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Exosome Research
Biochemistry, Biomarkers and Perspectives in Therapy

Edited by Sergey Sedykh

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Physiology

Volume 33

Aims and Scope of the Series

Modern physiology requires a comprehensive understanding of the integration of tissues and organs throughout the mammalian body, including the cooperation between structure and function at the cellular and molecular levels governed by gene and protein expression. While a daunting task, learning is facilitated by identifying common and effective signaling pathways mediated by a variety of factors employed by nature to preserve and sustain homeostatic life. As a leading example, the cellular interaction between intracellular concentration of Ca+2 increases, and changes in plasma membrane potential is integral for coordinating blood flow, governing the exocytosis of neurotransmitters, and modulating gene expression and cell effector secretory functions. Furthermore, in this manner, understanding the systemic interaction between the cardiovascular and nervous systems has become more important than ever as human populations' life prolongation, aging and mechanisms of cellular oxidative signaling are utilised for sustaining life. Altogether, physiological research enables our identification of distinct and precise points of transition from health to the development of multimorbidity throughout the inevitable aging disorders (e.g., diabetes, hypertension, chronic kidney disease, heart failure, peptic ulcer, inflammatory bowel disease, age-related macular degeneration, cancer). With consideration of all organ systems (e.g., brain, heart, lung, gut, skeletal and smooth muscle, liver, pancreas, kidney, eye) and the interactions thereof, this Physiology Series will address the goals of resolving (1) Aging physiology and chronic disease progression (2) Examination of key cellular pathways as they relate to calcium, oxidative stress, and electrical signaling, and (3) how changes in plasma membrane produced by lipid peroxidation products can affect aging physiology, covering new research in the area of cell, human, plant and animal physiology.

Meet the Series Editor

Prof. Dr. Thomas Brzozowski works as a professor of Human Physiology and is currently a Chairman at the Department of Physiology and is V-Dean of the Medical Faculty at Jagiellonian University Medical College, Cracow, Poland. His primary area of interest is physiology and pathophysiology of the gastrointestinal (GI) tract, with a major focus on the mechanism of GI mucosal defense, protection, and ulcer healing. He was a postdoctoral NIH fellow at the University of California and the Gastroenterology VA Medical Center, Irvine, Long Beach, CA, USA, and at the Gastroenterology Clinics Erlangen-Nuremberg and Munster in Germany. He has published 290 original articles in some of the most prestigious scientific journals and seven book chapters on the pathophysiology of the GI tract, gastroprotection, ulcer healing, drug therapy of peptic ulcers, hormonal regulation of the gut, and inflammatory bowel disease.

Meet the Volume Editor

Sergey Sedykh, Ph.D. in Biochemistry, graduated from the Faculty of Natural Sciences of Novosibirsk State University in 2008. His Ph.D. thesis focused on the polyreactivity of human milk antibodies. He has published more than 50 papers in journals indexed by Web of Science and Scopus, including more than 40 in the last 5 years. These publications have been cited more than 1,300 times. He is an active educator, teaching at the Faculty of Medicine and the Faculty of Natural Sciences of Novosibirsk State University. He has managed several projects supported by the Russian Foundation for Basic Research, the Russian Science Foundation, and other funding agencies. His research interests include the natural supramolecular structures of milk.

Contents

Preface **XI**

Section 1
Natural Exosomes in Health and Disease **1**

Chapter 1 **3**
Introductory Chapter: Exosomes – Its Structure, Biogenesis, Isolation, and Biological Functions
by Sergey Sedykh

Chapter 2 **11**
Exosomes in Diabetes, Cancer, and Inflammation Where Oxidative Stress Plays an Active Role
by Gözde Atila Uslu and Hamit Uslu

Chapter 3 **29**
Extracellular Vesicles in Hematological Malignancies
by Samir Zuberi and Jihane Khalife

Chapter 4 **59**
From Diagnosis to Prognosis: The Transformative Impact of Exosomal ncRNAs in Head and Neck Cancer
by Aditi Patel, Kanisha Shah, Vivek Tanavde and Shanaya Patel

Chapter 5 **75**
Harnessing the Therapeutic Potential of Mesenchymal Stem Cell-Derived Exosomes in Prostate Cancer: Current Insight and Perspective
by Mafewu Olga Raboshakga, Anh Duy Do, Ida Kurniawati, Chia-Ling Hsieh and Shian-Ying Sung

Section 2
Delivery of Therapeutic Molecules **103**

Chapter 6 **105**
Exosome-Based Drug Delivery Systems
by Ruotong Huang, Jianming Zhou and Shuying Chen

Chapter 7 **133**
Cytostatic Drugs Delivery to MCF-7 Cells Using Horse Milk Exosomes
by Anna Timofeeva, Victoria Cherenko, Sergey Sedykh and Georgy Nevinsky

Preface

Dear reader!

You are holding in your hands the book "Exosome Research – Biochemistry, Biomarkers and Perspectives in Therapy". The authors present reviews and original research on exosomes, natural extracellular vesicles. The study of exosomes has a history spanning several decades, but has developed particularly rapidly in the last twenty years. New methods that have enabled the isolation, study, and loading of these vesicles with therapeutically important molecules have developed alongside other fields of the natural sciences. The study of extracellular structures and the mechanisms by which they originate from intracellular structures has become one of the drivers of modern cell biology and physicochemical methods for studying cells.

This book is divided into two sections. The first section is devoted to natural exosomes in health and disease. In the first chapter, I briefly discuss the structure and biogenesis of exosomes, their isolation methods, and biological functions. In the second chapter, Gözde Atila Uslu and Hamit Uslu examine the role of exosomes in the development of diabetes, cancer, and inflammation, focusing on their contribution to oxidative stress. In the third chapter, Samir Zuberi and Jihane Khalife describe extracellular vesicles in hematopoietic malignancies. In the fourth chapter, Aditi Patel et al. examine the contribution of non-coding RNA exosomes to the development of head and neck tumors. In the final, fifth chapter, Mafewu Olga Raboshakga et al. describe the therapeutic potential of exosomes derived from mesenchymal stem cells for the treatment of prostate cancer.

Two chapters in the second section describe the delivery of therapeutic molecules using exosomes. In the first chapter, Ruotong Huang et al. describe exosome-based drug delivery systems. In the second chapter, Anna Timofeeva et al. examine the delivery of cytostatic drugs to MCF-7 cells using horse milk exosomes.

The number of studies on exosomes and other extracellular vesicles continues to grow each year. On behalf of the authors of this book, I welcome your interest in this topic!

I also want to express my sincere gratitude to Kristina Kardum Cvitan, who served as the Publishing Process Manager for this book.

Sincerely,

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Institute of Chemical Biology and Fundamental Medicine SB RAS,
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Section 1

Natural Exosomes in Health and Disease

Chapter 1

Introductory Chapter: Exosomes – Its Structure, Biogenesis, Isolation, and Biological Functions

Sergey Sedykh

1. Introduction

Exosomes are extracellular vesicles with a specific morphology. They are 40–120 nm in diameter and are secreted by cells from multivesicular bodies. Exosomes are distinguished from other extracellular vesicles, such as microvesicles (100–1000 nm in diameter) and apoptotic bodies (over 1000 nm in diameter), by specific proteins, lipids, nucleic acids, and other biomolecules [1]. The specific proteins and nucleic acids found in exosomes can serve as diagnostic markers for various diseases, primarily oncological ones, in a process known as liquid biopsy [2].

Natural exosomes are unique and universal vehicles for delivering therapeutically significant molecules for targeted delivery and treatment of various diseases. Exosomes isolated from sources, such as mesenchymal cell cultures and milk [3], are particularly important because exosomes from cancer cell cultures, blood, and urine have certain limitations in therapeutic use. Currently, no single, universal method for isolating exosomes has been proposed. Various methods and combinations of methods are used, including filtration and ultrafiltration, centrifugation and ultracentrifugation, different chromatographic approaches, and microfluidic technologies.

2. Structural features of exosomes

A distinctive feature that distinguishes exosomes from other extracellular vesicles is their bilayer phospholipid membrane [1]. Through the physical transport of various biomolecules (RNA, proteins, and possibly DNA and lipids), exosomes perform local and long-range intercellular communication, thus providing an indirect means of delivering cellular signals.

Exosome markers include the tetraspanins CD9, CD63, and CD81; the proteins ALIX, TSG101, and ESCRT; various microRNAs; and lipids such as phospholipids, phosphatidylserine, ceramides, and sphingolipids, see **Figure 1** [1, 4, 5].

Like other small extracellular vesicles, exosomes are structures whose integrity is maintained in the extracellular space. They circulate and are found in virtually all biological fluids [6]. Due to their biogenesis *via* endocytosis from multivesicular bodies, exosomes carry unique molecular signatures on their surface that are inherited from

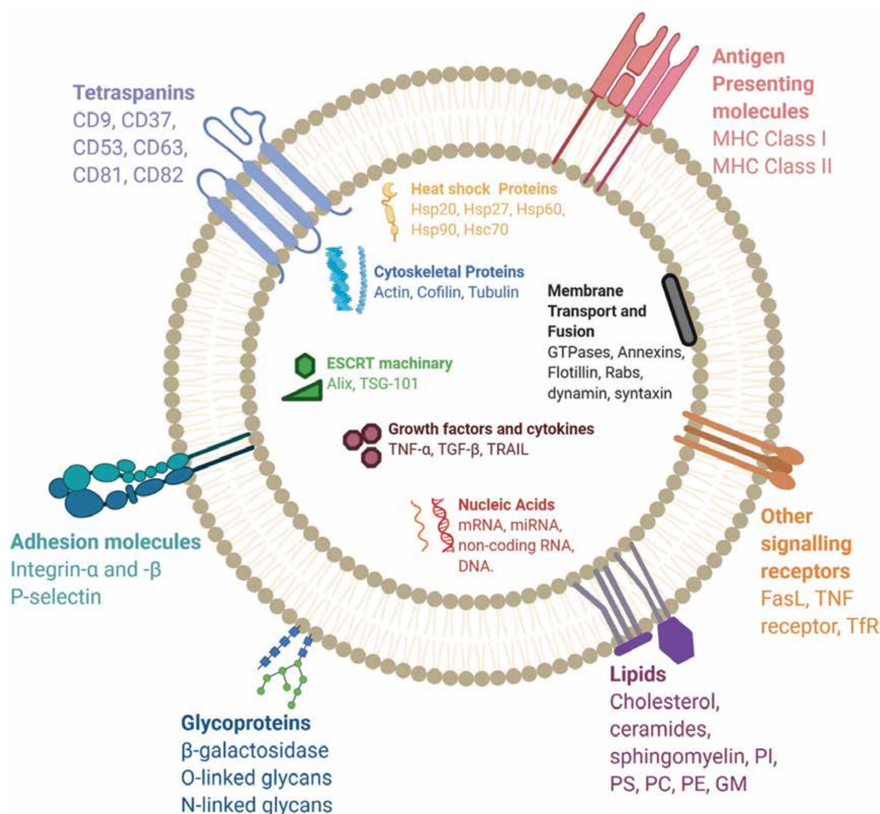

Figure 1.
Structure and biochemical components of exosomes. Hsp – heat shock proteins, ESCRT – endosomal sorting complex required for transport? PI – phosphatidylinositol, PS – phosphatidylserine, PC – phosphatidylcholine, PE – phosphatidylethanolamine, GM – gangliosides, Hsc – heat shock cognate, TSG – tumor susceptibility gene, TNF – tumor necrosis factor, TGF – transforming growth factor, TRAIL – TNF-related apoptosis-inducing ligand, FasL – fas ligand, TfR – transferrin receptor. Figure from Ref. [1].

their cell of origin. These signatures allow for the specific isolation and/or "loading" of exosomes using various physicochemical approaches [7].

Because of their origin, many exosome markers described in the literature for insufficiently purified exosomes are actually components of ribosomes, cytoplasmic proteins, and mitochondria. These markers are considered "negative" exosome markers. Recommendations for characterizing exosomes and other extracellular vesicles have been published in 2013 [8], 2018 [9], and 2023 [10]. These recommendations include requirements for the sources from which the vesicles were isolated, the presence of "positive" and "negative" structural markers, the quantitative characteristics of the drug isolated from the sample, and the mandatory presence of a double lipid membrane.

3. Methods for isolating and analyzing exosomes

A variety of basic and sensitive molecular techniques are employed in exosome analysis. Exosomes are therefore a tool for disease monitoring and the targeted delivery of therapeutically significant molecules.

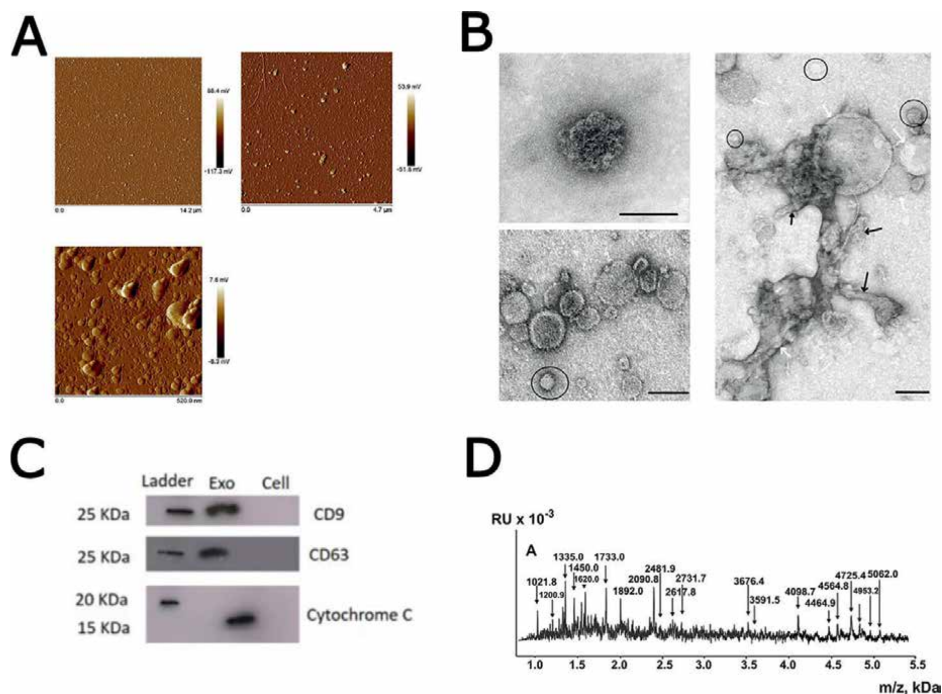

Figure 2.
Methods for milk exosome structure analysis: A – atomic force microscopy; B – transmission electron microscopy; C – Western blotting; D – MALDI-TOF-MS.

Two main methods for studying exosomes are microscopy, including transmission electron microscopy and atomic force microscopy. Two other universal approaches are used to count the number of vesicular particles in a sample: dynamic light scattering and nanotracking analysis [10] and surface plasmon resonance [5]. Protein markers are analyzed by Western blot and Electrospray ionization and Matrix-assisted laser desorption/ionization mass spectrometry. Examples of results obtained by these methods are shown in **Figure 2**. Nucleic acid content is usually analyzed using RT-qPCR and high-throughput sequencing. The Stem-Loop-RT-qPCR method deserves special mention for microRNA analysis.

The first step in exosome isolation is typically centrifugation (several steps from 100 to 10,000 × g) followed by ultracentrifugation (100,000 × g or higher) [2, 5]. However, the pellet is often incorrectly counted as exosomes because it contains not only extracellular vesicles, but also co-sedimented proteins and debris from cells and subcellular structures. Additional methods for exosome purification include gel filtration [11], precipitation, co-precipitation, and by magnetic particles containing antibodies to exosome surface marker proteins.

4. Fusion of exosomes with cell membranes: Exosomes - Pathogenesis and delivery

Exosomes can fuse directly with the membrane of recipient cells *via* macropinocytosis, phagocytosis, clathrin-dependent transport, caveolin-dependent transport, and lipid rafts mechanisms [1, 4]. Ultimately, the exosome ends up inside an early

endosome and, after several stages, in lysosomes. The contents of exosomes can appear in the cytoplasm of recipient cells, influencing their metabolism and molecular genetic processes through proteins, peptides, microRNA, RNA, and possibly DNA.

The most extensively studied role of exosomes in tumor diseases and in exosomes isolated from cancer cell cultures is their role in tumor development. They support tumor cell growth, invasion, and metastasis [2]. Along with chemokines, cytokines, small molecules, and growth factors, exosomes facilitate tumor cell communication with each other and with other cells, organs, and tissues [12].

Another well-studied source of exosomes and their potential biological functions is patients with neurodegenerative diseases, as well as the corresponding cell cultures and animal models. The transfer of toxic proteins, such as prionogenic, amyloidogenic, and α-synuclein proteins, *via* exosomes has been demonstrated in Parkinson's and Alzheimer's diseases [2].

Milk is a unique source of exosomes. It is inexpensive compared to cell culture, and its isolation is noninvasive compared to blood. Milk also has potential for delivery, unlike urine and blood, and for preparative production, unlike tears [13]. However, human milk is an unfavorable source due to its limited availability for isolation, and bovine milk may contain prions and allergens. Recently, more articles have appeared on exosomes isolated from horse and camel milk. So, delivery of therapeutically significant drugs *via* milk exosomes shows great promise.

5. Conclusions

Current trends in exosome research are: analysis of natural exosomes isolated from various sources, use of exosomes for the delivery of therapeutically important molecules, methods of exosome isolation, use of exosomes as disease markers for early diagnosis and personalized medicine, and the analysis of biomolecules specific to exosomes and other extracellular vesicles.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 2

Exosomes in Diabetes, Cancer, and Inflammation Where Oxidative Stress Plays an Active Role

Gözde Atila Uslu and Hamit Uslu

Abstract

Exosomes can be defined as membrane-enclosed extracellular vesicles produced by endosomal division secreted by almost all cells in the body. They can be produced by cells through the process of endocytosis, or they can be produced in other cells and directly enter other cells through different mechanisms. Exosome production and release, like the majority of bodily biomarkers, are influenced by a number of variables, such as radiation, oxidative stress, alterations in cellular pH, a drop in membrane cholesterol, and an increase in intracellular calcium levels. Oxidative stress is a process in which the balance between oxidants and antioxidants is disrupted in favor of oxidants, allowing uncontrolled free radicals to transform physiological conditions into pathological conditions. Exosomes, which are also crucial for intercellular communication, are strongly linked to oxidative stress, which alters cell signaling. In this review, we aim to address the changes that occur in the production and release pathways of exosomes with oxidative stress triggered by different physiopathological changes.

Keywords: oxidative stress, exosome, inflammation, cancer, reactive oxygen species

1. Introduction

The balance between oxidants and antioxidants is maintained by complex biochemical and genetic mechanisms under physiological conditions. However, uncontrolled increase in free radicals causes this balance to be disrupted in favor of oxidants, leading to the emergence of oxidative stress that transforms physiological conditions into pathological conditions. Increased and uncontrolled free radicals are a major factor in the pathophysiology of many organ and system dysfunctions such as lipid peroxidation, cell death, membrane and DNA damage, cardiovascular disorders, organ dysfunction, inflammation, sepsis, cancer, cognitive dysfunction, and cataracts.

Extracellular vesicles (EVs) are structures that form an important part of the intercellular signaling network. EVs can be divided into ectosomes and exosomes due to differences in their production mechanisms. Ectosomes are large extracellular vesicles that are 50–1000 nm in size. They are separated from cells by plasma membrane budding [1]. Microvesicles between 40 and 160 nm in size, and exosomes

are produced by endocytic cellular processes. In order to study the formation of exosomes, we must first look at how endocytic vesicles invaginate the cell membrane to form early shortening endosomes (ESEs), which then develop into late shortening endosomes (LSEs). Numerous intraluminal vesicles have been reported to accumulate within multivesicular bodies, which can then be destroyed by proteasomes, serve as a temporary storage space, or fuse with the plasma membrane to release exosomes [2–4]. The molecular load of extracellular vesicles (EVs) that act as mediators of intercellular communication varies depending on the physiological and pathological conditions. For instance, EVs released from healthy cells can transfer antioxidants to target cells, protecting them against oxidative stress. When oxidative stress occurs, EVs can also carry ROS-producing enzymes and oxidized molecules. Thus, it is necessary to clarify the function of EVs in the pathophysiology of illnesses linked to oxidative stress [5].

In this review, we summarize recent studies showing that oxidative stress induced by different inducers such as diabetes, cancer, and inflammation. can alter the release of exosomes and the changes in their components in exosomes derived from healthy cells or cells under oxidative stress, and even differences in exosomes released from immune cells and other target cells under oxidative stress.

2. Oxidative stress – diabetes and exosome

One of the most prevalent metabolic disorders, diabetes can be brought on by abnormal beta cell function, low levels of circulating insulin, and decreased insulin secretion, or by peripheral tissue insulin resistance and decreased insulin sensitivity. It has been reported that there is a close relationship between complications arising from diabetes and oxidative stress, that hyperglycemia and hyperlipidemia increase NAD and FAD levels, which in turn causes overloads in the electron transport chain and uncontrolled increases in ROS production due to the leakage of electrons from complexes I and III [6, 7]. β cells exhibit a markedly reduced antioxidant capacity in comparison with other cell types, making them more vulnerable to oxidative stress, according to a study examining the gene expression profiles of antioxidant enzymes in several cell types of human pancreatic islets. Additionally, it was found that after being exposed to oxidative stress, β cells have a poorer survival rate and more DNA damage than α cells [8]. Glucose can enter podocytes, mesangial cells, tubular cells, endothelial and immune system cells, neurons, and glial cells *via* facilitated diffusion through the insulin-independent glucose transporter; therefore, in cases of hyperglycemia, these cells may be exposed to excessive glucose load, an increase in cell workload may be observed, and cell damage may occur. Therefore, it is not surprising that the urinary system, cardiovascular system, and nervous system are the primary systems affected by diabetes [9, 10].

In nephropathy, which is one of the major complications of diabetes, kidney damage is seen due to progressive increase in albuminuria, glomerular damage and consequent decrease in glomerular filtration rate, increase in resistance of efferent arterioles, and the development of renal hypertension. Chronic exposure to hyperglycemia also leads to the activation of pro-inflammatory mediators and an increase in ROS levels, further progressing renal dysfunction and damage. These patients may have to undergo dialysis over time and may even need transplantation [11, 12]. In another study, it was reported that hyperglycemia-induced cardiomyocytes developed cardiac damage with apoptosis, increased ROS, DNA damage, NF-κB activation, and

increased pro-inflammatory cytokines [13]. Reports state that rats with experimental diabetes experience inflammation in their spinal cords due to oxidative stress, and this inflammation ultimately results in neuropathic pain [14].

Well, when the role, release, and function of exosomes in diabetes are investigated, it is obvious that this EV plays an active role in the pathogenesis and progression of the disease. It is known that miRNAs (a class of small non-coding RNAs), which are among the different components of exosomes, can bind to their target mRNAs and initiate their degradation or inhibit their translation, and therefore play active roles in critical roles [15]. Kamalden et al. [16] suggested that exosomes secreted from pancreatic β-cells cultured in a hyperglycemic environment may enter the bloodstream, and that these exosomes are the source of miR-15a increased in the blood and contribute to retinal damage. It has been reported that exposure of Müller cells to exosomes derived from INS-1 cells in a hyperglycemic environment induces miR-15a overexpression, causes oxidative stress *via* Akt3 pathway, and leads to apoptotic cell death. Given this knowledge, the researchers clarified how miRNAs released by one cell type can enter the bloodstream and contribute to the development of the disease by initiating multiple pathways that damage cells and induce oxidative stress by spreading to other cell types. Ying et al. [17] found that adipose tissue macrophages (ATMs) of obese mice secreted miRNA-containing exosomes that caused glucose intolerance and insulin resistance when applied to lean mice, while ATM exosomes obtained from lean mice increased glucose tolerance and insulin sensitivity when applied to obese mice. It has been suggested that ATM-exosome miRNAs, and proinflammatory and anti-inflammatory ATMs are important components of the paracrine and endocrine signaling system that can influence metabolic events in distant tissues, for example, proinflammatory macrophage accumulation and chronic tissue inflammation, which may trigger for obesity-induced insulin resistance. Moreover, Cianciaruso et al. [18] discovered that cytokine-induced endoplasmic reticulum stress induced exosomal release of the immunostimulatory chaperones calreticulin, Gp96, and ORP150, promoted exosomal stimulation of antigen-presenting cells, and increased the quantity of exosomes released by β-cells. It has been proposed that autoimmune reactions in type 1 diabetes may be triggered by stress-induced exosomal release of these intracellular autoantigens and immunostimulatory chaperones. mEXOs-siKeap1 was created by sonicating SiRNA-Keap1 (siKeap1) into milk-derived exosomes (mEXOs). When injected into diabetic wound-affected animals, it was found to enhance collagen synthesis and markedly speed up neovascularization and diabetic wound healing. Increased Keap1 expression may lead to increased ROS production and suppression of Nrf2. Therefore, it has been stated that decreasing Keap1 expression by siRNA treatment may be a new therapeutic strategy to reduce oxidative stress damage in diabetic wounds [19]. In the study investigating the exosomal miRNA profile of diabetic nephropathy patients, it was reported that miR-1246, miR-642a-3p, let-7c-5p, miR-1255b-5p, let-7i-3p, miR-5010-5p, and miR-150-3p were higher in these patients and miR-4449 was higher than in patients without nephropathy [20]. In another study, it has been reported that serum exosomes and miR-4449 cause an increase in ROS, proinflammatory cytokine levels and induce pyroptosis in renal tubular epithelial cells, and for these reasons, they play the effective factors in the development of diabetic kidney disease [21]. Zhang et al. [22] also demonstrated that adipose mesenchymal stem cell-origin exosomes (ADSC-exos) can reduce high glucose-induced oxidative stress, accelerate wound healing by increasing peri-wound vascularization, and reduce mitochondrial dysfunction and inflammatory response by increasing SIRT3 expression and SOD2 activity.

3. Oxidative stress – cancer and exosome

Because unchecked ROS formation can result in increased DNA mutations, DNA damage, genomic instability, and unchecked cell proliferation, oxidative stress is widely recognized as one of the primary factors that contribute to the development of cancer [23]. Malondialdehyde (MDA), protein carbonyl, myeloperoxidase (MPO), and glutathione S-transferase (GST) were shown to be significantly elevated, while catalase (CAT) and superoxide dismutase (SOD) activities were shown to be significantly decreased in hepatocellular carcinoma that was experimentally produced with diethylnitrosamine [24]. The oxidative damage resulting from ROS formation in N-nitrosomethylbenzylamine-induced esophageal squamous cell carcinogenesis was found to be active in all stages of this process in another study of rats. In comparison with normal animals, GPx and SOD2 expressions decreased, while NFκB-p65, IκBα, IKKα/β, p38 MPAK, ERK and SAPK/JNK, 8-OxoG, H_2O_2 and LPO levels, and ratios increased [25]. Oxidative stress is thought to be one of the main pathophysiological pathways in the development of gastric cancer because *H. pylori* reacts with nitric monoxide in gastric juice to produce compounds like azo compounds, peroxynitrite, and high levels of superoxide formation. It also causes the gastric mucosal epithelium to produce free radicals and macrophages to produce nitric monoxide [26, 27].

Exosomes are recognized to have a role in all phases of the development of cancer, including tumor growth, cancer cell dissemination, and the intricate communication system that develops between tumor and non-tumor cells [28]. Exosomes are even involved in the suppression of the tumor-induced immune system and in escaping the destruction mechanisms of the body's defense system by the structures that transform into tumor cells [29]. Signals from exosomes released from cancer cells have been reported to inhibit the function of T cells and natural killer cells (NK) and the differentiation of antigen-presenting cells, while increasing the number and activity of immunosuppressive cells [29]. The growth, metastasis, apoptosis, and interaction of cancer cells with immune system cells, along with the emergence of drug resistance, have all been connected to exosomes released into the extracellular space and tumor microenvironment by breast cancer and stromal/cancer-associated fibroblast cells [30].

There are many studies showings that exosomes of tumor origin can disrupt the functioning of T-B cells, monocytes (macrophages), NK cells, and dendritic cells [31–33]. Many studies have shown that cancer cell-derived exosomes have both activating and inhibitory properties on NK cells [31, 34, 35]. It has been reported that when cancer-derived exosomes contain heat shock protein 70 (Hsp70), Interleukin-15 (IL-15) /interleukin-15 receptor alpha (IL-15Rα), and NK cells are activated [36, 37], whereas when they contain Cluster of differentiation 33 (CD33), Cluster of differentiation 34 (CD34), C-kit (CD117), MHC class I chain-related protein-B (MICA-B), Transforming growth factor beta (TGF-β), microRNA 23a (miR-23A), and Natural killer group 2, member D (NKG2D) ligands, they cause inhibition of NK cells [36, 38]. In addition, it is known that exosomes expressed from NK cells activated by tumor cell-derived exosomes can contain Perforin, Granzyme, Fas ligand (FasL), and Interferon-gamma (IF-γ) and thus have anti-tumourogenic effects (**Figure 1**) [36, 39].

It appears inevitable that exosomes will be a significant area of study in the identification and management of various cancer types in the near future. Exosomes can help explain the growth and spread of cancer and cancerous cells, like those in many other diseases, because they can be isolated from blood and other body fluids. Exosomes can be modified for the treatment of various cancers or to boost immunity that has been compromised by cancer [40–42]. Using a preclinical animal model

(SK-MEL-28) of melanoma with the BRAF (V600E) mutation, Thakur et al. [43] introduced the cells beneath the skin of NOD/SCID mice in order to detected tumor-associated genetic mutations in circulating exoDNA. They isolated circulating and plasma exosomes when the tumor reached the appropriate size and discovered that the V600E mutation was also present in circulating exoDNA extracted from mice with melanoma. As a result of these results, they concluded that exoDNA is a biomarker that can be used to detect mutations in parental tumor cells and for early detection of cancers and monitoring of treatment response. It has been stated that melanoma exosomes affect endothelial tubule morphology, are involved in paracrine endothelial signaling that is effective in the regulation of inflammatory cytokines, and show functions such as signaling that affects melanoma cell aggregation, extracellular matrix accumulation, and vascular proliferation in lymph nodes, and therefore may be one of the effective pathophysiological actors in the process of preparing an exosome-mediated microanatomical niche that facilitates lymphatic metastasis by cancer cells [44].

Today, it is accepted that cancer cells communicate with each other through the exosomes they express. There is strong evidence that these exosomes used by cancer cells in communication contain molecules to keep cancer cells alive and stimulate their proliferation and metastasis in addition to the survival of target cells [28, 45–49]. Investigations have shown that pancreatic cancer cell-derived exosomes have an initiating effect on cellular transformation. Indeed, it has been reported that pancreatic cancer cell exosomes play a role in malignant cell transformation of NIH/3 T3 cells,

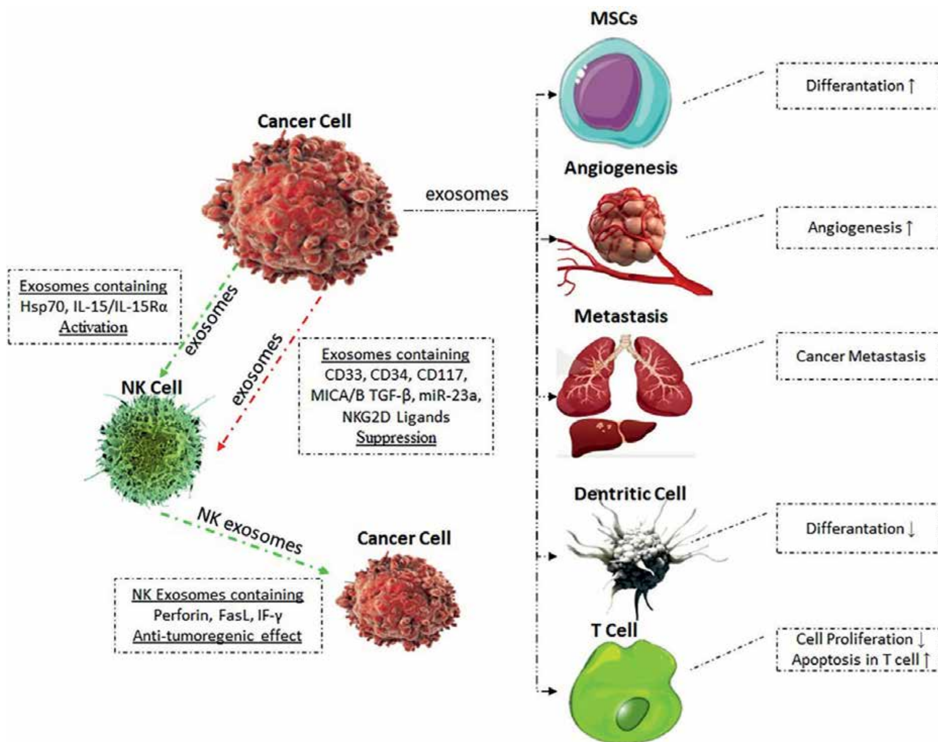

Figure 1.
Some roles of cancer cell-derived exosomes. ↑ denotes an increase and ↓ denotes a decrease. Red arrow shows exosome-induced NK cell inhibition, whereas green arrows show activation [36].

whereas healthy pancreatic cell exosomes do not cause such a situation [50]. Tumor cell-derived exosomes contain a variety of stimulatory and inhibitory factors that can affect the tumor microenvironment and thus promote both lung cancer formation and progression [51]. Studies have shown that lung cancer-derived exosomes carry the characteristics of the cells from which they originate [47] In addition, these properties of exosomes are extremely important in elucidating tumor cell biology including tumor cell development, proliferation, and metastasis [47, 48]. The presence of TGF-β, caveolin-1, hypoxia-inducible factor 1 alpha (HIF1α), and β-catenin in tumor-derived exosomes has been demonstrated to improve target cells' capacity for invasion and metastasis [49]. A study has shown that tumor-derived exosomes carry double-stranded DNA (dsDNA). In addition, it has been demonstrated that exosome dsDNAs isolated from many lung cancer cell lines containing epidermal growth factor receptor (EGFR) genetic alterations have a similar structure with the cell lines of origin [46, 52]. A study on a lung cancer cell line (A549) investigated the role of exosomes in the application of the drug Cisplatin, which is used in the chemotherapeutic treatment of testicular and lung cancers, and revealed that when Cisplatin is applied, it increases the expression of exosomes from the cell line. It was also shown that exosomes expressed from these cells increased the resistance of A549 cells to cisplatin when added to other A549 cells. It has been suggested that this may possibly be due to mRNAs and miRNAs exchanged by exosomes during intercellular communication [53].

Exosomes produced from tumors have been shown in numerous studies to stimulate angiogenesis in a variety of tumor types. In one of these studies, it was shown that exosomal miR-21 can increase the expression of Vascular Endothelial Growth Factor (VEGF) by activating STAT3 and eventually stimulate angiogenesis and tumor transformation of human bronchial epithelial cells [54]. In addition, different studies have revealed that exosomal miR-9 can stimulate angiogenesis in stromal cells by activating the JAK-STAT signaling pathway and [55], and exosomal MicroRNA 210 (miR-210) can stimulate angiogenesis in stromal cells by regulating ephrin A3. Exosomes stimulate angiogenesis by suppressing HIF-1 expression. Moreover, when tumor-derived exosomes are taken up by healthy endothelial cells, they activate angiogenic signaling pathways and cause the formation of new vasculature [56, 57].

Exosomes were found to be 40–100 nm in size and round or oval in shape when isolated from serum samples of patients with colon cancer. The results of mass spectrophotometry revealed that the primary RNAs in these exosomes were small RNAs, and their concentrations were noticeably higher than usual. In addition, these real-time PCR analyses revealed the presence of microRNA-21, microRNA-133a, and microRNA-181b in these exosomes. Indeed, the role of microRNA-21 in many cancers such as colon and colorectal cancer is very important and can be used in early diagnosis [58]. Mir-210 expression is frequently increased in colorectal cancer cases and has been shown to be associated with metastasis. Indeed, a study shows that miR-210 levels in the circulation of colorectal cancer patients are significantly elevated, which may be closely related to the formation of metastases. Indeed, Bigagli et al. [59] suggested that miR-210-containing exosomes originating from human colon cancer cells may be epithelial-mesenchymal transition (EMT) promoting signals that promote regional growth of cancer and migration of cancer cells to new areas. Indeed, normally epithelial cells have a certain polarity and thus serve for cell-cell adhesion. However, when EMT is stimulated in any way, it causes these cells to have an enhanced migratory capacity and increased resistance to apoptosis. In this process, immobilized solute cancer cells are transformed into mesenchymal cells that can migrate through some transformation processes and thus metastasize to other tissues [60].

It has been shown that small-interfering RNAs (siRNAs) isolated from brain endothelial bEND.3 cell culture media and added to exosomes can cross the blood-brain barrier. Although siRNAs have important therapeutic capabilities, problems in their delivery to the diseased area limit their ability. At this stage, the use of exosomes as a carrier may be an important way. In glioblastoma-astrocytoma U-87 MG cells treated with siRNA delivered by exosomes, inhibition of the expression of VEGF RNA and protein levels was demonstrated [61, 62].

Prostate cancer, like other cancers, uses exosomes for growth and metastasis. Studies have shown that exosomes have a double-layered lipid membrane and thus exhibit high durability. In fact, due to this durable structure, exosomes become resistant to the effects of many chemotherapeutic drugs targeting prostate cancer [63]. Another study showed that CD9 levels in exosomes isolated from the plasma of prostate cancer patients were much higher in advanced and chemotherapy-resistant prostate cancer patients than in healthy individuals without metastases [64].

As is well known, serum levels of the Cancer Antigen 125 protein (CA-125) in the blood are often used to diagnose ovarian cancer. However, in some cases of ovarian cancer, this parameter is not elevated; on the contrary, it may increase in different types of cancer, some inflammatory diseases, and benign tumors, and even in healthy women [65]. Due to this misdiagnosis, many unnecessary and incorrect treatments have been reported. In light of this information, it is thought that exosomes can be detected in different body fluids, especially blood, which are rich reservoirs of tumor-specific proteins, and thus can be used in the diagnosis of many cancers [63–65].

4. Oxidative stress – inflammation and exosome

Inflammation is considered to be a response to infection and tissue damage that occurs when highly complex inflammatory pathways are triggered by the immune system and is necessary for the restoration and maintenance of normal tissue homeostasis [66, 67]. Prolonged inflammation often causes detrimental side effects on health and is involved in the pathology of many diseases. Exosomes are also involved in inflammatory processes that play an important role in numerous pathological conditions. Determining the relationship between inflammation and some exosomal cargo alterations or changes in expression levels could contribute to the prediction and/or monitoring of pathological processes in inflammation-based diseases [67, 68].

Based on the ability of exosomes to present antigens and transfer pathogenic/non-pathogenic biomolecules, based on the ability of exosomes to present antigens and transfer pathogenic/non-pathogenic biomolecules, McDonald et al. [69] reported that exosomes secreted by LPS-stimulated macrophages carried higher levels of three murine homologs for human miRNAs (miR-21-3p, miR-146a, and miR-146b) known to prevent over-activation of the innate immune response. These three miRNAs also suppress NF-κB1 and other mRNAs involved in TLR signaling, which may inhibit the transcription and translation of proinflammatory cytokines and regulate the production of the anti-inflammatory cytokines IL-10 and IL-4. In another study, they reported that administration of Ag-containing exosomes to naive mice in the absence of conventional adjuvants elicited specific Ab responses across the MHC II haplotype barrier and that MC-exosomes stimulated immature dendritic cells to up-regulate MHC class II, CD80, CD86, and CD40 molecules and to acquire strong Ag-presenting capacity to T cells, and played an important role in the acquisition of Ag-presenting function by dendritic cells [70]. Exosomes from adipose-derived stem

cells were found by Shen and colleagues [71] to decrease ROS and pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 in macrophages, increase Nrf2 and nucleus translocation, decrease Keap1 expression, and regulate Nrf2/HO-1 expression in LPS-induced inflammation. As a result of these findings, they found that adipose-derived stem cell exosomes showed a protective effect against sepsis. In another study, it was determined that both adipose-derived and bone marrow-derived MSC exosomes reduced oxidative stress and inflammation, and even adipose-derived MSC exosomes improved kidney function and structure more significantly than bone marrow-derived MSC exosomes and protected against LPS-induced acute kidney injury [72]. Eshghi et al. [73] also found that in LPS-induced systemic inflammation, mesenchymal stem cell-derived exosomes decreased serum levels of ALT and AST liver enzymes after 4, 6 and 24 hours, decreased neutrophil/lymphocyte ratio, and decreased levels of inflammatory cytokines such as IL-6, IL-1β, and TNF-α, and decreased urea levels at 24th hours. Because of these findings, it has been proposed that exosomes made from mesenchymal stem cells could help repair damage to the kidney, liver, and lungs.

In Chagas disease caused by *Trypanosoma cruzi*, microvesicle release from blood cells is stimulated by Ca_2^+-mediated mechanism due to *T. cruzi* infection, and the released exosomes play a critical role in host-parasite interactions, intercellular communication, and parasite survival. By releasing glycoprotein 85 (gp85), trans-sialidase, and phosphatase, as well as by modulating the innate immune system, these exosomes have been proposed to bind to C3 convertase on the parasite surface, suppress C3 degradation, and shield extracellular trypomastigotes from the action of the complement system. Additionally, it has been proposed that the exosomes that are released might have structures that enable *T. cruzi* agents to evade complement-mediated lysis [74–77]. When considering the pathophysiological basis of intervertebral disc degeneration, the main causes are increased oxidative stress and inflammation. Thus, in a different study looking at the therapeutic impact of exosomes in this condition, exosome characteristics were generated; following analysis, it was found that exosomes may repair damaged mitochondria, suppress inflammatory mediators and NLRP3 inflammasome activation in pathological nucleus pulposus cells, and significantly slow the progression of intervertebral disc degeneration [78]. Exosomes from ARPE-19 cells secreted under rotenone-induced oxidative stress have been found to increase cell apoptosis and increase Apaf1 expression, which in turn causes oxidative damage and an inflammatory response *via* the caspase-9 apoptotic pathway [79].

The use of mesenchymal stem cell-derived exosomes (MSC-Exo) to prevent skin damage brought on by damaging stimuli like UV rays and oxidative stress is becoming more and more popular every day. It was found that MSC-Exo increases the antioxidant capacity of UV-irradiated mouse skin or H_2O_2-stimulated keratinocytes while reducing reactive oxygen species generation, DNA damage, abnormal calcium signaling, and mitochondrial changes. MSC-Exo has been shown to heal oxidative stress-induced skin damage by adaptively regulating the NRF2 defense system. It has been claimed that it can also be utilized to treat skin conditions as a nanotherapeutic agent [80]. In another study (human umbilical cord mesenchymal stem cells), it was reported that hucMSC-ex has significant potential in treating UV radiation-induced skin photodamage and that the 14-3-3ζ protein delivered by hucMSC-ex modulates a SIRT1-dependent antioxidant pathway and shows a cytoprotective effect [81]. Exosomes overexpressing miR-1246 (OE-EX), one of the nucleic acids present in exosomes derived from adipose-derived stem cells, were used in another study. It was found that OE-EX significantly reduced MMP-1 by blocking the MAPK/AP-1

signaling pathway, increased procollagen type I secretion by triggering the TGF-β/Smad pathway, and demonstrated anti-inflammatory effects by preventing the overexpression of NF-κB. Additionally, it was noted that in Kunming mice, OE-EX decreased collagen fiber loss and epidermal thickening [82]. Shen et al. [83] also found that UVB induced melanin production and increased the release of exosomes by melanocytes and that irradiated melanocyte-derived exosomes had higher levels of miR-4488, miR-320d, and miR-7704 compared to non-irradiated ones. Exome miR-29b-3p, derived from bone marrow mesenchymal stem cells-derived exosome, has been reported to attenuate UVB radiation-induced photodamage by regulating the viability, migration, apoptosis, oxidative stress, and matrix metalloproteinase levels of human dermal fibroblasts by targeting MMP-2 [84].

5. Conclusion

In conclusion, exosomes with unique properties are remarkable microvesicles that can easily pass through the cell membrane and transmit messages from one cell to another. The content of these microvesicles varies depending on which cells they originate from. Indeed, those originating from healthy cells differ from those secreted under oxidative stress, inflammation, and pathological processes (such as diabetes and cancer). In fact, the content of exosomes released from cells under oxidative stress differs from those released from immune system cells under these conditions, and hence the message they convey. Therefore, although it is known that exosomes have a very important potential for diagnosis, treatment, and monitoring the response to treatment, research on exosomes is still considered to be at the initial stage and more *in vitro* and *in vivo* studies are needed to be used safely and effectively in the clinical setting.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Chapter 3

Extracellular Vesicles in Hematological Malignancies

Samir Zuberi and Jihane Khalife

Abstract

Tumor-derived exosomes mirror the type and state of the cell of origin, and therefore represent specific pathogenic roles, therapeutic targets, and biomarkers of prognosis, drug resistance, and minimum residual disease. Because of their abundance in biological fluids and protection of their cargo from degradation, much of the translational exosome research revolves around finding biomarkers that can be used as precise diagnostic and prognostic tools in minimally invasive liquid biopsies, which is a particularly important in the context of minimal residual disease states and in cases where traditional solid biopsies are unavailable. Furthermore, in hematologic malignancies, exosomes play many pathogenic roles including remodeling their microenvironment, recruiting cancer supporting cells, facilitating drug resistance, and immunomodulation. This work will focus on two main aspects of exosomes in the most prevalent leukemias, myelomas, and lymphomas: the role of exosomes in pathogenesis and the use of exosomes as biomarkers and therapeutic targets. The roles and employment of exosomes hold true throughout the breadth of hematologic malignancies even though their specific cargo or biomarkers may vary between the cancer types.

Keywords: extracellular vesicles, exosomes, microparticles, hematological malignancies, microenvironment, cellular communication, leukemia, multiple myeloma, lymphoma

1. Introduction to extracellular vesicles and exosomes

Extracellular vesicles (EVs) are nano- or microscale particles secreted by cells that carry a wide array of cargo within a lipid bilayer or the aqueous core inside the bilayer. They are vastly heterogeneous and have a wide range of functions. However, exosomes, a 30–150-nm subset of EVs, are thought to be primarily for intercellular communication and are found in virtually all bodily fluids. They are exocytosed through the fusion of multivesicular bodies (MVBs) made in the endosomal pathway and this fusion of the outermost membrane with the plasma membrane (PM) releases the internal vesicle extracellularly [1, 2]. Unlike EVs, exosomes have specific surface makers including tetraspanins, MHC molecules, Cluster of Differentiation (CD)

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receptors, and various other surface markers that broadly correlate with the cell of origin and specific cargo [1]. The cargo they bear includes DNA, RNA, proteins, and small molecules. Their PMs can also be enriched in cholesterol or with certain lipid classes, like sphingolipids that add to their functionality and promote internalization. Exosomes play a role in maintaining both homeostasis and disease states. They have now been discovered to originate in a wide spectrum of cell types including epithelial cells, B and T cells, dendritic cells, mast cells, platelets, neurons, Schwann cells, and oligodendrocytes [3–7]. Exosomes were discovered in the mid-twentieth century observing the anucleate RBC progenitor, reticulocyte, differentiation [8, 9]. They discovered that reticulocytes released exosomes, with tetraspanin family surface proteins during differentiation into mature erythrocytes. These surface proteins have many types of binding partners and functions, like integrins, CD receptors, MHC molecules, and other tetraspanins directing extracellular trafficking and function [10]. Recent proteomics studies have shown that reticulocytes release these exosomes to shed these proteins. It is thought to promote differentiation by releasing proteins unnecessary for erythrocyte function including transporters, adhesins, and specific membranous and cytosolic proteins [11]. After the discovery of EVs during the 1980s, the 1990's were defined by the characterization and stratification of EVs. Because of improved purification and characterization methods, spearheaded in large part by Dr. Clotilde Théry, the distinction was made between EVs and exosomes, and the classification of the roles EVs began [12]. During this period, ultracentrifugation became the gold standard for EV isolation. Characterization of the functional roles of exosomes started in the 2000s and has made its way into many fields such as embryonic development, neurology, immunology, stem cell, and cancer biology. For example, Alzheimer's research was propelled by understanding neuronal exosome biology. We now know that exosomes play a substantial role in β-amyloid precursor protein metabolism, amyloid aggregation, plaque deposition, and clearance. They also contribute to neuroinflammation and neurological dysfunction [13].

These strides have licensed efforts to integrate exosome research into developing therapeutic, prognostic, and diagnostic tools. For example, exosomes have inspired the emergence of nanoparticle drug delivery, which has caused a paradigm shift in the drug development. The improved circulation time and safety of liposomal doxorubicin compared to doxorubicin has been the best example of clinical exosomal biomimicry. Exosomes can play a major part in cancer prognosis and diagnosis because of their abundance in bodily fluids and procurement ease. Exosome profiling tests are starting to make their way into the clinic. For example, ExoDx Prostate Test is a clinically used urine test to determine the risk of high-grade prostate antigen-positive prostate cancer. It was approved in 2018 in NCT03235687. The test generates a score of 0–100 determined from RNA sequencing (RNAseq) of PCA3, ERG, and SPDEF. A score of >15.6 indicates high-grade cancer. Longitudinal analysis of this test has proven that patients with low-risk scores remained low risk for 2.5 years post-testing, and 1.2–3.4% of low risk score patients developed high-grade prostate cancer, and < 0.1% of low-risk patients developed metastatic disease [14, 15].

2. Background on hematological malignancies

Hematological malignancies (HM) are myeloproliferative neoplasms that arise from lymphoid and myeloid cell lineages. They are distinct from other neoplasms because of the proliferative and circulatory nature of these cell lineages and make up

about 10% of cancers and cancer deaths. In the USA, about 157 people die of HMs every day. In general, dysregulated lymphoproliferation in lymph nodes (LNs) results in lymphoma and myeloproliferaiton in bone marrow (BM) in leukemia. Lymphomas and leukemias often share features and associate risk factors with immune and blood disorders, respectively. Tumors present on a wide spectrum, from circulating liquid tumors to solid nodal tumors occurring anywhere in the body [16]. Exosomes derived from HMs are typically enriched in CD surface markers, miRNA, mRNA, and protein cargo broadly correlating with the cell of origin. They play a major role in normal hematopoiesis and have significant emerging roles in HMs.

2.1 Introduction to leukemia

In normal hematopoiesis, hematopoietic stem cells (HSCs) differentiate into myeloid progenitors and subsequently into red blood cells (RBCs), thrombocytes, and granulocytes. In adults, these stem cells occupy the medullary cavity in the bone marrow. Exosomes play a major role in maintaining the niche *via* paracrine signaling allowing hematopoiesis to occur properly. HSCs release exosomes containing Wnt proteins to maintain HSC stemness and activate canonical kinase cascades to promote survival and proliferation [17, 18]. Acquisition of these features is desired by neoplastic cells and in leukemogenesis, neoplastic immature myeloid cells appropriate and destabilize exosomal singling to promote tumor growth. Chromosomal translocations resulting from aberrant DNA repair mechanisms, usually NHEJ, are a hallmark of HM particularly leukemias. As such, mutations in DNA damage repair machinery and sustained oxidative stress are common drivers of carcinogenesis. Leukemia patients present with anemia-like symptoms, leukopenia, and bone pain. The four most diagnosed leukemias include acute myeloid leukemia, chronic myeloid leukemia, acute lymphocytic leukemia, and chronic lymphocytic leukemia (AML, CML, ALL, and CLL, respectively) [16].

2.1.1 The role of exosomes in acute myeloid leukemia pathogenesis

AML pathogenesis is defined by clonal expansions of myeloid progenitors often referred to as blasts or myeloblasts. The 5-year survival for AML patients is 24% [16] Because of its high prevalence within leukemia, AML is one of the most well-studied blood cancers. As such, there are many studies characterizing the role of exosomes in pathogenesis, as well as the use of exosomes as biomarkers and therapeutic targets in AML. It is well known that AML cells have a marked increase in exosome secretion compared to normal myeloblasts; exosomes released by AML cells crosstalk with other tumor cells as well as a wide variety of cell types in the surrounding BM tissue. Similarly, endothelial and stem cells-derived exosomes can crosstalk with tumor cells to promote immune evasion and pathogenesis by, reprogramming the BM microenvironment, aiding the development of metastatic niches, and promoting drug resistance [19, 20].

In a series of studies, the Peter Kurre Group discovered and characterized AML-derived exosomes. Confirmed by DLS and TEM and purified by using ultracentrifugation followed by sucrose gradient centrifugation, J. Huan et al. successfully isolated cell line and patient-derived AML exosomes. Using PCR, they discovered that these exosomes were enriched with RNA transcripts including FLT3, NPM1, IGF-1R, MMP9, CXCR4, with both wild-type and mutant transcripts [21]. FLT3 overactivation can lead to hyperleukocytosis through downstream activation of

AKT; FLT3-isocitrate dehydrogenase (IDT) gene fusions are one of the most common mutations in AML, leading to constitutive activation of FLT3 and are associated with poor prognosis. FLT3-IDTs have been shown to cause decreased expression of many miRs, but interestingly not miR-155, a key miRNA for AML pathogenesis; its biosynthesis was proven to be regulated non-canonically [22, 23]. NPM1 is also commonly mutated in AML and often co-occurs with FLT3 mutations with synergistic effects. IGF-1R is a class II receptor tyrosine kinase with many downstream effectors. Its activation results in cell cycle progression and promotes cell differentiation and survival. MMP9 is an extracellular protease that breaks down the ECM, particularly collagen and laminin. Its expression in the BM enhances its permeability promoting increased diffusion of chemokine and cytokines that enhance AML cell survival and migration into the peripheral blood (PB). CXCR4 binds to CXCL12, a chemokine to anchor cells within the BM. Together MMP9 and CXCR4 expression act synergistically—MMP9 enhances the diffusion of CXCL12 and creates space for AML cells to grow. This specific protein cargo in exosomes functions as feed-forward autocrine and a pro-cancer paracrine signaling mechanism [21].

In addition to the mRNA transcripts, Huan et al. discovered multiple noncoding RNA transcripts, Let-7a, miR-9, miR-150, miR-155, mir-191, and miR-223, were enriched within exosomes of AML cell lines and primary patient-derived exosomes. RNAseq analysis found that treatment of Molm-14 exosomes shifted the transcriptome of the murine stromal cell line OP-1 to a proangiogenic profile and decreased growth factor signaling [21, 24]. In a follow-up from the 2013 study, Huan et al. further evaluated the effects of AML-derived exosomes in the BM niche by injecting AML cell line-derived exosomes into murine femurs. Their results indicated that protein and RNA from these exosomes transfer to cells in the BM niche (HPCS and stromal cells) correlating with broad transcriptomic and proteomic changes. Gene network analysis revealed the main downstream effects were the increase in expression of SYK, ICAM1, CD44, NF-kB1/2, and IL-1β, as well as the decrease of DNMT1, HELLS, PES1, RPL10A, and PAICS. AML-derived exosomes increased HSC migration from the BM and decreased overall hematopoiesis in conjunction with exosome-initiated BM remodeling. These effects were further exacerbated under hypoxic conditions, which resulted in feed-forward AML pathogenesis. Further work is being done in the Kurre group to characterize why AML cells incorporate specific RNA and proteins into their exosomes [24]. Highlighting the importance of miRs-150 and 155, the Kurre group further expanded on their roles in hematopoiesis. Correlating well with the anemic and leukopenic presentation of AML patients, they demonstrated that miR-155 delivery to healthy BM cells suppressed c-Myb expression and activated p53 by promoting cell cycle arrest and cell death, obstructing the proliferative clonal expansion and differentiation required of BM cells to make terminally differentiated leukocytes. Attenuation of miR-155 *via* anti-miR-155 shRNA rescued the normal hematopoietic phenotype and BM niche [25]. In 2018, these effects were confirmed and expanded upon by Kumar et al., who profiled stromal cells treated with AML-derived exosomes across a variety of cell lines. After treating cells with normal HSC-BM or AML HSC-BM-derived exosomes, they looked for transcriptomic changes in the stromal cells. Many of the differentially expressed genes overlap with the Kurre group studies, but interestingly the Kumar study identified new differentially expressed genes including decreased osteocalcin, IL-7, and COLIA1. AML engraftment in murine BM decreased osteocyte differentiation from the mesenchymal stroma by upregulating DKK1. DKK1 inhibition with small-molecule inhibitors rescued osteoporosis [26]. Another enzyme enriched in AML exosomes is DPP4.

Exosomes loaded with DPP inhibit hematopoietic progenitor proliferation. Exosomes were isolated from AML patient sera using anti-CD63 magnetic beads. Isolated exosomes were also positive for TSG101, CD123, CD96, CD117, CLL-1, DPP4, pro-TGF-β, and TNF-a. Exosomal DPP4 was shown to be enzymatically active and inhibit the proliferation of HSCs. AML patients in complete remission were shown to have decreased circulating exosomal DPP4 [27].

Many other studies of AML exosomes show that exosomes promote immune evasion and apoptosis of healthy tissue resident cells associated with exosome-mediated delivery of TGF-β, PD-L1 MCL-1, BCL-Xl, and BCL-2 protein delivery. Other important miRNAs identified include miR-99b, miR-146a, miR-191, and miR-10b. These exosomes are marked by heterogeneous surface expression of CD-33, CD-34, CD-117, and CD-13, which can be used as markers to identify AML-derived exosomes using flow cytometry or bead isolation methods. Strategies to use or target exosomes as therapies for AML are being evaluated in preclinical studies, stemming from the finding that exosomes derived from healthy BM compartments and stimulated immune cells have been shown to attenuate tumor growth [28]. Two other promising strategies include using small molecules to dysregulate exosome biosynthesis or release, as well as the elimination of AML blast-derived exosomes from circulation [29]. In a prospective, actively recruiting first-human study in Wuhan China (NCT06245746), AML patients will undergo allogeneic HLA-matched, umbilical cord-derived exosome translation to combat chemotherapy-induced myelosuppression, marking a big step in exosome-based therapy [30]. Circulating AML exosomes have been shown to reduce immunotherapy efficacy. Seeking to understand this phenomenon, Hong et al. purified blasts *via* SEC from venous blood collected from relapsed/refractory AML patients enrolled in NCT00900809 [31]. This completed phase I trial expanded and activated patients' natural killer (NK) cells *ex vivo* to better attack AML blasts. Circulating exosomal protein concentrations were decreased 21 days post-adoptive cell transfer therapy. CD34, CD123, CLL-1, and Fas were shown to be decreased in circulating exosomes 21-day post-transfer. They noticed that culturing NK cells with AML exosomes limited NK expansion, migration, and NKG2D expression; these changes correlated with increased SMAD2/3 and decreased T-bet activation in NK cells and were exacerbated by TGF-β signaling leading to reduced efficacy [32]. A recent study investigating exosome-targeted therapy showed pomegranate generates unique peptides (PGs 1-4), and PG2, specifically, decreased NB4 and MOLT-4 viability in a concentration-dependent manner and acted synergistically with daunorubicin. PG2 treatment had limited effects on healthy PBMCs. Analysis revealed that PG2 treated AML-derived exosomes had increased caspase 3 and miR-339-5p, and decreased CDK2 correlating with increased apoptosis of PG2-treated AML cells [33].

2.1.2 Exosomes as biomarkers for acute myeloid leukemia

Licensed by their previous work, another study from the Kurre group showed that drug-treated AML cells change the content of their exosomes relative to the drug treatment. They used that information to help better identify serum-based biomarkers of AML. Minimally invasive detection of MRD or early-stage disease is abhorrently lacking. By profiling exosomes with an RT-qPCR panel, the group developed a statistical model that correlated with high-fidelity circulating AML-derived exosomes containing miRNA before there were detectable circulating blasts. The most sensitive and specific marker for AML was the combination of miRNAs 150 and 155, followed closely by the combination of miRNAs 150 and 1246 [34]. In 2021, the Kurre Group

followed their biomarker work with further refinement of their predictive model, highlighted miR-1246 as a single predictive biomarker, and found that miR-1246 concentration correlated with disease state (stage/grade), particularly drug resistance. They identified 15 miRs that are differently expressed in AML (miRs: 25-5p, 27a-5p, 92a-1-5p, 96-5p, 144-5p, 145-5p, 181a-3p, 181b-5p, 199b-5p, 222-5p, 378a-5p, 1246, 3154, 6503-3p, and 6503-5p), and they found 7 miRs that remain relatively unchanged to use as controls for diagnosis/prognosis (miRs: 361-3p, 374b-3p, 589-5p, 855-5p, 942-5p, 3613-5p, and let-7i-5p) [35]. Another study identifying miRs in AML exosomes has shown over 10 differentially expressed miRNAs in exosomes from HL-60 and MOLM-14 AML cells. They focused on miR-548 ac as a miR of interest enriched in AML exosomes. Exosomal miR-548 ac is transferred to HSCs, inhibiting hematopoiesis by targeting TRIM28, a STAT3 activator. In addition, attenuation of miR-548sc decreases the AML cancer stem cell population and serum miR-458 is highly predictive of relapse and death rates in an AML patient cohort [36]. A follow-up study by Kang et al. looked at circulating EVs in general over a larger cohort (of majority Korean descent) and attempted to stratify EV miRNA with respect to disease prognosis. This study employed SEC with Sepharose beads. The analyzed fractions average 120 nm. They identified EVs bearing hsa-miRs 50, 130a, 143, 181b, 188, and 224 as prognostic biomarkers for AML [37].

2.1.3 The role of exosomes in chronic myeloid leukemia pathogenesis

CML makes up approximately 10% of all leukemia; patients present with anemia and splenomegaly as well as nondescript symptoms like fatigue, unexplained weight loss, satiety, and malaise [38]. The difference between CML and AML is the rate of disease onset—AML develops much faster than CML. The prototypical hallmark of CML is the ABL-BCR gene fusion (Philadelphia chromosome) resulting in the translation of a constitutively active tyrosine kinase chimeric oncoprotein [39, 40]. This fusion has been the target of many drug discovery labs and now FDA-approved ABL-BCR specific tyrosine kinase inhibitors (imatinib, nilotinib, dasatinib, and bosutinib) are part of the front-line standard of care for CML [38].

The first studies of exosomes in CML focused on their role in angiogenesis, which is well known to ameliorate CML progression. Using ultracentrifugation, the Alessandro group isolated exosomes from the CML cell line, K562. They showed that exosomes were internalized by HUVEC cells using fluorescence microscopy of fluorescently labeled exosomes. Using a creative carbon nanotube deposition assay as a proxy for angiogenesis, they showed that HUVECs, treated with K562, increased nanotube formation (angiogenesis) in a dose-dependent manner. However, TKI treatment (imatinib and dasatinib) decreased endosomal protein concentrations and cell proliferation but interestingly not nanotube formation. Shifting to a Matrigel-based vascularization assay in mice, however, did decrease vascularization upon TKi, dasatinib or imatinib, treatment. They discovered that K562 exosome-treated HUVEC had a marked increase in cytoplasmic Scr levels. Dasatinib is a dual inhibitor of ABL-BCR and Src (SRC family kinase). Cotreatment of CML exosomes with dasatinib rescued Src abundance, while imatinib did not. This effect was also true for p861-FAK, a substrate of Src. Src and p-FAK abundance correlated with AKT and MAPK activation, which was attenuated upon TKi treatment, respectively [41].

The following year, a study from the Alessandro Lab further explored the functional aspects of CML cell line, LAMA84-derived and patient-derived exosomes on HUVECs. They found that exosome treatment increased ICAM1, VCAM1,

and IL-8 in a time- and dose-dependent manner. Neutralizing IL-8 recapitulates non-exosome-treated expression levels of VCAM1 and ICAM1. LAMA84 and patient-derived exosome treatment correlated, likely *via* ICAM1 and VCAM1, with an increase in LAMA84 adhesion to a HUVEC monolayer, reversed by co-treatment with anti-IL-8. HUVEC migration was also increased with respect to exosome treatment and remained unaffected upon anti-actin Abs cotreated with exosomes. The independence from actin and dependency of IL-8 on exosome-induced angiogenesis was also recapitulated in murine studies. The increase in HUVEC migration makes sense; during angiogenesis, endothelia must line the neovascular lesions. Seeking to further explain these results they interrogated VE-cadherin and β-catenin subcellular distribution. In the exosome-treated HUVECs, VE-cadherin formed punctate/foci, as opposed to being membrane localized, while B-catenin nuclear localization markedly increased [42].

Follow-up studies sought to correlate their findings with the known functions of miRNAs from LAMA84-derived exosomes. A total of 124 miRNAs were differentially regulated; miR-126 was found to be enriched sixfold in exosomes compared to LAMA84 cells [43]. This finding confirmed a previous study [44]. miR-126 is known to have roles in angiogenesis and targets CXCL12 and VCAM1 mRNA transcripts. Clarifying their 2012 study showing increased VCAM1 mRNA levels in HUVECs with exosome treatment, they contrastingly showed decreased surface expression of VCAM1 on HUVECs only manifested about 24 hours post-exosome treatment. LAMA84 adhesion to HUVECs decreases upon exosome treatment, while LAMA84 trans endothelial migration transiently decreased then increased ~threefold over 24 hrs [43]. This study is a microcosm of the complexity of the bilateral crosstalk between cancer-derived exosomes and their microenvironment. .

2.1.4 Exosomes as therapeutic targets in chronic myeloid leukemia

An elaborate follow-up study from the Marcucci lab showed contradictory findings, they demonstrated that ABL-BCR deregulated miR-126 biosynthesis; they also demonstrated that TKi treatment restored miR-126 expression in CML cells. They designed a miR-126 inhibitor that could be used to attenuate miR-mediated transcript knockdown [45]. Exosomal miR-126 has also been shown to affect leukemia stem cell states. In CML cell lines and patient samples, BCR-ABL downregulates miR-126 biogenesis. miR-126 expression in HSC is necessary to maintain their quiescence. BM endothelia transfer miR-126 to HSCs *via* exosomes. In murine CLL models, miR-126 downregulation in BM endothelia renders TKi treatment more effective [45].

Attempts to target CML growth using IL-3 receptor targeted exosomes have shown potential in preclinical studies. HEK293Ts were transduced with a Lamp2b-IL-3 fusion protein such that IL-3 is presented extracellularly on exosomal membranes. HEK293Ts were treated with imatinib, and their exosomes were isolated and shown to contain imatinib and Lamp2b-IL3. CML cells overexpress the IL-3R; hence, IL-3 decorated exosomes will preferentially intake imatinib. For imatinib-resistant cells, the study also transfected cells with ABL-BCR siRNA, which permitted exosomal loading of the siRNA. To have efficacy against CML in xenograft mice, the exosomes needed the IL3 fusion protein and the drug, either siRNA or imatinib [46]. This clever approach could be built upon using primary cells and with well-developed combinations of therapeutics.

2.1.5 The role of exosomes in chronic lymphocytic leukemia pathogenesis

CLL is the most common leukemia in adults and is often asymptomatic in early stages. Presets with lymphocytosis, autoimmunity, BM fibrosis, and symptoms are associated with immune reactions—fatigue, malaise. Growing attached to stroma in BM and LNs, CLL emerges from mature clonal B-cells with 17p13 deletion and very rarely T-cell (<1–5%) [47]. CLL cells adapt their niche to support their growth using exosomes. One of the earliest reports of CLL cell line and clinical sample-derived exosomes was in 2015 by Paggetti et al. [48]. Exosomes were isolated by sequential ultracentrifugation followed by optiprep cushion centrifugation. Expressing MHCII and TSG101, these exosomes were internalized by myeloid, endothelial, and mesenchymal stem cells [48]. They used multiplex Illumina sequencing to identify miRNAs in the exosomes. About 55% of RNA was uncharacterized and 33% were miRNAs. Of the miRs: 29% was miR-21, 14% miR-155, 9% miR-146a, 8% miR-148a, 7% let-7 g, 4% miR-378a, and the rest were 2% or below. They also analyzed the proteome of these exosomes. Of the proteins analyzed, about 25% were associated with cell proliferation, 20% cell death/survival, 10% cell motility, 10% HM specific, 10% gene expression, and 7% RNA processing and protein synthesis. Some of the well-known proteins found include BCL-2, BCL-XL, MCL-1, XIAP, AGO2, PKC-b2, TCL1, VEGF, Hsp72, and Hsp90. BCL-Xl and VEGF were particularly enriched, correlating with apoptotic resistance and BM remodeling. Treating BM-mesenchymal stem cells (MSCs) with exosomes increased AKT phosphorylation over 16-fold, STAT3 phosphorylation ~eightfold, and β-catenin (β-cat) phosphorylation over ~fourfold. EC cells treated with exosomes increased pAKT by over fourfold, Phoshpo-CREB over fourfold, p-Lyn over twofold, as well as many other phosphoproteins. Transcriptomic analysis was employed to better understand the implications of these broad changes in BM-MSCs. RNA-seq analysis found that these cells adopted a partial CAF-like phenotype, concurrent with the expression of CCL2, CCL5, CXCL510, CXCL12, MMP1, ICAM1, promoting chemotaxis, CLL adhesion, and angiogenesis, which was not the case for BM-HSCs treated with healthy donor B-cell exosomes. These transcriptomic changes correlated increased cell survival, cytoskeleton remodeling, and cell migration; *in vivo* studies confirmed the proangiogenic role of the CAF like phenotype [48].

Compromising over 8 years of research, Uziel et al. gave an extensive analysis of CLL-derived exosomes from 45, mostly CD19/CD5 positive, treatment naive patient samples [49]. They isolated the exosomes using differential ultracentrifugation. The surface markers of these exosomes include CD81, CD9, CD63, and TSG101. Using flow cytometry showed that about 62% CD81 and CD19 double positivity, indicating they were derived from B-cells. HUVEC cells were treated with membrane dye and a membrane permeable FTIC-peptide conjugate labeled exosomes. The HUVECS internalized exosomes in a dose- and time-dependent manner, with the maximum signal at about 24 hours. They analyzed the exosomes ability to induce protein phosphorylation in HUVECs using a high-throughput mass spectroscopy approach. At least twofold increased phosphorylation was found in 53 proteins. B-catenin increased 3.6-fold. Interestingly, pathway analysis generated a network of all 53 phosphoproteins stemming from B-cat, yet their proteomics data did not reveal any Wnt/B-cat pathway proteins within the network. Knowing that activated B-cat binds to the promoter region of IL-6, which is required for CLL survival, they revealed and went on to prove, a mechanism where CLL cells use B-cat expression to induce IL-6 expression in bystander cells to promote their survival. IL-6 induces STAT3 to promote apoptotic resistance. They also associated IL-6 expression with CEBP, LEF/TCF,

and NF-kB expression which also promotes CLL growth [49] How B-cat expression or phosphorylation was regulated by exosomes, miRNA, mRNA, protein, etc., is a future step for these studies [49]. Other roles of exosomes in CLL described in the literature: promoting BM fibrosis, killing of CLL by NK-cell exosomes, inducing apoptosis in healthy B-cells, and impairing immune function [50–55].

The role of nurse-like cells (NLCs) (CD14+ monocyte-derived macrophages) in CLL survival is well established [56]. To interrogate this crosstalk with respect to exosomes, M2-like polarized THP-1 or NLC macrophage-derived exosomes were cultured with primary CLL cells grown from isolated PBMCs. Both THP-1 and NLC exomes were enriched with CD63 and CD81 and increased CLL mRNA expression, apoptosis resistance, and proliferation by increased expression of BCL-2, IGFBP-2, CD40, p53, and APRIL. RNA-seq GO of these CLL cells correlated with these findings. Additionally, CLL resistance to ibrutinib increased with NLC- or THP-1-derived exosome treatment [57].

2.1.6 Exosomes as biomarkers in chronic lymphocytic leukemia

A study aiming to identify a CLL in the context of MDR in PB turned to exosomal miRNA signatures in exosomes from 69 patients and 15 healthy volunteers isolated using differential ultracentrifugation. They analyzed the exosomes using flow cytometry with marker-specific labeled beads and found CD63 and CD9 to be reliable markers of CLL exosomes. CLL primary cells also secreted exosomes in increased concentrations, further stimulated by anti-IgM treatment and attenuated by ibrutinib treatment. Compared to healthy controls, they found at least twofold increases in miRs-29a, 29b, 29c, 630, and 155; miR-150 increased fivefold. BCR activation not only increases exosome release but also miR-150 and miR-155 loading. These differences, however, did not correlate with cellular miRNA concentrations of the same transcripts, indicting their enrichment in exosomes and ultimate paracrine function [58]. Further omics analysis of patient-derived CLL exosomes sought to cluster exosome content and stratify it across progressive or indolent CLL cohorts. Progressive CLL exosomes showed increased loading of S100-A9, which positively correlates with NF-kB pathway activation in CLL cells treated with exosomes. Inhibition of S100A9 decreases NF-kB signaling, which is known to drive CLL proliferation [59].

2.1.7 The role of exosomes in acute lymphocytic leukemia pathogenesis

ALL is a leukemia of immature lymphoid precursor of B- or T-cell origin. ALL is more common in children, whereas CLL typically affects older adults ~70 years old [16]. ALL onset is rapid and presents sudden fatigue, fever, and bone pain. Translocations such as BCR-ABL and ETV-RUNX as well as RAS mutations are common in B-ALL [60]. Like the previous leukemias, the role of the cancer exosome remains the same; however, there have been few studies of ALL exosomes compared to the previous HMs.

TEM of primary ALL blasts recovered by core needle biopsy revealed EVs with CD19 expression distinguishing them from platelet-derived EVs. Large EVs contained organelles and were internalized by labeled B-ALL and stromal cells in intrafemorally transplanted NSG mice. Exosomes from B-ALL cell lines NALM6 and SD-1 shifted the metabolic state of the stromal cell line, HS5, from oxidative phosphorylation to aerobic glycolysis concordant with lactate transporter MCT4 surface expression, increase in extracellular lactate concentrations, and activation of AKT. These

exosomes, however, did not affect stromal survival or proliferation [61]. Ostergaard et al. isolated exosomes from BM samples of ALL patients at different stages of treatment using ultracentrifugation followed by fractionation and subjected the microvesicular fraction to proteomics analysis. They identified CD97m PLXNB2, and ITG4 correlated with poor outcomes. Gene set enrichment showed that the EV proteome was enriched in ribosomal, PI3K/AKT pathway, epigenetic, metabolic, glycolytic proteins, complement, coagulation, and chaperone proteins. This study is limited in that it did not distinguish EVs by cell of origin [62].

PLEKHM1 regulates endosome trafficking, including exosome trafficking. Its dysregulation in osteoclasts induces osteoporotic effects to bone. A study from the Krause Lab investigated BM niche remodeling in B-ALL using PLEKHM1 KO mice with GFP+ ABL1-BCR+ immature BP-1 B-cells [63]. They isolated MSC exosomes using differential centrifugation. Proteomics analysis revealed that compared to normal MSCs, PLEKHM1 knockout MSCs had increased concentrations of syntenin, TSG101, and VPS28; proteins are part of the endosomal sorting complexes required for transport (ESCRT) machinery. The increase in KO MSC exosome syntenin corresponded with a cellular overexpression of syntenin and its binding partner syndecan-1. Treating ABL1-BCR+ B-ALL cell line Ba/F3 with KO MSC exosomes increased their expression of syntenin and syndecan-1 about twofold over wt-MSC exosomes. FAK activation was increased ~fourfold, and SKT activation ~1.5-fold. These changes corresponded with an increase in cell migration. Inhibition of the vesicular trafficking protein and dynamin decreased syntenin concentration and AKT activation in the PLEKHM1 KO MSC exosome-treated Ba/F3 cells. BP-1 B-ALL mice treated with PLEKHM1 KO MSC exosomes had decreased survival compared to wt-MSC treatment. B-ALL cells secrete TNF-α; coculture of Ba/F3 and wt-MSCs or treatment of MSCs with TNF-α increased syntenin exosome concentrations, like PLEKHM1 KO exosomes. Inhibition of TNF-α with the neutralizing antibody, adalimumab, rescued PLEKHM1 expression in coculture of MSCs with Ba/F3. Inflammatory cytokine IL-1β induced this effect, and it was also recapitulated in HS5 BM stromal cells and osteoblast precursor MC3T3 cells, but not murine and human endothelial cell lines, H5V and HUVEC, respectively. This highlights yet another cancer-promoting mechanism where B-ALL secretion of TNF-α increased exosome secretion and syntenin concentrations in MSCs. Delivery of TNF-α-transformed MSC exosomes to B-ALL cells increases their proliferation and migration *via* FAK and AKT activation [63]. This mechanism is interesting because it intersects a hallmark of chemotherapy drugs—the increase in inflammatory cytokines, particularly TNF-α and IL-1β that act as a double-edged sword in tumor growth [64].

A study investigating the biosynthesis of exosomes in B-ALL highlighted the importance of ActivinA, a TGF-β family cytokine, in CD81 and CD9+ exosome production in B-ALL cell lines NALM6 and 697. Treatment of B-ALL cells with ActivinA increased their total EV secretion as well as their uptake of EVs. B-ALL cells treated with Activin A primed B-ALL exosomes had increased cell counts compared to treatment with naive B-ALL exosomes. The cargo of the primed exosomes was also strikingly different; for example, miR-491-5p was increased ~threefold and let-7e-5p was increased ~twofold. ActivinA promotes paracrine cross-talk between B-ALL cells to increase exosome production and promote cell survival [65]. ALL is known to affect the quiescence of HSCs and hematopoietic progenitors. Investigating this concept in PDX T-ALL and B-ALL murine models, Georgievski et al. found exosomal, membrane anchored, Hsp70 to be involved in this process. ALL cells were shown to produce exosomes internalized by HSCs. However, artificial liposomal Hsp70 was

unable to be internalized by HSCs. This finding prompted the hypothesis that these exosomes were internalized by lipid raft-mediated mechanisms. Exosomes were able to bind to CTB-AF555 a known marker for lipid rafts. They injected mice with labeled exosomes isolated from ALL culture. They found reduced number and proliferative capacity of CD45+ progenitor and HSCs. These cells were stuck in G0/G1 phase. Mass spec-based lipidomics and metabolomics revealed ALL exosomes to be enriched with cholesterol, glycine, alanine, and many other metabolites, which seemingly promote quiescence and exhaustion of MSCs and HSCs, respectively. These cells had increased oxidative respiratory capacity, which may have interesting implications in hypoxic environments [66]. This study is particularly interesting because the roles of exosomes typically implicate RNA and protein cargo; this study underscores how lipid components can directly have pathological implications. Circulating exosomal miR-326 was found to be a potential prognostic biomarker for drug-resistant pediatric B-ALL. Drug-sensitive patients had ~fourfold decreases in miR-326 concentrations. miR-326 decreased RN95 viability; hence, the shedding of miR-326 by drug-resistant samples confers a protective effect to ALL cells [67].

2.2 The role of exosomes in multiple myeloma pathogenesis

MM originates from clonal expansions of mature plasma cells (plasmacytes), and is the most common, and one of the better-studied hematological malignancies. Myeloma is distinct from leukemia, the cells of origin are mature B cells, whereas leukemia originates from hematopoietic cells [68]. It has favorable survival outcomes, but complete long-term remission is nearly impossible [69]. Like AML and CML, the roles of MM exosomes share motifs of reprogramming the BM niche to favor tumor cell growth and immune evasion. The difference between these HMs is the respective cargo of the exosomes and subsequent downstream effectors. Plasmacytoid exosomes have been shown to induce pro-cancerous phenotypic shifts in endothelial, stromal, immune, and stem cell types [70, 71].

The Pichiorri lab was the first to discover MM-derived exosomes in cell lines using ultracentrifugation. Proteomics analysis revealed they were enriched in CD-44, CD-9, MHC-I, and BST-2 [70]. Using ultracentrifugation and miRNA array profiling, Zhang et al. stratified miRNA expression profiles of over 200 MM patients. Notwithstanding MM exosomes having much higher RNA content, they identified decreased levels of miR-16-5p, miR-15a-5p and miR-20a-5p, and miR-17-5p in bortezomib-resistant MM patient samples [70, 72]. Tumor suppressors, miR-15a and miR-16, have been identified as two key miRNAs that have decreased expression in MM exosomes relative to healthy plasma cell controls [70]. They cluster near the protomer region of exon 4 on 13q14.3., a commonly deleted chromosomal fragment in MM. miR-15a and miR-16 downregulate the expression of cell cycle (G0/G1) proteins; their downregulation leads to tumor progression by increasing BCL-2, CCND1, and WNT3a expression [73, 74]. In addition to shedding the miRNA through exosome release, to counteract the effects of miR-15a and miR-16, MM cells upregulate lnc00461 that is a sponge for mi-15a and miR-16. lnc00461 is also enriched in MM exosomes derived from patient samples, and its inhibition rescued the function of miRs 15a and 16 in part by restoring BCL-2 to normal levels [75]. Furthermore, miR-16 delivery to tissue-resident BM macrophages induced polarization to a pro-cancerous, immunotolerant M2-like phenotype. miR-16 inhibits canonical NF-kB signaling by inhibiting the IKK complex [76, 77]. IL-6, a driver of MM, is downstream of NF-kB and is highly expressed in the BM niche of MM patients. The lack of sufficient transfer of miR-16 deregulates

NF-kB and causes monocyte differentiation into tumor-associated macrophages. Complementary to the BM macrophages, endothelial cells increase IL-6 and VEGF secretion upon RNA transfer of miR-135 and piRNA-823 bearing MM exosomes, promoting tumor cell growth, BM remodeling, and immune evasion. Similarly, transfer of miR-21 to MSCs cause cancer-associated fibroblast (CAF) differentiation, which goes on to secrete IL-6, IP-10, and CCL2; IP-10 and CCL2 are chemokines that attract T-cell and innate immune cells [19, 78]. This milieu, thus, both recruits immune cells and polarizes them into cancer-promoting phenotypic states. Expanding on the pro-immunosuppressive role of MM exosomes, NK cell antitumor response is also attenuated by delivery of CD38 and ADAM10 from MM exosomes; Treg proliferation and CD-8 and CD-4 T-cell immune checkpoints PD-1 and CTLA-4 are increased upon transfer of MM exosome contents [20]. Combined with the fact that plasmacytes decrease surface presentation of MHC-I by loading them onto their exosomes, these soluble factors make the BM environment potently immunosuppressive, highlighting the potential for robust immunotherapy-based approaches in MM treatment [79, 80].

BM maintenance is also dysregulated though plasma cell exosome-mediated signaling. Increased DKK1, lncRUNX2-AS1, miR-103-3p, and miR-129 lead to HSC inhibition of osteoblast differentiation and osteoclast proliferation. The shift in osteoblasts to osteoclast ratio dysregulates bone mineralization correlating well with bone pain experienced by MM patients and increased immune cell chemotaxis in the BM niche [19]. An animal model study from the Ghobrial lab has shown that attenuating MM BM-derived exosome signaling or the addition of healthy BM exosomes to MM-bearing mice has been shown to significantly reduce tumor growth and maintain the BM niche as well as overall bone structure. Concurrently silencing miR-15a and miR-16 reversed the anticancer effects of the healthy BM exosome treatment. The cancer-promoting effects of MM exosomes correlated with increased fibronectin deposition, IL-6 and CCL2 secretion in the MM microenvironment [81]. Other studies used a similar approach and have recapitulated these outcomes and further investigated associated changes in the immunological processes at play.

Further probing MM exosome disruption of osteoclast and osteoblast maintenance, Faict et al. injected 5TGM1 murine MM cell-derived exosomes-bearing syntenin, TSG101, CD63, and CD81 in C57BL/KalwRij mice that are known to develop spontaneous myeloma. Mice treated with exosomes developed splenomegaly, a rare symptom of human MM, and a decreased trabecular bone volume and connective density. 5TGM1 exosomes promote osteoclast differentiation in RAW264.7 macrophages and increased their bone resorption. Likewise, 5TGM1 exosomes induce apoptosis of MC3T3-E1 osteoblasts, the bone-building osteoclast counterpart. Exosomal DKK1 inhibits Wnt signaling and attenuates osteoblast differentiation. Exosome biosynthesis inhibitor GW4869 in combination with bortezomib, the SOC drug for MM, reversed the inhibition of osteoblast differentiation and caused a reduced tumor burden in the mice [82].

2.2.1 Multiple myeloma exosomes as biomarkers and therapeutic targets

Exosomes have been shown to modulate immunotherapy efficacy. Exosomes were isolated using sequential centrifugation from culture media of MM cell lines with (or without) daratumumab (anti-CD38), a standard of care immunotherapy for MM, treated NK cells with purified exosomes, and profiled the NK cells using RNA-seq. Anti-CD38 treatment significantly increased the concentration of exosomes (four-fold), changed their miRNA profiles, and subsequent polarization of NK cells. Gene

ontology revealed that immune response, cell death regulation, and chemotaxis genes were upregulated, while mitotic genes were downregulated in the MM exosomes-treated NKs [83]. These data further our understanding of antibody-based immunotherapy in HM, implicating exosomes as critical for their efficacy.

A study by the Bianchi lab evaluated the intersection of anticancer agents with BM exosomes in MM. HDAC3 silencing or inhibition *via* small molecules decreased exosome concentration and stunted MM proliferation in cell lines and patient samples. A decrease in IL-6 signaling, not necessarily HDAC3 silencing, was shown to be necessary and sufficient to achieve the inhibition of MM growth. They identified a feedback loop whereby bone marrow HDAC3 signaling in BM stroma-derived exosomes drives changes in the soluble factors facilitating MM growth and subsequent exosome release to stromal cells [84]. MM endothelia are also affected by MM exosomes. piRNA-823, a PIWI RNA, has increased presence in the BM of MM patients and is highly enriched in ARH-77 MM cell line exosomes. piRNA-823 transfer to EA.hy926 endothelial cells increases their proliferation and oxidative stress as well as promotes angiogenesis and invasion. These effects were also recapitulated in a murine model of MM [85]. Murine BM-stromal cell-derived exosomes have been shown to confer drug resistance to 5T33MM MM cells. They promote migration, proliferation, and apoptotic resistance in MM *via* modulation of p38, p53, c-Jun, and AKT pathways. Cotreatment of bortezomib and BM-MSC exosomes decreased the potency of bortezomib to induce extrinsic apoptosis. Although MM priming or transformation of BM-stromal exomes has shown to alter exosomal cargo and function, in this study there was not a significant difference between naive and MM-primed BM stromal exosomes in conferring bortezomib resistance [86].

Hypoxia has a large role in shaping exosomal cargo and thus the BM-niche in MM. The Ohyashiki group generated a hypoxia-resistant MM cell lines to prove this concept. In these cell lines, HIF-1α was constitutively overexpressed regardless of oxygen levels compared to their hypoxia-sensitive counterparts. Hypoxia-resistant MM-derived exosomes had increased CD63 and CD1 surface expression and were released in increased concentrations. HUVECs treated with hypoxia-resistant MM exosomes had increased endothelial tube formation, a proxy for angiogenesis. Hypoxia-resistant exosomes were found to have consistently high concentrations of miR-135b and attenuation of miR-135b decreased angiogenesis in HUVECs. In a murine model, exosomal miR-135 originating from hypoxia-resistant MM cells targets the translation of HIF substrate, FIH-1, in HUVECs to promote their pro-angiogenic effects [87, 88]. In a subsequent study, they isolated BM-MSCs from 22 MM patients and two healthy volunteers. They purified exosomes using the ExoQuick-TC kit that reliably precipitates exosomes in a single step. Both MM and healthy MSC-exosomes were CD63, CD81, and TSG101 positive. Compared to the healthy control and intracellular concentrations, MM-MSC exosomes were particularly enriched with miR-10a, miR-346, and miR-135b. Inhibition of EV trafficking or release resulted in a buildup in of these miRs in MM-BM-MSCs, which resulted in increased rates of MM-MSC apoptosis regulated by miR-10a. They went on to show that BM-MSCs transfer miR-10a to MM cells and surprisingly increase MM proliferation. To corroborate their finding, they showed in murine models, that MSC EV release inhibition with FTY720 leads to decreased MM proliferation. To deconvolute this apparent contradiction, they postulated that miR-10a target genes differed in MSC and MM cells but have not yet proven their hypothesis [89].

Translational application exosomes using biomarkers of drug resistance, MRD, and prognosis can be well informed by the breadth of this research. The Ghobrial

group analyzed 156 patients circulating exosomal RNA array profiling to deduce MM prognostic risk factors. They used a combined centrifugation and reagent isolation method to purify the serum EVs followed by Illumina RNA sequencing. miRNAs made up at least ~50% of RNA content within the exosomes. After miRNA, uncharacterized RNA, mRNA, rRNA, and other ncRNAs made up the rest of the RNA content. Their statistical analyses identified miRs—16, 17, 18a, 20a, 106a, 106b, 155, let-7b, and let-7e as PFS risk factors. Among these RNAs, increased miR-18a or let-7b correlated the best with PFS and OS outcomes, with miR-18a slightly edging out at $p < 0.001$. let-7 functions to repress cell cycle progression and promote angiogenesis; it is likely shed from plasmacytes to promote cell growth [90]. miR-18a is known to increase tumor growth and vascularization, clearly explaining its prognostic value [73]. These biomarkers, and RNA profiles, are currently being evaluated for use in clinical settings. The role of neoplastic plasmacyte exosomes as potential biomarkers have been proven to be more sensitive and invasive compared to the clinical standards. Blocking cargo transfer or engineered transfer of tumor suppressor miRNAs, such as miR-15a and miR-16, have shown some hypothetical and preliminary value.

2.3 Introduction to lymphomas

Lymphomagenesis is similar to leukemogenesis; however, the role of exosomes in lymphoid progenitors and lymphoproliferation is not well established. Broad epigenetic reprogramming, CpG hypermethylation, DNA hypomethylation, and aberrant DNA acetylation caused in part by mutations in epigenetic regulators are also typical of lymphomas. Lymphomas are marked by enlarged LNs, inflammation, lymphocytosis, and nondescript physiologic changes. Many are Epstein-Barr Virus (EBV) infection-related and are broken down into Hodgkins (HL) and non-lymphomas (NHL). HL patients typically have enlarged B-cells originating in LN germinal centers. These B-cells have undergone VDJ recombination but do not express Ig genes; there are four subtypes. NHL, however, is far more heterogeneous than HL with B-cell neoplasms, mature T/NK-cell neoplasms, precursor lymphoblastic lymphomas, and others. As of 2022 mature T/NK-cell neoplasms have 36 WHO-recognized subtypes [91–94] A culmination of work in the 1990s on the MHC and viral immunology paved the way of our understanding of B- and T-cell lymphomas and exosomes; B-cell EVs were shown to be enriched with functional HMC-II and lysosomal markers [4, 95, 96].

2.3.1 The role of exosomes in virally induced lymphomas

Integrating this research in the context of EBV infection, which is a known lymphoma risk factor, the Middeldorp group sought to understand the role EBV infection plays in the function of B-cell exosomes and subsequently how transformed exosomes contribute to mechanisms of carcinogenesis of EBV-lymphomas. They purified exosomes from EBV transformed lymphoblastoid cell line, B95-8, using differential ultracentrifugation. They found that EVB-encoded miRNAs, BART and BHRF1 miRNAs, were enriched in activated B95-8 exosomes compared to spontaneous lymphoma cell line, IM1, or B-cell line, BJAB. In a transwell coculture assay, these exosomes were internalized by monocyte-derived dendritic cells (DCs) in a dose- and time-dependent manner but not by CD19+ B-cells. They found high copy numbers of EBV-miRs BHRF1-3, 1-5p. 2-5p and 3* transcripts in the DCs 48 hours post B95-8 exosome treatment. To prove the functionality of the miRs, they transduced HeLa cells with a CXCL11-3′UTR luciferase reporter such that EBV miRNAs would inhibit

luciferase translation. There was an 80% reduction in luciferase activity relative to non-EBV-infected exosomes or control 3'-UTRs. They showed the same reduction, with less efficiency, with BART1 cluster miRs using the LMP1 3′-UTR. To confirm the clinical relevance of this finding, they selected an asymptomatic EBV infected cohort and isolated, sorted, and sequenced their PBMCs for EBV miRNAs using multiplex PCR. Every patient selected had elevated EBV miRNA in their peripheral B-cells, and a slight majority of patients had EBV miRNA transcripts in their non-B cell PBMCs [97]. The effects of EBV miRs are known to be immunosuppressive, antiapoptotic, and proliferative as their role is to hijack cellular machinery to evade immune surveillance and promote viral particle synthesis [98]. A follow-up study of the Pegetel et al.'s 2010 work showed that EBER-1 exosomal RNA transferred from infected B-cells preferentially to TLR3/7+, and Tim-1/4+ DCs promotes antiviral immunity, notwithstanding miRNA transfer. GO revealed these exosomes caused DCs to increase inflammatory response and cholesterol biosynthesis mechanisms. They noticed the 5′ triphosphate on EBER-1 was required for the antiviral response induction. However, latent infection with cooccurring autoimmune conditions changes their effect. The lupus antigen, for example, is found in exosomes bound to EBER-1, stopping the induction of DC antiviral response [99].

An interesting study has shown that CAFs have an important role in the development of lymphomas using patient-derived CAFs. The patients include B-cell lymphomas, T-cell lymphomas, and follicular lymphomas. The coculture of these lymphocytes with CAFs improved their survival. The CAFs that improved lymphoma survival released increased concentrations of exosomes compared to the subset of CAFs, which did not improve survival. Enrichment of RAB27B and nSMase2 protein content also correlated with exosomes from the CAFs that improved lymphoma survival. Inhibition of RAB27B in CAFs decreased the survival advantage of lymphoma cells. In addition, this subset of CAFs enriched with RAB27B imparted pyrimidine analogs, gemcitabine, and cytarabine, resistance to the lymphoma cells. Yet, these exosomes conferred increased susceptibility to bendamustine, doxorubicin, and vincristine. They found miR-4717-5p responsible for the decreased nucleoside transporter, ENT2, limiting gemcitabine and cytarabine influx into the cells. This effect was recapitulated *in vivo* xenograft murine models [100]. This study beautifully highlights how understanding exosome biochemistry can directly inform clinical considerations for HM treatment.

2.3.2 The role of exosomes in B- and T-cell lymphoma pathogenesis

The roles of exosomes in T-cell lymphomas are difficult to decipher due to their innate heterogeneity and rarity, making it hard to obtain patient samples and subsequently develop a corpus of work in any specific T-cell lymphoma. Still, there have been a few studies probing the concept. A major role of T-cells is to regulate adaptive immunity through the engagement of antigen-presenting cells' MHC and costimulatory receptor complexes with the T-cell receptor (TCR). Dysregulated immune synapse formation is a common theme in T-cell lymphomas (up- and downregulated TCR expression) and can induce pathogenic mechanisms [101, 102]. The role of exosomes in the regulation and engagement of immune synapses were investigated in B- and T-cell lymphoma cell lines Raji and J77, respectively, expressing CD63-GFP knowing that it will label their exosomes [103]. They purified exosomes from media using differential ultracentrifugation. Exosomes derived from both cell lines were enriched with miR-760, miR-632, miR-654-5p, and miR-671-5p compared to their

cellular concentration. Exosomes from both cell lines were internalized by unlabeled Raji and J77 cells. They found J77 MVBs localized intracellularly *via* the actin cytoskeleton toward immune synapses formed between SEE peptide-pulsed Raji cells' MHC. J77 SEE antigen recognition was required to drive exosome release and Raji cell internalization. The transfer of contents included miR-335, which downregulates SOX4 expression in the antigen-presenting cells and is known to be prooncogenic [103]. Further work is evaluating the method of exosome internalization in antigen-presenting cells.

Diffuse large B-cell lymphoma (DLBCL) is driven by a small subpopulation of cancer stem cells in equilibrium with the lymphoma cells emerging from the LN germinal center. Knowing that Wnt signaling helps to maintain stem cell states and its role in many cancers, the Wulf group investigated how Wnt signaling is regulated by exosomes in DLBCL cancer stem cells that were shown to have increased Wnt3a concentrations. The release of Wtn3a containing exosomes was required to induce B-cat-driven proliferation in recipient cancer stem cells and DLBCL cells. Because Wnt promotes cell "stemness" that exosome release was able to increase stem cell proliferation and thereby increase DLBCL proliferation. IHC staining of B-cat in HIV/AIDS-induced DLBCL patients ($n = 258$) showed high nuclear positivity ($>30\%$) in the majority of patients. This finding indicates that cancer stem cells are releasing Wnt-containing exosomes to modulate the DLBCL-stem cell equilibrium [104]. The transfer of mutant RNAs from DLBCL exosomes in several DLBCL cell lines (LY1, LY3, LY7, HBL1, and TMC8) has been further characterized. The exosomes isolated from these cell lines as well as five primary samples were enriched with TSG101, CD63, CD81, Alix, snRNA, and protein-coding RNA transcripts. This cargo was transferred to stromal cells upon exosome internalization. These transcripts contained mutations corresponding to mutations in cellular RNA samples reflecting their cell of origin and thus highlighting prognostic potential [105].

Another study evaluated how macrophages are affected by EBV-exosomes from B95-8 and Burkitt's cell lymphoma line, Akata. Burkitt's cell lymphoma is a highly proliferative B-cell NHL acquired from EBV infection. They found that mice-bearing B95-8 tumors were far more survivable than Akata-derived tumors. This difference was attributed to the absence of BART miRNAs in the B95-8 genome. Murine monocytes/macrophages were shown to internalize the Akata exosomes. IHC revealed the survival disadvantage correlated with increased EBAR and IL-10 staining as well as macrophage infiltrates marked by CD68 and CD163. This was also the case in B95-8-bearing mice treated with Akata exosomes. Inhibition of CD163 reversed macrophage EBER positivity suggesting it mediates their internalization. BART miRNA transfer induced phenotypic shift in the macrophages to increase IL-10 and TNF-α expression, both of which have context-dependent immunostimulatory and immunosuppressive roles. They identified a ratio of BART/EBER (high>10 $n = 7$, low<10 $n = 5$) that correlates ($p = 0.0004$) with survival in DLBCL patients. They prove that EBV infection in B-cells polarizes macrophages to induce an inflammatory microenvironment, which promotes lymphoma growth [106]. Other studies have similarly implicated exosomes from infected T-cells in the carcinogenesis of lung cancer [107].

In Mantle cell lymphoma (MCL), driven by t(11;14)-induced cyclin-D overexpression, B-lymphocytes proliferate in the LN mantle zone and acquire secondary mutations leading to aggressive lymphoma. MCL Jeko-1 cells release exosomes

marked by TSG101, CD81, CD63, and CD19. MCL primary cells release exosomes marked by CD81, CD20, and CD63 expression. Jeko-1 and Mino cell line-derived exosomes, interestingly, are only taken up by their cell type of origin. However, monocytes, normal B-lymphocytes, and MCL B-lymphocytes uptake patient-derived exosomes but NK and T-cells do not. Inhibiting clathrin or caveolin did not impact exosome internalization, rather they hypothesized that MCL exosome internalization is dependent on cholesterol and lipid raft-derived mechanisms [108].

B-follicular lymphoma arises in LN germinal centers and t(14;18) is a hallmark of these neoplasms. EVs in B-follicular lymphoma prime a pro-metastatic microenvironment by reprogramming BM niches by shifting the BM stroma phenotype. This shift is distinct from the BM-MSC signature achieved by TNF-α plus lymphotoxin-α1β2 (Lt) stromal priming. They isolated exosomes using differential ultracentrifugation and did microarray Affymetrix sequencing and cytokine profiling. These exosomes are marked by TSG101 and CD81 coexpression. The most differentially regulated GO terms in exosome-treated stroma were cell motility (downregulated) and epithelium/tissue development (upregulated). They highlighted key-upregulated genes compared to TNF/Lt treatment: CXCL12, KITLG TGF-β, IL-7, ANGPT, PPARG, EBF1, RUNX2, and SP7. The key-downregulated genes were IL-1β, IL-6, CXCL8, CXCL10, ICAM1, VCAM1, and ITGAV. Repeating this experiment *in vivo* BM treated with follicular lymphoma cell line or patient primary cell-derived EVs also induced a similar set of upregulated genes: CXCL12, ANGPT1, TGF-β, IL7, EBF1, and FOXC1. These data suggest that priming BM stroma with EVs promotes B-follicular lymphoma cell adhesion with the BM stroma to promote metastasis [109]. This complex omics analysis of the stromal phenotype relates well to the leukemia-transformed BM phenotype discussed earlier. Although the role of exosomes in B-cell lymphomas is yet emerging, it has not been sufficiently characterized in T-cell lymphomas.

3. Conclusion

Although the role of exosomes in HMs is still developing, many of the themes remain the same across the disease subtypes. Exosomal paracrine signaling facilitates changes in cell adhesion, stemness, metabolism, vascularization, and extracellular niche structure to ameliorate cancer cell growth. In addition, exosomes can impact drug resistance and immune surveillance to stymie anti-cancer efforts. Essentially, these themes make room for cancer to grow and provide nutrients and security for their growth (**Table 1**). Consequently, efforts to understand the prognostic and diagnostic qualities of exosomes in liquid biopsies are being thoroughly investigated and will soon make their way into the clinic. Therapies using or targeting exosomes are now reaching late preclinical and early clinical trial stages but need considerable work before they can be translated into the clinic. Understanding the limitations of this research will be key to continue furthering our knowledge of exosomes in HMs and help to advance the research. Some of the key limitations are awareness of the biological niche concentration of exosomes, the mechanisms and desire for preferential cargo loading, and the exact functions of specific ncRNAs.

Cancer type	Notable extracellular vesicles- encapsulated- cargo ↑ increase ↓ decrease	Type of Cargo	Cell of origin	Role in pathogenesis	References
Acute myeloid leukemia	↑ FLT3	mRNA	Myeloid cell	Hyperleukocytosis	[21]
Acute myeloid leukemia	↑ NPM1	mRNA	Myeloid cell	Cell survival	[21]
Acute myeloid leukemia	↑IGF-1R	mRNA	Myeloid cell	Cell cycle progression	[21]
Acute myeloid leukemia	↑MM9, CXCR4	mRNA	Myeloid cell	Increase migration	[21]
Acute myeloid leukemia	↑Let-7a, miR-9, miR-150, miR-155, mir-191, and miR-223	Micro-RNA	Myeloid cell	Proangiogenesis	[21–24]
Acute myeloid leukemia	↑miR-150, miR-155	Micro-RNA	Myeloid cell	Disruption of normal hematopoiesis	[25]
Acute myeloid leukemia	↑DPP4	Protein	Myeloid cell	Inhibition of hematopoietic progenitor proliferation	[27]
Acute myeloid leukemia	↑ TGF-β, PD-L1	mRNA	Myeloid cell	Immune evasion	[28]
Chronic myeloid leukemia	↑Src Kinase	Protein	Myeloid cell	Proangiogenesis	[41]
Chronic myeloid leukemia	↑ICAM1, VCAM1, IL8	mRNA	Myeloid cell	Increase in HUVEC cell adhesion and migration	[42]
Chronic myeloid leukemia	↑miR-126	Micro-RNA	Myeloid cell, Bone marrow endothelial cell	Proangiogenesis, Hematopoietic stem cell quiescence	[43–45]
Chronic lymphocytic leukemia	↑miR-21, miR-155, miR-146a	Micro-RNA	Lymphocytic cell	Cell proliferation	[48]

Cancer type	Notable extracellular vesicles- encapsulated- cargo ↑ increase ↓ decrease	Type of Cargo	Cell of origin	Role in pathogenesis	References
Chronic lymphocytic leukemia	↑BCL-XL, VEGF	mRNA	Lymphocytic cell	Apoptosis resistance	[48]
Chronic lymphocytic leukemia	↑B-catenin	mRNA	B-lymphocytic cell	Cell survival, apoptosis resistance	[49]
Chronic lymphocytic leukemia	↑ CD63, CD81	Protein	Nurse-like cell	Cell proliferation, apoptosis resistance	[57]
Acute lymphoblastic leukemia	↓PLEKHM1	mRNA	Mesenchymal stem cell	Increase cell migration	[63]
Acute lymphoblastic leukemia	↑TNFα	Protein	Mesenchymal stem cell	Increase proliferation and migration	[63, 64]
Acute lymphoblastic leukemia	↑ActivinA	mRNA	B-lymphoblastic cell	Increase exosome production and cell survival	[65]
Acute lymphoblastic leukemia	↑Hsp70	Protein	B & T lymphoblastic cell	Progenitor and hematopoietic stem cell quiescence	[66]
Multiple myeloma	↓miR-16, miR-15a ↑lnc00461	MicroRNA Nc-RNA	Myeloma cell	Bortezomib resistance Tumor progression M2-macrophage polarization	[70–77]
Multiple myeloma	↑miR-135, piRNA-823	Micro-RNA Nc-RNA	Myeloma cell	Tumor growth Immune evasion	[78]
Multiple myeloma	↑miR-21	Micro-RNA	Myeloma cell	Immunosuppression	[78]
Multiple myeloma	↑ CD38, ADAM10	Protein	Myeloma cell	Immunoduppression	[20]
Multiple myeloma	↑ DKK1	mRNA	Myeloma cell	Dysregulation of bone mineralization	[19]
Multiple myeloma	↑ lncRUNX2-AS1, miR-103-3p, miR-129	Nc-RNA Micro-RNA	Myeloma cell	Dysregulation of bone mineralization	[19]

Cancer type	Notable extracellular vesicles- encapsulated- cargo ↑ increase ↓ decrease	Type of Cargo	Cell of origin	Role in pathogenesis	References
EBV-lymphoma	↑ BART, BHRF1	MicroRNA	EBV-lymphoma cell	Cell proliferation Immune suppression	[97, 98]
EBV-lymphoma	↑EBER-1	mRNA	EBV-B cell lymphoma	Antiviral response Inflammation	[99]
B, T, and follicular lymphoma	↑ RAB27B, nSMase2	Protein	Cancer-associated fibroblast	Lymphoma cell survival	[100]
B- and T-cell lymphoma	↑ miR-760, miR-632, miR-654-5p, miR-671-5p, miR-335	Micro-RNA	T-cell lymphoma cell	Immune synapse formation	[103]
Diffuse large B-cell lymphoma	↑Wnt3a	mRNA	Diffuse large B-cell lymphoma cancer stem cell	Cell proliferation	[104]
Burkitt's cell lymphoma	↑ EBAR, IL-10	Protein	Burkitt's cell lymphoma cell	Increase in tumor-associated macrophages/ lymphoma growth	[106]
B-follicular lymphoma	↑CXCL12, KITLG TGF-β, IL-7, ANGPT, PPARG, EBF1, RUNX2, SP7 ↓ IL-1β, IL-6, CXCL8, CXCL10, ICAM1, VCAM1, ITGAV	mRNA	Lymhoma cell	Lymphoma cell adhesion with the bone marrow stroma to promote metastasis	[109]

Table 1.
Role of extracellular vesicle cargoes in the pathogenesis of hematological malignancies.

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Chapter 4

From Diagnosis to Prognosis: The Transformative Impact of Exosomal ncRNAs in Head and Neck Cancer

Aditi Patel, Kanisha Shah, Vivek Tanavde and Shanaya Patel

Abstract

This chapter explores the potential of exosome-derived non-coding RNAs (ncRNAs) as minimally invasive biomarkers for the early diagnosis and prognosis of head and neck cancers (HNC). Late-stage diagnosis, lack of disease-specific biomarkers and disease heterogeneity contribute significantly to the poor survival rates of HNC patients, highlighting the urgent need for novel biomarkers. The chapter reviews the emerging role of exosome-derived ncRNAs in HNC in predicting early onset of HNC, with improved and accurate risk assessment and better prognosis. The chapter also offers insights into the future of ncRNA-based diagnostics and personalized patient management aimed at improving 5-year survival outcomes and reducing the burden of HNC.

Keywords: head and neck cancer, exosomes, non-coding RNAs, microRNA, lncRNA, circRNA, diagnosis, prognosis

1. Introduction

Head and neck cancer (HNC) is one of the most prevalent cancers worldwide, with 9,47,211 new cases and 4,82,428 deaths reported in 2022. The incidence of HNC continues to rise and is anticipated to increase globally by up to 30% by 2030 (Global Cancer Observatory (GLOBOCAN) [1–3]. The key etiological factors that contribute toward its high prevalence are consumption of smoking and smokeless tobacco, and/or excessive alcohol, betel-nut quid, infection with oncogenic strains of human papillomavirus (HPV-16 and HPV-18), Epstein-Barr virus (EBV) and herpes simplex virus type 1 (HSV1). Certain hereditary diseases that increase the risk of HNC include Fanconi's anemia (FA), ataxia, telangiectasia, Bloom's syndrome and Li-Fraumeni syndrome [4].

The histological progression to invasive HNC entails a systemic sequence of changes beginning with epithelial cell hyperplasia, advancing through various

degrees of dysplasia (mild, moderate and severe), carcinoma *in situ* and ultimately culminating in invasive carcinoma. HNC tumors are typically asymptomatic and are likely to be present as persistent ulceration, associated with leukoplakia (white patch), erythroplakia (red patch) or speckled leukoplakia (mixed red/white patch). HNC often has vague symptoms and few clear visible signs until late in the disease progression, resulting in delayed referral. HNC usually begins in the squamous cell lining of the mucosal surfaces inside the oral cavity, tongue, throat and voice box and is hence referred to as squamous cell carcinomas. While the rare type of squamous cell carcinomas also develops in the salivary glands, sinuses or muscles or nerves [5, 6]. Verrucous carcinoma is another low-grade variant of squamous cell carcinoma with specific clinical and morphological features which accounts for only 0.57–16.08% of HNC [7].

Despite the improvement in surgical and therapeutic approaches and ease of access to clinical examination, HNC patients often lose the opportunity for early diagnosis, leading to a poor overall survival rate (40–60%) [8]. At present, the conventional diagnostic approaches for HNC comprise physical examination, imaging techniques such as computed tomography (CT) scan, magnetic resonance imaging (MRI) and histopathological analysis of the tissue biopsies. Until now, tissue biopsy has been the gold standard method for diagnosis; however, it is invasive, painful, time-consuming, fairly challenging and possibly risky for the patient. Moreover, there is an un-accessibility of deeper sites, high false positive rates and intra-tumoral and metastatic heterogeneity remains undetected, affecting the specificity, sensitivity and accuracy of assessment [9]. Furthermore, the poor prognosis rate of HNC is also associated with lack of specific molecular biomarker(s) for early detection and real-time monitoring, locoregional aggressiveness, therapeutic refractoriness and high relapse rate [10]. Therefore, there is an urgent need to identify novel molecular biomarker(s) associated with the development, diagnosis and prognosis of HNC patients.

In recent years, liquid biopsy (LB) has emerged as a groundbreaking tool in cancer research, offering new avenues for diagnosis and prognosis of HNC. Unlike conventional tissue biopsies, which often require surgical procedures and can be uncomfortable for patients, LB allows for repeated sampling with minimal risk and discomfort. This makes it particularly useful in monitoring disease progression, treatment response and potential recurrence in HNC patients over time. The ability to conduct frequent tests without the need for invasive procedures holds great promise for improving patient care and outcomes [11].

The sensitivity of LB is another factor that has propelled its growth as a valuable diagnostic tool. Over the past decade, LB has attracted considerable attention as a non-invasive diagnostic and monitoring method that uses biological fluids—such as blood, saliva, pleural effusions, urine and cerebrospinal fluid (CSF), to detect and analyze a wide range of biomolecular markers. Each of these fluids has unique advantages and disadvantages depending on the type and location of HNC. Pleural effusions and CSF are not always readily accessible in HNC patients and are typically associated with advanced or metastatic disease, making these fluids less useful for early detection and limiting their prognostic value for the overall management of HNC [11–13].

Saliva is particularly promising due to its simple, non-invasive collection, ease of repeated sampling, proximity to tumor sites and cost-effectiveness, providing a more direct readout of disease status in HNC patients. LB has been successful in diagnosing and monitoring other solid tumors, and studies on HNC have shown promise for its use as a less-invasive, more affordable alternative to conventional diagnostic methods [14, 15].

A third notable advantage of LB is its ability to detect biomarkers that remain in the bloodstream for extended periods of time [16].

The persistence of these biomarkers in the system makes it possible to track changes over time, offering a dynamic and real-time health status. This feature is particularly useful for assessing treatment response, detecting minimal residual disease and identifying potential relapse before it becomes clinically evident. By extracting and analyzing genetic material such as circulating tumor DNA (ctDNA), tumor-derived exosomes, tumor-educated platelets (TEPs) and circulating cell-free RNA (cfRNA) from blood or other fluids, LB can provide real-time insights into a patient's disease status [17]. Furthermore, LB has proven to be highly specific in identifying molecular changes associated with HNC, allowing clinicians to tailor treatments based on the unique genetic profile of the tumor.

2. Exosomes in liquid biopsy for head and neck cancer

As liquid biopsy continues to emerge as a powerful, non-invasive tool for diagnosis, prognosis and monitoring of HNC, one of the most promising components of this approach is the analysis of *exosomes*. Exosomes are small, membrane-bound vesicles with a diameter of 40–160 nm, secreted by almost all cell types and stably present in various body fluids [18, 19]. These vesicles carry a rich cargo of bioactive molecules, including nucleic acids (e.g., DNA and RNA), proteins and lipids, which are reflective of the physiological and pathological state of their parent cells, offering valuable insights into the molecular processes underpinning HNC [20–23]. This cargo allows exosomes to serve as a potential goldmine of biomarkers for the diagnosis, prognosis and monitoring of HNC [24–27]. Beyond their direct role in tumor growth, exosomes contribute to the formation of the pre-metastatic niche, promote tumor angiogenesis and support immune suppression in the tumor microenvironment.

Compared to other liquid biopsy markers, such as circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), exosomes offer several advantages. First, exosomes are present in significantly higher quantities in biofluids—around 10^9 particles/mL—making them easier to isolate compared to CTCs, which are much less abundant in blood (only a few per mL) [23]. Second, exosomes are secreted by living cells and carry abundant biological information from their parental cells, making them more representative of the active tumor than ctDNA, which is primarily derived from apoptotic or necrotic tumor cells [18, 21]. Third, the lipid bilayer membrane of exosomes provides them with intrinsic stability, enabling them to circulate intact even in the harsh conditions of the tumor microenvironment. This high biological stability allows for long-term storage of exosome samples, facilitating both isolation and subsequent detection [28]. Despite their advantages, exosome-based LB faces challenges, particularly in isolating and purifying exosomes due to their nanoscale size and heterogeneity [29–31].

Cancer-derived exosomes represent only a small fraction of total exosomes, necessitating ultrasensitive and specific detection methods for accurate diagnostics. While progress has been made in exosome isolation and analysis of proteins and nucleic acids, issues with sensitivity, specificity, purity and throughput remain barriers to widespread clinical use [32–37]. As such, ongoing research is focused on developing more efficient, high-sensitivity and high-purity platforms for exosome separation and detection. Advancements in this area could significantly improve the use of exosomes in liquid biopsy, providing a powerful and non-invasive tool for the

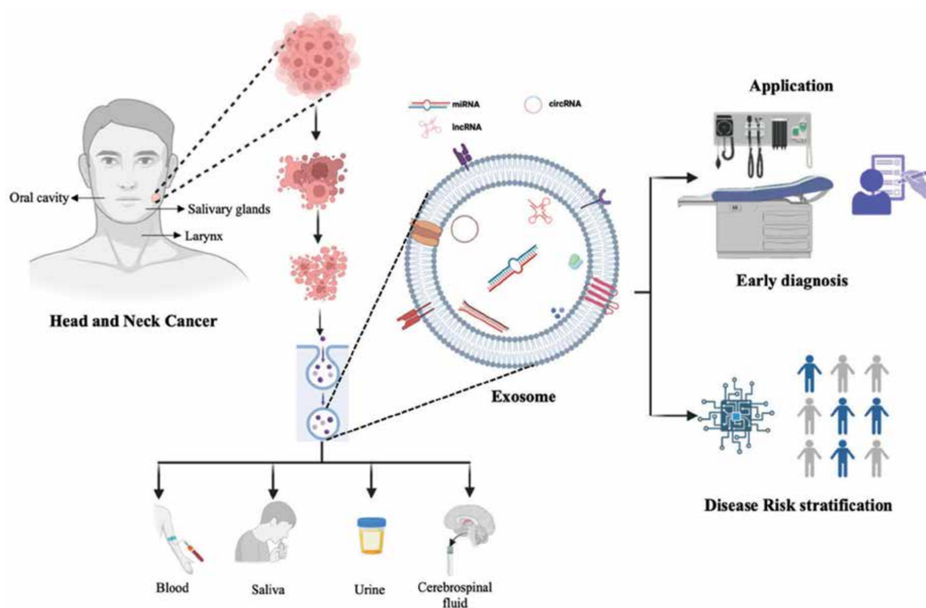

Figure 1.
A schematic representation of exosomal ncRNA involved in HNC diagnosis, prognosis and disease progression.

early detection, monitoring and prognosis of HNC [38]. Within the complex cargo of exosomes, ncRNAs have gained attention for their pivotal role in diagnosis and prognosis of HNC. ncRNAs enter exosomes *via* the double membrane invagination process associated with exosome formation. Studies have indicated that RNA-binding proteins (RBPs) are involved in the process by which exosomes selectively sort non-coding RNAs, including HnRNPA2B1, in addition to passively wrapping themselves around other substances within the cell. ncRNAs have been reported to play a role in cancer initiation, progression and metastasis, highlighting their potential as critical biomarkers in HNC management (**Figure 1**).

3. Exosomal non-coding RNAs: Classification, biogenesis and functions

Non-coding RNAs (ncRNAs) are functional RNA molecules that do not undergo protein translation. Approximately only 2% of RNAs are translated into proteins or are mRNAs, although 90% of the human genome is transcribed [39, 40].

The rest are called non-coding RNAs that were considered junk earlier but now are known to play a crucial role in regulating gene expression at the transcriptional, post-transcriptional and translational levels. The ncRNAs are divided into two main groups based on the length of the transcript. The short non-coding RNAs are less than 40 nt in length, and lncRNAs comprise more than 200 nt. The short non-coding RNAs include microRNAs (miRNAs), short interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs) and transfer RNA fragments (tRNAs) [41, 42].

The lncRNAs are found extensively in a large diversity of species, perform varied biological functions and are mostly categorized based on size, but current developments in transcriptome sequencing and analysis, have led to a further classification into different classes [43, 44].

Exosome-incorporated ncRNAs are released into the cytoplasm after being internalized by recipient cells, where they subsequently influence protein interactions, transcription activation, chromatin alteration and competitive splicing. By controlling cancer growth, metastasis, chemoresistance, immune evasion and angiogenesis, ncRNAs transported by exosomes have recently been discovered to play a crucial role in cancer development and progression [45]. Recent studies have investigated the biomarker potential of exosomal ncRNAs and a number of research teams have presented accumulating data supporting the use of ncRNA as biomarkers in HNC and its significance in oncogenic processes leading to growing evidence in diagnosis, risk assessment and monitoring of HNC.

3.1 Exosomal miRNA

MiRNAs are 20–25 nucleotides long single stranded RNAs encoded from introns that are involved in a number of biological functions. These coding regions are usually located in close proximity to one another referred to as poly-cistronic transcription unit. It is estimated that miRNAs regulate the expression of over 60% of all human genes and more than 1000 human miRNAs have been discovered to date. A growing body of evidence suggests that miRNAs are a crucial part of the intricate regulatory networks that govern a variety of cellular functions, such as the timing of developmental processes, immunological modulation, cell proliferation and differentiation, apoptosis and organ development [46].

miRNA dysregulation or their expression in tissues or biofluid samples, may represent an important diagnostic and prognostic factor for HNC. Extracellular miRNAs are found in different body fluids like saliva, serum plasma, breast milk and cerebrospinal fluid and they can be loaded into high-density lipoprotein (HDL) or bound by AGO2 protein. But exosomal miRNAs are packaged into exosomes or microvesicles that prevent miRNA from degradation and ensure their stability [47].

Due to the lack of endogenous RNase, a high concentration of multiple functional oncogenic miRNAs in HNC exosomes have been found [48, 49] miRNAs derived from exosomes have been extensively studied as potential biomarkers for HNC given their origin and representation of tumor cells [50, 51]. In a study by Li et al., they isolated miR-21 from serum exosomes of OSCC patients and found that significantly higher levels of circulating exosomal miR-21 was observed as compared to healthy individuals. Moreover, the level of circulating exosomal miR-21 was associated with late T stage ($p = 0.009$), positive HIFα expression ($p = 0.001$) and lymph node metastasis ($p = 0.021$). These results indicate that circulating serum exosomal miR-21 could be a potential biomarker for the diagnosis and predicting outcomes in OSCC patients [52].

Additionally, in another investigation miR-941 was significantly upregulated in Laryngeal Squamous Cell carcinoma (LSCC) serum exosomes compared to healthy controls with an area under the curve (AUC) of 0.797 (95% CI = 0.676–0.918) suggesting that serum exosomal miR-941 has the potential to be a therapeutic target and a promising oncogenic biomarker of LSCC [53]. In plasma exosomes the levels of miR-31 were found to be significantly higher in OSCC patients with an AUC of 0.82 compared to age and sex matched control individuals and a notable decrease in this marker expression was also observed following tumor resection indicating that miR-31 is associated with OSCC progression. Moreover, initial analysis for this study also highlighted the potential for detecting elevated miR-31 levels in the saliva of OSCC patients [54]. Consequently, these miRNAs may hold potential for use in the diagnostic, prognostic and therapeutic monitoring of HNC patients.

HPV has been considered to be an etiological factor for HNC and status of HPV infection can also be determined through specific miRNAs isolated from different body fluids. MiR-486-5p was elevated in salivary exosomes from patients with p16-positive oropharyngeal squamous cell carcinoma as compared to control subjects, while miR-10b-5p was linked to HPV-negative oropharyngeal SCC. Furthermore, higher expression levels of miR-486-5p were observed in later stages of the disease. Both miR-486-5p and miR-10b-5p dysregulations in salivary exosomes have been associated with oropharyngeal carcinoma and might serve as a potential prognostic marker [55]. However, the diagnostic significance of exosomal miRNAs in HNC is limited; thus, further research is essential to better define specific exosomal miRNAs and reassess their clinical relevance.

miRNAs have been thoroughly examined in saliva for their role in regulating various HNC-related processes through interactions with target mRNAs [56]. Moreover, saliva is a more accessible and non-invasive medium for collection, which significantly streamlines the identification process and serves as an effective method for monitoring OSCC, but it has a lot of background noise and microbial content, which contaminates the sample and hence isolating exosomes from saliva leads to a better way of monitoring miRNAs. Compared to other potential HNC biomarker-based detection methods, salivary exosomal miRNAs detection offers certain advantages. They can be found in trace amounts of saliva and identified by either whole saliva or just the supernatant [57, 58]. Salivary exosomal miR-134, miR-140-5p, miR-143-5p, miR-145-5p, miR-302b-3p, miR-517-3p, miR-512-3p, miR-24-3p, miR-184, miR-27-3p, miR-494-3p, miR-1307-5p and miR-412-3p exhibited varied expression levels in HNC when compared to normal [59]. Elevated expression of some of the above-mentioned miRNAs has been observed, among which miR-24-3p has been significantly elevated in salivary exosomes from preoperative patients compared to healthy individuals. Additionally, ROC analysis demonstrated that miR-24-3p exhibited excellent OSCC diagnostic accuracy (AUC = 0.738; P = 0.02), suggesting that salivary exosomal miR-24-3p could be a potential novel diagnostic biomarker for OSCC [60]. In a study by Patel et al., it was observed that miR-140-5p, miR-143-5p and miR-145-5p were found to be significantly downregulated in salivary exosomes from OSCC patients as compared to control. The identified 3-miRNA signature exhibited higher potency in predicting disease progression and was clinically associated with poor prognosis in OSCC ($p < 0.05$) [61]. Gai et al. also demonstrated that miR-302b-3p and miR-517b-3p were exclusively found in salivary exosomes derived from patients with OSCC. Additionally, there was a notable overexpression of miR-412-3p (AUC - 0.871) and miR-512-3p (AUC - 0.847) in the salivary exosomes of patients with oral cancer as compared to control subjects with the ROC curve indicating strong diagnostic capability of these miRs for OSCC [62].

In a study by Patel et al., salivary exosomal miRNA-1307-5p expression was upregulated as compared to controls and there was a significant clinical association with disease progression, local aggressiveness and chemotherapeutic refractoriness, making it an ideal prognosticator for OSCC [63]. In recent investigations, Langevin et al. evaluated the expression of 3 miRNAs that were differentially expressed in salivary exosomes collected from HNC patients and healthy controls. Importantly, a subset of HNC patients had significantly higher salivary levels of *miR-10b-5p, miR-486-5p* and *miR-486-3p*, as compared to controls with an accuracy of 85%, highlighting the potential clinical utility of exosomal miRNA as non-invasive salivary biomarkers [50]. Collectively, all these studies have demonstrated that these salivary exosomal miRNAs have the potential to be used as diagnostic or prognostic biomarkers for HNC.

Salivary exosomal miRNAs have also exhibited a correlation with both the disease stage and the histopathological classification and grade of HNC patients. Notably, the expression of miR-134 was elevated in high-grade OSCC, whereas miR-200a showed increased expression in low-grade tumors [64]. Moreover, salivary exosomal microRNAs also have the potential to serve as indicators for the progression of precancerous lesions to malignant stages. Significant example includes miR-4484 which signifies the transition from oral lichen planus (OLP) and oral dysplasia to OSCC [65].

miR-200a has been linked to epigenetic alterations induced by smoking, with its reduced expression correlating with an increased risk of developing oral cancer [64]. Furthermore, along with its malignant nature it was observed that miR-200a levels were found to be significantly elevated a year following radiotherapy [66].

3.2 Exosomal lncRNAs

Long non-coding RNAs (lncRNA) comprise more than 200 nucleotides and are primarily classified into five types according to their formation and activity—sense lncRNA, antisense lncRNA, bidirectional lncRNA, intronic lncRNA and intergenic lncRNA [67]. The extensive diversity of lncRNAs has recently been uncovered through full-length cDNA sequencing of the human genome and according to the GENCODE project, the human genome contains over 16,000 lncRNAs [40].

Exosomal lncRNAs as biomarkers offer several advantages: (I) lncRNAs within exosomes are protected from degradation by RNases, preserving their integrity and function [68]; (II) exosomes contain a higher quantity of lncRNAs compared to other types of extracellular vesicles [69] and (III) many lncRNAs exhibit tissue-specific expression patterns [70]. Moreover, it has been observed that dysregulation of exosomal lncRNA can adversely impact angiogenesis, metastasis and drug resistance, thereby facilitating the initiation and progression of HNC [71]. These factors make exosomal lncRNA profiling a promising approach for developing potential diagnostic markers. A growing number of studies suggests that various lncRNAs, including HOTAIR, UCA1, FOXC1, AFAP1-AS1, HNF1A-AS, ROR, LET, PlncRNA-1, GAS8-AS1, ADAMTS9-AS2, ESCCAL-1, PVT1, PTCSC2, PTCSC3, FIRRE, MEG3, MALAT1 and LOC541471, are involved in the initiation and progression of HNC [72].

These lncRNAs show potential as novel biomarkers and therapeutic targets, offering promise for improving diagnosis, prognosis and treatment strategies for HNC patients [73]. However, research on exosomal lncRNAs in the context of HNC remains limited, with one notable study by Li C et al. shedding light on this area. Serum exosomes are involved in rearranging the intercellular functional lncRNAs, which may also play a role in OSCC. Li C et al. investigated the efficacy of exosomal lncRNAs as a prognostic marker for recurrent OSCC (rOSCC) and OSCC with and without lymph node metastasis (OSCC-LNM and OSCC-NLNM, respectively). The expressions of the lncRNAs, namely MAGI2-AS3 and CCDC144NL-AS1, were significantly upregulated in rOSCC and OSCC-LNM, suggesting that these markers could be potential prognostic biomarkers, as well as therapeutic targets for OSCC [74]. lncRNAs hold potential as HNC biomarkers, however, most of the studies do not clarify whether these transcripts are contained within exosomes. Investigating how lncRNAs circulate in bodily fluids is crucial for understanding their roles and diagnostic and prognostic potential. Examining exosome membrane proteins can provide insights into the origin and uptake of lncRNAs, opening new research avenues. Exosomal lncRNAs may act as oncogenes or tumor suppressors, but further research is required to understand their mechanisms and functions.

3.3 Exosomal circRNAs

Circular RNA (circRNA), are a diverse class of closed-loop non-coding RNAs, lacking 5′capping or 3'poly(A) tail, with a more than 48 hours of average half-life. In 1976, these were first discovered in Sendai viruses and plant viroids [75]. Similar to lncRNAs, these are principally found in cytoplasm and nucleus. Numerous splicing-based models of circRNA biogenesis have been proposed: (1) circularization through canonical splicing-lariat or exon skipping, (2) circularization by splicing-intron pair or direct splicing. Majorly, circRNAs are of three different categories: (1) exonic circRNAs (EcRNAs): all introns are eliminated; (2) exon-intron circRNAs (EIcRNAs): few intronic sequences are conserved and (3) Circular intronic RNAs: derived from introns (ciRNAs) [24]. Moreover, it was first reported that c ircRNAs are enriched in exosomes with 2- to 6-fold higher expression than cells, indicating active incorporation of circRNAs into exosomes with high stability.

CircRNA has been stably present in exosomes derived from various cell lines including Oral Squamous cell carcinoma and Laryngeal carcinoma. Moreover, they have also been identified in exosomes from tumor tissues, patient saliva, serum and urine. Exosomal circRNAs may control tumor cell proliferation, invasion, metastasis, chemotherapeutic drug resistance and radiosensitivity, according to a number of studies that have linked them to the occurrence and progression of malignant tumors. These attributes position circRNA as a promising diagnostic biomarker or therapeutic target for HNC and many other cancers. Luo et al. demonstrated that elevated levels of circ_0000199 ($P < 0.001$) in circulating serum derived exosomes were significantly correlated with consumption of betel quid ($P < 0.01$), tumor size ($P < 0.01$), lymph node involvement ($P < 0.05$) and tumor staging ($P < 0.05$) in patients with OSCC. Furthermore, OSCC patients exhibiting high levels of exosomal circ_0000199 experienced higher rates of tumor recurrence and mortality. These findings indicate that high levels of circulating exosomal circ_0000199 may serve as an independent predictor of survival and disease recurrence in OSCC patients, although the specific regulatory mechanisms need to be further investigated [76].

In another study Tian et al. discovered that serum exosomal circRASSF2 was significantly overexpressed in LSCC patients. Additionally, serum exosomes from LSCC patients showed a strong inverse association between the expression levels of miR-302b-3p and circRASSF2 ($P = 0.003$) and promotes LSCC progression through its sponge effect on miR-302b-3p. Thus, understanding the exosomal circRASSF2 in association with miR-302b-3p may aid in developing a new treatment approach for LSCC in the future [77]. However, both these studies focus on circRNAs as targeted therapy. The clinical studies of exosomal circRNAs and their mechanism of action have not been identified and hence additional research is required to explore their diagnostic role in HNC.

4. Conclusions

In recent years, significant advancements have been made in the studies of exosomal ncRNAs, suggesting their immense potential in the diagnosis and prognosis of HNC. These molecules, carried within exosomes, reflect the molecular landscape of tumors, making them promising candidates for non-invasive biomarker development. Their high concentration and stability in biological fluids such as saliva, serum and plasma, attributed to the lipid bilayer's protection from RNase degradation,

positions exosomal ncRNAs as superior candidates compared to non-exosomal counterparts for early detection and prognostic evaluation.

This book chapter has explored the roles of exosomal ncRNAs—including miRNAs, lncRNAs and circRNAs—in HNC, emphasizing their diagnostic and prognostic relevance (**Table 1**). Evidence suggests that exosomal miRNA profiles, in particular, offer reliable insights into HNC progression, recurrence and response to therapy. By enabling stratification of patients based on disease monitoring, therapeutic response prediction, recurrence propensity and metastatic potential, exosomal ncRNAs can contribute to more precise and personalized management of HNC.

Nevertheless, there are significant challenges that remain to be resolved. The clinical utility of these biomarkers requires further validation through extensive studies and by establishing standardized protocols for their detection and quantification in HNC patients. Addressing these gaps is essential to harness their full potential and translate these findings into routine clinical practice.

Exosomal cargo	Located gene	Outcome	Application	References
miRNA	miR-486–5p, miR-486–3p, miR-10b-5p	Possible biomarker for HNSCC	Diagnosis	[50]
	miR-21	Proliferation, metastasis and invasion Cisplatin resistance	Prognosis & Diagnosis	[52]
	miR-140-5p, miR-143-5p, miR-145-5p	Disease progression	Prognosis	[61]
	miR-31	Possible biomarker for HNSCC	Diagnosis	[54]
	miR-9	Radiosensitivity	Prognosis	[35]
	miR-941	Proliferation and Invasion	Diagnosis	[53]
	miR-134	Higher expression in high-grade OSCC	Prognosis	[64]
	miR-200a	Higher expression in low-grade OSCC	Prognosis	[64]
	miR-24-3p	Proliferation	Diagnosis	[60]
	miR-4484	Disease progression	Prognosis	[65]
	miR-412-3p, miR-512-3p	Upregulation in salivary exosomes	Diagnosis	[62]
	miR-1307-5p	Disease progression, local aggressiveness and resistance to chemotherapy	Prognosis	[63]
lncRNA	MAGI2-AS3 and CCDC144NL-AS1	Therapeutic targets	Prognosis	[74]
circRNA	circ_0000199	Tumor stage, size and metastasis	Targeted therapy	[76]
	circRASSF2	Proliferation, migration and invasion	Targeted therapy	[77]

Table 1.
Potential ncRNA biomarkers for head and neck squamous cell carcinoma.

In conclusion, exosomal ncRNAs are emerging as powerful diagnostic and prognostic tools for HNC. Their unique attributes such as stability, non-invasive accessibility and ability to reflect tumor-specific changes, highlight their transformative potential for improving early detection, monitoring and prognostic stratification. With continued research and technological advancements, exosomal ncRNAs hold immense potential to advance both diagnostic precision and personalized therapeutic approaches in HNC.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

Harnessing the Therapeutic Potential of Mesenchymal Stem Cell-Derived Exosomes in Prostate Cancer: Current Insight and Perspective

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Abstract

Advanced prostate cancer is distinguished by substantial heterogeneity and intricacy, which present challenges in devising effective treatment strategies. The genetic landscape of prostate cancer is frequently altered, contributing to the development of resistance to conventional therapies and exacerbating systemic toxicity. These challenges necessitate more targeted and efficacious therapeutic modalities. Mesenchymal stem cells (MSCs) have been demonstrated to possess unique therapeutic properties and prostate tumor-homing potential. MSC-derived exosomes reflect the molecular composition and biological capabilities of their parent cells. These nanovesicles have emerged as a promising platform for drug delivery systems due to their biocompatibility and inherent ability to traffic bioactive molecules. Modification of exosomes by loading them with a therapeutic agent or incorporating surface modifications for targeted delivery further enhances the precision of therapy, enabling direct delivery to prostate cancer cells while minimizing off-target effects. Herein, we review the therapeutic effects of naïve MSC-derived exosomes in prostate cancer. Furthermore, we explore prostate cancer-specific exosome modifications, emphasizing targeted delivery and cargo-loading strategies, with particular focus on their emerging roles in gene therapy, sonodynamic therapy, vaccine-based exosome therapeutics, and potential clinical applications.

Keywords: mesenchymal stem cells, exosomes, prostate cancer, targeted therapy, therapeutic vehicle/cargo

1. Introduction

Prostate cancer represents the second most commonly diagnosed cancer and the fifth leading cause of cancer-related mortality among men on a global scale. As reported by GLOBOCAN, there are over 1.4 million new cases of prostate cancer

diagnosed annually, with the disease affecting approximately one in six men at some point during their lifetime. The risk is markedly elevated in men over the age of 50 and among African American men, who experience higher incidence rates and poorer outcomes [1, 2]. In 2022, prostate cancer ranked as the fourth most commonly diagnosed cancer worldwide, accounting for 1,466,680 new cases and 396,792 deaths [3].

The treatment and management of metastatic prostate cancer have proven to be a significant challenge due to the disease's complex nature and inherent adaptability. Prostate cancer is fundamentally a hormone-dependent malignancy, driven and exacerbated by androgen hormones. Androgen deprivation therapy (ADT) represents the standard initial intervention. However, its efficacy is constrained to cases of hormone-sensitive prostate cancer (HSPC). Prolonged exposure frequently results in the development of resistance, characterized by the persistence of malignant advancement despite the attainment of hormone castration levels. This phenomenon is referred to as castration-resistant prostate cancer (CRPC) [4, 5]. Furthermore, neuroendocrine prostate cancer (NEPC) introduces additional complexities, posing a significant clinical challenge due to its more aggressive phenotype [6–9]. A number of FDA-approved drugs are currently in practice, including docetaxel [10], abiraterone acetate [11], enzalutamide [12], and apalutamide [13]. Nevertheless, despite the conventional therapeutic interventions that are currently in place, researchers continue to face significant challenges in addressing the aggressive progression and systemic toxicity associated with this disease.

The biological complexity and heterogeneity of prostate tumors represent a significant challenge for the advancement of therapeutic development. In light of the current landscape, there is a clear need for a more precise targeting approach and therapeutic delivery platform that can meet these needs. Stem cell therapy has emerged as a prominent biological delivery system. MSCs exhibit distinctive characteristics, including self-renewal properties and multipotency. These distinctive characteristics enable them to play a dual role in cancer progression, either as a facilitator of angiogenesis [14], immune evasion [15], or extracellular matrix remodeling [16]. It is noteworthy that studies have reported the therapeutic outcomes of MSC treatment despite minimal engraftment or physical presentation of the cell in the tumor site [17, 18]. It is postulated that the regulation is effected remotely, potentially through extracellular vesicle (EV) crosstalk, such as exosome secretome. The molecular constitution and therapeutic efficacy of MSC-derived exosomes are analogous to those of their parent cells, exhibiting comparable regenerative, anti-inflammatory, and immunomodulatory effects [19, 20]. The various inherent characteristics of these vesicles, including their size, biocompatibility, endogenous production, natural cargo inclusion, minimal display of macromolecules, and more, render them an appealing alternative for therapeutic delivery [21–23]. Moreover, the modification versatility ensures targeted delivery and sustained efficacy in cancer therapeutics.

Several reviews have explored the emerging roles of MSCs and their derivatives, such as exosomes, in cancer therapy, focusing on their biological properties and therapeutic applications [24, 25]. Review documentation has highlighted the advancements of MSC-derived exosomes in prostate cancer therapeutics and management [26, 27]. However, there remains a gap in detailed analyses of the prostate cancer-specific targeting capabilities of these nanovesicles and the diverse cargo-loading strategies in different prostate cancer models. This chapter delves into the mechanisms by which MSC-derived exosomes influence prostate cancer. Additionally, it discusses the engineering of exosomes for targeted prostate cancer therapy, emphasizing their potential in delivering conventional or chemotherapeutic drugs, therapeutic proteins, genetic materials, vaccines, and other therapeutic agents.

2. MSC-derived exosomes therapeutic potential in prostate cancer

MSCs are multipotent stromal cells with unique therapeutic potential due to their ability to self-renew and differentiate into multiple cell types, including osteoblasts, chondrocytes, and adipocytes. These properties make MSCs a valuable regenerative therapy for a wide range of diseases and injuries. In addition, their ability to modulate the immune response and secrete bioactive factors contributes to their therapeutic efficacy in tissue repair, immune modulation, and resolution of inflammation [28, 29]. These stromal cells exhibit a complex dual role in cancer initiation and progression, acting as promoters and inhibitors of tumor development. MSCs promote cancer progression by regulating angiogenesis [14], immune response [15], and extracellular matrix remodeling to support tumor growth and metastasis [16]. In addition to the aforementioned pro-tumorigenic contribution, MSCs also modulate antitumor activities. Albeit contradictory, the dual function of MSCs in cancer presents attractive therapeutic implications [30, 31].

2.1 MSC recruitment and infiltration in the prostate tumor site

MSCs are found in both healthy and malignant prostate tissue across all age groups, and their infiltration into prostate tissue is driven mainly by inflammation [32, 33]. Prostate inflammation is often caused by pathogenic exposure, carcinogenesis, or physical trauma, among other factors [32, 33]. The anatomical location of the prostate in close proximity to the urinary system predisposes it to frequent inflammatory episodes. Factors such as exposure to acidic urinary secretions, infectious agents, and chemical trauma contribute to this susceptibility, which is exacerbated by its partial exposure to the external environment [34, 35]. These inflammatory signals act as a chemotactic stimulus for MSC recruitment to prostate tissue. In prostate tumors, MSC levels are elevated and present in approximately 1% of the stromal cell population [34, 35]. Once in the tumor microenvironment, MSCs can differentiate into specialized cells that contribute to tissue repair and regeneration. However, direct physical proximity between MSCs and their target region is not always necessary for their therapeutic effects [17, 18]. Their secretome mediates the crosstalk between MSCs and stromal cells, a diverse array of bioactive molecules, including cytokines, chemokines, and growth factors. In addition, MSCs release extracellular vesicles (EVs) into the extracellular space, such as exosomes, which play critical roles in cell-cell communication and therapeutic signaling [36–38]. MSC-derived exosomes closely mirror the molecular composition of their parent cells, including surface proteins, lipids, and bioactive molecules. This similarity allows them to exhibit analogous chemotactic trafficking properties, enabling efficient migration to the prostate tumor site in response to chemokines and cytokines present in the tumor microenvironment [39].

2.2 MSC-derived exosomes as a potential cell-free therapeutic vehicle

Although MSCs have a natural affinity for tumor sites and can modulate the tumor microenvironment, MSC-derived exosomes offer a more refined, efficient, and safer approach for therapeutic delivery and tumor targeting in prostate cancer [39]. Exosomes are enclosed, lipid bilayer, membrane-bound bodies that bud from the plasma membrane. These 30–130 nm diameter lipid vesicles are naturally produced endogenously, encapsulating and harboring specific molecules such as lipids, proteins,

DNA fragments, mRNA, lncRNA, miRNA, or tRNA [40]. They serve as protective carriers, safeguarding their cargo from enzymatic degradation by proteases or nucleases [41]. Although initially thought to function primarily as cellular waste disposal vehicles, exosomes are now recognized as key mediators of intercellular communication. They play significant roles in physiological and pathological processes, acting as a platform for the exchange of molecular information between cells and contributing to homeostasis, disease progression, and therapeutic modulation [42]. The composition, properties, and function of exosomes represent the secretory cell; therefore, MSC-derived exosomes are postulated to exert similar therapeutic effects on recipients [43].

2.2.1 Therapeutic cargo loading

Exosomes naturally encapsulate molecular cargo from their donor cells. However, due to the limited profiling of naïve exosomes, their therapeutic outcomes can be unpredictable [44]. Therefore, it is crucial to engineer exosomes to encapsulate known and desired therapeutic agents to ensure more reliable and targeted therapeutic effects. These vesicles serve as carriers for the delivery of a broad spectrum of therapeutic agents, including, but not limited to, pro-apoptotic factors, anti-angiogenic compounds, immunomodulatory agents, cytokines, and growth factors. Additionally, exosomes can be loaded with a range of pharmaceutical compounds, including conventional drugs, prodrugs, and prodrug-converting enzymes [19, 45–47]. Modification of exosomes can be performed before isolation, on the parent cell during exosome biogenesis, and after isolation. Pre-isolation modification is performed through natural cargo incorporation or transfection of cells with expression vectors. In post-isolation modification, cargoes are loaded after exosome isolation. Various techniques are used in post-isolation modification, including drug co−/ incubation, electroporation, sonication, freeze-thaw cycles, saponin permeabilization, and extrusion (**Figure 1**) [48–50].

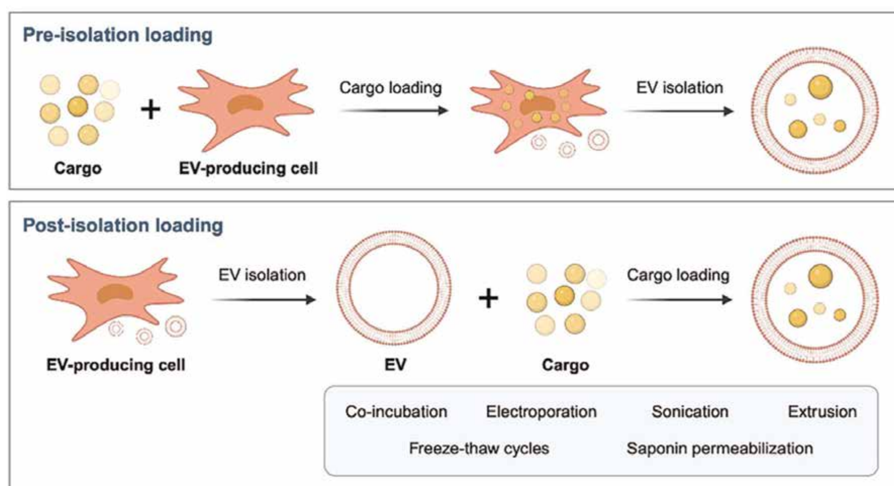

Figure 1.
Extracellular vesicles (exosomes) cargo-loading methods/techniques.

2.2.2 Modification of exosomes for prostate cancer targeting

Beyond their role as passive carriers, exosomes are being modified to enhance their therapeutic potential. Modifications can include surface modifications to target specific receptors on prostate cancer cells, facilitating precise delivery of their cargo [19, 51]. Advanced prostate cancer is characterized by inherent heterogeneity and variability in marker expression across subtypes [52]. This malignancy exhibits diverse molecular profiles, with different levels of expression of key surface markers such as prostate-specific membrane antigen (PSMA) [53], prostate-specific-antigen (PSA) [54], AR [55], and epithelial cell adhesion molecule (EpCAM) [56], depending on the stage and subtype of the cancer. Additionally, markers such as EpCAM, Her2/neu [57], and neuroendocrine markers are associated with aggressive subtypes, such as poorly differentiated and NEPC [58], which are more resistant to conventional therapies. Furthermore, carcinoembryonic antigen-related cell adhesion molecule (CEACAM)1 has been shown to be expressed in prostate cancer, particularly in NEPC [59]. Therefore, the one-size-fits-all approach falls short in this malignancy. Decorating the exosome membrane with surface ligands specific for multiple prostate tumor markers exploits these variations for precise treatment delivery.

2.2.3 Exosome infiltration and uptake into the tumor stroma

Therapeutic efficacy in prostate cancer is driven by exosomal involvement in pathogenesis through modulation of the tumor microenvironment, targeting specific key regulatory molecules or pathways involved in cancer maintenance and cell survival for selective destruction [60, 61]. Therefore, infiltration and concentration within the intra-tumor region is critical for subsequent efficacy enhancement [51]. However, drug uptake in the prostate stroma is significantly hindered by the dense extracellular matrix (ECM) and irregular vascularization. The stromal ECM, rich in collagen, fibronectin, and proteoglycans, creates a physical barrier that limits the diffusion of therapeutic agents into the tumor. Additionally, while prostate cancer induces angiogenesis, the resulting vasculature is often disorganized, leading to reduced therapeutic penetration within the stromal and epithelial compartments [62–64]. Due to their nanoscale size, exosomes can penetrate the dense ECM in prostate cancer and reach tumor cells more efficiently. These vesicles can navigate through the fibrotic and stromal barriers of the prostate tumor microenvironment. In addition, exosomes are inherently less immunogenic and more stable in the acidic and hypoxic conditions of the tumor site, ensuring better delivery of the therapeutic payload [65–67].

Exosomes exhibit remarkable penetration efficacy in prostate cancer models. Lee and colleagues [68] reported efficient exosome penetration in a PC-3 three-dimensional (3D) sphere prostate cancer model. Using DiD-labeled exosomes encapsulating an ALK5 inhibitor (SD-208) and R848, the study observed increased fluorescence intensity, indicating successful exosome infiltration into the 3D sphere structure [68]. Furthermore, the biodistribution and targeting of exosomes *in vivo* has been reported with intravenous administration of Cy5.5-labeled exosomes in a mouse model showing systemic distribution and targeted penetration into prostate tissue [68]. Similarly, Wang et al. reported effective tumor infiltration and localization of resiquimod (R848)- and chlorin e6 (Ce6)-encapsulated, dye-labeled exosomes delivered by both tail vein and intra-tumoral injections [69].

3. Preclinical analysis of exosomes in prostate cancer therapeutics

Exosomes are a growing versatile therapeutic tool, with their natural ability to transport cargo to specific sites offering significant potential. By leveraging this capability, exosomes can enhance treatment precision, reduce side effects, and overcome the limitations of conventional therapies. A growing body of studies has highlighted their effectiveness in targeted drug delivery, gene therapy, immunotherapy, and peptide-based approaches in prostate cancer, collectively paving the way for their future clinical applications (**Table 1**).

3.1 Exosome-mediated nucleic acid delivery

Gene therapy is a critical approach in precision medicine that allows the inhibition of disease-causing genes or the delivery of therapeutic genes [82]. However, genetic material is prone to degradation. The delivery of exogenous nucleic acids and the maintenance of their integrity are challenging due to the impermeability of the cell membrane and the fragile nature of these structures. Although the use of viral or non-viral vectors addresses some of these barriers, their use *in vivo* tends to elicit an immune response that risks their degradation as well as that of the incorporated gene of interest. The exosomal packaging ensures maintained stability and safe delivery of the cargo [83–86].

Han et al. developed E3 aptamer-modified exosomes carrying sirtuin 6 (SIRT6) siRNA to silence SIRT6, a therapeutic target for metastatic prostate cancer. E3 aptamer-modified exosomes had a higher binding affinity to prostate cancer cells than to non-cancerous cells and a potent targeting effect. Silencing of SIRT6 by engineered exosomes effectively inhibited tumor proliferation, growth, and metastasis [76]. The expression pattern of dehydrogenase/reductase 2 (DHRS2) is minimal in prostate cancer cells compared to normal prostate tissue. Overexpression of the gene contributes to the reduction of cell proliferation. Delivery of the human umbilical cord (hUC)-MSC-derived DHRS2-modified exosomes (pcDNA3.1-DHRS2) to prostate cancer cell lines has shown a significant reduction in the malignant behavior of the cancer through G0/G1 cell culture arrest as well as induction of apoptosis [77]. Gan et al. co-incubated hUC-MSC-derived exosomes with miR-375 antisense PMOs (e-375i) and evaluated the targeting effects *in vitro* and *in vivo*. E-375i reduced miR-375 levels, a tumor-promoting miRNA, suppressed prostate cancer cell proliferation, migration, and invasion, and enhanced apoptosis. The *in vivo* study also showed reduced tumor size and weight in DU-145 cell-derived tumor-bearing xenograft mice [71]. Another study also showed that exogenous miR-let-7c can be packed into human bone marrow (BM)-MSC-derived exosomes to target CRPC cells, PC3, and CWR22Rv1. Treatment with miR-let-7c exosomes inhibited cell proliferation and migration *in vitro* [70]. Meanwhile, exosomes derived from HEK293 cells were shown to carry miR-143 targeting prostate cancer cells. The results showed that non-cancerous cells could deliver tumor-suppressing miRNA and inhibit prostate cancer PC-3 M-luc cells *in vitro* and *in vivo* [75].

3.2 Drugs-loaded exosomes represent a novel approach to combating prostate cancer

While docetaxel and cabazitaxel, two commonly utilized chemotherapy agents for metastatic CRPC (mCRPC), have demonstrated efficacy in suppressing tumor

Cargo type	Study model	Cargo	Cargo-loading method	Targeting	EV cell source	Ref.
miRNA	*In vitro*, CRPC cell lines (PC3 and CWR22Rv1)	miR-let-7c	Pre-isolation (synthetic miRNA transfection)	N/A	Human bone marrow-derived MSCs (hBM-MSCs)	[70]
miRNA	*In vitro*, PCa cell lines (DU-145 and PC3) *In vivo*, DU-145 cell-derived tumor-bearing nude mice	miR-375 antisense PMO	Post-isolation (co-incubation)	N/A	Human umbilical cord-derived MSCs (hUC-MSCs)	[71]
Drug	*In vitro*, PCa cell (PC3) *In vivo*, Luc2-PC3 cell-derived tumor-bearing nude mice	Docetaxel	Pre-isolation (pre-incubation, extrusion)	N/A	Induced pluripotent stem cell-derived MSCs (iPSC-MSCs)	[72]
Drug	*In vitro*, PCa cell lines (LNCaP and PC3)	Paclitaxel	Post-isolation (co-incubation)	N/A	PCa cell lines (LNCaP and PC3)	[73]
Peptide	*In vitro*, PCa cell lines (LNCaP, C4-2B, and PC3) *In vivo*, C4-2B NSG-xenograft-bearing mice	N/A	Pre-isolation (nucleofection, lentiviral transduction)	Anti-PSMA peptide	U937 cells	[74]
miRNA	*In vitro*, PCa cells (PC-3 M-luc cells) *In vivo*, PC-3 M-luc cell-derived tumor-bearing nude mice	miR-143	Pre-isolation (transfection)	N/A	HEK293 cells	[75]
siRNA	*In vitro*, non-cancerous prostate epithelial cell line (BPH-1) and PCa cell lines (DU145 and PC3) *In vivo*, DU-145 and PC3 cells-derived tumor-bearing nude mice	SIRT6	Post-isolation (electroporation)	E3 aptamer	HEK293T cells	[76]
DNA	*In vitro* Human PCa cell lines DU145, PC-3, MDA PCa 2b, 22Rv1	DHRS2	Pre-isolation (pcDNA3.1 transfection)	N/A	hUC-MSCs	[77]
Drug	*In vitro*, PCa cell (DU-145) *In vivo*, DU-145 cell-derived tumor-bearing nude mice	Fe-HSA@DOX	Post-isolation (electroporation)	PMA	Urinary exosomes	[78]
Drug	*In vitro*, NCl-H660 and NEPC PCa model LNCaP *In vivo*, NE PDX LNCaP145.1	Enzalutamide Tazemetostat	Post-isolation (Sonication)	CEACAM5 Ab (NEPC)	HEK293T	[79]
Sonosensitizer	*In vitro*, RM-1, DC2.4, and RAW264.7 *In vivo*, male C57BL/6 mice	Ce6 + R848	Post-isolation (co-incubation)	N/A	HEK293T	[69]

Cargo type	Study model	Cargo	Cargo-loading method	Targeting	EV cell source	Ref.
Vaccine	*In vivo*, male C57BL mice	IFN-γ	Post-isolation (co-incubation)	N/A	RM-1	[80]
Vaccine	*In vivo*, male BALB/c or C57BL/6	MVA-BN-PSA-C1C2 MVA-BN-PAP-C1C2	Pre-isolation	PSA, PAP	HEK293-F	[81]

Table 1.
Preclinical studies of exosome-mediated prostate cancer therapeutics.

growth, they frequently induce adverse effects such as neutropenia, peripheral neuropathy, and gastrointestinal toxicity, including nausea and diarrhea [87, 88]. It should be noted that abiraterone, when used in conjunction with ADT, has been associated with an increased risk of cardiovascular complications and sexual dysfunction. Such toxicities frequently restrict the efficacy of the treatment regimen [89]. One promising solution to reduce systemic toxicity is the use of exosome-based drug delivery systems to minimize the exposure of healthy tissues. Exosomes can encapsulate toxic or cytotoxic agents, thereby enhancing the stability and efficacy of the drugs while minimizing systemic toxicity. The encapsulation of toxic substances in exosomes ensures a more selective and efficacious therapeutic approach.

The application of prostate cancer cell-derived exosomes as drug delivery systems, particularly for the chemotherapeutic agent paclitaxel, exhibited the capacity to effectively deliver the drug to target prostate cancer cells, namely LNCaP and PC3 cells. The mechanism of action involved cellular uptake via endocytosis, facilitating intracellular drug release. This targeted approach resulted in a reduction in cell viability, which validates the potential of cancer-derived exosomes in therapeutic applications. However, the utilization of cancer-derived exosomes devoid of encapsulated drugs has been observed to enhance cellular viability, a phenomenon that persists even in the presence of the encapsulated drug [73]. Given the beneficial therapeutic properties of these cells, utilization of MSC-derived exosomes represents an attractive alternative in the context of prostate cancer therapeutics. Zhao et al. employed induced pluripotent stem cell-derived MSCs (iPSC-MSCs) to develop EV-mimicking iPSC-MSC nanovesicles for the treatment of metastatic prostate cancer. The *in vitro* analysis demonstrated a markedly elevated uptake of the exosomes by PC3 prostate cancer cells in comparison to non-tumor cells, resulting in accumulation within the prostate tumor region. The nanovesicle-encapsulated docetaxel exhibited more pronounced cytotoxicity on docetaxel-resistant prostate cancer cells than the free docetaxel. This analysis of subcutaneous and bone metastatic prostate cancer xenograft models demonstrated that the nanovesicle-encapsulated docetaxel treatment group exhibited a greater degree of tumor growth suppression compared to the control group [72].

Exosome-mediated drug combination therapy has the potential to address the limitations of single-drug regimens. The encapsulation of multiple therapeutic agents within exosomes has been demonstrated to enhance the efficacy and specificity of treatment while reducing systemic toxicity [90, 91]. The co-delivery of enzalutamide, an androgen receptor inhibitor, and tazemetostat, an Enhancer of Zeste Homolog 2 (EZH2) inhibitor, via encapsulation, has been demonstrated to synergistically inhibit androgen signaling and neural gene expression, leading to tumor regression in NEPC models [79]. This dual therapeutic approach disrupts the interplay between AR and EZH2, which cooperatively regulate neural and stem cell gene expression in NEPC [79].

Wang and colleagues developed a sonodynamic therapy (SDT) approach utilizing the combination of Ce6 and R848 in exosomes for enhanced therapeutic efficacy while minimizing systemic toxicity in prostate cancer. This was achieved by engineering exosomes derived from HEK293T cells (ExoCe6 + R848) through a process of co-incubation. Engineered exosomes were then injected into subcutaneous RM-1 prostate cancer-bearing mice, followed by ultrasound exposure. The treatment resulted in enhanced immune responses, including the maturation of dendritic cells (DCs), polarization of M1 macrophages, activation of effector T cells, and inhibition of regulatory T cells (Tregs). This method effectively combined SDT with immune

modulation, offering a promising therapeutic strategy for prostate cancer without inducing significant systemic side effects [69]. In another study, Pan and colleagues developed an innovative nanoparticle system, PMA/Fe-HSA@DOX, encapsulated within urinary exosomes derived from prostate cancer patients. This system incorporated multiple therapeutic components with the objective of enhancing targeted treatment. Doxorubicin (DOX), a widely used chemotherapeutic drug, was adsorbed onto human serum albumin (HSA) for drug delivery. Additionally, Fe_3O_4 nanoparticles, cross-linked with polymethacrylate (PMA), were designed to catalyze the production of toxic hydroxyl radicals (OH) by reacting with hydrogen peroxide in the acidic prostate tumor microenvironment, specifically targeting tumor cells. The engineered exosome-based delivery system demonstrated superior efficacy, with enhanced uptake into DU145 prostate cancer cells, increased penetration into DU145 3D tumor spheroids, and improved retention in DU145 xenograft models in BALB/c nude mice compared to treatments with either DOX or PMA/Fe-HSA@DOX alone [78].

3.3 Exosome-targeted vaccines for prostate cancer immunotherapy

Prostate cancer cells often evade immune surveillance by impairing antigen presentation and altering tumor-associated antigens. A key mechanism is the downregulation of major histocompatibility complex (MHC) class I molecules, which are indispensable for the presentation of tumor antigens to CD8+ T cells. This reduction in MHC I expression impairs T cell recognition and targeting of cancer cells, allowing the tumor to escape immune detection [92]. Furthermore, prostate cancer cells can lose or modify the expression of tumor-specific antigens, such as PSMA and PSA, which are normally targeted by immune cells [93]. Such alterations in these antigens diminish the ability of the immune system to recognize and eradicate the cancerous cells, thereby further facilitating immune evasion.

Exosomes have been implicated in the modulation of several immunological processes, such as antigen presentation [94] and immune surveillance [95]. Tumor-derived exosomes can be employed as a vaccine platform to present tumor antigens and to elicit an immune response. Additionally, engineering and encapsulation of immune-stimulating molecules may potentially enhance the efficiency [96]. Xiaojun Shi and colleagues explored the potential of exosomes as a novel vaccine platform for prostate cancer treatment. These exosomes were genetically modified to carry and express interferon-γ (IFN-γ), a potent immunomodulatory molecule. The delivery of prostate cancer-derived exosomes loaded with IFN-γ effectively upregulated the expression of MHC molecules on malignant cells, enhancing their visibility to cytotoxic T lymphocytes (CTLs). This sensitization to CTLs amplifies the immune response against the tumor. Additionally, the internalization of these exosomes by target cells contributed to a significant reduction in Tregs, which play a role in immunosuppression within the tumor microenvironment. This approach demonstrates the potential of exosome-based platforms to enhance antitumor immunity while reducing immune evasion by cancer cells [80].

Two prostate tumor-associated antigens, PSA and prostatic acid phosphatase (PAP), play a central role in prostate cancer immunotherapeutics. By cloning these antigens into a recombinant vector derived from a highly attenuated strain of the modified vaccinia Ankara (MVA) virus, known as MVA-BN, researchers have developed innovative vaccine candidates. To further enhance the therapeutic potential, the fusion of these antigens with the C1C2 domain of lactadherin, a protein that facilitates exosomal

incorporation, ensures precise exosomal localization of the vaccines, MVA-BN-PSA-C1C2 and MVA-BN-PAP-C1C2. Preclinical studies in mice demonstrated that these exosome-targeted vaccines significantly enhance the immune response against PSA and PAP, effectively targeting prostate tumor models that express these antigens [81].

3.4 Peptide loading and targeting on exosomes

Despite the efficacy of recombinant therapeutic molecules, lack of specificity poses a risk of toxicity to untargeted cells. The incorporation of a target cell-specific molecule can ensure specific toxicity while providing another layer of specificity in addition to the exosomal tropism. Genetic modification of exosomes with a peptide against the target can ensure tropism and interaction that is specific and selective to prostate cancer cells, thus enhancing precision [97, 98].

PSMA is a well-established biomarker and therapeutic target for prostate cancer, particularly in the advanced stages of the disease. The protein is highly expressed on the surface of prostate cancer cells, including those that have developed resistance to hormone therapy and those that have metastasized, while exhibiting limited expression in normal tissues. This selective overexpression renders PSMA an optimal target for precision therapy, facilitating the targeted delivery of therapeutic agents to cancer cells [99, 100]. Severic et al. engineered exosome mimics to express PSMA-targeting peptides (PSMA-EMs) produced from anti-PSMA peptide-expressing U937 monoblastic cells. PSMA-EMs demonstrated targeting capabilities in both *in vitro* and *in vivo* settings, exhibiting heightened cellular internalization in PSMA-positive prostate cancer cell lines, namely LNCaP and C4-2B cells. Additionally, the *in vivo* study demonstrated enhanced tumor targeting in solid C4-2B tumors following intravenous administration [74].

Prostate cancer is distinguished by its intricate and diverse protein expression profiles, which impede the efficacy of conventional therapeutic modalities. Among the molecular markers are CEACAM5, a distinct marker present in NEPC and CRPC [4, 9, 101]. CEACAM5 has emerged as a promising therapeutic target, particularly in the context of NEPC, which exhibits elevated surface expression of this protein. Targeting this marker has been demonstrated to enhance the specificity and cytotoxicity of therapeutic approaches. Saini and colleagues engineered HEK293T-derived exosomes decorated with CEACAM5 antibodies, anchored using DMPE-PEG-STVD, to achieve NEPC-specific drug delivery [59, 79, 102]. The loading of both enzalutamide, the androgen deprivation drug, and tazemetostat, a competitive inhibitor of EZH2, effectively targets and combats NEPC, evidenced by the downregulation of neural markers in NCI-H660 and tumor regression in LuCaP145.1 NE PDX [79].

4. Mechanistic analysis of mesenchymal stem cell exosomes on prostate cancer

Naïve MSC-derived exosomes exert biochemical effects on malignant cells through their endogenous inclusions [103]. Although MSCs exhibit common properties, inherent biological and functional variations exist across MSCs from different sources, driven by genetic and epigenetic programming [104]. Furthermore, tumors display considerable heterogeneity, which results in disparate responses to exosomal intercommunication. MSC-derived exosomes have been demonstrated to exert a remarkable inhibitory effect on prostate carcinoma, primarily through their miRNA-rich secretome, which regulates a wide range of oncogenic pathways (**Table 2**).

Tumorigenic effect	Exosome/ secretome	Downstream gene regulation	Pathways analysis	Prostate cancer model	Mesenchymal stem cell source	Ref.
Prostate cancer inhibition	miR-187	Ki-67, Bcl2, and N-Cadherin downregulation E-Cadherin and Bax upregulation	Apoptosis induction, proliferation, and EMT inhibition	*In vitro*: 22Rv1, LNCaP, Du145, and PC-3 PCa cell lines	Human bone marrow MSC	[105]
	miR-205	MMP-2 and MMP-9 downregulation	Proliferation, invasion, and migration. Promotes apoptosis Tumor growth inhibition	*In vitro*: LNCaP PCa cell line *In vitro*: male BALB/c nude mice inoculated subcutaneously with LNCaP	Human bone marrow MSC	[106]
	miR-99b-5p	Ki-67	Proliferation reduction Tumor progression inhibition	*In vitro*: LNCaP, DU145, and PC3 PCa cell line *In vivo*: male BALB/c nude mice subcutaneously inoculated with LNCaP cells	Human bone marrow MSC	[107]
	mi-R145	Casp3/7 downregulation BclxL upregulation	Proliferation reduction Apoptosis induction Reduced tumor growth	*In vitro*: luciferase-expressing metastatic PCa cell line (PC3M-luc2) *In vivo*: PC3M-luc2-bearing male athymic nude mice	Human adipose-derived stromal cell (ASC)	[108]
	miR-375	PCNA, MMP-2/9 upregulation	Migration and invasion inhibition Apoptosis induction		Human bone marrow MSC	[109]
	miR-114		Apoptosis induction; Migration, proliferation, invasion inhibition	*In vitro*: LNCaP PCa cell line	Human bone marrow MSC	[110]
	Secretome	TP53 upregulation BCL2 downregulation	Apoptosis and proliferation	*In vitro*: LNCaP and PC3 cell PCa cell lines	Human abdominal adipose tissue	[111]
	Secretome	AKT and PI3K downregulation p53 upregulation	Reduced viability, proliferation, and motility	*In vitro*: PC3 and LNCaP PCa cell lines	Human umbilical cord (UC) MSC	[112]
	Secretome	Caspase 3/7 upregulation	Proliferation inhibition Apoptosis induction Tumor growth inhibition	*In vitro*: LNCaP and PC3 PCa cell lines *In vivo*: male athymic nude mice bearing LNCaP or PC3-derived subcutaneous tumors	Human adipose-derived stromal cells	[113]

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Tumorigenic effect	Exosome/ secretome	Downstream gene regulation	Pathways analysis	Prostate cancer model	Mesenchymal stem cell source	Ref.
Prostate cancer promotion	Secretome	TGF-β, VEGF, IL-6, and MIP-2 upregulation in bone-marrow-derived MSC	Vascularization and endothelial cell tubule formation Tumor-enhanced growth	*In vitro*: DU145 PCa cell line *In vivo*: nude mice subcutaneously injected with DU145	Human bone marrow-derived MSC	[114]
	Secretome	TGF-β upregulation in bone marrow-derived MSC	Immune suppression/ evasion	*In vitro*: RM-1 murine PCa cell line *In vivo*: Male Balb/c and C57BL/6 mice subcutaneously injected with RM-1	Mouse bone marrow-derived MSC pre-stimulated by IL-1α	[115]
	miR-200c	Wnt/β-catenin	Enhance tumor growth	—	Human bone marrow MSC	[116]

Table 2.
Molecular mechanism(s) of action of MSC-derived exosomes in prostate cancer.

Immune evasion represents a pivotal mechanism that drives tumor progression. CD276, also known as B7-H3, is frequently overexpressed in various cancers, including prostate cancer. Its upregulation facilitates immune evasion by suppressing the activation of T cells and dendritic cells, thereby promoting tumor immune tolerance [117]. Exosomal miR-187, derived from BM-MSC, has been observed to interfere with the translation of CD276 in prostate cancer. Additionally, this therapeutic intervention impedes prostate carcinoma progression by downregulating Ki-67, Bcl-2, and N-cadherin, while upregulating pro-apoptotic and epithelial markers, including E-cadherin and Bax [105].

Prostate cancer is distinguished by its genetic complexity and extensive heterogeneity, driven by the accumulation of genetic, epigenetic, and transcriptomic alterations during disease progression [4]. Alteration of RHPN2 plays a significant role by promoting the epithelial-to-mesenchymal transition (EMT), as well as the enhancement of cell motility and invasion [118]. Exosomes encapsulating miR-205 have been demonstrated to mediate significant alterations in prostate cancer cells through the regulation of RHPN2, thereby inhibiting cell migration in the population [106]. Similarly, BM-MSC exosomes have been observed to downregulate trefoil factor 3 (TFF3), a protein associated with cancer progression via the action of miR-275. This downregulation counteracts prostate cancer progression by modulating key markers such as proliferating cell nuclear antigen (PCNA) and matrix metalloproteinases MMP-2 and MMP-9 [109].

Yang and colleagues demonstrated that prostate cancer growth and progression are impeded. The BM-MSC-derived exosomal miRNA (miR-114) has been demonstrated to induce apoptosis through the downregulation of p53 [110]. While BM-MSC exosomal miR-99b-5p has been shown to attenuate prostate cancer progression by targeting and downregulating insulin-like growth factor 1 receptor (IGF1R), a key driver of cancer cell growth and survival. This downregulation leads to reduced proliferation of prostate cancer cells and a significant inhibition of tumor growth [107].

Adipose-derived stem cells have been demonstrated to suppress prostate tumorigenicity through miR-145, a microRNA with known anti-cancer properties. miR-145, exerting its effects via the activation of the caspase 3/7 apoptotic pathway [108, 113]. Similarly, a tumor-suppressive effect has been observed in the prostate, renal, and bladder cancers, with significant anti-proliferative activity specifically against prostate cancer cells, suggesting an enhanced uptake and therapeutic efficacy in the prostate model in comparison to other tissue types [111]. Exosomes derived from hUC-MSCs have been indicated to regulate the inhibition of PI3K/AKT activation and to decrease mRNA levels of pro-inflammatory cytokines. Moreover, subsequent p53 upregulation suggests the possibility of hindering the cell cycle [112].

5. Clinical implications of mesenchymal stem cell-derived exosomes in prostate cancer

The clinical application of MSC-based therapy has been vastly explored in several degenerative diseases (for example: NCT01765634 NCT04208646). The evaluation has demonstrated the safety of the administration of these cells in circulation; however, the efficacy has not been satisfactory. Moreover, the information on oncological malignancies is still scanty. A phase I clinical trial (NCT01983709) of MSCs cell-based therapy in the prostate cancer region was carried out in patients who were scheduled for prostatectomy. The said patients were infused intravenously with MSCs harvested from healthy individuals, and the analysis of the presence of the MSCs was carried out *ex vivo*, following the prostatectomy. Although no successful homing was observed in

the specimen, thereby a shortfall in feasibility, this study has proven the safety of the practice. Moreover, it leaves room for a more strategic approach, such as mode of administration and duration [119]. Due to the lack of correlation between the cell functionality and engraftments, the clinical application of MSC cell-based therapy is being redirected to the use of exosomes. Exosomes are an extension of their secreting cells. As such, cancer-derived exosomes are most likely to exert tumor-promoting properties, as reported by Saari and colleagues in prostate cancer preclinical analysis [73]. Therefore, the use of MSC-derived exosomes remains an attractive alternative due to the cell's regenerative and therapeutic properties.

5.1 Feasibility of cell-free therapy and current status

Clinical applications of exosomes in therapeutics have been proven feasible. This approach is currently employed in several clinical trials. Those include the use in drug delivery for malignant ascites (NCT01854866) and malignant pleural effusion (NCT02657460), the delivery of siRNA in metastatic pancreatic adenocarcinoma (NCT03608631), and others. The sustainability of this therapeutic approach is challenged by the rapid clearance upon systemic injection and macrophage uptake, shortening the half-life. Currently, there are no records of clinical use of MSC-derived exosomes in prostate cancer therapy. However, the rigorous preclinical analysis, both *in vitro* and *in vivo,* has demonstrated the efficacy of this therapeutic approach. Clinical translation of this phenomenon is a promising prostate cancer therapeutic intervention that can address some of the shortfalls in mCRPC treatment.

5.2 Exosomes used in management and monitoring of prostate cancer

In biomarker studies, exosomes also have some benefits. Exosomes have adequate sources due to their presence in most body fluids with less invasive sampling techniques [120]. Exosome storage is relatively straightforward and less influenced by cryopreservative agents that prevent potential loss [121, 122]. Exosomes offer notable advantages in biomarker studies due to their widespread availability in various body fluids, enabling less invasive sampling techniques [120]. Around 488 studies about EVs, exosomes, or secretomes and about 15 relevant studies on exosomes and prostate cancer have been registered in clinicaltrials.gov (accessed on 7 July 2023). Exosomes isolated from body fluids were mainly used to determine the diagnostic, risk classification, and prognostic of prostate cancer (NCT02702856, NCT04720599, NCT04556916, NCT04100811, NCT05572099, NCT03957252, NCT04661176, NCT03911999, NCT04340245). Urinary exosomes become simple and convenient liquid biopsy tests not limited to diagnosis but include decision-making in performing invasive procedures such as prostate biopsy (NCT03031418, NCT03235687). Current clinical trials also identify exosome roles in prostate cancer pathogenesis related to other pathologic conditions or during therapeutic interventions (NCT04167722, NCT02928432). Finally, the exosomes are also utilized to monitor the effects of therapy (NCT03824275, NCT05192694).

6. Challenges and limitations of the use of MSC-derived exosomes in prostate cancer

Numerous preclinical studies have explored the potential of exosomes in prostate cancer, focusing on their roles in intercellular communication, tumor progression, and

therapeutic delivery. Clinically, trials have primarily emphasized the diagnostic, risk stratification, and prognostic utility of exosomes in prostate cancer, with significant progress in developing non-invasive liquid biopsy approaches. However, the application of exosomes in therapeutic clinical trials remains limited. Shortfalls include a source of exosomes, mass production, and standardization.

6.1 Challenges with sourcing and large-scale production

Bone marrow MSCs are commonly utilized in MSC investigations owing to their easy accessibility and reproducibility, making them a good therapeutic source [105]. Additionally, human umbilical cord MSCs represent a readily accessible MSC source, with collection posing no risk to donors as the tissue would have otherwise been discarded [112]. Attractive as it may be, sourcing exosomes from MSCs also presents challenges, such as ethical implications and other limitations, which impede clinical application. Utilizing induced iPSCs offers a solution by providing unlimited expandability without moral implications [72]. Large-scale experimental studies are essential to evaluate exosome efficiency and safety profiles. However, mass and standardized production is prone to microvesicles, lipoproteins, and chylomicrons contamination, among other factors [123–126]. More extensive studies are therefore of paramount importance in the standardization and applicability of exosomes in prostate cancer.

6.2 Tumor-educated exosome and tumorigenic promontory effect

Prostate cancer-derived exosomes are advantageous in prostate tissue homing; however, they are most likely to exert tumor-promoting properties [73]. This phenomenon makes mesenchymal stem cells an attractive alternative due to their therapeutic characteristics. However, the broach constituency and heterogeneity of MSC exosomes potentiate undesirable tumor-promoting effects. These promontory effects can be inherent or acquired through tumor education from cancer cells, immune cells, or other elements within the tumour microenvironment [127, 128]. Several studies have reported a cancer-promoting effect of MSC-derived exosomes in prostate cancer, contrary to the anticipated tumor-inhibitory role, possibly due to their regenerative properties (**Table 2**). This phenomenon was observed through different mechanisms. This unpredictability highlights the challenge of controlling MSC-derived exosomes in cancer therapy, as their potential to promote tumor growth complicates their therapeutic use.

6.2.1 In vitro *studies of MSC exosomal mechanisms in cancer progression*

MSCs can be recruited to the tumor niche, where they become an integral part of the tumor stroma. In the orchestration of this occurrence, naïve MSCs are educated to acquire cancer-promoting activities that enable tumor progression, such as invasion and immune evasion, among others. Elucidation of the mechanism behind this phenomenon is crucial in developing therapeutic strategies to impede tumor growth [127, 128]. Co-incubation or co-inoculation of MSCs with prostate cancer cells has been shown to result in genetic alterations of the MSCs. The interaction between MSCs and the tumor stroma leads to changes in the genetic make-up of MSCs, suggesting a process of molecular transfer and genetic reprogramming influenced by the signaling from prostate cancer cells. This crosstalk involves the exchange of bioactive molecules, which alter the gene expression profiles of MSCs, promoting tumor-promoting behaviors like enhanced migration, invasion, and immune modulation. Co-incubation/co-inoculation of MSCs with prostate

cancer results in MSCs genetic alteration. The variation of the genetic make-up following the interaction of MSCs with tumor stroma and the capacity thereof insinuates molecular transfer and subsequent genetic reprogramming of MSC, influenced by prostate cancer cells [129, 130]. The crosstalk between prostate cancer and BM-MSC overexpresses transforming growth factor-beta (TGF-β), VEGF, IL-6, and MIP-2 pro-angiogenic factors in BM-MSCs, perpetuating tubular formation and vascularization, enabling tumor expansion [114]. Pre-treatment of MSCs with inflammatory cytokines promotes the proliferation of prostate cancer cells. The infusion of conditioned medium from MSCs into prostate cancer tumor-bearing mice significantly enhances tumor growth. The crosstalk between MSCs and prostate cancer cells leads to the upregulation of TGF-β in MSCs, a critical mediator of immune evasion. TGF-β potentiates immune surveillance evasion by modulating immune cell function, thus promoting the survival of malignant prostate tumor cells in the tumor microenvironment [115].

Prostate tumorigenicity promotion by BM-MSC exosomes is associated with the activity of exosomal miR-200c, enhancing tumor invasion and growth in prostate cancer. The tumor-promoting effects are attributed to regulating cortactin (CTTN), a protein involved in cytoskeletal remodeling and cell motility. By influencing CTTN expression, exosomal miR-200c contributes to increased invasiveness and the expansion of prostate tumors [116].

7. Conclusion

MSCs are acknowledged for their valuable therapeutic attributes and the diverse nature of their composition. Exosomes derived from MSC demonstrate the properties of the parent cell in prostate cancer tumor infiltration, homing, and therapeutics. However, naïve exosomes exhibit a dual pro- or anti-tumorigenic effect in prostate malignancy. A myriad of studies reported desirable therapeutic effects, attributing a wide variety of secretomes, mostly miRNAs. However, a subset of studies also documented the prostate tumor-promoting effect stemming from the broad inherent regenerative properties of MSCs' broad composition, tumor education from cancer cells, immune cells, and other tumor microenvironment components. Nevertheless, the positive therapeutic outcomes were evident from modifying exosomes, as reported by several preclinical analyses in diverse prostate models, signifying the emergence of a novel therapeutic avenue with the potential for feasible clinical translation. Prostate cancer-specific exosome modification yielded a more improved binding and efficacious uptake. Furthermore, various loaded cargoes such as siRNAs, DNA, vaccines, androgen-deprivation therapeutic drugs, and chemotherapeutics have yielded prostate tumor regression. The clinical applicability of exosomes has been proven feasible in prostate cancer monitoring and management. However, the clinical therapeutic application is still met with some challenges and limitations, such as exosome sourcing, scalability, techniques, and standardization. Further research and clinical exploration to harness the full therapeutic potential of modified MSC-derived exosomes in prostate cancer is of paramount importance.

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Conflict of interest

The authors declare no conflict of interest in relation to this work.

Acronyms and abbreviations

ADT	androgen deprivation therapy
CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5
CRPC	castration-resistant prostate cancer
CTTN	cortactin
CTLs	cytotoxic T lymphocytes
DCs	dendritic cells
DOX	doxorubicin
EZH2	enhancer of zeste homolog 2
EpCAM	epithelial cell adhesion molecule
EMT	epithelial-to-mesenchymal transition
ECM	extracellular matrix
EV	extracellular vesicle
MHC	histocompatibility complex
HSPC	hormone-sensitive prostate cancer
HSA	human serum albumin
OH	hydroxyl radicals
IFN-γ	interferon-γ
Fe	iron
MMPs	matrix metalloproteinases
MSCs	mesenchymal stem cells
MVA	modified vaccinia Ankara
NEPC	neuroendocrine prostate cancer
iPSC-MSCs	pluripotent stem cell-derived MSCs
PMA	polymethacrylic acid
PCNA	proliferating cell nuclear antigen
PSA	prostate-specific antigen
PSMA	prostate-specific membrane antigen
PAP	prostatic acid phosphatase
Tregs	regulatory T cells
Sirtuin 6	SIRT6
SDT	sonodynamic therapy
TGF-β	transforming growth factor-beta
TFF3	trefoil factor 3

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Section 2

Delivery of Therapeutic Molecules

Chapter 6

Exosome-Based Drug Delivery Systems

Ruotong Huang, Jianming Zhou and Shuying Chen

Abstract

Extracellular vesicles, especially exosomes, have attracted widespread attention in the biomedical field in recent years. They have a unique ability to efficiently transport a variety of bioactive molecules, a property that makes them show great potential in precision medicine. In addition, exosomes can evade detection by the immune system, providing a new solution for drug delivery and cancer research. This manuscript provides an overview of exosome biogenesis, isolation-related techniques, and their potential for application as therapeutic vehicles. We discuss various strategies for loading exosomal cargo and engineering them for targeted delivery, highlighting recent advances in exosome-based vaccines and personalized cancer therapies. This book chapter concludes by emphasizing the transformative impact of exosome-based therapeutics on precision medicine, outlining the future direction of this field and its potential to overcome traditional therapeutic limitations.

Keywords: extracellular vesicles, exosomes, drug delivery systems, engineered exosomes, targeted therapy, cancer therapy

1. Introduction

Extracellular vesicles (EVs) have recently garnered substantial interest in cancer research. According to the MISEV2018 guidelines [1], EVs are categorized into three primary types based on their size and mode of biogenesis: exosomes, which typically measure less than 150 nm in diameter; microvesicles (MVs), sometimes referred to as ectosomes, which are formed by direct budding from the plasma membrane and generally range from 100 nm to 1000 nm in size; and apoptotic bodies, which range from 1 to 5 μm in diameter [2]. For the purposes of this review, we will focus primarily on exosomes, the smallest subclass of EVs, and will not delve further into microvesicles or apoptotic bodies, as these are released directly from the plasma membrane of viable and apoptotic cells, respectively. Exosomes are enriched with a variety of bioactive molecules, including proteins, lipids, metabolites, and nucleic acids (**Figure 1**).

Exosomes were first identified in sheep reticulocytes in the 1980s and initially considered as cellular debris [3]. However, subsequent research has revealed their pivotal role in intercellular communication. The significance of exosomes is further

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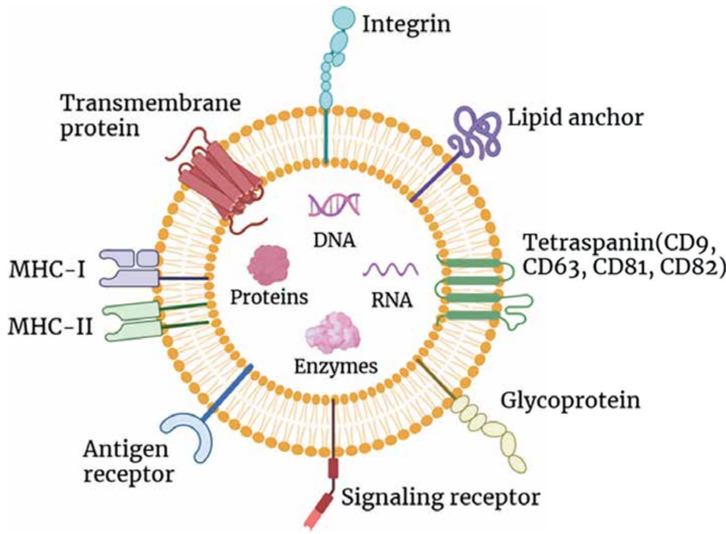

Figure 1.
Structure and composition of exosomes. Created with BioRender.com.

underscored by their involvement in a broad range of pathological and physiological processes [4]. Exosome biogenesis occurs in three stages, beginning with the inward budding of the plasma membrane (**Figure 2**). This process leads to the formation of early endosomes, which sequester cellular proteins and genetic material from the cytoplasm. As these endosomes mature into late endosomes, they give rise to multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) [5]. MVBs are intermediates that have the ability to merge with the plasma membrane or be targeted for lysosomal breakdown. ILVs are released into the extracellular space by MVBs after they fuse with the plasma membrane, where they are identified as exosomes [6].

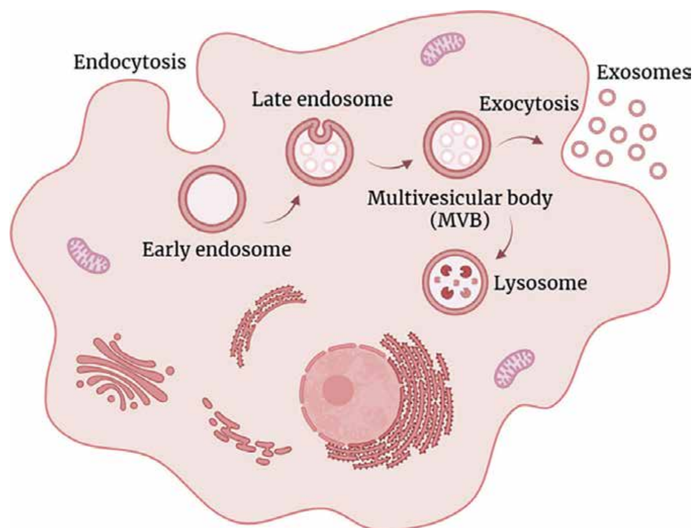

Figure 2.
Biogenesis of exosomes. Created with BioRender.com.

Exosome biogenesis is completed in this last stage, which sets them apart as a distinct group of exosomes that come from the endocytic route.

A promising therapeutic strategy that has the potential to entirely alter the way numerous diseases are treated is exosome-mediated medication delivery. Exosomes can transfer therapeutic cargo straight into the cytoplasm, avoiding both lysosomal degradation and the endosomal route, because of their low immunogenicity [7]. Furthermore, exosomes have the extraordinary capacity to pass across biological barriers, including cell membranes and the blood–brain barrier, which makes it simpler for them to enter tumor tissues [8]. This distinctive feature paves the way for precision-targeted therapies. Furthermore, exosomes can be engineered to selectively target specific cells, enhancing the long-term stability and efficacy of therapeutic agents and imaging probes while increasing cellular uptake [9].

In this review, we provide an overview of the fundamental concepts of exosomes and offer a comprehensive analysis of current strategies for exosomal cargo loading. We also examine engineering techniques for targeted delivery and highlight the advantages and limitations of these approaches. Additionally, we discuss recent advancements in exosome-based vaccines and their potential in personalized cancer therapy. This review aims to bridge the gap between foundational knowledge and emerging research, offering new insights into the future of exosome-based therapeutics.

2. Isolation techniques of exosomes

Exosomes were first isolated using ultracentrifugation-based methods, which remain the gold standard for isolation [10]. Although alternative techniques have been developed, most fail to effectively separate exosomes from lipoproteins with similar physicochemical properties or exosomes derived from non-endosomal pathways, leading to low purity [11]. As a result, developing isolation methods that are rapid, efficient, reproducible, and clinically feasible remains a significant challenge. An overview of some of these methods is provided in **Table 1**.

2.1 Ultracentrifugation

Ultracentrifugation (UC) remains the most commonly used isolation technique and is considered the gold standard. It is divided into two primary methods: Differential ultracentrifugation and density gradient ultracentrifugation [16]. Differential ultracentrifugation involves multiple steps of low-speed centrifugation to remove cellular debris and apoptotic fragments, followed by two cycles of high-speed centrifugation to eliminate larger vesicles and precipitate exosomes and other extracellular vesicles from the matrix [12]. However, this approach is time-consuming, labor-intensive, costly, and often results in low yields and compromised purity.

In contrast, density gradient ultracentrifugation employs an inert gradient medium, such as linear sucrose or iodixanol, into which the sample is added for centrifugal sedimentation or equilibration. This method separates sample components based on their density, improving exosome purity while preserving their structure [13]. However, it is also a highly time-consuming technique that yields relatively low amounts of exosomes.

Techniques	Principle	Advantages	Disadvantages	References
Differential ultracentrifugation	Size-based sequential separations	High yield, simple, suitable for large sample volumes	Time-consuming, difficult to upscale potential for sample aggregation and contamination	[12]
Density gradient ultracentrifugation	Density- and size-based sequential separations	High yield, high purity, effective for body fluids	Difficult to upscale, risk of gradient damage	[13]
Size-based techniques	Size	Easy to operate, quick, low-cost, maintains exosome structure and biological activity	Low purity, possible contamination with similar-sized particles	[14]
Immunoprecipitation	Uses antibodies for specific capture	High specificity, sensitivity, purity	Low yield, high reagent cost, limited use	[15]
Polymer precipitation	Solubility or dispersibility	Easy to operate, does not require specialized equipment, short analysis time, suitable for large samples	Low purity and recovery rate, potential for false positives, difficulty in removing polymer	[14]

Table 1.
Isolation techniques of exosomes.

2.2 Size-based techniques

Size-based techniques isolate exosomes by exploiting the size differences between exosomes and other components in biological samples. Ultrafiltration uses a membrane with a specific molecular weight cut-off to separate exosomes based on size, allowing for the use of smaller sample volumes. However, the application of force during the process may deform or rupture larger vesicles, potentially leading to inaccurate results.

Exosomes and other particles are distinguished using size exclusion chromatography (SEC) according to their capacity to either enter or be excluded from a porous gel matrix. Smaller molecules are held in the porous matrix and then eluted with an eluent, whereas larger macromolecules that cannot pass through the gel pores are flushed through with the mobile phase. SEC is an isolation technique that maintains the biological integrity of exosomes and is comparatively easy, quick, and economical [14].

In addition to these methods, other techniques such as sequential filtration, flow field-flow fractionation (FFFF), and hydrostatic filtration dialysis (HFD) are also employed for exosome separation [15].

2.3 Methods based on immunoaffinity capture

Exosomes can be selectively separated and purified from complex biological mixtures using immunoaffinity capture-based approaches that use antibodies specific to exosome surface markers. Various solid substrates, including microplates, magnetic

beads, resins, and microfluidic devices, can be conjugated to these antibodies [15]. The enzyme-linked immunosorbent assay (ELISA), which isolates exosomes from a sample by binding an antibody that targets a particular antigen on the surface of a microplate, is an example of an immunoaffinity capture technique. Exosomes can be captured more precisely and effectively using a similar method called immuno-based microfluidic isolation, which is performed on a microfluidic chip.

2.4 Other isolation techniques

Polyethylene glycol (PEG) is used in polymer precipitation to make exosomes more insoluble so that they can be collected by centrifugation [14]. Commercial kits, which are based on conventional methods and have benefits like high yield and time efficiency, are another type of isolation method. Furthermore, the potential of advanced technologies like micro-vortex chips, acoustic fluid platforms, and precise filtration techniques to improve isolation efficiency is being investigated.

When many tactics are combined, isolation efficiency may be higher than when one strategy is used alone. Consequently, several research teams have started combining different approaches to improve yield, purity, enrichment, and separation efficiency [17]. However, variability in preanalytical factors, such as sample collection, the use of anticoagulants, the presence of contaminants, and the time required for sample processing, can significantly impact the isolation and characterization of exosomes, posing technical barriers to their analysis [18]. In addition to technological advancements, achieving sufficient yield and accurate identification is crucial for the clinical application of exosomes.

3. Engineering exosome strategies for targeted delivery

Exosomes are naturally occurring; however, they may be readily modified for certain uses. Different cell types' exosomes can be guided to particular target areas under precise conditions. By choosing suitable exosome donors or using bioengineering methods, the potential of exosomes as drug delivery vehicles can be increased. It is feasible to develop tailored carriers that carry treatments to the intended cells or organs by encapsulating medications in modified exosomes, which enhances clinical results [19]. Exosome surface changes can be accomplished chemically or genetically, as explained and summed up below (**Figure 3** and **Table 2**).

3.1 The application of genetic engineering

Transmembrane proteins produced on the exosomal surface are frequently combined with ligands or homing peptides in genetic engineering. Targeting ligands are seen on the membranes of exosomes secreted by donor cells transfected with plasmids producing these fusion proteins. Although this technique successfully displays proteins and peptides on the exosomal surface, it does have certain drawbacks. It limits targeting motifs to those that are genetically encoded, to start. Second, it cannot be readily used on cells that are difficult to transfect, including red blood cells and stem cells, nor is it appropriate for pre-isolated exosomes, such as those obtained from tissues or bodily fluids. Additionally, the method is costly and time-consuming, which limits its viability for clinical use [20].

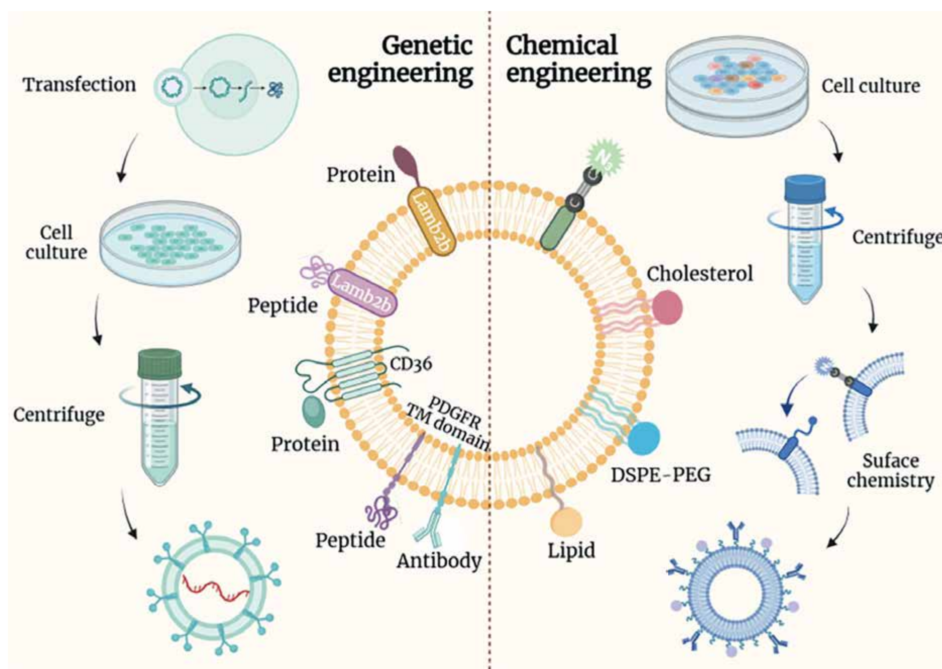

Figure 3.
Engineering exosome strategies for targeted delivery. Created with BioRender.com.

Since it does not affect the structural integrity or characteristics of exosomes, lysosome-associated membrane glycoprotein 2b (Lamp2b) is now the most often employed surface protein for genetic engineering [21]. To deliver doxorubicin to αv integrin-positive breast cancer cells in vitro, for example, researchers fused the iRGD peptide to the extracellular N-terminus of Lamp2b. By specifically delivering doxorubicin to tumor sites in vivo, these modified exosomes reduced toxicity and stopped tumor development [22]. The N-terminus of Lamp2b has also been fused with a HER2-binding affibody (zHER), producing exosomes that have a high binding affinity and selectivity for HCT-116 colon cancer cells. In vivo, these exosomes effectively transported anti-miRNA-21 and 5-FU medications to tumors that expressed HER2 [23]. The tetraspanin superfamily proteins CD63, CD9, and CD81, which have two extracellular loops, provide additional chances for surface functionalization and protein fusion in addition to Lamp2b. To target liver cancer cells that express scavenger receptor class B type 1, Liang et al. [24] created exosomes with functional miR-26a. They accomplished tumor cell-specific targeting by producing Apo-A1 as a fusion protein in donor 293 T cells and introducing it into the short extracellular loop of CD63. The platelet-derived growth factor receptor (PDGFR) is another transmembrane protein that is frequently employed for surface display. Exosomes laden with miRNA were able to target breast cancer cells that expressed the epidermal growth factor receptor (EGFR) by modifying donor cells to express the transmembrane domain of PDGFR linked to the GE11 peptide [25].

While the specificity of these methods offers promising in vivo applications, the main drawbacks are the synthetic challenges and high costs associated with presenting functional ligands on exosomes [26].

Strategy	Classification	Advantages	Disadvantages
Genetic engineering of donor cells	Fusion of ligands or peptides with transmembrane proteins	Enhance target specificity Improve uptake by target cells	Limited applicability Genetic coding limitation Laborious and expensive
	Lamp2b	Maintain the integrity of exosomes Efficient drug delivery Enhance targeting selectivity	Complex genetic engineering Potential toxicity
	CD63/CD9/CD81	Maintain the integrity of exosomes Highly targeted Use natural ligand	Complex genetic engineering Limited by the transfection efficiency Limited by cell types
	Receptor membrane proteins such as PDGFR and EGFR	Highly efficient targeting Enhance selective delivery of exosomes	Limited to specific receptors Need engineering of donor cells
Chemical modification	Conjugation reactions	Stable modification of exosomal surface proteins	Low reaction efficiency Lack of site specificity control
	Surface charge	Control targeting efficiency toward desired organs	Complex optimization process required
	Amphipathic molecule insertion	Enhance target cell uptake and therapeutic efficiency Can cross the blood-brain barrier	Complex modification process Risk of changing the structure of exosomes Need to optimize the insertion and stability of different molecules

Table 2.
Advantages and disadvantages of engineering exosome strategies.

3.2 Chemical modification

The benefit of chemical alteration over genetic engineering is that it avoids the dangers of gene transfer and the difficulty of multi-step manipulation. By using covalent conjugation procedures and non-covalent modifications, this method makes it possible to offer a wide variety of ligands, including both natural and synthesized molecules [20].

3.2.1 The reaction of conjugation

While exosomal surface proteins can be covalently and permanently modified by conjugation reactions, the intrinsic complexity of the exosome surface may restrict reaction efficiency, and the absence of site specificity control may be a drawback. For instance, copper-catalyzed azide-alkyne cycloaddition “click” chemistry allows alkyne groups to easily alter the amine groups of exosomal proteins, which may then be bio-orthogonally attached to molecules containing azide. By engineering exosomes with neuropilin-1-targeted peptides using click chemistry, Jia et al. [27] were able to deliver curcumin and superparamagnetic iron oxide nanoparticles for concurrent brain cancer detection and treatment uses.

3.2.2 Surface charge

The surface charge of exosomes plays a critical role in their cellular internalization, distribution, and targeting to specific organs or cells [28]. By optimizing the surface charge, the targeting efficiency of exosomes can be directed toward the desired tissues or organs [29]. In one study [30], charge-variable exosomes were engineered by conjugating their surface proteins with near-infrared fluorophores to modulate in vivo distribution and clearance. The results showed that zwitterionic fluorophore-labeled exosomes exhibited rapid renal clearance with minimal nonspecific tissue uptake, while anionic exosomes were predominantly excreted via the hepatobiliary route, demonstrating high liver uptake.

3.2.3 Amphipathic molecule insertion

A promising chemical modification approach involves incorporating amphipathic molecules into the lipid bilayer of exosomes. Ye et al. [31] employed ApoA-1 mimetic peptides to functionalize the lipid bilayer, resulting in methotrexate-loaded exosomes decorated with low-density lipoprotein (LDL) peptides. Compared to unmodified exosomes, in vitro experiments demonstrated that this modification enhanced selective uptake by the human glioma cell line U87, thereby significantly improving therapeutic efficacy. Additionally, LDL peptides facilitated the exosomes' ability to penetrate the blood-brain barrier (BBB) and target glioma cells, as confirmed by both in vitro and in vivo imaging studies.

Similarly, another study explored the modification of exosomal membranes with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG), which can anchor polyethylene glycol (PEG) onto the exosome surface. Moreover, a targeted approach aimed at sigma receptors, which are overexpressed in lung cancer cells, introduced an aminoethylanisamide-polyethylene glycol (AA-PEG) vector into the exosomal membrane. AA-PEG-modified exosomes showed enhanced cellular uptake in lung cancer cell lines, and the targeted delivery of paclitaxel resulted in improved therapeutic outcomes in vivo [32]. In another strategy, cholesterol, due to its hydrophobic nature, was integrated into exosome membranes to enable more precise targeting. For example, exosomes were functionalized with cholesterol conjugated to RNA ligands or folate to facilitate targeted siRNA delivery to specific cells [33].

4. Drug-loading approaches of exosomes

Exosomes exhibit distinctive properties that make them highly promising candidates for drug delivery applications. To date, a variety of strategies have been devised for loading therapeutic cargo into exosomes, which are summarized below (**Figure 4** and **Table 3**).

4.1 Incubation

The simplest and most direct preloading technique is incubation, which entails co-incubating the desired cargo with donor cells or exosomes. Because exosomes and plasma membranes are lipid-rich and hydrophobic, cargo, especially hydrophobic substances like paclitaxel and curcumin, can naturally interact with and integrate into exosomes or cells that secrete exosomes [34].

Figure 4.
Drug-loading approaches of exosomes. Created with BioRender.com.

However, despite efforts to optimize cargo ratios, concentration, incubation times, and conditions, this method typically results in low loading efficiencies [28]. Additionally, larger cargoes, such as proteins and nanomaterials, face difficulties penetrating the exosomal membrane without external assistance.

4.1.1 Donor cell and cargo incubation

This approach involves exposing donor cells to therapeutic agents, thereby altering the culture conditions and cellular environment, which in turn affects the biological properties of the exosomes produced. Once stimulation triggers exosome secretion, the cargo-loaded exosomes can be identified and isolated using techniques such as ultrasonication, polymer-based precipitation, immunoaffinity capture, and microfluidics [44].

Small-molecule drugs can cross the lipid bilayer of donor cells and become encapsulated within intraluminal vesicles, which are subsequently secreted as exosomes. Pascucci et al. [45] incubated mesenchymal stromal cells (MSCs) with paclitaxel for 24 hours, resulting in the production of paclitaxel-loaded exosomes with potent antitumor activity. Similarly, Wang et al. [46] incubated macrophages with curcumin to generate curcumin-loaded exosomes, which improved the solubility and bioavailability of curcumin and enhanced its ability to cross the BBB to alleviate Alzheimer's disease symptoms.

Nanomaterials can also be packaged into exosomes via co-incubation. Silva et al. [47] demonstrated that macrophages incubated with iron oxide nanoparticles internalized these particles and incorporated them into exosomes. The study revealed that the uptake of these exosomes by cancer cells could be modulated kinetically and spatially, controlled using a magnetic field, enhancing cancer cell death through magnetic targeting.

Approach	Principle	Advantages	Disadvantages	References
Incubation	Donor cell and cargo incubation: Subject donor cells to medications to change exosome properties Exosome and cargo incubation: Allow drugs to enter exosomes based on concentration gradient	Simple and straightforward	Low loading efficiency difficulty in cargo management potential toxicity of cargo to cells	[34]
Transfection	Transfer cargo into parent cells by introducing protein-expressing plasmids or nucleic acids	High loading efficiency	Costly and time-consuming risk of damage or contamination	[35]
Electroporation	Use high-voltage pulses to create pores in the exosome membrane for efficient cargo migration and loading	High loading efficiency controllable	Requires optimization of parameters affect stability and integrity of membrane	[36]
Sonication	Use ultrasonic waves to reduce lipid layer's microviscosity, allowing hydrophobic medications to flow through	High loading efficiency	Possible active agent damage and heat generation extra mechanical strain can affect exosome structure	[37]
Extrusion	Using an extruder device, exosomes are mechanically disrupted and reconstructed into nanovesicles containing the desired drug	High loading efficiency uniform size	Alter exosome immune-privileged state risk of membrane damage	[38]
Freeze-thaw cycles	The exosomes and drug are frozen in liquid nitrogen at −80°C, followed by multiple cycles of thawing at room temperature.	Simple and mild can produce simulated exosome nanoparticles	Low loading efficiency aggregation protein inactivation	[39]
Surfactants	Redistribute exosome lipid layer particles with surfactants to create surface holes and enhance permeability	High loading efficiency	Possible cargo breakdown or inactivity additional purification steps	[40]
Hypotonic dialysis	Dialyzing exosomes and cargo by mixing them within a dialysis membrane or tube	High loading efficiency	Breakdown of peptide and protein	[41]
pH gradient loading	Establish an acidic gradient across exosome membrane to encapsulate drugs	High loading efficiency	Protein degradation, aggregation	[42]
In situ synthesis	Load nanomaterials onto the surface or interior of exosomes without damaging their physical integrity	Preserves integrity of exosomes	Complex, load precious metals	[43]

Table 3.
Drug-loading techniques of exosomes.

Despite being simple and practical, this approach has drawbacks like poor loading efficiency and difficulties with cargo management. These problems are exacerbated by the challenge of accurately regulating the amount of cargo that is packaged into exosomes or integrated into cells. Furthermore, the payload itself might be cytotoxic, which could damage cells or interfere with exosome secretion's normal course. Consequently, even though this method's simplicity is beneficial, its drawbacks need to be carefully evaluated [48].

4.1.2 Exosome and cargo incubation

The incubation method involves exposing isolated exosomes to cargo for a predetermined amount of time at a certain temperature, such as body temperature (37°C) or room temperature (20°C) [49]. This technique makes use of the concentration gradient to make it easier for cargo to enter the exosomes. A variety of cargo types, such as tiny molecules, proteins, peptides, and therapeutic nucleic acids, have been effectively loaded into exosomes using this approach.

For instance, Saari et al. [50] demonstrated that cancer cell-derived exosomes effectively delivered the small-molecule drug paclitaxel to prostate cancer cells via an endocytic pathway, enhancing the drug's cytotoxicity. Similarly, doxorubicin, another chemotherapeutic agent, can be efficiently packaged into exosomes using the incubation method [51]. Proteins and peptides can also be incorporated into exosomes through membrane interactions under controlled conditions. Yuan et al. [52] used macrophage-derived exosomes to systemically deliver brain-derived neurotrophic factors to inflamed brain tissues in Parkinson's disease models.

This strategy is equally applicable for loading therapeutic nucleic acids. Gong et al. [53] incubated exosomes with cholesterol-modified miR-159 at room temperature with shaking, enabling co-delivery of miRNA and doxorubicin for targeted therapy against triple-negative breast cancer.

Notably, this technique minimally disrupts the structural integrity of exosomes and is both simple and cost-effective. However, because it primarily relies on diffusion and hydrophobic interactions between the cargo and the exosome lipid membrane, loading efficiency is limited [54]. Additionally, factors such as pH can influence loading efficiency. The physicochemical properties of both the cargo and the exosomes may result in insufficient cargo release, posing challenges for clinical applications [55].

4.2 Transfection

Another widely used preloading technique is transfection. In this method, cargo is introduced into parent cells, leading to the incorporation of the therapeutic agents into or onto exosomes during their formation. Despite its utility, transfection faces the significant challenge of low loading efficiency due to difficulties in controlling cargo uptake. Moreover, it carries the risk of cell and exosome damage or contamination due to the use of transfection reagents. The transfection process can be performed using various methods, including chemical approaches, electroporation, and viral vector-mediated strategies.

Chemical transfection reagents are commonly employed to introduce specific plasmids into cells, prompting the expression of desired cargoes. For instance, Lou et al. [56] used Lipofectamine to transfect adipose tissue-derived mesenchymal stem cells (AMSCs) with a miR-122-expressing plasmid. The resulting AMSC-derived exosomes were used to enhance chemosensitivity in hepatocellular carcinoma.

While chemical transfection can also introduce nucleic acids directly into isolated exosomes, it often leads to contamination, posing potential risks to both cells and exosomes. Moreover, the use of transfection reagents may cause cellular damage or introduce impurities, making this method less suitable for drug-loading applications [35].

4.3 Physical methods

To enhance cargo diffusion, physical methods are employed. Techniques like sonication, electroporation, and surfactant treatment create micropores on the exosomal surface via mechanical, electrical, or chemical means. Extrusion and freeze–thaw cycles further facilitate membrane recombination and fusion. These methods significantly improve cargo loading compared to incubation [57]. However, they also introduce potential risks, including membrane damage or aggregation, potential contamination and toxicity, and damage to cargo. Therefore, careful control over parameters is essential.

4.3.1 Electroporation

Electroporation, regarded as the gold standard method for exosome loading, employs short, high-voltage electrical pulses to create temporary nanopores on the membrane of isolated exosomes, facilitating the efficient incorporation of cargoes [36]. This technique can be used to load a wide range of payloads, including drugs, nucleic acids, and nanomaterials.

Optimal loading efficiency with electroporation depends on precise fine-tuning parameters such as capacitor capacity, voltage, pulse frequency, pulse duration, and interval length. However, despite its effectiveness, electroporation can compromise the stability and integrity of exosome membranes, leading to potential aggregation and reduced efficiency, thereby limiting its broader applicability [8].

4.3.2 Sonication

The sonication technique leverages ultrasonic waves to significantly reduce the microviscosity of the exosomal lipid bilayer—typically by more than two orders of magnitude—facilitating the diffusion of hydrophobic molecules [37]. In this method, exosomes derived from donor or target cells are mixed with specific drugs or protein labels and subjected to ultrasonic waves via a homogenizer probe. The mechanical stress induced by sonication temporarily distorts the exosome membrane, allowing biologically active compounds to be efficiently loaded into the exosomes.

Drugs, proteins, and nanomaterials can all be loaded using this flexible technique. Large-scale applications may encounter difficulties, though, as the mechanical strain caused by sonication may weaken the exosomal lipid bilayer's structural integrity [9].

4.3.3 The process of extrusion

A combination of exosomes and cargo is run through a syringe-based lipid extruder that has nanopores that range in size from 100 to 400 nanometers as part of the extrusion process. By repeatedly extruding the payload under carefully regulated conditions, this technique breaks the exosome barrier and permits effective and uniform loading of the contents. Extrusion has a high loading efficiency, but it can also

change the shape of the exosome membrane and jeopardize its immune-privileged status, which makes the immune system more likely to recognize it [38].

4.3.4 Freeze-thaw cycles

In the freeze-thaw cycle method, exosomes are treated with selected drugs for a specified duration at room temperature, and then rapidly frozen in liquid nitrogen or at −80°C. After freezing, the mixture is thawed at room temperature. Typically, at least three cycles of freezing and thawing are performed to enhance drug encapsulation [39]. However, this method exhibits lower drug-loading efficiency compared to ultrasound or extrusion techniques. Furthermore, repeated freeze-thaw cycles can induce aggregation of exosomes, potentially leading to protein inactivation. This technique can also be used to generate simulated exosome nanoparticles by fusing exosome and liposome membranes [43].

4.3.5 Surfactants

Under the surface modification technique, surfactants such as saponin are used to induce redistribution of the exosomal lipid bilayer, creating surface pores that enhance membrane permeability. This approach significantly increases the capacity for cargo loading into exosomes. However, the use of surfactants may lead to the degradation or inactivation of the loaded cargo, potentially reducing its therapeutic efficacy. Moreover, saponin exhibits hemolytic activity in vivo, which necessitates an additional purification step to remove residual surfactant and ensure the safety and effectiveness of the system [40].

4.3.6 Hypotonic dialysis

The hypotonic dialysis approach involves dialyzing vesicles and cargo by placing them in a specialized membrane or tube. This method significantly enhances the encapsulation of drugs and RNA, particularly miRNA and siRNA. However, due to the acidity gradients that develop during the dialysis process, there is a risk of degradation of peptide and protein payloads. As a result, while dialysis is an effective technique for packaging cargo, its successful application requires careful consideration of both the experimental setup and cargo selection [41].

4.3.7 pH gradient loading

By establishing an acidic gradient across the exosome membrane, the pH gradient loading approach encapsulates drugs. The pH level within the vesicles is typically maintained at about 9. The medicine is successfully encapsulated within exosomes by transferring the vesicles into a drug solution with a pH of 4.5. It has been demonstrated that this method may increase loading efficiency by up to three times. It is noteworthy that following the encapsulation procedure, the exosomes' size and zeta potential do not change [42].

4.3.8 Synthesis in situ

Nanomaterials may be loaded onto the membrane or inside of exosomes using the in situ synthesis method, a chemical production process that preserves the structural

integrity of the exosomes. The capacity of this method to maintain the physical integrity of the exosome is a major benefit. Its wider use is constrained by the fact that it is mainly relevant to the loading of precious metals and entails quite an intricate operating process [43].

In general, when integrating materials onto or within exosomes, the primary factors to consider are maximizing loading efficiency while minimizing damage to the exosome surface. The challenge facing the scientific community is to leverage the strengths of the above-mentioned strategies while mitigating their drawbacks, which requires further comprehensive studies to deepen our understanding of exosome biogenesis, content sorting, and packaging mechanisms.

5. Application of drug delivery based on exosomes

5.1 Small-molecule drugs

Exosomes' remarkable biological compatibility, tissue-specific targeting, and effective drug release in targeted cells have made them extremely attractive delivery platforms for tiny molecular medicines. Exosomes provide longer bloodstream circulation periods, improved medication stability, and the opportunity to get around some of the drawbacks of conventional drug delivery techniques. These features significantly enhance the pharmacokinetic profiles and effectiveness of small-molecule medications, including curcumin, doxorubicin, and paclitaxel (**Table 4**).

5.1.1 Paclitaxel

Clinical issues with paclitaxel (PTX), the first FDA-approved natural anticancer medication, include low water solubility and restricted availability. PTX delivery in cancer therapy has been investigated using a variety of nanocarriers, including lipids, proteins, polymers, solid nanoemulsions, and hybrid systems, in order to overcome these problems [72]. Three different loading techniques were examined by Kim et al. [57] in order to encapsulate PTX into exosomes made from RAW 264.7 macrophages: electroporation, mild ultrasonic, and room temperature incubation. According to their research, exosomes boosted by ultrasound had a longer release profile and better drug-loading abilities. In vitro, these PTX-loaded exosomes demonstrated improved cytotoxicity against drug-resistant cancer cells as well as notable accumulation in cancer cells.

5.1.2 Doxorubicin

A common anticancer antibiotic, doxorubicin (Dox), efficiently prevents the formation of both RNA and DNA, with RNA being most strongly inhibited. Its cardiotoxicity, however, poses serious problems; therefore, improving its tumor-specific targeting while reducing its accumulation in cardiomyocytes is essential. Wei et al. [73] looked into the possibility of doxorubicin delivery using exosomes made from bone marrow MSCs. They discovered that exosome-encapsulated doxorubicin reduced its harmful effects on cardiomyocytes while increasing absorption and improving anticancer activity in human osteosarcoma cells (MG63). Additionally, in vivo research showed that doxorubicin was efficiently transported by MSC-derived exosomes, resulting in increased anticancer efficacy against osteosarcoma and decreased cardiotoxicity [74].

Cargo	Exosome source	Disease	References
Paclitaxel	RAW 264.7 macrophages	Pulmonary metastases	[32]
Paclitaxel	Human brain glioblastoma–astrocytoma U-87 cells	glioblastoma	[58]
Doxorubicin	Immature mouse dendritic cell transfected by vector expressing iRGD-Lamp2b fusion protein	Breast cancer	[22]
Doxorubicin	Non-small-cell lung cancer H1299 cells and MRC9 lung fibroblasts	Lung cancer	[59]
Doxorubicin and paclitaxel	Human brain glioblastoma–astrocytoma U-87 cells, endothelial bEND.3 cells	Brain cancer	[60]
Doxorubicin or paclitaxel	RAW 264.7 macrophages	Breast cancer	[61]
curcumin	Pancreatic adenocarcinoma PANC-1 or MIA PaCa-2 cell	Pancreatic cancer	[62]
Curcumin	RAW 264.7 macrophages	Glioma	[27]
atorvastatin	human endometrial stem cells (hEnSCs)	Glioblastoma	[63]
Antisense oligonucleotide	HEK 293 T cell	Colorectal cancer, hepatocellular carcinoma	[64]
CRISPR–Cas9 and miRNA	Human red blood cells	Leukemia, breast cancer	[65]
siRNA and curcumin	Immature dendritic cells (imDCs)	Parkinson's disease (PD)	[66]
miRNA	γδ T cell	Oral squamous cell carcinoma	[67]
siRNA	HEK 293 T cell	Chronic myelogenous leukemia	[68]
5-Fluorouracil and miR-21 inhibitor oligonucleotide	HEK 293 T cell	Colorectal cancer	[23]
siTPD52	HEK 293 T cell	HER2-positive breast cancer	[69]
Oncolytic virus, CD40 Ligand, and 4-1BB Ligand	Mel 526 cell	Melanoma	[70]
oncolytic virus Ad5D24	LL/2 mouse lung cancer cell	Lung cancer	[71]

Table 4.
Exemplary exosome-based drug delivery in cancer.

5.1.3 Curcumin

Among the many medicinal qualities of curcumin, a traditional Chinese medication, are anticancer, anti-inflammatory, antibacterial, antimalarial, and neuroprotective benefits. Its quick elimination and low bioavailability, however, restrict its therapeutic use. A possible method for enhancing curcumin's physicochemical and pharmacokinetic characteristics, such as its stability, solubility, and bioavailability,

is the use of exosome-based delivery methods [75]. Li et al. [76] effectively delivered hydrophobic curcumin to tumor locations by integrating $CaCO_3$ nanoparticles into tumor-derived exosomes. The $CaCO_3$ nanoparticles' therapeutic potential was increased by the exosomal membrane's homologous targeting capabilities.

5.2 Nucleic acids

Nucleic acids, unlike small-molecule drugs, are highly susceptible to deactivation and degradation in vivo when encountering biological barriers such as endosomes and lipid membranes. Exosomes, however, offer a promising solution as they are natural carriers of bioactive molecules involved in intercellular communication. Their intrinsic ability to navigate biological barriers, including cell membranes, enhances their potential as vehicles for nucleic acid delivery. Additionally, exosomes provide a novel approach by co-delivery of both nucleic acids and small-molecule drugs, opening new avenues for combinatory therapeutic strategies (**Table 4**).

5.2.1 DNA delivery

Exosomes have shown great promise as DNA delivery vehicles, especially for antisense oligonucleotides (ASOs), which are becoming more and more well-known as powerful gene therapy agents that alter RNA-level gene expression [77]. By preventing the expression of the target gene, exosomes that have been engineered to transport ASOs that target STAT6 (exoASO-STAT6) have demonstrated remarkable effectiveness in converting human M2 macrophages to M1 macrophages. ExoASO-STAT6 treatment has enhanced hepatic and colon cancer recovery and decreased tumor growth. [64].

5.2.2 CRISPR/Cas9 delivery

Delivering the groundbreaking gene-editing technology CRISPR/Cas9 using traditional viral or non-viral vectors presents difficulties. Numerous tactics, such as chemical alterations, physical interactions, and the use of biological carriers, have been devised to get around these restrictions. Exosomes are a potential delivery method that has been successfully used to distribute the CRISPR/Cas9 system [78]. For example, Kim et al. [79] demonstrated that exosomes produced from cancer helped transport CRISPR/Cas9 plasmids in vivo, allowing for their selective accumulation in mouse ovarian cancer tumors. Through the suppression of poly (ADP-ribose) polymerase-1 (PARP-1) expression, this delivery method caused cancer cells to undergo apoptosis and increased chemosensitivity to cisplatin. Furthermore, it has been shown that hybrid exosomes—which are created by combining exosomes and liposomes—have the ability to transport CRISPR/Cas9 plasmids to specific cells [80, 81].

5.2.3 RNA delivery

RNA plays a significant role in cancer progression, influencing processes such as cell proliferation, migration, and invasion [82]. Exosomes, due to their ability to carry RNA, are emerging as a promising tool for delivering therapeutic RNA molecules.

siRNA can silence oncogenes, correct mutations in tumor suppressor genes, and impact cancer cell signaling pathways [83]. However, siRNA's instability and

degradation in vivo have highlighted the need for an effective delivery system, making exosomes an attractive vehicle due to their natural ability to transport RNA. As a result, exosomes have become a potentially effective and safe way to deliver siRNA. For example, Liu et al. [66] developed an engineering core-shell hybrid system named RVG peptide-modified exosome curcumin/small interfering RNA targeting SNCA, which serves as a platform for neurodegenerative disease treatment. It is a nano-scavenger for clearing α-synuclein aggregates and reducing their cytotoxicity in Parkinson's disease neurons.

Exosomes are also being explored as delivery systems for mRNA, which can produce functional proteins or peptides in the human body [84]. The advantages of using mRNA include its activity in the cytoplasm, which minimizes the risk of genomic integration, thereby reducing the potential for gene mutations and cumulative toxicity [85]. Researchers tested modified exosomes for delivering mRNA to glioma cells [86]. In mouse models, exosomes successfully delivered mRNA across the BBB, restored the tumor-suppressive function, and exhibited potent anti-tumor effects. This treatment led to enhanced tumor growth inhibition and improved survival in glioma-bearing mice, with no significant toxicity or immunogenicity observed in vivo.

5.2.4 Co-delivering systems

Exosomes have shown great potential as co-delivery systems, capable of simultaneously transporting both nucleic acids and drugs [87]. This dual delivery enhances therapeutic efficacy by targeting multiple pathways or mechanisms of disease simultaneously. For instance, Liang et al. [23] engineered exosomes that could co-deliver the chemotherapy drug 5-Fluorouracil (5-FU) and a miR-21 inhibitor (miR-21i) to Her2-positive colorectal carcinoma cells. The combination of 5-FU and miR-21i delivered by exosomes significantly increased the cytotoxic effects in drug-resistant colorectal carcinoma cells, effectively overcoming the resistance to 5-FU. The use of exosomes for co-delivery represents an exciting avenue in the development of more effective cancer therapies, particularly in overcoming chemoresistance, and may pave the way for future advancements in personalized medicine.

5.3 Proteins and peptides

Protein and peptide therapeutics hold immense promise in medicine, but their susceptibility to degradation in vivo presents a significant challenge. Exosomes are increasingly recognized as effective delivery vehicles for these biomolecules due to their ability to protect proteins and peptides from degradation while facilitating their targeted delivery.

For example, Yim et al. [88] developed a system known as EXPLORs (optically reversible protein-protein interactions) to load proteins into exosomes for intracellular delivery. This innovative approach enabled the efficient encapsulation of cargo proteins, resulting in significantly increased intracellular levels of functional proteins both in vitro and in vivo. This method offers great potential for the delivery of therapeutic proteins, enhancing their stability and bioavailability.

In another study [89], the vesicular stomatitis virus glycoprotein (VSVG) was fused into exosomes to load protein cargo. This fusion increased the exosomes' delivery capability through a pseudotyping mechanism, enhancing their ability to transfer proteins to target cells. Additionally, engineered exosomes have been used to deliver

membrane proteins. By incorporating fusogenic proteins such as viral fusogens and VSVG, exosomes can fuse with plasma membranes, facilitating the transfer of biologically active membrane proteins into target cells both in vitro and in vivo [90].

5.4 Viral vectors

Oncolytic adenoviruses (OAs) represent a promising therapeutic strategy for cancer treatment, leveraging viruses that selectively replicate in and destroy tumor cells. However, challenges such as pre-existing neutralizing antibodies and poor delivery specificity hinder the effectiveness of systemically administered OAs. To overcome these obstacles, researchers have explored the use of exosomes and exosomes as delivery vehicles for oncolytic viruses (OVs).

For example, Lv et al. [91] developed cell membrane nanovesicles that incorporate targeting ligands, enhancing the antiviral immune shielding and targeting capabilities for oncolytic virotherapy. In another study [71], exosomes released by tumor cells infected with armed OVs were shown to transduce and stimulate dendritic cell activation. This process locally activates immune responses at the tumor site, contributing to the broader immune response and potentially increasing the effectiveness of oncolytic virotherapy.

5.5 Cancer vaccine

Exosomes hold significant promise as innovative agents in cancer immunotherapy, potentially becoming one of the most effective cancer vaccines. Their growing interest as vaccine candidates is primarily attributed to their ability to induce tumor-specific immunity. For instance, dendritic cell (DC)-based vaccination strategies can be enhanced by utilizing tumor-derived exosomes (TEX) as tumor antigens. In preclinical models of myeloid leukemia and renal cell carcinoma, TEX-loaded DCs have demonstrated robust efficacy in eliciting a targeted immune response. The advantages of using TEX include improved antigen presentation to T cells and the upregulation of key immune markers such as CD11c, MHC II, and IL-12, indicating that TEX can serve as a personalized antigen source for DC-based vaccines [92]. Additionally, engineered DC-derived exosomes containing targeting peptides, antigenic epitopes, and immunostimulatory domains have been developed as "trigger" vaccines for hepatocellular carcinoma (HCC). These vaccines have successfully enhanced dendritic cell activity and T cell responses in mice, resulting in significant tumor growth inhibition and the generation of immune memory [93]. However, despite their potential, TEXs can also pose challenges in cancer immunotherapy by potentially inducing immunosuppressive effects alongside stimulating anti-tumor immunity.

6. Conclusion

Exosomes offer many advantages as drug delivery vehicles, including low immunogenicity, excellent biocompatibility, and stability. However, there are still challenges in using them clinically, such as unclear mechanisms behind their behavior and difficulties in producing high-quality, clinical-grade exosomes [49]. Proper storage and ensuring their longevity are also ongoing issues. Additionally, the immune response to exosomes in the body is not well understood, and further research is needed to evaluate their safety, pharmacokinetics, and possible unintended

interactions with healthy cells. Exosomes may also carry unwanted substances from their parent cells, which could pose risks [94]. Although methods like hypotonic treatment have shown promise in removing harmful components from macrophage-derived exosomes [95], more research is needed to ensure their safety. Hybrid exosomes are gaining attention for future use, but their safety and effectiveness need thorough investigation before clinical applications [7]. The immune response to hybrid or animal-derived exosomes must also be studied, as they could be involved in cancer progression. Despite efforts to improve circulation time, exosomes may still face challenges in delivering enough payload to tumor sites. While exosome-based therapies are still in early development, progress has been made in clinical trials and by pharmaceutical companies [96]. In conclusion, exosome-based drug delivery has great potential, offering a new way to overcome limitations of traditional therapies. With continued advancements in exosome engineering and drug delivery strategies, these therapies could pave the way for more effective and personalized treatments in the future.

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Conflict of interest

The authors declare no conflict of interest.

Notes/thanks/other declarations

Not applicable.

Appendices and nomenclature

EVs	extracellular vesicles
MVBs	multivesicular bodies
ILVs	intraluminal vesicles
UC	ultracentrifugation
SEC	size exclusion chromatography
ELISA	enzyme-linked immunosorbent assay
PEG	polyethylene glycol
Lamp2b	lysosome-associated membrane glycoprotein 2b
PDGFR	platelet-derived growth factor receptor
EGFR	epidermal growth factor receptor
LDL	low-density lipoprotein
BBB	blood-brain barrier
MSCs	mesenchymal stromal cells

PTX	paclitaxel
Dox	doxorubicin
ASOs	antisense oligonucleotides
5-FU	5-fluorouracil
VSVG	vesicular stomatitis virus glycoprotein
OAs	oncolytic adenoviruses
OVs	oncolytic viruses
DC	dendritic cell
TEX	tumor-derived exosomes
HCC	hepatocellular carcinoma

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Chapter 7

Cytostatic Drugs Delivery to MCF-7 Cells Using Horse Milk Exosomes

Anna Timofeeva, Victoria Cherenko, Sergey Sedykh and Georgy Nevinsky

Abstract

We isolated exosomes from horse milk by sequential centrifugations and ultracentrifugations and additionally purified by gel filtration. Exosomes preparation, which did not contain any co-isolating proteins, was loaded with cytostatic drugs: Doxorubicin, Tamoxifen, Docetaxel, Paclitaxel. MCF-7 cell culture was treated with the cytostatic-loaded exosomes and unloaded drugs. The conditions for loading cytostatic drugs into horse milk exosomes were determined and a change in the size of exosomes as a result of loading was shown. It was shown that loading of drugs into exosomes leads to a statistically significant increase in the cytotoxicity of drugs and an increase in the expression of pro-apoptotic genes p53, BCL-2, and BAX. The obtained results indicate the prospects of using horse milk exosome preparations for delivery of cytostatic drugs and treatment of oncological pathology.

Keywords: exosomes, milk exosomes, horse milk, drug delivery, doxorubicin, tamoxifen, docetaxel, paclitaxel, MCF-7

1. Introduction

Chemotherapy is the classical method of cancer treatment [1], and systemic administration of chemotherapeutic drugs has revealed several problems such as low specificity, low efficacy, high toxicity and development of drug resistance [2]. Most chemotherapeutic drugs have high systemic toxicity and low water solubility. Limited penetration of cytotoxic agents through the biological barrier prevents the successful local accumulation [3], necessitating repeated administration, which can lead to the acquisition of drug resistance. As a result, post-treatment response rates remain very low for many types of malignancies [4, 5].

Therefore, new approaches are currently being developed to improve the effectiveness of antitumor therapy [6], one of such approaches to improve the effectiveness of a drug is to develop drugs targeting tumor cells. Thus, antitumor therapy aimed at increasing the concentration of a drug in tumor foci while avoiding systemic effects is becoming increasingly attractive. In most studies, cytostatic drugs are encapsulated in drug delivery vehicles to reduce the concentration of free drugs in the bloodstream [7, 8].

Chemotherapeutic drug delivery vehicles: Micelles, liposomes, polymeric nanoparticles, and other types are complex to manufacture and can cause unwanted side effects [9]. Exosomes have attracted attention as "natural nanoparticles" with potential for use as chemotherapeutic drug delivery vehicles [10, 11].

Exosomes are extracellular vesicles secreted different cells with an average size of 40–200 nm [12, 13]. Exosomes are promising delivery vehicles due to their biocompatibility, long circulation time in the blood, low immunogenicity, and side toxic effects. Exosomes have very good permeability and can cross most biological membranes [14]. Although MISEV guidelines are against calling a fraction of extracellular vesicles as exosomes, given that this chapter is part of a book entitled "Exosomes," we use this designation. Furthermore, we have no doubt that the structures we obtained are exosomes morphologically and by other MISEV-approved criteria.

Due to their structure, exosomes can carry functional molecules both internally (e.g. proteins or small molecule drugs) and by anchoring to the membrane layer [15]. In addition, exosomes have a higher capacity for cellular uptake due to the proteins expressed on their surface [16, 17]. Typically, all exosomes contain annexins that regulate the processes of fusion of their membrane with the cell membrane, Rab GTPases, adhesion molecules, and receptors that help the exosome to dock to the target cell [18]. There is evidence that exosomes are capable of expressing the CD47 receptor, which protects exosomes from being "eaten" by phagocytes through interaction with the signal regulatory protein α (SIRPα) [19].

Exosomes are produced and secreted by almost all cells and are found in all body fluids including blood, urine, saliva, amniotic fluid, lymph, and milk [20]. However, horse milk has several advantages as a source of exosomes. Milk is an inexpensive, scalable source from which exosomes can be isolated in high yields [21]. Milk is a biocompatible and cost-effective source for obtaining exosomes that can be used to deliver the therapeutically important substances [22, 23].

In our previous work, we isolated exosome preparations from horse milk, and showed that exosome preparations purified by gel filtration [24] and affinity chromatography [25, 26] were virtually free of contaminants from co-excreted proteins (e.g., caseins, beta-lactoglobulin, and others), and could be used to deliver various drugs to cell cultures. Horse milk exosomes represent a next generation of delivery vehicles [27], combining natural origin, scalability of isolation source, and low immunogenicity.

Currently, there is a need to develop an effective approach to obtain agents for the delivery of chemotherapeutic drugs based on milk exosomes. In this study, we isolated highly pure horse milk exosomes, obtained a complex of cytostatic drugs with exosomes, and showed that exosomes in complex with cytostatic drugs can effectively suppress tumor cell growth. Here, we tested the hypothesis that exosomes in complex with drugs can enhance their toxicity and cause the death of more cells. The results of the analysis of changes in the expression levels of pro-apoptotic p53, BAX, and BCL-2 genes suggest that exosomes may be a promising delivery platform for the treatment of neoplasms.

2. Materials and methods

2.1 Materials

The MCF-7 cell line (ATCC catalog number: HTB-22) (ATCC, Manassas, VA, USA) was used in this study. This cell line was cultured in modified Eagle's medium DMEM/F12 (1:1) with high glucose (11,320,033, Thermo Fisher

Scientific, Waltham, MA, USA), supplemented with 10% FBS, with the addition of NEAA, L-GlutoMax, sodium pyruvate, 1% antibiotic antifungal (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C, and 5% CO_2.

The following cytostatics were used in the work: Doxorubicin hydrochloride (Dox), catalog number: 25316-40-9; Tamoxifen (Tam), catalog number: 10540-29-1; Docetaxel (Doc), catalog number: 148408-66-6; Paclitaxel (Ptx), catalog number: 33069-62-4, and the chemicals and Ultrogel A4 were purchased from Sigma-Aldrich (Missouri, USA) was.

2.2 Exosome isolation, purification, and characterization

Horse milk exosomes were prepared as described in Ref. [24]. Briefly, milk was collected from healthy horses (Irmen Breeding Farm, Novosibirsk, Russia). Milk samples were cooled to 4°C and then centrifuged at 100,000 g for 40 minutes at 4°C. Milk samples (500 ml) were centrifuged twice for 40 minutes at 12,000 rpm (Beckman Coulter Avanti J-E, JA-14 rotor), and after each centrifugation the lipid layer on top and the cells and protein pellet on the bottom were removed. The supernatants were centrifuged three times at 30,000 rpm (Beckman Coulter Avanti J-30I, JA-30.50Ti rotor). After each centrifugation, the pellet was resuspended with 10 ml TBS (20 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl). The resulting pellet corresponded to the crude preparation of horse milk exosomes. Exosome preparations were purified by gel filtration on Ultrogel A4 (Sigma-Aldrich, Missouri, USA), the resulting preparations did not contain contamination from the co-excreted proteins. Analysis of the isolated exosomes included electron microscopy and immunoblotting of surface tetraspanins as described in Ref. [24]; thus, the isolated preparations met the MISEV requirements for exosomes [28]. Size distribution and homogeneity of drug-loaded and unloaded exosomes were assessed by dynamic light scattering (DLS). Samples were diluted 1:2000 in deionized water. DLS measurements were performed using a Malvern Zetasizer Nano (Instruments, Worcestershire, UK) at 25°C.

2.3 Preparation of a complex of cytostatic drugs with exosomes or encapsulation of cytostatic drugs and characterization of loaded exosomes

The final concentration of cytostatic drugs was selected based on cytotoxicity tests for IC50 and was: Dox – 5 μg/ml, Tam – 6 μg/ml; Doc – 5 μg/ml; Ptx – 6 μg/ml. Complexes of exosomes with cytostatic drugs were obtained as described in [29]. Complexes of exosomes with doxorubicin (ExoDox), tamoxifen (ExoTam), docetaxel (ExoDoc), and paclitaxel (ExoPtx) were obtained by adding exosome solution to cytostatic drugs at dilutions of 1:1000, 1:500, 1:100, and 1:50. The mixture was incubated at room temperature for 30 minutes with mixing on a laboratory vortex.

2.4 Analysis of MCF-7 cell viability

The cytotoxicity of cytostatic drugs (not encapsulated) and in complex with exosomes against tumor cells was investigated on human breast adenocarcinoma MCF-7 cell culture using MTT assay.

MCF-7 cells were cultured in DMEM/F-12 medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) in the presence of (1x) 1 μl/ml antibiotic antifungal (Sigma) and 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C until a

monolayer was formed. The medium was then decanted and washed several times with PBS buffer (150 mM NaCl, 17 mM KH_2PO_4, and 52 mM Na_2HPO_4). About 1 ml of 0.25% trypsin-EDTA solution was added to remove cells from the surface of the culture flask. The cell monolayer was then incubated at 37°C for 5 minutes to detach the cells from the surface. After the cells were completely detached from the surface of the culture flask, 10 ml of DMEM/F12 medium was added, mixed by pipetting, and the cells were seeded in a 96-well plate at 100 µl per well, at a concentration of 2 × 105 cells/ml, and incubated for 24 hours in DMEM/F12 medium with an antibiotic antifungal at 37°C and 5% CO_2. The resulting ExoDox, ExoTam, ExoDoc; ExoPtx complexes were then added to the medium with MCF-7 cells and incubated for 24 hours at 37°C, 5% CO_2. Cells grown without any treatment were used as the negative control. A cell culture grown in the presence of final concentrations of cytostatic drugs only was used as the positive control.

The antitumor efficacy of ExoDox; ExoTam; ExoDoc; ExoPtx was evaluated using a standard MTT assay. After 24 hours of incubation with the complexes, MTT reagent was added to the cell culture and incubated for 2 hours at 37°C and 5% CO_2. The assay used is based on the ability of mitochondrial dehydrogenases to convert colorless, water-soluble MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) into colored formazan, which crystallizes inside the cell. The enzymes do not function in non-viable cells, so these cells do not stain with MTT. Absorbance at 570 nm was measured using a Shimadzu RF5000 fluorescence spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Survival values were calculated using the formula: (OD of experimental wells/OD of control wells) * 100%, where OD is the optical density. Results were presented as a series of five independent experiments. The standard deviation was calculated using the formula: $\sqrt{\sum \frac{(x-\underline{x})^2}{(n-1)}}$, where x is the number of the sample (number 1, number 2, ...), and n is the sample size.

Untreated cells were used as negative control. Exosomes were diluted in DMEM/F12 cell culture medium. Exosomes were diluted 1000-fold (1:1000), 500-fold (1:500), 100-fold (1:100), and 50-fold (1,50). Cells treated with Dox, Tam, Doc; Ptx at the final concentration (see Section 2.3) were used as positive controls.

2.5 Isolation of total RNA

Isolation of mRNA was performed using "Kit for isolation of RNA from animal cells/bacteria, smear/scraping of epithelial cells, viruses on silica columns" according to the manufacturer's protocol (Biolabmix, Novosibirsk, Russia). After the MTT test, 400 µl of LB lysis buffer (with 20 µl of 2 M dithiothriol (DTT) added to 1 ml of the buffer) was added to each well with the cell culture, mixed and incubated for 10 minutes. Then, 400 µL of 96% ethanol was added to the lysate and transferred to the column. The column was centrifuged for 1 minutes at 10,000 g, the liquid was removed, and 500 µL of WB1 wash buffer was added to the column and centrifuged for 1 minutes at 10,000 g. The column was then washed again with 500 µL of WB2 wash buffer and centrifuged twice for 1 minutes at 10,000 g until the buffer was completely removed. The column was transferred to a clean tube, and 50 µL of elution buffer water was added and centrifuged for 1 minutes. About 10,000 g and again 50 µL of elution buffer water were added and centrifuged for 1 minutes at 10000 g to increase the yield of RNA. The concentration was measured on a Qubit 4 instrument (Invitrogen, USA).

2.6 Analysis of changes in differential gene expression

Changes in relative gene expression after the MMT assay were assessed by RT-PCR using TaqMan fluorescent probes. The following genes were analyzed: p53, BAX, BCL-2. Normalization was performed using the reference gene GAPDH. Primers were selected using the Primer BLAST resource (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). The selected primer variants were tested for the presence of hairpins, self- and heterodimers using the OligoAnalyzer tool (https://eu.idtdna.com/calc/analyzer). The primers were tested for specificity by alignment to human mRNA and DNA (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). **Table 1** shows the primers and TaqMan probes used in this work.

PCR was performed using the BioMaster RT-PCR Standard (2×) kit (Biolabmix, Novosibirsk, Russia). The PCR mixture consisted of 12.5 μl 2× RT-qPCR buffer; 1 μl 25× Bio-Mastermix; 1 μl forward primer; 1 μl reverse primer; 0.5 μl fluorescent probe; and 9 μl RNA at a concentration of 5 ng/μl. The primers were annealed at the following temperatures: 55°C (bcl-2), 54.6°C (BAX), 54°C (p53), 50°C (GAPDH).

The PCR temperature profile was: 94°C, 2 minutes – 1 cycle; 94°C – 34 cycles; 55°C – 34 cycles; 72°C – 34 cycles; final elongation 72°C – 5 minutes. The gene expression of each sample was represented by the threshold cycle (C_T) value. The PCR efficiency of all target and endogenous genes used in this study was in the range of 100 ± 10%. PCR products were tested for specificity by melting curve analysis and electrophoretic separation in 1.6% agarose gel. The results were calculated using the formulas:

$$\text{Comparative expression level} = 2^{-\Delta\Delta C_T} \tag{1}$$

$\Delta\Delta C_T = \Delta C_T$ (treated sample) – ΔC_T (untreated sample),
ΔC_T (treated sample) = ΔC_T (target) – ΔC_T $\left(\text{endogenous reference}\right)_{(treated)}$,
ΔC_T (untreated sample) = ΔC_T (target) – ΔC_T) $\left(\text{endogenous reference}\right)_{(untreated)}$.
ΔC_T – threshold number of cycles

2.7 Statistical analysis

Experimental results are presented as median [Q1, Q3]. The results are presented for a series of three independent experiments. The measurement error did not exceed

Primer names	Primer sequences	Probe	qPCR product length, bp
BAX For	5’-CCCTTTTGCTTCAGGGTTTCAT-3′	5’-CGAGTGTCTCAAGCGCATCG-3’	103
BAX Rev	5’-CTGCCACTCGGAAAAAGACC-3’		
BCL-2 For	5’-ATGTGTGTGGAGAGCGTCAA-3’	5’-TGCACACCTGGATCCAGGA –3’	127
BCL-2 Rev	5’-TTCCACAAAGGCATCCCAGC-3’		
GAPDH For	5’-CGAGATCCCTCCAAAATCAA-3’	5’-TGGAGAAGGCTGGGGCTCAT-3’	131
GAPDH Rev	5’-TTCACACCCATGACGAACAT-3’		
p53 For	5’-CTCCTCAGCATCTTATCCGAGT-3’	5’-TGGTGGTGCCCTATGAGC -3’	128
p53 Rev	5’-ACAGTCAGAGCCAACCTCA-3’		

Table 1.
Probes and primers used to measure the changes in relative gene expression.

10%. The normality of the data obtained was tested using the Shapiro-Wilk test. Most of the variables did not meet the assumptions of normality ($p < 0.05$).

The Wilcoxon-Mann-Whitney test and the Kruskal-Wallis one-way analysis of variance test were used for non-normally distributed variables. A two-tailed $p < 0.05$ value was considered statistically significant. Statistical analysis was performed with Statistica 10 (StatSoft. Inc., Tulsa, OK, USA). Graphs were generated using Origin 2021 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1 Isolation and characterization of horse milk exosomes

Many protocols were been developed for the isolation of exosomes and their subsequent loading with cargo [27, 30, 31]. Among the purification methods, the most commonly used protocols are based on: centrifugation [32, 33], microfiltration [34, 35], density gradient separation [32], immunoaffinity capture using antibodies specific for exosome surface proteins [36, 37], and microfluidics [38–40]. Previously, our paper [24] showed that the combination of centrifugation and ultracentrifugation methods allows the isolation of preparative quantities of milk exosomes. In this work, exosomes were isolated from the milk of healthy horses.

In accordance with the recommendations of the International Society for Extracellular Vesicles (ISEV) [28, 41], we performed several methods to identify isolated exosomes, including transmission electron microscopy, DLS, flow cytometry, and Western blotting. Electron microscopy revealed numerous membranous structures down to 200 nm (**Figure 1A**). These results are consistent with the size range typically defined for exosomes (30–200 nm) [42]. Western blotting revealed the expression of some common canonical exosome markers such as CD81 and CD63. The absence of cytochrome C, in contrast to the whole cell, confirmed the nature of the collected extracellular vesicles as exosomes (**Figure 1B**). Tetraspanin proteins, especially CD63, CD81, and CD9, are widely distributed in exosome membranes from different sources and are considered as classical exosome markers [43]. Taken together, our results indicate that our exosome sample isolated from horse milk is rich in exosomes. In addition, particle size distribution and homogeneity measured by DLS showed a size distribution of approximately 200 nm (**Figure 1C**). These results are consistent with the size range typically reported for exosomes (30–200 nm).

The DLS method we used for exosome characterization allows for rapid vesicle size determination. We believe this method is suitable and provides sufficient information to identify the resulting preparations as exosomes, rather than other extracellular vesicles. The existing limitations [44] 8 are overcome by using transmission electron microscopy and other physicochemical methods.

3.2 Loading of exosomes with cytostatic drugs

The optical density of A280 of the exosome preparation after the gel filtration was 2 AU, and we studied exosomes in four dilutions: in 1000, 500, 100, and 50 times. The final concentration of exosomes was: 0.002, 0.004, 0.02, and 0.04, respectively. During the preliminary study based on MTT analysis, it was found that the IC 50 values for Tam and Doc are 6 μg/ml, and for Dox and Ptx – 5 μg/ml; therefore, these final concentrations were used to obtain the complex.

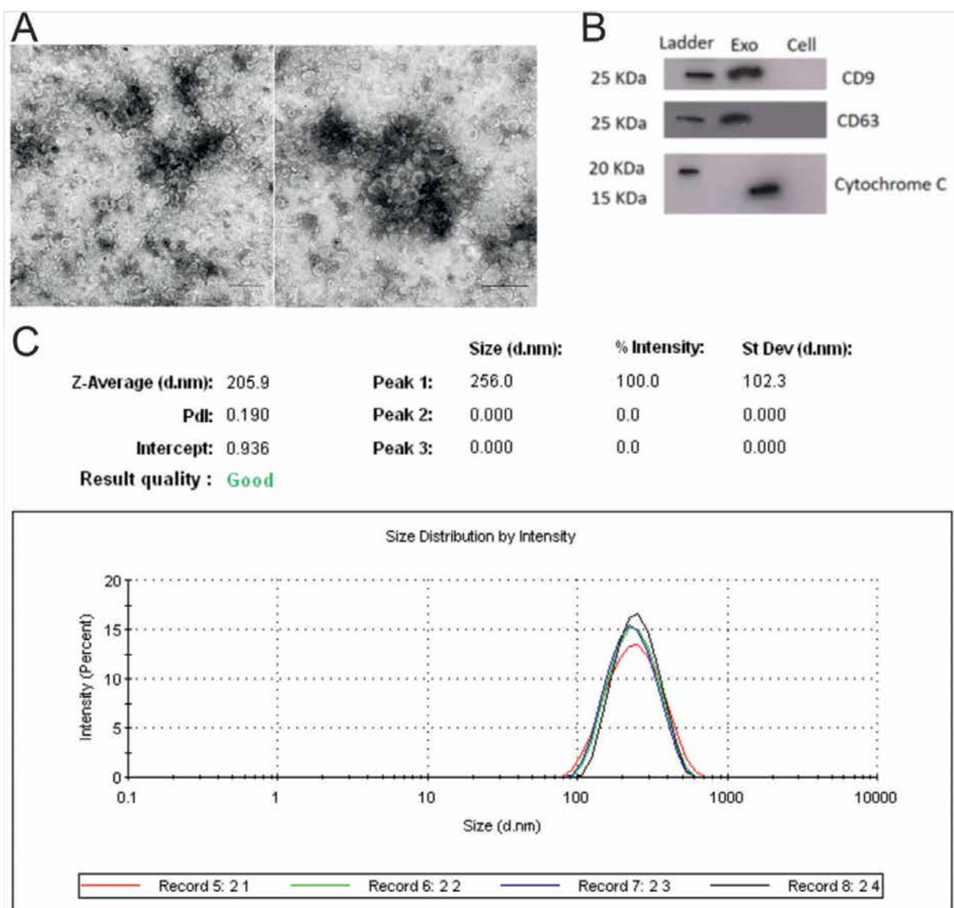

Figure 1.
Characterization of exosome-enriched samples from horse milk: (A) Transmission electron microscopy images show exosome-like vesicles with scale bars of 500 and 200 nm. (B) Western blot analysis of protein expression for negative control markers (cytochrome c) at 15 kDa and exosomal markers (CD63 and CD9) at 25 kDa. (C) Dynamic light scattering (DLS) size distribution of exosome samples.

Among the various methods of drug encapsulation in exosomes, incubation is easy to perform, capable of protecting the integrity of exosomes, in which drugs diffuse through the membrane along the concentration gradient. However, the main drawbacks of the method are low-loading efficiency and difficulty in controlling the amount of cargo packed into exosomes due to the physicochemical properties of exosomes and drugs [45]. We chose the strategy of co-incubation of exosomes with the cytostatic drugs for 30 minutes.

The dynamic light scattering method is suitable for analyzing the diameter of vesicles that do not contain fluorescent dyes, such as milk exosomes loaded with cytostatic drugs in this work. Comparison of the hydrodynamic size of the exosome before and after Dox loading by DLS revealed an expansion of approximately 50%, from 200 to 300 nm (approximately 0.5-fold) (**Figure 2B**). Similarly, a nearly twofold increase in PDI was observed before (0.365) and after loading (Dox-exo, 0.634). These results confirmed the successful encapsulation of Dox, and the increase in size probably indicates the integration of doxorubicin into the exosomes, which increases the polydispersity index

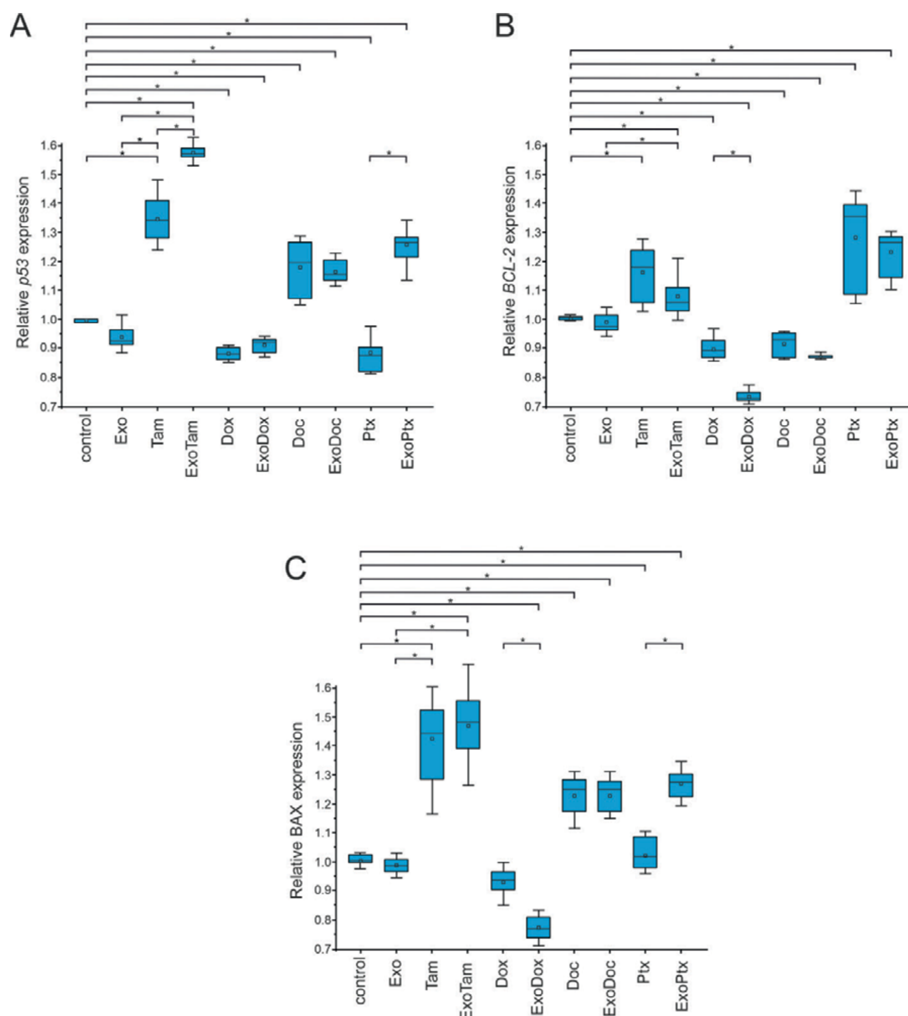

Figure 2.
*Relative gene expression of p53 (***Figure 2A***), BCL-2 (***Figure 2B***), and BAX (***Figure 2C***) mRNAs in MCF-7 cells treated with unloaded exosomes and cytostatic drugs, and exosomes loaded with the cytostatic drugs. Measurement was performed by RT-qPCR. Results are expressed as fold change relative to untreated control.*

due to increased nanoparticle size heterogeneity and the formation of a polydisperse complex with a size distribution ranging from 145.6 (free exosomes) to 205.8 (Dox-Exo). Similarly, the other study by showed an increase in exosome size from 112.4 nm to 152.7 nm after Dox loading [46]. Nanoparticle size plays a critical role in controlling circulation and biodistribution as it directly affects the fate of drugs *in vivo*. Based on previous studies, the size range of 20–200 nm has been recognized as the optimal size range for therapeutic nanoparticles because they not only cannot be filtered out by the kidney, but also accumulate effectively at tumor sites [47]. The final hydrodynamic size of our formulation determined by DLS is 300 nm, which is still in the appropriate particle size range that can actively deliver the drug to the target site. Such nanoparticles can easily reach the tumor site and accumulate for a long period of time where tumor blood vessels are larger and more numerous than normal blood vessels [48].

3.3 Evaluation of MCF-7 cell viability after treatment with cytotoxic drugs in complex with exosomes

To compare the efficiency of milk exosomes in delivering cytostatic drugs to MCF-7 cells, we used a standard cell viability assay that measures the cellular metabolic activity (MTT test). The MTT assay is a colorimetric assay used to assess the metabolic activity of cells. NADPH-dependent cellular oxidoreductase enzymes can reflect the number of viable cells under certain conditions. These enzymes are able to reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide to insoluble formazan, which has a purple color. We evaluated the effect of the obtained complexes of exosomes with cytostatic drugs on the viability of MCF-7 cells. Untreated cells and MCF-7 cells incubated with unloaded exosomes were used as controls.

Figure 3 shows the viability of MCF-7 cells after treatment with exosomes (**Figure 3A**), as well as exosomes loaded with tamoxifen (**Figure 3B**), doxorubicin (**Figure 3C**), docetaxel (**Figure 3C**), and paclitaxel (**Figure 3D**) in comparison with unloaded cytostatic drugs. Incubation with unloaded exosomes had no significant effect, but the treatment by exosomes loaded with cytostatic drugs enhanced the cytotoxic effects. The viability of MCF-7 cells after Tam, Dox, and Ptx treatment was twice lower in the highest concentration of exosomes (0.040 ou/ml). Since the greatest cytotoxic effect was observed at the highest concentration of exosomes, we suggest the loading of cytostatic drugs into exosomes increase the bioavailability of the drugs. This fact supports our hypothesis.

3.4 Evaluation of changes in expression of pro-apoptotic genes

The expression of p53 (**Figure 2A**), BCL-2 (**Figure 2B**), and BAX (**Figure 2C**) mRNA of corresponding pro-apoptotic genes was assessed by RT-qPCR; the preparations of treated and untreated MCF-7 cells were analyzed. Expression of cells treated with unloaded exosomes or cytostatic drugs was assessed as the corresponding controls. Expression of mRNA from the untreated cell line was taken as one (1). Treatment of cells with the unloaded exosomes did not result in a significant change in gene expression compared to the control.

The relative expression level of p53 gene decreased after Doc, Exo, and ExoDox treatment to 0.85–0.91, 0.77–0.85, and 0.87–0.95, respectively, compared to the level of the expression in control group. The relative expression of p53 gene increased by 1.24–1.48 and 1.52–1.63 after Tam and ExoTam treatment of cells, respectively. The relative expression of p53 gene increased with Doc and ExoDoc treatment of cells by 1.05–1.28 and 1.01–1.2, respectively. The relative expression of p53 gene decreased with the Ptx treatment of cells to 0.81–1.97 and 0.82–1.03, respectively. With ExoPtx treatment, the relative expression increased to 1.06–1.34.

After treatment of the cell culture with Dox and ExoDox, the relative expression of the BCL-2 gene decreased to 0.83–0.95 and 0.67–0.73, respectively. Cells have, treated with Tam and ExoTam, shown the increase of the relative expression of the BCL-2 gene by 1.02–1.2 and 1.03–1.21, respectively, while the relative expression of BCL-2 gene decreased when the cells were treated with Doc and ExoDoc by 0.81–0.96 and 0.87–0.97, respectively. The relative expression of BCL-2 gene increased when cells were treated with Ptx and ExoPtx to 1.05–1.44 and 1.1–1.3, respectively.

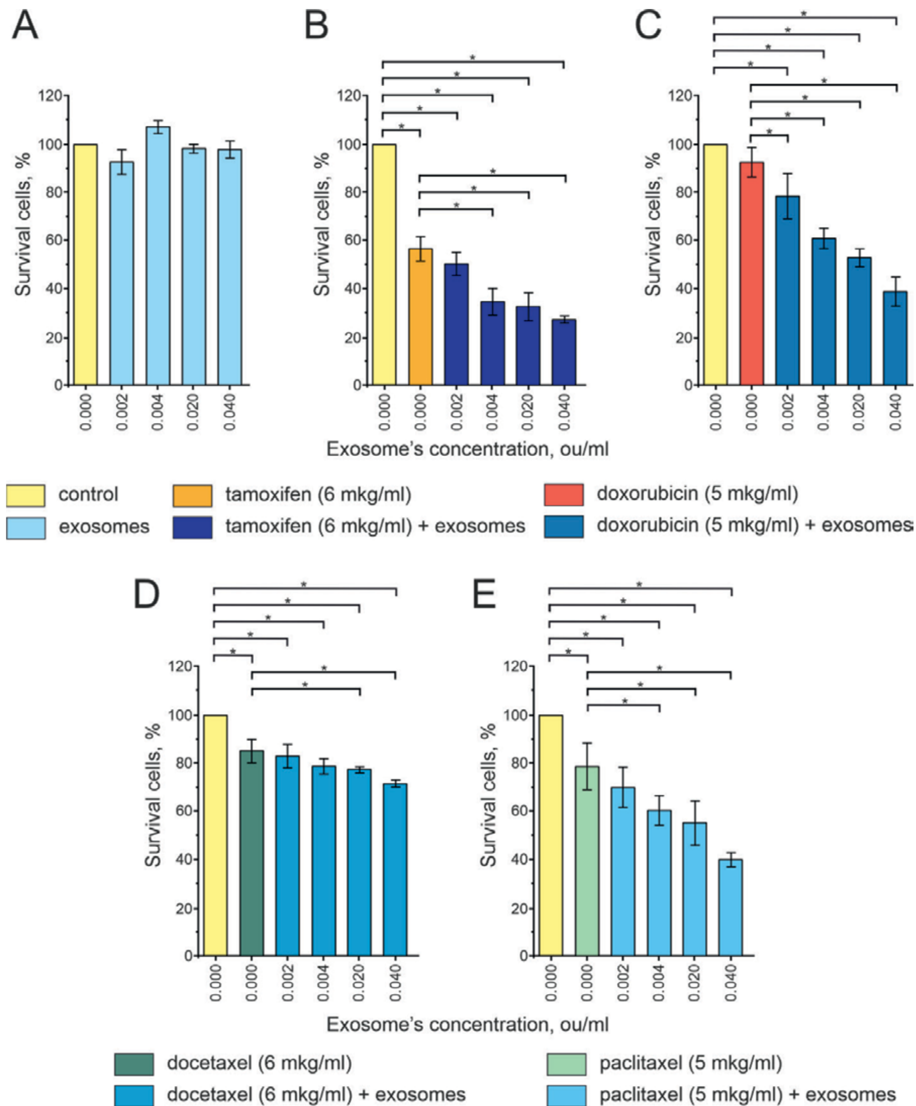

Figure 3.
*Analysis of cytotoxicity against culture of MCF-7 (human breast adenocarcinoma) cells using the MTT test. Unloaded exosomes (A), cytostatics loaded into exosomes: Tam (B), Dox (C), Doc (D), Ptx (E). * - p-value <0.05. Viability of intact cells was taken as 100%.*

The relative expression of BAX decreased to 0.7–0.80 with ExoDox treatment. With Doc cell treatment, the expression decreased to 0.85–1 without significant differences from the expression in untreated cells. The relative expression of the BAX gene increased with Tam cell treatment and ExoTam treatment by 1.17–1.6 and 1.29–1.67, respectively. The relative expression of the BAX gene increased with Doc and ExoDoc cell treatment by 1.19–1.32 and 1.12–1.32, respectively. No significant differences in BAX gene expression were observed with Ptx cell treatment.

Since Tam and Tam-Exo show the most pronounced effect on the expression of the apoptotic genes studied in this work, the change in the expression of these genes in MCF-7 cell lines was further analyzed depending on the time of exposure to the cytostatic drug.

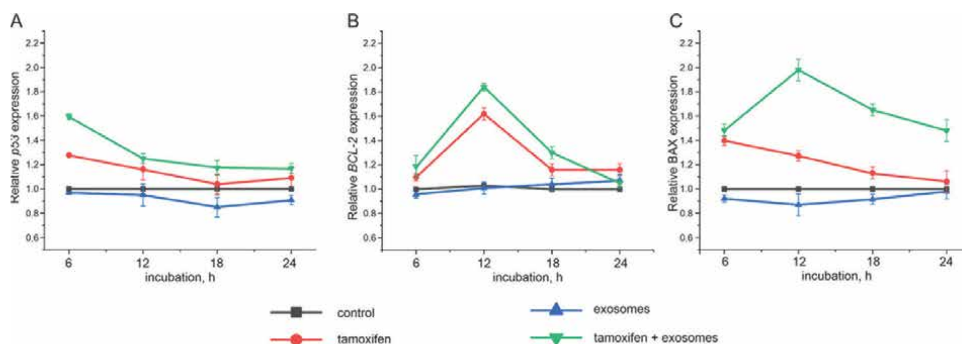

Figure 4.
*Relative gene expression of p53 (**Figure 4A**), BCL-2 (**Figure 4B**), and BAX (**Figure 4C**) in MCF-7 cultures treated with exosomes, cytostatic drugs, and exosomes loaded with cytostatic drugs. Measurements were performed by RT-qPCR, and the results are expressed as fold change compared to untreated control.*

3.5 Changes in gene expression according to the tamoxifen exposure

Changes in gene expression of the MCF-7 cell culture were analyzed depending on the time of exposure to the Tam, and the data are shown in **Figure 4**.

As shown in **Figure 4**, Tamoxifen increased the expression of all three genes. The most pronounced effect was observed at 6 hours for the p53 and BAX genes (**Figure 4A**, **C**), and at 12 hours for the BCL-2 gene (**Figure 4B**). Loading of Tamoxifen in exosomes increased the expression of all three genes, and in the case of the BAX gene, it led to a longer period of increased expression.

4. Discussion

Research in the field of cancer chemotherapy focuses on either the development of new chemotherapeutic agents or combination therapy to improve the efficacy of the chemotherapeutic agents used [49, 50]. We tested a new drug delivery system based on natural horse milk exosomes to deliver four cytostatic drugs. Horse milk exosomes can provide the advantages of both nanoparticles and cell-mediated drug delivery. Extracellular vesciles were isolated from horse milk and characterized by a combination of three methods: TEM, Western blotting, and DLS were corresponded morphologically to exosomes. One of the major challenges is the efficient loading of exosomes without significantly altering the structure and composition of exosomal membranes.

In this work, we used incubation to load substances into exosomes. All selected substances are highly hydrophobic compounds that are likely to be incorporated into the interior of the relatively dense and well-structured lipid bilayers of exosomes. As a result, we observed an increase in therapeutic efficacy when using milk exosomes as delivery agents compared to pure cytotoxic drugs.

In this study, we used the MCF7 cell line to investigate the effect of encapsulating cytotoxic drugs in milk exosomes. We were the first to: prepare and characterize a new complex of exosomes loaded with Dox, Tam, Doc, Ptx (ExoDox, ExoTam, ExoDoc, ExoPtx), to demonstrate the efficacy of exosomes loaded with cytostatic drugs for antitumor therapy. Our data show that horse milk exosomes can be used to deliver various chemotherapeutic drugs to cancer cells and that loading of exosomes with chemical substances can be done by a passive method—incubation of exosomes

with cytostatic drugs. We determined the mechanisms underlying the increased efficiency of exosome-based complexes in cancer cells. The increased efficiency of the complexes is caused by increased cytotoxicity of the drugs toward the MCF-7 cell line. Exosomes also enhance the expression of pro-apoptotic genes, thereby reducing cell viability.

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Conflict of interest

The authors declare no conflict of interest

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Exosomes are extracellular vesicles with specific morphology and diameter that are secreted by cells from multivesicular bodies. Exosomes have specific proteins, lipids, nucleic acids, and other biomolecules that distinguish them from other extracellular vesicles. These proteins, nucleic acids, and other molecules can serve as diagnostic markers for various diseases, particularly cancer. Natural exosomes are unique and universal vehicles for delivering therapeutic molecules, including targeted therapies for various diseases. Of particular importance are exosomes isolated from sources such as mesenchymal cell cultures and, for example, milk. Currently, no single, universal method has been proposed for isolating exosomes; methods and combinations of methods are widely used. In this book, we discuss the latest research on natural exosomes, their use for the delivery of therapeutics, methods for isolating exosomes, and their use as disease markers for early diagnosis and personalised medicine.

Tomasz Brzozowski, Physiology Series Editor

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