

AWARD NUMBER: W81XWH-19-1-0647

TITLE: Exosomes in Immunotherapy Resistance

PRINCIPAL INVESTIGATOR: Rajagopal Ramesh

CONTRACTING ORGANIZATION: University of Oklahoma Health Sciences Center
Oklahoma City, OK

REPORT DATE: October 2021

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2021		2. REPORT TYPE Annual		3. DATES COVERED 15Sep2020-14Sep2021	
4. TITLE AND SUBTITLE Exosomes in Immunotherapy Resistance				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-19-1-0647	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Rajagopal Ramesh E-Mail: rajagopal-ramesh@ouhsc.edu				5d. PROJECT NUMBER 0011350685	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Board of Regents of the University of Oklahoma Health Sciences Center 865 Research Parkway, STE 530 Oklahoma City, OK 73104-3609				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of the funded project is to test the role of tumor-derived exosomes (TDE) expressing immune checkpoint proteins in abrogating the ability of anti-PD1 or anti-PD-L1 antibodies from disrupting the tumor cell mediated suppression of T-cell cytotoxicity. To test our hypothesis, <i>specific aim 1</i> focused on examining the expression of PD-L1 and other immune checkpoint proteins in lung tumor cell derived exosomes. <i>Specific aim 2</i> focused investigating the immune modulation properties of tumor derived exosomes in vivo receiving anti-PD-L1 immunotherapy. Results from aim 1 studies showed TDE isolated from human non-small cell lung cancer (NSCLC) cell lines (H1299 and A549) were 100-150 nm in size, spherical with bilayer membrane, and had a negative charge. Transmission electron microscope showed TDE expressed PD-L1 on their surface. Immune checkpoint protein array optimization studies for determining the expression of immune-related proteins by TDE was performed. <i>In vitro</i> binding studies revealed anti-PD-L1 antibody bound to TDE as determined by changes in size and number of free exosomes. Finally, optimization of T-cell culture conditions for assessing TDE binding in the presence and absence of anti-PD-1/anti-PD-L1 antibody <i>in vitro</i> is completed.					
15. SUBJECT TERMS None listed.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	126	19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	8
5. Changes/Problems	9
6. Products	10
7. Participants & Other Collaborating Organizations	12
8. Special Reporting Requirements	14
9. Appendices	14

1. INTRODUCTION:

Immunotherapy has demonstrated limited efficacy and clinical benefit to patients diagnosed with lung cancer. Recent studies indicate development of resistance against immunotherapeutic agents. Therefore, unraveling the resistance mechanism will lead to new and improved therapeutics. In this study, we hypothesized that tumor-derived exosomes (TDE) expressing immune checkpoint proteins against which therapeutic antibodies have been developed contribute to therapy resistance by engaging the antibodies and preventing their binding to tumor and/or T cells thereby disrupting the tumor cell mediated suppression of T-cell cytotoxicity. To test our hypothesis we put forth conducting preclinical studies using *in vitro* and *in vivo lung tumor models*. Our study will demonstrate a role for TDE in immunotherapy resistance and provide opportunities and strategies for overriding TDE-mediated resistance.

2. KEYWORDS:

Lung cancer, exosomes, tumor-derived exosomes, resistance, immunotherapy, PD-1, PD-L1, cytotoxicity, T-cell, NK cells.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Two specific aims were proposed in the funded application and each specific aim had one major task along with milestones/timeline identified for activities to be completed. Provided below are the details:

Specific aim 1. The major goal of aim 1 was to test whether tumor cell derived exosomes expressing PD-L1 abrogate the efficacy of anti-PD-L1 therapy and contribute to resistance. To accomplish aim 1, two sub-tasks were listed.

Subtask 1 – isolation of exosomes from lung cancer cell lines, physical and structural characterization of exosomes and analysis for PD-L1 and immune checkpoint proteins by western blotting and flow cytometry.

Time line identified – 1-3 months; *Percentage completion* – 100%

Subtask 2 – molecular immune-studies investigating tumor-T-cell interaction and efficacy studies using anti-PD-L1 antibody.

Time line identified – 2-6 months; *Percentage completion* – 50%

Specific aim 2. The major goal of aim 2 was to investigate the immune modulating properties of tumor derived exosomes *in vivo* receiving anti-PD-L1 immunotherapy. To accomplish aim 2, two sub-tasks were identified:

Subtask 1 – IACUC and ACURO approval

Time line identified – 4 months; *Percentage completion* – 100%

Subtask 2 – establish experimental lung tumor in immunocompetent mice primed with tumor derived exosomes and test for antitumor efficacy of anti-PD-L1 antibody therapy.

Time line identified – 5-12 months; *Percentage completion* – 0%

What was accomplished under these goals?

1. **Major activities:** The major activities has been to recruit a post-doctoral fellow; submit and receive approvals from institution (IACUC); and conduct the *in vitro* studies
2. **Specific objectives:** The objectives of the study was to demonstrate that exosomes derived from tumor cells hereafter referred to as tumor-derived exosomes (TDE) contribute to therapy resistance against immune checkpoint targeted antibodies. The objective is to be achieved was by demonstrating that TDE express proteins on their outer surface that acts as sink and bind to the immunotherapeutic antibodies thereby abolishing the therapeutic activity. To accomplish this objective, we planned to conduct preclinical studies using *in vitro* and *in vivo* lung tumor models.
3. **Significant results:**

Specific aim1. Subtask 1. We have completed subtask 1of specific aim 1. Exosomes were successfully isolated from lung tumor cells and characterized for shape, size and demonstrated expression of PD-L1 on the surface of exosomes by transmission election microscope (TEM) and by western blotting [Figure 1]. To increase the exosome production from lung tumor cell lines, during the current reporting period we established a bioreactor system in the laboratory. The advantage of this is that we can actively maintain cell lines for a period of 1-2 months by growing them in 3D hollow fiber columns in the bioreactor and produce large quantities of exosomes. We have established the protocol and have been able to obtain exosomes in the range of 10¹⁵-10¹⁶ per ml. We also established a protocol for having concentrated and pure exosomes by incorporating the tangential flow filtration (TFF) system. Using this we could concentrate 100 ml of exosomes containing culture medium to 5 ml that resulted in having large concentrated exosomes per ml of medium. As reported in prior report, we characterize each batch of exosome preparation for size, shape, charge and exosome specific molecular markers using the nanotracker analysis (NTA), transmission electron microscope (TEM), zeta analyzer, and western blotting.

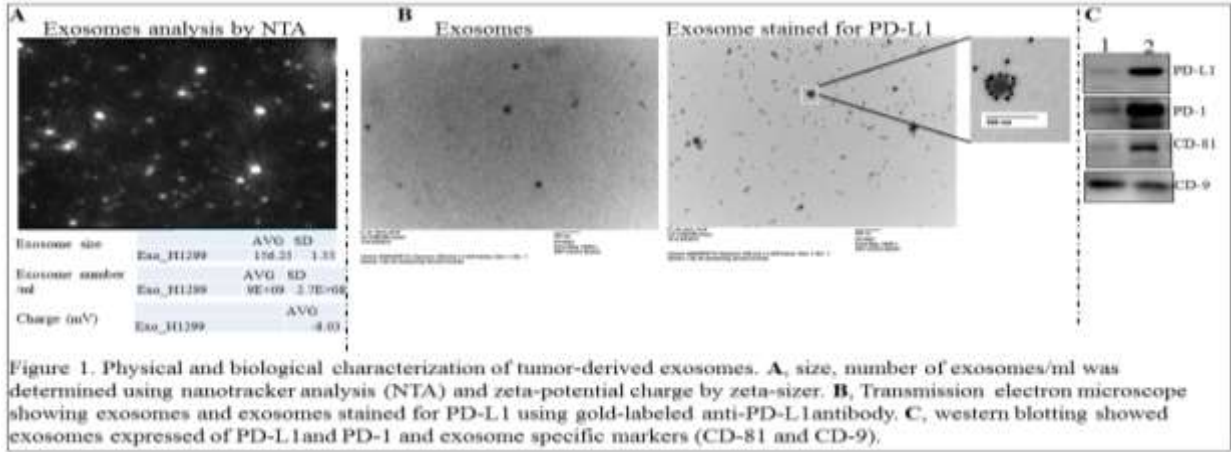


Figure 1. Physical and biological characterization of tumor-derived exosomes. **A**, size, number of exosomes/ml was determined using nanotracker analysis (NTA) and zeta-potential charge by zeta-sizer. **B**, Transmission electron microscope showing exosomes and exosomes stained for PD-L1 using gold-labeled anti-PD-L1 antibody. **C**, western blotting showed exosomes expressed of PD-L1 and PD-1 and exosome specific markers (CD-81 and CD-9).

In vitro binding studies revealed anti-PD-L1 antibody bound to TDE [Table 1].

Sample	No.	Size (nm)	SD (for size)
Exo Control	6.9 x 10e8	125.4	32.5
0.5ug anti-PD-L1	6.46 x 10e8	154.6	3.4
1.5ug anti-PD-L1	2.3 x 10e8	194.6	13.1

Table 1. Exosomes were analyzed for size, and number of free exosomes available after incubating with anti-PD-L1 antibody for 24 h at room temperature. Increasing concentration of anti-PD-L1 antibody resulted in increased size and reduction in the number of free exosomes indicating binding and aggregation of exosomes with the antibody.

While we continue to analyze for immune checkpoint protein on exosomes by conventional western blotting, we have incorporated testing of commercially available immune checkpoint array kit. Using the manufacturer recommended guidelines, we the assay has been optimized for determining the expression of immune-related checkpoint proteins by exosomes [Figure 2]. We now routinely utilize this rapid assay system for each batch of exosomes that we isolate and purify in the laboratory.

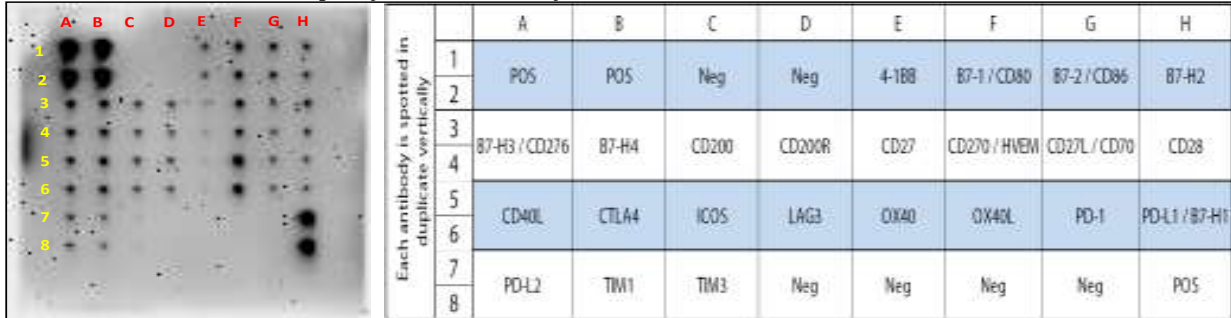


Figure 2. Optimization study for human immune checkpoint array analysis using exosomes. *Left panel* shows protein detection and *right table* shows proteins blotted on the membrane.

Subtask 2. We previously reported our ability to establish a co-culture system for lung tumor cells and human T-cell interaction studies for assessing T-cell apoptosis as determined by Annexin V staining combined with flow-cytometry (**Figure 3**). However, this assay is time consuming and cumbersome. As an alternate, we have been working in establishing a co-culture protocol using the Incucyte Live Imaging Analysis System that is fast and captures the interaction and outcomes in real time by capturing images of tumor and T-cell apoptosis. Using the Incucyte System, we can conduct time-point analysis from minutes to hours-to days without disturbing the cells. As a result we can acquire data with minimal artifact and quantitate the number of apoptotic cells over time. As shown in **Figure 4**, we have been able establish the protocol and optimize the cell growth conditions. We are currently conducting co-culture experiments to determine the efficacy in the presence and absence of TDE and anti-PD-L1 antibody.

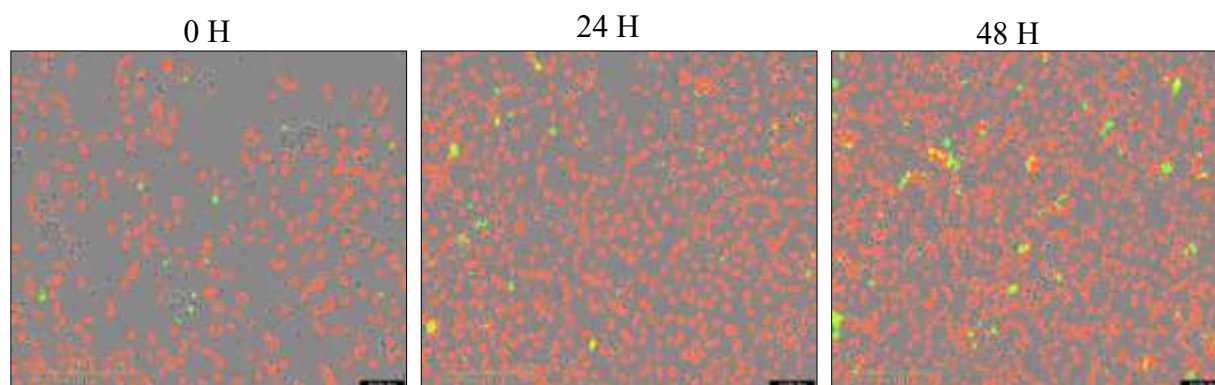
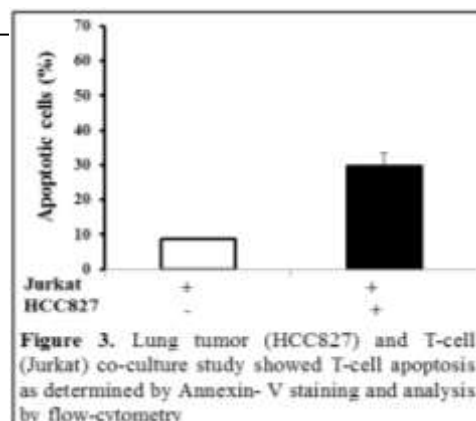


Figure 4. Co-culture experiments and analysis using the Incucyte Live Imaging Analysis System. Human lung cancer (H1299; labeled Red) and human T-cells (Jurat; unlabeled) were co-cultured in 48-well plates and analyzed over time for tumor (**Red**) and T-cell apoptosis. Apoptotic tumor cells appear green to yellowish orange in color.

4. *Stated goals not met.* We have thus far been unable to accelerate the progress of the project and complete *in vivo* studies planned in aim 2. This is partly due to the COVID-19 pandemic, delay in hiring of post-doctoral fellow, and departure of a key personnel from the laboratory. As a result, pending studies of aim 1 and aim 2 has been delayed. We however have a post-doctoral fellow from India who will soon be joining the laboratory in March 2022 pending visa approval and no further recurrence and increase in the pandemic incidence that will delay his joining. We also have a graduate student in the laboratory who will be participating in the project and assist in completing the objectives before the end of the funding in September 2022.

What opportunities for training and professional development has the project provided?

The University of Oklahoma Health Sciences Center (OUHSC) has implemented an individual development plan (IDP) for all graduate students and post-doctoral fellows to set and meet individual career goals. The IDP is implemented through an “Annual Performance Evaluation” that occurs as a one-on-one meeting between the mentor (PI) and the mentee (students and fellows).

The annual evaluation process: The mentor and mentee meet and have a detailed discussion about the project goals and milestones to be achieved. Further, discussion related to short-term and long-term goals are discussed to ensure a successful career in academic/industry setting. After mutual agreement, the goals are set and the accomplishments made are evaluated the following year. Deficiencies in one or more areas are then identified and procedures to rectify or improve are made and evaluated the following year again. This process enables the individuals to continuously improve and become successful. The goals are tailored based on individual level of experience (0, 1, 2-year, etc.), and the stage (graduate student or post-doctoral fellow) of their training.

A summary of the IDP and evaluation process is given below.

Post-doctoral fellows and graduate students have defined milestones set at the beginning of the year and evaluated annually. In the annual evaluation, the milestones achieved for prior year and new short and long-term goals set for the following year discussed. Within the individual development plan (IDP), issues pertaining to acquisition of new techniques and methodologies, improvement of oral presentation skills, scientific writing skills and ability to critically think through the projects were particularly focused on.

The individuals are allowed to participate and complete mandatory training courses on Laboratory safety, general biosafety, fire safety, viral vector risk assessment and safety, and animal biosafety levels I and II. They also are required to complete institutional mandated training (HIPPA, Sexual harassment and discrimination guideline training). They are encouraged to attend additional courses (grant writing, presentation, interview, etc.) offered by the institute.

Meeting with Dr. Ramesh (PI), on a one to one basis when needed, to discuss experimental design, troubleshooting, and data interpretation is encouraged. Attendance and participation in formal lab meeting held once a week gives the opportunity to sharpen presentation skills and to receive feedback on the research in progress, from fellow postdocs and invited faculties. Additionally, participation in institutional Cancer Research Seminar Series and Departmental Research Seminars is highly recommended. These meetings and seminar series provide many opportunities to interact with other investigators and share ideas. The individuals to encouraged to meet invited speakers to discuss their research and career plans in academia/industry. Finally, they present their research work at the institutional, regional, and national conferences.

All of these training opportunities provide enhanced career opportunities in the biomedical field in academia and biotechnology industries.

How were the results disseminated to communities of interest?

“Nothing to Report.”

What do you plan to do during the next reporting period to accomplish the goals?

We plan to accomplish the following during the final reporting period:

- i) Complete the studies pending in specific aim 1 and all of the studies of aim 2.
- ii) Prepare study results and submit for publication in peer-reviewed journal
- iii) Use the data generated submitting large grant applications to NIH, DOD, VA etc.
- iv) Present study results in national conferences (AACR, ASGCT, ISEV, etc.)
- v) Submit final progress report to DOD

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

"Nothing to Report"

What was the impact on other disciplines?

"Nothing to Report."

What was the impact on technology transfer?

"Nothing to Report."

What was the impact on society beyond science and technology?

"Nothing to Report."

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

"Nothing to Report."

Actual or anticipated problems or delays and actions or plans to resolve them

The grant funding started in September 2019 and institutional approval in October of 2020. However, we encountered two problems related to the occurrence of the COVID-19 pandemic.

The first problem has been in the recruitment of a post-doctoral fellow. Initially, recruitment was delayed to COVID-19 pandemic (2019-2020) and university closing. With reopening of the university in fall of 2020, we tried to reopen the recruitment and since majority of the potential applicants that fit the required expertise were international, it was difficult for their travel as there were many visa restrictions due to COVID. With the re-emergence of COVID-19 variants in spring of 2021, recruitment was again a challenge and couple of individuals interviewed within the country accepted position elsewhere.

The second problem some of the studies planned and initiated in February 2020 was terminated, and all research activities university wide was shut down in March 2020. This made a major impact in advancing the studies. With the university reopening, the laboratory research resumed, albeit at reduced workforce. However, the unanticipated short supply and delay in ordering and receiving reagents and plasticware has continued and has contributed to slow progress of the study.

The third problem contributing to delay in the progress of the project was that the key personnel (Dr. Akhil Srivastava) working on the project accepted a faculty position at University of Missouri Columbia and departed in June 2021. This halted the project progress. We are currently in the process of hiring a post-doctoral fellow from India and anticipate that he will arrive in March 2022 to join the project and contribute to completing the study.

Changes that had a significant impact on expenditures

A significant impact on the project and expenditure has not only been in the delay in hiring a post-doctoral fellow but also the departure of the key personnel (Dr. Srivastava) in June of 2021. Additionally, significant delay in receiving reagents and plasticware and having most of the company having the shipments "backordered" has contributed in making an impact on expenditure.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

"Nothing to Report."

Significant changes in use or care of human subjects

"Nothing to Report."

Significant changes in use or care of vertebrate animals

"Nothing to Report."

Significant changes in use of biohazards and/or select agents

"Nothing to Report."

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Articles Published (total of 5 published; 2 are under review)

1. Srivastava, A., Amreddy, N., Pareek, V., Razaq, M., Chinnappan, M., Ahmed, R., Azeem, A., Munshi, A., Ramesh, R. A decade of extracellular vesicles research in cancer biology and its application in medicine. Wiley Interdiscip Rev. Nanomed Nanobiotechnol. 12(4): e1621, 2020. doi:10.1002/wnan.1621. PMID: 32131140; *Status of publication (published); Acknowledgement of federal support (Yes)*
2. Chinnappan, M., Srivastava, A., Amreddy, N., Razaq, M., Pareek, V., Ahmed, R., Peterson J. E., Mehta, M., Munshi, A., Ramesh, R. Exosomes as a drug delivery vehicle and contributor to treatment resistance. Cancer Lett. 486:18-28, 2020. PMID: 32439419; *Status of publication (published); Acknowledgement of federal support (Yes)*
3. Srivastava, A., Rathore, S., Munshi, A., Ramesh, R. Extracellular vesicles in oncology: from immune suppression to immunotherapy. AAPS J. 23:30, 2021. doi:10.1208/s12248-021-00554-4. PMCID: PMC7882565; *Status of publication (published); Acknowledgement of federal support (Yes)*
4. Raguraman, R., Srivastava, A., Munshi, A., Ramesh, R. Therapeutic approaches targeting molecular signaling pathways common to diabetes, lung diseases and cancer. Adv. Drug Deliv Rev. 178: 113918, 2021, doi.org/10.1016/j.addr.2021.113918. PMID: 34375681. *Status of publication (published); Acknowledgement of federal support (Yes)*
5. Raguraman, R., Shanmugarama, S., Mehta, M., Peterson, J.E., Zhao, Y.D., Munshi, A., Ramesh, R. Drug delivery approaches for HuR-targeted therapy for lung cancer. Adv. Drug Deliv Rev. 180: 114068, 2022, doi.org/10.1016/j.addr.2021.114068; PMID: 34822926. *Status of publication (published); Acknowledgement of federal support (Yes)*
6. Srivastava, A., Rathore, S., Munshi, A., Ramesh, R. Organically derived exosomes as carriers of anticancer drugs and imaging agents for cancer treatment. Semin. Cancer Biol. 2022 *Status of publication (revised and resubmitted and under review); Acknowledgement of federal support (Yes)*
7. Amreddy, N., Srivastava, A., Gali, H., Riedinger, N., Raguraman, R., Johnston, S.E., Rathore, S., Zhao, Y.D., Raguraman, R., Peterson, J.E., Munshi, A., Ramesh, R. Tumor-targeted multifunctional exosomes as drug carriers for lung cancer therapy. Mol Pharm. *Status of publication (under review); Acknowledgement of federal support (Yes)*

Books or other non-periodical, one-time publications.

Book chapters (total of 3 published; 2 are in preparation)

1. Amreddy, N., Munshi, A., Ramesh, R. Multifunctional dendrimers for theranostic applications. In: Kesharwani, P. (ed.). Dendrimers based nanotherapeutics. Chapter 22; pp: 385-416; Elsevier Science and Technology, Academic Press, USA, 2021. doi.org/10.1016/B978-0-12-821250-9.00010-X. *Status of publication (published); Acknowledgement of federal support (Yes)*
2. Amreddy, N., Chinnappan, M., Munshi, A., Ramesh, R. Dendrimer-based nanoformulations as drug carriers for cancer treatment. In: Bajpai, A.K., and Saini, R. (eds.). Advances in polymeric nanomaterials for biomedical applications. pp: 137-159; Elsevier, USA, 2021. DOI: doi.org/10.1016/B978-0-12-814657-6.00003-3. *Status of publication (published); Acknowledgement of federal support (Yes)*
3. Amreddy, N., Babu, A., Munshi, A., Ramesh, R. Dendrimers as drug carriers for cancer therapy. In: Yata, V.K., Ranjan, S., Dasgupta, N., Lichtfouse, E. (eds.). Nanopharmaceuticals: Principles and Applications Vol. 3. Vol 48; pp: 245-269; Springer Press, USA, 2020. *Status of publication (published); Acknowledgement of federal support (Yes)*
4. Ramesh, R., Raghuraman, R., Rathore, S., Mehta, M., Munshi, A. Exosomes-based drug delivery for lung cancer treatment. In: Narang, A., and Mahato, R. (Eds.). Drug delivery targeting to the lung. pp: XX; CRC Press/Taylor & Francis Publishers, USA, 2022. *Status of publication (In preparation)*.
5. Ramesh, R., Rathore, S., Mehta, M., Munshi, A. Exosomes: Opportunities for use as biomarker source and as drug carrier in cancer progression and treatment. In: Sobti, R.C., Ganguly, N.K., Kumar, R. (Eds). Handbook of Oncobiology: From basic to clinical sciences. pp: XX; Springer Nature Singapore Ptd Ltd, 2022. *Status of publication (In preparation)*

Other publications, conference papers and presentations.

"Nothing to report"

- **Website(s) or other Internet site(s)**

"Nothing to Report."

- **Technologies or techniques**

"Nothing to Report."

- **Inventions, patent applications, and/or licenses**

"Nothing to Report."

- **Other Products**

“Nothing to Report.”

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

<i>Name:</i>	<i>Rajagopal Ramesh</i>
<i>Project Role:</i>	<i>Principal Investigator</i>
<i>Researcher Identifier (e.g. ORCID ID):</i>	<i>0000-0002-7658-5338</i>
<i>Nearest person month worked:</i>	<i>2</i>
<i>Contribution to Project:</i>	<i>Dr. Ramesh was responsible for writing and getting approval of IACUC protocol; ordering cell lines and reagents; designing the studies and overseeing the experiment outcome; troubleshoot problems; weekly meetings to discuss project progress; performed work in the area of exosome characterization.</i>
<i>Funding Support:</i>	<i>N/A</i>
<i>Name:</i>	<i>Akhil Srivastava</i>
<i>Project Role:</i>	<i>Assistant Professor, Research</i>
<i>Researcher Identifier (e.g. ORCID ID):</i>	<i>0000-0003-2015-259X</i>
<i>Nearest person month worked:</i>	<i>4</i>
<i>Contribution to Project:</i>	<i>Dr. Srivastava has performed work in the area of isolating exosomes, growing cell lines, characterizing exosomes, and conducting immune checkpoint array and western blotting.</i>
<i>Funding Support:</i>	<i>CDMRP-DOD & Oklahoma Center for Advancement of Science and Technology (OCAST)</i>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The changes in the “Other Support” of the PD/PI is listed below.

New/Active Grant

Title: Exosomes as carriers of cancer therapeutics
Grant # -1 R01 CA254192
Time Commitment: 1.8 Calendar Months (15% OUHSC Effort)
Supporting Agency: National Institutes of Health/National Cancer Institute
Performance Period: 09/14/2021– 08/31/2026
Role: PI

Completed Grants

Title: Exosome as Drug Carriers for Cancer Therapy
Time Commitment: 0.06 Calendar Months (1% OUHSC Effort)
Supporting Agency: Presbyterian Health Foundation
Performance Period: 09/01/2020 – 09/13/2021
Role: PI

Title: END2CANCER: Emerging Nanotechnology and Drug Delivery Applications for Cancer
Time Commitment: 0.06 Calendar Months (1% OUHSC Effort)
Supporting Agency: Presbyterian Health Foundation - Symposium Grant Program
Performance Period: 07/01/2019 – 06/30/2020
Role: PI

What other organizations were involved as partners?

“Nothing to Report”

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

N/A

QUAD CHARTS:

N/A

9. APPENDICES:



Review Article

Theme: Identification and Implementation of Predictive Biomarkers for Checkpoint Targeted Immunotherapy

Guest Editors: Baolin Zhang and Mario L. Rocci Jr.

Extracellular Vesicles in Oncology: from Immune Suppression to Immunotherapy

Akhil Srivastava,^{1,2} Shipra Rathore,^{1,2} Anupama Munshi,^{2,3} and Rajagopal Ramesh^{1,2,4,5}

Received 17 August 2020; accepted 5 January 2021

Abstract. Exosomes are involved in cell-to-cell communication and play a crucial role in cellular physiology. The role of exosomes in cancer has been widely explored. Tumor cells have evolved and adapted to evade the immune response. The study of the immune system's modulations in favor of rogue tumor cells led to the development of a novel immunotherapeutic strategy targeting the immune checkpoint proteins (ICPs). In clinical settings, the response to ICP therapy has been inconsistent and is difficult to predict. Quantitating the targeted ICPs through immunohistochemistry is one approach, but is not pragmatic in a clinical setting and is often not sensitive. Examining the molecules present in bodily fluids to determine ICP treatment response, “liquid biopsy” is a convenient alternative. The term “liquid biopsy” refers to circulating tumor cells (CTCs), extracellular vesicles (EVs), non-coding (nc) RNA, circulating tumor DNA (ctDNA), circulating free DNA (cfDNA), etc. EVs includes exosomes, microvesicles, and oncosomes. Herein, we focus on exosomes isolated from bodily fluids and their use in liquid biopsy. Due to their unique ability to transfer bioactive molecules and perturb the physiology of recipient cells, exosomes have garnered attention for their immune modulation role and as a resource to identify molecules associated with liquid biopsy-based diagnostic methods. In this review, we examine the putative role of exosomes and their cargo in influencing the immune system. We discuss the immune and tumor cells present in the tumor microenvironment (TME), and the exosomes derived from these cells to understand how they participate in creating the immune-suppressive TME. Additionally, use of exosomes in liquid biopsy-based methods to measure the treatment response elicited by immunotherapy is discussed. Finally, we describe how exosomes have been used to develop immune therapies, especially cell-free vaccines, for cancer treatment.

KEY WORDS: cancer; exosomes; liquid biopsy; immunotherapy; tumor microenvironment.

INTRODUCTION

Cancer is a unique pathological condition in which marked dysregulation of a series of molecular events and processes allows healthy cells to behave abnormally and, in

many cases, aggressively. Since these changes are not caused by foreign molecules, it is interesting to observe how the body's immune system, which is designed to eliminate such cancerous cells, is perturbed and allows these cells to go unchecked and display their effects by initiating cancer and its progression through metastases. Recent studies in the areas of immuno-oncology and the tumor microenvironment (TME) have revealed that immune cells and their soluble secretions bring about changes in the TME that allow cancer cells to establish and propagate (1,2). Research has confirmed the immune-suppressive activities of immunogenic cells, such as T cells, B cells, natural killer (NK) cells, and dendritic cells (DC), but the exact mechanism of their regulation is still unclear. However, a critical role of intercellular communication and signaling has been well established (3,4).

At a multicellular organizational level, the essential communication between cells is maintained through various mechanisms. One such mechanism is through tiny cellular vesicles that are collectively called extracellular vesicles

Guest Editors: Baolin Zhang and Mario L. Rocci Jr.

¹ Department of Pathology, University of Oklahoma Health Sciences Center, 975 N.E., 10th Street, Oklahoma City, Oklahoma 73104, USA.

² Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, 73104, Oklahoma, USA.

³ Department of Radiation Oncology, University of Oklahoma Health Sciences Center, 975 N.E., 10th Street, Oklahoma City, 73104, Oklahoma, USA.

⁴ Graduate Program in Biomedical Sciences, University of Oklahoma Health Sciences Center, Oklahoma City, 73104, Oklahoma, USA.

⁵ To whom correspondence should be addressed. (e-mail: rajagopal-ramesh@ouhsc.edu)

(EVs). One subclass of EVs is exosomes. Exosomes are tiny, nano-sized (50 nm to 150 nm in diameter) vesicles produced and released by all cells through a well-defined and regulated endosomal pathway. Exosomes, due to their nature as cellular cargo carriers, were previously considered vesicles that removed toxic materials from the cellular lumen (5).

Although exosomes were discovered in the sixties, their functional importance was not recognized until recent. However, scientists recently discovered that exosomes carry several biologically active regulatory molecules, such as proteins, nucleic acids (DNA, several species of coding and non-coding long and small RNAs), and lipids, in their lipid bilayered membrane structure (5). The pivotal role of exosomes in cellular communication led to several studies and discoveries demonstrating exosomes as an important mediator in immunomodulation in the TME and their involvement in cancer pathophysiology (6–8). Immune cells and tumor cells in the TME secrete exosomes, which influence early and late events related to carcinogenesis. In 1996, the first evidence of the role of exosomes in immunogenicity was documented in B cell-derived exosomes that showed an immunomodulatory effect in major histocompatibility complex (MHC) class II restricted CD4⁺ T lymphocytes (9,10). Two years later, the Zitovgel group published their work showing the potential of DC-derived exosomes (DEX) in immunotherapy (11).

Tumor cells are an important component of the TME. The exosomes released by tumor cells, hereafter referred to as “TEX” are enriched in molecules that are purportedly involved in immunosuppressive and inflammatory effects aiding in the development and establishment of favorable TME for tumor cells and metastatic cells (Fig. 1). DEX, B-cell, and macrophage-released exosomes promote the anti-tumor immune response (12,13). Thus, studying the exosomes produced by various cellular components present in the TME will enhance our understanding of cancer pathology and physiology, and aid in developing novel immunotherapeutic interventions. Immunotherapy has been successful in treating many cancers, including lung, breast, colon, cervix, head and neck cancer, and melanoma. Despite immunotherapy offering a new hope for cancer patients, especially those who have failed chemotherapy, treatment response has been variable when administered alone or in combination with conventional therapies.

Currently, the programmed death-ligand (PD-L1) levels estimated through immunohistochemistry (IHC) performed on tissue biopsy specimens is an accepted biomarker to predict the treatment response. However, conventional tissue biopsies are not sensitive enough. Since exosomes in the TME are involved in immunomodulation and can be isolated from different bodily fluids, use of exosomes in liquid biopsy-based diagnosis is a new strategy to develop predictive diagnostic biomarkers for immunotherapy.

This review describes the role of exosomes and their contents in immune stimulation, possible immunotherapeutic aspects, and their role in novel liquid biopsy modalities to develop sensitive and specific predictive biomarkers for a given immunotherapy. The use of exosomes in translational cancer research, their application in clinical trials, and various challenges associated with applying exosomes in cancer diagnosis and therapy are discussed herein. For more detailed information

on exosomes influencing the immune response, readers are directed to read reviews by Yan *et al.* and Xie *et al.* (14,15).

CONTENT OF EVS FOR IMMUNOMODULATION AND AS CANDIDATES OF LIQUID BIOPSY

The presence of molecular entities in the exosomes makes them unique in their functioning and significance. Studies have shown that the packaging of molecules into the lumen of exosomes is not a random process and occurs through a tightly orchestrated process during the biogenesis of exosomes in the late endosomal pathway. Exosome biogenesis begins with the formation of endosomes by endocytosis. As the early endosome transitions to late endosomes, the inner membrane starts folding or invaginating forming small vesicle-like structures inside the maturing endosomes. These small vesicles inside the endosomes are called intraluminal vesicles (ILVs) and the endosomes bearing them are called multi-vesicular bodies (MVBs). These MVBs are an important site for packaging of biomolecules into the ILVs that are released as exosomes when MVBs fuse with plasma membrane (16). During the process of MVB maturation and until ILVs are released, a battery of proteins under the umbrella of endosomal sorting complexes required for transport (ESCRT) play an important role in the packaging of proteins into the lumen of exosomes and release of exosomes from cells. This ESCRT machinery includes the ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III protein complexes. In addition, the Rab proteins, which include Rab 27a, Rab27b, Rab35, and Rab11, are involved in the formation of ILVs and sorting of proteins and biological molecules (17–19). It is well established that the content of exosomes mirrors its cell of origin and hence one can examine the exosome content to determine its function and significance (20).

In the next sub-section, we describe important molecules present in the immunogenic exosomes and their putative functioning in cancer immunology. The exosomes derived from melanoma cells regulate the tumor immune response, mostly by instigating immune evasion by tumor cells. Hence, the exosomes and their contents can also be a therapeutic target (21). Using microscopy and flow cytometry techniques, CD8⁺ T cells were shown to internalize exosomes from different tumor types, even if these T cells do not internalize EVs as efficiently as do other immune cells. Further, the function of melanoma-derived exosomes in CD8⁺ T cells was examined. Researchers showed that these exosomes downregulated the T cell response through decreased T cell receptor (TCR) signaling and diminished cytokine and granzyme B secretions, which reduced the cells' cytotoxic activity (22,23).

Protein

In 1996, exosomes were first reported as nanovesicles that were enriched in major MHC-II molecules secreted from B-lymphoblastoid cells (9). This discovery led to the identification of exosome-like structures that were associated with all types of immunocytes, including B and T lymphocytes, macrophages, DCs, NK cells, mast cells, and thymocytes (10,24,25). Following the premise that exosomes play an

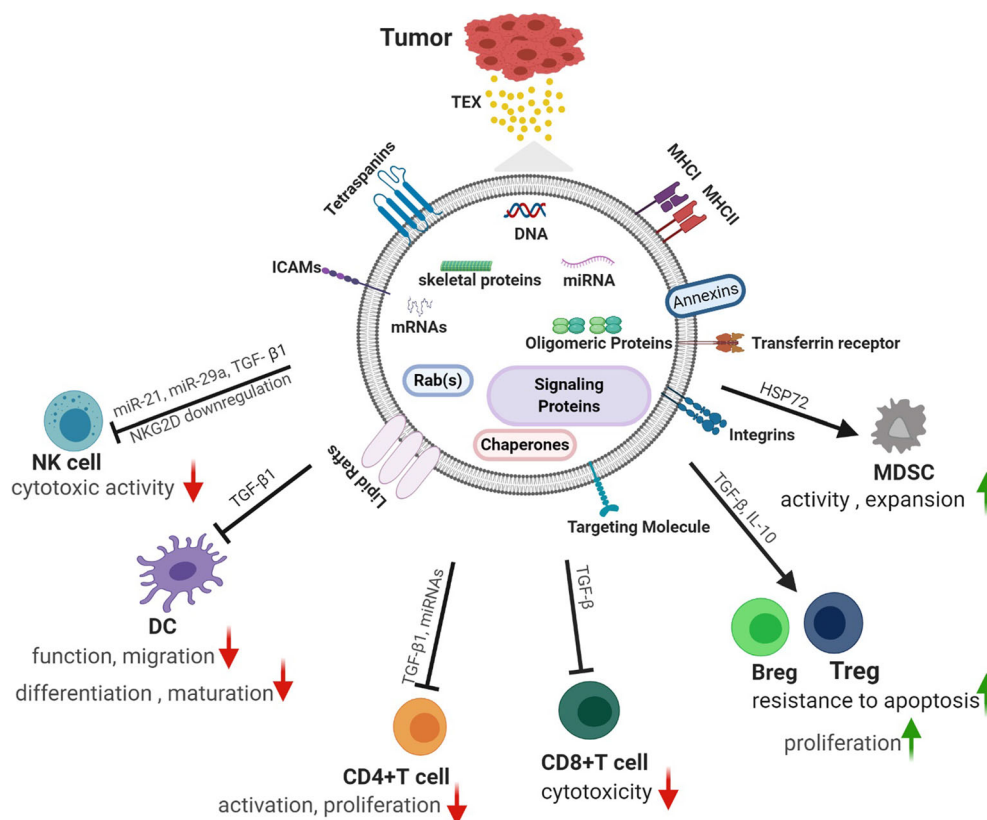


Fig. 1. Exosomes from tumor cells (TEX) play an important role in modulating immune cells in the TME and promote different activities resulting in favorable condition for tumor propagation. Figure created with BioRender.com

integral role in immune modulation, the presence of numerous proteins derived from the immunocytes and enriched in the exosomes have been reported (26). In addition to cytokines, cytokine receptors, and integrin's, the proteins that are present in the exosomes include adhesion molecules (CD11b, CD54/ICAM-1), antigen-presenting molecules (MHC class I, MHC class II, and CD1), T and B cell receptors, and costimulatory proteins (CD86) (10,27,28).

Checkpoint Inhibitor as a Contributor to Liquid Biopsy

Immune checkpoint proteins (ICPs) function to restrict the immune system by providing inhibitory signals, and block the activation of T cells to enact self-tolerance (29). Tumor cells express these checkpoint proteins and successfully use them to evade the immune response (30). Novel immunotherapeutic interventions attempt to block these checkpoint proteins through antibodies. Higher expression of checkpoint proteins, such as programmed cell death-1 (PD-1), programmed death-ligand1 (PD-L1), cytotoxic T lymphocyte antigen-4 (CTLA-4), B7 homolog 4 protein (B7-H4), T cell membrane protein-3 (TIM-3), and lymphocyte activation gene 3 (LAG-3), has been observed in tumors. Based on the nature of exosome biogenesis, it is predicted that TEX should also have these checkpoint proteins present in their lumens and membrane surface (31–33). Chen *et al.* recently showed that PD-L1 is enriched in exosomes, when compared with that in melanoma microvesicles, suggesting that exosomes are the primary source of PD-L1 among EVs in this cancer type (34). Other studies have confirmed exosomal PD-L1 in melanoma, prostate cancer, breast cancer, glioblastoma, head and neck cancer, lung cancer, and other tumors

(35,36). However, the success of immunotherapy depends on the expression profile of these different ICPs.

Liquid biopsy utilizes circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating cell-free DNA (cfDNA), micro (mi) RNA, and non-coding (nc) RNA for cancer diagnosis and in determining disease progression and treatment response. Exosomes isolated from bodily fluids carry the above-mentioned biomolecules. Moreover, exosomes have been successfully isolated in a non-invasive fashion from bodily fluids, such as urine and saliva. In our lab, we have explored the use of urine-derived exosomes from non-small cell lung cancer (NSCLC) patients receiving immunotherapy to develop a non-invasive liquid biopsy tool to predict treatment response. We successfully detected many ICPs in urinary exosomes from NSCLC patients treated with anti-PD-1 and chemotherapy (Fig. 2). We also observed a change in the ICP expression profile in response to the treatment, suggesting the putative role of exosomes in immune modulation and the possibility of testing their role as predictive markers for measuring therapeutic outcomes. Concurring with our findings, Cardonniere *et al.* (37) showed that in comparison to tissue biopsies, exosomal PD-L1 isolated from plasma of melanoma patients showed improved detection of disease incidence (67% in tissue biopsies vs 100% in blood plasma). Further this study also showed a correlation between exosome PD-L1 post treatment (Δ ExoPD-L1) and tumor response to treatment with an 83% sensitivity, 70% specificity, 91% positive predictive value, and 54% negative predictive value for disease progression (37). Song *et al.* in their study speculated the probable mechanism of PD-L1 upregulation in exosomes in response to immunotherapy is by interferon- γ (IFN- γ) (38). Through mechanistic

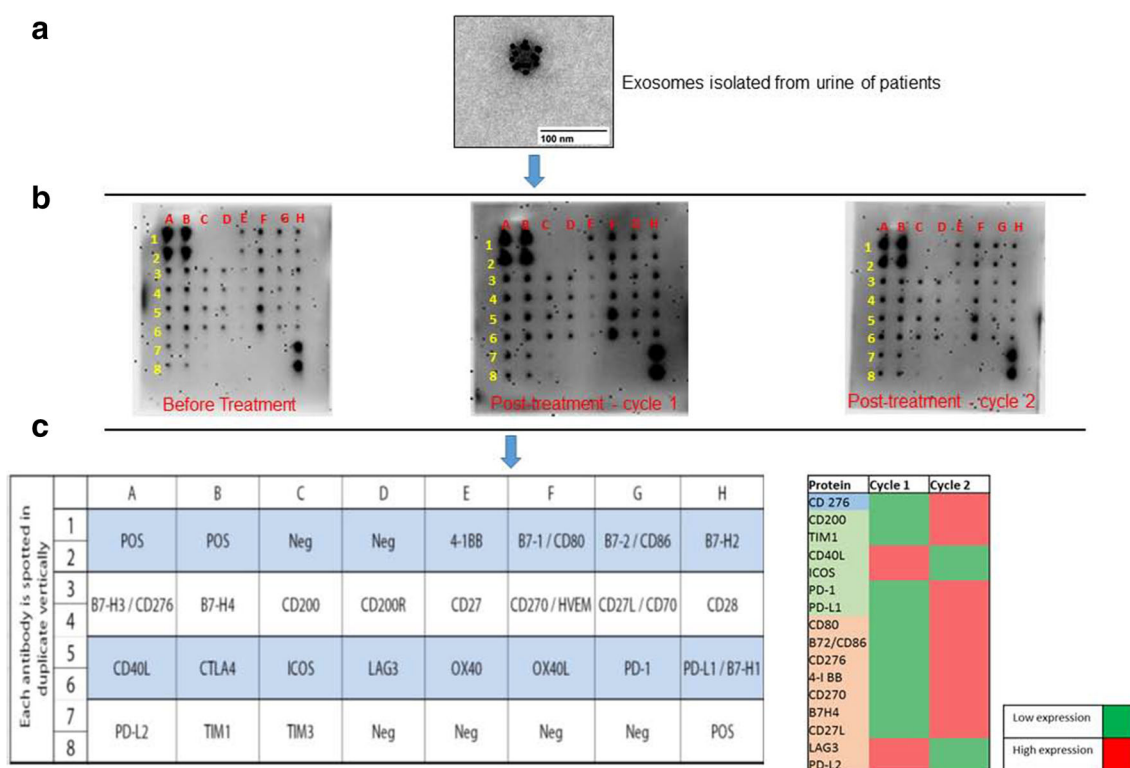


Fig. 2. The exosomes contains various immunogenic proteins including immune checkpoint proteins. It has been hypothesized that these checkpoint proteins play crucial role in treatment outcomes. **(a)** Detection of PD-L1 in exosomes derived from the urine of non-small cell lung cancer (NSCLC) patients. Ant-PD-L1 antibody conjugated to gold was used to detect the presence of PD-L1 on the surface of exosomes. **(b)** A protein array detects twenty-three different immune checkpoint proteins on exosomes. **(c)** Expression analysis of immune checkpoint proteins show change in expression profile in response to the immune therapy

studies, the authors tried to explain melanoma patients who failed pembrolizumab therapy showed increased levels of exosomal PD-L1 post treatment. The study also established the role of exosome PD-L1 isolated from blood in stratifying patients as responders and non-responders indicating the utility of exosome PD-L1 as a treatment prediction marker (38). Gunasekaran *et al.* investigated the role of exosomal PD-L1 as a non-invasive tool for predicting the treatment response to immune checkpoint inhibitors on a small cohort ($n = 25$) of NSCLC patients (39).

Apart from PD-L1, PD-L2 expression on exosomes has also been reported (40). Examination of exosomes from IL-10 treated murine dendritic cells showed reduction in exosomal PD-L1 and PD-L2 did not contribute to the immune-suppressive function in a murine model of sepsis (40). It is evident from this study that exosomal PD-L2 could also serve as potential biomarker in cancer diagnosis and therapy. Finally, testing of additional ICP proteins on exosomes as predictive liquid biopsy biomarker in cancer patients receiving various ICP inhibitors would improve understanding the molecular mechanisms of resistance and failure to immunotherapy (41,42).

Lipids

In addition to unique sets of proteins, the lipid composition of exosomes is distinct from other cellular membranes.

Exosomes are enriched in membrane lipids and are deprived of cytosolic lipids. More than 2000 lipid molecules have been described to be present in the exosomes (43). Sphingomyelin, glycosphingolipids, phosphatidylserine, and cholesterol are enriched two to three times in exosomes compared to cells. Exosomes have no transmembrane phospholipids present in them. In contrast, phosphatidylcholine and phosphatidylinositol are more enriched in cells compared to exosomes (44). Lipidomics study of immunocytes like B cells and mast cells showed the presence of cholesterol (45). The high content of lipids in the exosome membrane is implicated in facilitating the interaction between exosomes and cells (46,47). Like nucleic acids and proteins, packaging of lipids into exosomes occurs through a highly regulated process and often reflects the state of cells. Several studies have explored the possibility of using lipid profiles for the identification of putative biomarkers. Skotland *et al.* (46) identified one hundred and seven lipid species in the exosomes of urine samples collected from prostate cancer patients and healthy controls. In this study, the authors identified nine lipid species that showed significant differences in the urinary exosomes of prostate cancer patients compared to normal individuals. Phosphatidylserine (PS) 18:1/18:1 and lactosylceramide (d18:1/16:0) showed the highest patient- to-control ratio with 93% sensitivity and 100% specificity (46). Brzozowski *et al.* profiled lipidomics on exosomes from non-tumorigenic (RWPE1), tumorigenic (NB26), and metastatic (PC-3) prostate cancer cell lines. They identified 187 unique signatures in

lipid profiles and identified fatty acids, glycerolipids, prenol lipids, sterol lipids, sphingolipids, and glycerophospholipids as candidate biomarkers (48). As a unique strategy of harnessing lipid profiling as biomarkers, Cheng *et al.* combined the proteomic and lipidomics of exosomes isolated from ovarian cancer cell lines and ovarian surface epithelial cells. They identified 1433 proteins and 1227 lipids in the exosomes derived from the two cell lines and observed significant differences in several lipids and proteins in the two exosomes populations. Their study implied combination of proteomic and lipidomic profiles could be a promising approach in the early diagnosis of ovarian cancer (49). Application of such unique approaches make exosomes excellent candidates for developing biomarkers and strategies for liquid biopsies.

Nucleic Acids

Different species of nucleic acids (gDNA, miRNA, lncRNA, mRNA, etc.) have been identified in tumor-derived exosomes (TEXs). Several non-coding small RNAs, such as miRNAs, are present in high levels in the exosomes and are considered potential circulating diagnostic biomarkers for many cancer types, such as glioblastoma, melanoma, and prostate cancer, and thus forms a major contributor of liquid biopsies (50–52). The presence of coding mRNAs and an array of non-coding small RNAs, such as Piwi-interacting RNA (piRNA), small non-coding RNA (sncRNA), short interfering RNA (siRNA), and miRNAs, have also been reported (53). Additionally, the presence of DNA, including single-stranded (ss) and double-stranded (ds) DNA, genomic (g) DNA, and even mitochondrial (mt) DNA, in the exosomes have been reported (54). However, the presence of gDNA in the exosomes is under debate due to conflicting results presented warranting additional studies with thorough investigation (55). The presence of nucleic acids in the exosomes has led to their investigation in immunosuppression during tumorigenesis, molecular cues in generating an immunocompromised TME, and favorable metastatic niche. In the following sub-sections, different nucleic acid molecules present in the exosomes and their role in immuno-oncology are discussed.

miRNAs

miRNAs are small 22–24 nucleotide non-coding RNAs that regulate gene expression and are present in cells and body fluids, and constitute a major component of the exosomal cargo. miRNAs packaged in the membrane-encapsulated vesicles are protected from RNA-digesting enzymes and participate in several regulatory functions in the recipient cells (56,57). The packaging of miRNAs into the exosomes occurs through a well-regulated mechanism. Research has shown that certain guiding sequences dictate the loading of miRNAs into the exosomal lumen (58). Additionally, the putative role of some RNA binding proteins in packaging the miRNAs into exosomes is currently under investigation.

miRNAs are functionally important species among all other molecules present in the exosome cargo (59). Hence, exosomal miRNAs are important in imparting immune

evasion by tumor cells. The miRNAs from exosomes can stage this immune evasion in several ways, including immune cell modulation and altering the immune response. Further, exosomes derived from several immune cell types contain miRNAs to perform the function of immune modulation in the TME (23,60). The exosomal miRNAs in addition to influencing the oncogenic behavior of tumor cells can serve as molecular targets for cancer therapeutics; in the identification of new diagnostic biomarkers; and in the assessment of treatment response. Table 1 lists various miRNAs isolated from different TEX and their influence on the immune system during cancer pathogenesis.

DNAs

The presence of DNA in the exosome cargo has underscored the importance of DNA in exosome physiology (78,79). However, there are also studies that challenge the existence of DNA in exosome lumen (55). The functional significance of exosomal DNA (exoDNA) compared with exosomal RNA has not been widely studied. Nevertheless, exoDNA is involved in transmitting crucial genetic information from the donor cell to recipient cells, bringing about changes, including immunogenic changes, in the recipient cells. Some studies have provided insights into its putative role in immune cell response. The mechanism of DNA packaging to exosomes is unknown, but initial experiments on astrocytes and glioblastoma cells by Guescini *et al.* indicate the presence of mitochondrial DNA (mtDNA) in exosomes (80). Thakur *et al.* showed that TEXs contain exoDNA, the constituents of which were similar to those of the cell (cancer cell) of origin (81). Similar observations were reported in lung, breast, melanoma, pancreatic, and prostate cancer. *In vitro* and *in vivo* studies in glioblastoma and medulloblastoma cells demonstrated the presence of single-stranded DNA (ssDNA), gDNA, complementary (c) DNA, and transposon DNA (82,83,84). Two studies reported that exoDNA can play a pivotal role in modulating innate immune response. Lian *et al.* (85) and Kitai *et al.* (86) showed that cells under stress tend to produce more exoDNA by activation of DCs through cGAS-STING and the AIM-2 cell signaling pathway. These findings show that in addition to RNAs, DNA can have functional significance in regulating the immune system and could be novel immunotherapy targets.

EXOSOMES IN IMMUNE SUPPRESSION THROUGH MODULATION OF IMMUNOCYTES

The TME is a dynamic region involved in the initiation of malignant lesions and creation and establishment of the metastatic niche. Exosomes that are derived from antigen-presenting cells (APCs) and other immunocytes can present antigens to T cells and activate immune responses *via* classical MHC-restricted mechanisms, involving multiple cell–cell interactions (15,87–89). Exosomes function in initial T cell priming, differentiation of mature T cells, and in the development of effector functions. Antigen presentation by exosomes can be accomplished by transfer of MHC peptide complexes between naïve DCs, uptake and presentation of exosome cargo by mature DCs, and direct T cell activation without the need for uptake and further processing by APCs

Table I. Exosome-Derived miRNAs in Tumor Immunology

miRNA ID	Exosome origin	Functional role	Reference
(hsa)-miR-3187-3p, hsa-miR-498, hsa-miR-122, hsa-miR149, and hsa-miR-181a/b	Melanoma	Downregulate CD 8 ⁺ T cell response by decreasing T cell receptor (TCR) signaling and TNF- α secretion	(23)
miR-21 and miR-29a	Tumor cell	Bind to Toll-like receptors (TLRs) expressed on macrophages, and stimulation of NF- κ B-mediated secretion of cytokines to produce a pro-inflammatory TME	(61)
miR-223	Tumor-associated macrophages (TAMs)	Development of aggressive and invasive phenotypes in breast cancer.	(62)
miR-23a	Tumor cell	Interference with the functioning of NK cells	(63)
miR212-3p	Pancreatic cancer	Target MHC class II promoting immune evasion of tumor cells	(63,64)
miR-203	Pancreatic cancer	Regulate TLR4 to influence the functioning of DCs	(65)
miR-24-3p	Nasopharyngeal cancer	Impaired T cell proliferation and differentiation to Th17 cells.	(66)
miR-152	Non-small cell lung cancer	Regulates fibroblast growth factor (FGF)2, affecting the cell growth and migration	(67)
miR-24-3p, miR-891a, miR-106a-5p, miR-20a-5p, and miR-1908	Nasopharyngeal cancer	Development of tumor immune-suppressive TME by promoting regulatory T cells (Tregs)	(68)
miR-92b	Hepatocellular carcinoma	Suppress CD69 expression and NK cell-mediated cytotoxicity	(69)
miR-222-3p	Epithelial ovarian cancer	Suppress cytokine signaling (SOCS3), polarize macrophage to M2 phenotype, and create an immunosuppressive niche for tumor development and metastasis	(70)
miR-let-7a-5p, miR-10a-5p, miR-1246, and miR-125b-5p	Lung adenocarcinoma	Promote polarization of macrophages (M1 to M2) to create an immunosuppressive pro-inflammatory environment.	(6,69)
miR-210	Lung cancer	Develop immunosuppressive TME	(68)
miR-155 and miR-125b-2	Pancreatic cancer	Promote polarization of macrophages (M1 to M2) to create an immunosuppressive pro-inflammatory environment	(71,72)
miR-214	Lewis lung carcinoma	Promote Tregs cells	(71,73)
miR-940	Epithelial ovarian cancer	Activate M2 phenotype to create an immunosuppressive pro-inflammatory environment	(74)
miR-301a-3p	Pancreatic cancer	Activate PTEN to trigger M2 polarization of TAMs	(6)
miR-24-3p	Tumor cell	Block T cell proliferation to induce immune suppression via regulating the ERK- and STAT-signaling pathways	(66)
miR-1246	Colon cancer	Reprogram macrophages into a tumor-supporting state.	(75)
miR-92a	Leukemia	Pro-tumor TME and immune evasion	(76)
miR-142-5p	Pancreatic cancer	Increase cytotoxic CD4 ⁺ and CD8 ⁺ T cells	(77)

(10,90,91). Immunogenic cells, such as CD4⁺ T, CD8⁺ T, regulatory T cells (Tregs), NK cells, and DCs, are populated in TMEs. These cells and their respective exosomes have demonstrated key functions in stimulating or attenuating immune responses that rely on the functional molecules in the cargo and the microenvironment in which the exosomes are produced.

DCs are the major type of APCs involved in both innate and adaptive immunity. In cancer pathophysiology, DC-derived exosomes promote and oppose oncogenic activities through two subtypes: exosomes from mature DCs (mDex) and exosomes from immature DCs (imDex). Researchers have shown high levels of MHC class II and other ICP

proteins and reduced levels of milk fat globule EGF factor 8 (MFG-E8) in exosomes from imDex result in antigen-specific T cell proliferation and *in vivo* immune responses. Under malignant conditions, the TEXs perturb the maturation and differentiation of DCs (28,92,93). TEXs modulate the functioning of DCs through the presence of heat shock protein (HSP)72 and HSP105 proteins on their surfaces, inducing interleukin (IL)-6 secretion, which culminates in tumor metastases (94). TEXs can inhibit both differentiation and maturation of DCs. In pancreatic adenocarcinoma (PDAC), tumorigenesis is promoted by suppression of the function of DCs by exosomes carrying miR-203, which downregulates Toll-like receptor 4 (TLR4), tumor necrosis factor- α (TNF- α),

and IL-12 expression, and ultimately prevents antigen presentation by DCs (95). In a similar fashion, exosomes enriched in miR-212-3p and isolated from pancreatic cancer cells when taken up by DC cells downregulate the regulatory factor X-associated protein (RFXAP) expression, which subsequently decreases MHC class II expression and results in the inactivation of CD4⁺ T cells, contributing to the generation of an immunotolerant microenvironment in PDAC (64). Therefore, studies suggest that targeting the release of miR-203- and miR-212-3p-positive TEXs could result in positive treatment outcomes in PDAC patients (64,95).

TMEs are enriched in Tregs that create an immunosuppressive environment to promote oncogenic properties. Treg-derived exosomes have also been shown to maintain immune tolerance (96). Interaction of DCs with exosomes produced by Tregs and transfer of miRNA-150-5p and miR-142-3p from the Treg exosomes to DCs suppresses DCs functions and leads to an immunosuppressive TME (97).

Macrophages are another class of tumor-infiltrating immunocytes that are abundant in the TME. These cells are designed to engulf and destroy bodies of foreign origin or those that work against the normal physiology of the cell. Malignant tumors hijack this potential of macrophages in the TME and re-engineer them as tumor-associated macrophages (TAMs), which are then exploited by tumors for sustainability and successful proliferation. Macrophages can change polarization from conventional pro-inflammatory (M1) to immunosuppressive (M2) cells depending on the physiological conditions. Studies have revealed that functional differences in M1 and M2 cells are attributed to their molecular contents. M1 cells express stimulatory molecules such as CD80, CD86, iNOS, TLR2, TLR4, cytokines (IFN- γ , TNF- α , IL-12, IL-23), and chemokines (CCL5, CCL9, CCL10). M2 cells express high levels of CD163, CD23, CD204, CD206, VEGF, and arginase (Arg1), in addition to cytokines like IL-10, TGF- β , TGF- α , TNF, and chemokines such as CCL16, CCL17, and CCL22. The transition from M1 to M2 is very dynamic in nature and regulated by cellular signaling pathways in the TME. In a recent review, Cheng *et al.* have elegantly discussed the differentiation of M1 to M2 macrophages and their regulation and implications in cancer (98). Recent evidence from several studies has established that exosomes are intimately involved in the various cellular signaling mechanisms; hence, it becomes obvious that they will have important role in the polarization of macrophages under specific physiological conditions, such as cancer. A review by Baig *et al.* extensively discuss the role of TEXs in macrophage polarization and differentiation (99).

Conventionally, M2 macrophages promote pro-oncogenic activities, anti-inflammatory responses, and metastases. TAMs are largely regarded as M2 macrophages. The presence of M2 macrophages has been reported in many invasive cancers (e.g., pancreatic cancer, brain cancer), contributes to tumor progression by lymphangiogenesis, and correlates with lymphatic metastases and poor survival (100–103). Chemokines and other molecular cues in the TME are considered responsible for TAMs/M2 phenotypes' appearance. Recently, exosomes' role in TAM/ M2 polarization and its underlying effect on metastasis and other cancer-related activities have been discussed. Tumor cells produce a hypoxic microenvironment, and the TEXs produced under hypoxia

can polarize macrophages to the M2 phenotype in a HIF-1 α - or HIF-2 α -dependent manner (104). In ovarian cancer, TAM/ M2-derived exosomes have been shown to carry and deliver miRNAs that induced a Treg/Th17 cell imbalance to promote disease development (105). It is also reported that exosomes can activate T cells such as CD8⁺ and CD3⁺ cells. Consequently, exosomes produced from these activated T cells participate in IL-2-mediated immune response (106,107). Exosomes produced from the activated T cells interestingly are large and is presumably due to the packaging of a large number of cytokine molecules. These studies suggest that immunocyte-derived exosomes might have both beneficial and deleterious effects on the immune response and their functions dictated by the *in situ* TME (108).

T cell-derived exosomes are known to be involved in the invasion and metastasis of different cancers. As an example, exosomes expressing FasL isolated from activated CD8⁺ T cells though did not show any effect on apoptosis and proliferation of tumor cells promoted the invasion of B16 melanoma and 3LL lung cancer cells via the Fas/FasL pathway (109).

All of these study results demonstrate exosomes from immune cells present within the TME contribute in the remodeling of the tumor stroma, specifically by establishing an immunosuppressive microenvironment.

EVS IN IMMUNOTHERAPY

Apart from studies investigating exosomes in immune modulation and their contribution in reconfiguring TME and promoting tumorigenesis, the use of exosomes as drug carriers for cancer therapy has also been examined (110). The exosomes due to their small size (50–150 nm) and being non-immunogenic due to their bodily origin make them an ideal alternative as drug carriers in cancer therapeutics (111–114). Further, the endogenous origin of exosomes and the ability to load desired molecules into their lumen (exogenously) has opened avenues for the use of exosomes as nanocarriers for targeted delivery of tumor-specific antigens and immunomodulatory drugs for cancer treatment (5,115). Preclinical studies demonstrate exosomes derived from normal cells (e.g., fibroblasts, DCs, macrophages, mesenchymal stem cells) can be designed to operate as carriers of anticancer drugs, imaging agents, and their combination to serve as a theranostic (116,117). Additionally, attachment of various tumor-targeted ligands on the exosomes surface resulted in tumor-targeted drug delivery and anticancer activity both *in vitro* and *in vivo* (118,119). The results from these studies demonstrate exosomes are stable and are not susceptible to physical and chemical manipulation. An added advantage of using exosomes as drug carriers is that they can avoid phagocytosis by circulating macrophages and monocytes via CD47 (120). Finally, exosome derived from immune cells (DCs, T cells, macrophages) have been used as cancer vaccines and for immunotherapy (121,122). Studies show that these exosomes elicit robust immune response independent of MHC molecules (123). Additionally, no marked therapeutic benefits were observed when exosomes from autologous cells were used and compared to exosomes from allogeneic cells (124). These observations open avenues for testing allogeneic cell-derived

exosomes in immunotherapy and vaccine-based cancer therapies. Srivastava *et al.* have extensively described several strategies for engineering parental cells for increased exosome production and loading the exosomes with anticancer drugs and immunotherapeutics for use as nanocarriers for cancer treatment (110). The use of exosomes for combined delivery of immunomodulatory agents and chemotherapy could be another approach to develop a potent therapeutic intervention with minimal side effects. Currently, there are several phase 1/2 clinical trials testing exosomes as cancer vaccine, combinatorial therapy, and as a source of biomarkers to monitor and predict disease progression and treatment response (Table II). Thus, exosomes hold promise in the areas of cancer diagnosis and treatment.

EV-BASED LIQUID BIOPSY TOOL FOR CANCER

In the previous section, we noted that exosomes could be used to improve the efficacy of immune therapeutics. The success of these immunotherapies depends on the ability of cancer cells to express a number of receptor proteins and ligands on their surfaces. These proteins can be used as targeting moieties or therapeutic targets, and feasibility studies to quantify these molecules' expression can tremendously aid in enhancing the efficacy of immunotherapy. Based on this premise, researchers have worked to develop tissue- and cell-free or exosome-based liquid biopsies. Exosomes can be conveniently isolated from bodily fluids, such as blood (minimally invasive) and urine (non-invasive), and reflect the status of the cell of origin. Thus, investigating the content of exosomes is expected to provide specific information about critical components, including miRNA levels, ICP levels, and tumor mutational burden, which can be utilized as a prognostic or predictive biomarker for immunotherapy.

Additionally, exosomes offer many advantages to be explored for biomarkers and liquid biopsies. Several studies have shown exosomes are very stable and can be stored at -80°C for a very long period of time, translating that the unique signature molecules carried by them are also stable and can be studied over time (125,126). The extraordinary stability bestowed to biomolecules enclosed in the lumen of exosomes also enhances the accuracy in those molecules' diagnostic potential increasing their utility in liquid biopsies. cfDNA and CTCs are highly unstable and difficult to isolate from body fluids. In contrast, exosomes can be isolated in large quantities from body fluids like blood serum, cerebrospinal fluids, and urine. Finally, exosomes also show uniqueness as they, as a single particle, can simultaneously carry multiple biomolecules such as miRNA, lncRNA, mRNA, DNAs, lipids, cytokines, and proteins allowing investigation of more than one molecule as a candidate biomarker. This property will further enhance their precision and rigor in diagnosis or predicting treatment response. In thoracic malignancies and several other cancers, exosome-based liquid biopsy predictive biomarkers have already been described (127,128). In a recent study, Chen *et al.* discovered PD-L1 is packaged in the lumen of exosomes, and the levels of PD-L1 in exosomes played a crucial role in dictating the treatment response of anti-PD-1 antibody pembrolizumab immunotherapy in patients diagnosed with metastatic melanoma (34). The general liquid biopsy approach utilizes invasive and non-

invasive or minimally invasive methods. The invasive methods primarily included plasma- and serum-derived exosomes to explore the encapsulated molecules for candidate biomarkers; however, some other bodily fluids have also been studied to identify biomarkers. In a recent study, Rodríguez *et al.* demonstrated that the quantity of exosomes was significantly increased in the bronchoalveolar lavage samples of cancer patients compared to that of non-cancer subjects (129). Pleural effusion is also a rich source of liquid biopsies. Lin *et al.* explored pleural effusion-derived exosome miRNA in pneumonia and lung cancer. The study discovered 27 differentially expressed miRNAs among which the miR-205-5p and miR-200b were significantly upregulated in lung cancer, suggesting that these two miRNAs could be used for distinguishing lung cancer from pneumonia (130). In another study Hydring *et al.* evaluated pleural effusions from 18 adenocarcinoma patients and 18 with a benign inflammatory condition. They compared the exosomal miRNA profile between the groups and found miR-200 as a putative candidate for diagnosis of adenocarcinoma (131).

The second category of liquid biopsy is non-invasive methods consisting of bodily fluids such as urine, saliva, and breast milk. Li *et al.* examined the diagnostic potential of urine-derived exosomes in the diagnosis of NSCLC. The study revealed that in urinary exosomes, leucine-rich α -2-glycoprotein (LRG1) was expressed at higher levels as compared to matched cancer-free control indicating the potential use of urine-based exosomal diagnostic biomarkers (132). Studies conducted in our laboratory also demonstrated the diagnostic potential of miR-200c, which was enriched in the urine-derived exosomes of endometrial cancer patients compared to asymptomatic normal individuals (114). Urine thus has proven to be a useful resource for developing exosome-based liquid biopsy modalities for bladder, renal, and prostate cancers. The exosome content, especially proteins and miRNAs, has been extensively studied to identify putative diagnostic or prognostic biomarkers for bladder cancer. Proteins alpha-1 antitrypsin (SERPINA1) and histone H2B type 1-K (H2B1K) have been identified from urinary exosomes as promising biomarkers for prognosis in bladder cancer (133). Similarly, miRNAs like miR-21, miR-93, miR-200c, and miR-940 present in urine-derived exosomes were identified as a molecular signature for urothelial carcinoma of the bladder (UCB). The high degree of specificity determined in this study holds urinary exosome miRNA as a strong contender for developing a promising clinical diagnostic tool (134). Clinical assessment of prostate cancer through urine-derived exosomes has also been investigated in numerous studies (135–138). Similarly, in renal carcinoma, mass spectrometry analysis of urine-derived exosomes have helped in the identification of multiple proteins as candidate biomarkers (139). Human breast milk is a rich source of exosomes representing the status of tissues of the breast. Thus, the probability of obtaining breast-specific exosomes is much greater in human milk than in serum, blood, or urine. Biomarkers for several breast-related ailments can be investigated in breast milk and develop as a liquid biopsy modality (56). Exosomes from breast milk collected from involuting breast have shown to carry proteins that have pro-oncogenic effects. Matrix metalloproteinase 2 (MMP-2), MMP-3, and MMP-9, which are involved in reorganizing breast tissues to

Table II. List of Exosomes and Exosome-Based Liquid Biopsy Clinical Trials (CT) in Immune Oncology (www.clinicaltrials.gov)

NCT number	Title	Status	Conditions	Interventions	Phase
NCT01159288	Trial of a Vaccination With Tumor Antigen-loaded Dendritic Cell-derived Exosomes	Completed	Non-small cell lung cancer (NSCLC)	Sequential treatment of chemotherapeutic agent; metronomic cyclophosphamide (mCTX) or mCTX and immunotherapy using antigen-loaded dendritic cell-derived exosomes (Dex).	Phase 2
NCT04427475	Prediction of Immunotherapeutic Effect of Advanced Non-small Cell Lung Cancer	Recruiting	NSCLC	This CT explores response of immunotherapy (pablolizumab and nafilizumab) by measuring the PD-L1 and miRNA levels in plasma exosomes in pre- and post-treated patients and correlate it with the treatment outcome.	Not applicable
NCT02869685	Clinical Research for the Consistency Analysis of PD-L1 in Lung Cancer Tissue and Plasma Exosome Before and After Radiotherapy	Unknown status	NSCLC	This CT is based on the premise that radiation therapy can enhance the effect of immunotherapy. The study involves measuring PD-L1 expression levels in tissues and plasma exosomes (pExo) of patients before and after radiation treatment.	Not applicable
NCT02890849	Clinical Research for the Consistency Analysis of PD-L1 in Cancer Tissue and Plasma Exosome	Unknown status	NSCLC	This CT tends to explore the consistency of PD-L1 levels in cancer tissues and plasma exosomes (pExo) so that pExo can be utilized as a modality to monitor response of radiotherapy and immunotherapy.	Not applicable
NCT02507583	Antisense102: Pilot Immunotherapy for Newly Diagnosed Malignant Glioma	Active, not recruiting	Malignant glioma	This CT uses the unique property of exosomes where they carry tumor antigens. The investigators of the study planted exosomes along with an anti-	Phase 1
NCT02439008	Early Biomarkers of Tumor Response in High Dose Hypofractionated Radiotherapy Immune Response	Terminated	Hepatocellular carcinoma, colorectal neoplasms melanoma kidney neoplasms	To use blood plasma derived exosomes (nanovesicles) collected before, during, and after radiation therapy to determine the treatment response and develop a liquid biopsy modality	Not applicable
NCT01550523	Pilot Immunotherapy Trial for Recurrent Malignant Gliomas	Completed	Malignant glioma of the brain	This CT is based on similar premise of NCT02507583 described above. The modification included in this study is use of a new oligodeoxynucleotide sequence and treatment at initial diagnosis	Phase 1
NCT03985696	Exosomes and Immunotherapy in Non-Hodgkin B-cell Lymphomas	Recruiting	Lymphoma, B cell, aggressive non-Hodgkin (B-NHL)	This CT explores the role of exosomes in immunotherapy escape by investigating therapeutic targets CD20, and PDL-1 carried by exosomes derived from B cell non-Hodgkin lymphoma (B-NHL) cells. The CT also evaluates exosome composition for identification of potential diagnostic markers	Not applicable
NCT03854032	Nivolumab and BMS986205 in Treating Patients With Stage II-IV Squamous Cell Cancer of the Head and Neck	Recruiting	oral cavity, larynx, hypopharynx, nasal cavity/paranasal sinuses, stage 1 oropharyngeal with lymphadenopathy	This CT basically explores the therapeutic efficacy of nivolumab and BMS986205 in combination or as standalone therapy. Exosome content and the number of exosomes present in peripheral blood will be used to assess the interactions between the immune and metabolic microenvironment. The exosomes will be isolated before, during, and after treatment.	Phase 2

the pre-pregnancy stage, have also established their role in promoting breast metastasis (140). Transforming growth factor beta (TGF β) isoforms TGF β 1 and TGF β 2 are also enriched in breast milk–derived exosomes and involved in tumorigenesis (141).

In addition to proteins, Sauter *et al.* has also discussed the differentially enriched miRNAs in breast milk and their putative role in breast cancer (140). Although these molecules have been investigated for their influence in the pathophysiology of breast cancer, in addition, their enrichment under a specific condition and its correlation with the development of cancer can be explored for the discovery of predictive biomarker (142).

Saliva has long been traditionally studied for tumor biomarkers for oropharyngeal cancer (OPC) and human papilloma virus (HPV) associated OPC. Wang *et al.* explored saliva exosomes for the identification of candidate biomarkers. Exosomes from saliva were isolated using an acoustofluidic platform and investigated for small RNA and DNA content using droplet digital RT-PCR. The study demonstrated the enrichment of HPV16 DNA in the exosomes isolated from the saliva of HPV-OPC patients suggesting their putative application in saliva exosome–based liquid biopsies (143). In their review article, Nonaka and Wong have discussed in detail the current status of salivary exosomes and their molecular content in the context of developing diagnostic and prognostic biomarkers and developing saliva exosomes as a novel modality of liquid biopsies. This context in the article has coined the term “Saliva-Exosomics” (144). We strongly recommend readers to refer the article to assess the potential of saliva exosomes in developing biomarkers and their application in cancers.

Despite the availability of many novel avenues, tissue biopsies are still the gold standard for cancer diagnosis. Nevertheless, it is not a perfect method as the issues with sensitivity in tissue biopsy are still lingering. In addition to the tedious process of obtaining tissue specimens, its storage and transportation are peripheral issues that make this process cumbersome. As mentioned before, exosomes are directly produced from the tumor cells and are present in all bodily fluids. Hence, their efficient isolation and follow-up analysis bring them to the forefront of diagnostic modalities. The current attention that exosomes receive from the scientific community will undoubtedly open new avenues to develop, test, and establish exosome-based liquid biopsy methods for diagnosis and assess the treatment outcome in the near future.

FUTURE ROLE

In the sections above, we described the tremendous progress made in the field of EVs, in particular on exosomes and their application in liquid biopsy as a source of biomarkers for cancer diagnosis and as a drug delivery vehicle for cancer therapy. We have also seen how assessment through liquid biopsy presents many advantages, such as sample collection through a non-invasive or minimally invasive procedure (145,146). In addition, since exosome-based liquid biopsies rely on proteomic or genomic analysis, availability of a robust automated protocol will make the process of diagnosis fast and convenient. However, several challenges in advancing exosomes as a diagnostic tool and drug carrier exists. In the area of diagnosis, the method of isolation and

characterization of exosomes needs to be addressed. It is difficult to discriminate TEXs from exosomes derived from normal cells when both populations are mixed and circulating in bodily fluids. While antibody capture method that relies on expression of specific antigen on TEXs has been developed, inconsistencies in the expression levels of the antigens on the exosomes have hindered advancement of this technology. Exosomes like CSCs continue to present varying antigens on their surface and hence pose challenge in reproducibility. Another area that need to be addressed for ensuring rigor and reproducibility is in defining exosomes quantity. Majority of the studies report exosomes quantity in terms of protein concentration and is per recommendation by ISEV. However, for clinical use in particular in the area of drug delivery, it is impractical to express exosome quantity in terms of protein concentration. Rather, it will need to be expressed in terms of particles per milliliter akin to the expression of viral particles used in cancer gene therapy studies. This approach will ensure rigor and reproducibility among different laboratories and clinical studies. Third concern that needs to be addressed is whether to use exosomes from autologous cells or from allogeneic cells for cancer vaccine and drug delivery as concerns of cross-reactivity and eliciting deleterious immune response exist. Use of autologous cells is beneficial for personalized medicine and recognition of specific tumor antigens. However, scaling-up of exosomes from autologous cells in particular if it has to be from individual patients could be time consuming. In contrast, use of exosomes from allogeneic cells can be easily scaled-up and in fact, better antigen presentation and cross-antigen presentation can occur resulting in enhanced immune response. However, detailed studies comparing exosomes from allogeneic and autologous cells are warranted. Improvisation in exosome production in large scale is another challenge to be overcome for clinical translation (147,148). Finally, since exosomes are heterogeneous in size (50–150 nm), it will be of interest to study if size matters for drug delivery and anticancer activity. This is especially important, as size and shape of various nanomaterials have been shown to impact half-life, cell uptake, biodistribution, and efficacy. Continued advances made in the biology of exosomes and technology development should result in overcoming the current limitations and result in the broad use of exosomes in clinical applications in the next few years.

DISCLAIMER

The content is solely the responsibility of the authors. The opinions, interpretations, conclusions and recommendations are those of the authors and not necessarily endorsed by or representative of the official views by the NIH, DOD, Department of Veterans Affairs, or the Oklahoma Tobacco Settlement Endowment Trust.

FUNDING

This study was supported in part by grants received from the National Institutes of Health (NIH), (R01 CA167516 and R01CA233201); from the National Institute of General

Medical Sciences (P20 GM103639) of the National Institutes of Health; a Merit Grant (101BX003420A1) from the Department of Veterans Affairs; a Pilot Grant and Seed Grant funded by the National Cancer Institute Cancer Center Support Grant P30CA225520 awarded to the University of Oklahoma Stephenson Cancer Center; from the Department of Defense (DOD) through the Lung Cancer Research Program (LCRP) under award no.W81XWH-18-1-0637 & W81XWH-19-1-0647; a grant (HR18-088) from the Oklahoma Center for Advanced Science and Technology (OCASST); funds received from the Presbyterian Health Foundation Seed Grant, Presbyterian Health Foundation Bridge Grant, Oklahoma Tobacco Settlement Endowment Trust (TSET) awarded to the University of Oklahoma Stephenson Cancer Center, and the Jim and Christy Everest Endowed Chair in Cancer Developmental Therapeutics.

Rajagopal Ramesh is an Oklahoma TSET Research Scholar and holds the Jim and Christy Everest Endowed Chair in Cancer Developmental Therapeutics.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflicts of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

REFERENCES

1. Arneth B. Tumor microenvironment. *Medicina* (Kaunas). 2019;56(1). <https://doi.org/10.3390/medicina56010015>.
2. Bregenzler ME, Horst EN, Mehta P, Novak CM, Raghavan S, Snyder CS, et al. Integrated cancer tissue engineering models for precision medicine. *PLoS One*. 2019;14(5):e0216564. <https://doi.org/10.1371/journal.pone.0216564>.
3. Luan YY, Dong N, Xie M, Xiao XZ, Yao YM. The significance and regulatory mechanisms of innate immune cells in the development of sepsis. *J Interf Cytokine Res*. 2014;34(1):2–15. <https://doi.org/10.1089/jir.2013.0042>.
4. Pallmer K, Oxenius A. Recognition and regulation of T cells by NK cells. *Front Immunol*. 2016;7:251. <https://doi.org/10.3389/fimmu.2016.00251>.
5. Srivastava A, Amreddy N, Pareek V, Chinnappan M, Ahmed R, Mehta M, et al. Progress in extracellular vesicle biology and their application in cancer medicine. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*. 2020;12(4):e1621. <https://doi.org/10.1002/wnan.1621>.
6. Wang M, Yu F, Ding H, Wang Y, Li P, Wang K. Emerging function and clinical values of exosomal microRNAs in cancer. *Mol Ther Nucleic Acids*. 2019;16:791–804. <https://doi.org/10.1016/j.omtn.2019.04.027>.
7. Javeed N, Mukhopadhyay D. Exosomes and their role in the micro-/macro-environment: a comprehensive review. *J Biomed Res*. 2017;31(5):386–94. <https://doi.org/10.7555/JBR.30.20150162>.
8. Azambuja JH, Ludwig N, Yerneni S, Rao A, Braganhol E, Whiteside TL. Molecular profiles and immunomodulatory activities of glioblastoma-derived exosomes. *Neurooncol Adv*. 2020;2(1):vdaa056. <https://doi.org/10.1093/naojnl/vdaa056>.
9. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med*. 1996;183(3):1161–72. <https://doi.org/10.1084/jem.183.3.1161>.
10. Li Q, Wang H, Peng H, Huyan T, Cacalano NA. Exosomes: Versatile Nano Mediators of Immune Regulation. *Cancers* (Basel). 2019;11(10). <https://doi.org/10.3390/cancers11101557>.
11. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med*. 1998;4(5):594–600. <https://doi.org/10.1038/nm0598-594>.
12. Othman N, Jamal R, Abu N. Cancer-derived exosomes as effectors of key inflammation-related players. *Front Immunol*. 2019;10:2103. <https://doi.org/10.3389/fimmu.2019.02103>.
13. Bae S, Brumbaugh J, Bonavida B. Exosomes derived from cancerous and non-cancerous cells regulate the anti-tumor response in the tumor microenvironment. *Genes Cancer*. 2018;9(3–4):87–100. doi: <https://doi.org/10.18632/genesandcancer.172>.
14. Yan W, Jiang S. Immune cell-derived exosomes in the cancer-immunity cycle. *Trends Cancer*. 2020;6(6):506–17. <https://doi.org/10.1016/j.trecan.2020.02.013>.
15. Xie F, Zhou X, Fang M, Li H, Su P, Tu Y, et al. Extracellular vesicles in cancer immune microenvironment and cancer immunotherapy. *Adv Sci* (Weinh). 2019;6(24):1901779. <https://doi.org/10.1002/adv.201901779>.
16. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci*. 2018;75(2):193–208. <https://doi.org/10.1007/s00018-017-2595-9>.
17. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. 2013;200(4):373–83. <https://doi.org/10.1083/jcb.201211138>.
18. Tschuschke M, Kocherova I, Bryja A, Mozdziak P, Angelova Volponi A, Janowicz K, et al. Inclusion biogenesis, methods of isolation and clinical application of human cellular exosomes. *J Clin Med*. 2020;9(2). <https://doi.org/10.3390/jcm9020436>.
19. Alenquer M, Amorim MJ. Exosome biogenesis, regulation, and function in viral infection. *Viruses*. 2015;7(9):5066–83. <https://doi.org/10.3390/v7092862>.
20. Kalluri R. The biology and function of exosomes in cancer. *J Clin Invest*. 2016;126(4):1208–15. <https://doi.org/10.1172/JCI81135>.
21. Tucci M, Mannavola F, Passarelli A, Stucci LS, Cives M, Silvestris F. Exosomes in melanoma: a role in tumor progression, metastasis and impaired immune system activity. *Oncotarget*. 2018;9(29):20826–37. <https://doi.org/10.18632/oncotarget.24846>.
22. Gurnathan S, Kang MH, Jeyaraj M, Qasim M, Kim JH. Review of the isolation, characterization, biological function, and multifarious therapeutic approaches of exosomes. *Cells*. 2019;8(4). doi: <https://doi.org/10.3390/cells8040307>.
23. Vignard V, Labbe M, Marec N, Andre-Gregoire G, Jouand N, Fonteneau JF, et al. MicroRNAs in tumor Exosomes drive immune escape in melanoma. *Cancer Immunol Res*. 2020;8(2):255–67. <https://doi.org/10.1158/2326-6066.CIR-19-0522>.
24. Chaput N, Thery C. Exosomes: immune properties and potential clinical implementations. *Semin Immunopathol*. 2011;33(5):419–40. <https://doi.org/10.1007/s00281-010-0233-9>.
25. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol*. 2002;2(8):569–79. <https://doi.org/10.1038/nri855>.

26. Pegtel DM, Gould SJ. Exosomes. *Annu Rev Biochem*. 2019;88:487–514. <https://doi.org/10.1146/annurev-biochem-013118-111902>.
27. Chettimada S, Lorenz DR, Misra V, Dillon ST, Reeves RK, Manickam C, et al. Exosome markers associated with immune activation and oxidative stress in HIV patients on antiretroviral therapy. *Sci Rep*. 2018;8(1):7227. <https://doi.org/10.1038/s41598-018-25515-4>.
28. Yang H, Sun L, Mao Y. The role of exosomes in tumor immunity. *Ann Transl Med*. 2018;6(Suppl 2):S116. <https://doi.org/10.21037/atm.2018.12.03>.
29. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. 2012;12(4):252–64. <https://doi.org/10.1038/nrc3239>.
30. Grywalska E, Pasiarski M, Gozdz S, Rolinski J. Immune-checkpoint inhibitors for combating T-cell dysfunction in cancer. *Onco Targets Ther*. 2018;11:6505–24. <https://doi.org/10.2147/OTT.S150817>.
31. Dong Y, Sun Q, Zhang X. PD-1 and its ligands are important immune checkpoints in cancer. *Oncotarget*. 2017;8(2):2171–86. doi: <https://doi.org/10.18632/oncotarget.13895>.
32. Ni L, Dong C. New B7 family checkpoints in human cancers. *Mol Cancer Ther*. 2017;16(7):1203–11. <https://doi.org/10.1158/1535-7163.MCT-16-0761>.
33. Xie F, Xu M, Lu J, Mao L, Wang S. The role of exosomal PD-L1 in tumor progression and immunotherapy. *Mol Cancer*. 2019;18(1):146. <https://doi.org/10.1186/s12943-019-1074-3>.
34. Chen G, Huang AC, Zhang W, Zhang G, Wu M, Xu W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. *Nature*. 2018;560(7718):382–6. <https://doi.org/10.1038/s41586-018-0392-8>.
35. Tang Y, Zhang P, Wang Y, Wang J, Su M, Wang Y, et al. The biogenesis, biology, and clinical significance of exosomal PD-L1 in cancer. *Front Immunol*. 2020;11:604. <https://doi.org/10.3389/fimmu.2020.00604>.
36. Olejarczyk W, Dominiak A, Zolnierzak A, Kubiak-Tomaszewska G, Lorenc T. Tumor-derived exosomes in immunosuppression and immunotherapy. *J Immunol Res*. 2020;2020:6272498–11. <https://doi.org/10.1155/2020/6272498>.
37. Cordonnier M, Nardin C, Chanteloup G, Derangere V, Algros MP, Arnould L, et al. Tracking the evolution of circulating exosomal-PD-L1 to monitor melanoma patients. *J Extracell Vesicles*. 2020;9(1):1710899. <https://doi.org/10.1080/20013078.2019.1710899>.
38. Song Y, Wu L, Yang C. Exosomal PD-L1: an effective liquid biopsy target to predict immunotherapy response. *National Science Review*. 2018;6(6):1103–4. <https://doi.org/10.1093/nsr/nwy154>.
39. Gunasekaran M, Russo A, Cardona AF, de Miguel PD, Lapidus R, Cooper B, et al. Exosomal PD-L1 expression as non-invasive biomarker for immune checkpoint inhibitors in non-small cell lung cancer. *J Immunol*. 2020;204(1 Supplement):90.10–0.
40. Ruffner MA, Kim SE, Bianco NR, Francisco LM, Sharpe AH, Robbins PD. B7-1/2, but not PD-L1/2 molecules, are required on IL-10-treated tolerogenic DC and DC-derived exosomes for in vivo function. *Eur J Immunol*. 2009;39(11):3084–90.
41. Zhou K, Guo S, Li F, Sun Q, Liang G. Exosomal PD-L1: new insights into tumor immune escape mechanisms and therapeutic strategies. *Front Cell Dev Biol*. 2020;8:569219. <https://doi.org/10.3389/fcell.2020.569219>.
42. Knox MC, Ni J, Bece A, Buccì J, Chin Y, Graham PH, et al. A Clinician's guide to cancer-derived exosomes: immune interactions and therapeutic implications. *Front Immunol*. 2020;11:1612. <https://doi.org/10.3389/fimmu.2020.01612>.
43. Haraszti RA, Didiot MC, Sapp E, Leszyk J, Shaffer SA, Rockwell HE, et al. High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. *J Extracell Vesicles*. 2016;5:32570. <https://doi.org/10.3402/jev.v5.32570>.
44. Skotland T, Sagini K, Sandvig K, Llorente A. An emerging focus on lipids in extracellular vesicles. *Adv Drug Deliv Rev*. 2020;159:308–21. <https://doi.org/10.1016/j.addr.2020.03.002>.
45. Skotland T, Sandvig K, Llorente A. Lipids in exosomes: current knowledge and the way forward. *Prog Lipid Res*. 2017;66:30–41. <https://doi.org/10.1016/j.plipres.2017.03.001>.
46. Skotland T, Ekroos K, Kauhanen D, Simolin H, Seierstad T, Berge V, et al. Molecular lipid species in urinary exosomes as potential prostate cancer biomarkers. *Eur J Cancer*. 2017;70:122–32. <https://doi.org/10.1016/j.ejca.2016.10.011>.
47. Hough KP, Wilson LS, Trevor JL, Strenkowski JG, Maina N, Kim YI, et al. Unique lipid signatures of extracellular vesicles from the airways of asthmatics. *Sci Rep*. 2018;8(1):10340. <https://doi.org/10.1038/s41598-018-28655-9>.
48. Brzozowski JS, Jankowski H, Bond DR, McCague SB, Munro BR, Predebon MJ, et al. Lipidomic profiling of extracellular vesicles derived from prostate and prostate cancer cell lines. *Lipids Health Dis*. 2018;17(1):211. <https://doi.org/10.1186/s12944-018-0854-x>.
49. Cheng L, Zhang K, Qing Y, Li D, Cui M, Jin P, et al. Proteomic and lipidomic analysis of exosomes derived from ovarian cancer cells and ovarian surface epithelial cells. *J Ovarian Res*. 2020;13(1):9. <https://doi.org/10.1186/s13048-020-0609-y>.
50. Cheng J, Meng J, Zhu L, Peng Y. Exosomal noncoding RNAs in glioma: biological functions and potential clinical applications. *Mol Cancer*. 2020;19(1):66. <https://doi.org/10.1186/s12943-020-01189-3>.
51. Zhou X, Xie F, Wang L, Zhang L, Zhang S, Fang M, et al. The function and clinical application of extracellular vesicles in innate immune regulation. *Cell Mol Immunol*. 2020;17(4):323–34. <https://doi.org/10.1038/s41423-020-0391-1>.
52. Mills J, Capece M, Cocucci E, Tessari A, Palmieri D. Cancer-derived extracellular vesicle-associated microRNAs in intercellular communication: one cell's trash is another cell's treasure. *Int J Mol Sci*. 2019;20(24). <https://doi.org/10.3390/ijms20246109>.
53. Turchinovich A, Drapkina O, Tonevitsky A. Transcriptome of extracellular vesicles: state-of-the-art. *Front Immunol*. 2019;10:202. <https://doi.org/10.3389/fimmu.2019.00202>.
54. Pezzuto F, Buonaguro L, Buonaguro FM, Tornesello ML. The role of circulating free dna and microRNA in non-invasive diagnosis of HBV- and HCV-related hepatocellular carcinoma. *Int J Mol Sci*. 2018;19(4). doi: <https://doi.org/10.3390/ijms19041007>.
55. Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, Liebler DC, Ping J, Liu Q, Evans R, Fissell WH, Patton JG, Rome LH, Burnette DT, Coffey RJ. Reassessment of exosome composition. *Cell*. 2019;177(2):428–445 e18. doi: <https://doi.org/10.1016/j.cell.2019.02.029>.
56. Halvaei S, Daryani S, Eslami SZ, Samadi T, Jafarbeik-iravani N, Bakhshayesh TO, et al. Exosomes in cancer liquid biopsy: a focus on breast cancer. *Mol Ther Nucleic Acids*. 2018;10:131–41. <https://doi.org/10.1016/j.omtn.2017.11.014>.
57. Tai YL, Chu PY, Lee BH, Chen KC, Yang CY, Kuo WH, et al. Basics and applications of tumor-derived extracellular vesicles. *J Biomed Sci*. 2019;26(1):35. <https://doi.org/10.1186/s12929-019-0533-x>.
58. Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borrás FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles*. 2015;4:27066. <https://doi.org/10.3402/jev.v4.27066>.
59. Schwarzenbach H, Gahan PB. MicroRNA shuttle from cell-to-cell by exosomes and its impact in cancer. *Noncoding RNA*. 2019;5(1). <https://doi.org/10.3390/ncrna5010028>.
60. Bhome R, Del Vecchio F, Lee GH, Bullock MD, Primrose JN, Sayan AE, et al. Exosomal microRNAs (exomiRs): small molecules with a big role in cancer. *Cancer Lett*. 2018;420:228–35. <https://doi.org/10.1016/j.canlet.2018.02.002>.
61. Fabbri M, Paone A, Calore F, Galli R, Croce CM. A new role for microRNAs, as ligands of Toll-like receptors. *RNA Biol*. 2013;10(2):169–74. <https://doi.org/10.4161/ma.23144>.
62. Yang M, Chen J, Su F, Yu B, Su F, Lin L, et al. Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells. *Mol Cancer*. 2011;10:117. <https://doi.org/10.1186/1476-4598-10-117>.
63. Berchem G, Noman MZ, Bosseler M, Paggetti J, Baconnais S, Le Cam E, et al. Hypoxic tumor-derived microvesicles negatively regulate NK cell function by a mechanism involving

- TGF-beta and miR23a transfer. *Oncoimmunology*. 2016;5(4):e1062968. <https://doi.org/10.1080/2162402X.2015.1062968>.
64. Ding G, Zhou L, Qian Y, Fu M, Chen J, Chen J, et al. Pancreatic cancer-derived exosomes transfer miRNAs to dendritic cells and inhibit RFXAP expression via miR-212-3p. *Oncotarget*. 2015;6(30):29877–88. <https://doi.org/10.18632/oncotarget.4924>.
 65. Zhou M, Chen J, Zhou L, Chen W, Ding G, Cao L. Pancreatic cancer derived exosomes regulate the expression of TLR4 in dendritic cells via miR-203. *Cell Immunol*. 2014;292(1–2):65–9. <https://doi.org/10.1016/j.cellimm.2014.09.004>.
 66. Ye SB, Zhang H, Cai TT, Liu YN, Ni JJ, He J, et al. Exosomal miR-24-3p impedes T-cell function by targeting FGF11 and serves as a potential prognostic biomarker for nasopharyngeal carcinoma. *J Pathol*. 2016;240(3):329–40. <https://doi.org/10.1002/path.4781>.
 67. Cheng Z, Ma R, Tan W, Zhang L. MiR-152 suppresses the proliferation and invasion of NSCLC cells by inhibiting FGF2. *Exp Mol Med*. 2014;46:e112. <https://doi.org/10.1038/emmm.2014.51>.
 68. Alfonsi R, Grassi L, Signore M, Bonci D. The double face of exosome-carried microRNAs in cancer immunomodulation. *Int J Mol Sci*. 2018;19(4). <https://doi.org/10.3390/ijms19041183>.
 69. Nakano T, Chen IH, Wang CC, Chen PJ, Tseng HP, Huang KT, et al. Circulating exosomal miR-92b: its role for cancer immunoediting and clinical value for prediction of posttransplant hepatocellular carcinoma recurrence. *Am J Transplant*. 2019;19(12):3250–62. <https://doi.org/10.1111/ajt.15490>.
 70. Ying X, Wu Q, Wu X, Zhu Q, Wang X, Jiang L, et al. Epithelial ovarian cancer-secreted exosomal miR-222-3p induces polarization of tumor-associated macrophages. *Oncotarget*. 2016;7(28):43076–87. <https://doi.org/10.18632/oncotarget.9246>.
 71. Czernek L, Duchler M. Functions of cancer-derived extracellular vesicles in immunosuppression. *Arch Immunol Ther Exp (Warsz)*. 2017;65(4):311–23. <https://doi.org/10.1007/s00005-016-0453-3>.
 72. Su MW, Yu SL, Lin WC, Tsai CH, Chen PH, Lee YL. Smoking-related microRNAs and mRNAs in human peripheral blood mononuclear cells. *Toxicol Appl Pharmacol*. 2016;305:169–75. <https://doi.org/10.1016/j.taap.2016.06.020>.
 73. Yin Y, Cai X, Chen X, Liang H, Zhang Y, Li J, et al. Tumor-secreted miR-214 induces regulatory T cells: a major link between immune evasion and tumor growth. *Cell Res*. 2014;24(10):1164–80. <https://doi.org/10.1038/cr.2014.121>.
 74. Nowak M, Klink M. The role of tumor-associated macrophages in the progression and chemoresistance of ovarian cancer. *Cells*. 2020;9(5). <https://doi.org/10.3390/cells9051299>.
 75. Cooks T, Pateras IS, Jenkins LM, Patel KM, Robles AI, Morris J, et al. Mutant p53 cancers reprogram macrophages to tumor supporting macrophages via exosomal miR-1246. *Nat Commun*. 2018;9(1):771. <https://doi.org/10.1038/s41467-018-03224-w>.
 76. Eichmüller SB, Osen W, Mandelboim O, Seliger B. Immune modulatory microRNAs involved in tumor attack and tumor immune escape. *J Natl Cancer Inst*. 2017;109(10). <https://doi.org/10.1093/jnci/djx034>.
 77. Jia L, Xi Q, Wang H, Zhang Z, Liu H, Cheng Y, et al. miR-142-5p regulates tumor cell PD-L1 expression and enhances anti-tumor immunity. *Biochem Biophys Res Commun*. 2017;488(2):425–31. <https://doi.org/10.1016/j.bbrc.2017.05.074>.
 78. Jablonska J, Pietrowska M, Ludwig S, Lang S, Thakur BK. Challenges in the isolation and proteomic analysis of cancer exosomes-implications for translational research. *Proteomes*. 2019;7(2). <https://doi.org/10.3390/proteomes7020022>.
 79. Fernando MR, Jiang C, Krzyzanowski GD, Ryan WL. New evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes. *PLoS One*. 2017;12(8):e0183915. <https://doi.org/10.1371/journal.pone.0183915>.
 80. Guescini M, Guidolin D, Vallorani L, Casadei L, Gioacchini AM, Tibollo P, et al. C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction. *Exp Cell Res*. 2010;316(12):1977–84. <https://doi.org/10.1016/j.yexcr.2010.04.006>.
 81. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res*. 2014;24(6):766–9. <https://doi.org/10.1038/cr.2014.44>.
 82. Balaj L, Lessard R, Dai L, Cho YJ, Pomeroy SL, Breakefield XO, et al. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat Commun*. 2011;2:180. <https://doi.org/10.1038/ncomms1180>.
 83. Taghikhani A, Farzaneh F, Sharifzad F, Mardpour S, Ebrahimi M, Hassan ZM. Engineered tumor-derived extracellular vesicles: potentials in cancer immunotherapy. *Front Immunol*. 2020;11:221. <https://doi.org/10.3389/fimmu.2020.00221>.
 84. Kurywchak P, Tavormina J, Kalluri R. The emerging roles of exosomes in the modulation of immune responses in cancer. *Genome Med*. 2018;10(1):23. <https://doi.org/10.1186/s13073-018-0535-4>.
 85. Lian Q, Xu J, Yan S, Huang M, Ding H, Sun X, et al. Chemotherapy-induced intestinal inflammatory responses are mediated by exosome secretion of double-strand DNA via AIM2 inflammasome activation. *Cell Res*. 2017;27(6):784–800. <https://doi.org/10.1038/cr.2017.54>.
 86. Kitai Y, Kawasaki T, Sueyoshi T, Kobiyama K, Ishii KJ, Zou J, et al. DNA-containing exosomes derived from cancer cells treated with topotecan activate a STING- dependent pathway and reinforce antitumor immunity. *J Immunol*. 2017;198(4):1649–59. <https://doi.org/10.4049/jimmunol.1601694>.
 87. Maia J, Caja S, Strano Moraes MC, Couto N, Costa-Silva B. Exosome-based cell-cell communication in the tumor micro-environment. *Front Cell Dev Biol*. 2018;6:18. <https://doi.org/10.3389/fcell.2018.00018>.
 88. Maheshwari S, Singh AK, Arya RK, Pandey D, Singh A, Datta D. Exosomes: emerging players of intercellular communication in tumor microenvironment. *Discoveries (Craiova)*. 2014;2(3):e26. <https://doi.org/10.15190/d.2014.18>.
 89. Gao D, Jiang L. Exosomes in cancer therapy: a novel experimental strategy. *Am J Cancer Res*. 2018;8(11):2165–75.
 90. Lindenberg MFS, Stoorvogel W. Antigen presentation by extracellular vesicles from professional antigen-presenting cells. *Annu Rev Immunol*. 2018;36:435–59. <https://doi.org/10.1146/annurev-immunol-041015-055700>.
 91. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol*. 2014;14(3):195–208. <https://doi.org/10.1038/nri3622>.
 92. Lu M, Huang B, Hanash SM, Onuchic JN, Ben-Jacob E. Modeling putative therapeutic implications of exosome exchange between tumor and immune cells. *Proc Natl Acad Sci U S A*. 2014;111(40):E4165–74. <https://doi.org/10.1073/pnas.1416745111>.
 93. Ning Y, Shen K, Wu Q, Sun X, Bai Y, Xie Y, et al. Tumor exosomes block dendritic cells maturation to decrease the T cell immune response. *Immunol Lett*. 2018;199:36–43. <https://doi.org/10.1016/j.imlet.2018.05.002>.
 94. Shen Y, Guo D, Weng L, Wang S, Ma Z, Yang Y, et al. Tumor-derived exosomes educate dendritic cells to promote tumor metastasis via HSP72/HSP105-TLR2/TLR4 pathway. *Oncoimmunology*. 2017;6(12):e1362527. <https://doi.org/10.1080/2162402X.2017.1362527>.
 95. Batista IA, Melo SA. Exosomes and the future of immunotherapy in pancreatic cancer. *Int J Mol Sci*. 2019;20(3). <https://doi.org/10.3390/ijms20030567>.
 96. Tung SL, Fanelli G, Matthews RI, Bazoer J, Letizia M, Vizcay-Barrena G, et al. Regulatory T cell extracellular vesicles modify T-effector cell cytokine production and protect against human skin allograft damage. *Front Cell Dev Biol*. 2020;8:317. <https://doi.org/10.3389/fcell.2020.00317>.
 97. Tung SL, Boardman DA, Sen M, Letizia M, Peng Q, Cianci N, et al. Regulatory T cell- derived extracellular vesicles modify dendritic cell function. *Sci Rep*. 2018;8(1):6065. <https://doi.org/10.1038/s41598-018-24531-8>.
 98. Cheng H, Wang Z, Fu L, Xu T. Macrophage polarization in the development and progression of ovarian cancers: an overview. *Front Oncol*. 2019;9:421. <https://doi.org/10.3389/fonc.2019.00421>.

99. Baig MS, Roy A, Rajpoot S, Liu D, Savai R, Banerjee S, et al. Tumor-derived exosomes in the regulation of macrophage polarization. *Inflamm Res*. 2020;69(5):435–51. <https://doi.org/10.1007/s00011-020-01318-0>.
100. Smith HA, Kang Y. The metastasis-promoting roles of tumor-associated immune cells. *J Mol Med (Berl)*. 2013;91(4):411–29. <https://doi.org/10.1007/s00109-013-1021-5>.
101. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol*. 2017;14(7):399–416. <https://doi.org/10.1038/nrclinonc.2016.217>.
102. Yang M, McKay D, Pollard JW, Lewis CE. Diverse functions of macrophages in different tumor microenvironments. *Cancer Res*. 2018;78(19):5492–503. <https://doi.org/10.1158/0008-5472.CAN-18-1367>.
103. Ran S, Montgomery KE. Macrophage-mediated lymphangiogenesis: the emerging role of macrophages as lymphatic endothelial progenitors. *Cancers (Basel)*. 2012;4(3):618–57. <https://doi.org/10.3390/cancers4030618>.
104. Meng W, Hao Y, He C, Li L, Zhu G. Exosome-orchestrated hypoxic tumor microenvironment. *Mol Cancer*. 2019;18(1):57. <https://doi.org/10.1186/s12943-019-0982-6>.
105. Zhou J, Li X, Wu X, Zhang T, Zhu Q, Wang X, et al. Exosomes released from tumor-associated macrophages transfer miRNAs that induce a Treg/Th17 cell imbalance in epithelial ovarian cancer. *Cancer Immunol Res*. 2018;6(12):1578–92. <https://doi.org/10.1158/2326-6066.CIR-17-0479>.
106. Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol*. 2002;168(7):3235–41. <https://doi.org/10.4049/jimmunol.168.7.3235>.
107. Wahlgren J, Karlson Tde L, Glader P, Telemo E, Valadi H. Activated human T cells secrete exosomes that participate in IL-2 mediated immune response signaling. *PLoS One*. 2012;7(11):e49723. <https://doi.org/10.1371/journal.pone.0049723>.
108. Zhang L, Yu D. Exosomes in cancer development, metastasis, and immunity. *Biochim Biophys Acta Rev Cancer*. 2019;1871(2):455–68. <https://doi.org/10.1016/j.bbcan.2019.04.004>.
109. Cai Z, Yang F, Yu L, Yu Z, Jiang L, Wang Q, et al. Activated T cell exosomes promote tumor invasion via Fas signaling pathway. *J Immunol*. 2012;188(12):5954–61. <https://doi.org/10.4049/jimmunol.1103466>.
110. Srivastava A, Babu A, Filant J, Moxley KM, Ruskin R, Dhanasekaran D, et al. Exploitation of exosomes as Nanocarriers for gene-, chemo-, and immune-therapy of cancer. *J Biomed Nanotechnol*. 2016;12(6):1159–73. <https://doi.org/10.1166/jbn.2016.2205>.
111. Kim MS, Haney MJ, Zhao Y, Mahajan V, Deygen I, Klyachko NL, et al. Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine*. 2016;12(3):655–64. <https://doi.org/10.1016/j.nano.2015.10.012>.
112. Yong T, Zhang X, Bie N, Zhang H, Zhang X, Li F, et al. Tumor exosome-based nanoparticles are efficient drug carriers for chemotherapy. *Nat Commun*. 2019;10(1):3838. <https://doi.org/10.1038/s41467-019-11718-4>.
113. Srivastava A, Amreddy N, Razaq M, Towner R, Zhao YD, Ahmed RA, et al. Exosomes as theranostics for lung cancer. *Adv Cancer Res*. 2018;139:1–33. <https://doi.org/10.1016/bs.acr.2018.04.001>.
114. Srivastava A, Moxley K, Ruskin R, Dhanasekaran DN, Zhao YD, Ramesh R. A non-invasive liquid biopsy screening of urine-derived exosomes for miRNAs as biomarkers in endometrial cancer patients. *AAPS J*. 2018;20(5):82. <https://doi.org/10.1208/s12248-018-0220-y>.
115. Chinnappan M, Srivastava A, Amreddy N, Razaq M, Pareek V, Ahmed R, et al. Exosomes as drug delivery vehicle and contributor of resistance to anticancer drugs. *Cancer Lett*. 2020;486:18–28. <https://doi.org/10.1016/j.canlet.2020.05.004>.
116. Tan A, Rajadas J, Seifalian AM. Exosomes as nano-theranostic delivery platforms for gene therapy. *Adv Drug Deliv Rev*. 2013;65(3):357–67. <https://doi.org/10.1016/j.addr.2012.06.014>.
117. He C, Zheng S, Luo Y, Wang B. Exosome theranostics: biology and translational medicine. *Theranostics*. 2018;8(1):237–55. <https://doi.org/10.7150/thno.21945>.
118. Zhan Q, Yi K, Qi H, Li S, Li X, Wang Q, et al. Engineering blood exosomes for tumor-targeting efficient gene/chemo combination therapy. *Theranostics*. 2020;10(17):7889–905. <https://doi.org/10.7150/thno.45028>.
119. Tian Y, Li S, Song J, Ji T, Zhu M, Anderson GJ, et al. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials*. 2014;35(7):2383–90. <https://doi.org/10.1016/j.biomaterials.2013.11.083>.
120. Kamerkar S, LeBleu VS, Sugimoto H, Yang S, Ruivo CF, Melo SA, et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature*. 2017;546(7659):498–503. <https://doi.org/10.1038/nature22341>.
121. Naseri M, Bozorgmehr M, Zoller M, Ranaei Pirmardan E, Majid Z. Tumor-derived exosomes: the next generation of promising cell-free vaccines in cancer immunotherapy. *Oncoimmunology*. 2020;9(1):1779991. <https://doi.org/10.1080/2162402X.2020.1779991>.
122. Nikfarjam S, Rezaie J, Kashanchi F, Jafari R. Dexosomes as a cell-free vaccine for cancer immunotherapy. *J Exp Clin Cancer Res*. 2020;39(1):258. <https://doi.org/10.1186/s13046-020-01781-x>.
123. Hiltbrunner S, Larssen P, Eldh M, Martinez-Bravo MJ, Wagner AK, Karlsson MC, et al. Exosomal cancer immunotherapy is independent of MHC molecules on exosomes. *Oncotarget*. 2016;7(25):38707–17. <https://doi.org/10.18632/oncotarget.9585>.
124. Forsberg MH, Kink JA, Hematti P, Capitini CM. Mesenchymal stromal cells and exosomes: progress and challenges. *Front Cell Dev Biol*. 2020;8:665. <https://doi.org/10.3389/fcell.2020.00665>.
125. Cheng Y, Zeng Q, Han Q, Xia W. Effect of pH, temperature and freezing-thawing on quantity changes and cellular uptake of exosomes. *Protein Cell*. 2019;10(4):295–9. <https://doi.org/10.1007/s13238-018-0529-4>.
126. Jeyaram A, Jay SM. Preservation and storage stability of extracellular vesicles for therapeutic applications. *AAPS J*. 2017;20(1):1. <https://doi.org/10.1208/s12248-017-0160-y>.
127. Reclusa P, Taverna S, Pucci M, Durendez E, Calabuig S, Manca P, et al. Exosomes as diagnostic and predictive biomarkers in lung cancer. *J Thorac Dis*. 2017;9(Suppl 13):S1373–S82. <https://doi.org/10.21037/jtd.2017.10.67>.
128. Revelo AE, Martin A, Velasquez R, Kulandaisamy PC, Bustamante J, Keshishyan S, et al. Liquid biopsy for lung cancers: an update on recent developments. *Ann Transl Med*. 2019;7(15):349. doi: <https://doi.org/10.21037/atm.2019.03.28>.
129. Rodriguez M, Silva J, Lopez-Alfonso A, Lopez-Muniz MB, Pena C, Dominguez G, et al. Different exosome cargo from plasma/bronchoalveolar lavage in non-small-cell lung cancer. *Genes Chromosomes Cancer*. 2014;53(9):713–24. <https://doi.org/10.1002/gcc.22181>.
130. Lin J, Wang Y, Zou YQ, Chen X, Huang B, Liu J, et al. Differential miRNA expression in pleural effusions derived from extracellular vesicles of patients with lung cancer, pulmonary tuberculosis, or pneumonia. *Tumour Biol*. 2016;37:15835–45. <https://doi.org/10.1007/s13277-016-5410-6>.
131. Hydbring P, De Petris L, Zhang Y, Branden E, Koyi H, Novak M, et al. Exosomal RNA- profiling of pleural effusions identifies adenocarcinoma patients through elevated miR-200 and LCN2 expression. *Lung Cancer*. 2018;124:45–52. <https://doi.org/10.1016/j.lungcan.2018.07.018>.
132. Li Y, Zhang Y, Qiu F, Qiu Z. Proteomic identification of exosomal LRG1: a potential urinary biomarker for detecting NSCLC. *Electrophoresis*. 2011;32(15):1976–83. <https://doi.org/10.1002/elps.201000598>.
133. Lin SY, Chang CH, Wu HC, Lin CC, Chang KP, Yang CR, et al. Proteomic profiling of urinary exosomes identifies alpha 1-antitrypsin and H2B1K as diagnostic and prognostic biomarkers for urothelial carcinoma. *Sci Rep*. 2016;6:34446. <https://doi.org/10.1038/srep34446>.
134. Long JD, Sullivan TB, Humphrey J, Logvinenko T, Summerhayes KA, Kozinn S, et al. A non-invasive miRNA

- based assay to detect bladder cancer in cell-free urine. *Am J Transl Res*. 2015;7(11):2500–9.
135. Fujita K, Nonomura N. Urinary biomarkers of prostate cancer. *Int J Urol*. 2018;25(9):770–9. <https://doi.org/10.1111/iju.13734>.
 136. McKiernan J, Donovan MJ, O'Neill V, Bentink S, Noerholm M, Belzer S, et al. A novel urine exosome gene expression assay to predict high-grade prostate cancer at initial biopsy. *JAMA Oncol*. 2016;2(7):882–9. <https://doi.org/10.1001/jamaoncol.2016.0097>.
 137. Overbye A, Skotland T, Koehler CJ, Thiede B, Seierstad T, Berge V, et al. Identification of prostate cancer biomarkers in urinary exosomes. *Oncotarget*. 2015;6(30):30357–76. <https://doi.org/10.18632/oncotarget.4851>.
 138. Rodriguez M, Bajo-Santos C, Hessvik NP, Lorenz S, Fromm B, Berge V, et al. Identification of non-invasive miRNAs biomarkers for prostate cancer by deep sequencing analysis of urinary exosomes. *Mol Cancer*. 2017;16(1):156. <https://doi.org/10.1186/s12943-017-0726-4>.
 139. Raimondo F, Morosi L, Corbetta S, Chinello C, Brambilla P, Della Mina P, et al. Differential protein profiling of renal cell carcinoma urinary exosomes. *Mol Biosyst*. 2013;9(6):1220–33. <https://doi.org/10.1039/c3mb25582d>.
 140. Sauter ER, Reidy D. How exosomes in human breast milk may influence breast cancer risk. *Transl Cancer Res*. 2017;6:S1384–S8.
 141. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev*. 2002;12(1):22–9. [https://doi.org/10.1016/s0959-437x\(01\)00259-3](https://doi.org/10.1016/s0959-437x(01)00259-3).
 142. Rontogianni S, Synadaki E, Li B, Liefwaard MC, Lips EH, Wesseling J, et al. Proteomic profiling of extracellular vesicles allows for human breast cancer subtyping. *Commun Biol*. 2019;2:325. <https://doi.org/10.1038/s42003-019-0570-8>.
 143. Wang Z, Li F, Rufo J, Chen C, Yang S, Li L, et al. Acoustofluidic salivary exosome isolation: a liquid biopsy compatible approach for human papillomavirus-associated oropharyngeal cancer detection. *J Mol Diagn*. 2020;22(1):50–9. <https://doi.org/10.1016/j.jmoldx.2019.08.004>.
 144. Nonaka T, Wong DTW. Saliva-Exosomics in cancer: molecular characterization of cancer-derived exosomes in saliva. *Enzymes*. 2017;42:125–51. <https://doi.org/10.1016/bs.enz.2017.08.002>.
 145. Perez-Callejo D, Romero A, Provencio M, Torrente M. Liquid biopsy based biomarkers in non-small cell lung cancer for diagnosis and treatment monitoring. *Transl Lung Cancer Res*. 2016;5(5):455–65. <https://doi.org/10.21037/tlcr.2016.10.07>.
 146. De Rubis G, Rajeev Krishnan S, Bebawy M. Liquid biopsies in cancer diagnosis, monitoring, and prognosis. *Trends Pharmacol Sci*. 2019;40(3):172–86. <https://doi.org/10.1016/j.tips.2019.01.006>.
 147. Li X, Corbett AL, Taatizadeh E, Tasnim N, Little JP, Garnis C, et al. Challenges and opportunities in exosome research: perspectives from biology, engineering, and cancer therapy. *APL Bioeng*. 2019;3(1):011503. <https://doi.org/10.1063/1.5087122>.
 148. Ludwig N, Whiteside TL, Reichert TE. Challenges in exosome isolation and analysis in health and disease. *Int J Mol Sci*. 2019;20(19). <https://doi.org/10.3390/ijms20194684>.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Exosomes as drug delivery vehicle and contributor of resistance to anticancer drugs

Mahendran Chinnappan^{a,d}, Akhil Srivastava^{a,d}, Narsireddy Amreddy^{a,d}, Mohammad Razaq^{b,d}, Vipul Pareek^{b,d}, Rebaz Ahmed^{a,e}, Meghna Mehta^{c,d}, Jo Elle Peterson^{a,d}, Anupama Munshi^{c,d}, Rajagopal Ramesh (PhD)^{a,d,e,*}

^a Department of Pathology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA

^b Department of Medicine, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA

^c Department of Radiation Oncology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA

^d Stephenson Cancer Center, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA

^e Graduate Program in Biomedical Sciences, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA

ARTICLE INFO

Keywords:

Extracellular vesicles
Nanocarriers
Tumor-derived exosome
Tangential flow filtration
Tumor-microenvironment

ABSTRACT

Exosomes are small membranous vesicles implicated in intercellular signalling. Through their uncanny ability to carry and deliver donor cellular cargo (biomolecules) to target cells, they exert a profound effect on the regular functioning of healthy cells and play a significant role in pathogenesis and progression of several diseases, including cancer. The composition and number of endogenously circulating exosomes frequently vary, which is often reflective of the pathophysiological status of the cell. Applicability of exosomes derived from normal cells as a drug carrier with or without modifying their intraluminal and surface components are generally tested. Conversely, exosomes also are reported to contribute to resistance towards several anti-cancer therapies. Therefore, it is necessary to carefully evaluate the role of exosomes in cancer progression, resistance and the potential use of exosomes as a delivery vehicle of cancer therapeutics. In this review, we summarize the recent advancements in the exploitation of exosomes as a drug delivery vehicle. We also discuss the role of exosomes in conferring resistance to anti-cancer therapeutics. While this review is focused on cancer, the exosome-based drug delivery and resistance is also applicable to other human diseases.

1. Introduction

Efficacy of anticancer drugs is limited by poor drug solubility, short half-life, and inefficient drug delivery to the tumor cells resulting in drug resistance, significant local and systemic toxicity [1]. To override these clinical challenges, testing of various nanoformulations (liposome, biodegradable polymers, microspheres, hydrogels, etc.) has been undertaken. While each of the formulations has shown promise in preclinical studies, their clinical translation has been hampered by off-target effects and toxicity, albeit lower than that observed with chemotherapeutic drugs. Therefore, new and improved nanocarriers having desirable properties such as biocompatibility, ability to carry hydrophobic and hydrophilic drugs, have longer half-life *in vivo* are warranted. Ultimately, these nanocarriers need to efficiently target and deliver anticancer drugs to the tumor cells [2]. In this set up, researchers were attracted to test extracellular vesicles (EVs) including

exosomes as natural drug delivery vehicles for cancer therapy.

EVs are secreted by almost all cell types and play significant roles in intercellular communication [3,4]. EVs are classified into three types; exosomes, microvesicles (MVs), and apoptotic bodies based primarily on their size and pathway of generation. Among them, exosomes have garnered greater attention and are the most widely studied in cancer biology and therapy. Exosomes are 30–150 nm sized vesicles generated by the endocytic process and secreted by the fusion of multi-vesicular bodies (MVBs) with the plasma membrane [5]. Exosomes are enriched with distinct membrane proteins called tetraspanin proteins such as CD9, CD37, CD63, CD81 and CD82. Additionally, proteins such as the tumor susceptibility gene (TSG)-101 and Alix of exosome sorting complex required for transport (ESCRT) are also routinely used as markers to characterize exosomes [6].

The ever-increasing body of literature suggests that exosomes are involved in the regulation of normal functioning of cells. They have also

* Corresponding author. Department of Pathology, Stanton L. Young Biomedical Research Center, Suite 1403, 975 NE, 10th Street, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA.

E-mail address: rajagopal-ramesh@ouhsc.edu (R. Ramesh).

<https://doi.org/10.1016/j.canlet.2020.05.004>

Received 1 March 2020; Received in revised form 21 April 2020; Accepted 4 May 2020

0304-3835/ © 2020 Elsevier B.V. All rights reserved.

been reported to be involved in the pathology of several human diseases that include cardiovascular, neurodegenerative, autoimmune diseases and cancer [7–10]. The exosome composition (lipids, nucleic acids, metabolites, proteins, and peptides) reflects its cellular origin. However, the composition of exosomes from diseased cells is dysregulated compared to normal healthy cells [11]. Efforts to characterize the exosomal composition necessitated the emergence of databases like ExoCarta, Vesiclepedia, and EV miRNA which document and present the relevant information from various publications [12–14]. A growing number of reports have demonstrated that exosomes also contribute to resistance against anticancer drugs via autocrine and paracrine mode of communication. These exosome mediated resistance mechanisms eventually result in therapy failure and disease recurrence [15].

In this short review, we will first discuss the accumulating knowledge on the strategies applied to use exosomes as drug delivery systems and in targeted delivery of therapeutics; and in the latter part about their role in carting factors that contribute to resistance to a variety of anti-cancer therapies.

2. Exosomes in cancer drug delivery

Exosomes are suitable as drug delivery vehicles due to their small size, biocompatibility, and reduced toxicity in comparison with synthetic nanoformulations such as liposomes, dendrimers, and polymers [2]. Additionally, delivery of anticancer drugs contained in exosomes demonstrated improved pharmacokinetic and pharmacodynamic properties and enhanced anticancer activity *in vivo* compared to free drug [16]. Similarly, loading of therapeutic nucleic acids such as small interfering (si) RNA into exosomes protects the RNA from nucleases and increases cellular uptake and therapeutic effect compared to free RNAs that are rapidly degraded by nucleases [17,18]. Above all, exosomes have the ability to cross the blood brain barrier and penetrate deep tissues with improved efficacy compared to that of synthetic nano-carriers [19]. Thus, exosomes are demonstrably an effective and necessary tool for carrying and delivering the anti-cancer therapeutics. In the sections outlined below, we discuss the current status and research strides made in using exosomes as delivery vehicles for cancer treatment.

2.1. The cellular source of exosomes. Does it matter?

Exosomes can serve as a natural and potential carrier for cancer drug delivery. However, exosomes obtained from different biological sources and cell types behave differently *in vivo* thus impacting the anticipated antitumor activity. So, it is imperative to find an appropriate biological or cellular source for exosome isolation with desirable characteristics for use as a drug carrier. For instance, exosomes isolated from macrophages had better doxorubicin loading capacity than did exosomes isolated from pancreatic cancer cells or pancreatic stellate cells [20]. Similarly, autologous exosomes (same cell type) were reported to function with higher potential in drug loading capacity and delivering gemcitabine to pancreatic cells than did heterologous (different cell type) exosomes [21]. Additionally, a study by Emam et al., demonstrated that the autologous exosomes of C26 colorectal cancer cells were taken up more efficiently than the allogeneic exosomes from B16-BL6 melanoma cells in a murine model [22]. Thus, it is evident that exosomes from the same cell type are taken up effectively than the exosomes from another cell type from the same species. However, this is not always a beneficial criterion for selecting the cell source for exosome isolation. Sometimes, exosomes isolated from donor cell that is different from the recipient cell function better as drug delivery vehicle. Indeed, exosomes isolated from certain cell types show organotropism towards certain cancers wherein the exosomes get accumulated selectively in the major cell type of an organ rather than in adjacent cell types. For instance, overexpression of integrin beta 4 (ITG-B4) on the surface of human MDA-MB-231 breast cancer cell line, and its

subsequent interaction with surfactant protein C (SPC+) on the surface of human A549 lung cancer cells are reasoned for the observed lung-tropism of exosomes from breast cancer cells. This finding could be explored for developing a drug delivery vehicle for lung cancer [23].

Studies report tumor-derived exosomes (TDEs) as drug carrier for cancer therapy. The rationale is that TDEs express different kinds of ligands and receptors on their surface that can be utilized to target the actively growing *in situ* tumor. By this approach, surface modification of exosomes for tumor-targeted drug delivery which is the norm for synthetic drug carriers, is avoided. As an example, TDEs loaded with the heat shock protein (HSP)-90 inhibitor geldanamycin exhibited improved antitumor activity compared to the administration of free drug [24]. Similarly, induction of robust cytotoxic T-cell response against lung cancer was observed with tumor-associated exosomes compared to tumor cell lysate suggesting exosomes as better cancer vaccine candidate [25]. Contrary to these reports, studies demonstrate exosomes are notorious in their function and could operate as both pro-tumorigenic and anti-tumorigenic based on the source from which exosomes are derived. For example, exosomes from menstrual mesenchymal stem cells (MenSc) exhibited antiangiogenic properties *in vitro* and *in vivo* while exosomes from bone marrow derived stem cells promoted tumor growth *in vivo* [26]. Adding to the literature, studies from our laboratory have observed exosomes from human lung tumor cells are pro-tumorigenic (*unpublished*) and could be deleterious in their use as drug carriers. Therefore, we have resorted to using exosomes from normal lung fibroblast cells as drug carriers for cancer treatment [27].

Taken together, it is evident that the source of exosomes to be isolated and used for anticancer drug delivery needs to be prudently chosen.

2.2. Methods to obtain a high yield of exosomes

Although, exosomes possess favorable characteristics for drug delivery, producing them in adequate quantity for pre-clinical and clinical trial applications remains a challenge. Conventional methods applied for isolating exosomes include differential ultracentrifugation, ultra-filtration, precipitation, immunoaffinity capture beads-based methods. Application of these methods, while improving the quality of exosomes, is limited by the low yield and occasionally contains contaminants including microvesicles in the preparation [28,29]. Recently, acoustic and fluidic device-based purification methods were described which again focus primarily in improving the quality [30]. Application of tangential flow filtration (TFF) has also been shown to improve the quality and more importantly useful for concentrating the exosome number. Since cell culture supernatant is often the common source of exosomes, multiple methods were described to increase the secretion of exosomes from cell culture into the medium thereby the above mentioned purification methods can be exploited efficiently to achieve adequate quantity of exosomes. For example, application of chemical, electrical stimulation, hypoxia and silencing of specific endo-lysosomal traffic proteins have shown to enhance exosome production and secretion from cells (Fig. 1). Large-scale exosome production using laboratory and industrial grade bioreactors are also being evaluated for clinical testing.

In the case of chemical induction of exosome production, paraformaldehyde and dithiothreitol (DTT)-mediated blocking of sulfhydryl group on the receptor proteins increased exosome blebbing and quantity [31]. Exosomes prepared this way showed improved release of Dox to the recipient cells leading to reduced tumor growth in mice compared to mice receiving liposome preparations. Similarly, calcium phosphate (CaP) treatment of THP-1 monocytes like cells or RAW264.7 macrophage cells increased the production of exosomes [32]. Along these lines, inhibition of endo-lysosomal trafficking by silencing the N-Myc downstream regulated-1 (*NDRG1*) gene increased exosome secretion [33]. On the other hand, exosomes secreted from stress induced cells were able to facilitate antitumor function. As such, hepatocellular

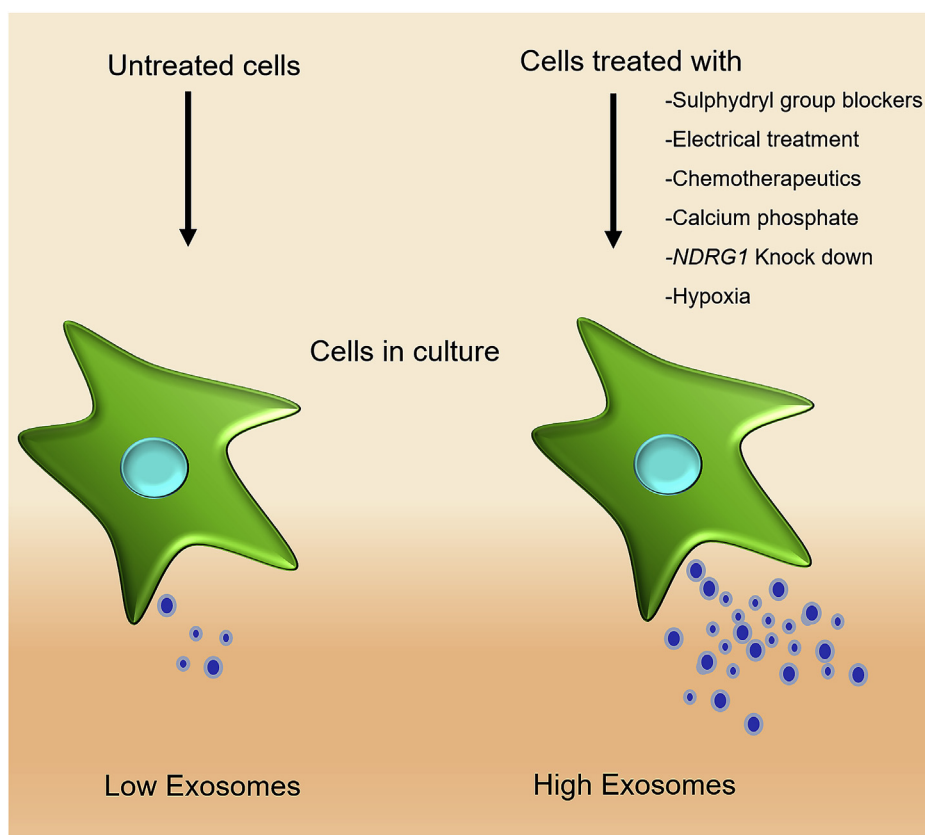


Fig. 1. Strategies to induce exosome induction from cells. Exosome production can be increased by subjecting the cells to electrical signal; knockdown of endolysosomal gene (*NDRG1*); treating cells with calcium phosphate; membrane protein sulphydryl group blockers; chemotherapy agents; and hypoxia.

carcinoma (HCC) cells that are resistant to chemotherapeutic drugs secreted exosomes with increased levels of Hsp-60, -70 and -90 proteins. These exosomes eventually bolstered the function of natural killer (NK) cells thereby boosting immune surveillance [34]. Similarly, hypoxia-induced stress is known to activate exosome production and secretion by multiple cell lines *in vitro* that can be further explored for their applicability as drug delivery vehicles. However, it is to be noted that hypoxia induced exosomes carry proteins such as protein-lysine-6-oxidase (LOX), thrombospondin-1 (TSP-1) and vascular derived endothelial growth factor (VEGF) that are known to play a role in metastasis, invasion, angiogenesis, and tumor progression. Hence, phenotypic outcome of these hypoxia associated exosomes on the recipient cells needs to be determined before being used as a drug carrier [35–38]. Another novel approach used electrical signal to induce exosomes in cell culture. Minimal electric treatment (0.34 mA/cm^2) to murine melanoma and fibroblast cells ended up in yielding more exosomes. This noticeable increase in the production of exosomes operate through the activation of Rho GTPase signalling [39]. These methods while useful in enhancing the exosome production, continue to be inadequate for industrial scale production. To fill this gap, microcarriers and hollow-fiber bioreactors with increased surface area are being developed and tested for producing exosome in large-scale quantities [40,41]. However, these bioreactor-based methods have intrinsic steps that influence and modify the phenotype of the cells over time thereby reducing the quality of the exosomes. For example, interleukin (IL)-2 was reduced in the exosomes from T cells when subjected to agitation at 180 rpm [42]. Thus, it is important to carefully select and optimize the method of large-scale exosome production that ensures the quality of exosomes is maintained without influencing the exosome source adversely.

Ensuring the quality and quantity of exosomes isolated from bodily fluids such as blood and urine is another challenging task. Enrichment

of exosomes with specific receptor protein is explored besides using conventional methods to address this requirement. For example, purification of transferrin receptor (TfR+) containing exosomes from blood using the superparamagnetic nanoparticle cluster method with transferrin (Tf) surface conjugated nanoparticles was shown to result in exosomes with increased biocompatibility and deliverability [43]. Recently, an exosome total isolation chip (ExoTic) was launched that works primarily by filtration to isolate exosomes from bodily fluids and resulted in a 1000-fold increased yield [44]. Altogether, these methods can be used to produce sufficient quantities of exosomes for their subsequent use in the drug delivery applications.

2.3. Methods of exosome preservation with the retention of quality and function

To successfully utilize exosomes as a delivery vehicle it is important to optimize the storage methods for the isolated exosomes. It is necessary because the native exosomes lose quality and functionality during standard storage conditions such as freezing at -80°C and repeated freeze thaw cycles rupture or aggregate the exosomes [45]. Multiple storage conditions were tested to find a suitable method. For instance, Frank et al., studied the enzyme activity of beta-glucuronidase loaded onto exosomes isolated from mesenchymal stem cells, endothelial cells and cancer cells. They stored the exosomes at -80°C , $+4^\circ\text{C}$, room temperature and lyophilized form; of which lyophilization worked most efficiently [46]. Similarly, a recent study demonstrated that exosomes modified with a peptide rich in arginine for enhanced penetration of the cell retained their quality during lyophilization [47]. Furthermore, Charoenviriyakul et al., demonstrated that the lyophilization of B16BL6 melanoma cell-derived exosomes with the cryoprotectant, trehalose, reduces their aggregation during the lyophilization step [48]. However, Akers et al., argued that lyophilization reduced the EV number and

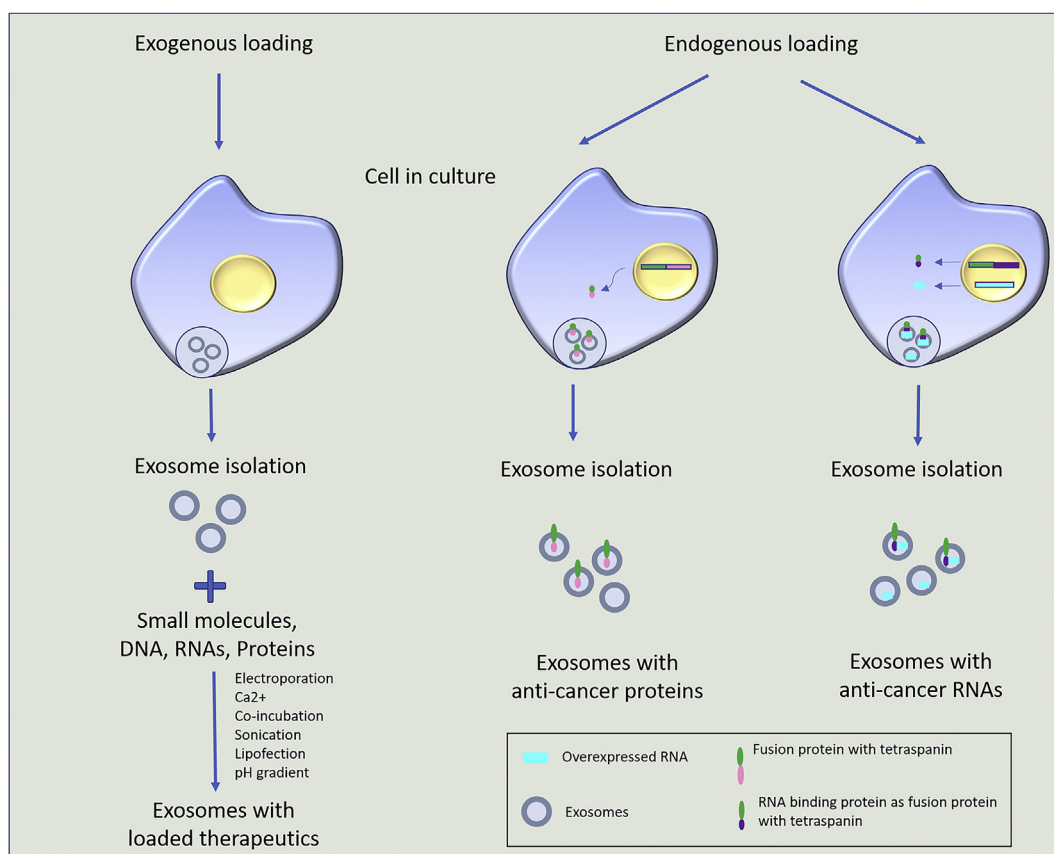


Fig. 2. Method of loading therapeutics in exosomes. In exogenous loading, exosomes are loaded with therapeutic proteins, nucleic acids, and small molecules by application of chemical, electrical and biological methods. Endogenous loading of exosomes is achieved using a plasmid vector based expression of protein as a fusion protein with the tetraspanin protein.

their RNA content when compared to exosome storage at room temperature [45]. Thus, prior consideration of the appropriate method for long-term storage of exosomes specific to a cellular source would increase the beneficial applications.

2.4. Exosome loading with desired content

Once the exosomes are isolated from the cellular or biological source of choice, they are loaded with the therapeutic drug of interest, thereby making them more effective for drug delivery. Different types of synthetic and natural biomolecules, including nucleic acids, lipids, proteins and small chemical drugs are loaded into the exosomes. There are two kinds of strategies followed in the exosomal loading of any content that includes the endogenous and exogenous loading. In the endogenous loading method, overexpression of nucleic acid and/or protein in the source (donor) cells results in the generation of exosomes pre-loaded or enriched with the component of interest (Fig. 2). In the exogenous loading method, exosomes are directly loaded with nucleic acids and small molecular therapeutics using various methods including chemical transfection, electroporation, sonication and co-incubation.

Each of the loading methods have pros and cons of their own. The required method is often decided based on the therapeutic component that is to be loaded into the exosomes. For instance, loading the mRNA transcripts into exosomes often results in a reduced yield than when loading them with short RNAs such as siRNAs and miRNAs. In this scenario, loading the exosomes with mRNA is preferably done through an endogenous method. A recent study utilized a nanoporation method where-in the source cells were transfected with plasmid DNA to express the desired biomolecule and transiently stimulated with an electric signal. This method yielded a 50-fold increase in exosome production

and 1000-fold increase in the exosomes enriched in mRNAs and peptides compared with other standard methods [49].

Efforts to further enhance the endogenous loading of exosomes with desired RNA were also attempted. A recent study described an enhanced RNA packaging into exosomes by overexpressing RNA binding protein, HuR, as a fusion protein with tetraspanins such as CD9 in the host cells used for exosome isolation. Co-overexpression of HuR-CD9 fusion protein with either miR-155 inhibitor RNA or guide RNA (gRNA) for CRISPR/Cas9 resulted in the specific enrichment of these RNAs in the isolated exosomes. Such exosomal systems were demonstrated to be efficient for *in vitro* and *in vivo* gene therapy applications [50]. The endogenous loading of exosomes with the protein of interest is often carried out as a fusion protein along with the surface marker proteins of exosomes. A recent study tested four exosomal membrane localization proteins such as CD63, Palm, PB and CAAX and determined the loading efficiency of fusion proteins. The authors individually expressed these proteins as fusion protein with mCherry reporter protein for tracking of exosome loading. The results showed that CD63 followed by Palm had the highest sorting and packaging of cargo (mCherry) protein in the exosome. Fusion with PB and CAAX led to the expression of the reporter protein mCherry on the plasma membrane rather than in the exosomes [51]. This study revealed a strategy for enhancing endogenous packaging of desired therapeutic proteins into exosomes for achieving maximum treatment outcomes.

To simplify the exosomal loading with desired therapeutic components, researchers developed and successfully demonstrated methods that enabled direct loading of exosomes with therapeutic compounds. For instance, exosomes transfected with miRNA mimics or inhibitors using the modified calcium chloride-based method resulted in an efficient overexpression or depletion of the target miRNAs in the recipient

cells [52]. As an alternative, sonication was also shown to be effective in loading the short nucleic acid sequences into exosomes. For instance, the loading of siRNA against HER2 in exosomes resulted in a significant reduction in HER2 mRNAs in the recipient cells making it a viable option for breast cancer therapy [53]. On the other hand, treatment of SKOV3 ovarian cancer cells with exosomes loaded via electroporation with plasmid DNA expressing sgRNA for poly (ADP-ribose) polymerase-1 (PARP-1) suppressed cell growth and sensitized the cells to cisplatin [54]. Another study reported pH gradient based direct loading of RNAs, especially small RNAs into exosomes [55]. Above all, a simple co-incubation of hydrophobic synthetic siRNAs with exosomes yielded successful encapsulation within exosomes [56]. Thus, exogenous loading of exosomes with different biomolecules offers a rapid production of therapeutic exosomes. However, direct loading of exosomes with siRNA and DNA were also shown to result in inefficient loading and delivery of the contents that became in-effective in the recipient cells [57]. Altogether, direct loading of exosomes with therapeutic content requires further optimization and scale-up methods to make it a viable approach for cancer therapy.

2.5. Surface engineering of exosomes

Exosomes while proven to be useful as anti-cancer drug carrier *in vitro*, its use for delivering anticancer drugs *in vivo* could be limited due to non-specific toxicity and off-target effects similar to that observed with conventional chemodrugs. This necessitates equipping the drug-loaded exosome's surface with tumor-targeted ligands (eg: peptide, antibody, aptamer) for achieving tumor-specific drug delivery and reduced non-specific toxicity. The ligands used are often targeted against receptors that are overexpressed in cancer cells. Such ligand/antibody displaying exosomes were reported to function effectively in suppressing tumor growth since they are taken up by recipient tumor cells through a receptor mediated endocytosis process that avoids endosome encapsulation and trapping [54]. Several strategies for surface modification of the exosome with ligands have been tested. For instance, exosomes obtained from the non-cancerous HEK293T cells were anchored with lipophilic hyaluronic acid (lipHA-hEVs) as a ligand to target CD44 overexpressing MCF7/ADR breast cancer cells to deliver doxorubicin. These lipHA-hEVs were shown to enrich doxorubicin specifically in breast cancer cells thereby reducing the tumor mass by 89% and increasing the animal survival by 50% [58]. Similarly, lipophilic linker mediated surface tagging of exosomes with monoclonal antibodies (mAb) is another common strategy widely endorsed. For example, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine lipophilic linker conjugated with polyethylene glycol (PEG) and SSTR2 mAb was used to modify the exosome surface to deliver FK228 histone deacetylase inhibitor to pancreatic neuroendocrine xenograft model in mouse where the SSTR2 is overexpressed [59]. Another example is the exosomal surface modification with antibodies capable of releasing checkpoint inhibition for improved cancer therapy. Overexpression of CD47 is observed in many cancer cells that enables them to escape from being engulfed by macrophages. Exosomes derived from M1 macrophages anchored on their membrane with dibenzocyclooctyne-modified antibodies against CD47 or SIRP α via a pH responsive benzoic imine bond resulted in the enhanced targeting of tumor cells by macrophages. Notably, this modification stimulated a conversion of M2 pro-tumoral macrophage phenotype to M1 anti-tumoral phenotype characteristics [60,61].

Receptor protein engineering is another strategy for preparing exosomes for targeted delivery. Recently, a novel method was described in HEK293 cells that enabled partitioning of reporter proteins into the inner or outer surface of exosome by utilizing specific locations on the tetraspanin proteins for fusion protein engineering. Such methods immensely allow engineering of exosomes to suit better for the targeted delivery of therapeutics [62]. On these lines, chimeric antigen receptor (CAR)-T therapy is becoming an appealing and promising approach for

cancer treatment. Exosomes obtained from CAR-T cells contain high number of CAR on the surface, which elicits enhanced tumor cell cytotoxicity. These CAR exosomes lack programmed cell death protein 1 (PD-1) expression on their membrane and hence bypass the immunosuppressive mechanisms to which CAR-T cells are subjected. Moreover, CAR exosomes do not show the acute toxicities observed with CAR-T cell therapy. These characteristics make the exosomes derived from CAR-T cells an ideal candidate for anti-cancer therapy [63,64]. Likewise, utilizing the sulfhydryl groups on the exosome membrane proteins for the targeted delivery has also been described in the literature. Engineering the surface of exosomes with functionalized DNA linked to quantum dots (EXO-DNA-QDs) enhances the specific uptake of these exosomes by tumor cells up to 85%. Thus, this method can be another effective strategy for improved tumor targeted delivery [65].

All these methods significantly improve the tumor-targeted drug delivery of the native exosomes and helps reduce the systemic toxicity otherwise observed with free anticancer drugs.

2.6. Exosome hybrids in drug delivery

The composition of native exosomes varies widely and as stated earlier their yield is usually low, thus restricting their potential for translational adoption. To address this, exosome-mimetic nanosystems (EMNs) were produced by sequential extrusion of the cells through multiple filters with downgraded pore size [66]. Such EMNs result in 100 times more yield in nanovesicles and with defined composition. Alternatively, incorporating the liposome technology for generating these EMNs was shown to result in 1000 times more yield in nanovesicles. Liposomes based on various lipids such as DOTAP, POPC, DPPC and PPG are commonly fused with exosomes to produce these EMNs hybrid particles with desired size range of 100 nm. These EMNs can further be improved for an exosome like properties such as tagging them with integrin $\alpha 6 \beta 4$ to enable organotropism [67]. Fusion of membrane proteins or exosomes obtained from red blood cells (RBCs) and leukocytes were also reported to improve these liposomes based EMNs in exerting antitumor activity. Moreover, these EMNs were effective in delivering the benefits of natural exosomes such as improved tumor enrichment, stability, controlled production, and drug loading capacity [68–70].

On the other hand, combining exosomes with nanoparticles has been reported to reduce the immunogenicity associated toxicity of the nanoparticles. For example, Wang et al. [71], used a “cocktail therapy”, that combined exosomes from natural killer cells with their biomimetic core shell nanoparticles. This cocktail therapy efficiently delivered miRNAs to neuroblastoma cells in an *in vivo* model resulting in increased tumor prevention. In another study, exosomes were loaded with gold nanoparticles pre-conjugated with doxorubicin via a pH sensitive hydrazine linker to achieve tumor-specific and controlled release of drug to lung cancer cells [29]. This approach specifically is useful when chemotherapeutics are planned for cancer therapy.

2.7. Exosomes of non-human origin for cancer therapy

It's intriguing to note that exosomes obtained from non-human origin such as from bacteria and plants showed anti-tumor functionality. They were suggested to be useful in cancer therapy mainly due to their reduced toxicity and comparative ease in large scale production. Moreover, these exosomes retained the functionality comparable to the exosomes prepared from animal and human origin. EVs or outer membrane vesicles in the size range of 40 nm obtained from gram-negative and gram-positive bacteria accumulated in the tumor environment and stimulated the production of anti-tumor cytokines, CXCL10 and interferon- γ [72]. Likewise, EVs from a gram-positive bacterium, *Lactobacillus rhamnosus* induced apoptosis in hepatic cancer cells through the regulation of anti-apoptotic Bcl-2 and pro-

Table 1

List of anticancer drugs with reported resistance mechanisms mediated by exosomes.

Drug	Resistance factor	Expression change in resistant cells	Target	Cancer	Reference
1. Oxaliplatin	hsa_circ_0005963	Up	miR-122/PKM2	Colorectal cancer	[118]
2. Palbociclib	Thymidine Kinase 1 (TK1), CDK9	Up	-	Breast cancer	[119]
3. 5-fluorouracil (5-FU)	p-STAT3 and GSTP1	Up	-	Colorectal Cancer	[120]
4. Temozolomide (TMZ)	lncRNA SBF2-AS1	Up	miR-151a-3p/XRCC4	Glioblastoma	[121]
	miR-1238	Up	CAV1/EGFR	Glioblastoma	[122]
5. Docetaxel	miR-9-5p,	Up	ONECUT2	Breast cancer	[123]
	miR-195-5p,				
	miR-203a-3p				
6. Doxorubicin	miR-501-5p	Up	BLID	Gastric cancer	[79]
7. Melfalan or Bortezomib	Acid sphingomyelinase (ASM)	Up	Converts sphingomyelin into ceramide	Multiple myeloma	[124]
8. Vincristine	Chloride intracellular channel 1 (CLIC1)	Up	-	Gastric cancer	[125]

apoptotic Bax proteins [73]. On the other hand, plant derived EVs have been well documented and their drug delivery applications reported as well. Particularly, intranasal delivery of grapefruit derived EVs loaded with miR-17 and decorated with folic acid were selectively taken up by tumor cells and inhibited brain tumor in mice [71]. Similarly, citrus-derived exosomes induced TRAIL-mediated apoptosis and reduced growth of chronic myeloid leukemia (CML) in NOD/SCID mice [74]. Thus, such extended sources of exosomes are a valuable alternative that can be availed as a carrier for encapsulating the anti-cancer therapeutics for an effective cancer therapy in humans.

2.8. Exosome mediated delivery vehicles in pre-clinical and clinical trials

Since exosomes have tremendous potential to serve as drug delivery vehicles *in vitro* and *in vivo*, their translation into clinical application have been initiated and several are in progress. Currently, a phase I clinical trial in progress utilizes a plant derived exosome to deliver curcumin for colon cancer treatment (www.clinicaltrials.gov; NCT01294072). Similarly, dexosomes are small vesicles secreted by the dendritic cells with an enriched membrane content of major histocompatibility complex (MHC) class I and II molecules along with tetraspanins and CD86 proteins. These dexosomes are implicated in stimulating the immune response. Results from preclinical studies and phase I clinical trial demonstrated the dexosomes functioned as efficient activator of the innate and adaptive immune response towards antitumor surveillance [75]. In fact, a phase II clinical trial with tumor antigen loaded dexosomes for cancer vaccination is currently in progress (NCT01159288). Similarly, a phase II clinical trial using IFN- γ on dexosomes showed that they could elicit anti-tumor function through natural killer (NK) cells [76]. The results from these clinical trials highlight the significant strides made with exosome as drug delivery vehicles and provide enormous promise for additional clinical trials in the future that utilize exosomes for therapeutic applications.

3. Contribution of exosomes in anticancer drug resistance

Common cancer treatment options include monotherapy or a combination of the modalities, such as chemotherapy, immunotherapy, cellular therapy, radiotherapy, hormonal therapy, and surgery. However, development of resistance to these treatments is common and the disease on relapse often exhibits an aggressive behavior. While several factors contributing to drug resistance have been studied in the past, the role of exosomes in drug resistance is relatively new. For example, enhanced metastasis post-surgery has been documented, and exosomes are thought to be a contributing factor [77,78]. Similarly, exosomes produced by drug resistant tumor cells can reshape and reconfigure the infiltrating immune cells (T-cell, macrophage, dendritic cells) present within the tumor microenvironment and perturb their

function resulting in their inability to induce host immune response [79,80]. Furthermore, exosomes undeniably mediate drug resistance as evidenced by a study that showed exosomes from drug-resistant human microvascular endothelial cells (HMVEC) promoted epithelial-mesenchymal transition (EMT) and growth of nasopharyngeal carcinoma cells [81]. However, the tumor growth promoting effects of exosomes from the drug resistant HMVEC was abrogated when treated with the exosome inhibitor, GW4869. In the section below, we discuss exosome-mediated resistance mechanisms to currently used anti-cancer therapies.

3.1. Exosomes in chemotherapy resistance

Chemotherapy is widely used in cancer treatment. There have been a variety of classes of chemotherapies described in the literature based on their mode of action such as alkylating agents, inhibitors of mitosis, and corticosteroids to name a few [82]. However, occurrence of resistance (innate and acquired) to chemotherapy is common causing disease recurrence. While several mechanisms of drug resistance have been reported in the past, more recently resistance contributed by TDEs has emerged. Table 1 lists exosome-mediated drug resistance towards commonly used anti-cancer drugs. Factors involved in drug resistant mechanisms for a select list of drugs are discussed below.

Cisplatin (cis-diamminedichloridoplatinum; CDDP) is an alkylating agent that is widely used against multiple human solid tumors [82]. In ovarian cancer cells, exosome mediated transfer of cytosolic gelsolin (pGSN) protein caused CDDP-sensitive cells to become resistant [15]. Similarly, treatment of lung cancer cells with miR-425-3p packaged exosomes isolated from CDDP-treated cells conferred resistance by stimulating the autophagic process via AKT1 [80]. In MDA-MB-231 triple negative breast cancer cells, addition of miR (miR-370-3p, miR-423-5p, and miR-373) enriched exosome conferred CDDP resistance [81].

Oxaliplatin is another platinum-based alkylating agent and anti-cancer drug used in the treatment of cancers. Poor response to the oxaliplatin treatment is attributed to the long-noncoding RNA (lncRNA) cargo in the exosomes from drug resistant cells. In colorectal cancer (CRC) cells, transfer of exosomes containing colorectal cancer-associated lncRNA (CCAL) from cancer associated fibroblasts (CAF) to CRC cells resulted in suppression of apoptosis, activation of beta-catenin and drug resistance. Functional studies showed CCAL interacted with the mRNA binding human antigen R (HuR) and increased β -catenin mRNA and protein levels contributing to chemoresistance [83]. Similarly, miR-46146 was observed to be enriched in exosomes obtained from CRC cells and was shown to influence PDCD10 to exert chemoresistance in the recipient cells [84]. Resistance to combinatorial therapies have also been reported in CRC. Oxaliplatin combined with the anti-metabolite 5-fluorouracil (5-FU) was reported to be challenged by the generation of a resistant mechanism through transcription factor activating enhancer

binding protein 2e (TFAP2E) mediated upregulation of miRNAs miR106a5p and miR421 in MGC803/5FU CRC cells [81,85].

Sorafenib, a multi-kinase inhibitor that inhibits tumor growth by blocking the Raf kinase, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) is widely used in the treatment of liver, kidney, and thyroid cancers. Resistance to sorafenib was reported to be mediated by exosomes in human renal cell carcinoma (RCC) via miR-31-5p and MLH1 mediated mechanisms both *in vitro* and *in vivo*. Furthermore, RCC patients with progressive disease receiving sorafenib treatment showed increased amounts of miR-31-5p in the exosomes [86]. Similarly, resistance towards sorafenib due to reduced miR-744 content in exosomes was demonstrated in hepatocellular carcinoma (HCC) [87]. In the same study, the authors showed that addition of miR-744 enriched exosomes to HCC reverted to chemosensitization and inhibited tumor cell proliferation. Exosome-mediated resistance mechanisms to multiple anti-cancer drugs including docetaxel, temozolomide, bortezomib and doxorubicin were also reported [Table 1].

Another mechanism by which exosomes can contribute to drug resistance is by carrying the drug efflux components that attribute for multi-drug resistance (MDR). Frequently, more than 50% of cancer patients with MDR are linked with elevated levels of adenosine triphosphate (ATP)-binding cassette transporter (ABC) family of proteins such as, P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). The ABC transporters are reported to impart resistance to over 20 different anti-cancer drugs [88,89]. Conceivably, transfer of constituents of these multi-drug efflux pumps can be a viable mechanism for cancer cells to combat anti-cancer therapy. Notably, exosomes either directly carry these ABC transporter proteins or carry miRNA/mRNA components from drug resistant cells that can stimulate expression of the ABC transporter proteins in drug-sensitive cancer cell population and make them become resistant. Intriguingly, if the ABC transporters are placed inverted in the exosome membrane, they can remove anti-tumor drugs from the intercellular space as well [90–92]. Conversely, direct transfer of chemotherapeutic drugs from resistant to susceptible tumor cells via exosomes is another substantial mode of development of chemoresistance. Such exosome mediated resistance was conferred for cisplatin, pixantrone and doxorubicin drugs in melanoma, breast and ovarian cancers respectively [93–96]. Thus, multiple-modes of resistance mechanisms are operational to escape chemotherapy.

3.2. Exosomes contribute to immunotherapy resistance

Until recently, cancer treatment options were limited to conventional chemotherapy, radiotherapy, and surgery. However, the recent application of immunotherapy has seen great success due to efficacious and durable response observed in cancer patients. Immunotherapy strategies include the usage of monoclonal antibodies, vaccines, immune checkpoint inhibitors, lymphocyte-activating cytokines, CAR-T cells, bispecific antibodies, and oncolytic viruses [97–99]. Immune checkpoint proteins restrict both the immune responses to pathogens and self-antigens generated by the carcinogenesis process. Cancer cells are known to overexpress checkpoint proteins such as programmed death ligand (PDL)-1 that interacts with programmed cell death (PD)-1 receptor to inactivate T cells. Application of antibodies to regulate the immune checkpoint has improved response to therapy by 20–30%. Below are some of the recent immunotherapy approaches and the reported resistance mechanisms mediated by exosomes.

Exosomes are reported to disseminate resistance either by carrying receptor proteins that compete with the targeted therapy antibodies or deliver a cargo of noncoding RNAs and proteins to the sensitive cells. For instance, increasing evidence supports that the tumor secreted exosomes contain increased levels of PD-L1 that function as a decoy for the PD-L1 targeted therapy [100]. A similar phenomenon was observed with anti-CD20 targeted therapy. Exosomes secreted by B-cell lymphoma cells have been shown to carry CD-20 to block antibodies

targeted against CD-20. Furthermore, these exosomes complexed with anti-CD20 antibodies attracted complement proteins, thereby evading the antibody mediated lysis of cancer cells [101]. Contribution of noncoding RNAs and protein cargo present in the exosomes have also been shown to contribute to resistance against trastuzumab-targeted therapy. Trastuzumab is a monoclonal antibody against human epidermal growth factor receptor (HER)-2 that is used as a targeted therapy for breast, metastatic gastric, and other HER2 overexpressing cancers [102]. In breast cancer, overexpression of lncRNA-small nucleolar RNA host gene 14 (SNHG14) was associated with resistance to trastuzumab targeted therapy [103]. Similarly, overexpression of lncRNAs such as the actin filament associated protein 1 antisense RNA 1 (AFAP1-AS1) and AGAP2 antisense RNA 1 (AGAP2-AS1) in trastuzumab resistant breast cancer cells and their exosomes contributed to therapy resistance [104,105]. Exosome mediated resistant mechanisms were reported not only for HER2-targeted therapy but also for other cancer targeted therapies. For example, Cetuximab is a human and mouse chimeric monoclonal antibody against the epidermal growth factor receptor (EGFR). This antibody is widely used for treatment of colon cancer patients carrying the wild-type *KRAS* gene. In RKO colon cancer cells, exosome conferred resistance to cetuximab by regulating the phosphatase and tensin homolog (PTEN)/Akt pathway [106]. Similarly, exosome mediated resistance to the anti-apoptotic survivin protein was observed in glioma patients receiving anti-survivin vaccine (SurVaxM) [107]. The survivin containing exosomes were higher in glioma patients than in healthy control individuals. Overall, the resistance mechanisms contributed by the exosomes significantly reduce the effectiveness of immunotherapy in cancer patients.

3.3. Radiotherapy/proton therapy resistance mediated by exosomes

Radiation therapy or radiotherapy uses ionizing radiation to kill cancer cells. Although, radiotherapy alone is effective in killing cancer cells, it is often combined with other cancer treatments such as chemotherapy, hormonal therapy and surgery to contain cancer more efficiently [108]. Nevertheless, exosome mediated resistance to radiotherapy results in the recurrence of metastatic cancers. For instance, exposing BHY and FaDu head and neck cancer cells to gamma radiation stimulated increased secretion of exosomes. Co-culturing of these exosomes with BHY cells conferred radioresistance and promoted proliferation of recipient cells as early as 6 h post exosome treatment. Mechanistically, these exosomes exerted their resistance function through enhanced DNA double strand break repair [109]. Similarly, latent membrane protein-1 (LMP1) containing exosomes from CNE1 nasopharyngeal carcinoma cells were observed to induce radioresistance by activating the p38 MAPK pathway in recipient cells [110]. Also, exosomes from hypoxic glioblastoma cells were found to contain miR-301a which targeted the tumor-suppressor TCEAL7 gene and conferred radioresistance to corresponding normoxic recipient cells [111]. Likewise, Ni et al., have extensively discussed the aspects of exosome mediated radiation-resistance to multiple cancer treatments [112]. Conversely, exosomes also carry tumor suppressor miRNAs that suppressed the radioresistance of nasopharyngeal carcinoma [113].

Proton beam therapy (PBT) is a type of radiotherapy with improved therapeutic effects and reduced side effects compared with conventional X-ray based radiotherapy. Notably, PBT has been increasingly employed as a treatment option for multiple cancer types [114]. However, exosome mediated PBT resistance mechanisms have been reported as well. Proton irradiated HeLa cervical carcinoma cells were shown to secrete exosomes with higher content of survivin [115] thereby favoring tumor growth and treatment resistance. Thus, it is evident that exosomes play an important role in conferring resistance to radiation and proton therapy and strategies overriding radiotherapy resistance are warranted.

3.4. Involvement of exosomes in the resistance to anti-cancer hormonal therapy

Pathogenesis of certain malignancies such as breast and prostate cancer are influenced by hormones. Hence, first and second-generation drugs targeting hormone specific pathways are recommended for treating these malignancies. However, exosome mediated resistance mechanisms have been reported that decrease the efficiency of hormonal therapies. Notably, MCF-7 breast cancer cells resistant to the anti-estrogen, tamoxifen, secrete exosomes that confer resistance to tamoxifen treatment upon its delivery to sensitive cells [116]. Moreover, transfer of mitochondrial DNA by exosomes to oxidative phosphorylation-deficient breast cancer cells enabled the cells to bypass hormonal therapy-induced dormancy and develop resistance to hormonal therapy [117].

4. Conclusion and perspectives

Within the past decade, the field of exosome research has disclosed enormous information on these nanosized natural particles that can be harnessed in a broad range of basic and translational applications. This was achieved due to the keen interest in the field combined with parallel discovery and evolution of supportive isolation and purification technologies; direct/indirect exosome drug loading/manipulating methods; *in vivo* imaging and high-throughput methods for exosomal RNA/protein profiling. However, limitations exist in the use of exosomes as drug delivery vehicle. Major issues related to their use as drug carriers include isolation methods, purity, homogeneity, immunogenicity, and scalability. For example, exosomes have been shown to exhibit low immunogenicity compared to synthetic nanocarriers. However, whether exosomes derived from different cell types exhibit varying immunogenicity is not known. Thus, a comparative understanding of the immunogenicity of exosomes isolated from different cellular and biological sources will allow researchers to choose appropriate exosome source for use as drug delivery vehicle and advance to clinical translation. Similarly, information about why exosomes from certain cell types demonstrate high intrinsic drug loading capacity and efficient drug delivery to tumor sites than others can potentially improve exosome's application for clinical use.

In the context of drug resistance, improved understanding of the exosome biology has allowed in demonstrating their role in resistance and therapy failure. However, majority of the studies have focused on resistance towards a single agent. For clinical relevance where combination treatments are applied, it is important to investigate the contribution of exosome-mediated resistance towards combination therapies. Therefore, designing studies to closely monitor the emergence of exosome mediated resistance to numerous current and novel anti-cancer therapies, will enable researchers to quickly re-engineer these therapies to reduce or avoid emergence of exosome mediated therapy resistance. These necessary steps will stimulate the application of exosomes as a drug carrier and for the targeted delivery of anti-cancer therapeutics.

Declaration of competing interest

The authors report no conflict of interest in this work.

Acknowledgments

This study was supported by grants received from the National Institutes of Health (NIH), (R01 CA167516 and R01CA233201); from the National Institute of General Medical Sciences (P20 GM103639) of the National Institutes of Health; a Merit Grant (101BX003420A1) from the Department of Veterans Affairs; a Pilot Grant, Seed Grant, and Student Trainee Grant funded by the National Cancer Institute Cancer

Center Support Grant P30CA225520 awarded to the University of Oklahoma Stephenson Cancer Center; from the Department of Defense (DOD) through the Lung Cancer Research Program (LCRP) under award no.W81XWH-18-1-0637 & W81XWH-19-1-0647; a grant (HR18-088) from the Oklahoma Center for Advanced Science and Technology (OCAT); funds received from the Presbyterian Health Foundation Seed Grant (AM), Presbyterian Health Foundation Bridge Grant, and the Jim and Christy Everest Endowed Chair in Cancer Developmental Therapeutics.

The authors thank Ms. Kathy Kyler at the Office of the Vice President for Research, OUHSC, for editorial assistance. Rajagopal Ramesh is an Oklahoma TSET Research Scholar and holds the Jim and Christy Everest Endowed Chair in Cancer Developmental Therapeutics.

The content is solely the responsibility of the authors. The opinions, interpretations, conclusions and recommendations are those of the author and not necessarily endorsed by or representative of the official views by the NIH, DOD, or Department of Veterans Affairs.

References

- [1] Z. Livshits, R.B. Rao, S.W. Smith, An approach to chemotherapy-associated toxicity, *Emerg. Med. Clin.* 32 (2014) 167–203.
- [2] S. Hossen, M.K. Hossain, M.K. Basher, M.N.H. Mia, M.T. Rahman, M.J. Uddin, Smart nanocarrier-based drug delivery systems for cancer therapy and toxicity studies: a review, *J. Adv. Res.* 15 (2019) 1–18.
- [3] B. Adem, P.F. Vieira, S.A. Melo, Decoding the biology of exosomes in metastasis, *Trends Cancer* 6 (2020) 20–30.
- [4] L. Dini, S. Tacconi, E. Carata, A.M. Tata, C. Vergallo, E. Panzarini, *Microvesicles and Exosomes in Metabolic Diseases and Inflammation*, Cytokine Growth Factor Rev, 2020.
- [5] S. Gurunathan, M.H. Kang, M. Jeyaraj, M. Qasim, J.H. Kim, Review of the isolation, characterization, biological function, and multifarious therapeutic approaches of exosomes, *Cells* 8 (2019) 307.
- [6] Y. Yoshioka, Y. Konishi, N. Kosaka, T. Katsuda, T. Kato, T. Ochiya, Comparative marker analysis of extracellular vesicles in different human cancer types, *J. Extracell. Vesicles* 2 (2013) 20424.
- [7] Y. Bei, T. Chen, D.D. Banciu, D. Cretoiu, J. Xiao, Circulating exosomes in cardiovascular diseases, *Adv. Exp. Med. Biol.* 998 (2017) 255–269.
- [8] J. Howitt, A.F. Hill, Exosomes in the pathology of neurodegenerative diseases, *J. Biol. Chem.* 291 (2016) 26589–26597.
- [9] H. Baharloo, M. Azimi, Z. Salehi, M. Izad, Mesenchymal stem cell-derived exosomes: a promising therapeutic ace card to address autoimmune diseases, *Int J Stem Cells* 13 (2020) 13–23.
- [10] T.B. Steinbichler, J. Dudas, H. Riechmann, Skvortsova II, The role of exosomes in cancer metastasis, *Semin. Canc. Biol.* 44 (2017) 170–181.
- [11] L.M. Doyle, M.Z. Wang, Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis, *Cells* (2019) 8.
- [12] S. Keerthikumar, D. Chisanga, D. Ariyaratne, H. Al Saffar, S. Anand, K. Zhao, M. Samuel, M. Pathan, M. Jois, N. Chilamkurti, L. Gangoda, S. Mathivanan, *ExoCarta: a web-based compendium of exosomal cargo*, *J. Mol. Biol.* 428 (2016) 688–692.
- [13] M. Pathan, P. Fonseka, S.V. Chitti, T. Kang, R. Sanwlani, J. Van Deun, A. Hendrix, S. Mathivanan, *Vesiclepedia 2019: a compendium of RNA, proteins, lipids and metabolites in extracellular vesicles*, *Nucleic Acids Res.* 47 (2019) D516–D519.
- [14] T. Liu, Q. Zhang, J. Zhang, C. Li, Y.R. Miao, Q. Lei, Q. Li, A.Y. Guo, *EVmiRNA: a database of miRNA profiling in extracellular vesicles*, *Nucleic Acids Res.* 47 (2019) D89–D93.
- [15] M. Asare-Werehene, K. Nakka, A. Reunov, C.T. Chiu, W.T. Lee, M.R. Abedini, P.W. Wang, D.B. Shieh, F.J. Dilworth, E. Carmona, T. Le, A.M. Mes-Masson, D. Burger, B.K. Tsang, The exosome-mediated autocrine and paracrine actions of plasma gelsolin in ovarian cancer chemoresistance, *Oncogene* 39 (2020) 1600–1616.
- [16] C. Melzer, V. Rehn, Y. Yang, H. Bahre, J. von der Ohe, R. Hass, Taxol-loaded MSC-derived exosomes provide a therapeutic vehicle to target metastatic breast cancer and other carcinoma cells, *Cancers (Basel)* 798 (2019) 11, <https://doi.org/10.3390/cancers11060798>.
- [17] T. Musacchio, V.P. Torchilin, siRNA delivery: from basics to therapeutic applications, *Front. Biosci.* 18 (2013) 58–79.
- [18] S.F. Dowdy, Overcoming cellular barriers for RNA therapeutics, *Nat. Biotechnol.* 35 (2017) 222–229.
- [19] C.C. Chen, L. Liu, F. Ma, C.W. Wong, X.E. Guo, J.V. Chacko, H.P. Farhoodi, S.X. Zhang, J. Zimak, A. Segaliny, M. Riazifar, V. Pham, M.A. Digman, E.J. Pone, W. Zhao, Elucidation of exosome migration across the blood-brain barrier model in vitro, *Cell. Mol. Bioeng.* 9 (2016) 509–529.
- [20] R. Kanchanapally, S.K. Deshmukh, S.R. Chavva, N. Tyagi, S.K. Srivastava, G.K. Patel, A.P. Singh, S. Singh, Drug-loaded exosomal preparations from different cell types exhibit distinctive loading capability, yield, and antitumor efficacies: a comparative analysis, *Int. J. Nanomed.* 14 (2019) 531–541.
- [21] Y.J. Li, J.Y. Wu, J.M. Wang, X.B. Hu, J.X. Cai, D.X. Xiang, Gemcitabine loaded autologous exosomes for effective and safe chemotherapy of pancreatic cancer,

- Acta Biomater. 101 (2020) 519–530.
- [22] S.E. Emam, A.S. Abu Lila, N.E. Elsadek, H. Ando, T. Shimizu, K. Okuhira, Y. Ishima, M.A. Mahdy, F.S. Ghazy, T. Ishida, Cancer cell-type tropism is one of crucial determinants for the efficient systemic delivery of cancer cell-derived exosomes to tumor tissues, *Eur. J. Pharm. Biopharm.* 145 (2019) 27–34.
 - [23] H. Nie, X. Xie, D. Zhang, Y. Zhou, B. Li, F. Li, F. Li, Y. Cheng, H. Mei, H. Meng, L. Jia, Use of lung-specific exosomes for miRNA-126 delivery in non-small cell lung cancer, *Nanoscale* 12 (2020) 877–887.
 - [24] A. Dhayapalay, M. Kanapathipillai, Exosomes based geldanamycin delivery to cancer cells with increased therapeutic efficacy, *J. Biomed. Nanotechnol.* 15 (2019) 2202–2208.
 - [25] C. Wang, X. Huang, Y. Wu, J. Wang, F. Li, G. Guo, Tumor cell-associated exosomes robustly elicit anti-tumor immune responses through modulating dendritic cell vaccines in lung tumor, *Int. J. Biol. Sci.* 16 (2020) 633–643.
 - [26] L. Rosenberger, M. Ezquer, F. Lillo-Vera, P.L. Pedraza, M.I. Ortuzar, P.L. Gonzalez, A.I. Figueroa-Valdes, J. Cuenca, F. Ezquer, M. Khoury, F. Alcayaga-Miranda, Stem cell exosomes inhibit angiogenesis and tumor growth of oral squamous cell carcinoma, *Sci. Rep.* 9 (2019) 663.
 - [27] A. Srivastava, N. Amreddy, A. Babu, J. Panneerselvam, M. Mehta, R. Muralidharan, A. Chen, Y.D. Zhao, M. Razaq, N. Riedinger, H. Kim, S. Liu, S. Wu, A.B. Abdel-Mageed, A. Munshi, R. Ramesh, Nanosomes carrying doxorubicin exhibit potent anticancer activity against human lung cancer cells, *Sci. Rep.* 6 (2016) 38541.
 - [28] G.K. Patel, M.A. Khan, H. Zubair, S.K. Srivastava, M. Khushman, S. Singh, A.P. Singh, Comparative analysis of exosome isolation methods using culture supernatant for optimum yield, purity and downstream applications, *Sci. Rep.* 9 (2019) 5335.
 - [29] A. Srivastava, N. Amreddy, V. Pareek, M. Chinnappan, R. Ahmed, M. Mehta, M. Razaq, A. Munshi, R. Ramesh, Progress in Extracellular Vesicle Biology and Their Application in Cancer Medicine, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* (2020) e1621, <https://doi.org/10.1002/wnan.1621>.
 - [30] P. Li, M. Kaslan, S.H. Lee, J. Yao, Z. Gao, Progress in Exosome Isolation Techniques, *Theranostics* vol. 7, (2017) 789–804.
 - [31] D. Ingato, J.A. Edson, M. Zakharian, Y.J. Kwon, Cancer cell-derived, drug-loaded nanovesicles induced by sulphydryl-blocking for effective and safe cancer therapy, *ACS Nano* 12 (2018) 9568–9577.
 - [32] Y.J. Shyong, K.C. Chang, F.H. Lin, Calcium phosphate particles stimulate exosome secretion from phagocytes for the enhancement of drug delivery, *Colloids Surf. B Biointerfaces* 171 (2018) 391–397.
 - [33] F.G. Ortega, M.T. Roefs, D. de Miguel Perez, S.A. Kooijmans, O.G. de Jong, J.P. Sluijter, R.M. Schiffelers, P. Vader, Interfering with endolysosomal trafficking enhances release of bioactive exosomes, *Nanomedicine* 20 (2019) 102014.
 - [34] L.H. Lv, Y.L. Wan, Y. Lin, W. Zhang, M. Yang, G.L. Li, H.M. Lin, C.Z. Shang, Y.J. Chen, J. Min, Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro, *J. Biol. Chem.* 287 (2012) 15874–15885.
 - [35] R.A. Kore, J.L. Edmondson, S.V. Jenkins, A. Jamshidi-Parsian, R.P.M. Dings, N.S. Reyna, R.J. Griffin, Hypoxia-derived exosomes induce putative altered pathways in biosynthesis and ion regulatory channels in glioblastoma cells, *Biochem Biophys Res* 14 (2018) 104–113.
 - [36] Y.L. Hsu, J.Y. Hung, W.A. Chang, Y.S. Lin, Y.C. Pan, P.H. Tsai, C.Y. Wu, P.L. Kuo, Hypoxic lung cancer-secreted exosomal miR-23a increased angiogenesis and vascular permeability by targeting prolyl hydroxylase and tight junction protein ZO-1, *Oncogene* 36 (2017) 4929–4942.
 - [37] G.K. Panigrahi, P.P. Praharaj, T.C. Peak, J. Long, R. Singh, J.S. Rhim, Z.Y. Abd Elmageed, G. Deep, Hypoxia-induced exosome secretion promotes survival of African-American and Caucasian prostate cancer cells, *Sci. Rep.* 8 (2018) 3853.
 - [38] J.E. Park, B. Dutta, S.W. Tse, N. Gupta, C.F. Tan, J.K. Low, K.W. Yeoh, O.L. Kon, J.P. Tam, S.K. Sze, Hypoxia-induced tumor exosomes promote M2-like macrophage polarization of infiltrating myeloid cells and microRNA-mediated metabolic shift, *Oncogene* 38 (2019) 5158–5173.
 - [39] T. Fukuta, A. Nishikawa, K. Kogure, Low level electricity increases the secretion of extracellular vesicles from cultured cells, *Biochem Biophys Res* 21 (2020) 100713, <https://doi.org/10.1016/j.bbrep.2019.100713>.
 - [40] J.C. Gerlach, Y.C. Lin, C.A. Brayfield, D.M. Minter, H. Li, J.P. Rubin, K.G. Marra, Adipogenesis of human adipose-derived stem cells within three-dimensional hollow fiber-based bioreactors, *Tissue Eng. C Methods* 18 (2012) 54–61.
 - [41] M. Mendt, S. Kamerkar, H. Sugimoto, K.M. McAndrews, C.C. Wu, M. Gagea, S. Yang, E.V.R. Blanco, Q. Peng, X. Ma, J.R. Marszalek, A. Maitra, C. Yee, K. Rezvani, E. Shpall, V.S. LeBleu, R. Kalluri, Generation and testing of clinical-grade exosomes for pancreatic cancer, *JCI Insight* 3 (2018) e99263, <https://doi.org/10.1172/jci.insight.99263>.
 - [42] K.S. Carswell, E.T. Papoutsakis, Culture of human T cells in stirred bioreactors for cellular immunotherapy applications: shear, proliferation, and the IL-2 receptor, *Biotechnol. Bioeng.* 68 (2000) 328–338.
 - [43] L. Yang, D. Han, Q. Zhan, X. Li, P. Shan, Y. Hu, H. Ding, Y. Wang, L. Zhang, Y. Zhang, S. Xue, J. Zhao, X. Hou, Y. Wang, P. Li, X. Yuan, H. Qi, Blood TIR+ exosomes separated by a pH-responsive method deliver chemotherapeutics for tumor therapy, *Theranostics* 9 (2019) 7680–7696.
 - [44] F. Liu, O. Vermesh, V. Mani, T.J. Ge, S.J. Madsen, A. Sabour, E.C. Hsu, G. Gowrishankar, M. Kanada, J.V. Jokerst, R.G. Sierra, E. Chang, K. Lau, K. Sridhar, A. Bermudez, S.J. Pitteri, T. Stoyanova, R. Sinclair, V.S. Nair, S.S. Gambhir, U. Demirci, The exosome total isolation chip, *ACS Nano* 11 (2017) 10712–10723.
 - [45] J.C. Akers, V. Ramakrishnan, I. Yang, W. Hua, Y. Mao, B.S. Carter, C.C. Chen, Optimizing preservation of extracellular vesicular miRNAs derived from clinical cerebrospinal fluid, *Canc. Biomarkers* 17 (2016) 125–132.
 - [46] J. Frank, M. Richter, C. de Rossi, C.M. Lehr, K. Fuhrmann, G. Fuhrmann, Extracellular vesicles protect glucuronidase model enzymes during freeze-drying, *Sci. Rep.* 8 (2018) 12377, <https://doi.org/10.1038/s41598-018-30786-y>.
 - [47] K. Noguchi, M. Hirano, T. Hashimoto, E. Yuba, T. Takatani-Nakase, I. Nakase, Effects of lyophilization of arginine-rich cell-penetrating peptide-modified extracellular vesicles on intracellular delivery, *Anticancer Res.* 39 (2019) 6701–6709.
 - [48] C. Charoenriyayakul, Y. Takahashi, M. Nishikawa, Y. Takakura, Preservation of exosomes at room temperature using lyophilization, *Int. J. Pharm.* 553 (2018) 1–7.
 - [49] Z. Yang, J. Shi, J. Xie, Y. Wang, J. Sun, T. Liu, Y. Zhao, X. Zhao, X. Wang, Y. Ma, V. Malkoc, C. Chiang, W. Deng, Y. Chen, Y. Fu, K.J. Kwak, Y. Fan, C. Kang, C. Yin, J. Rhee, P. Bertani, J. Otero, W. Lu, K. Yun, A.S. Lee, W. Jiang, L. Teng, B.Y.S. Kim, L.J. Lee, Large-scale generation of functional mRNA-encapsulating exosomes via cellular nanoporation, *Nat Biomed Eng* 4 (2020) 69–83.
 - [50] Z. Li, X. Zhou, M. Wei, X. Gao, L. Zhao, R. Shi, W. Sun, Y. Duan, G. Yang, L. Yuan, In vitro and in vivo RNA inhibition by CD9-HuR functionalized exosomes encapsulated with miRNA or CRISPR/dCas9, *Nano Lett.* 19 (2019) 19–28.
 - [51] L. Huang, D. Wang, N. Gu, X.E. Zhang, [Construction of engineered exosomes with high loading efficiency of cellular endogenous proteins], *Sheng Wu Gong Cheng Xue Bao* 35 (2019) 1537–1545.
 - [52] D. Zhang, H. Lee, Z. Zhu, J.K. Minhas, Y. Jin, Enrichment of selective miRNAs in exosomes and delivery of exosomal miRNAs in vitro and in vivo, *Am. J. Physiol. Lung Cell Mol. Physiol.* 312 (2017) L110–L121.
 - [53] T.N. Lamichhane, A. Jeyaram, D.B. Patel, B. Parajuli, N.K. Livingston, N. Arumugasamy, J.S. Schardt, S.M. Jay, Oncogene knockdown via active loading of small RNAs into extracellular vesicles by sonication, *Cell. Mol. Bioeng.* 9 (2016) 315–324.
 - [54] S.M. Kim, Y. Yang, S.J. Oh, Y. Hong, M. Seo, M. Jang, Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting, *J. Contr. Release* 266 (2017) 8–16.
 - [55] A. Jeyaram, T.N. Lamichhane, S. Wang, L. Zou, E. Dahal, S.M. Kronstadt, D. Levy, B. Parajuli, D.R. Knudsen, W. Chao, S.M. Jay, Enhanced loading of functional miRNA cargo via pH gradient modification of extracellular vesicles, *Mol. Ther.* 28 (2019) 975–985.
 - [56] M.C. Didiot, L.M. Hall, A.H. Coles, R.A. Haraszti, B.M. Godinho, K. Chase, E. Sapp, S. Ly, J.F. Alterman, M.R. Hassler, D. Echeverria, L. Raj, D.V. Morrissey, M. DiFiglia, N. Aronin, A. Khvorova, Exosome-mediated delivery of hydrophobically modified siRNA for huntingtin mRNA silencing, *Mol. Ther.* 24 (2016) 1836–1847.
 - [57] S.A.A. Kooijmans, S. Stremersch, K. Braeckmans, S.C. de Smedt, A. Hendrix, M.J.A. Wood, R.M. Schiffelers, K. Raemdonck, P. Vader, Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles, *J. Contr. Release* 172 (2013) 229–238.
 - [58] J. Liu, Z. Ye, M. Xiang, B. Chang, J. Cui, T. Ji, L. Zhao, Q. Li, Y. Deng, L. Xu, G. Wang, L. Wang, Z. Wang, Functional extracellular vesicles engineered with lipid-grafted hyaluronic acid effectively reverse cancer drug resistance, *Biomaterials* 223 (2019) 119475, <https://doi.org/10.1016/j.biomaterials.2019.119475>.
 - [59] Y. Si, S. Kim, E. Zhang, Y. Tang, R. Jaskula-Sztul, J.M. Markert, H. Chen, L. Zhou, X.M. Liu, Targeted exosomes for drug delivery: biomanufacturing, surface tagging, and validation, *Biotechnol. J.* 15 (2020) e1900163.
 - [60] W. Nie, G. Wu, J. Zhang, L.L. Huang, J. Ding, A. Jiang, Y. Zhang, Y. Liu, J. Li, K. Pu, H.Y. Xie, Responsive exosome nano-bioconjugates for synergistic cancer therapy, *Angew Chem. Int. Ed. Engl.* 59 (2020) 2018–2022.
 - [61] E. Koh, E.J. Lee, G.H. Nam, Y. Hong, E. Cho, Y. Yang, I.S. Kim, Exosome-SIRPalpha, a CD47 blockade increases cancer cell phagocytosis, *Biomaterials* 121 (2017) 121–129.
 - [62] Z. Stickney, J. Losacco, S. McDevitt, Z. Zhang, B. Lu, Development of exosome surface display technology in living human cells, *Biochem. Biophys. Res. Commun.* 472 (2016) 53–59.
 - [63] W. Fu, C. Lei, S. Liu, Y. Cui, C. Wang, K. Qian, T. Li, Y. Shen, X. Fan, F. Lin, M. Ding, M. Pan, X. Ye, Y. Yang, S. Hu, CAR exosomes derived from effector CAR-T cells have potent antitumor effects and low toxicity, *Nat. Commun.* 10 (2019) 4355.
 - [64] X.J. Tang, X.Y. Sun, K.M. Huang, L. Zhang, Z.S. Yang, D.D. Zou, B. Wang, G.L. Warnock, L.J. Dai, J. Luo, Therapeutic potential of CAR-T cell-derived exosomes: a cell-free modality for targeted cancer therapy, *Oncotarget* 6 (2015) 44179–44190.
 - [65] Z. Fan, K. Xiao, J. Lin, Y. Liao, X. Huang, Functionalized DNA enables programming exosomes/vesicles for tumor imaging and therapy, *Small* 15 (2019) e1903761.
 - [66] S.C. Jang, O.Y. Kim, C.M. Yoon, D.S. Choi, T.Y. Roh, J. Park, J. Nilsson, J. Lotvall, Y.K. Kim, Y.S. Cho, Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors, *ACS Nano* 7 (2013) 7698–7710.
 - [67] A.J. Vazquez-Rios, A. Molina-Crespo, B.L. Bouzo, R. Lopez-Lopez, G. Moreno-Bueno, M. de la Fuente, Exosome-mimetic nanoplateforms for targeted cancer drug delivery, *J. Nanobiotechnol.* 17 (2019) 85.
 - [68] K.L. Zhang, Y.J. Wang, J. Sun, J. Zhou, C. Xing, G. Huang, J. Li, H. Yang, Artificial chimeric exosomes for anti-phagocytosis and targeted cancer therapy, *Chem. Sci.* 10 (2019) 1555–1561.
 - [69] Y.Y. Jhan, D. Prasca-Chamorro, G. Palou Zuniga, D.M. Moore, S. Arun Kumar, A.K. Gaharwar, C.J. Bishop, Engineered extracellular vesicles with synthetic lipids via membrane fusion to establish efficient gene delivery, *Int. J. Pharm.* 573 (2020) 118802.
 - [70] Q. Chen, Y. Chen, Y. Sun, W. He, X. Han, E. Lu, X. Sha, Leukocyte-mimicking

- Pluronic-lipid nanovesicle hybrids inhibit the growth and metastasis of breast cancer, *Nanoscale* 11 (2019) 5377–5394.
- [71] G. Wang, W. Hu, H. Chen, X. Shou, T. Ye, Y. Xu, Cocktail Strategy Based on NK Cell-Derived Exosomes and Their Biomimetic Nanoparticles for Dual Tumor Therapy, *Cancers (Basel)* (2019) 11.
- [72] O.Y. Kim, H.T. Park, N.T.H. Dinh, S.J. Choi, J. Lee, J.H. Kim, S.W. Lee, Y.S. Gho, Bacterial outer membrane vesicles suppress tumor by interferon-gamma-mediated antitumor response, *Nat. Commun.* 8 (2017) 626.
- [73] E. Behzadi, H. Mahmoodzadeh Hosseini, A.A. Imani Fooladi, The inhibitory impacts of *Lactobacillus rhamnosus* GG-derived extracellular vesicles on the growth of hepatic cancer cells, *Microb. Pathog.* 110 (2017) 1–6.
- [74] S. Raimondo, F. Naselli, S. Fontana, F. Monteleone, A. Lo Dico, L. Saieva, G. Zito, A. Flugi, M. Manno, M.A. Di Bella, G. De Leo, R. Alessandro, Citrus limon-derived nanovesicles inhibit cancer cell proliferation and suppress CML xenograft growth by inducing TRAIL-mediated cell death, *Oncotarget* 6 (2015) 19514–19527.
- [75] M.A. Morse, J. Garst, T. Osada, S. Khan, A. Hobeika, T.M. Clay, N. Valente, R. Shreenivas, M.A. Sutton, A. Delcayre, D.H. Hsu, J.B. Le Pecq, H.K. Lyster, A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer, *J. Transl. Med.* 3 (2005) 9.
- [76] B. Besse, M. Charrier, V. Lapierre, E. Dansin, O. Lantz, D. Planchard, T. Le Chevalier, A. Livartoski, F. Barlesi, A. Laplanche, S. Ploix, N. Vimond, I. Peguillet, C. Thery, L. Lacroix, I. Zoernig, K. Dhodapkar, M. Dhodapkar, S. Viaud, J.C. Soria, K.S. Reiners, E. Poggendorf, von Strandmann, F. Vely, S. Rusakiewicz, A. Eggermont, J.M. Pitt, L. Zitvogel, N. Chaput, Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC, *Oncolimmunology* 5 (2016) e1071008.
- [77] Q. Fu, Q. Zhang, Y. Lou, J. Yang, G. Nie, Q. Chen, Y. Chen, J. Zhang, J. Wang, T. Wei, H. Qin, X. Dang, X. Bai, T. Liang, Primary tumor-derived exosomes facilitate metastasis by regulating adhesion of circulating tumor cells via SMAD3 in liver cancer, *Oncogene* 37 (2018) 6105–6118.
- [78] Q. Fu, Q. Zhang, Y. Lou, J. Yang, G. Nie, Q. Chen, Y. Chen, J. Zhang, J. Wang, T. Wei, H. Qin, X. Dang, X. Bai, T. Liang, Correction: primary tumor-derived exosomes facilitate metastasis by regulating adhesion of circulating tumor cells via SMAD3 in liver cancer, *Oncogene* 38 (2019) 5740–5741.
- [79] X. Liu, Y. Lu, Y. Xu, S. Hou, J. Huang, B. Wang, J. Zhao, S. Xia, S. Fan, X. Yu, Y. Du, L. Hou, Z. Li, Z. Ding, S. An, B. Huang, L. Li, J. Tang, J. Ju, H. Guan, B. Song, Exosomal transfer of miR-501 confers doxorubicin resistance and tumorigenesis via targeting of BLID in gastric cancer, *Cancer Lett.* 459 (2019) 122–134.
- [80] J.Y. Jang, J.K. Lee, Y.K. Jeon, C.W. Kim, Exosome derived from epigallocatechin gallate treated breast cancer cells suppresses tumor growth by inhibiting tumor-associated macrophage infiltration and M2 polarization, *BMC Cancer* 13 (2013) 421.
- [81] L. Huang, C. Hu, H. Chao, Y. Zhang, Y. Li, J. Hou, Z. Xu, H. Lu, H. Li, H. Chen, Drug-resistant endothelial cells facilitate progression, EMT and chemoresistance in nasopharyngeal carcinoma via exosomes, *Cell. Signal.* 63 (2019) 109385, <https://doi.org/10.1016/j.cellsig.2019.109385>.
- [82] C.Y. Huang, D.T. Ju, C.F. Chang, P. Muralidhar Reddy, B.K. Velmurugan, A review on the effects of current chemotherapy drugs and natural agents in treating non-small cell lung cancer, *Biomedicine (Taipei)* 7 (2017) 23, <https://doi.org/10.1051/bmdcn/2017070423>.
- [83] X. Deng, H. Ruan, X. Zhang, X. Xu, Y. Zhu, H. Peng, X. Zhang, F. Kong, M. Guan, Long noncoding RNA CCAL transferred from fibroblasts by exosomes promotes chemoresistance of colorectal cancer cells, *Int. J. Cancer* 146 (2020) 1700–1716.
- [84] Y. Xu, M. Zhu, Novel exosomal miR-46146 transfer oxaliplatin chemoresistance in colorectal cancer, *Clin. Transl. Oncol.* (2019), <https://doi.org/10.1007/s12094-019-02237-1>.
- [85] S. Jingyue, W. Xiao, Z. Juanmin, L. Wei, L. Daoming, X. Hong, TFAP2E methylation promotes 5-fluorouracil resistance via exosomal miR106a5p and miR421 in gastric cancer MGC803 cells, *Mol. Med. Rep.* 20 (2019) 323–331.
- [86] J. He, J. He, L. Min, Y. He, H. Guan, J. Wang, X. Peng, Extracellular vesicles transmitted miR-31-5p promotes sorafenib resistance by targeting MLH1 in renal cell carcinoma, *Int. J. Cancer* 146 (2020) 1052–1063.
- [87] G. Wang, W. Zhao, H. Wang, G. Qiu, Z. Jiang, G. Wei, X. Li, Exosomal MiR-744 inhibits proliferation and sorafenib chemoresistance in hepatocellular carcinoma by targeting PAX2, *Med. Sci. Mon.* 25 (2019) 7209–7217.
- [88] R. Januchowski, K. Sterzynska, K. Zaorska, P. Sosinska, A. Klejewski, M. Brazert, M. Nowicki, M. Zabel, Analysis of MDR genes expression and cross-resistance in eight drug resistant ovarian cancer cell lines, *J. Ovarian Res.* 9 (2016) 65, <https://doi.org/10.1186/s13048-016-0278-z>.
- [89] K. Moitra, H. Lou, M. Dean, Multidrug efflux pumps and cancer stem cells: insights into multidrug resistance and therapeutic development, *Clin. Pharmacol. Ther.* 89 (2011) 491–502.
- [90] D. Sousa, R.T. Lima, M.H. Vasconcelos, Intercellular transfer of cancer drug resistance traits by extracellular vesicles, *Trends Mol. Med.* 21 (2015) 595–608.
- [91] C. Corcoran, S. Rani, K. O'Brien, A. O'Neill, M. Principe, R. Sheikh, G. Webb, R. McDermott, W. Watson, J. Crown, L. O'Driscoll, Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes, *PloS One* 7 (2012) e50999, <https://doi.org/10.1371/journal.pone.0050999>.
- [92] M. Bebawy, V. Combes, E. Lee, R. Jaiswal, J. Gong, A. Bonhoure, G.E. Grau, Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells, *Leukemia* 23 (2009) 1643–1649.
- [93] R. Safaei, B.J. Larson, T.C. Cheng, M.A. Gibson, S. Otani, W. Naerdemann, S.B. Howell, Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells, *Mol. Cancer Therapeut.* 4 (2005) 1595–1604.
- [94] K. Shedden, X.T. Xie, P. Chandary, Y.T. Chang, G.R. Rosania, Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles, *Canc. Res.* 63 (2003) 4331–4337.
- [95] C. Federici, F. Petrucci, S. Caimi, A. Cesolini, M. Logozzi, M. Borghi, S. D'Ilio, L. Lugini, N. Violante, T. Azzarito, C. Majorani, D. Brambilla, S. Fais, Exosome release and low pH belong to a framework of resistance of human melanoma cells to cisplatin, *PloS One* 9 (2014) e88193, <https://doi.org/10.1371/journal.pone.0088193>.
- [96] R. Koch, T. Aung, D. Vogel, B. Chapuy, D. Wenzel, S. Becker, U. Sinzig, V. Venkataramani, T. von Mach, R. Jacob, L. Truemper, G.G. Wulf, Nuclear trapping through inhibition of exosomal export by indomethacin increases cytostatic efficacy of doxorubicin and pixantrone, *Clin. Canc. Res.* 22 (2016) 395–404.
- [97] Y. Yang, Cancer immunotherapy: harnessing the immune system to battle cancer, *J. Clin. Invest.* 125 (2015) 3335–3337.
- [98] V. Velcheti, K. Schalper, Basic overview of current immunotherapy approaches in cancer, *Am Soc Clin Oncol Educ Book* 35 (2016) 298–308.
- [99] B. Tang, Z.S. Guo, D.L. Bartlett, D.Z. Yan, C.P. Schane, J. Liu, G. McFadden, D.L. Thomas, J.L. Shisler, E.J. Roy, Synergistic combination of oncolytic virotherapy and immunotherapy for glioma 26 *Clin Cancer Res.* 2020, pp. 2216–2230.
- [100] F. Xie, M. Xu, J. Lu, L. Mao, S. Wang, The role of exosomal PD-L1 in tumor progression and immunotherapy, *Mol. Canc.* 18 (2019) 146, <https://doi.org/10.1186/s12943-019-1074-3>.
- [101] T. Aung, B. Chapuy, D. Vogel, D. Wenzel, M. Oppermann, M. Lahmann, T. Weinlage, K. Menck, T. Hupfeld, R. Koch, L. Trumper, G.G. Wulf, Exosomal evasion of humoral immunotherapy in aggressive B-cell lymphoma modulated by ATP-binding cassette transporter A3, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 15336–15341.
- [102] T. Sarosiek, P. Morawski, [Trastuzumab and its biosimilars], *Pol. Merkur. Lek.* 44 (2018) 253–257.
- [103] H. Dong, W. Wang, R. Chen, Y. Zhang, K. Zou, M. Ye, X. He, F. Zhang, J. Han, Exosome-mediated transfer of lncRNASHG14 promotes trastuzumab chemoresistance in breast cancer, *Int. J. Oncol.* 53 (2018) 1013–1026.
- [104] M. Han, Y. Gu, P. Lu, J. Li, H. Cao, X. Li, X. Qian, C. Yu, Y. Yang, X. Yang, N. Han, D. Dou, J. Hu, H. Dong, Exosome-mediated lncRNA AFAP1-AS1 promotes trastuzumab resistance through binding with AUF1 and activating ERBB2 translation, *Mol. Canc.* 19 (2020) 26, <https://doi.org/10.1186/s12943-020-1145-5>.
- [105] Z. Zheng, M. Chen, P. Xing, X. Yan, B. Xie, Increased expression of exosomal AGAP2-AS1 (AGAP2 antisense RNA 1) in breast cancer cells inhibits trastuzumab-induced cell cytotoxicity, *Med. Sci. Mon.* 25 (2019) 2211–2220.
- [106] S. Zhang, Y. Zhang, J. Qu, X. Che, Y. Fan, K. Hou, T. Guo, G. Deng, N. Song, C. Li, X. Wan, X. Qu, Y. Liu, Exosomes promote cetuximab resistance via the PTEN/Akt pathway in colon cancer cells, *Braz. J. Med. Biol. Res.* 51 (2017) e6472, <https://doi.org/10.1590/1414-431X20176472>.
- [107] P.M. Galbo Jr., M.J. Ciesielski, S. Figel, O. Maguire, J. Qiu, L. Wilsie, H. Minderman, R.A. Fenstermaker, Circulating CD9+/GFAP+/survivin+ exosomes in malignant glioma patients following survival vaccination, *Oncotarget* 8 (2017) 114722–114735.
- [108] C. Hennequin, I. Barillot, D. Azria, Y. Belkacemi, M. Bollet, B. Chauvet, D. Cowen, B. Cutuli, A. Fourquet, J.M. Hannoun-Levi, M. Leblanc, M.A. Mahe, [Radiotherapy of breast cancer], *Canc. Radiother.* 20 (Suppl) (2016) S139–S146.
- [109] L. Mutschelknaus, C. Peters, K. Winkler, R. Yentrappalli, T. Heider, M.J. Atkinson, S. Moertl, Exosomes derived from squamous head and neck cancer promote cell survival after ionizing radiation, *PloS One* 11 (2016) e0152213, <https://doi.org/10.1371/journal.pone.0152213>.
- [110] Z. Zhang, X. Yu, Z. Zhou, B. Li, J. Peng, X. Wu, X. Luo, L. Yang, LMP1-positive extracellular vesicles promote radioresistance in nasopharyngeal carcinoma cells through P38 MAPK signaling, *Canc. Med.* 8 (2019) 6082–6094.
- [111] X. Yue, F. Lan, T. Xia, Hypoxic glioma cell-secreted exosomal miR-301a activates wnt/beta-catenin signaling and promotes radiation resistance by targeting TCEAL7, *Mol. Ther.* 27 (2019) 1939–1949.
- [112] J. Ni, J. Bucci, D. Malouf, M. Knox, P. Graham, Y. Li, Exosomes in cancer radioresistance, *Front Oncol* 9 (2019) 869, <https://doi.org/10.3389/fonc.2019.00869>.
- [113] F.Z. Wan, K.H. Chen, Y.C. Sun, X.C. Chen, R.B. Liang, L. Chen, X.D. Zhu, Exosomes overexpressing miR-34c inhibit malignant behavior and reverse the radioresistance of nasopharyngeal carcinoma, *J. Transl. Med.* 18 (2020) 12, <https://doi.org/10.1186/s12967-019-02203-z>.
- [114] M. Hu, L. Jiang, X. Cui, J. Zhang, J. Yu, Proton beam therapy for cancer in the era of precision medicine, *J. Hematol. Oncol.* 11 (2018) 136, <https://doi.org/10.1186/s13045-018-0683-4>.
- [115] S. Khan, J.M. Jutzy, J.R. Aspe, D.W. McGregor, J.W. Neidigh, N.R. Wall, Survivin is released from cancer cells via exosomes, *Apoptosis* 16 (2011) 1–12.
- [116] S.E. Semina, A.M. Scherbakov, A.A. Vnukova, D.V. Bagrov, E.G. Evtushenko, V.M. Safronova, D.A. Golovina, L.N. Lyubchenko, M.V. Gudkova, M.A. Krasil'nikov, Exosome-Mediated Transfer of Cancer Cell Resistance to Antiestrogen Drugs, *Molecules* 23 (2018) 829, <https://doi.org/10.3390/molecules23040829>.
- [117] P. Sansone, C. Savini, I. Kurelac, Q. Chang, L.B. Amato, A. Strillacci, A. Stepanova, L. Iommarini, C. Mastroleo, L. Daly, A. Galkin, B.K. Thakur, N. Soplog, K. Uryu, A. Hoshino, L. Norton, M. Bonafe, M. Cricca, G. Gasparre, D. Lyden, J. Bromberg, Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer, *Proc. Natl. Acad. Sci. U. S. A.* 114 (2017) E9066–E9075.
- [118] X. Wang, H. Zhang, H. Yang, M. Bai, T. Ning, T. Deng, R. Liu, Q. Fan, K. Zhu, J. Li, Y. Zhan, G. Ying, Y. Ba, Exosome-delivered circRNA promotes glycolysis to induce chemoresistance through the miR-122-PKM2 axis in colorectal cancer 14 *Mol*

- Oncol, 2020, pp. 539–555.
- [119] M. Del Re, I. Bertolini, S. Crucitta, L. Fontanelli, E. Rofi, C. De Angelis, L. Diodati, D. Cavallero, G. Gianfilippo, B. Salvadori, S. Fogli, A. Falcone, C. Scatena, A.G. Naccarato, M. Roncella, M. Ghilli, R. Morganti, A. Fontana, R. Danesi, Overexpression of TK1 and CDK9 in plasma-derived exosomes is associated with clinical resistance to CDK4/6 inhibitors in metastatic breast cancer patients, *Breast Canc. Res. Treat.* 178 (2019) 57–62.
- [120] Q. Zhang, R.X. Liu, K.W. Chan, J. Hu, J. Zhang, L. Wei, H. Tan, X. Yang, H. Liu, Exosomal transfer of p-STAT3 promotes acquired 5-FU resistance in colorectal cancer cells, *J. Exp. Clin. Canc. Res.* 38 (2019) 320, <https://doi.org/10.1186/s13046-019-1314-9>.
- [121] Z. Zhang, J. Yin, C. Lu, Y. Wei, A. Zeng, Y. You, Exosomal transfer of long non-coding RNA SBF2-AS1 enhances chemoresistance to temozolomide in glioblastoma, *J. Exp. Clin. Canc. Res.* 38 (2019) 166, <https://doi.org/10.1186/s13046-019-1139-6>.
- [122] J. Yin, A. Zeng, Z. Zhang, Z. Shi, W. Yan, Y. You, Exosomal transfer of miR-1238 contributes to temozolomide-resistance in glioblastoma, *EBioMedicine* 42 (2019) 238–251.
- [123] M. Shen, C. Dong, X. Ruan, W. Yan, M. Cao, D. Pizzo, X. Wu, L. Yang, L. Liu, X. Ren, S.E. Wang, Chemotherapy-induced extracellular vesicle miRNAs promote breast cancer stemness by targeting ONECUT2, *Canc. Res.* 79 (2019) 3608–3621.
- [124] S. Faict, I. Oudaert, L. D'Auria, J. Dehairs, K. Maes, P. Vlummens, K. De Veirman, E. De Bruyne, K. Fostier, I. Vande Broek, R. Schots, K. Vanderkerken, J.V. Swinnen, E. Menu, The Transfer of Sphingomyelinase Contributes to Drug Resistance in Multiple Myeloma, (2019), p. 11 *Cancers* (Basel).
- [125] K. Zhao, Z. Wang, X. Li, J.L. Liu, L. Tian, J.Q. Chen, Exosome-mediated transfer of CLIC1 contributes to the vincristine-resistance in gastric cancer, *Mol. Cell. Biochem.* 462 (2019) 97–105.

Seminars in Cancer Biology

Organically derived exosomes as carriers of anticancer drugs and imaging agents for cancer treatment --Manuscript Draft--

Manuscript Number:	
Article Type:	SI: Exosomes in Cancer
Keywords:	exosomes, drug delivery, theranostic, cancer, extracellular vesicles; exosomes, drug delivery, theranostic, cancer, extracellular vesicles
Corresponding Author:	Rajagopal Ramesh, Ph.D The University of Oklahoma Health Sciences Center Oklahoma City, OK UNITED STATES
First Author:	Akhil Srivastava
Order of Authors:	Akhil Srivastava
	Shipra Rathore
	Anupama Munshi, PhD
	Rajagopal Ramesh, Ph.D
Abstract:	Extracellular vesicles (EVs), or exosomes, perform intercellular communication by carrying several biologics from donor or parental cells and delivering them to recipient cells. Their unique cargo-carrying capacity has recently been explored for use as drug delivery vehicles. Being naturally produced, exosomes are considered to have many advantages over synthetic lipid-based nanoparticles currently used in clinics to treat cancer and other diseases. The discovery of the role of exosomes has led to numerous preclinical and clinical studies exploring their use as an amenable drug delivery vehicle and as a theranostic in cancer diagnosis and treatment. However, there are certain limitations associated with exosomes. Among these limitations, the most important is in selecting the biological source for producing highly biocompatible exosomes on a large scale. This review article explores the various sources from which exosomes of therapeutic interest can be isolated and used for as drug carriers for cancer treatment. The method of exosome isolation and their loading with cancer therapeutics and imaging agents are also discussed in the follow-up sections. Finally, the article concludes with future directions for exosome-based applications in cancer diagnosis and treatment.
Suggested Reviewers:	Subree Subramanian, PhD Associate Professor subree@umn.edu Expertise in extracellular vesicles
	Arash Hatefi, PhD Associate Professor, Rutgers University Board of Governors: Rutgers The State University of New Jersey ahatefi@pharmacy.rutgers.edu Expertise in drug delivery
	Bingliang Fang, MD, PhD Professor, The University of Texas MD Anderson Cancer Center bfang@manderson.org Expertise in cancer- lung cancer



Rajagopal Ramesh, Ph.D., DBA, AIMBE
Professor of Pathology
Jim and Christy Everest Endowed Chair
in Cancer Developmental Therapeutics
Prebyterian Health Foundation Presidential Professor
Director, Experimental Therapeutics and
Translational Cancer Medicine
Director, Small Animal Bioluminescence Imaging
Core
Co-Leader, Cancer Biology Program
Co-Director, Nanomedicine Program
Chair, Fellowship Training and Mentoring Program
Phone: Office – (405) 271-6101
Lab - (405) 271-6104
Fax: (405) 271-2472
Email: rajagopal-ramesh@ouhsc.edu

Date: May 20, 2021

To: Editorial Office
Seminars in Cancer Biology

Ref: *Invited manuscript submission for “Clinical Relevance of Exosomes in Cancer” special issue*

Dear Editorial Office,

Submitted is an invited review article for publication in “Seminars in Cancer Biology” for the special issue “*The Clinical Relevance of Exosomes in Cancer*” edited by guest editors, Dr. Stefano Fais and Dr. Francesco Cappello. The article is titled “**Organically derived exosomes as carriers of anticancer drugs and imaging agents for cancer treatment**” by Srivastava, A. *et al.* In the submitted review article, we discuss strategies for utilizing organically derived exosomes as a carrier of anticancer drugs and imaging agents for cancer treatment. The authors believe that the submitted article will be of interest to the general audience and individuals working in the areas of cancer biology, therapy, drug delivery and nanomedicine.

The submitted article has not been previously published or submitted for publication to any other journal.

The authors declare there is no conflict of interest.

Thanking you for your time in reviewing the submitted manuscript and look forward to hearing from your office.

Should you have any questions, please contact me by Phone, 405-271-6101 or by Email: rajagopal-ramesh@ouhsc.edu.

Sincerely

R. Ramesh
Rajagopal Ramesh, Ph.D.

Organically derived exosomes as carriers of anticancer drugs and imaging agents for cancer treatment

Akhil Srivastava^{a,c}, Shipra Rathore^{a,d}, Anupama Munshi^{b,c}, Rajagopal Ramesh^{a,c,d*}

Department of ^aPathology, and ^bRadiation Oncology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

^cStephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

^dGraduate Program in Biomedical Sciences, University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, USA

* Corresponding author: contact email address: rajagopal-ramesh@ouhsc.edu

***Address for Correspondence.** Rajagopal Ramesh, Department of Pathology, University of Oklahoma Health Sciences Center, 975 N.E., 10th Street, Oklahoma City, OK 73104, USA; Phone +1 (405) 271-6101; Email address: rajagopal-ramesh@ouhsc.edu

Abstract

Extracellular vesicles (EVs), or exosomes, perform intercellular communication by carrying several biologics from donor or parental cells and delivering them to recipient cells. Their unique cargo-carrying capacity has recently been explored for use as drug delivery vehicles. Being naturally produced, exosomes are considered to have many advantages over synthetic lipid-based nanoparticles currently used in clinics to treat cancer and other diseases. The discovery of the role of exosomes has led to numerous preclinical and clinical studies exploring their use as an amenable drug delivery vehicle and as a theranostic in cancer diagnosis and treatment. However, there are certain limitations associated with exosomes. Among these limitations, the most important is in selecting the biological source for producing highly biocompatible exosomes on a large scale. This review article explores the various sources from which exosomes of therapeutic interest can be isolated and used for as drug carriers for cancer treatment. The method of exosome isolation and their loading with cancer therapeutics and imaging agents are also discussed in the follow-up sections. Finally, the article concludes with future directions for exosome-based applications in cancer diagnosis and treatment.

Keywords. Extracellular vesicles, exosomes, cancer, drug delivery, theranostic

Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
AFM	Atomic Force Microscopy
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CpGDNA	5'—C—phosphate—G—3'DNA
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9
CSF	Cerebrospinal fluid
CUR	Curcumin
DARP	Dopamine-Releasing Protein
DARP	Designed ankyrin repeat proteins
DC	Dendritic Cells
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DOC	Docetaxel
EGFR	Epidermal Growth Factor Receptor
EPR	Enhanced Permeability and Retention
ESCRT	Endosomal Sorting Complex Required for Transport
EV	Extracellular Vesicles
FA	Folic Acid
FCS	Fetal Calf Serum
FTIR	Fourier-Transform Infrared Spectroscopy
GBM	Glioblastoma multiforme
GDV	Gene Delivery Vector
HCl	Hydrochloric Acid
ILV	Intra Luminal Vesicles
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
miR	microRNA
MRD	Multi-Drug Resistance
MRI	Magnetic Resonance Imaging
MS	Mass Spectroscopy
MSC	Mesenchymal Stem Cells
MVB	Multi Vesicular Bodies
NMR	Nuclear Magnetic Resonance
NTA	Nanotracker Analysis
OMV	Outer Membrane Vesicles
OSCC	Oral Squamous Cell Carcinoma
PAMP	Pathogen-Associated Molecular Pattern
PEG	Polyethylene Glycol
PRR	Pattern Recognition Receptor

PTX	Paclitaxel
qRT-PCR	quantitive Real Time Polymerase Chain Reaction
RBC	Red Blood Cells
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
siRNA	Small interfering RNA
SPIONs	Super Paramagnetic Iron Oxide Nanoparticles
TEM	Transmission Electron Microscopy
TE _x	Tumor Exosomes
TLR	Toll-Like Receptor
TMZ	Temozolomide
TNBC	Triple-Negative Breast Cancer
UHT	Ultra Heat Treated
VEGF	Vascular Endothelial Growth Factor
WFA	Withaferin A

1. Introduction

The success of a chemotherapeutic treatment regimen largely depends on the efficient and precise delivery of therapeutic agents to cancer cells with minimal side effects or cytotoxicity to normal cells of the body [1]. An efficient and safe drug delivery system is urgently required to reduce the side effects of chemotherapy and lead to a better cure for the patients. For many years, researchers in nanomedicine have developed several kinds of synthetic nanomaterials that can function as drug carriers and deliver anti-cancer therapeutics in a targeted fashion to cancer cells [2, 3]. Although significant progress has been made in this direction, few of the drug carriers have reached the clinic. Poor biocompatibility, inefficient bio-distribution, and immunogenic response are few reasons for the failure of many synthetic drug delivery complexes. Liposomes, which are formulated by the use of phospholipids and cholesterol of various composition, have been the most successful drug delivery system developed and tested for cancer treatment in the clinic. With a composition similar to that of the cell membrane, liposomes show better biocompatibility than do other synthetic drug delivery systems. Furthermore, the liposomes are amenable to modifications to accommodate multiple therapeutic payload (eg: plasmid DNA, hydrophilic and hydrophobic drugs, siRNA, miRNA, etc.) and tumor-targeting moieties (e.g., monoclonal antibodies, aptamers, protein ligands, etc.) [4, 5]. Incorporation of tumor-targeted ligands facilitates tumor-targeted drug delivery, increased drug accumulation in the tumor, and enhanced antitumor activity with minimal off-target side effects. Active targeting in combination with the inherent enhanced permeability and retention (EPR) effect contributes to the enhanced antitumor activity [6]. These unique properties have made liposomes one of the most acceptable drug delivery methods. Several such liposome formulations, such as Doxil®, DaunoXome®, and Marqibo®, and recently approved liposomal formulations Onivyde®, Vyxeos®, and Onpattro®, are available in clinics for the

treatment of cancer and other diseases [6, 7]. A quick survey of clinical trials involving liposomes and cancer at “clinicaltrials.gov” indicates a total of 1059 clinical trials have used or will use liposomes in cancer (www.clinicaltrials.gov last accessed on May 7th 2021). However, despite improved therapeutic carrying capability, liposomes suffer from many drawbacks due to their synthetic nature. These include inferior targeting, generation of a cytokine storm and immune response, morphological instability, and their inability to pass through many stringent biological barriers, such as the blood brain barrier (BBB) [8-10].

As an alternative to synthetic liposomes, ‘natural nanoparticles,’ such as the extracellular vesicle (EV’s), produced by plants, microbes, or the body’s own cells, are being investigated as carriers of anti-cancer drugs. EV’s is a generic and broad term used for defining a spectrum of vesicle-like structures produced and released by the cells into the extracellular milieu [11]. EVs are heterogeneous, and their classification is mainly based on the mode of biogenesis, size, and morphology (**Table 1, Figure 1**). Each type of EV has a functional role in the cell and contributes to different physiological processes [12]. Exosomes form the largest group of EVs and are classically defined as nano-sized (30-150 nm), lipid bilayer vesicles produced by the endosomal pathway by all types of cells [13, 14]. The International Society of Extracellular Vesicles (ISEV) published Minimal Information for Studies of Extracellular Vesicles (“MISEV”) in 2018 that refrains from using the term exosomes in absence of an exosome specific marker and rather suggests to classify EVs on the basis of size (small EVs < 100 nm or < 200 nm; medium / large EVs > 200 nm) [15]. Based on transmission electron microscopy (TEM) imaging, several studies report exosomes as cup-shaped organelles; however, the cryoTEM images of a freshly frozen exosomes show a near-circular structure [16]. The biogenesis and hence packaging of exosomes

occurs through a highly orchestrated endocytic pathway and the matured exosomes are released into the extracellular milieu by fusion of multivesicular bodies and the plasma membrane [17].

During synthesis, exosomes are packaged with different types of bioactive molecules, such as lipids, proteins, DNA, RNA, and several species of small non-coding (nc) RNAs and long non-coding (lnc) RNAs, as cargo. The surfaces of the exosomes are embedded with several fusion proteins that are involved in navigation (targeting) and attachment to the surface of recipient cells (cellular adhesions). The most enriched proteins in exosomes are tetraspanins (CD63, CD81, and CD9) and targeting proteins (integrins) [18, 19]. Lipids also form a substantial part of exosome structure and cargo. Lipids that are enriched in the exosome membrane include cholesterol, sphingomyelins (SM), ceramide, lyso(bis)phosphatidic acid, lysophosphatidylcholines, phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines (PS), phosphatidylinositols, phosphatidic acid, and phosphatidylglycerol. The composition of the lipids akin to proteins and nucleic acids present in the exosomes resembles that of their parental cells. [20, 21].

The content of exosomes is significant as it is the repertoire of composition and physiological status of its cell of origin. Since exosomes are present in all bodily fluids (e.g., urine, blood plasma/serum, cerebrospinal fluid (CSF)) and can be easily purified from different bodily fluids, they are used to study the pathobiology of disease and to develop novel liquid biopsy modalities for the diagnosis of diseases, including cancer [22, 23]. Although exosomes were discovered in the early forties, they were primarily regarded as a mode of discarding unwanted toxic materials from cells. However, in the late 2000s, several seminal discoveries highlighted the potential of exosomes in pathobiology and biomedical research [24]. This article aims to discuss the potential of exosomes isolated from several natural resources in the areas of cancer therapy and diagnosis.

2. Biogenesis of exosomes

The biogenesis of exosomes occurs as early endosomes mature to late endosomes. During this time, the inner membrane starts folding inwards and forms a small, vesicle-like structure. These vesicles are called intraluminal vesicles (ILVs), and the endosomes carrying them are known as multivesicular bodies (MVBs). There exists a cascade of signaling pathways that regulates the packaging of molecules into the lumen of ILVs [25]. The site of parental cells in the body and its physiological condition determines the biosynthesis of exosomes and their composition [26]. In general, endosomal sorting complex required for transport (ESCRT) proteins TSG101, STAM1, Alix, and CD9 have been implicated in regulating pathways involved in the packaging and biosynthesis process [27]. Subsequently, the Rab-GTPase family of proteins, such as Rab2B, Rab5A, Rab7, Rab11, Rab27, and Rab35, are involved in the release of ILVs as exosomes in an extracellular cellular milieu as a result of a fusion of MVPs with the plasma membrane [28, 29]. Finally, the SNARE family of proteins and other adhesion and targeting proteins guide and steer the exosomes to attach and release their content to recipient cells [30]. Understanding the complex process involved during the biosynthesis of exosomes has helped us to explore strategies to regulate metastasis and tumor growth. Using mouse models, Ghoroghi *et al.* delineated the effect of GTPases of the Ral family on regulating exosome biogenesis by controlling the maturation of MVBs. The researchers further described how members of the Ral family, RalA and RalB or Ral A/B, are involved in the packaging of produced exosomes and dictate organotropic capacities, which finally enables the exosomes to reconfigure tissue microenvironment in forming a metastatic niche. The authors demonstrated this by studying the exosomes involved in lung metastasis of breast cancer cells [31].

Serum starvation has also shown to impede exosome biosynthesis. THP-1 cells cultured in RPMI-1640 cell culture medium supplemented with 10% FCS secreted a low level of exosomes compared with cells grown in cell culture medium supplemented with 1% FCS [32]. Likewise, hypoxia influences the biosynthesis of exosomes, and studies have shown that any kind of cellular stress induces the production of exosomes. This is probably why many studies claim that cancer cells produce more exosomes than do their normal counterparts [33]. ESCRT independent exosome biosynthesis have also been reported indicating multiple mechanisms are operational in exosome biogenesis [34-36]. In addition to proteins, lipids and several other molecules have also been associated with the biosynthesis pathway and have been extensively described in review articles by Théry and Kowal [37, 38].

Knowledge of the mechanisms and constituents involved in exosome synthesis is essential for their use in the diagnosis and therapeutic interventions, and hence reaching the goal of developing a potent theranostic for cancer treatment.

3. Characteristics of exosomes similar to liposomes

Exosomes can stably carry and transfer different biomolecules to recipient cells across the body, suggesting a new mode of intercellular communication. Building on this notion, scientists also engineered the composition of exosome content to realize its potential to be used as a natural drug delivery system [39]. Exosomes show many similarities with synthetic liposomes. Most prominent among them is the composition of their membrane, which in both cases consist of lipoproteins. Additionally, both liposomes and exosomes share a similarity in size range [40]. Both are sub-micrometers in diameter (20-200 nm), enabling them to move easily inside the body. Their unique size and composition also help in the packaging of cargo into their lumens.

4. Advantage of exosomes as a delivery vehicle

Exosome are naturally produced by all kind of cells in all forms of living systems [41]. Despite sharing many similarities with synthetic drug delivery systems, such as liposomes as mentioned in above section, exosomes have certain unique characteristics that make them stand out for drug delivery. The exosomes *de novo* are designed to transport cellular material from parental cell to recipient cells, and their membranes are often composed of a constellation of proteins, such as tetraspanins and other adhesive proteins. The presence of such proteins on their outer membranes aid in testing exosomes as a drug delivery vehicle and reduce the off-target effects of the administered therapy [42].

Exosomes intrinsically possess many desirable traits of an ideal drug delivery vehicle. These include: (A) They do not elicit measurable host immune response and thus are non-immunogenic, (B) their uptake by recipient cells occurs through various mechanisms including receptor mediated binding and internalization, (C) their surface can be functionalized for cell specific targeting, (D) they are stable and can be lyophilized without impacting their structural or biological properties, (E) they inherently carry small molecules and cargoes (eg: mRNA, miRNA, protein, etc.) and operate as biological carriers and cell signal inducers, and finally (F) use of cross-species exosomes has shown no ill effect on recipient cells, which suggests the possibilities of using several alternative sources for isolation and use of exosomes as drug carriers [43, 44]. Hence, exosomes have the potential to replace liposomes as a drug delivery modality.

5. Source of exosomes

5.1. Cell-derived exosomes

Cell-derived exosomes are the most preferred resource for developing drug delivery vehicles. Cells can be cultured in a controlled and optimized environment to produce a near-homogenous population of exosomes in large quantity. Because exosomes exhibit similar composition to that

of their cells of origin, researchers can explore different kinds of cells to identify the most appropriate candidate source to produce a therapeutic-grade exosomes. Various parameters, such as production capacity, immunogenicity, ability to be genetically manipulated to produce and load exosomes with desired cargos, and the ability to maintain a continuous supply of exosomes for a long time, are critical parameters that have to be examined prior to selecting the cell source for exosomes. Following are some cell types that are being investigated as a cell based source for exosomes in drug delivery.

5.1.1. Dendritic cells (DCs)

DCs are antigen-presenting cells that have been used in several studies to produce exosomes that are often termed as Dexosomes, or Dex. Alvarez–Erviti *et al.* were the first to show that small regulatory RNA, like siRNA, can be successfully transferred to recipient cells and confer therapeutic effect by engineering DC-derived exosomes [45]. DC-derived exosomes were shown to mediate MHC-dependent immune responses and hence have been explored as cell-free vaccines for cancer treatment. Pitt *et al.* noted several advantages of Dex over DCs in devising potent immunotherapy interventions. These advantages included their ability to carry and maintain essential immunostimulatory properties of DCs, the stability of Dex over DCs, and a relatively simplistic approach lacking the complexities that arise when cells are used for treatment [46].

5.1.2. Mesenchymal stem cells (MSCs)

MSCs are multipotent stem cells produced by different tissues in the body. In general, MSC-derived exosomes are involved in various processes related to the development and progression of cancer. They have been implicated in processes including the initiation of the tumor niche, tumorigenesis, and cancer-promoting phenomena, such as angiogenesis. MSC-derived exosomes have also received great interest as drug delivery vehicles in cancer treatment due to their

immunomodulatory properties. The propensity of MSC-derived exosomes towards tumors imparts the property of tumor tropism and has been exploited in several studies. Pascucci *et al.* packaged paclitaxel (PTX) into MSC-derived exosomes and successfully demonstrated its anti-cancer activity against in the human pancreatic cell line CFPAC-1 [47]. Kalimuthu *et al.* also packaged PTX into exosomes *via* loading MSC with PTX and then isolating exosomes from treated MSCs, demonstrating a mechanism of isolating PTX-loaded exosomes that they termed PTX-loaded exosome mimetics (PTX-loaded Ems) [48]. MSC-derived exosomes have also been used to load small RNAs. Munoz *et al.* reported loading and transport of Cy5-tagged anti-miR-9 in temozolomide (TMZ)-resistant glioblastoma multiforme (GBM) cells [49]. In a similar study by Kamerkar *et al.*, MSC-derived exosomes loaded with siRNA against KRAS demonstrated a therapeutic effect in mouse pancreatic tumor models [43].

5.1.3. Tumor cells

Exosomes from tumor cells have been considered an ideal source with which to develop delivery vehicles suitable for transporting and delivering anti-cancer therapeutics in a tumor-targeted fashion. Tumor cell-derived exosomes, or TEx, can be used to target specific tissues, as they display many proteins and molecules that are responsible for their preferred and efficient uptake by tumor cells [50, 51]. The homing effect by TEx further strengthens the idea of using them as a modality to develop an effective cell vaccination strategy. Efficient delivery of antigens and adjuvant to antigen-presenting cells is a challenge, and is required to develop an effective immunotherapy modality for cancer treatment. Morishita *et al.* evaluated the use of murine melanoma (B16BL6) cell-derived exosomes genetically modified to express endogenous tumor antigens and immune stimulatory CpG DNA for cancer immunotherapy. The *in vitro* and *in vivo* studies demonstrated treatment with the modified exosomes resulted in the activation of mouse

dendritic (DC2.4) cells and efficient antigen presentation that resulted in greater antitumor response in B16BL6 tumor-bearing mice compared to treatment with a mixture of exosomes and CpG DNA. The study results suggested that the use of engineered tumor-derived exosomes can be a useful tool for cancer immunotherapy [52]. In contrast, Rong *et al* showed exosomes from human breast cancer cells suppressed T-cell proliferation and was mediated by transforming growth factor (TGF)- β present in the tumor-derived exosomes [53]. These results demonstrate the immunomodulatory properties of exosomes and that the use of TEx for cancer therapy has to be handled with caution.

Qiao *et al.* explored the idea of using TEx to exploit the tumor tropism for drug delivery. The study used exosomes isolated from human colon cancer (HT1080) and cervical (Hela) cancer cell lines and tested for their homing properties toward HT1080 tumor cells. Exosomes from the two cell lines were loaded with Doxil (Doxorubicin) and tested for exosome uptake and antitumor activity *in vitro* and *in vivo* using HT1080 cells. Both, *in vitro* and *in vivo* study results demonstrated drug loaded exosomes from HT1080 cells exhibited greater tumor tropism and enhanced antitumor activity towards the parental HT1080 cells compared to drug loaded exosomes from Hela cells [51]. The findings revealed that in cancer cell-derived exosomes fuse preferentially with their parent cancer cells and thus could potentially be exploited as drug carriers for cancer therapy. In another study, Sun *et al* using exosomes from mouse lymphoma (EL-4) cell line demonstrated the anti-inflammatory properties of curcumin loaded exosomes in suppressing inflammation in lipopolysaccharide (LPS)-induced septic shock mouse model [54, 55]. The study results revealed exosomes from tumor cells can also be useful in delivering anti-inflammatory agents.

Studies from our laboratory showed addition of TEx isolated from human lung cancer cell lines to parental tumor cells and to normal lung fibroblast resulted in increased cell proliferation of both cell types. Additionally, molecular studies revealed activation of signaling pathways that favored cell survival and drug resistance (*unpublished data*). The results from all of the studies described above while providing evidence for use of TEx in the treatment of cancer and other indications, advancing TEx to clinical translation will need careful consideration and caution.

5.1.4. Other cell-derived exosomes

In addition to the above mentioned cell types, other cell types have also been explored as sources of exosomes. Stable (transformed) human embryonic kidney, HEK 293T cells, are the most common cell line involved in studies related to exosome-mediated delivery of therapeutic molecules. Ohno *et al.* demonstrated the tumor-targeting property of exosomes isolated from HEK 293 cells by decorating them with the GE11 peptide or epidermal growth factor (EGF) on their surfaces. They used this to deliver let-7a miRNA to EGFR-expressing breast cancer xenograft tissues and observed an enhanced therapeutic effect [56]. Similarly, HER-2 positive breast cancer cells were treated with therapeutic siRNA using engineered HEK298T-derived exosomes that displayed synthetic peptides DARP in G3 on the surface to confer tumor-targeting ability [57]. HEK293-derived exosomes have also shown remarkable ability to carry and deliver chemotherapeutic drugs, such as doxorubicin, in such a manner that the drug causes minimal toxicity to non-targeted cells [58].

We in our studies have explored the use of normal lung fibroblast-derived exosomes as drug delivery carriers. We used normal human lung fibroblast (MRC-9)-derived exosomes and combined them with doxorubicin-conjugated gold nanoparticles (Dox-GNP) to develop a unique hybrid drug delivery vehicle that we termed ‘nanosomes.’ We reported enhanced tumor cell death

and also demonstrated the protection of cardiac cells (non-targeted cells) when nanosomes were used to deliver doxorubicin (**Figure 2**) [59]. Recently, we have developed a multifunctional tumor-targeted drug delivery system by loading MRC-9 derived exosomes with cisplatin (CDDP) conjugated to iron-oxide nanoparticles (IONP) via a pH-sensitive linker. The rationale to incorporate pH-sensitive linker is to exploit the intracellular pH difference between tumor cells (acidic) and normal cells (basic) and have selective drug release under acidic conditions, a feature common in the tumor microenvironment. The CDDP-IONP loaded exosomes were further decorated on their surface with transferrin for achieving tumor-targeted drug delivery in transferrin-receptor (TfR) overexpressing lung cancer cells. These multifunctional tumor-targeted exosomes have the ability to operate as theranostic by exerting CDDP-induced tumor cell cytotoxicity and the IONP facilitating monitoring of tumor cell killing by magnetic resonance imaging (MRI). Additionally, it is anticipated that these exosomes will exhibit minimal to no toxicity to normal cells. The theranostic potential of these exosomes are currently being evaluated and the study results are anticipated to be available in the next one-year.

5.1.5. 3D cell cultures for exosome production

Studies to develop exosome-based therapeutic modalities commonly use exosomes produced by cells growing in cell culture dishes or flasks in 2D. Although the cells grow optimally in 2D space on a plastic-coated base, they seldom resemble the real growing conditions present under *in vivo* conditions. Conventionally, cells grown in 3D as organoid or spheroids more closely resemble the real *in vivo* compositions. Investigators recently explored the isolation of exosomes from 3D-cultured cells and registered an increase in production of exosomes. They also compared the molecular composition of exosomes and showed that the small RNA profile of 3D- culture derived exosomes exhibited a higher similarity (~96%) to *in vivo* circulating exosomes derived from

cervical cancer patient plasma [60, 61]. This finding demonstrates the feasibility of using 3D cell-derived exosomes as drug delivery vehicles for precise therapeutic interventions.

Although cell-derived exosomes appear to be a straightforward choice, changes in the physiology of cells as a consequence of growing them over plastics under artificial conditions could impact exosome production and composition. Hence, scientists are now exploring other more natural resources for exosomes.

5.2. Natural sources of exosomes

There are two major avenues from which naturally produced exosomes can be isolated: 1) plants and microbes (e.g., bacteria, fungi) and 2) bodily fluids. The exosomes produced by these resources are considered to be more amenable, less toxic, and show high absorption potential in the recipient cells. These resources also bear the tremendous potential to produce exosomes on a large scale. In the following section, these natural reservoirs of exosomes are described and some studies that have used these natural exosomes as delivery vehicles are also discussed.

5.2.1. Plant-derived exosomes

Plant-derived extracellular vesicles are a group of nano-scaled vesicles that are produced by all kinds of plants and have been isolated from various edible vegetables and fruits [62]. These are membrane-bound vesicles, like exosomes, and can be considered as a potential alternative to synthetic liposomes or nanoparticles. Hence, plant-derived vesicles or exosomes can be used for developing green, sustainable, and biocompatible materials for the delivery of bioactive compounds. Natural extracellular vesicles, including exosomes, have been identified in plants and to be involved in various physiological processes and in cell-to-cell communication [63, 64]. The composition of plant-derived nanovesicles (exosomes) is similar to that of animal exosomes and is made up of lipids, proteins, and nucleic acids. Plant-derived exosomes show a high degree of

biocompatibility with animal systems and can be used in cross-kingdom communications. Li *et al.* showed that plant-derived exosomes called as ‘botanosomes’ can carry small (s) RNAs and transfer it into humans. In their study, they showed exosome-like nanoparticles present in drinking decoctions and referred to as “decoctosomes” comprised of lipids, proteins, sRNAs, and chemical compounds. Oral administration of decoctosomes containing sRNAs (HJT-sRNA-m7 and PGY-sRNA-6) offered therapeutic benefits in bleomycin-induced lung fibrosis and poly(I:C)-induced inflammation in mice [65]. Garaneva *et al.* used grapefruit juice-derived exosomes to successfully transport exogenous Alexa Fluor 647-labeled bovine serum albumin (BSA) and heat shock protein (HSP)70 to human peripheral blood mononuclear cells (PBMCs) and colon cancer cells. The presence of exosomes was also confirmed through various methods, including Nanotracker Analysis (NTA), Dynamic Light Scattering (DLS), Atomic Force Microscopy (AFM), and cryo-electron microscopy (Cryo-EM) [66]. A variety of plant components have been used to isolate exosomes using conventional ultracentrifugation, filtration, and other methods (**Table 3**). Since plant-derived exosomes can be easily scaled up for mass production and harvested in bulk, compared to synthetic nanoparticles, they hold great promise as a resource for exosomes production and use as drug carrier.

Consumption of green vegetables and fruits and a vegetarian diet has been suggested as a way to reduce cancer incidence. Even certain epidemiological studies have demonstrated that consumption of plant-based foods helps in reducing cancer incidence [67]. Studies have demonstrated the beneficial effect of various plant extracts and citrus fruit juice in exerting anticancer activity. Lemon-derived exosomes have been shown to have anti-cancer activity in several solid tumors and hematological cancers [68]. Similarly, Zhang *et al* explored edible ginger-derived exosomes that showed an inhibitory effect in colitis-associated cancer [63]. In melanoma,

ginseng-derived nanoparticles showed remarkable anti-cancer activity by effectively inhibiting tumor growth [69]. The plant-derived exosomes also show notable stability in different conditions of pH and temperature. Grapefruit-derived exosomes have adhered to a wide range of pH, suggesting its capacity to work as an efficient delivery vehicle in tumor microenvironment which is acidic in nature [62]. Researchers have demonstrated that algae produce exorbitant numbers of exosomes, which suggests that scalability of exosomes needed for developing delivery vehicle can be easily achieved [70]. Thus, plant-derived exosomes not only have inbuilt anti-cancer activities, but also show remarkable stability, biocompatibility, and ability for large-scale production, strongly indicating that these exosomes can be a good resource with which to develop carrier vehicles.

5.2.2. Microbes and Bacteria

Small nano-sized vesicles that resemble EVs or exosomes are produced by several microbes, including bacteria, protozoans, and fungi. These vesicles are present in different groups or microbial kingdoms, and are often termed membrane vesicles, outer membrane vesicles (OMVs), exosomes, or shedding microvesicles. Regardless of the term used, the fundamental process of biogenesis that largely uses ESCRT proteins and its homologues to facilitate formation of vesicles/exosomes and their release is conserved [71]. The major function of these small vesicles has been to transfer virulence factors from these microbes to host cells, thereby aiding infections. Interestingly, the virulence factors are primarily made up of proteins, lipids, nucleic acids, and other bioactive molecules. In other words, microbe-derived exosomes have the capability to deliver exogenic cargo to host cells, breaking the kingdom barrier. Physiologically, microbe-derived exosomes are also involved in promoting infections. Pathogen-associated molecular patterns (PAMPs) are key molecules involved in eliciting the host immune system by activation

of pattern recognition receptors (PRRs) and Toll-like receptors (TLRs). It is now recognized that pathogens release PAMPS through exosomes or EVs and other immunomodulatory molecules to promote the infectious cycle [72].

The most extensively studied microbe-derived exosomes are those that are isolated from gram-negative bacteria cells. The first report of exosomes in gram-negative bacteria was *E.coli* in the 1960s. In gram-negative bacteria, exosomes are called outer membrane vesicles (OMVs), as they have no membrane-bound organelles and are usually generated from the outer membrane. The gram-negative OMVs are well characterized for transferring biomolecules to host cells, including immune cells, to promote virulence and infections, develop resistance to antibiotics, and in some cases to degrade the host cells. OMVs have also been secreted in larger amounts when bacteria are under stress [73]. Gram-positive bacteria also produce OMVs into their extracellular space to perform various biological activities. Species such as *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Bacillus anthracis* have been reported to produce OMVs [74].

For more than 20 years, protozoans have been known to produce extracellular vesicles. *Leishmania* spp. and *Trypanosoma cruzi* EVs (or exosomes) have recently been recognized to have a physiological role. Protozoan-derived EVs also carry and transfer various biomolecules that act like virulence factors and promote infection in the host cells. In a proteomic analysis of *T. cruzi*, a number of proteins containing nucleic acid-binding sites and ribosomal proteins were identified, suggesting that the exosomes from this protozoan can successfully carry nucleic acids to the host cells [75, 76].

Fungal cells are prolific producers of extracellular vesicles that are similar to exosomes. Unlike bacteria, fungi are eukaryotic cells. Still, the exact mechanism of EV/exosome biogenesis is not fully understood. One reason is that, like bacteria, fungal cells are enclosed in a sturdy cell wall.

Nevertheless, fungi-derived EVs have been reported to carry enzymes, proteins, and other biomolecules required to enhance fungal pathogenesis [77, 78]

In summary, microbe-derived EVs efficiently pass genetic and other biomolecules across kingdoms. Further they can be easily isolated in high numbers from continuously growing microbial cultures and can be manipulated to transfer exogenous material, suggesting that they could be explored as ideal drug carriers and vaccines.

5.2.3. Bodily fluids

5.2.3.1. Milk-derived exosomes

Milk is an excellent source of exosomes, and studies have shown that milk is enriched in exosomes. Milk-derived exosomes have been shown to have an enhanced uptake by recipient cells, suggesting that an efficient delivery system can be formed with them [79]. In general, there are two sources of exosomes: human breast milk and bovine milk. Since humans consume both types of milk, they have no immunogenic effect and retain bio-compatibility. Samuel *et al.* extensively compared the quality and quantity of exosomes isolated from bovine mature milk and colostrum samples at 24, 48, and 72 h postpartum. The study revealed the presence of many exosome-associated common and unique proteins. Interestingly, they also detected several proteins involved in vesicle docking and fusion with the cell membrane during release of exosomes. Rab family proteins and certain integrins responsible for anchorage and uptake of exosomes were also found to be enriched in all of the milk samples tested [80]. Milk-derived exosomes are very robust, as they can survive in harsh and degrading conditions present in the gut, and can cross biological barriers to reach peripheral tissues and deliver their content to those cells. Additionally, milk-derived exosomes have successfully shown inter-organismal and cross-species communication and attributes, including being a natural source of exosomes, abundance, scalability, economic

viability, and lack of immunologic reactions that make them a significant candidate as drug carriers and vaccines for cancer therapy [81].

Anti-cancer drugs are generally infused intravenously due to their poor oral bioavailability. The mode of administration, although necessary, involves drawbacks such as high cost, physical inconvenience, and issues associated with sterility. To circumvent the shortcomings, administration of therapeutics via the oral route is preferred and is being tested. Milk-derived exosomes are an attractive option as they show nontoxicity and in abundance, and have the built-in property of being easily absorbed from the gastrointestinal tract in humans thereby increasing the bioavailability of drugs in the body. Built on this premise, studies have explored the potential of milk-derived exosomes to develop an effective drug delivery vehicle. Some of these aspects will be discussed in the following sections [82].

Munagala *et al.* reported for the first time the utility of bovine milk as a potentially scalable source of exosomes that can be used as a drug delivery vehicle. The study successfully demonstrated efficient loading and delivery of hydrophilic compounds, lipophilic agents, and chemotherapeutic drugs. Compounds, including curcumin (CUR), Withaferin A (WFA), anthocyanidins (Anthos), paclitaxel (PTX), and docetaxel (DOC), that vary in their lipophilicity, molecular weights, and functional groups were loaded onto the exosomes. These loaded exosomes were used to treat human lung (A549 and H1299) and breast (MDA-MB-231 and T47D) cancer cell lines. The treatment with exosome-loaded vehicle showed an improved treatment response than did treatment with free compounds/drugs. The study further explored the efficacy of the milk exosome drug delivery vehicle in lung cancer A549 xenograft models. The therapeutic response was enhanced when administered intraperitoneally (i.p.) or orally. The therapeutic effect was further increased by adding the tumor-targeting ligand folic acid (FA) to the drug containing milk-

derived exosomes [83]. Milk-derived exosomes membranes were conjugated to doxorubicin via a pH-responsive imine bond to leverage the acidic nature of the tumor micro-environment in aiding sustainable drug release. In addition to doxorubicin, administration of milk-derived exosomes loaded with anthracene endoperoxide derivative (EPT1) and chlorin e6 (Ce6) showed controlled drug release and biocompatibility, and was effective in treating oral squamous cell carcinoma (OSCC) [84]. Casein is a protein that is abundant in milk and causes a reduction in exosome release. Separation of casein by acid treatment using hydrochloric acid (HCl) or acetic acid facilitates exosome isolation and purification [85].

High quality bovine milk-derived exosomes isolated by casein chelation were engineered with modular surface tunability to enable loading of small interfering RNA (siRNA) and administration via the oral route [86]. The study showed that polyethylene glycol (PEG) coating of exosomes can reduce their degradation in the acidic gastric environment and enhanced their permeability through mucin. These findings show that milk-derived exosomes may be useful for the development of an effective, scalable platform technology for oral drug delivery of siRNA [86]. siRNA-based therapeutics and their delivery through exosomes is a promising treatment strategy. However, the loading of negatively charged molecules like siRNA through methods like electroporation is cumbersome and is accompanied by issues such as siRNA aggregation, toxicity, and experimental complexities. To mitigate this, Shandilya, *et al.* exploited the use of bovine lactoferrin which is a natural ligand for its receptor, GAPDH present in milk exosomes [87]. In their study, lactoferrin with poly-L-lysine was used to conjugate electrostatically with negatively charged siRNA to load siRNA into milk exosomes. The lactoferrin-poly-L-lysine complexed with siRNA when added onto milk exosomes showed excellent loading into exosomes resulting in transfection efficiency, colocalization percentage, and colocalization threshold equivalent to the electroporation method.

Aquil *et al.* using milk-derived exosomes demonstrated successful loading of siRNAs against a panel of genes (VEGF, EGFR, AKT, MAPK, and KRAS) albeit varying in their loading efficiency. Use of these siRNA-loaded exosomes resulted in 2- to 10-fold knockdown of the target protein expression levels in various cancers [88]. In the same study, the authors demonstrated the therapeutic effect of targeting KRAS^{G12S} in A549 lung tumors both *in vitro* and *in vivo*. Functionalization of the KRAS^{G12S} siRNA containing exosomes with folic acid further enhanced the therapeutic effect and resulted in significant suppression of A549 tumor growth *in vivo*.

miRNA is another class of regulatory small RNA molecule that can be used as anticancer drugs. Xhe *et al.* loaded raw bovine milk-derived exosomes with exogenous hsa-miR148a-3p and tested the delivery capability of the loaded system against hepatic (HepG2) and intestinal (Caco-2) cell lines. Results from this study strongly indicated that bovine milk-derived exosomes could be a cost-effective resource for the production of nanocarriers of functional miRNAs and could be used in RNA-based therapy [89].

One of the important criteria of a successful drug delivery system is its bioavailability in the recipient system and the effective distribution of its cargo. Manca *et al.* used bovine, porcine and murine milk-derived exosomes labeled with fluorophores or fluorescent fusion proteins and studied its biodistribution within and across species boundaries. Their study revealed preferred accumulation of exosomes and their microRNA cargos in liver, spleen, and brain after suckling, oral gavage, and intravenous administration in mice and pigs. The study further showed unique distribution profiles and cross-species distribution when synthetic, fluorophore-labeled microRNAs carrying bovine milk-derived exosomes were administered to mice [90].

Through the studies mentioned above and many others, it has been demonstrated that milk (human breast or bovine) is an excellent source of exosomes, and its scaling can easily be

controlled. However, a few questions prevent milk-derived exosomes from being explored at commercial level for devising drug delivery modalities. One such question is regarding the purity of exosomes [91]. The second question is regarding the status of exosomes in commercially available milk. Since commercially available milk undergoes a number of processing steps, these steps are speculated to change the exosome properties or its content. Kleinjan *et al.* compared exosomes (EVs) isolated from raw bovine milk with exosomes (EVs) from commercially available processed milk, such as pasteurized and ultra-heat treated (UHT) milk. The study concluded that the processing of milk affects the number of exosomes and their components. In UHT milk, no exosomes were detected. Although pasteurization had no effect on the number and morphology of exosomes, their protein and RNA profiles were different from those of raw milk, suggesting that industrial processing affects exosome characteristics [92].

5.2.3.2. Blood

Blood is a connective tissue that is circulated throughout the body and plays an important role in communication between various organs/parts of the body. Blood is also a central mechanism for transporting useful biological material and removing toxic substances from organs. All of these characteristics position blood as an enriched reservoir of exosomes. Indeed, blood is one of the most common bodily fluids used for isolation of exosomes. The first report of isolation of exosomes from blood came from Marie-Pierre Caby and co-workers. Their study reported the detection of exosomes in the blood plasma samples collected from 15 healthy donors. The results were substantiated by performing electron microscopy and identifying proteins characteristically present in exosomes, such as tetraspanins CD63, CD9, CD81, class I and class II MHC molecules, and Lamp-2 proteins [93]. Following this report, several studies have focused on using blood-derived exosomes in identifying novel biomarkers for cancer diagnosis and prognosis and as drug

delivery vehicle. The benefits of using blood-derived exosomes for drug delivery is that they are biocompatible and exhibit reduced immunogenicity.

Wahlgren *et al.* investigated plasma-derived exosomes as a gene delivery vector (GDV). In their study, treatment of PBMC-derived monocytes and lymphocytes with mitogen-activated protein kinase (MAPK) 1 siRNA loaded exosomes resulted in effective suppression of the MAPK1 protein expression [94]. Jakubec *et al.* reported human plasma-derived exosomes efficiently cross the blood brain barrier (BBB) and bind to endothelial cells [95]. In the same study, the authors showed that the human plasma-derived exosomes were enriched in lyso-phospholipids but lacked phosphatidylserine (PS) compared to exosomes derived from established PC3 cell line. The presence of PS on cells, an indicator of cells undergoing apoptotic death, and on micro-particles, and micro-vesicles results in their rapid clearance by macrophages and removal from circulation. Thus, the minimal to no PS presence on plasma-derived exosomes explains for their ability to evade the macrophages and have a longer circulation time *in vivo*. This offers a plausible explanation for plasma-derived exosomes being preferred over liposomes for drug delivery.

Red blood cells (RBCs) also serves as a rich source of exosomes that have been tested as drug carriers for cancer therapy. The benefit of RBC-derived exosomes is that they lack nuclear and mitochondrial DNA thus minimizing potential toxicity and elicitation of immune response. Additionally, concerns related to exosome scale-up is alleviated as exosomes can be easily isolated from blood samples, especially from group O-RBCs that are available from established blood banks. Since blood which contains billions of exosomes when transfused to patients have not resulted in any adverse events, it is accepted that the blood-derived exosomes are safe and could be used for drug delivery [96]. Using RBC-derived exosomes, delivery of antisense oligonucleotide against miR-125b resulted in suppression of leukemia and breast cell growth *in*

vitro and *in vivo* [97]. All of these results demonstrate exosomes from RBCs or plasma can be developed as drug carriers for cancer therapy.

5.2.3.4. Urine

In the previous section, we discussed the possibilities of using blood (plasma and RBCs)-derived exosomes as drug carriers. One of the current areas of emphasis in cancer research is on precision medicine that heavily relies on innovations such as noninvasive "liquid biopsy" to reduce the dependency on tumor biopsies. Urine is a valuable resource for exosomes, and studies have explored the utility of urinary exosomes for the discovery of biomarkers to detect urologic cancers, prostate, and other urinary tract malignancies [98-101]. In addition, urine, unlike blood is a relatively clean liquid, free of proteins and cells. Hence, the proteomic or molecular content of urinary exosomes is usually free of any other peripheral markers. Further, urinary exosomes are in more direct contact with tumor tissues than are blood-derived exosomes, which circulate throughout the body before they are harvested for diagnostic or therapeutic use. These urinary exosomes are supposed to closely represent the characteristics of (tumor) cell of origin and hence can be harnessed for a direct read about the tumor molecular profile and membrane proteins. In high-precision theranostics, this property can be useful for designing tumor-targeted drug delivery vehicles and identifying and characterizing tumor-specific biomarkers. Studies done with urinary exosomes isolated from bladder cancer patients and healthy controls showed that the concentration of urinary exosomes was significantly higher in urine from patients with bladder cancer than in urine from healthy individuals [102].

miRNAs have been investigated for diagnostic biomarkers in bladder cancer. Armstrong *et al.* profiled miRNAs in tumor tissues and body fluids, including urinary exosomes, in bladder cancer patients. Their study identified miR-205, miR-2003c-3p, and miR-29b-3p as candidate

biomarkers, as they showed similar profiles in tissue and urinary exosomes, suggesting that urinary exosomes can be used as a source to discover diagnostic biomarkers in bladder cancer [103]. In a similar study, urinary exosomes showed that SLC2A1, GPRC5A, and KRT17 were enriched in higher-stage bladder cancer and could be used as biomarkers [104]. Our group has extensively explored the diagnostic potential of miRNAs and proteins contained in urinary exosomes in predicting treatment outcomes in non-small cell lung cancer patients treated with chemotherapy $\pm$ radiation. We have identified a panel of miRNA signature in urinary exosomes that are being validated with clinical outcomes (*unpublished data*). In a separate ongoing study, we are investigating changes in the immune checkpoint (ICP) protein landscape in urinary exosomes isolated from lung cancer patients receiving anti-PD-1/anti-PD-L1 immunotherapy. The objective of this study is to identify changes in a set of ICP proteins during the course of the immunotherapy treatment that can be used to predict resistance and treatment failure (*unpublished data*). Besides identifying genetic regulators, enrichment of specific metabolites also occurs in the urinary exosomes from cancer patients compared with their normal counterparts. In a study of patients with prostate cancer, the levels of glucuronate, D-ribose 5-phosphate, and isobutyryl-L-carnitine were lower in all pre-prostatectomy samples compared with healthy control and post-prostatectomy samples ($p < 0.05$) [105]. It is evident from these study results that urinary exosomes can be used for cancer prognosis and diagnosis.

5.2.3.5. Saliva

Saliva is a colorless biofluid that shares many characteristics with blood and has been extensively explored to discover biomarkers. Saliva is considered significant for developing diagnostic modalities because the salivary glands are densely surrounded by blood vessels. Hence, there is an intense molecular exchange between circulating blood and salivary gland cells. This

exchange results in a significant overlap between molecular characteristics and biological information shared between them. Saliva offers many other advantages over blood; it can be easily be collected in a non-invasive manner, we can get an unlimited supply of saliva, and, importantly, unlike blood, saliva does not coagulate and can be stored and transported easily [106]. A number of studies have shown that saliva is an enriched source of exosomes containing DNA, several species of RNA, and proteins that can serve as prognostic and diagnostic biomarkers for several oral diseases, including oral cancer [107,108]. Ogawa *et al* reported presence of exosome-like vesicles in human saliva that contained dipeptidyl peptidase IV (DPP IV), galectin-3, and immunoglobulin A. The DPP IV present in the vesicles was observed to be metabolically active and able to catabolize proteins into peptides. Additionally, DPP IV, galectin-3, and immunoglobulin A are all reported to participate in regulating the immune response. The presence of these molecules in the vesicles of saliva and the role of saliva in preventing oral infection it is speculated that these exosome-like vesicles have putative role in promoting local immunity [109].

Oral squamous cell carcinoma (OSCC) is one of the most prevalent forms of oral cancer that has generally poor prognosis because it is diagnosed at an advanced stage. He *et al.* evaluated the miRNA content of salivary exosomes from OSCC patients and compared to salivary exosomes from healthy controls. A total of 109 miRNAs were found to be enriched by 2-fold and were altered in the saliva of patients with OSCC compared with healthy controls. Of significance, miR-24-3p was identified to be one of top three miRNAs enriched in OSCC exosomes (121.54 fold over control) and reported to upregulated and operate as an oncogene in head and neck squamous cell carcinoma (HNSCC). This observation was further substantiated when elevated levels of miR-24-3p were detected in OSCC neoplastic tissues, suggesting that circulating miR-24-3p may originate

from tumor cells [110]. Based on these findings, salivary exosomal mir-24-3p was indicated to serve as a biomarker in OSCC screening.

Zlotogorski-Hurvitz applied a unique approach for diagnosing oral cancer. The study used a Fourier-transform IR equipped (FTIR) and machine learning approach to study salivary exosomes and developed models to differentiate the absorbance levels of nucleic acids, proteins, and lipids that can accurately differentiate between oral cancer and healthy individuals [111]. Similarly, many other studies have shown the utility of salivary exosomes in the diagnosis of oral cancers. Therefore, the use of salivary exosomes is recognized as a promising non-invasive diagnostic modality that can accurately detect premalignant lesions and early-stage oral cancers. To describe the importance of salivary exosomes, Nonaka *et al.* coined the term "saliva-exosomics" as the next-generation salivaomics, i.e., the detailed molecular study of saliva exosomes and exploration of its molecular landscape that can be used for developing diagnostic modalities and studying the pathobiology of the disease [112]. Thus, salivary exosomes have demonstrated significance in diagnostic space, but their potential as therapeutic carriers has yet to be investigated. Nevertheless, the ability to isolate exosomes from saliva in bulk and in a non-invasive manner positions salivary exosomes as a strong candidate for developing theranostics for oral and other cancers.

6. Methods of loading therapeutics

The unique ability of exosomes to carry molecules has paved the way for their use as an alternate vehicle for carrying anticancer drugs and molecular imaging agents. Exosomes inherently have the ability to carry many molecules in their lumen and deliver to cells at distant sites in the body. Exploiting this feature for cancer therapy however requires some modifications of the exosomes to ensure they specifically reach the tumor and delivery the therapeutic with minimal off-target effects to normal cells. These reengineered exosomes referred to as “designer's

exosomes,” have been subjected to different techniques for loading molecules into their lumen. This aspect of exosome research has put them on the edge of being used as theranostics for cancer and other diseases. The various approaches undertaken to load biomolecules can be broadly classified as active or direct manipulation of exosomes or passive or indirect loading. Both methods have shown interesting results and promise.

6.1. Sonication

Sonication uses high-frequency ultrasound energy that causes agitation and partly disrupts the exosome membrane. This partial disruption of membrane helps in the diffusion of therapeutic cargoes (drugs, proteins, or small RNAs). Kim *et al* showed low water-soluble drugs like PTX can be successfully packaged into exosomes by sonication [113]. without producing any major structural changes in the exosomes that affected its function. Sonication while briefly disrupting the exosomes membrane was shown to recover quickly within one hour of post sonication when incubated at 37° C. Utilizing this approach, treatment of multi-drug resistant (MDR) murine lung cancer (3LL-M27) and Madin-Darby canine kidney (MDCK_{MDR1}) cancer cells with PTX loaded exosomes resulted in enhanced antitumor activity. Similarly, Hanney *et al* also showed superior incorporation of both hydrophilic (doxorubicin) and hydrophobic (PTX) drugs into the lumens of macrophage-derived exosomes after sonication [114]. Treatment of triple-negative breast cancer (TNBC) cells with these drug loaded exosomes resulted in antitumor activity. In another study, Li *et al.* demonstrated loading of gemcitabine (GEM) onto autologous exosomes by sonication resulted in higher drug loading efficiency and was superior over drug loading by the incubation method [115]. In their study, treatment of pancreatic cancer with GEM loaded autologous exosomes (ExoGEM) resulted in antitumor activity and increased animal survival with no measurable non-specific toxicity.

Though, sonication appears to be a straightforward method with favorable outcomes, its effect on membrane integrity and the mechanism to control the precise amount of drug loading remains a road block in developing this technique as a standard loading method for exosomes.

6.2. Electroporation

Loading of drugs, and especially hydrophobic drugs and therapeutic nucleic acids such as siRNA and miRNAs, can be significantly enhanced by using the method of electroporation. The applied electric charge disrupts the phospholipid bilayer membrane of exosomes and creates transient, temporary micro-pores on the membrane of exosomes. The micro-pores created on the membrane increase its permeability and develop an electrical potential, which allows efficient loading of drugs, nucleic acids, and nanomaterials. This method has been extensively used for loading siRNA or miRNA into exosomes. Alvarez-Elveti *et al* applied electroporation method for loading therapeutic siRNA on dendritic cell-derived exosomes [45]. In their study, the exosomal membrane protein Lamp2b was fused to the neuron-specific RVG peptide for targeted delivery to mouse brain. Using this approach, they demonstrated BACE1 specific siRNA loaded onto the Lamp2b/RGV targeted exosomes when injected intravenously produced significant reduction in BACE1 mRNA and protein expression in mice. Pomatto *et al* demonstrated that loading of antitumor miRNAs (miR-31 and miR-451a) onto plasma-derived EVs was superior to the conventional incubation method because electroporation not only showed increased efficiency of miRNA loading, but also showed better protection of miRNA from RNase degradation and did not produce changes in the characteristics of exosomes that would hamper their ability to be taken up by the target cells [116]. Similarly, Liang *et al.* showed exosomes loaded with 5-Fluorouracil (5-FU) and anti-miR for miR-21 (miR-21i) by electroporation when administered to mice bearing 5-

FU resistant colon tumors (HCT116^{5FR}) resulted in enhanced cytotoxicity and antitumor activity compared to individual treatment with 5-FU or miR-21i [117].

The major drawback of electroporation is substantial cell death caused by high voltage pulses and only partly successful membrane repair, requiring the use of more significant quantities of cells compared with the chemical transfection method. This method also requires the additional step of exosomes purification after electroporation. Aggregation of RNA and partial recovery of exosome membrane are other reported drawbacks of electroporation [118].

6.3. Freeze-thaw

Freeze-thaw is a conventional method of cellular transfection and loading of drugs in liposomes [119]. This involves incubation of exosomes and drug at room temperature and at freezing temperature (-80° C) in multiple cycles. These freeze-thaw cycles allow the membrane to permeabilize and allows the external materials (i.e., drug) to enter. This method is not very successful with exosomes and optimal conditions must be met to use this method of loading. However, Sato *et al.* used the freeze-thaw method to fuse exosomes and liposomes to develop a novel exosome mimetic. This method can be useful once it is optimized for large-scale use [120].

6.4. Click chemistry or surface functionalization

The surfaces of exosomes can be functionalized with several molecules that can be used for therapeutic or diagnostic purposes. Small molecules and macromolecules of interest can be attached to the surfaces of exosomes *via* chemical bonds, such as covalent bonds. One form of such bioconjugation is copper-catalyzed azide-alkyne cycloaddition, or click chemistry [121]. This is a relatively faster technique that is specific in nature and suitable for reaction between an alkyne and an azide to form a triazole linkage. In their study involving bioconjugation of azide-fluor 545 to mouse breast cancer cell line 4T1-derived exosomes, Smyth *et al.* found that the conjugation

was efficient and did not produce changes in the properties or structure of exosomes. [122]. Wang *et al.* proposed a copper-free click chemistry to load mouse mesenchymal stem cells derived (mMSCs) EVs (exosomes) with Alendronate (Ale), a bone-targeting ligand, and used this complex to treat osteoporosis [123]. Although little has been done on using click chemistry for loading molecules of interest on exosomes, the encouraging results produced suggest that this method can be used for loading drugs and imaging agents and developing a powerful theranostic tool.

In addition to these strategies, many other physical methods, such as extrusion, which involves breaking the exosome membrane under physical pressure and mixing the exosome solution with drug and allowing the membrane to recombine, result in drug loading. Dialysis and use of surfactants to modify the exosomal membrane for loading drugs are additional strategies that are being actively studied to improve the loading of therapeutics into exosomes.

6.5 Physical loading

6.5.1. Incubation

Like cell membranes, the exosome membrane is dynamic in nature and allows passive movement of materials across it following a concentration gradient. This property has been exploited by many studies to propose physical incubation as a more simple and straightforward strategy of cargo loading into exosomes. Hydrophobic drug curcumin has been efficiently loaded by incubating with mouse lymphoma-derived exosomes at 22° C in 1X PBS [54]. In our laboratory, we have used the incubation method to load hydrophilic drug doxorubicin (DOX) conjugated to gold nanoparticles (GNP) and tested its therapeutic efficacy in lung cancer treatment *in vitro*. The GNP-DOX loaded exosomes exhibited selective tumor cell killing with minimal toxicity towards normal cells. [59]. We further tested the imaging capability of exosomes by loading normal human lung fibroblast-derived exosomes with superparamagnetic iron oxide particles (SPIONS) and

subjected to MRI. *In vitro* phantom and cellular studies showed excellent T1 contrast images without losing the magnetic properties of the SPIONS (*unpublished data*). In addition to chemotherapeutic drugs, small nucleic acids like miRNAs have been loaded onto exosomes by direct incubation. Brynsaki *et al.* and Gong *et al.* showed successful fabrication of exosomes by loading them with miR 150 and miR 159, respectively [122, 123].

The incubation method is simple and can be adapted in any laboratory settings as it does not require special instrumentation. However, the challenge with this method is ensuring consistency in drug loading for achieving reproducible results.

6.5.2. Transfection

In the transfection method, the cargo of interest is transfected into the parent cells and the cell packages the transfected molecules into the lumens of exosomes. This is also a conventional way to load exosomes, especially with small proteins, siRNA, and miRNA molecules. Kooijmans *et al.* demonstrated transfecting the parental mouse neuroblastoma cell line, Neuro2A, with human glycosylphosphatidylinositol (GPI)-anchored protein decay-accelerating factor (GPI-DAF, or CD55) fused to targeting ligands for epidermal growth factor receptor (EGFR) resulted in Neuro2A cells producing exosomes displaying EGFR-targeting ligands on their surface [126]. Along similar lines, Cho *et al* expressed tumor-targeting human MUC1 (hMUC1) in mouse cell lines CT26 and TA3HA. Exosomes isolated from these cell lines were found to have the target MUC1 on their surface, and both autologous and allogeneic exosomes were able to stimulate the activation of immune cells and suppress hMUC1-expressing tumor growth in a MUC1-specific and dose-related manner [127]. In addition to proteins and small peptides, several studies have reported packaging of siRNA or miRNA by transfecting the parental cells, followed by isolation of the engineered exosomes.

The CRISPR/Cas9 system is a powerful tool to change the genome structure, resulting in changes in gene expression. Conventionally, the CRISPR/Cas9 system works *via* bulky plasmids. Scientists have been exploring ways to use exosomes as carriers for delivering the CRISPR/Cas9 system into the cells. To achieve this, two studies used an interesting strategy. Li *et al.* first engineered exosomes to express CD9-HuR by transfecting protein fusion constructs. The HuR is an RNA-binding protein that was used to improve the loading efficiency of dCas9 mRNA into CD9-HuR exosomes. In this process, 3×AU rich elements (AREs) were cloned downstream of the dCas9 stop codon dCas9, with AREs more efficiently encapsulated into CD9-HuR exosomes. Consistently, exosomes from the C/ebpα gRNA, CD9-HuR, and dCas9-ARE co-expressing cells significantly reduced C/ebpα expression in the recipient adipogenic stem cells and liver cells [128]. Lin *et al* using a different approach, developed hybrid exosome system by fusing HEK293FT cell-derived exosomes with liposomes for encapsulating large plasmids, including the CRISPR–Cas9 expression vectors [129]. The authors elegantly demonstrated successful loading of large nucleic acids such as the CRISPR-Cas9 by simple incubation of the plasmid with the hybrid exosomes. Addition of the CRISPR-Cas9 loaded hybrid exosomes to mesenchymal stem cells (MSCs) resulted in their expression and regulation of the target gene expression. This approach although exciting is in its infancy and additional studies are required prior to considering this gene delivery approach for clinical translation.

7. Isolation methods

Enrichment of exosomes is a bottleneck for their application in the development of theranostic or drug delivery vehicles. To have clinically viable applications and follow the requirements of precision and personalized medicine, exosomes should be isolated from bodily fluids. All of the bodily fluids described above are complex and differ widely in their composition. The presence of

proteins, lipids, or enzymes often needs a specific process to enrich exosomes. For academic purposes, isolation by ultracentrifugation method is still considered the gold standard method, but several studies still do not consider this as an ideal method. Polymer-based precipitation is the second most widely used method, but it is often unable to differentiate between various-sized particles present in the isolates. The development of an ideal therapeutic vehicle requires a uniformly sized, homogenous population of exosomes; a continuous supply of large quantities of exosome particles; and the methodology should be simple, economic and require minimum input materials. To overcome challenges and bring the various methods described above into clinics, researchers in exosome science are exploring novel ways to isolate exosomes from different resources efficiently. **Table 4** describes some such methods, but at present, no universal method for isolation has been reported in the public domain. Exosome-based biotech companies like Codiak Biosciences have claimed to have developed a platform technology for the enrichment of a homogenous population of exosomes for their use and application in the treatment human diseases including cancer. Currently, several exosomes-based products are in clinical testing and results from these clinical trials are awaited for determining their effectiveness in efficacy and treatment outcomes.

8. Conclusion and future directions

In the present article, we discussed the development and use of exosomes for cancer diagnosis and treatment. Highlighting the information presented by several studies, we present the premise of exosomes as a potential alternative to synthetic liposomes as drug carriers or delivery vehicles. Exosomes are naturally produced vesicles and thus have many advantages over synthetic delivery vehicles, which is currently the core idea behind using exosomes as drug delivery vehicles or carriers. Organotropism, non-immunogenicity, their efficient uptake by recipient cells, and the

simultaneous packaging of multiple payloads in the same vesicle are some exemplary properties of exosomes that make them suitable as drug delivery vehicles. Nevertheless, important challenges must be overcome before exosome-based delivery systems can be translated into the clinic and replace the existing successful liposome-based modalities. Pertinent questions related to efficient isolation methods for purifying homogenous population of exosomes on a large scale and identification of a particular population of exosomes that shows maximum efficiency for therapeutic loading and transportation must be explored. Since exosome science is a relatively new area of study, there is a need to enhance our understanding of the biogenesis and basic physiology of exosome synthesis so that we can develop processes for loading molecules of interest. Finally, the selection of starting material for isolation of large scale exosomes must be addressed. In this article, we present the idea of using exosomes derived from biological materials for the development of cancer theranostics, as they are non-immunogenic, exhibit organotropism and offer the opportunity for scale up and mass production. It is also worth mentioning that the scientific community have recently started exploring many of the described biological sources for exosome isolation and developing drug delivery vehicles out of them. The idea of having an 'organic exosomes' is still in its infancy but is nevertheless an attractive area to study and develop products of clinical relevance and patient care. There are several milestones yet to achieve. However, with the tremendous progress and advances made in the field of EVs, application of exosomes for cancer therapy, diagnosis and as a theranostic in the clinic is not too distant in the future.

Acknowledgments

This study was supported in part by grants received from the National Cancer Institute (NCI) of the National Institutes of Health (NIH; R01 CA167516 and R01CA233201); from the National Institute of General Medical Sciences (P20 GM103639) of the National Institutes of Health; a Merit Grant (101BX003420A1) from the Department of Veterans Affairs; a Pilot Grant and Seed Grant funded by the National Cancer Institute Cancer Center Support Grant P30CA225520 awarded to the University of Oklahoma Stephenson Cancer Center; from the Department of Defense (DOD) through the Lung Cancer Research Program (LCRP) under award no.W81XWH-18-1-0637 & W81XWH-19-1-0647; a grant (HR18-088) from the Oklahoma Center for Advanced Science and Technology (OCAST); funds received from the Presbyterian Health Foundation Seed Grant, Presbyterian Health Foundation Bridge Grant, Oklahoma Tobacco Settlement Endowment Trust (TSET) awarded to the University of Oklahoma Stephenson Cancer Center, and the Jim and Christy Everest Endowed Chair in Cancer Developmental Therapeutics.

Rajagopal Ramesh is an Oklahoma TSET Research Scholar and holds the Jim and Christy Everest Endowed Chair in Cancer Developmental Therapeutics.

The content is solely the responsibility of the authors. The opinions, interpretations, conclusions and recommendations are those of the authors and not necessarily endorsed by or representative of the official views by the NIH, DOD, Department of Veterans Affairs, or the Oklahoma Tobacco Settlement Endowment Trust.

Conflict of interest

The authors report no conflicts of interest in this work

Table 1. List of exosomes-based clinical trials completed

NCT Number	Title	Status	Conditions	Interventions	Phases
NCT02702856	Clinical Validation of a Urinary Exosome Gene Signature in Men Presenting for Suspicion of Prostate Cancer	Completed	Prostate Cancer	Exosome scoring	
NCT04529915	Multicenter Clinical Research for Early Diagnosis of Lung Cancer Using Blood Plasma Derived Exosome	Active, not recruiting	Lung Cancer	Diagnostic Test: Exosome sampling	
NCT01294072	Study Investigating the Ability of Plant Exosomes to Deliver Curcumin to Normal and Colon Cancer Tissue	Active, not recruiting	Colon Cancer	Curcumin conjugated with plant exosomes	Phase 1
NCT01159288	Trial of a Vaccination With Tumor Antigen-loaded Dendritic Cell-derived Exosomes	Completed	Non Small Cell Lung Cancer	Biological: Dex2	Phase 2

NCT01668849	Edible Plant Exosome Ability to Prevent Oral Mucositis Associated With Chemoradiation Treatment of Head and Neck Cancer	Active, not recruiting	Head and Neck Cancer and Oral Mucositis	Grape extract Drug: Lortab, Fentanyl patch, mouthwash	Phase 1
NCT03109873	Metformin Hydrochloride in Affecting Cytokines and Exosomes in Patients With Head and Neck Cancer	Completed	Larynx, Lip, Oral Cavity and Pharynx Cancer	Radiation: External Beam Radiation Therapy Drug: Metformin Hydrochloride	Early Phase 1
NCT03032913	Diagnostic Accuracy of Circulating Tumor Cells (CTCs) and Onco-exosome Quantification in the Diagnosis of Pancreatic Cancer - PANC-CTC	Completed	Pancreatic Ductal Adenocarcinoma (PDAC)	Blood samples for developing diagnostics	
NCT03488134	Predicting Prognosis and Recurrence of Thyroid Cancer Via New Biomarkers, Urinary Exosomal Thyroglobulin and Galectin-3	Active, not recruiting	Thyroid Cancer		

NCT02662621	Pilot Study With the Aim to Quantify a	Completed	Cancer	blood samples	Not
	Stress Protein in the Blood and in the			Urine samples	Applicable
	Urine for the Monitoring and Early				
	Diagnosis of Malignant Solid Tumors				
NCT03031418	Clinical Evaluation of the 'ExoDx Prostate IntelliScore' (EPI)	Completed	Cancer of Prostate	ExoDx Prostate Intelliscore	
NCT03317080	Dynamic Monitoring Circulating Tumor DNA in Surgical Patients With Lung Cancer	Active, not recruiting	Lung Cancer		
NCT02862470	Anaplastic Thyroid Cancer and Follicular Thyroid Cancer-derived Exosomal Analysis Via Treatment of Lovastatin and Vildagliptin and Pilot Prognostic Study Via Urine Exosomal Biological Markers in Thyroid Cancer Patients	Active, not recruiting	Thyroid Cancer		

NCT03791073	New Biomarkers in Pancreatic Cancer Using EXPEL Concept	Active, not recruiting	Oncology		
NCT02063464	Blood Collection From People With Ovarian Cancer	Completed	Ovarian Cancer, Cancer of the Ovary, Ovarian Neoplasms		
NCT03235687	Decision Impact Trial of the ExoDx Prostate (IntelliScore)	Active, not recruiting	Cancer of the Prostate	ExoDx Prostate (IntelliScore)	Not Applicable
NCT02921854	Detection of Circulating Biomarkers of Immunogenic Cell Death	Completed	Non Small Cell Lung Cancer	Blood withdrawal	Not Applicable
NCT02507583	Antisense102: Pilot Immunotherapy for Newly Diagnosed Malignant Glioma	Completed	Malignant Glioma, Neoplasms	Drug: IGF-1R/AS ODN; Implanted for 48 hours.	Phase 1
NCT01550523	Pilot Immunotherapy Trial for Recurrent Malignant Gliomas	Completed	Malignant Glioma of Brain	Drug: IGF-1R/AS	Phase 1

NCT02928432	SWITCH: Study of the Prednisone to Dexamethasone Change in mCRPC Patients Treated With Abiraterone	Completed	Prostate Cancer	Steroids switch	Phase 2
NCT03228277	Olmudinib Trial in T790M (+) NSCLC Patients Detected by Liquid Biopsy Using BALF Extracellular Vesicular DNA	Completed	Non Small Cell Lung Cancer	Olmudinib	Phase 2
NCT02971761	Pembrolizumab and Enobosarm in Treating Patients With Androgen Receptor Positive Metastatic Triple Negative Breast Cancer	Active, not recruiting	Androgen Receptor Positive, Estrogen Receptor Negative, HER2/Neu Negative Metastatic Triple-Negative Breast Carcinoma, Progesterone Receptor Negative,	Enobosarm Laboratory Biomarker Analysis, Biological: Pembrolizumab	Phase 2

			Stage IV Breast Cancer		
NCT00578240	Molecular Studies and Clinical Correlations in Human Prostatic Disease	Active, not recruiting	Prostate Cancer		
NCT02051101	Pathogenic Mechanisms of Port Wine Stain and Repository of Port Wine Stain Biopsy Samples	Completed	Port-Wine Stain	Biopsy sample from Port Wine Stain Birthmark	Not Applicable
NCT02535247	Study of MK-3475 Alone or in Combination With Copanlisib in Relapsed or Refractory NK and T-cell Non-Hodgkin Lymphoma	Active, not recruiting	Lymphoma, T-Cell, Peripheral	MK-3475 Drug: Copanlisib	Phase 1 Phase 2

Table 2. Types of extracellular vesicles

Type of EV	Production Mode	Size (Diameter in nm)	Morphology	References
Small EVs	MVBs fusion with plasma membrane	< 100 nm < 200 nm	Spherical	[15]
Medium/large EVs	MVBs fusion with plasma membrane	> 200 nm	Spherical	[15]
Apoptotic bodies	Blebbing	50–5,000 nm	Heterogeneous	[130]
Microvesicles	Outward budding and shedding	100–1,000 nm	Spherical	[130]
Exomeres	MVBs fusion with plasma membrane	< 50 nm	Spherical	[131]
Migrasomes	Formed during cell migration	up to 3000 nm	Pomegranate-like structures-oval shaped	[132]
Oncosomes	vesicle budding and membrane scission	1000–10,000 nm	Spherical	[133]

Table 3. Plant derived exosomes and their isolation methods

Source	Method	Study	Reference
Plant-derived Extracellular Vesicles-PDEVs (Lemon)	Ultracentrifugation and electrophoresis combined with Dialysis (named ELD)	Gastric cancer	[134]
Lemon	Differential centrifugation	Bone marrow and lung cancer	[68]
Ginger rhizome root juice	Sucrose gradient ultracentrifugation	Bio-distribution and uptake by mouse hepatocytes	[135]
Ginger juice	Ultracentrifugation	Colitis and colitis-associated colon cancer	[67]
Plant EVs: <i>D. morbifera</i> , <i>P. densiflora</i> , <i>T. occidentalis</i> , and <i>C. obtusa</i>	Filtration and concentrator	Breast and skin cancer	[136]
Algae	Ultracentrifugation	Isolation and characterization	[70]
apoplastic fluids of Arabidopsis (<i>Arabidopsis</i>	Ultracentrifugation	PEN protein involved biotic and abiotic stress	[137]

<i>thaliana</i>)			
leaves			
Grapefruit juice	Differential centrifugation	Colon cancer	[66]
Grapefruit juice	Sucrose gradient centrifugation	Colitis in mice	[139]
Grapefruit juice	Differential centrifugation	Colon cancer mouse model	[139]
Arabidopsis <i>thaliana</i> and Brassicaceae vegetables	Sucrose gradient centrifugation	Characterization of exosomes	[140]
Herbal plants		Transfer of stable (s)RNA for treatment of lung fibrosis and inflammation	[65]
Broccoli	Centrifugation and high pressure filtration	Mouse colitis	[141]

Table 4. Exosome isolation methods

Isolation Techniques	Source	Advantages	Disadvantages	References
Differential centrifugation	Cell line	Isolation from large sample volumes, Simple method	Time-consuming. Requires expensive equipment	[142]
Density-gradient centrifugation	Cell line	High purity without the requirement of additional reagent	Time-consuming and complex procedure	[143]
Size-exclusion chromatography	Plasma	High yield and purity	Not suitable for large sample volumes. Time-consuming	[144]
Ultrafiltration	Cell line, plasma	No requirement of additional reagent Simple procedure	Risk of contamination Sample loss	[145]
Sequential filtration	Cell line	High purity Homogeneous exosome size	Time-consuming, requires skilled operator. Expensive	[146]
Hydrostatic Filtration Dialysis (HFD)	Urine	Minimum interference of soluble proteins. High yield	Time-consuming	[147]

Tangential flow filtration (TFF)	Stem Cells	Higher yield of exosomes. Homogeneous exosome Size	Time-consuming Expensive	[148]
Fractionation	Stem cells	Homogeneous exosome size	Expensive	[149]
Flow Field-Flow Fractionation (FFFF)	Stem Cells	Homogeneous exosome size. Less requirement of starting material	Expensive	[149]
Magneto- immunoprecipitation	Plasma, Cell line	Quick Method. No requirement of expensive instrument	Less Yield	[150]
Polymer-based precipitation	Serum	Easy to use Biological activity of exosomes is retained	Risk of contamination. Interference of contaminants during detection	[151]
Lectin-induced agglutination	Cell line, Urine	Less expensive	Co-precipitation of other soluble components may happen	[152]

Microfluidic-Based Isolation Techniques	Blood	Rapid, requires of input	Expensive	[153]
Acoustic Nanofilter	Red blood cells	Simple and quick method	In developmental stage	[154]
ExoQuick Method	Serum	Less time-consuming	Expensive Not suitable for large volume of sample	[155]
Deterministic lateral displacement (DLD) pillar array	Urine	Inexpensive and sensitive method	Technology still in developmental stage	[156]
Integrated double-filtration microfluidic device	Urine	Less expensive	Not validated for clinical use	[156]
On-chip immunoelectrophoresis	Cell line	EVs and their subpopulation can be separated	Technology still in developmental stage	[158]
Sepharose and HiTrap Heparin HP column	Serum	Selective isolation of exosomes		[148]
Cation- and anion-exchange chromatography	Cell line	Requires less time	Not suitable for all samples	[159]

Figure Legends

Figure 1. Different types of extracellular vesicles produced by the cell. These vesicles are categorized based on their mode of synthesis and size. Figure created with BioRender.com

Figure 2. Schematic to show the synthesis of nanosomes. An exosome-based hybrid delivery system consisting of cell-derived exosomes and gold nanoparticles carrying doxorubicin was synthesized for lung cancer treatment. The bottom panel shows TEM images of each step of synthesis.

Figure modified from: Srivastava, A., Amreddy, N., Babu, A., Panneerselvam, J., Mehta, M., Muralidharan, R., et al. (2016). Nanosomes carrying doxorubicin exhibit potent anticancer activity against human lung cancer cells. *Scientific Reports*, 6, 38541.

<https://doi.org/10.1038/srep38541>. Copyright © 2016 Rights Managed by Nature Publishing Group.

References

- [1] Senapati, S., Mahanta, A. K., Kumar, S., & Maiti, P. (2018). Controlled drug delivery vehicles for cancer treatment and their performance. *Signal transduction and targeted therapy*, 3, 7. doi:10.1038/s41392-017-0004-3
- [2] Li, Z., Tan, S., Li, S., Shen, Q., & Wang, K. (2017). Cancer drug delivery in the nano era: An overview and perspectives (Review). *Oncology reports*, 38(2), 611–24. doi:10.3892/or.2017.5718
- [3] Chen, F., Shi, Y., Zhang, J., & Liu, Q. (2020). Nanoparticle-based drug delivery systems for targeted epigenetics cancer therapy. *Current drug targets*, 21(11), 1084–98. doi:10.2174/1389450121666200514222900
- [4] Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S. W., Zarghami, N., Hanifepour, Y. et al. (2013). Liposome: classification, preparation, and applications. *Nanoscale research letters*, 8(1), 102, doi:10.1186/1556-276X-8-102
- [5] Sercombe, L., Veerati, T., Moheimani, F., Wu, S. Y., Sood, A. K., & Hua, S. (2015). Advances and challenges of liposome assisted drug delivery. *Frontiers in pharmacology*, 6, 286, doi:10.3389/fphar.2015.00286
- [6] Almeida, B., Nag, O. K., Rogers, K. E., & Delehanty, J. B. (2020). Recent progress in bioconjugation strategies for liposome-mediated drug delivery. *Molecules (Basel, Switzerland)*, 25(23), 5672, doi:10.3390/molecules25235672
- [7] Pillai, G. (2014). Nanomedicines for cancer therapy: An update of FDA approved and those under various stages of development. *SOJ Pharmacy & pharmaceutical sciences*, 1(2), 13, doi:10.15226/2374-6866/1/2/00109

- [8] Yue, X., & Dai, Z. (2014). Recent advances in liposomal nanohybrid cerasomes as promising drug nanocarriers. *Advances in colloid and interface science*, 207, 32–42, doi:10.1016/j.cis.2013.11.014
- [9] Raemdonck, K., Braeckmans, K., Demeester, J., & De Smedt, S. C. (2014). Merging the best of both worlds: hybrid lipid-enveloped matrix nanocomposites in drug delivery. *Chemical society reviews*, 43(1), 444–72, doi:10.1039/c3cs60299k
- [10] Shao, J., Zaro, J., & Shen, Y. (2020). Advances in exosome-based drug delivery and tumor targeting: from tissue distribution to intracellular fate. *International journal of nanomedicine*, 15, 9355–71, doi:10.2147/IJN.S281890
- [11] Bongiovanni, L., Andriessen, A., Wauben, M., Hoen, E., & de Bruin, A. (2021). Extracellular Vesicles: novel opportunities to understand and detect neoplastic diseases. *Veterinary pathology*, 58(3), 453–71, doi:10.1177/0300985821999328
- [12] Abels, E. R., & Breakefield, X. O. (2016). Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. *Cellular and molecular neurobiology*, 36(3), 301–12, doi:10.1007/s10571-016-0366-z
- [13] György, B., Szabó, T. G., Pásztói, M., Pál, Z., Misják, P., Aradi, B., et al. (2011). Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cellular and molecular life sciences*, 68(16), 2667–88, doi:10.1007/s00018-011-0689-3
- [14] Ailuno, G., Baldassari, S., Lai, F., Florio, T., & Caviglioli, G. (2020). Exosomes and extracellular vesicles as emerging theranostic platforms in cancer research. *Cells*, 9(12), 2569, doi:10.3390/cells9122569
- [15] Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R. et al. (2018). Minimal information for studies of extracellular vesicles 2018

- (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of extracellular vesicles*, 7(1), 1535750. doi:10.1080/20013078.2018.1535750
- [16] Emelyanov, A., Shtam, T., Kamyshinsky, R., Garaeva, L., Verlov, N., Miliukhina, I., et al. (2020). Cryo-electron microscopy of extracellular vesicles from cerebrospinal fluid. *PloS one*, 15(1), e0227949, doi:10.1371/journal.pone.0227949
- [17] Srivastava, A., Filant, J., Moxley, K. M., Sood, A., McMeekin, S., & Ramesh, R. (2015). Exosomes: a role for naturally occurring nanovesicles in cancer growth, diagnosis and treatment. *Current gene therapy*, 15(2), 182–92, doi:10.2174/1566523214666141224100612
- [18] Mathivanan, S., Ji, H., & Simpson, R. J. (2010). Exosomes: extracellular organelles important in intercellular communication. *Journal of proteomics*, 73(10), 1907–20, doi:10.1016/j.jprot.2010.06.006
- [19] Andreu, Z., & Yáñez-Mó, M. (2014). Tetraspanins in extracellular vesicle formation and function. *Frontiers in immunology*, 5, 442, doi:org/10.3389/fimmu.2014.00442
- [20] Makler, A., & Asghar, W. (2020). Exosomal biomarkers for cancer diagnosis and patient monitoring. *Expert review of molecular diagnostics*, 20(4), 387–400, doi:10.1080/14737159.2020.1731308
- [21] Subra, C., Laulagnier, K., Perret, B., & Record, M. (2007). Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. *Biochimie*, 89(2), 205–12. doi:10.1016/j.biochi.2006.10.014.
- [22] Soung, Y. H., Ford, S., Zhang, V., & Chung, J. (2017). Exosomes in cancer diagnostics. *Cancers*, 9(1), 8, doi:10.3390/cancers9010008

- [23] Li, W., Li, C., Zhou, T., Liu, X., Liu, X., Li, X., & Chen, D. (2017). Role of exosomal proteins in cancer diagnosis. *Molecular cancer*, 16(1), 145, doi:10.1186/s12943-017-0706-8
- [24] Srivastava, A., Amreddy, N., Pareek, V., Chinnappan, M., Ahmed, R., Mehta, M. et al. (2020). Progress in extracellular vesicle biology and their application in cancer medicine. *Wiley interdisciplinary reviews. nanomedicine and nanobiotechnology*, 12(4), e1621. doi:10.1002/wnan.1621
- [25] Mashouri, L., Yousefi, H., Aref, A. R., Ahadi, A. M., Molaei, F., & Alahari, S. K. (2019). Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. *Molecular cancer*, 18(1), 75, doi:10.1186/s12943-019-0991-5
- [26] Gurung, S., Perocheau, D., Touramanidou, L., & Baruteau, J. (2021). The exosome journey: from biogenesis to uptake and intracellular signaling. *Cell communication and signaling*, 19(1), 47. doi:10.1186/s12964-021-00730-1
- [27] Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P. et al. (2013). Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *Journal of cell science*, 126(Pt 24), 5553–65, doi:10.1242/jcs.128868
- [28] Blanc, L., & Vidal, M. (2018). New insights into the function of Rab GTPases in the context of exosomal secretion. *Small GTPases*, 9(1-2), 95–106, doi:10.1080/21541248.2016.1264352
- [29] Hsu, C., Morohashi, Y., Yoshimura, S., Manrique-Hoyos, N., Jung, S., Lauterbach, M. A. et al. M. (2010). Regulation of exosome secretion by Rab35 and its GTPase-activating

- proteins TBC1D10A-C. *The Journal of cell biology*, 189(2), 223–32, doi:10.1083/jcb.200911018
- [30] Bebelman, M. P., Smit, M. J., Pegtel, D. M., & Baglio, S. R. (2018). Biogenesis and function of extracellular vesicles in cancer. *Pharmacology & therapeutics*, 188, 1–11. doi:10.1016/j.pharmthera.2018.02.013
- [31] Ghoroghi, S., Mary, B., Larnicol, A., Asokan, N., Klein, A., Osmani, N., et al. V. (2021). Ral GTPases promote breast cancer metastasis by controlling biogenesis and organ targeting of exosomes. *eLife*, 10, e61539, doi:10.7554/eLife.61539
- [32] Gurunathan, S., Kang, M. H., & Kim, J. H. (2021). A Comprehensive review on factors influences biogenesis, functions, therapeutic and clinical implications of exosomes. *International journal of nanomedicine*, 16, 1281–312, doi:10.2147/IJN.S291956
- [33] Kumar, A., & Deep, G. (2020). Hypoxia in tumor microenvironment regulates exosome biogenesis: molecular mechanisms and translational opportunities. *Cancer letters*, 479, 23–30, doi:10.1016/j.canlet.2020.03.017
- [34] Wei, D., Zhan, W., Gao, Y., Huang, L., Gong, R., Wang, W. et al. (2021). RAB31 marks and controls an ESCRT-independent exosome pathway. *Cell research*, 31(2), 157–77. doi:10.1038/s41422-020-00409-1
- [35] Wei, H., Chen, Q., Lin, L., Sha, C., Li, T., Liu, Y. et al. (2021). Regulation of exosome production and cargo sorting. *International journal of biological sciences*, 17(1), 163–77. doi:10.7150/ijbs.53671
- [36] Li, S. P., Lin, Z. X., Jiang, X. Y., & Yu, X. Y. (2018). Exosomal cargo-loading and synthetic exosome-mimics as potential therapeutic tools. *Acta pharmacologica sinica*, 39(4), 542–51, doi:10.1038/aps.2017.178

- [37] Skryabin, G. O., Komelkov, A. V., Savelyeva, E. E., & Tchevkina, E. M. (2020). Lipid rafts in exosome biogenesis. *Biochemistry moscow*, 85(2), 177–91, doi:10.1134/S0006297920020054
- [38] Kowal, J., Tkach, M., & Théry, C. (2014). Biogenesis and secretion of exosomes. *Current opinion in cell biology*, 29, 116–25, doi:10.1016/j.ceb.2014.05.004
- [39] Elkhoury, K., Koçak, P., Kang, A., Arab-Tehrany, E., Ellis Ward, J., & Shin, S. R. (2020). Engineering smart targeting nanovesicles and their combination with hydrogels for controlled drug delivery. *Pharmaceutics*, 12(9), 849. doi:10.3390/pharmaceutics12090849
- [40] Antimisiaris, S. G., Mourtas, S., & Marazioti, A. (2018). Exosomes and exosome-inspired vesicles for targeted drug delivery. *Pharmaceutics*, 10(4), 218, doi:10.3390/pharmaceutics10040218
- [41] Zhang, Y., Liu, Y., Liu, H., & Tang, W. H. (2019). Exosomes: biogenesis, biologic function and clinical potential. *Cell & bioscience*, 9, 19, doi:10.1186/s13578-019-0282-2
- [42] Jafari, D., Shajari, S., Jafari, R., Mardi, N., Gomari, H., Ganji, F. et al. (2020). Designer exosomes: a new platform for biotechnology therapeutics. *BioDrugs*, 34(5), 567–86. doi:10.1007/s40259-020-00434-x
- [43] Kamerkar, S., LeBleu, V. S., Sugimoto, H., Yang, S., Ruivo, C. F., Melo, S. A. et al. (2017). Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature*, 546(7659), 498–503. doi:10.1038/nature22341
- [44] Kibria, G., Ramos, E. K., Wan, Y., Gius, D. R., & Liu, H. (2018). Exosomes as a drug delivery system in cancer therapy: potential and challenges. *Molecular pharmaceutics*, 15(9), 3625–633. doi:10.1021/acs.molpharmaceut.8b0027

- [45] Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhali, S., & Wood, M. J. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nature biotechnology*, 29(4), 341–45. doi:10.1038/nbt.1807
- [46] Pitt, J. M., André, F., Amigorena, S., Soria, J. C., Eggermont, A., Kroemer, G. et al. (2016). Dendritic cell-derived exosomes for cancer therapy. *The journal of clinical investigation*, 126(4), 1224–32, doi:10.1172/JCI81137
- [47] Pascucci, L., Coccè, V., Bonomi, A., Ami, D., Ceccarelli, P., Ciusani, E., et al. (2014). Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit in vitro tumor growth: a new approach for drug delivery. *Journal of controlled release*, 192, 262–70, doi:10.1016/j.jconrel.2014.07.042
- [48] Kalimuthu, S., Gangadaran, P., Rajendran, R. L., Zhu, L., Oh, J. M., Lee, H. W. et al. (2018). A new approach for loading anticancer drugs into mesenchymal stem cell-derived exosome mimetics for cancer therapy. *Frontiers in pharmacology*, 9, 1116. doi:10.3389/fphar.2018.01116
- [49] Munoz, J. L., Bliss, S. A., Greco, S. J., Ramkissoon, S. H., Ligon, K. L., & Rameshwar, P. (2013). Delivery of functional anti-miR-9 by mesenchymal stem cell-derived exosomes to glioblastoma multiforme cells conferred chemosensitivity. *Molecular therapy nucleic acids*, 2(10), e126. doi:10.1038/mtna.2013.6
- [50] Sun, Y., & Liu, J. (2014). Potential of cancer cell-derived exosomes in clinical application: a review of recent research advances. *Clinical therapeutics*, 36(6), 863–72. doi:10.1016/j.clinthera.2014.04.018

- [51] Qiao, L., Hu, S., Huang, K., Su, T., Li, Z., Vandergriff, A. et al. (2020). Tumor cell-derived exosomes home to their cells of origin and can be used as Trojan horses to deliver cancer drugs. *Theranostics*, 10(8), 3474–87. doi:10.7150/thno.39434
- [52] Morishita, M., Takahashi, Y., Matsumoto, A., Nishikawa, M., & Takakura, Y. (2016). Exosome-based tumor antigens-adjuvant co-delivery utilizing genetically engineered tumor cell-derived exosomes with immunostimulatory CpG DNA. *Biomaterials*, 111, 55–65. doi:10.1016/j.biomaterials.2016.09.031
- [53] Rong, L., Li, R., Li, S., & Luo, R. (2016). Immunosuppression of breast cancer cells mediated by transforming growth factor- β in exosomes from cancer cells. *Oncology letters*, 11(1), 500–04. doi:10.3892/ol.2015.3841
- [54] Ha, D., Yang, N., & Nadithe, V. (2016). Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. *Acta pharmaceutica sinica. B*, 6(4), 287–96, doi:10.1016/j.apsb.2016.02.001
- [55] Sun, D., Zhuang, X., Xiang, X., Liu, Y., Zhang, S., Liu, C. et al. (2010). A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Molecular therapy* 18(9), 1606–14. doi:10.1038/mt.2010.105
- [56] Ohno, S., Takanashi, M., Sudo, K., Ueda, S., Ishikawa, A., Matsuyama, N., et al. (2013). Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Molecular therapy*, 21(1), 185–91, doi:10.1038/mt.2012.180
- [57] Limoni, S. K., Moghadam, M. F., Moazzeni, S. M., Gomari, H., & Salimi, F. (2019). Engineered exosomes for targeted transfer of siRNA to HER2 positive breast cancer cells.

- Applied biochemistry and biotechnology, 187(1), 352–64. doi:10.1007/s12010-018-2813-4
- [58] Schindler, C., Collinson, A., Matthews, C., Pointon, A., Jenkinson, L., Minter, R. R. et al. (2019). Exosomal delivery of doxorubicin enables rapid cell entry and enhanced in vitro potency. *PloS one*, 14(3), e0214545, doi:10.1371/journal.pone.0214545
- [59] Srivastava, A., Amreddy, N., Babu, A., Panneerselvam, J., Mehta, M., Muralidharan, R. et al. (2016). Nanosomes carrying doxorubicin exhibit potent anticancer activity against human lung cancer cells. *Scientific reports*, 6, 38541. doi:10.1038/srep38541
- [60] Thippabhotla, S., Zhong, C., & He, M. (2019). 3D cell culture stimulates the secretion of in vivo like extracellular vesicles. *Scientific reports*, 9(1), 13012, doi:10.1038/s41598-019-49671-3.
- [61] Abdollahi S. (2021). Extracellular vesicles from organoids and 3D culture systems. *Biotechnology and bioengineering*, 118(3), 1029–49, doi:10.1002/bit.27606
- [62] Yu, L., Deng, Z., Liu, L., Zhang, W., & Wang, C. (2020). Plant-derived nanovesicles: a novel form of nanomedicine. *Frontiers in bioengineering and biotechnology*, 8, 584391. <https://doi.org/10.3389/fbioe.2020.584391>
- [63] Zhang, M., Xiao, B., Wang, H., Han, M. K., Zhang, Z., Viennois, E. et al. (2016). Edible ginger-derived nano-lipids loaded with doxorubicin as a novel drug-delivery approach for colon cancer therapy. *Molecular therapy* 24(10), 1783–96, doi:10.1038/mt.2016.159
- [64] Yang, C., Zhang, M., & Merlin, D. (2018). Advances in plant-derived edible nanoparticle-based lipid nano-drug delivery systems as therapeutic nanomedicines. *Journal of materials chemistry*, 6(9), 1312–1321, doi:10.1039/C7TB03207B

- [65] Li, X., Liang, Z., Du, J., Wang, Z., Mei, S., Li, Z. et al. (2019). Herbal decoctosome is a novel form of medicine. *Science china. life sciences*, 62(3), 333–48, doi:10.1007/s11427-018-9508-0
- [66] Garaeva, L., Kamyshinsky, R., Kil, Y., Varfolomeeva, E., Verlov, N., Komarova, E., et al. (2021). Delivery of functional exogenous proteins by plant-derived vesicles to human cells in vitro. *Scientific reports*, 11(1), 6489, doi:10.1038/s41598-021-85833-y
- [67] Zhang, M., Viennois, E., Xu, C., & Merlin, D. (2016). Plant derived edible nanoparticles as a new therapeutic approach against diseases. *Tissue barriers*, 4(2), e1134415. doi:10.1080/21688370.2015.1134415
- [68] Raimondo, S., Naselli, F., Fontana, S., Monteleone, F., Lo Dico, A., Saieva, L. et al. (2015). Citrus limon-derived nanovesicles inhibit cancer cell proliferation and suppress CML xenograft growth by inducing TRAIL-mediated cell death. *Oncotarget*, 6(23), 19514–27. doi:10.18632/oncotarget.4004
- [69] Cao, M., Yan, H., Han, X., Weng, L., Wei, Q., Sun, X. et al. (2019). Ginseng-derived nanoparticles alter macrophage polarization to inhibit melanoma growth. *Journal for immunotherapy of cancer*, 7(1), 326, doi:10.1186/s40425-019-0817-4
- [70] Kuruvinashetti, K., Pakkiriswami, S, & Packirisamy, M. (2020) Algal extracellular vesicles for therapeutic applications. *IEEE 20th international conference on nanotechnology (IEEE-NANO)*, pp. 354-357, doi: 10.1109/NANO47656.2020.9183452.
- [71] Deatherage, B. L., & Cookson, B. T. (2012). Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. *Infection and immunity*, 80(6), 1948–57 doi:10.1128/IAI.06014-11

- [72] Kuipers, M. E., Hokke, C. H., Smits, H. H., & Nolte-'t Hoen, E. (2018). Pathogen-derived extracellular vesicle-associated molecules that affect the host immune system: An overview. *Frontiers in microbiology*, 9, 2182. doi:10.3389/fmicb.2018.02182
- [73] Brown, L., Wolf, J. M., Prados-Rosales, R., & Casadevall, A. (2015). Through the wall: extracellular vesicles in gram-positive bacteria, mycobacteria and fungi. *Nature reviews. microbiology*, 13(10), 620–30. doi:10.1038/nrmicro3480
- [74] Yu, Y. J., Wang, X. H., & Fan, G. C. (2018). Versatile effects of bacterium-released membrane vesicles on mammalian cells and infectious/inflammatory diseases. *Acta pharmacologica sinica*, 39(4), 514–33. doi:10.1038/aps.2017.82
- [75] de Souza, W., & Barrias, E. S. (2020). Membrane-bound extracellular vesicles secreted by parasitic protozoa: cellular structures involved in the communication between cells. *Parasitology research*, 119(7), 2005–23. doi:10.1007/s00436-020-06691-7
- [76] Szempruch, A. J., Dennison, L., Kieft, R., Harrington, J. M., & Hajduk, S. L. (2016). Sending a message: extracellular vesicles of pathogenic protozoan parasites. *Nature reviews microbiology*, 14(11), 669–75, doi:10.1038/nrmicro.2016.110
- [77] Rizzo, J., Rodrigues, M. L., & Janbon, G. (2020). Extracellular vesicles in fungi: past, present, and future perspectives. *Frontiers in cellular and infection microbiology*, 10, 346. doi:10.3389/fcimb.2020.00346
- [78] Samuel, M., Bleackley, M., Anderson, M., & Mathivanan, S. (2015). Extracellular vesicles including exosomes in cross kingdom regulation: a viewpoint from plant-fungal interactions. *Frontiers in plant science*, 6, 766. doi:10.3389/fpls.2015.00766

- [79] Melnik, B. C., John, S. M., & Schmitz, G. (2013). Milk is not just food but most likely a genetic transfection system activating mTORC1 signaling for postnatal growth. *Nutrition journal*, 12, 103. <https://doi.org/10.1186/1475-2891-12-103>
- [80] Samuel, M., Chisanga, D., Liem, M., Keerthikumar, S., Anand, S., Ang, C. S. et al. (2017). Bovine milk-derived exosomes from colostrum are enriched with proteins implicated in immune response and growth. *Scientific reports*, 7(1), 5933, doi:10.1038/s41598-017-06288-8
- [81] Sanwlani, R., Fonseka, P., Chitti, S. V., & Mathivanan, S. (2020). Milk-derived extracellular vesicles in inter-organism, cross-species communication and drug delivery. *Proteomes*, 8(2), 11, doi:10.3390/proteomes8020011
- [82] Betker, J. L., Angle, B. M., Graner, M. W., & Anchordoquy, T. J. (2019). The potential of exosomes from cow milk for oral delivery. *Journal of pharmaceutical sciences*, 108(4), 1496–505. doi:10.1016/j.xphs.2018.11.022
- [83] Munagala, R., Aqil, F., Jeyabalan, J., & Gupta, R. C. (2016). Bovine milk-derived exosomes for drug delivery. *Cancer letters*, 371(1), 48–61. doi:10.1016/j.canlet.2015.10.020
- [84] Zhang, Q.; Xiao, Q.; Yin, H.; Xia, C.; Pu, Y.; He, Z. et al. (2020). Milk-exosome based pH/light sensitive drug system to enhance anticancer activity against oral squamous cell carcinoma. *RSC adv.* 10, 28314– 23, doi: 10.1039/D0RA05630H
- [85] Rahman, M. M., Shimizu, K., Yamauchi, M., Takase, H., Ugawa, S., Okada, A. et al. (2019). Acidification effects on isolation of extracellular vesicles from bovine milk. *PloS one*, 14(9), e0222613, doi:10.1371/journal.pone.0222613

- [86] Warren, M. R., Zhang, C., Vedadghavami, A., Bokvist, K., Dhal, P. K., & Bajpayee, A. G. (2020). Milk exosomes with enhanced mucus penetrability for oral delivery of siRNA. *Biomaterials science*, 10.1039/d0bm01497d, doi:10.1039/d0bm01497d
- [87] Shandilya, S., Rani, P., Onteru, S. K., & Singh, D. (2020). Natural ligand-receptor mediated loading of siRNA in milk derived exosomes. *Journal of biotechnology*, 318, 1–9. doi:10.1016/j.jbiotec.2020.04.015
- [88] Aqil, F., Munagala, R., Jeyabalan, J., Agrawal, A. K., Kyakulaga, A. H., Wilcher, S. A. et al. (2019). Milk exosomes - natural nanoparticles for siRNA delivery. *Cancer letters*, 449, 186–95, doi:10.1016/j.canlet.2019.02.011
- [89] Del Pozo-Acebo, L., Hazas, M., Tomé-Carneiro, J., Gil-Cabrero, P., San-Cristobal, R., Busto, R. et al. (2021). Bovine milk-derived exosomes as a drug delivery vehicle for miRNA-based therapy. *International journal of molecular sciences*, 22(3), 1105. doi:10.3390/ijms22031105
- [90] Manca, S., Upadhyaya, B., Mutai, E., Desaulniers, A. T., Cederberg, R. A., White, B. R. et al. (2018). Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. *Scientific reports*, 8(1), 11321, doi:10.1038/s41598-018-29780-1
- [91] Somiya, M., Yoshioka, Y., & Ochiya, T. (2018). Biocompatibility of highly purified bovine milk-derived extracellular vesicles. *Journal of extracellular vesicles*, 7(1), 1440132. doi:10.1080/20013078.2018.1440132
- [92] Kleinjan, M., van Herwijnen, M. J., Libregts, S. F., van Neerven, R. J., Feitsma, A. L., & Wauben, M. H. (2021). Regular industrial processing of bovine milk impacts the integrity

- and molecular composition of extracellular vesicles. The Journal of nutrition, doi:10.1093/jn/nxab031
- [93] Caby, M. P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G., & Bonnerot, C. (2005). Exosomal-like vesicles are present in human blood plasma. International immunology, 17(7), 879–87, doi:10.1093/intimm/dxh267
- [94] Wahlgren, J., De L Karlson, T., Brisslert, M., Vaziri Sani, F., Telemo, E., Sunnerhagen, P. et al. (2012). Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. Nucleic acids research, 40(17), e130, doi:10.1093/nar/gks463
- [95] Jakubec, M., Maple-Grødem, J., Akbari, S., Nesse, S., Halskau, Ø., & Mork-Jansson, A. E. (2020). Plasma-derived exosome-like vesicles are enriched in lyso-phospholipids and pass the blood-brain barrier. PloS one, 15(9), e0232442. doi:10.1371/journal.pone.0232442
- [96] Aslan, C., Kiaie, S. H., Zolbanin, N. M., Lotfinejad, P., Ramezani, R., Kashanchi (2021). Exosomes for mRNA delivery: a novel biotherapeutic strategy with hurdles and hope. BMC biotechnology, 21(1), 20, doi:10.1186/s12896-021-00683-w
- [97] Usman, W.M., Pham, T.C., Kwok, Y.Y. *et al.* (2018). Efficient RNA drug delivery using red blood cell extracellular vesicles. Nature Communications, 9, 2359. <https://doi.org/10.1038/s41467-018-04791-8>
- [98] Franzen, C. A., Blackwell, R. H., Foreman, K. E., Kuo, P. C., Flanigan, R. C., & Gupta, G. N. (2016). Urinary exosomes: the potential for biomarker utility, intercellular signaling and therapeutics in urological malignancy. The Journal of urology, 195(5), 1331–39. doi:10.1016/j.juro.2015.08.115

- [99] Lourenço, C., Constâncio, V., Henrique, R., Carvalho, Â., & Jerónimo, C. (2021). Urinary extracellular vesicles as potential biomarkers for urologic cancers: an overview of current methods and advances. *Cancers*, 13(7), 1529, doi:10.3390/cancers13071529
- [100] Erozeñci, L. A., Böttger, F., Bijnsdorp, I. V., & Jimenez, C. R. (2019). Urinary exosomal proteins as (pan-) cancer biomarkers: insights from the proteome. *FEBS letters*, 593(13), 1580–97. doi:10.1002/1873-3468.13487
- [101] Kang, J. S. (2020). The potential of exosomes as theragnostics in various clinical situations. *Exosomes*, 467–86. doi:10.1016/B978-0-12-816053-4.00020-1
- [102] Panfoli I. (2017) Cancer exosomes in urine: a promising biomarker source. *Translational cancer research*; 6(Suppl 8):S1389-S1393. doi: 10.21037/tcr.2017.10.17
- [103] Armstrong, D. A., Green, B. B., Seigne, J. D., Schned, A. R., & Marsit, C. J. (2015). MicroRNA molecular profiling from matched tumor and bio-fluids in bladder cancer. *Molecular cancer*, 14, 194, doi:10.1186/s12943-015-0466-2
- [104] Chen, C. K., Liao, J., Li, M. S., & Khoo, B. L. (2020). Urine biopsy technologies: Cancer and beyond. *Theranostics*, 10(17), 7872–88. doi:10.7150/thno.44634
- [105] Puhka, M., Takatalo, M., Nordberg, M. E., Valkonen, S., Nandania, J., Aatonen, M. et al. (2017). Metabolomic profiling of extracellular vesicles and alternative normalization methods reveal enriched metabolites and strategies to study prostate cancer-related changes. *Theranostics*, 7(16), 3824–41. doi:10.7150/thno.19890
- [106] Cheng, J., Nonaka, T., & Wong, D. (2019). Salivary exosomes as nanocarriers for cancer biomarker delivery. *Materials (Basel, Switzerland)*, 12(4), 654. doi:10.3390/ma12040654
- [107] Nair, S., Tang, K. D., Kenny, L., & Punyadeera, C. (2018). Salivary exosomes as potential biomarkers in cancer. *Oral oncology*, 84, 31–40. doi:10.1016/j.oraloncology.2018.07.001

- [108] Zhan, C., Yang, X., Yin, X., & Hou, J. (2020). Exosomes and other extracellular vesicles in oral and salivary gland cancers. *Oral diseases*, 26(5), 865–75. doi:10.1111/odi.13172
- [109] Ogawa, Y., Kanai-Azuma, M., Akimoto, Y., Kawakami, H., Yanoshita, R. (2008). Exosome-like vesicles with dipeptidyl peptidase IV in human saliva. *Biol Pharm Bull.* Jun;31(6):1059-62. doi: 10.1248/bpb.31.1059.
- [110] He, L., Ping, F., Fan, Z., Zhang, C., Deng, M., Cheng, B. et al. (2020). Salivary exosomal miR-24-3p serves as a potential detective biomarker for oral squamous cell carcinoma screening. *Biomedicine & pharmacotherapy*, 121, 109553. doi:10.1016/j.biopha.2019.109553
- [111] Zlotogorski-Hurvitz, A., Dekel, B. Z., Malonek, D., Yahalom, R., & Vered, M. (2019). FTIR-based spectrum of salivary exosomes coupled with computational-aided discriminating analysis in the diagnosis of oral cancer. *Journal of cancer research and clinical oncology*, 145(3), 685–94. doi:10.1007/s00432-018-02827-6
- [112] Nonaka, T., & Wong, D. (2017). Saliva-exosomics in cancer: Molecular characterization of cancer-derived exosomes in saliva. *The Enzymes*, 42, 125–151. doi:10.1016/bs.enz.2017.08.002
- [113] Kim, M. S., Haney, M. J., Zhao, Y., Mahajan, V., Deygen, I., Klyachko, N. L. et al. (2016). Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine: nanotechnology, biology, and medicine*, 12(3), 655–64. doi:10.1016/j.nano.2015.10.012
- [114] Haney, M. J., Zhao, Y., Jin, Y. S., Li, S. M., Bago, J. R., Klyachko, N. L. et al. (2020). Macrophage-derived extracellular vesicles as drug delivery systems for triple negative

- breast cancer (TNBC) therapy. *Journal of neuroimmune pharmacology* 15(3), 487–500. doi:10.1007/s11481-019-09884-9
- [115] Li, Y. J., Wu, J. Y., Wang, J. M., Hu, X. B., Cai, J. X., & Xiang, D. X. (2020). Gemcitabine loaded autologous exosomes for effective and safe chemotherapy of pancreatic cancer. *Acta biomaterialia*, 101, 519–30. doi:10.1016/j.actbio.2019.10.022
- [116] Pomatto, M., Bussolati, B., D'Antico, S., Ghiotto, S., Tetta, C., Brizzi, M. F. et al. (2019). Improved loading of plasma-derived extracellular vesicles to encapsulate antitumor miRNAs. *Molecular therapy. Methods & clinical development*, 13, 133–44. doi:10.1016/j.omtm.2019.01.001
- [117] Liang, G., Zhu, Y., Ali, D. J., Tian, T., Xu, H., Si, K. et al. (2020). Engineered exosomes for targeted co-delivery of miR-21 inhibitor and chemotherapeutics to reverse drug resistance in colon cancer. *Journal of nanobiotechnology*, 18(1), 10. doi:10.1186/s12951-019-0563-2
- [118] Li, X., Corbett, A. L., Taatizadeh, E., Tasnim, N., Little, J. P., Garnis, C. et al. (2019). Challenges and opportunities in exosome research-Perspectives from biology, engineering, and cancer therapy. *APL bioengineering*, 3(1), 011503, doi:10.1063/1.5087122
- [119] Gangadaran, P., & Ahn, B. C. (2020). Extracellular vesicle- and extracellular vesicle mimetics-based drug delivery systems: new perspectives, challenges, and clinical developments. *Pharmaceutics*, 12(5), 442. doi:10.3390/pharmaceutics12050442
- [120] Sato, Y. T., Umezaki, K., Sawada, S., Mukai, S. A., Sasaki, Y., Harada, N. et al. (2016). Engineering hybrid exosomes by membrane fusion with liposomes. *Scientific reports*, 6, 21933 doi:10.1038/srep21933

- [121] Luan, X., Sansanaphongpricha, K., Myers, I., Chen, H., Yuan, H., & Sun, D. (2017). Engineering exosomes as refined biological nanoplatfroms for drug delivery. *Acta pharmacologica sinica*, 38(6), 754–63, doi:10.1038/aps.2017.12
- [122] Smyth, T., Petrova, K., Payton, N. M., Persaud, I., Redzic, J. S., Graner, M. W. et al. (2014). Surface functionalization of exosomes using click chemistry. *Bioconjugate chemistry*, 25(10), 1777–84, doi:10.1021/bc500291r
- [123] Wang, Y., Yao, J., Cai, L., Liu, T., Wang, X., Zhang, Y., Zhou, Z., Li, T., Liu, M., Lai, R., & Liu, X. (2020). Bone-targeted extracellular vesicles from mesenchymal stem cells for osteoporosis therapy. *International journal of nanomedicine*, 15, 7967–77. doi:10.2147/IJN.S263756
- [124] Bryniarski, K., Ptak, W., Martin, E., Nazimek, K., Szczepanik, M., Sanak, M. et al. (2015). Free extracellular miRNA functionally targets cells by transfecting exosomes from their companion cells. *PloS one*, 10(4), e0122991, doi:10.1371/journal.pone.0122991
- [125] Gong, C., Tian, J., Wang, Z., Gao, Y., Wu, X., Ding, X. et al. (2019). Functional exosome-mediated co-delivery of doxorubicin and hydrophobically modified microRNA 159 for triple-negative breast cancer therapy. *Journal of nanobiotechnology*, 17(1), 93. doi:10.1186/s12951-019-0526-7
- [126] Kooijmans, S., Schiffelers, R. M., Zarovni, N., & Vago, R. (2016). Modulation of tissue tropism and biological activity of exosomes and other extracellular vesicles: New nanotools for cancer treatment. *Pharmacological research*, 111, 487–500. doi:10.1016/j.phrs.2016.07.006

- [127] Cho, J. A., Yeo, D. J., Son, H. Y., Kim, H. W., Jung, D. S., Ko, J. K. et al. (2005). Exosomes: a new delivery system for tumor antigens in cancer immunotherapy. *International journal of cancer*, 114(4), 613–22. doi:10.1002/ijc.20757
- [128] Li, Z., Zhou, X., Wei, M., Gao, X., Zhao, L., Shi, R. et al. (2019). In vitro and in vivo RNA inhibition by CD9-HuR functionalized exosomes encapsulated with miRNA or CRISPR/dCas9. *Nano letters*, 19(1), 19–28. doi:10.1021/acs.nanolett.8b02689
- [129] Lin, Y., Wu, J., Gu, W., Huang, Y., Tong, Z., Huang, L. et al. (2018). Exosome-liposome hybrid nanoparticles deliver CRISPR/Cas9 system in MSCs. *Advanced science*, 5(4), 1700611, doi:10.1002/advs.201700611
- [130] Jaiswal, R., & Sedger, L. M. (2019). Intercellular vesicular transfer by exosomes, microparticles and oncosomes - implications for cancer biology and treatments. *Frontiers in oncology*, 9, 125. doi:10.3389/fonc.2019.00125
- [131] Zhang, H., Freitas, D., Kim, H. S., Fabijanic, K., Li, Z., Chen, H. et al. (2018). Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nature cell biology*, 20(3), 332-43. doi:10.1038/s41556-018-0040-4
- [132] Ma, L., Li, Y., Peng, J., Wu, D., Zhao, X., Cui, Y. et al. (2015). Discovery of the migrasome, an organelle mediating release of cytoplasmic contents during cell migration. *Cell research*, 25(1), 24-38. doi:10.1038/cr.2014.135
- [133] Li, B., Antonyak, M. A., Zhang, J., & Cerione, R. A. (2012). RhoA triggers a specific signaling pathway that generates transforming microvesicles in cancer cells. *Oncogene*, 31(45), 4740-49. doi:10.1038/onc.2011.636

- [134] Yang, M., Liu, X., Luo, Q., Xu, L., & Chen, F. (2020). An efficient method to isolate lemon derived extracellular vesicles for gastric cancer therapy. *Journal of nanobiotechnology*, 18(1), 100, doi:10.1186/s12951-020-00656-9
- [135] Zhuang, X., Deng, Z. B., Mu, J., Zhang, L., Yan, J., Miller, D. et al. (2015). Ginger-derived nanoparticles protect against alcohol-induced liver damage. *Journal of extracellular vesicles*, 4, 28713, doi:10.3402/jev.v4.28713
- [136] Kim, K., Yoo, H. J., Jung, J. H., Lee, R., Hyun, J. K., Park, J. H. et al. (2020). Cytotoxic effects of plant sap-derived extracellular vesicles on various tumor cell types. *Journal of functional biomaterials*, 11(2), doi:10.3390/jfb11020022
- [137] Rutter, B. D., & Innes, R. W. (2017). Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins. *Plant physiology*, 173(1), 728-41, doi:10.1104/pp.16.01253
- [138] Wang, B., Zhuang, X., Deng, Z. B., Jiang, H., Mu, J., Wang, Q. et al. (2014). Targeted drug delivery to intestinal macrophages by bioactive nanovesicles released from grapefruit. *Molecular therapy*, 22(3), 522-34, doi:10.1038/mt.2013.190
- [139] Wang, Q., Zhuang, X., Mu, J., Deng, Z. B., Jiang, H., Zhang, L. et al. (2013). Delivery of therapeutic agents by nanoparticles made of grapefruit-derived lipids. *Nature communications*, 4, 1867, doi:10.1038/ncomms2886
- [140] Liu, Y., Wu, S., Koo, Y., Yang, A., Dai, Y., Khant, H. et al. (2020). Characterization of and isolation methods for plant leaf nanovesicles and small extracellular vesicles. *Nanomedicine*, 29, 102271, doi:10.1016/j.nano.2020.102271
- [141] Deng, Z., Rong, Y., Teng, Y., Mu, J., Zhuang, X., Tseng, M. et al. (2017). Broccoli-derived nanoparticle inhibits mouse colitis by activating dendritic cell AMP-activated protein kinase. *Molecular therapy*, 25(7), 1641-54. doi:10.1016/j.ymthe.2017.01.025

- [142] Jeppesen, D. K., Hvam, M. L., Primdahl-Bengtson, B., Boysen, A. T., Whitehead, B., Dyrskjot, L. et al. (2014). Comparative analysis of discrete exosome fractions obtained by differential centrifugation. *Journal of extracellular vesicles* 3, 25011, doi:10.3402/jev.v3.25011
- [143] Wang, B., Zhuang, X., Deng, Z. B., Jiang, H., Mu, J., Wang, Q. et al. (2014). Targeted drug delivery to intestinal macrophages by bioactive nanovesicles released from grapefruit. *Molecular therapy*, 22(3), 522-34. doi:10.1038/mt.2013.190
- [144] Boing, A. N., van der Pol, E., Grootemaat, A. E., Coumans, F. A., Sturk, A., & Nieuwland, R. (2014). Single-step isolation of extracellular vesicles by size-exclusion chromatography. *Journal of extracellular vesicles*, 3, doi:10.3402/jev.v3.23430
- [145] Lobb, R. J., Becker, M., Wen, S. W., Wong, C. S., Wiegman, A. P., Leimgruber, A., & Moller, A. (2015). Optimized exosome isolation protocol for cell culture supernatant and human plasma. *Journal of extracellular vesicles*, 3, 4, 27031, doi:10.3402/jev.v4.27031
- [146] Heinemann, M. L., Ilmer, M., Silva, L. P., Hawke, D. H., Recio, A., Vorontsova, M. A. et al. (2014). Benchtop isolation and characterization of functional exosomes by sequential filtration. *Journal of chromatography. A*, 1371, 125-35, doi:10.1016/j.chroma.2014.10.026
- [147] Musante, L., Tataruch, D., Gu, D., Benito-Martin, A., Calzaferri, G., Aherne, S., & Holthofer, H. (2014). A simplified method to recover urinary vesicles for clinical applications, and sample banking. *Scientific reports*, 4, 7532, doi:10.1038/srep07532
- [148] Haraszti, R. A., Miller, R., Stoppato, M., Sere, Y. Y., Coles, A., Didiot, M. C. et al. (2018). Exosomes produced from 3D cultures of MSCs by tangential flow filtration show higher yield and improved activity. *Molecular therapy*, 26(12), 2838-47, doi:10.1016/j.ymthe.2018.09.015

- [149] Kang, D., Oh, S., Ahn, S. M., Lee, B. H., & Moon, M. H. (2008). Proteomic analysis of exosomes from human neural stem cells by flow field-flow fractionation and nanoflow liquid chromatography-tandem mass spectrometry. *Journal of proteome research*, 7(8), 3475-80, doi:10.1021/pr800225z
- [150] Wang, B., Zhuang, X., Deng, Z. B., Jiang, H., Mu, J., Wang, Q. et al. (2014). Targeted drug delivery to intestinal macrophages by bioactive nanovesicles released from grapefruit. *Molecular therapy*, 22(3), 522-34. doi:10.1038/mt.2013.190
- [151] Helwa, I., Cai, J., Drewry, M. D., Zimmerman, A., Dinkins, M. B., Khaled, M. L. et al. (2017). A Comparative study of serum exosome isolation using differential ultracentrifugation and three commercial reagents. *Plos one*, 12(1), e0170628. doi:10.1371/journal.pone.0170628
- [152] Samsonov, R., Shtam, T., Burdakov, V., Glotov, A., Tsyrlina, E., Berstein, L. et al. 2016). Lectin-induced agglutination method of urinary exosomes isolation followed by mi-RNA analysis: application for prostate cancer diagnostic. *Prostate*, 76(1), 68-79, doi:10.1002/pros.23101
- [153] Davies, R. T., Kim, J., Jang, S. C., Choi, E. J., Ghoo, Y. S., & Park, J. (2012). Microfluidic filtration system to isolate extracellular vesicles from blood. *Lab chip*, 12(24), 5202-10. doi:10.1039/c2lc41006k
- [154] Lee, K., Shao, H., Weissleder, R., & Lee, H. (2015). Acoustic purification of extracellular microvesicles. *ACS nano*, 9(3), 2321-27, doi:10.1021/nn506538f
- [155] Cheng, Y., Qu, X., Dong, Z., Zeng, Q., Ma, X., Jia, Y. et al. (2020). Comparison of serum exosome isolation methods on co-precipitated free microRNAs. *PeerJ*, 8, e9434. doi:10.7717/peerj.9434

- [156] Zeming, K. K., Salafi, T., Shikha, S., & Zhang, Y. (2018). Fluorescent label-free quantitative detection of nano-sized bioparticles using a pillar array. *Nature communication*, 9(1), 1254, doi:10.1038/s41467-018-03596-z
- [157] Liang, L. G., Sheng, Y. F., Zhou, S., Inci, F., Li, L., Demirci, U., & Wang, S. (2017). An integrated double-filtration microfluidic device for detection of extracellular vesicles from urine for bladder cancer diagnosis. *Methods in molecular biology*, 1660, 355-64. doi:10.1007/978-1-4939-7253-1_29
- [158] Akagi, T., Kato, K., Kobayashi, M., Kosaka, N., Ochiya, T., & Ichiki, T. (2015). On-chip immunoelectrophoresis of extracellular vesicles released from human breast cancer cells. *Plos one*, 10(4), e0123603, doi:10.1371/journal.pone.0123603
- [159] Kim, D. K., Nishida, H., An, S. Y., Shetty, A. K., Bartosh, T. J., & Prockop, D. J. (2016). Chromatographically isolated CD63+CD81+ extracellular vesicles from mesenchymal stromal cells rescue cognitive impairments after TBI. *Proceedings of the national academy of sciences of the united states of america*, 113(1), 170-75, doi:10.1073/pnas.1522297113

Graphical abstract

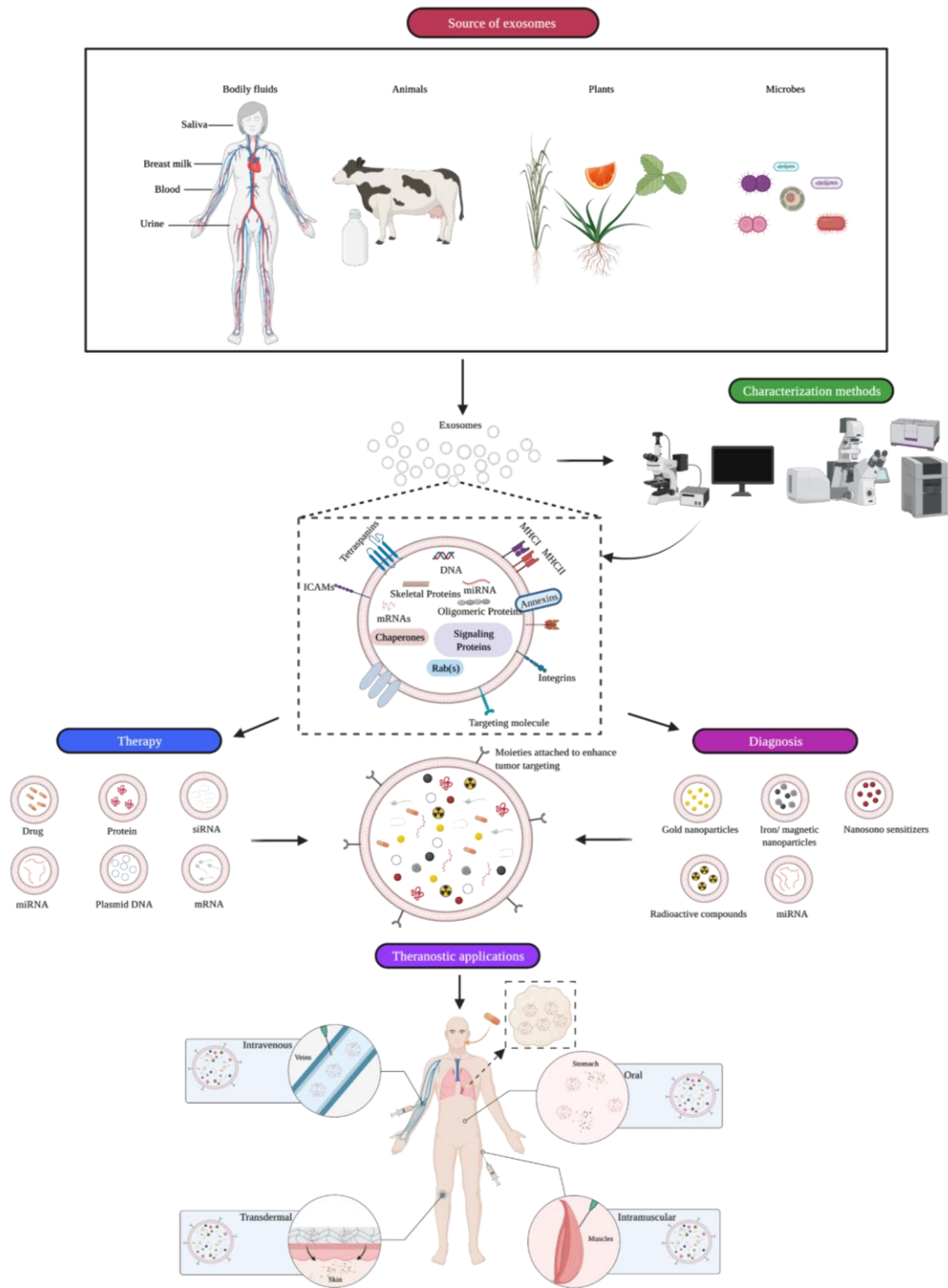


Figure 1

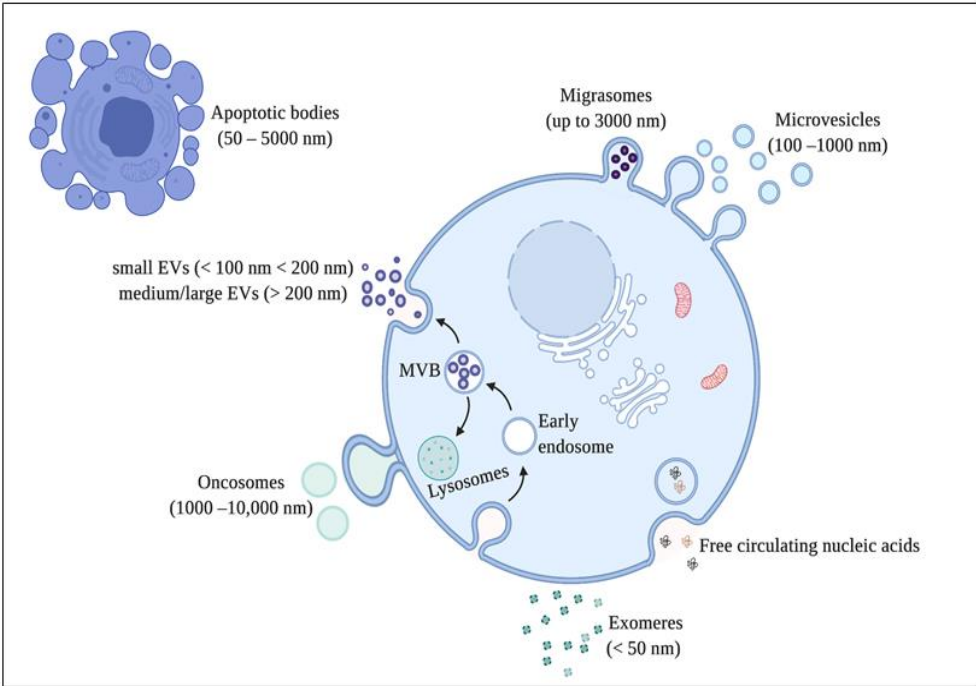
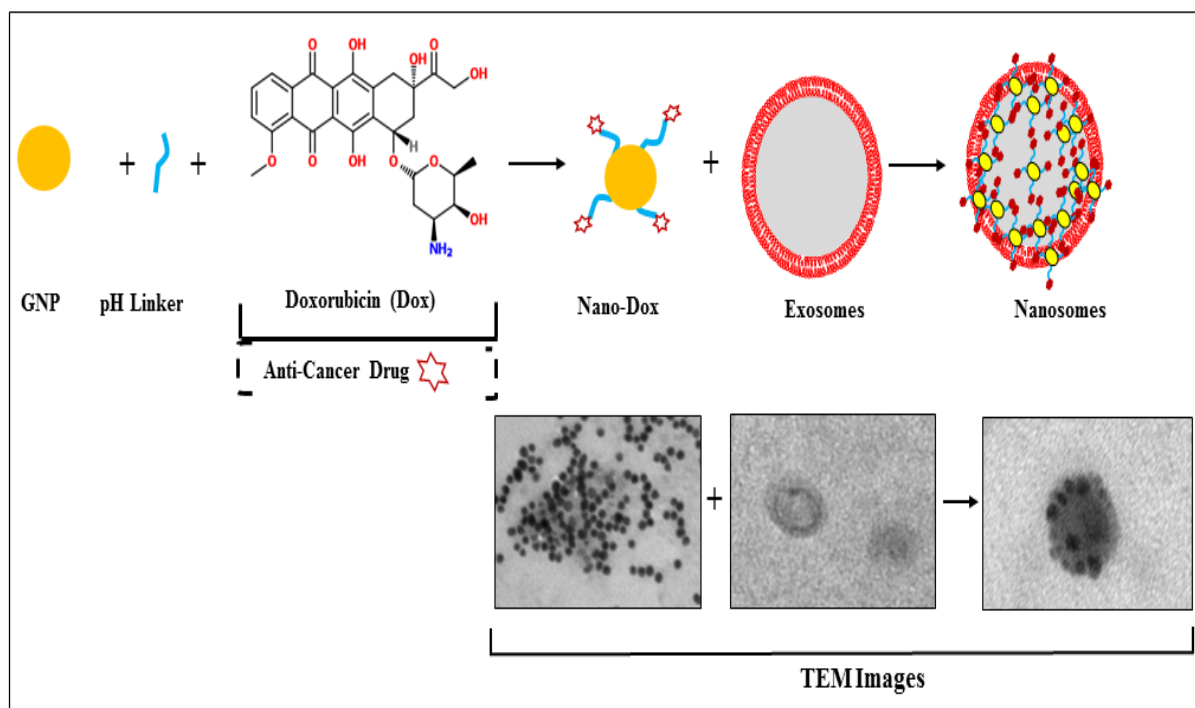


Figure 2



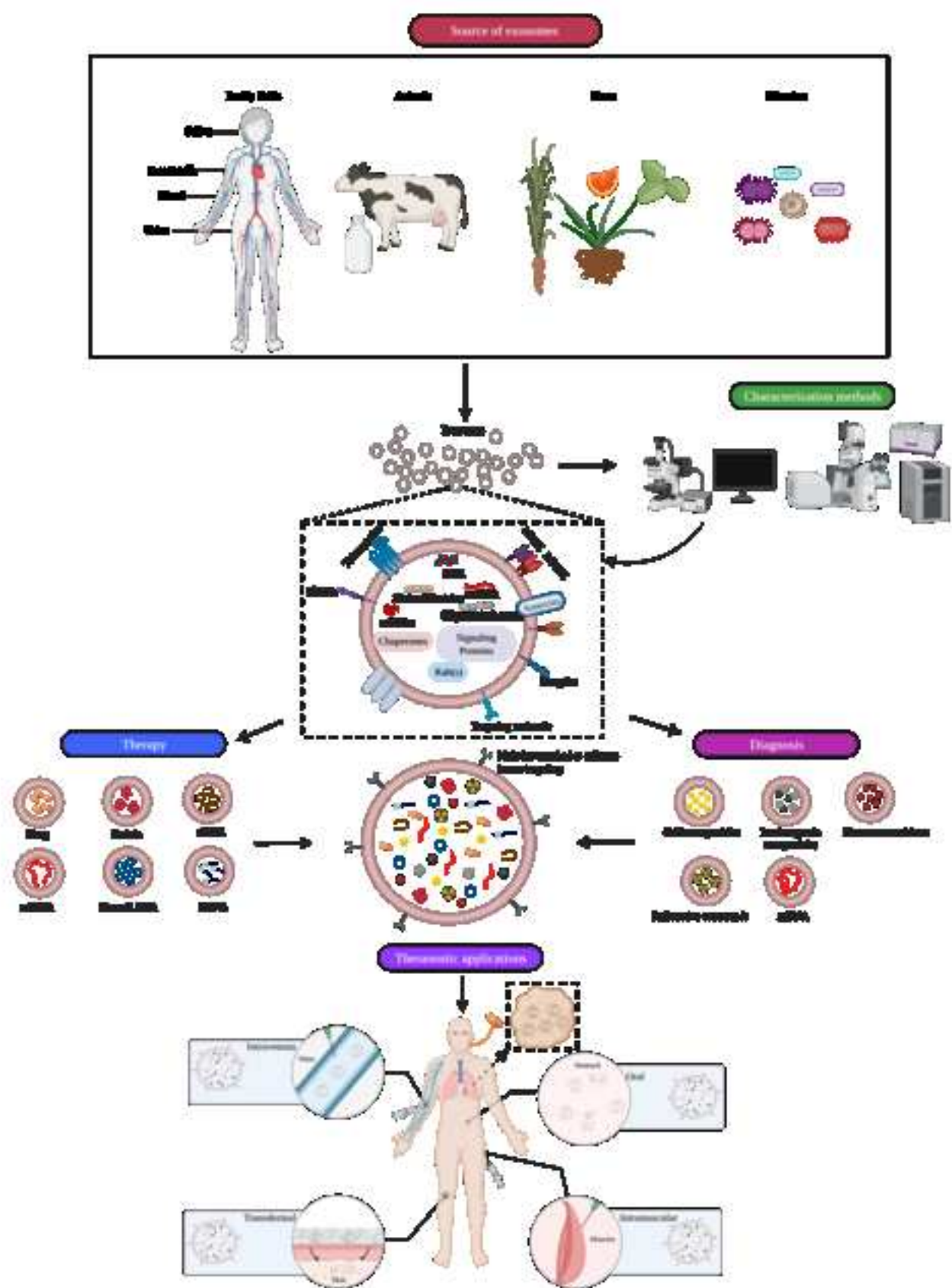


Figure 1

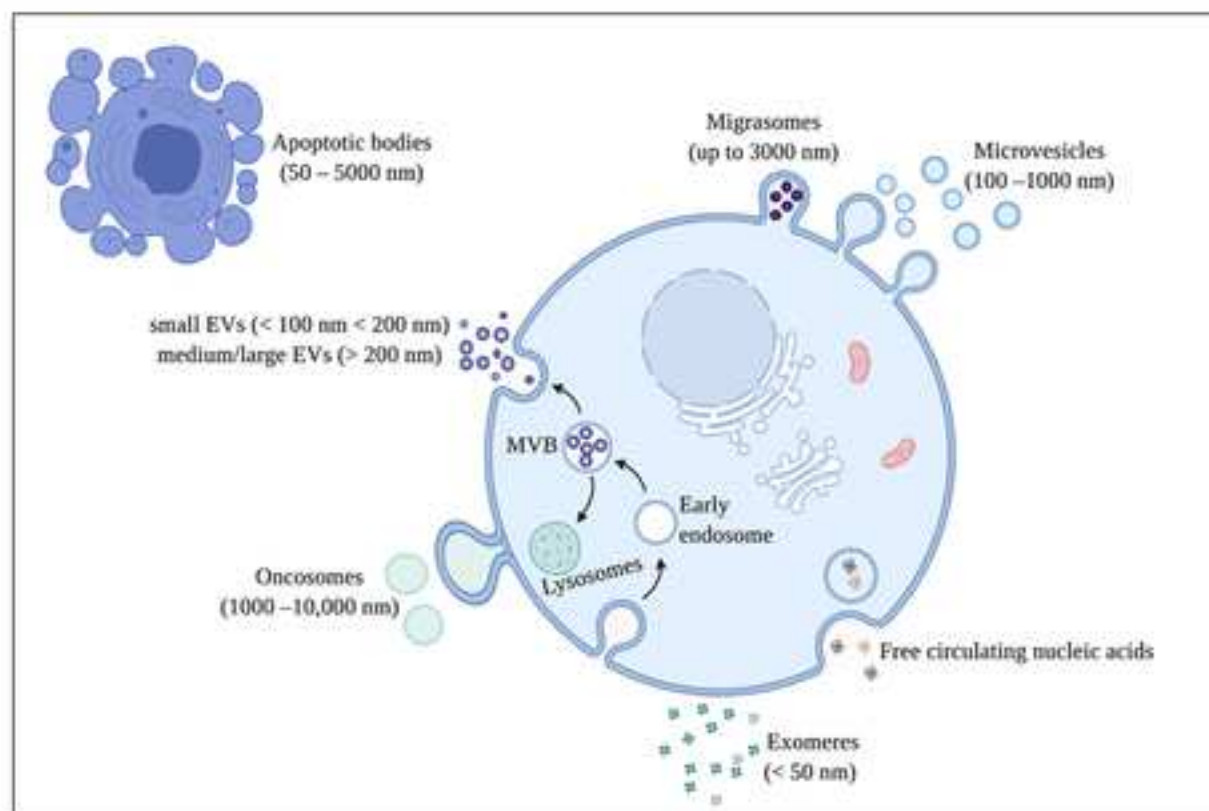
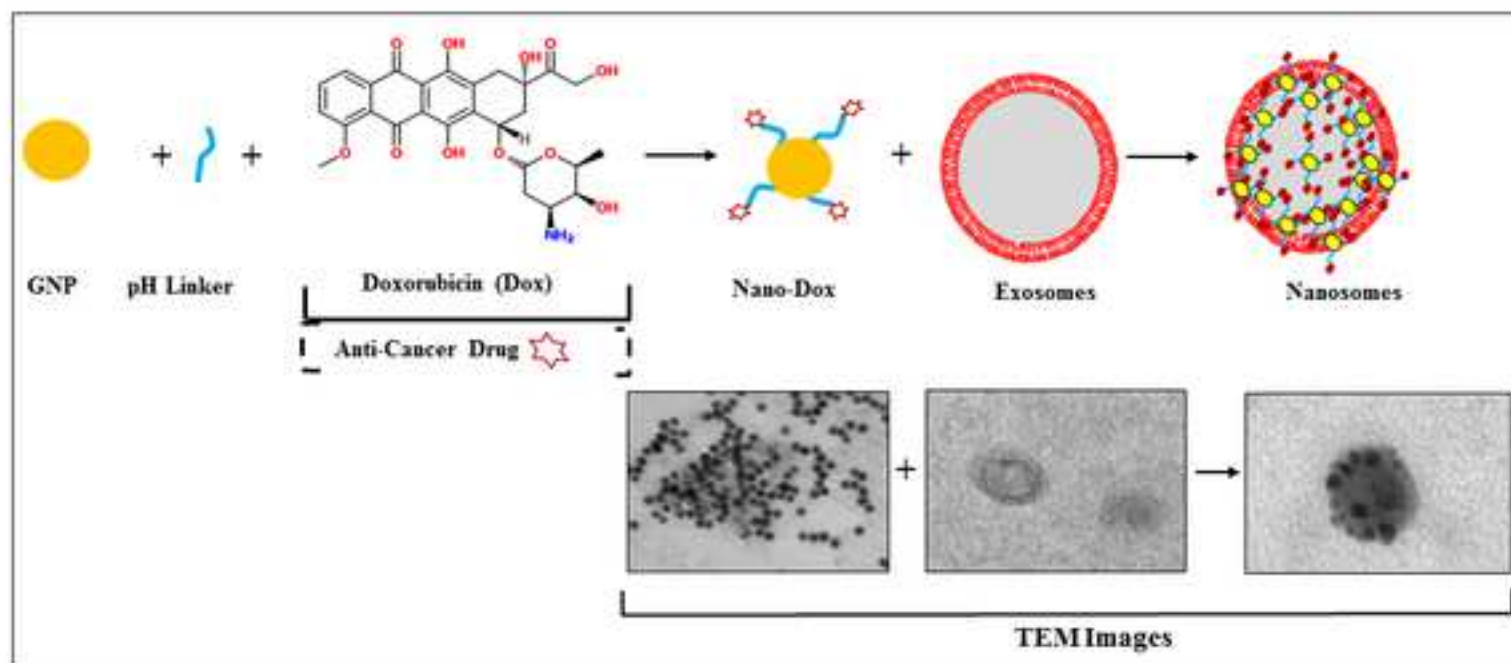


Figure 2





Seminars in Cancer Biology

Conflict of Interest Policy

Article Title: Organically derived exosomes as carriers of anticancer drugs and imaging agents for cancer treatment

Author name:
Rajagopal Ramesh

Declarations

Seminars in Cancer Biology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgment concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

N/A

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

National Cancer Institute (NCI) of the National Institutes of Health
National Institute of General Medical Sciences of the National Institutes of Health
Department of Veterans Affairs
National Cancer Institute Cancer Center Support Grant
Department of Defense (DOD) through the Lung Cancer Research Program (LCRP)
Oklahoma Center for Advanced Science and Technology (OCAST)
Presbyterian Health Foundation

Signature (a scanned signature is acceptable, but each author must sign)

R. Ramesh.

Print name

Rajagopal Ramesh



Seminars in Cancer Biology

Conflict of Interest Policy

Article Title: Organically derived exosomes as carriers of anticancer drugs and imaging agents for cancer treatment

Author name:
Anupama Munshi

Declarations

Seminars in Cancer Biology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgment concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

N/A

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

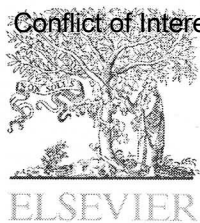
Presbyterian Health Foundation
National Cancer Institute Cancer Center Support Grant

Signature (a scanned signature is acceptable, but each author must sign)

Anupama Munshi

Print name

Anupama Munshi



Seminars in Cancer Biology

Conflict of Interest Policy

Article Title: **ORGANICALLY DERIVED EXOSOMES AS CARRIERS OF ANTI CANCER DRUGS & IMAGING AGENTS FOR CANCER TREATMENT**

Author name:
Akhil Srivastava

Declarations

Seminars in Cancer Biology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgment concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

N/A

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

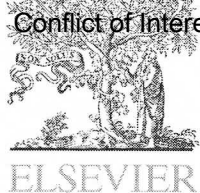
Please state any sources of funding for your research

Department of Defense (DOD) through the Lung Cancer Research Program (LCRP)

Signature (a scanned signature is acceptable, but each author must sign)

Print name

Akhil Srivastava



Seminars in Cancer Biology

Conflict of Interest Policy

Article Title: **ORGANICALLY DERIVED EXOSOMES AS CARRIERS OF ANTI-CANCER DRUGS & IMAGING AGENTS FOR CANCER TREATMENT**

Author name:
Shipra Rathore

Declarations

Seminars in Cancer Biology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgment concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

N/A

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

N/A

Signature (a scanned signature is acceptable, but each author must sign)

Shipra Rathore

Print name

Shipra Rathore