known that an increase in calcium levels triggered by the local responses in the cytosol [116], p38 MAPK [117], the Rho-ROCK axis [118], and acid sphingomyelinase [119] are involved in the shedding of MVs. Furthermore, it has also been suggested that ATP-mediated activation of TNF-α signaling in endothelium cells and activation of the P2X7 receptor in macrophages can induce increases in calcium levels as well as MAPK activation thus leading to the shedding of MVs [115]. However, further research is needed to better understand the underlying mechanism involved in MV secretion.

ABs are released as membrane blebs from cells undergoing apoptosis. The formation of membrane blebs can occur during various stages of cell death [120], and it is dependent on the phosphorylation of myosin light chains [121, 122]. Even though blebbing occurs on a large scale on the cell surface, only a few blebs are released from cells by exocytic budding. While the exact function of membrane blebbing is not known, it might be to add to cell volume regulation when apoptotic cells shorten and debacle during cell death [123]. Further, it has been shown that some cell types can release ABs during injury or normal growth and that some diseased cells release ABs [26, 124, 125], but the precise molecular mechanisms behind the release of these ABs have yet to be determined. However, it has been shown that the release of ABs can be inhibited by overexpression of Bcl-2 or by caspase inhibition [126].

Uptake

EV uptake from different cellular origins can be visualized directly. The most commonly used fluorescent lipid membrane dyes for detecting EVs include PKH26 [127-130], PKH67 [127, 131-136], DiI [128, 137, 138], rhodamine B

(also known as R18) [89, 114, 137, 139-142], and DiD [143], and most of these are lipophilic in nature. There are also membrane-permeable compounds that are used to stain EVs, and these include carboxyfluorescein succinimidyl ester [138, 143-149] and 5(6)-carboxyfluorescein diacetate [149]. These chemical compounds become restricted to the lumen and fluoresce as a result of esterification. Thus the entry of EVs into the recipient cells can be measured by fluorescence/confocal microscopy as well as by flow cytometry.

There is direct and indirect evidence showing that EVs internalize in the recipient cells. EVs have been shown to transfer miRNA and mRNA from mouse mast cells to human mast cells, and the mouse mRNA was functional in the recipient cells [8]. siRNA delivery via EVs has been shown to knock down target genes in recipient cells [150], the horizontal transfer of oncogenes and DNA has been observed [151, 152], and the delivery of luciferin substrate in EVs results in bioluminescence in luciferase-expressing cells [139]. These results provide evidences that EVs either merge with the plasma membrane through membrane fusion to deliver their cargo into the cytoplasmic compartment or are internalized by fusion with the endosomal membrane [139].

Due to the large amount of evidence for direct EV uptake, it has become important once again to study the mechanism of EV internalization. The studies that have demonstrated EV uptake have proposed the following mechanisms for such uptake: a) macropinocytosis, b) clathrin-mediated endocytosis, c) phagocytosis, d) lipid raft-mediated endocytosis, e) caveolin-mediated endocytosis, and f) plasma or endosomal membrane fusion. Many techniques have been used to study the molecular mechanisms of EV uptake, including the use of antibodies for testing specific receptors or ligands and the use of inhibitors to block specific EV uptake pathways. For example, Feng and colleagues have shown that the proteins that are involved in endocytosis do not affect EV uptake; rather, the internalization is dependent mainly on actin and PI3-kinase – two important components required during phagocytosis – suggesting that EV internalization occurs through phagocytosis and not by endocytosis or macropinocytosis. [135]. In addition, the use of chemical blockers that affect EV uptake has also been reported, including cytochalasin (inhibits phagocytosis), choloropromazine (inhibits clathrin-mediated endocytosis), methyl-beta-cyclodextrin (caveolae mediated endocytosis, and 5-(N-ethyl-N-isopropyl) amiloride (EIPA; inhibits macropinocytosis) [144]. Although these findings did not shed much light on the routes of EV uptake, they did suggest that EV uptake can either occurs by

membrane fusion or by internalization of whole EVs through different mechanisms that might differ in different cell types.

1.1.4 Isolation and characterization

The isolation of EVs requires differential centrifugation from cell culture supernatants (conditioned media) and body fluids. The experimental procedure of isolating these EVs does not tell us about the origin of the EVs, but it can distinguish EV subsets based on their size, density, composition, and morphology. It is recommended that these samples be processed using high-quality isolation and characterization methods to distinguish these EV subsets. The effects of storage and different processing methods have not been extensively studied, thus it is also recommended that EVs be isolated immediately from fresh samples.

A position paper that describes the processing of samples and the isolation of EVs was recently published that addresses important considerations about sample collection, handling, storage, dilution of samples, etc. [153, 154]. EVs can be isolated through different methods, but the most commonly used method for isolating different subsets of EVs uses a differential centrifugation protocol as the gold standard method [155]. The centrifugation protocol starts with pelleting cells at $300 \times g$, and this is followed by pelleting ABs at $2,000–3,000 \times g$, pelleting the MVs at $10,000–20,000 \times g$, and then, with or without use of filtration, pelleting the EXOs at $100,000–120,000 \times g$ [72, 156-158]. The first step of centrifugation at $300 \times g$ is essential to pellet the cells that might burst during the isolation procedure and release intracellular vesicles that can contaminate the EXO pellet. The use of a filtration step (0.2 μm) is recommended to eliminate larger contaminating vesicles [158]. To eliminate the contaminating soluble proteins, EXOs are further washed in PBS and ultracentrifuged again at $100,000–120,000 \times g$ to re-pellet the EXOs. For further purification, EXOs and other vesicles can be loaded onto a sucrose or iodixanol gradient or cushion or they can be captured by binding to antibody-coated beads, in which case only a specific subpopulation of vesicles can be purified [18, 24, 157-159]. Other isolation techniques include size-exclusion chromatography [160-162], polymer-based precipitation [163-165], concentration protocols [162, 166, 167], ExoQuick™ (System Biosciences), microfluidics, and immuno-affinity bead capture [24, 29, 82].

The characterization and identification of EVs involves several methods, including electron microscopy, western blot, flow cytometry, and NTA. Due

to the small size of EVs, they can only be visualized by electron microscopy to describe their morphological characteristics. In addition, immuno-staining with gold particles can be used to observe the presence of EXOs with cell surface proteins. It has recently been shown that EXOs have a cup shaped morphology, but other techniques like cryo-electron microscopy have suggested a different morphology [4, 72, 168, 169].

Another way of characterizing EVs is by using western blot or flow cytometry. To date, no specific EV marker exists, but due to their intracellular origin the enrichment of specific proteins such as tetraspanins (CD63, CD81, CD82, CD9) and proteins associated with the endocytic pathway such as TSG101, ALIX, and Flotillin-1 are often use as EV markers. The absence of endoplasmic reticulum proteins such as calnexin indicates that the vesicles, especially EXOs, are not contaminated with vesicles derived from the endoplasmic reticulum, which is often used as a negative control [23, 45, 170]. With flow cytometry, EVs are usually bound to beads that are coated with tetraspanins such as CD63 or CD81 to be able to distinguish them from the background noise because these vesicles are very small in size. A recent study showed that EVs can be directly detected by using high-resolution flow cytometry, and this will likely become a very beneficial purification technique in future [171, 172].

Size characterization by NTA, dynamic light scattering (DLS), ZetaViewer, and scanning ion occlusion sensing are some suggested techniques to determine the size of the vesicles and to determine the concentration by particle count analysis. NTA measurements are generally used to determine the protein/particle concentration ratios as well as size distribution. It has also been suggested that these techniques can be used to investigate the effect of storage conditions on EV isolates.

1.1.5 Functions of EVs

Many cells, including fibroblasts [173], mesenchymal stem cells [174], cancer cells [175], epithelial cells [176], and neuronal cells [177], release EVs into the extracellular milieu *in vitro* [178]. EVs are also found to be present *in vivo* in biological fluids, including synovial fluid [179], blood [180], urine [181], breast milk [136], saliva [160], nasal lavage [23], bronchoalveolar lavage [182], amniotic fluid [181], malignant effusions of ascites [183], and seminal fluid [184]. The first report to show the biological role of EXOs demonstrated that they transport proteins that are expelled from reticulocytes during erythrocyte maturation [11, 13]. Later, numerous studies

showed that EVs are secreted by many cell types and have different functions in different physiological settings. Even though the exact mechanism of how EVs interact with recipient cells is still not clear, it has been shown that EVs have a diverse range of functions that includes the exchange of genetic material between cells [8, 9, 185], immuno-stimulatory functions [16], immuno-suppressive functions [186], the removal of unwanted proteins [11, 187, 188], educating the pre-metastatic niche [173, 189, 190], and spreading infectious agents [191-193]. This thesis will further discuss the potential role of EVs in tumorigenesis.

Role of EVs in tumorigenesis

Tumor cells have developed several defense mechanisms against various treatments that prevent their elimination from an organ. EVs released by the tumor cells have recently been shown to aid in these defense mechanisms within the tumor microenvironment. They play a crucial role in intercellular as well as intracellular communication and in controlling the local microenvironment. It has been shown that EVs carry various sets of cargo, including proteins, lipids, miRNA, and mRNA that regulate the function of the recipient cells, and there are extensive data showing the role of EVs, especially EXOs, in driving metastasis and cancer progression. It has further been shown that EVs are involved in multi-drug resistance mechanisms [194-196], epithelial to mesenchymal transition [197-199], and the radiation-induced bystander effect [200, 201]. Moreover, EXOs are able to educate bone to form a pre-metastatic niche thus enabling the cancer cells to escape from the host immune cells [173, 189, 190]. In addition, EVs secreted by mesenchymal stem cells and tumor cells are responsible for formation of blood vessels (angiogenesis) via sphingomyelin [31, 202]. This shows the importance of secreted EVs in the development and progression of cancer.

Pro-tumorigenic effects of tumor-derived EVs

Lindoso and colleagues have recently shown that EVs derived from renal cancer stem cells induce persistent phenotypical changes in mesenchymal stem cells (MSCs) and that these changes are characterized by alterations in gene expression related to cell migration (CXCR4, CXCR7), angiogenesis, and tumor growth. They further showed that the migratory phenotype of MSCs is enhanced when they are incubated with tumor-conditioned media. This suggest that EVs from renal cancer cells induce a pro-tumorigenic phenotype in MSCs [203].

Another study by Clayton *et al* showed that tumor-derived EXOs that carry NKG2D ligands down-regulate NKG2D expression and result in impairment of cytotoxic CD8^{+} T-cell function [204, 205]. Other studies have also confirmed the role of the NKG2D ligands carried by tumor-derived EXOs that allow tumors to evade the immune system [206, 207]. Hedlund and colleagues have shown that EVs derived from the tumor cells express NKG2D ligands and down-regulate the NGKGD2 receptor-mediated cytotoxicity and thus impair the natural killer cell function, which consequently promotes the immune evasion by these tumor cells. They also showed that the EXO secretion was enhanced under oxidative and thermal stress, which could explain the impairment of natural killer cell function in patients suffering from leukemia and lymphoma [207, 208].

Tumor-derived EVs have also been shown to induce the differentiation of T-helper cells as well as regulatory T-cells, which suggests a possible mechanism for evading immune surveillance. This could possibly be explained by the regulatory T-cells that mediate the immune tolerance of the tumors by regulating the tolerance of self-antigens [209, 210]. Further, it has also been shown that Interleukin 2-mediated inhibition by tumor-derived EVs not only inactivates natural killer cells but also supresses T-cells via induction of adenosine [31, 211, 212]. In addition, it has also been shown that Fas-ligand and tumor necrosis factor (TNF)-bearing EXOs from tumor cells induce apoptosis in CD[+] T-cells [213, 214].

Anti-tumorigenic effects of tumor-derived EVs

In contrast to the pro-tumorigenic effect of tumor-derived EVs, other research has demonstrated that some EVs derived from tumor cells do not suppress the immune system but rather activate it [183, 215]. These studies demonstrated that tumor-derived EVs from mast cells and effusions have a high enrichment of heat shock and MHC class 1 proteins and also carry tumor-specific antigens gp100 and MART-1/Melan-A, thus transferring the tumor-specific antigen to the dendritic cells and subsequently activating cytotoxic T-cell reactivity *in vitro.*

Another study demonstrated the use of MHC class II-positive EXOs when treating dendritic cells, and these enhanced the splenocyte proliferation and interferon-γ (INF-γ) production of CD4^{+} T-cells and subsequent inhibition of IL-10 secretion [216]. They further showed that the tumor-derived EVs induce higher TNF-α and IL-12 mRNA levels suggesting an anti-tumor immune response [216]. Yang and colleagues also demonstrated that interleukin-2-modified tumor cell-derived EXOs efficiently induce anti-

tumor responses in the antigen-specific Th1 polarized immune response and cytotoxic T-cells, which suggests that these EXOs could potentially be used as a tumor vaccine [217].

Role of EVs in pre-metastatic niche formation

Tumor-derived EXOs have shown promise as therapeutic vaccines by activating the immune system, but these EVs have also been shown to promote tumor progression by being involved in pre-metastatic niche formation. The tumor microenvironment plays an important role in cancer progression, and several soluble factors are released by the cells such as growth factors and cytokines, and these factors are known to play a key role in the initiation of the metastatic cascade. Many studies have thus shown a supporting role of EVs in the tumor microenvironment as mediators of metastasis favoring the conditions for pre-metastatic niche formation [189, 218, 219].

Jung *et al* were the first to report the role of cancer-associated EXOs in the formation of the pre-metastatic niche in a rodent pancreatic cancer model [219]. Another report showed that MVs secreted from CD105-positive renal cancer cells activate endothelial cells to establish capillary-like structures on Matrigel and induce enhanced chemo-resistance *in vitro*. Moreover, they also showed that CD105-positive MVs up-regulate MMP2, MMP9, and VEGFR1 in SCID mice thus promoting pre-metastatic niche formation in the lung microenvironment [220, 221]. In similar settings, another study led by Lyden's group showed that melanoma-derived EXOs induce lung permeability and increase lung metastasis in mice [189]. In addition, they demonstrated that these melanoma cell-derived EXOs educate the bone marrow to initiate pre-metastatic niche formation. Two recent studies from the same group showed that EXOs from pancreatic ductal adenocarcinoma cells induce pre-metastatic niche formation and subsequently increase the liver metastasis burden [190]. They further showed that uptake of EXOs by Kupffer cells increases TGF-β secretion and upregulates fibronectin expression in hepatic cells. Another study showed that EXOs derived from liver, lung, and brain tropic tumor cells fuse with the resident cells and prepare the organ-specific cells to form the pre-metastatic niche [173]. Moreover, proteomics of these tumor EXOs revealed tumor integrins linked specifically to lung and liver metastasis [173]. Hood *et al* also demonstrated the formation of micro-anatomic niche preparation facilitated by EXOs from melanoma cells thus enhancing the lymphatic metastasis of cancer cells [218].

It has been discussed that the intracellular communication between cancer cells and stromal cells is reciprocal and not unidirectional. Luga *et al* showed that EXOs derived from CD81+ fibroblasts promote the motility and protrusive activity of breast cancer cells via Wnt/planar cell polarity signaling, and stimulation of Wnt signaling was associated with increased motility in lung metastasis in an orthotropic mouse model of breast cancer [222]. Overall, these studies demonstrate that tumor-derived EVs play a crucial role in manipulating the tumor microenvironment and thereby benefiting the cancer cells.

1.2 Diagnostic and Therapeutic Potential of EVs

1.2.1 The use of EVs in diagnosis and prognosis

EVs contain a wide range of RNA and protein molecules, thus detection of tumor signatures in cancer-derived vesicles could provide biomarker information. These vesicles are very stable in different storage conditions from 4°C to −80°C, and such characteristics could make them useful for the early detection of serum vesicles as potential biomarkers of the expected cancer burden and thus could have an impact on personalized cancer care. Patients with different malignant diseases have been shown to have circulating EVs containing tumor markers on them, thus reflecting the tumor profiles in these tumor tissues [9, 29, 82, 90, 189, 223-225]. Circulating tumor EVs can easily be sampled non-invasively from patients with mild and severe tumor burden, and the quality and the content of the EVs reflect their origin and make these circulating tumor EVs diagnostic and prognostic markers for several malignant diseases [82, 189, 223-226].

The first ever report to show the potential use of EVs as biomarkers was by Skog *et al* who showed that mRNA variants and characteristic miRNAs were detected in serum MVs of glioblastoma patients, and these specific mRNA and miRNA were absent in healthy controls. Further, tumor-specific EGFRvIII variants were also detected in serum MVs in 25% of glioblastoma patients thus providing diagnostic information that could aid in therapeutic applications. They also showed that mRNA detected in serum MVs was no longer found after the tumor was removed indicating that the tumor was the source for the exosomal mRNA [9]. In addition, another report showed that prostate cancer-derived vesicles contain mRNA and that these vesicles are

found in urine and are a potential source of biomarkers for prostate cancer [226].

It has been suggested that the miRNAs found in EVs might reflect the source of tumor tissue as well as their respective miRNA profiles in tumor tissue. For example, Rabinowits and colleagues have shown that serum isolated from lung cancer patients is highly enriched in miRNA in EVs compared with healthy controls and thus that these could potentially be used as diagnostic biomarkers in lung cancer [82]. A report by Silva *et al* showed that the non-small cell lung cancer patients and healthy controls differed in EV-related miRNA in plasma suggesting that these miRNAs could serve as a circulating biomarker of prognostic value to predict survival [227].

Such applications of EVs in tumor diagnosis have been described in many studies of ovarian cancer [29], colorectal cancer [228], and melanoma [189]. Silva *et al* showed that the levels of EXOs in cancer patients was significantly higher than in healthy controls and that high levels of vesicles in plasma correlated with poorly differentiated tumors and that patients with low levels of vesicles in plasma tended to have shorter survival times. This suggests that the circulating tumor-derived vesicles could be used as a marker for prognosis for patients [228]. Another study led by Taylor and Gercel-Taylor demonstrated that EpCAM-positive EVs were found in patients with benign tumors as well as ovarian cancer but showed distinct miRNA profiles in ovarian cancer patients compared with benign tumor patients. These EV-miRNAs were not found in the healthy controls suggesting that these circulating EV-miRNAs could potentially be used as diagnostic markers for ovarian cancer [29]. Altogether, these results points towards the tumor-derived vesicle-associated miRNA and mRNA as useful tools to categorize patients into separate classes that might enable more personalized cancer therapies.

1.2.2   The therapeutic use of EVs

The recent use of EVs as drug carriers has demonstrated advantages over cell-based drug delivery and nanotechnology [229, 230]. Similar to the size of viruses, these small vesicles are capable of transferring genetic information between cells and can easily pass their cargo across the cell membrane, thus delivering their cargo in a biologically active form. These tiny vesicular packets are also able to cross the blood brain barrier, which is one of the most difficult biological barriers in the body.

EVs have been shown to be effective therapeutic drug delivery vehicles in several investigations [231-237]. In the very first report by Sun and colleagues, they demonstrated that EXOs loaded with curcumin, an anti-inflammatory compound, protected mice from lipopolysaccharide-induced brain inflammation [231, 232]. The incorporation of curcumin in EXOs not only improved their solubility but also increased their circulation time and subsequent transport across the blood brain barrier. In another study, EVs and EXO-like vesicles called nanovesicles (NVs) were loaded with different chemotherapeutic agents such as doxorubicin (Dox) and paclitaxel (PTX), and these were shown to traffic to the tumor tissue and to significantly reduce the tumor growth in mice without any of the adverse effects observed with the free drug [233-235]. In addition, Jang *et al* also demonstrated that the therapeutic effect of Dox-loaded liposomes was inefficient in treating tumors compared to Dox-loaded NVs [233]. Furthermore, PTX-loaded vesicles secreted by macrophages were used to treat multidrug-resistant cancers [238]. The loading of PTX in vesicles using mild sonication resulted in highly efficient loading and sustained drug release [238]. A very interesting study was recently published showing that EXOs are able to deliver anticancer agents that significantly reduce the fluorescence intensity of cancer cells and reduce tumor growth [237]. In addition, this study showed that EVs loaded with hydrophobic photosensitizers had significant phototherapeutic effects compared to polymer-based nanoparticles. These vesicles were able to fuse with the plasma membrane of cancer cells more efficiently and effectively than the polymer-based nanoparticles thereby enhancing the therapeutic efficacy of the drugs *in vivo* [237].

Another therapeutic aspect involves the use of EVs to deliver small interfering RNAs (siRNAs). The pioneering report by Alvarez-Erviti *et al* demonstrated the use of dendritic cell-derived vesicles that were loaded with siRNAs using electroporation [150]. Similar to this method, EXOs were also loaded with miRNAs against the epidermal growth factor receptor that is abundantly expressed in breast cancer cells [185]. Because EVs carry a negative charge, siRNAs cannot be complexated electrostatically. This was shown by Wahlgren *et al* who suggested that the loading efficiency of siRNAs could be achieved by fusion of vesicles with pre-complexation of siRNA via cationic liposomes [239]. This group also reported that EXOs derived from the peripheral blood loaded with siRNAs could efficiently silence the *MAPK* gene in monocytes and lymphocytes. In another report, Shtam *et al* introduced two different siRNAs against the *RAD51* and *RAD52* genes into vesicles derived from HeLa cells, and this resulted in significant killing of recipient cancer cells [240]. O'Brien *et al* also demonstrated that engineering the donor cells with miR-134, which is heavily down regulated

in breast cancer tissue, could lead to the production of EVs that are highly enriched with miR-134. These EVs loaded with miR-134 were able to down regulate STAT5B and Hsp90 in breast cancer cells and reduce cellular migration and invasion [241]. Moreover, the sensitivity of these cancer cells to anti-Hsp90 drugs increased drastically, but this did not affect the cellular proliferation and/or cisplatin-induced apoptosis unlike the miR-134-transfected cells. In addition to these, several other studies have reported the therapeutic importance of EVs [242-244].

It has also been suggested that EVs can be used as vaccines for immunotherapy. In 1998, it was shown that EVs produced from dendritic cells that had been pulsed with tumor peptides were able to suppress the growth of tumor tissues in mice [245]. The first phase I clinical trials took place in 2005 by Escudier *et al* where dendritic cell-derived EXOs directly loaded with antigens were injected into malignant melanoma patients [246]. Other phase I and phase II studies have shown promising results using dendritic cell-derived EXO immunotherapy to treat advanced non-small cell lung cancer patients [247, 248]. In another study, EVs derived from ascites combined with granulocyte macrophage colony-stimulating factor as an adjuvant were used as immunotherapy to treat colon cancer [249].

Taken together, these studies show that the EV-based formulations have the potential to treat several disorders. Nevertheless, these methods still need to be optimized and further efforts are required to develop these therapies for clinical use.

1.3 Melanoma

Malignant melanoma is one of the most aggressive and deadliest forms of skin cancer. It develops in the melanocytes that give the skin its color, and it has a very high tendency to spread to other parts of the body. It occurs among all ethnic and racial groups, but its prevalence among these groups differs depending on geography. Risk factors include high exposure to the sun (UV radiation) with low levels of skin pigment, especially during childhood, exposure to artificial UV lights, and socioeconomic status. The American Cancer Society estimates that around 135,000 melanoma cases are diagnosed every year. In 2016, an estimated 76,380 invasive melanoma's were diagnosed in 46,870 men and 29,510 women [250].

The development and progression of melanoma involves a stepwise process of clonal succession that is driven by the acquisition of genomic alterations [251]. Genome sequencing approaches have revealed thousands of mutations, deletions, translocations, amplifications, and DNA methylation changes that are present in the individual tumors thus showing the massive genetic complexity of melanoma [252-256]. Only small molecular alterations are required for melanomagenesis – the so-called "driver" mutations. The known major driver mutations in melanoma along with the associated regulatory signaling pathways are listed in Table 1.

One of the most commonly reported oncogenic mutations in melanoma occurs in the *BRAF* (v-Raf murine sarcoma viral oncogene homolog) gene that encodes a protein kinase that regulates the mitogen-activated protein kinase (MAPK) signaling pathway. The first identified *BRAF* mutation was reported by Davies *et al* who reported that a valine to glutamic acid substitution at residue 600 (V600E) occurs in 50% of melanomas [257]. Other oncogenic mutations include the upstream components of the MAPK pathways such as *KIT* (v-Kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog) [258], *NRAS* (neuroblastoma RAS viral oncogene homolog) [259], *GNA11* (guanine nucleotide-binding protein, α11) [260], and *GNAQ* (guanine nucleotide-binding protein, q polypeptide) [261]. *BRAF* mutations have also been found in benign and dysplastic naevi in higher frequencies [262, 263]. A classic example of oncogene-induced senescence is the development of naevi in response to *BRAF* activation [264], which is a cellular mechanism that prevents normal cells from transforming into a malignant state by inducing growth arrest.

It has been reported that overexpression of mutated BRAF^{V600E} in melanocytes promotes the expression of p16^{INK4A} [262]. p16^{INK4A} is a key negative regulator of the cell cycle that is encoded by the *CDKN2A* locus and is known to be mutated in 25% of melanoma families [265]. Genetic aberrations in the p16^{INK4A}–cyclin D/CDK4-RB cell cycle checkpoint have been found to be present in all melanoma cell lines, underlying the importance of this checkpoint in inhibiting melanoma growth [266, 267]. Moreover, p16^{INK4A} is a component of the senescence barrier in human melanocytes. Although its potential role in *BRAF*-induced senescence is not very clear, it has been suggested that bypass of the p16^{INK4A}-independent senescence barrier is required for melanoma development [262, 268-270].

Table 1. Molecular drivers in melanoma.

Gene	Signaling pathway	Alterations in melanoma (frequency)	Refs
AKT1, AKT2, AKT3	PI3K–AKT–mTOR	Oncogenic mutations (<3%)	[271]
BRAF	MAPK	Oncogenic mutations (~50%)	[257]
NRAS	MAPK, PI3K–AKT–mTOR	Oncogenic mutations (15–20%)	[259]
GNAQ	MAPK	Oncogenic mutations in uveal melanomas (~30%)	[261, 272]
GNA11	MAPK	Oncogenic mutations in uveal melanomas (~40%)	[260, 272]
KIT	MAPK, PI3K–AKT–mTOR, JAK–STAT	Promoter hypermethylation in cutaneous melanomas (25–40%) Activating mutations and amplifications in mucosal and acral melanomas (10–20%)	[258, 273]
MITF	MITF–PGC1α	Amplifications (15–20%) Germline mutations in familial melanoma	[274-276]
CDKN2A (p16^{INK4A})	p16^{INK4A}–cyclin D/CDK4–RB checkpoint	Homozygous deletions, inactivating mutations, and promoter hypermethylation (50–80%)	[266, 277]
CDKN2A (p14^{ARF})	MDM2–p53	Homozygous deletions and inactivating mutations (~50%)	[277, 278]
CDK4	p16^{INK4A}–cyclin D/CDK4–RB checkpoint	Oncogenic mutations and amplifications (5–10%)	[267, 279]
MYC		Amplifications (5–17%)	[280]
LKB1	LKB1–AMPK	Inactivating mutations (~5%)	[281, 282]
PIK3CA	PI3K–AKT–mTOR	Oncogenic mutations (~3%)	[283]
PTEN	PI3K–AKT–mTOR	Homozygous deletions, inactivating mutations, and promoter hypermethylation (40–70%)	[284-286]

Adapted and modified from [249].

Another report demonstrated that in BRAF-initiated melanomagenesis there is constitutive activation of the PI3K (phosphoinositide 3-kinase) – AKT-mTOR (mammalian target of rapamycin) signaling pathway. Hyper-activation of this signaling pathway in melanoma often results in inactivation of *PTEN* (phosphatase and tensin homolog), which is a negative regulator of this pathway [284, 285]. *NRAS* and *PTEN* mutations are also mutually present in melanomas with the oncogenic role of RAS being to activate the PI3K–AKT–mTOR pathways [287]. However, the role of AKT in activating PI3K in melanoma cells is still in question based on a report that showed that inhibition of PI3K blocks the downstream signaling and prevents proliferation much better than AKT inhibitors [288]. Several other studies have shown that non-random co-occurrence of *BRAF* mutations and *PTEN* mutations in the same melanoma suggest a possible collaboration during melanomagenesis. These findings have been further verified by functional studies showing that inactivation of PTEN abolishes BRAF^{V600E}-induced senescence in p16^{INK4A}-deficient melanocytes [270].

There are several inhibitors available to treat malignant melanoma with different mutation status. Out of these, the first selective BRAF inhibitor, vemurafenib (PLX4032), is 10-fold more selective and potent against the mutated form of BRAF compared to wild type BRAF [289]. The clinical efficacy of vemurafenib in a metastatic setting has been validated thoroughly in clinical trials [290, 291]. There are other BRAF inhibitors that are being tested and currently undergoing clinical testing [292], including GSK2118436, which has shown potential benefits for patients with brain metastasis [293]. In BRAF wild type tumors, where NRAS is frequently mutated, CRAF appear to play a key role and can be a therapeutic target in these melanomas [294, 295].

Despite vemurafenib's success as a potent BRAF inhibitor, several questions were raised that resulted in deeper investigations, including how vemurafenib stimulates MEK-ERK signaling in BRAF wild type or RAS-mutated cells and how BRAF-mutated cells escape BRAF-inhibitor suppression. Heidorn *et al* and Poulikakos *et al* suggested a hint to answering these questions by demonstrating that RAF activation involves side-to-side dimerization as a possible mechanism [296, 297]. In the presence of an oncogenic mutation or in response to growth factor receptor engagement, RAS localizes to the plasma membrane, which further induces the formation of homodimers and heterodimers of CRAF and BRAF that lead to the phosphorylation and activation of MEK. In BRAF^{V600E} cells, BRAF is constitutively activated, and this drives the phosphorylation of MEK and ERK that subsequently activates the MAPK signaling pathways leading to uncontrolled cell growth. Thus it is

still not clear why there is paradoxical activation of MAPK signaling in BRAF wild type cells. In one of the reports, it was suggested that inhibition of RAF could lead to inactivation of a single monomer in RAF dimers that could then trans-activate another monomer of the RAF dimer that in turn could trigger the MAPK signaling [297]. In another report, it was demonstrated that wild type BRAF could translocate to the cell plasma membrane upon vemurafenib binding and that BRAF could then dimerize with CRAF and stimulate the CRAF-associated pathway signaling [296]. A pan-RAF inhibitor could suppress both CRAF and BRAF and thus inactivate MAPK signaling. CRAF mutations (e.g., CRAF (T421N)) could interfere with RAF binding to inhibitors of CRAF, and this could re-establish MAPK signaling. The bottom line is that RAS-mutated cells might be stimulated by vemurafenib inhibition in wild-type BRAF cells, which might explain the poor response to vemurafenib among patients and the observed HRAS-driven squamous cell carcinomas that develop when these patients are on selective BRAF inhibitors or other first-generation RAF inhibitors [298]. Thus, the use of vemurafenib requires knowledge of the patient's genetic background in order to avoid enhancement of disease and potential negative side effects.

Multiple mechanisms have been suggested to explain how cells escape vemurafenib inhibition and develop resistance to the drug. One of the possible explanations is the acquisition of NRAS or MEK mutations or the upregulation of insulin growth factor receptor (IGFR) or platelet derived growth factor receptor beta (PDGFRβ) that can lead to re-activation of MAPK signaling [299-301]. It has also been shown that ectopic expression of both COT/TPL2/MAP3K8 and CRAF is associated with higher resistance to PLX4720, another BRAF inhibitor [302]. Recently, it has been demonstrated that the 61 kDa variant form of BRAF^{V600E} that lacks the RAS binding domain promotes more efficient dimerization of RAF compared to the full-length BRAF^{V600E} [297]. Furthermore, it has been demonstrated that ectopic expression of p61BRAF^{V600E} results in constitutive ERK signaling that is not affected by BRAF inhibitors. Splice variants of BRAF that are missing the RAS-binding domain were detected in 6 out of 19 tumors from patients who had acquired BRAF inhibitor resistance, and it has also been reported that BRAF amplification is associated with vemurafenib resistance [303]. In another recent study by Das *et al*, vemurafenib resistance could be forestalled through a discontinuous dosing strategy in drug-resistant melanoma cells [304]. These results clearly show that vemurafenib generates a high selection for survival, and any epigenetic mechanism that allows re-activation or bypass of ERK signaling is likely to generate resistance. One clinically important situation is the development of distinct resistance mechanisms

within the metastatic deposits that make secondary therapeutic interventions nearly impossible.

1.4 Non-coding RNA

Non-coding RNAs (ncRNAs, also referred to as non-protein coding RNAs) represent a diverse group of regulatory transcripts that affect various stages of gene expression. Recent advances have led to the discovery and unraveling of critical roles for ncRNA in cancer pathogenesis. For instance, over expression or under expression of several miRNAs have been shown to drive tumorigenesis in cancer cells and mouse models. More recently, long noncoding RNAs (lncRNAs) have shown to be linked in many cancer phenotypes. Further establishment of the molecular functions of different classes of ncRNAs and their roles in development and physiology will be needed to understand their role in human malignancies.

High-throughput technologies such as deep sequencing and tiling arrays in combination with in-depth bioinformatic analysis have not only produced increased amounts of expression data, but also functional information in regards to the characterization of ncRNAs [305]. Currently, there are several databases that define the ncRNA families in terms of secondary structures, tissue-related expression, functional annotation, species-specific expression, phylogeny, taxonomy and alignment analysis, related diseases, putative target genes, and several other aspects [305]. ncRNAs can be broadly classified based on size into short ncRNAs (<200 nucleotides) and long ncRNAs (≥ 200 nucleotides). Small ncRNAs includes miRNA, small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), small Cajal body RNA (scaRNA), siRNA, Piwi-interacting RNA (piRNA), extracellular RNA (exRNA), Y RNA, vault RNA, tRNA, etc. [306]. lncRNAs include the functionally important ribosomal RNA (rRNA) – which consists of 18S and 28S subunits and is essential for protein synthesis in all living organism – and long intergenic ncRNA (lincRNA) [306, 307].

The functional roles of ncRNAs have been very well documented and demonstrated. For example, lncRNAs such as HOTAIR and MALAT1 have been shown to be associated with cancer [308], and several other lncRNAs have been associated with neurodegenerative diseases and other diseases of the central nervous system [309]. Macro lncRNAs that are bigger in size can affect imprinting or gene expression in cis, and the telomeric repeat

containing the TERRA transcript has been associated with telomere syndromes and cancer [310, 311]. Small ncRNAs, especially miRNAs, have been well studied based on their common mechanisms. Due to their common function of post-transcriptionally downregulating mRNAs, miRNAs can be classified based on their targets and their physiological and pathological functions [307]. Given that these ncRNAs are expressed in a highly tissue-specific manner and in specific cellular compartments, and due to their heterogeneous mechanisms of action, it is a challenge to determine the diverse regulatory functions of these transcripts in many disease models, and this is a truly exciting field of research.

1.4.1   ncRNA in EVs

Since the discovery of miRNA and mRNA in EVs that acts as vehicles to carry genetic information between cells [8], extracellular small regulatory RNAs circulating in the body fluids as well as in the conditioned media of cell cultures have been the focus of tremendous attention in recent years. In order to maintain small regulatory ncRNAs in the extracellular milieu, these RNAs are encapsulated in different EV subsets that protect them from different RNases [312].

Several studies have documented the presence of coding RNAs as well as ncRNAs in subsets of EVs [85, 86, 90, 124, 165, 313-318]. The first report of the sequencing approach to EVs was by Zhou and colleagues who determined the miRNA content in breast milk EXOs [165]. They identified 603 unique miRNA sequences out of which 68% were related to the immune system. In another study led by Hill's group during the same period, they identified a diverse range of ncRNAs in EXOs released by neuronal cells. Interestingly, they found mRNA fragments, small ncRNA, retroviral RNA repeat regions, small cytoplasmic RNA, siRNA, and a novel type of miRNA. In addition, they found that prion-infected cells release EXOs that are significantly enriched in let-7b, let-7i, miR-128a, miR-21, miR-222, miR-29b, miR-342-3p, and miR-424 compared to non-infected cells [86]. Nolte-'t Hoen *et al* also established another interesting unbiased sequencing approach with immune cells [85]. They found several pervasive transcripts, RNA cleavage products of protein-coding genes, and repeat sequences that were enriched in EXOs relative to cells indicating that the cells were releasing these RNAs into the extracellular space. In addition, they also found a large abundance of vault RNA, Y RNA, and tRNA fragments in EXOs secreted by immune cells. Lunavat *et al* showed the presence of ncRNA in subsets of EVs, including ABs, MVs, and EXOs, and they demonstrated that small RNA profiling

discriminates the subsets of EVs based on the presence of unique miRNAs in EXOs compared to ABs and MVs [124].

With so many studies in relation to ncRNAs in EVs, researchers can now verify the presence of EVs based on the RNAs that are enriched in EVs compared to cells. By using novel normalization strategies, many studies have shown the differential regulation of ncRNAs and their respective correlation patterns by comparing EVs with cells. In one report, Tosar *et al* validated several tRNA fragments that were found by deep sequencing approaches [313], and they used small RNA sequencing techniques to demonstrate the sorting of small regulatory RNA fragments into MVs, EXOs, and ribonucleoprotein complexes in breast cancer cell lines. Another interesting study characterized the small RNAs, especially miRNAs, in human plasma-derived EXOs [315]. They found around 600 miRNAs that were present in plasma EXOs out of which 5 miRNAs were in high abundance. They also detected enrichment of other RNA species in the EXOs, including rRNA, lncRNA, piRNA, snRNA, tRNA, snoRNA, and fragmented mRNA. Furthermore, they identified some miRNA fractions that were in greater abundance in the EXOs compared with the cells.

Overall, these studies suggest that the ncRNA species, especially miRNA, are present in high abundance in EVs, including ABs, MVs, and EXOs. These EVs can potentially be used as biomarkers for the diagnosis of many malignant diseases based on their enrichment of ncRNAs.

1.4.2 RNA interference

RNA interference (RNAi) is biological process in which small RNA molecules inhibit gene expressions by causing the destruction of target mRNA. In 2006, Andrew Fire and Craig Mello shared the Nobel Prize in Physiology and Medicine for their groundbreaking work in RNAi in *Caenorhabditis elegans* [319]. Endogenous RNAi can regulate many cellular processes. Endogenous, double stranded RNA (dsRNA) generated from long RNA transcripts are cut into pieces by enzyme Dicer and are loaded into a RISC complex (RNA-inducing silencing complex) that comprises several proteins, including Argonaute and RNA binding proteins. The guide strand in dsRNA is paired with a complimentary mRNA sequence via the RISC complex, and upon binding it induces RNA-mediated degradation or translational repression.

RNAi can be found in many eukaryotes, including animals, and miRNA and siRNA molecules play central roles in the process. RNAi is an RNA-

dependent gene silencing process that is initiated by dsRNA molecules in the cytoplasm of the cells and is regulated by the RISC complex [320]. dsRNA is broken down into siRNAs by the action of the DICER enzyme complex in the host cells where they interact with the catalytic RISC complex. When the dsRNA is exogenous, for example, coming from an RNA virus infection or by laboratory manipulations, the RNA is imported directly into the cytoplasm of the cells and then cleaved into short fragments by DICER. In another mechanism, the dsRNA can originate endogenously. For example, pre-miRNAs that are expressed from the intronic and intergenic RNA transcript in the genome can be processed to form characteristic stem loop structures in the nucleus that are exported into the cell cytoplasm. These two endogenous and exogenous dsRNA mechanisms both use the RISC complex [320].

RNAi is a fundamental part of the immune response to viruses and other foreign genetic material. It has been very well studied and documented in plants such as *Arabidopsis thaliana* that expresses multiple DICER homologs and is specialized to undergo distinct reactions when the plant is exposed to different viruses [321]. Animals express fewer DICER enzymes than plants, but RNAi is still part of the antiviral response in many animals [322]. The role of RNAi in mammalian innate immunity is not fully understood and still requires extensive study. Some viruses have been reported to encode important genes that might be able to suppress the RNAi response in mammalian cells [323], but this hypothesis has been challenged due to a lack of validation [324]. Other functions of RNAi using siRNA and miRNA in the downregulation and upregulation of many vital genes have been studied extensively [325, 326].

miRNA and siRNA biogenesis

miRNA and siRNA are families of short ncRNA molecules that regulate target gene expression in a sequence-specific manner. miRNAs are generally 20–25 nt in length and are usually derived from larger precursors that form imperfect stem loop structures. The mature miRNA is produced from one arm of the precursor hairpin and is released through stepwise processing by two ribonuclease-III enzymes. The main function of the mature miRNA-leading strand is to bind to the 3´ untranslated region (UTR) of target gene and function as a translational repressor. siRNAs are generally produced from dsRNA molecules of approximately 500 bp in length when RNAi is triggered in worms, plants, and flies. This dsRNA is processed *in vivo* into small siRNAs of approximately 20–25 bp in length, which are similar in length to that of miRNAs [327].
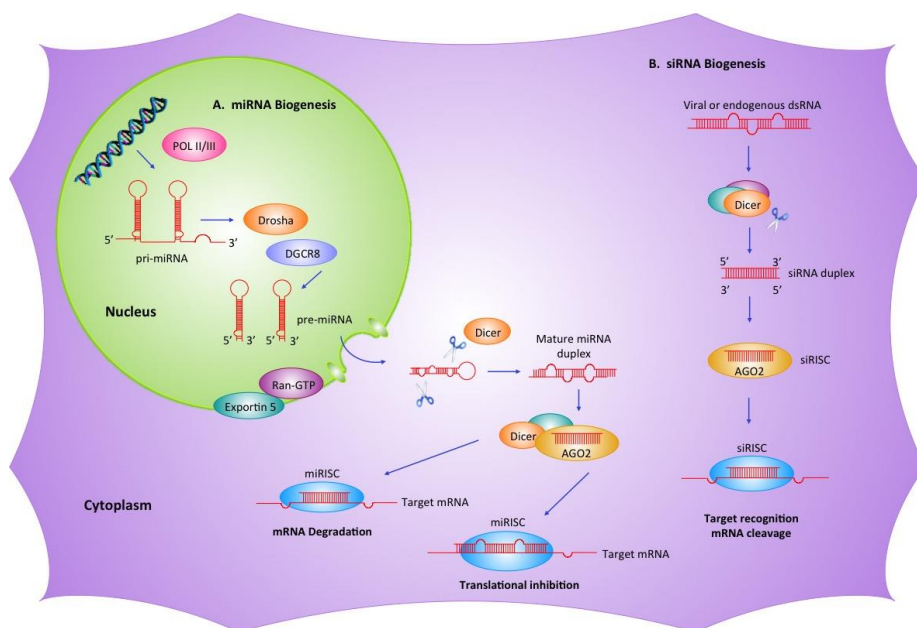
Figure 3. miRNA and siRNA biogenesis. *A) miRNA biogenesis begins in the nucleus where Drosha and DGCR8 cleave the pri-miRNA resulting in a miRNA precursor that is exported to the cytoplasm by Exportin5 and Ran-GTP61 where it is then processed by Dicer and TRBP. Next, the mature miRNA duplex is incorporated to the RISC where it subsequently binds to the 3' UTR of a target RNA leading to either mRNA degradation or translational repression. (B) siRNA biogenesis depends on Dicer activity by forming siRNA duplex and eventually incorporation into the RISC. Given the specificity of base pairing, the final outcome of the siRNA interference is the cleavage and degradation of their target mRNAs. pre-miRNA – premature miRNA, pri-miRNA – primary miRNA, RISC – RNA inducing silencing complex, dsRNA – double-stranded RNA. Adapted from [328].*

The biogenesis of miRNA occurs from nascent primary miRNA (pri-miRNA) transcripts of around 200 nt in length, and these are processed further into premature miRNA (pre-miRNA) of 70 nt in length by Drosha inside the nucleus (Figure 3). Pre-miRNAs are then transported into the cytoplasm by Exportin-5 where DICER processes them into miRNA duplexes, which also processes long dsRNA molecules into siRNA duplexes. Only one strand of the miRNA and siRNA duplex is preferentially assembled into the RISC complex, and this strand subsequently regulates its target gene expression by translational repression or by mRNA cleavage [329]. The translational repression or mRNA cleavage completely depends on the level of complementarity between the small RNA and its target genes.

In principle, miRNA and siRNA both have similar end point functional effects, and both have similarities in terms of their molecular characteristics, biogenesis, and effector functions. Both share the common RNase-III processing enzyme DICER and RISC complex for post-transcriptional repression (Figure 3), and much of the current knowledge about miRNA biochemistry stems from what we know about siRNA and the RNAi pathway.

The primary difference between miRNAs and siRNAs is in their molecular geneses and their mode of target recognition. In contrast to miRNAs, siRNAs are generated randomly from long dsRNAs that can be introduced exogenously or produced by endogenously transcribed RNA that anneals to form dsRNA. In most cases, miRNAs negatively regulate their target gene expression by binding to the 3´ UTR of the target mRNA through imperfect complementarity at multiple sites, whereas siRNAs often form a perfect duplex with their targets at only one perfectly complementary site, which results in cleavage of the target mRNA by RISC (Figure 3) [329].

There are several reports describing the use of EVs enriched in miRNA/siRNA to target specific tissues or genes of interest. Following the discovery of EXOs containing functional miRNA and mRNA, many studies have shown great interest in using these EVs as carriers of genetic information for targeting oncogenes and tumor suppressor genes. Using several miRNA overexpression systems in donor cells, researchers have found high enrichment of miRNAs of interest in EVs, which could be used to target and regulate gene expression. Furthermore, there are studies that have tried electroporating siRNA into EVs and then using these EVs as vehicles to target tissue-specific genes in mice as well as in cell culture-based systems. There are still limitations to the use of EVs as carriers because it is difficult to electroporate siRNAs into EVs due to the rigid morphology of the EVs. It would be very interesting to see if there are any synergistic effects of miRNA-enriched EVs that are electroporated with siRNA against several oncogenes that play a role in disease progression.

1.5   EXO-mimetic NVs

EXO-mimetic NVs – also referred to as artificial NVs – are generated by serial extrusion of cells through a series of polycarbonate membranes with several pore sizes [233]. These NVs have characteristic features similar to

those of EXOs, but they are produced in 100-fold greater numbers than EXOs [233]. In addition, they also have lipid bilayer membranes with diameters of around 100–200 nm. These bio-engineered cell-derived nano-carriers retain the natural ability to target the cells by maintaining a similar topology of the plasma membrane as the cells.

Artificial NVs can be generated through microfluidics, micro-channel fabrication, centrifugation-based extrusion, or mechanical serial extrusion through polycarbonate membrane filters [233, 330-333]. NVs can be loaded with chemotherapeutics for use against malignant tumors, and in a very interesting study by Jang *et al* they demonstrated that chemotherapeutic-loaded NVs can traffic to tumor tissue and reduce tumor growth without any of the side effects observed with equal concentrations of free drug [233]. In another recent therapeutic study, Keunhee and colleagues showed that the EXO-mimetic NVs from murine pancreatic β-cells are able to detect insulin-producing cells in the *in vivo* microenvironment by the presence of key pancreatic β-cell markers [330]. This suggests that EXO-mimetic NVs could efficiently detect therapeutic insulin-producing cells *in vivo* and could maintain physiological glucose levels. In addition to NVs derived from mammalian cell cultures, Kim *et al* demonstrated that bacterial protoplast-derived NVs can be used as a vaccine delivery system to prevent bacterial infection [333]. These protoplast-derived NVs not only showed higher productivity and safety compared to the currently used vaccine delivery vehicles, but they also induced antigen-specific humoral and cellular immune responses. Moreover, Kim *et al* also showed that immunization with NVs loaded with bacterial antigens provided protection against bacterial sepsis in mice. Taken together, these results suggest that NVs might not only be used for therapeutics in malignant diseases but can also be used as vaccine delivery vehicles to prevent infectious diseases.

This advancement in nanotechnology has shown great potential for the development of therapeutics and vaccinations. These particles are nano-sized, derived from cellular origins, and can be produced in amounts 100-fold greater than traditional cell-derived or conditioned media-derived EXOs, and these bio-engineered and bio-inspired NVs show great potential for various clinical applications such as targeted delivery of chemotherapeutics, tissue regeneration, theragnosis, and epigenetic reprogramming against several human diseases, including cancer.

2 AIMS

EVs, including ABs, MVs, EXOs, and cell-derived artificial NVs, are important mediators of cell-to-cell communication, partly by transferring bioactive molecules such as DNA, mRNA, miRNA, siRNA, proteins, and lipids. These EVs are released by many cell types, including melanomas, and are found in many body fluids, including saliva, plasma, urine, cerebrospinal fluid, breast milk, etc. EVs derived from various cell types differ in their molecular composition making them as important diagnostic and prognostic markers. The overall aim of this thesis was to define the RNA cargo in these subsets of EVs as well as to determine the functional relevance of EV-associated miRNA and siRNA in the recipient cells.

More specifically, the aims of this thesis were to

- Determine the RNA species of different EV subtypes.

- Evaluate the *in vitro* and *in vivo* differences in the RNA cargo upon oncogene treatment in melanoma.

- Determine the functional effects of vesicles derived from oncogene-treated melanoma cells.

- Determine if NV-loaded siRNA can be taken up and have functional effects in the recipient cells.

3 MATERIALS AND METHODS

3.1 Cell culture (Papers I, II, and III)

3.1.1 Cell cultures for EV isolation

The human malignant melanoma cell line A375 (papers I and II) (ATCC, Manassas, VA, USA) was cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA), the MML-1 cell line (**papers I and II**) (CLS, Eppelheim, Germany) was cultured in RPMI-1640 (Sigma-Aldrich), the SK-MEL-28 cell line (**paper I**) (ATCC) was cultured in DMEM (Lonza, Basel, Switzerland), and the MeWo cell line (**paper II**) (ATCC) was cultured in EMEM (Sigma). Complete culture media contained 10% fetal bovine serum (FBS) (Sigma-Aldrich), 100 U/ml penicillin (HyClone, Logan, UT, USA), 100 µg/ml streptomycin (HyClone), and 2 mM L-glutamine (HyClone). In addition, the A375 cell culture medium also contained 1% non-essential amino acids (PAA Laboratories, Pasching, Austria) and 5 mM β-mercaptoethanol (Sigma-Aldrich). The FBS was ultracentrifuged using a Type 45 Ti rotor (Beckmann Coulter, Brea, CA, USA) at $120,000 \times g$ for 18 hours to pellet the bovine EXOs prior to use.

In paper II, MML-1 cells were subjected to increasing concentrations of vemurafenib (PLX4032) to determine the optimal dose for downstream experimental applications.

3.1.2 Cell cultures for NV construction

In **paper III**, NIH3T3 cells were cultured in DMEM (HyClone) and λ820 cells were cultured in RPMI-1640 (Lonza) with stable glutamine. Both media were supplemented with regular 10% FBS (Sigma-Aldrich), 1% L-glutamine (HyClone), 100 U/ml penicillin (HyClone), and 100 µg/ml streptomycin (HyClone). In addition, the λ820 cell culture medium also contained 50 µM β-mercaptoethanol (Invitrogen). HMEC-1 cells expressing GFP and U937 cells were cultured in RPMI-1640 (Lonza or Sigma-Aldrich) supplemented with 200 µg/ml hygromycin, 10% FBS, and 1% antibiotic-antimycotic.

All cells were tested for the presence of mycoplasma prior to use. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. The viability of the cells was assessed by trypan blue exclusion assay.

3.2 Development of xenografts (Paper II)

All animal experiments were performed in accordance with EU directive 2010/63 and had approval of the regional animal ethics committee of Gothenburg (#287/289-12 and #36-2014). The establishment of patient-derived xenografts (PDX) and information regarding the mouse strains has been described previously [334]. Briefly, equal volumes of Matrigel and dispersed patient cells were mixed and injected subcutaneously into the flanks of immuno-compromised NOG mice (non-obese severe combined immune deficient interleukin-2 chain receptor γ knockout mice; Taconic, Denmark) to form xenografts. PDX were passaged twice until they reached a size of 100–150 mm^3 sizes as measured by a caliper during the treatment phase. The mice were randomized into two treatment groups – one receiving vehicle and other receiving vemurafenib (240 mg·kg^{-1}·day^{-1}; Zelboraf, Roche) mixed in the fodder (Research Diets Inc, New Brunswick, NJ). After the treatment, tumors were harvested, measured with a caliper, and weighed.

The tumors from vehicle-treated and vemurafenib-treated groups were placed onto tissue culture plates with 3 ml of serum-free medium with antibiotics. The tumors were chopped into fine pieces, and collagenase D (2 mg/ml; Roche) and DNase (400 U/ml; Sigma) was added to the medium. The chopped tumors and cells were incubated for 30 minutes at 37ºC in the culture medium to achieve a uniform suspension of tumor cells. The medium with the tumor tissues and cells was filtered through a 70 µm filter, and fresh medium containing 10% exosome-depleted FBS and 1% penicillin-streptomycin was added to the required volume and cultured for 24 hours.

For the establishment of cell line xenografts, 2×10^5 MML-1 cells were mixed in equal volumes of Matrigel and transplanted subcutaneously into the flank of each mouse. Tumors were harvested and weighed after the treatment. Each tumor from PDX and MML-1 cell-derived xenografts was suspended in RPMI 1640 medium containing penicillin and streptomycin until further use.

3.3 EV isolation (Papers I and II)

ABs, MVs, and EXOs were isolated from the supernatant of A375, MML-1, and SK-MEL-28 cells (80–90% confluence) using differential centrifugation. In addition, culture supernatant from vehicle-treated and vemurafenib-treated MML-1 cells was also used for the isolation of EVs. The protocol has previously been described as "the modified protocol-2B" by Crescitelli *et al* [26]. Briefly, the supernatant from the cell pellet at $300 \times g$ was used for

pelleting ABs at 2,000 × *g* for 20 minutes. The supernatant from ABs was used to isolate MVs by centrifuging at 16,500 × *g* for 20 minutes. The resulting supernatant was passed through a 0.22 µm filter (Sarstedt, Nümbrecht-Rommelsdorf, Germany), and EXOs were pelleted at 120,000 × *g* for 70 minutes. All of the EVs were re-suspended in the appropriate buffer (PBS or lysis buffer) depending on downstream analysis.

3.4 Preparation of NVs (Paper III)

Cells (5×10^6 cells/ml) in PBS were serially extruded three times through 10 µm, 5 µm, and 1 µm polycarbonate membrane filters (Whatman) using a mini-extruder (Avanti Polar Lipids). To isolate the NVs, two-step OptiPrep density gradient ultracentrifugation was performed. From the bottom to the top, 50% iodixanol (Axis-Shield PoC AS), 10% iodixanol, and the extruded samples were placed in an ultracentrifuge tube and then ultracentrifuged at 100,000 × *g* for 2 hours at 4°C. NVs were obtained from the interface of the 50% and 10% iodixanol layers. For the exogenous loading of siRNAs, the amount of protein was adjusted to 1 mg/ml with PBS. For exogenous encapsulation, siRNA (20 µM) was added and electroporated at 200 V. A two-step OptiPrep density gradient ultracentrifugation was performed once more as described above. The protein concentrations in the NVs were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

3.5 RNA isolation, RNase treatment, and analysis (Papers I, II, and III)

Cellular and vesicular RNAs were isolated using the miRCURYTM RNA Isolation Kit (Exiqon, Vedbaek, Denmark) following the manufacturer's protocol. All of the RNA samples were stored at −80°C until use. The quality, size, and concentration of the isolated RNA was determined using capillary electrophoresis with the total RNA 6000 Nano Chip and the Small RNA Chip (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions.

In paper I, EVs suspended in PBS were used for the RNase treatment. All of the EVs were isolated from equal volumes having similar seeding densities of cells and dissolved in equal volumes. A small portion of the vesicles re-suspended in PBS was used for particle count analysis using the ZetaView® NTA device (Particle Matrix, Meerbusch, Germany). Cellular RNA was used as a positive control for the RNase treatment. Equal volumes of RNA

samples were used in non-treated and RNase-treated (5 μg/ml) assays. RNase-treated samples were incubated for 20 min at 37ºC, while the non-treated samples were kept on ice. RNA was immediately extracted from these samples using the Exiqon Total RNA Isolation Kit (miRCURY™). These samples were analyzed with the total RNA Nano Chip on an Agilent 2100 Bioanalyzer. The RNA concentration was used as the normalizing factor for the RNA load-per-particle analysis.

3.6 Small RNA sequencing, library preparation, and data analysis (Papers I and II)

The total RNA quality as assessed by the Bioanalyzer was used for preparing the small RNA library. For each library, 50 ng of RNA was ligated to adapters containing a unique index barcode (Ion Xpress™ RNA-Seq Barcode 1-16 Kit, Life Technologies, Carlsbad, CA, USA). Using adaptor-specific primers, cDNA was prepared from RNA using reverse transcription in a protocol designed for short RNA sequencing. cDNA products were size-selected from 94 to 200 nt using the Magnetic Bead Purification Module (Life Technologies), and this included the adaptor sequences of around 25 bp. The cDNA library was then PCR amplified followed by a library cleanup step using nucleic acid beads (Life Technologies). The size distribution and yield of the amplified products was assessed using the High-sensitivity DNA chip with the Agilent 2100 Bioanalyzer.

Pooled cDNA libraries were clonally amplified onto Ion Sphere™ Particles (ISPs) supplied with the Ion OneTouch™ 200 Template Kit v2 (Life Technologies) and enriched using the One Touch 2 ES system (Life Technologies). Enriched ISPs loaded with cDNA libraries were sequenced on the Ion Torrent PGM™ using Ion™ 318 V2 chips (Life Technologies) and the Ion PGM™ 200 V2 Sequencing Kit (Life Technologies). Each chip was pooled with two libraries. Pre-processing of reads and the removal of adapters and barcodes were performed with the Torrent Suite software (v.3.4.1). Sequences were analyzed for quality control (FASTQC) and aligned to the Human genome (HG19) using the Torrent Suite. Output files (.bam) were uploaded, mapped to miRBase V.20, and further analyzed using the Partek Genomic Suite (Partek Incorporated, Singapore). Reads were normalized to reads per million reads.

miRNAs identified with at least two reads were used for further analysis. Sequences were also mapped to Ensembl Release 74 to identify other non-coding and coding RNA species.

3.7   Bioinformatics (Papers I and II)

The clean sequences generated from the Ion Torrent sequencer were assessed by FASTQC for quality of reads [335]. Files were aligned to the human genome (HG19) using Torrent Suite and transferred to the Partek Genomic Suite and Flow (Partek Incorporated, Singapore) for mapping against miRBase V.21 and Ensembl Release 75 to identify miRNA and non-coding and coding RNA species. Reads were normalized to reads per million reads. miRNAs identified with at least 10 reads were used for further analysis in the Partek Genomic Suite, which included statistical analysis and hierarchical clustering.

Files were also trimmed using PRINSEQ-lite (the minimum fragment length = 10 and Q = 20) [336]. Reads were then aligned using the STAR 2-pass alignment [337] and were counted using HTSeq [338] with the Gencode V19 annotation [339]. Differential expression of aligned ncRNA was analyzed using DESeq2 [340].

Reads per million reads was normalized by quantile normalization in MATLAB (version 2010a) to compare the miRNA profiles among EV types and cells. Correlation among the sample types was performed using MATLAB. The gene expression distribution was modeled into a two-component Gaussian mixture distribution for a uniform cut-off using the gmdistribution function in MATLAB [341, 342]. All statistical analysis was performed using MATLAB.

3.8   Immunoblotting (Papers I, II, and III)

Cells, EVs, and NVs were lysed in RIPA buffer by sonicating for 5 minutes three times with vortexing between each sonication. For cells, the debris was pelleted at 14,500 x g for 5 min and the supernatant was collected for further application. The protein concentration was determined with the BCA Protein assay kit (Pierce, Thermo Scientific, Rockford, IL, USA). For each western blot, 30–50 µg of lysed samples was loaded into the wells of 10% gels.

Proteins were resolved on 10% SDS-PAGE gels using the loading buffer with reducing agent, transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA), blocked with 5% non-fat dry milk (Bio-Rad Laboratories) for one hour, and then washed three times with washing buffer. The membranes were incubated with primary antibodies against Nucleoporin-p62 (1:1000 dilution), BCL-2 (1:1,000; from Santa Cruz

Biotechnology, Dallas, TX, USA) for **paper I,** TSG101 (1:1,000; clone 4A10; Abcam, Cambridge, UK) for **papers I and II**, Melan-A (1:1000) and CD81 (1:1000) from Santa Cruz Biotechnology for **paper II**, β-Actin (1:1000) from Cell Signalling Technology (Danvers, MA, USA), for **papers II and III,** BRAFV600E (1:1000) for **paper II,** Flotillin-1 (1:1000) for **papers I and III,** Calnexin (1:1000) for **papers I, II and III,** PDGFR (1:1000), CD9 (1:1000), c-Myc (1:1,000) from Santa Cruz Biotechnology, cleaved PARP (Asp214) (Mouse Specific), cleaved Caspase 3 (Asp175) (1:1,000; CST), GFP (1:1000), and α-Tubulin (1:1000) all for **paper III** at 4°C overnight. The membranes were washed three times in TBST buffer for five minutes and incubated with secondary antibodies for one to two hours at room temperature. The membranes were washed again three times for five minutes in TBST buffer. The secondary antibodies were detected with the ECL Prime Western Blotting Detection (GE HealthCare) and analyzed on a VersaDoc 4000 MP (Bio-Rad Laboratories).

3.9   EV and NV characterization

3.9.1   Cytospins (Paper I)

ABs (~50 μg) were pre-dissolved in 100 μl of PBS for equal loading. At the start, the filters for the cytospins were pre-wet with PBS. The samples were loaded onto the fixed filters having glass slides in the bucket of the CYTOSPIN 3 rotor, and the samples were run at maximum speed for 25 minutes in the CYTOSPIN 3 for pelleting the ABs. The samples were methanol fixed and stained with Giemsa for 20 minutes and air-dried. The samples were imaged on an Axioplan 2 microscope (Carl Zeiss Jena GmbH, Eching, Germany) with a 100× oil immersion lens.

3.9.2   Transmission electron microscopy (Papers I and III)

Ten micrograms of MVs, EXOs, and NVs were dissolved in equal volumes of PBS and loaded onto Formvar/carbon-coated grids (Ted Pella Inc., Redding, CA, USA) that had been UV treated for 15 minutes. The samples were incubated on the grids and then fixed in 2.5% glutaraldehyde and contrasted in 2% uranyl acetate. Samples were examined with a LEO 912AB Omega electron microscope (Carl Zeiss NTS, Jena, Germany). The size of EVs were measured with the iTEM® software (Olympus-SiS, Münster, Germany).

3.9.3 Size analysis (Paper III)

NVs (5 µg/ml total protein) were dispersed in equal volumes of PBS, and NTA and DLS (Nano ZS, Malvern) were used to determine the size of the NVs. The zeta potential was also measured using DLS to evaluate the charge distribution over the NVs.

3.10 Quantitative PCR (Papers I, II, and III)

Total RNA was assessed for its quality using a Bioanalyzer. Total RNA from cultured cells and EVs was treated with DNase (Ambion) to remove genomic DNA contamination from the samples. For validation of miRNAs in cells and tissues and in EVs from cells and PDX (**paper I and II**), 5 ng/µl of cDNA was prepared using the Universal cDNA Synthesis Kit (Exiqon) to make the final volume 10 µl. The diluted cDNA was then used for qRT-PCR using the PCR starter kit. *C. elegans* miR-39-3p was used as a normalizing control. The UniSp6 RNA Spike-in template was also converted to cDNA and amplified using the UniSp6 RNA Spike-in control primer set as an internal control. Furthermore, miR-103a-3p was used as a positive control for cells, which was provided in the starter kit. The plate was run on a Bio-Rad CFX96 (Bio-Rad Laboratories, Inc) real-time detection system for 40 cycles following the provided protocol. Data were analyzed by using the Ct values exported from the CFX manager software. ΔΔCt values were plotted as fold change differences between the treated and non-treated groups.

cDNA was also synthesized using ExiLERATE Starter Kit (Exiqon). Following manufacturing instructions, diluted cDNA was used further to perform qPCR using specific set of gene primers to check the targeting efficiency by exosomes enriched miR-211 in recipient cells. The primers used are as follows a) TRPM1: *for* – TGCGAAGGCTGCTGGAAA,
rev – CAAGACGATGGACACCACGTTAGG
b) NUAK1: *for* – ATATACTGCTCGATGACAACTGC,
rev – GCATAGAGTGGACTCCCACAAAA
c) TRP1: *for* – GGGAGACCAGCAGTGCAGCG,
rev – CCGTGGGAGCACTGTAACCTTC
d) KCNMA1: *for* – GCACACGGTCCACAGGTACTT,
rev – GCCTCCTCCATGGTGACTTTC
e) IGF2R: *for* – ACTCCTTGCTTAGCGGACAA,
rev – TCGCTGGTCCTTAACAGCTT
f) GAPDH: *for* – TCCCTGAGCTGAACGGGAAG,
rev – GGAGGAGTGGGTGTCGCTGT. Data were analyzed using relative

expression normalized with endogenous control GAPDH; by using the ΔΔCt values exporting Ct values from the CFX manager software (BIORAD).

For **paper III,** two different total RNA samples were prepared from cultured cells, and cDNA was prepared from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was performed using KAPA SYBR FAST ABI Prism 2X qPCR Master Mix (Kapa Biosystems). Data analysis was performed by comparing ΔΔCt values using ubiquitin as the reference gene and with a control sample set for measuring relative expression. For real-time PCR against GFP and GAPDH, total RNA was amplified with the Perkin-Elmer Gene Amp PCR system 2400 and One Step SYBR RT-PCR Kit (TaKaRa Bio) using a Bio-Rad CFX96 real-time detection system and LightCycler 2.0 PCR system (Roche Diagnostics). The comparative Ct method was used for relative quantification of target gene expression against beta-actin as the control.

3.11   BrdU cell proliferation assay (Paper II)

Cell proliferation was determined with a BrdU labeling ELISA kit (Calbiochem, San Diego, CA, USA) following the manufacturer's protocol. Briefly, MeWo cells were seeded at a density of 5000 cells/well in a 96-well plate and incubated overnight to allow cells to adhere to the plate. Cells received BrdU (20 μl) 4 h prior to the termination and 30 μg/ml of treated or non-treated EXOs. After 24 h or 48 h of incubation, the cells were fixed and washed following the manufacturing protocol and the absorbance was measured on a dual wavelength (450 nm and 595 nm) spectrophotometer (Spectra Max; Molecular Devices, Sunnyvale, CA, USA).

3.12 NV uptake (Paper III)

The NVs derived from shRNA-transduced NIH3T3 cells were used for labeling with the PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma-Aldrich) according to the manufacturer's protocol, with minor modifications in the washing process as described previously [22]. A total of 20 μg of PKH67-labelled NVs or the same volume of PKH67-PBS control was added to λ820 cells and incubated for 12 hours at 37ºC. The uptake of NVs in the λ820 cells was analyzed with a flow cytometer and visualized with a confocal microscope (LSM 700 Carl Zeiss). For flow cytometry, the cells were washed twice with PBS, washed twice with 1% FBS in PBS, and analyzed with the FlowJo software. For confocal microscopy, the cells were washed twice with PBS, fixed with 4%

formaldehyde for 15 minutes, and washed twice with PBS. The samples were then pelleted with the cytospin. The cells on the glass slide were mounted using the mounting solution (ProLong® Gold Antifade Mountant) with DAPI (Thermo Fisher Scientific, Inc.) to label the nuclei blue.

3.13 NV treatment (Paper III)

3.13.1   In vitro treatment of HUVECs with NVs

GFP-expressing HMEC-1 cells were grown to a density of 3×10^4 cells on a gelatin-coated cover glass in a 24 well plate. TNF-α (10 ng/ml, R&D systems) was added for 16 h. The siRNA-loaded NVs or other samples were added for 1 h with 10% FBS-containing medium and then exchanged with new medium. After 12 h, NVs were added again and allowed to incubate for another 12 h. Cells were washed once with PBS, and the total RNA was isolated with the RNeasy Mini Kit (Qiagen).

3.13.2   In vitro treatment of λ820 cells with NVs

The NVs from NIH3T3 cells were used to treat λ820 cells three times for 12 hours. In brief, 20 µg/ml of NVs was used to treat the lymphoma cells for all experiments, including qPCR, western blot, and cell counting.

3.14 siRNA quantification (Paper III)

To quantify the siRNA, NVs were loaded with FITC-labeled siRNA. The siRNA-loaded NVs (100 µl) were placed into the wells of 96-well plates, and the FITC fluorescence was detected using a Wallac 1420 VICTOR plate reader (Perkin-Elmer Life Sciences) with excitation/emission at 488 nm/530 nm.

3.15 Statistical analysis (Papers I, II and III)

To determine the statistical significance of the RNA and protein contents, rRNA ratios between the treated and non-treated groups were compared, and a 2-tailed Student's paired *t*-test was used to calculate *p*-values. Student's paired *t*-test was used for *in vitro* qPCR analysis, and an unpaired Mann–Whitney U-test was performed for *in vivo* qPCR analysis. The data were shown as the mean ± SD, and $p < 0.05$ was considered to be significant. All analyses were performed with GraphPad Prism 6.0 (GraphPad Software) and Microsoft Excel spreadsheets.

4 RESULTS AND DISCUSSION

This section presents and discusses the main findings of this thesis. For detailed information, please refer to **papers I, II, and III**.

4.1 EVs contain diverse RNA molecules with distinct RNA profiles

The discovery of RNA in EVs has led to further investigations of different subsets of vesicles obtained using differential centrifugation. Recently, Crescitelli *et al* demonstrated that different subsets of vesicles, including ABs, MVs, and EXOs, show distinct RNA profiles [26]. They also characterized these EV subsets with electron microscopy to view the vesicular structures. In the work presented in this thesis, we have aimed to determine the RNA contents in these subsets of vesicles released by the melanoma cells.

ABs, MVs, and EXOs were isolated the MML-1 cell model of melanoma harboring the BRAF^{V600E} mutation using a previously published and validated sequential centrifugation protocol [26]. Characterization of EVs by western blot using several markers showed distinct profiles. These vesicles were positive for Tsg101 and flotillin-1, but calnexin, an endoplasmic reticulum marker, was only detected in ABs, not in MVs or EXOs, suggesting that the EXO fraction was derived from the endosome and not from the endoplasmic reticulum. The mitochondrial marker Bcl-2 and the nuclear marker nucleoporin p62 were absent in MVs and EXOs but expressed in ABs, which is similar to previously published data [86]. These results show that our protocol for isolating EXOs results in no contamination of nuclear, mitochondrial, or endoplasmic reticulum membrane components, whereas the other two EV subsets seem to contain such markers.

The presence of different types of vesicles in the different EV isolates was also confirmed by electron microscopy (Figure 4A). ABs showed larger vesicular structures as viewed by Giemsa staining, and MVs and EXOs also showed characteristic vesicular structures as demonstrated with electron microscopy (Figure 4A). RNA profiling using the Bioanalyzer showed the presence of distinct 18S and 28S rRNA peaks with relatively moderate levels of small RNA in ABs and MVs (Figure 4B). This supports the data suggesting that the ABs and MVs isolated with differential centrifugations showed distinct RNA profiles harboring rRNA
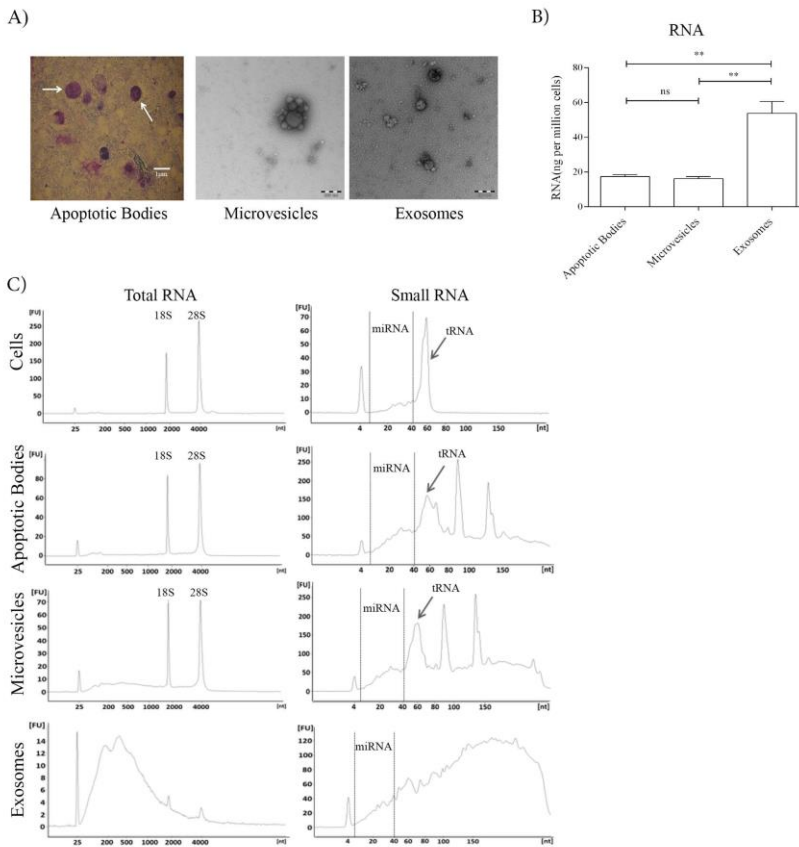
Figure 4. Characterization of EVs and RNA profiles*. A) Characterization of EVs by Giemsa staining (Cytospins) for ABs and transmission electron microscopy for MVs and EXOs. B) Total RNA and small RNA profiling using a Bioanalyzer showing distinct rRNAs in ABs and MVs and no rRNA in EXOs. C) Representation of the RNA content in subsets of EVs normalized per million cells (n = 3). Data are presented as mean ± SEM. **p < 0.01, ns- non-significant.*

compared to the EXOs that lack rRNA [26]. This supports the many findings that EXOs lack rRNA but show enrichment of other RNAs compared to other vesicular types. When comparing the RNA content, we also found that EXOs contained significant amounts of RNA as compared to ABs and MVs indicating that these tiny packets contain much more genetic information than other vesicular subsets (Figure 4C).

To evaluate the repertoire of diverse RNA species, we performed small RNA sequencing in the cells and subsets of EVs derived from MML-1 melanoma cells. We detected the presence of several ncRNAs in these subsets that were annotated and mapped to the human genome (HG19). The ncRNA species

identified in the sequencing analysis included snRNA, snoRNA, miRNA, tRNA, vault RNA, Y RNA, mitochondrial associated RNA, and many other ncRNAs. In addition to this, there were several reads that were not mapped and aligned to the genome indicating that there is a lot yet to discover about RNA species in these EV subsets. Differential enrichment of ncRNA showed a large difference for several RNA species between the cells and EV subtypes. Similarly, there were many new findings with respect to ncRNAs in EVs that have been published recently [85, 87, 315, 316].

Analysis of the miRNA in these EV subsets showed that around 1041 miRNAs were found in all of the biological samples. By comparing the overlap of 252 miRNA species between cells and EVs as determined by the integrative statistical hypothesis-testing method, we found that 113 miRNAs were shared between all the samples (Figure 5). It is noteworthy
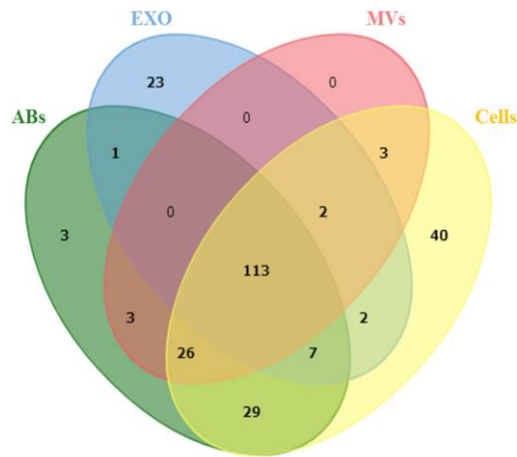
Figure 5. Overlap of miRNAs between cells and EVs. Venn diagram analysis showing uniquely identified miRNAs in different EV isolates and cells.

that EXOs contained 23 unique miRNAs that were not present in other samples indicating unique sorting of miRNAs into EXOs. These miRNAs include miR-199a-3p, miR-150-5p, miR-142-3p, miR-486-5p, and miR-223-3p, which have been shown to be associated with uveal melanoma [343], melanoma tissues [344], primary cutaneous melanoma [345], and blood samples from melanoma patients [346, 347]. Enrichment of miR-214-3p, which has been shown to play a key role in melanoma progression, was also found in EXOs [348, 349]. Furthermore, it has also been reported that miR-214 and miR-199a, which are related to melanoma progression, are found in miRNA clusters that are regulated by the transcription factor TWIST1 [350]. MVs did not have any unique miRNAs, but ABs had 3 unique miRNAs compared to cells and other EV subsets (Figure 5). Taken together, different isolates of EVs have distinct sets of RNAs, and the most highly expressed miRNAs in EXOs from the MML-1 cells play a role in melanoma progression.
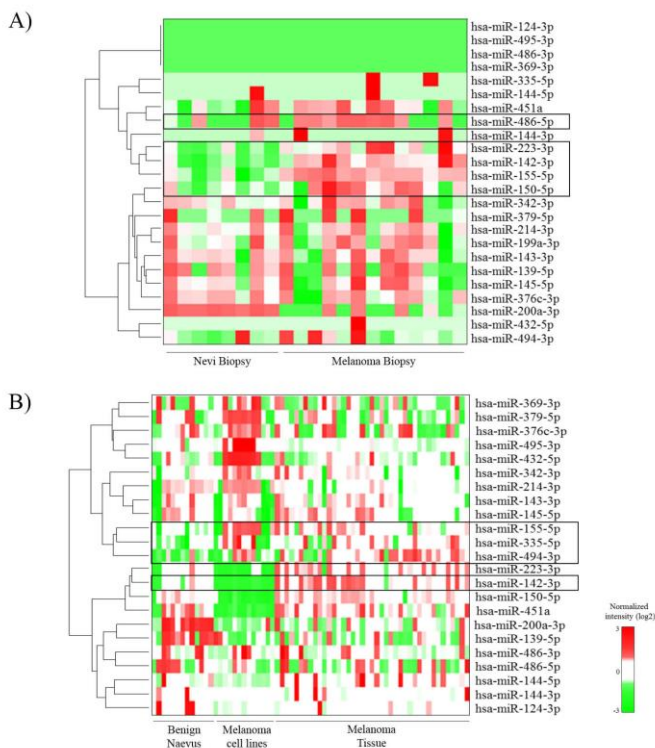
Figure 6. Comparative analysis of exosome-specific miRNA and miRNA identified previously in cell lines and melanoma tissues and biopsies*. Comparison of unique MML-1 exosomal miRNAs with databases of melanoma tissue and benign naevi miRNAs*

Next, the unique miRNAs found in EXOs were compared with clinical microarray datasets (GSE34460 and GSE35579) in the GEO database. Comparing the GSE34460 data showed differentially expressed miRNAs such as hsa-miR-142-3p, hsa-miR-150-5p, hsa-miR-155-5p, hsa-miR-223-3p, and hsa-miR-486-5p in melanoma biopsies as compared to benign naevi (Figure 6A). In the GSE35579 dataset, we additionally found differential expression of hsa-miR-335-5p and hsa-miR-494-3p in melanoma tissues and melanoma cell lines compared to benign naevi (Figure 6B). There is no documented evidence for miR-335-5p and miR-494-3p being associated with melanoma, and this suggests that these might be new melanoma-associated miRNAs. The other five miRNAs found in the clusters are known to be associated with melanoma [344, 345, 347, 351-353], thus our findings demonstrate that the miRNAs that are significantly enriched in EXOs from MML-1 cells are associated with disease progression in patients.

4.2 BRAF inhibition regulates miR-211 expression in melanoma EVs and has functional consequences.

The next question was to test if the RNA cargo in subsets of EVs changes upon treatment with vemurafenib (PLX4032). MML-1 melanoma cells were treated with 200 nM vemurafenib as determined by the dose response curve. Treatment of melanoma cells with vemurafenib reduced the cell growth by 50%. The RNA and protein contents were analyzed in the EVs isolated with and without vemurafenib treatment. The RNA and protein contents in EVs from treated cells were significantly greater compared to the EVs from the non-treated cells, and the RNA content in EXOs was much higher compared to the other vesicle subsets as shown previously by Lunavat *et al* [124]. However, the RNA/protein ratio was not increased in the EV subsets after vemurafenib treatment, and this further argues against increased loading of RNA into EVs.

Characterization of EVs using several markers showed enrichment of TSG101 and CD81 in EXOs from both treated and non-treated cells. EXOs from treated cells showed enrichment of melan-A/MART-1, which is a known melanoma marker, although this marker was also present in the other EV subsets. Eldh *et al* showed the presence of melan-A in EXOs isolated from melanoma metastasis liver perfusates from uveal melanoma patients [354] indicating the presence of melanoma-derived EXOs that are released into the circulation. Total RNA profiling of EV subsets using nano chips also showed degradation of rRNA in ABs and MVs isolated from vemurafenib-treated cells compared to ABs and MVs from non-treated cells. EXOs did not show any difference in the RNA profile between treated and non-treated cells. In addition, small RNA analysis did not show any major difference in the profiles except for the miRNA region in the Bioanalyzer trace that was slightly higher in ABs and MVs from treated cells compared to those from untreated cells.

Given that the cells release greater quantities of EV subsets after BRAF inhibition, we hypothesized that vemurafenib treatment would alter the RNA cargo in these EV subsets. Indeed, small RNA sequencing of EVs from treated and non-treated cells showed a distinct repertoire of ncRNAs between the EV subsets. We found Y RNA, snRNA, tRNA, snoRNA, rRNA, lincRNA, piwiRNA, miRNA, and mRNA fragments in all of the samples, including cells. Comparing the sequencing reads between the EV types, we found that EXOs contained a high percentage of miRNA reads compared to the ABs and MVs. In addition to this, the EXOs carried a unique set of

snoRNA clusters compared to those from the cells, ABs, and MVs again suggesting that EXOs carry much more information compared to other EV subtypes. Furthermore, a larger proportion of tRNA was found to be present in EVs compared to cells, showing that the EVs encapsulate a majority of the tRNA that is processed in the cytoplasm of the EV-producing cell. A few studies have also shown the presence of tRNA fragments in neuronal cells [86], plasma EXOs [315], immune cells [85], and semen [87]. This suggests that tRNA is enriched in EVs derived from many different cell types and that this tRNA might have potential functions in normal physiological processes.

Sequencing analysis revealed distinct miRNAs in the vesicles from vemurafenib-treated cells, and we found that several miRNAs were differentially regulated in the EV subsets as well as in the cells after the treatment. Moreover, a Venn diagram analysis showed several unique miRNAs that were present in the EXOs from treated cells compared to EXOs from non-treated cells. In addition, hierarchical clustering of differentially expressed miRNAs revealed unique sets of clusters between cells and EXOs and between the treatments. This indicates that BRAF inhibition regulates the miRNA loading in the subsets of EVs. Validation of the miRNAs by qPCR showed that miR-211 was significantly upregulated in the treated cells as well as in the ABs, MVs, and EXOs derived from the cells. To further test this upregulation *in vivo*, we transplanted patient melanoma cells harboring BRAF^{V600K} mutations and MML-1 melanoma cells harboring BRAF^{V600E} mutations and generated a xenograft platform in NOG mice [334]. The mice were randomized into two groups when the tumor volume reached about 100–150 mm^3. Three days after the treatment, a significant reduction in tumor size was observed. After sacrificing the mice, we harvested the tumors, confirmed their reduced size, dispersed them into single cells, and expanded the cells *in vitro* as a monolayer cell suspension as shown in the manuscript. The EVs that were released during this 24 h culture were used for RNA isolation and subsequent qPCR analyses. There was a significant upregulation of miR-211-5p in cells and EVs after vemurafenib treatment. These results were all generated in cell lines that have been in culture for decades, and therefore these results need to be confirmed *in vivo* under physiologically relevant conditions. In addition to this, we also observed differential expression of miR-15b-5p, miR-574-3p, and miR-16-5p in MML-1 xenografts and in the PDX model, although the validation of these miRNAs was not evident in all settings.

Next we wanted to test whether EXOs harvested from BRAF^{V600E} mutated cells have functional effects on wild-type BRAF cells. We chose the MeWo melanoma cell line, which carries NF1 mutations. Treatment of wild-type

BRAF cells with EXOs from non-treated cells showed a significant increase in proliferation at 24 h and 48 h using a BrdU assay and cell counting. In contrast, treatment of MeWo cells with EXOs from vemurafenib-treated cells significantly enhanced proliferation at 24 h, similar to that of EXOs from untreated cells, but this response was attenuated at 48 h. This kind of blunted effect could be due to the release of their molecular contents after the uptake of EXOs from treated cells, which have significant enrichment of miR-211. The release of miR-211 in the cells thus targets genes that are known to be involved in proliferation and metastasis.

To further determine that these vesicles enriched in miR-211 indeed have some targeting molecules, we performed a literature search and selected experimentally validated miR-211 interactomes [355, 356]. We found that incubating MeWo cells with EXOs from vemurafenib-treated cells showed significant downregulation of KCNMA1 and IGF2R genes, which are known miR-211 targets and are known to play roles in tumor progression [357, 358]. Other targets such as TRP1, TRPM, and NUAK1 were not affected by the EXOs. Incubation of MeWo cells with synthetic miR-211 resulted in a similar response, and this supports the results obtained with the EXOs.

In this study, we showed that inhibition of BRAF mutations with vemurafenib (PLX4032) is associated with increased secretion of EVs from melanoma cells and increased RNA and protein cargo within the EXOs. The fact that secreted EVs shuttle significant amounts of functional proteins and biologically active RNA cargo between cells might be an important clue to unraveling many of the mysteries of melanoma progression. We also observed increased secretion of miRNAs, specifically miR-211, which might be very important for melanoma progression. Further, we showed that EXOs with altered miRNA cargo influence the expected target gene products in recipient wild type cells. Our findings of significant changes in RNA cargo in subsets of EVs with targeted oncogene mutation treatment might indeed explain why these treatments only have temporary effects. Until now, no one has analyzed the effects of vemurafenib treatment on BRAF mutant melanoma cells and the molecular contents in the EVs that are secreted from these cells. Our data provide the starting point for further studies on the role of EVs in malignant melanoma undergoing treatment with targeted therapies because these vesicles might have significant regulatory effects on disease progression and metastasis.

4.3   Therapeutic implications of EXO-mimetic NVs

To develop efficient RNA-based therapeutics, it is crucial to develop delivery vehicles that help the siRNA reach the cell cytoplasm. Naturally released EXOs have been proposed as possible RNAi carriers, but the yield of EXOs is small in cell culture systems. It has been shown previously that EXO-mimetic NVs generated by serial extrusions of cells through nano-sized filters gives 100-fold higher yields than isolating EXOs from cell culture [233]. In this study, we generated NVs that were endogenously and exogenously loaded with siRNA.

In the first model system, NVs were generated from the monocytic cell line U937 by serial extrusion as described previously [233]. The NVs were mixed with different concentrations of FITC-labeled siRNA using electroporation. The results indicated that the siRNA loading in NVs was directly proportional to the siRNA concentration and electroporation voltage, and 20 μM of siRNA at 200 V gave the most efficient loading into the NVs. It has been suggested that electroporation of siRNA into EXOs might cause aggregation of siRNA that might co-pellet with EXOs during the isolation process [359]. To address this further, electroporation of siRNA was performed with or without NVs, and the NVs were purified by Optiprep density gradient ultracentrifugation to determine whether any such aggregates end up at the same density as the NVs. As expected, siRNA fluorescence was observed only at the interface of 10% and 50% OptiPrep after electroporation in the presence of NVs, which suggests that the siRNA does not aggregate with NVs during purification.

DLS analysis showed that NVs both with and without siRNA had similar size distributions of around 150 nm. In addition, it has previously been reported that siRNAs carry a net negative charge due to their phosphate backbone and thus the zeta potential changes to a more negative charge [360]. The zeta potential of NVs with and without siRNA also remain unchanged, which again suggests that the siRNA is encapsulated within the NVs.

To further determine the function of siRNA-loaded NVs, we used activated endothelial cells that we have previously shown to readily take up NVs derived from monocytes [233]. Activation of endothelial cells by TNF-α induces the expression of cell adhesion molecules and promotes monocyte adhesion [361], and this activation leads to the efficient uptake of monocyte-derived siRNA-loaded NVs in HMEC-1 cells expressing GFP. [U937]NV[GFP] siRNA suppressed the GFP expression in a dose-dependent manner, and this effect was greater in TNF-α-treated HMEC-1 cells when measuring the

fluorescence intensity, mRNA levels, and protein levels. In contrast, scrambled siRNA-loaded NVs did not show any change in the expression of GFP. Clathrin-mediated and caveolin-mediated endocytic pathway inhibitors (monodansyl cadaverine and Filipin, respectively) attenuated the [U937]NV[GFP] siRNA-mediated GFP gene knockdown suggesting that the uptake of NVs occurs through both clathrin and caveolin-mediated endocytosis. Overall, this model of exogenous loading of siRNAs is efficient in delivering the cargo and down regulating the target gene expression in recipient cells.

Another model that was developed was the use of endogenous expression of shRNA against human cMyc in mouse fibroblasts. First, different clones of shRNA against cMyc were tested in mouse fibroblast cells infected with human cMyc. The efficiency of these clones was tested by measuring the active proliferation rate of the mouse fibroblasts and the levels of human cMyc expressed in these cells. Only two clones of shRNA (cMyc #12 and cMyc #4) along with scrambled shRNA (non-template control: NTC) were used for further generation of NVs.

The NVs produced by serial extrusions were purified with an Optiprep density gradient using 10% and 50% gradient concentrations. NTA revealed a size distribution of around 180–200 nm, and transmission electron microscopy indicated vesicular structures with lipid bilayer membranes. Western blot analysis showed the presence of Flotillin-1 and CD9 as exosomal markers that were enriched in NV fractions compared to the donor cells. In addition, calnexin, which is an endoplasmic reticulum marker, was present in both cells and NVs indicating that the NVs could be derived from multiple membrane compartments of the cells. Also, NVs showed characteristic enrichment of PDGFR, which is one of the primary protein molecules associated with the plasma membrane of the parent cells. This suggests that the NVs mimic EXOs in several ways, including protein characteristics and size distribution as previously described [233].

Further experiments were performed to determine if [NIH3T3]NVs could be taken up by λ820 lymphoma cells. To test this, the NVs were labeled with Pkh67 lipid dye and incubated with λ820 cells for 12 h. Confocal imaging confirmed the uptake of all of the NVs derived from NIH3T3 cells into the λ820 cells. In addition, flow cytometry confirmed the efficient uptake of these NVs in the lymphoma cells. This suggests that NVs can be taken up in a similar fashion as EXOs or other naturally occurring EVs [362-364].

To test if the NVs derived from endogenously expressing shRNA in the cells has a functional therapeutic effect in lymphoma cells, we treated λ820 cells

with NVs three times every 12 h and isolated the protein and RNA for further analysis. The c-Myc expression, as analyzed by qPCR, was significantly reduced relative to mouse ubiquitin after treating with $^{NIH3T3}NV_{c\text{-Myc \#4}}$ and $^{NIH3T3}NV_{c\text{-Myc \#12}}$ compared to $^{NIH3T3}NV_{WT}$ and $^{NIH3T3}NV_{NTC}$. This suggests that the siRNA-loaded NVs are able to down-regulate human cMyc expression in recipient cells. In addition, the protein levels of Myc were reduced in $^{NIH3T3}NV_{c\text{-Myc \#12}}$-treated lymphoma cells compared to $^{NIH3T3}NV_{WT}$ and $^{NIH3T3}NV_{NTC}$. Treatment of these lymphoma cells also activated the apoptosis pathways by inducing the expression of cleaved PARP and cleaved caspase-3 in $^{NIH3T3}NV_{c\text{-Myc \#12}}$-treated cells. In addition, treatment of lymphoma cells with NVs showed significantly lower cell counts as analyzed by trypan blue exclusion assay. This supports the conclusion that treatment with NVs containing c-Myc-targeting siRNA can function as therapeutic vesicles targeting diseases associated with c-Myc over-expression, including cancer.

Overall, this strategy of NV generation and loading with oncogenic inhibitors could potentially be used in developing therapeutics. NVs are similar in size to EXOs, and these bio-engineered and bio-inspired NVs show promise for developing various clinical applications for targeted drug delivery.

5 CONCLUSION

Paper I: EVs derived from melanoma cells, including ABs, MVs, and EXOs, show distinct RNA profiles with significantly different RNA contents in EXOs compared to ABs and MVs. Small RNA sequencing reveals unique miRNA signatures in EXOs that are not found in ABs and MVs. These miRNA signatures unique to EXOs are differentially expressed in melanoma tissues compared to benign naevi. This study provides unique insights into the contribution of EV-associated extracellular RNA in cancer.

Paper II: Vemurafenib treatment of melanoma cells significantly increases the RNA and protein content in EVs compared to the non-treated cell-derived EVs. Further, the BRAF inhibition by vemurafenib affects miRNA loading in the subsets of EVs. Vemurafenib treatment leads to significant upregulation of miR-211 in cells and EVs both *in vitro* and *in vivo*, and EXOs derived from vemurafenib-treated cells attenuate the growth of BRAF^{WT} melanoma cells by down-regulating the KCNMA1 and IGF2R genes, which are known to play key roles in cancer progression. Overall, treatment with the BRAF inhibitor vemurafenib induces miR-211 upregulation in melanoma cells and xenografts and their subsequent subsets of EVs, and this has potential for use as a biomarker of response in patients suffering from BRAF mutant melanoma.

Paper III: EXO-mimetic NVs are efficiently loaded with siRNA both endogenously and exogenously. The siRNA can then be efficiently delivered to the recipient cells and can suppress the target gene expression regardless of which approach is used. EXO-mimetic NVs have several advantages as delivery vehicles, including high yield during production compared to EXOs as well as the possibility of producing them from engineered cells expressing specific surface molecules. This technology thus provides a valuable platform for siRNA delivery vehicles that could be applied to treat numerous diseases.

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APPENDIX