Diagnostic and Therapeutic Potential of Extracellular Vesicles

Sai Priyanka Kodam, MD1,2 and Mujib Ullah, MD, PhD1,2

Abstract
Extracellular vesicles (EVs) are naturally phospholipid enclosed nanovesicles released by many cells in the body. They are stable in circulation, have low immunogenicity, and act as carriers for functionally active biological molecules. They interact with target organs and bind to the receptors. Their target specificity is important to use EVs as noninvasive diagnostic and prognostic tools. EVs play a vital role in normal physiology and cellular communication. They are known to protect their cargo from degradation, which makes them important drug carriers for targeted drug delivery. Using EVs with markers and tracking their path in systemic circulation can be revolutionary in using them as diagnostic tools. We will discuss the scope of this in this paper. Although there are limitations in EVs isolation and storage, their high biocompatibility will fuel more innovations to overcome these challenges.

Keywords
extracellular vesicles, regenerative medicine, cytokine, artificial intelligence, exosomes, innovative technologies, drug delivery, cancer

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Extracellular Vesicles (EVs) and Therapeutic Cargo
EVs are nano-sized, vesicles known for cellular communication and drug delivery. They are the key mediators for tissue regeneration, inflammatory regulation, and immune response. They play a significant role in preventing the accumulation of toxic substances and drugs. Their role as carriers can be exploited for chemotherapy and to study the efficacy of chemotherapeutic drugs.

Studying the pharmacokinetics and permeability of labeled EVs is useful to understand the systemic uptake of medications that can lead to chemotherapeutic side effects. This information makes experimentation with various drug combinations and dosage recommendations possible. The studies can be repeated with adjusted doses and medication combinations till the desired result that is, maximum therapeutic benefit with minimal side effects is achieved.

EVs are present in all biological fluids. EV-based biomarkers are sensitive for the detection of mutations in cancers. As EVs can efficiently protect their cargo against degradation, they can be used as biomarkers for diagnostic purposes. They are well studied for their targeting ability and biodistribution in vivo.

EVs is a common term used to describe exosomes, microvesicles, and apoptotic bodies. Microvesicles are released by outward vesiculation of the cell membrane. Exosomes are formed by endocytosis of cell membrane forming microvesicular bodies (MVBs). These MVBs fuse with the plasma membrane and are released into the extracellular space. They carry cell membrane lipoproteins, miRNAs. Apoptotic bodies are released during apoptosis. The EVs are heterogeneous compounds. They vary in size, composition, and cargo based on the mechanism of production.

1 Institute for Immunity and Transplantation, Stem Cell Biology and Regenerative Medicine, School of Medicine, Stanford University, Palo Alto, California, USA
2 School of Medicine, Stanford University, Palo Alto, California, USA

Corresponding Author:
Mujib Ullah, MD, PhD, Senior Medical Investigator, Institute for Immunity and Transplantation, Stem Cell Biology and Regenerative Medicine, School of Medicine, Stanford University, Palo Alto, California 94304, United States.
Email: ullah@stanford.edu
Need for Standardization of EV Purification Methods

After extraction from their natural environment, EVs are subjected to centrifugation to remove debris and dead cells. To be used as drug carriers, they are injected with specific medications.

After release from cells in their natural environment, there are mechanisms for cell-to-cell communication with adjacent tissues and distant organs. Given their role in biological communication between different tissues, they can serve as drug carriers for targeted therapy. EVs are able to transfer cytokines, bioactive compounds, miRNAs, and repair proteins in their cargo. They are effective in carrying the therapeutic cargo for targeted therapy. EVs are able to transfer cytokines, bioactive compounds, miRNAs, and repair proteins in their cargo. They are effective in carrying the therapeutic cargo for targeted therapy. EVs are able to transfer cytokines, bioactive compounds, miRNAs, and repair proteins in their cargo. They are effective in carrying the therapeutic cargo for targeted therapy. EVs are able to transfer cytokines, bioactive compounds, miRNAs, and repair proteins in their cargo. They are effective in carrying the therapeutic cargo for targeted therapy. EVs are able to transfer cytokines, bioactive compounds, miRNAs, and repair proteins in their cargo. They are effective in carrying the therapeutic cargo for targeted therapy. EVs are able to transfer cytokines, bioactive compounds, miRNAs, and repair proteins in their cargo. They are effective in carrying the therapeutic cargo for targeted therapy.

Tissue-specific receptors and biosensors can be lodged on the EVs for tracking the drug path. The bioactive cargo of nanovesicles will increase the efficacy of drug delivery. The target specificity is useful in preventing drug penetration into other organs that can lead to systemic side effects.

The current concerns with EV therapeutic applications are, that the complete biochemical profile of EVs is not known. Further, there are no standardized isolation and purification methods, and the lack of efficient drug loading systems for clinical-grade production.

Multiple novel technologies have emerged in the past decade for EV isolation and characterization, but there is still a need for reproducible, cheap, and simpler alternatives that can be adapted for large-scale production. Continuous attempts to develop more effective protocols are ongoing.

Isolation methods that are currently available include ultracentrifugation, filtration, size-exclusion chromatography, density gradient, and immunoaffinity-based isolation strategies. Ultracentrifugation isolates the EVs by differential centrifugation. Filtration uses membranes with specific pore sizes for different EVs. The isolated yield can be poor with this method. Size-exclusion chromatography uses the hydrodynamic column to reduce contamination, thus yielding a low EV output, but less protein contamination. Density gradient separates them based on density after initial isolation. Immune-affinity-based isolation uses antibodies to capture EVs. This method is not a cost-effective approach. Table 1.

The current gold standard for EV isolation is “Ultracentrifugation-linked immunoprecipitation method.” This method yields smaller sized EVs with lesser apolipoprotein contamination, compared to other methods. The EVs need enrichment before processing. Currently, different labs have been using different preprocessing protocols. There is a need to develop an easily reproducible and standardized method.

The newer technique size-exclusion chromatography purifies the EVs by removing the contaminating plasma proteins and high-density lipoproteins. It has been successfully used in the small-scale analysis of EVs. The column preparation, washing, and purification make it a time-consuming procedure. A novel membrane affinity spin column with better efficacy, ease, and reproducible workflow was released. The efficacy of purification using the new spin column is better compared to the optimized ultracentrifugation procedure, which is the current gold standard. Using this method, EV isolation can be coupled with EV RNA extraction. This procedure captures nearly 100% mRNA. This provides a purified EV isolate without undesired protein-bound extracellular RNA co-precipitate. The analysis of EV RNA is useful to understand the genetic markers of EVs.

Clinical Applications of EVs

The biggest advantage of EVs is the fact that they are composed of non-immunogenic substances. Therefore, the tissues do not identify the EVs as foreign, thus protecting their cargo from degradation. This makes it a safe method for drug delivery. The nano-size of EVs makes it one of the ideal carriers for the delivery of active molecules and drugs. They have successfully been applied for cancer therapy, inflammatory modulation, and immune response generation. They can also be used to administer nutrients, minerals, and oxygen in stroke patients. This can reduce the recovery period and early rehabilitation of patients.

Surface protein bioengineering, biosensor development, drug loading, coupled with placement of ligands on the surface of EVs reprogram EVs into smart multifunctional drug-loaded systems.

EVs in Diagnostic Imaging

EVs are also used in imaging studies. The 2 major methods used in detecting EVs in the visible light spectrum are Bioluminescence imaging and fluorescence imaging. Bioluminescence imaging involves protein-based labeling and has a high signal-to-noise ratio as it is transmitted without any light source. An ultra-sensitive camera is needed to detect the EVs. Fluorescence imaging uses organic dyes or protein to emit signals under excitation with an external light source. Fluorescence is more easily detected by a charged coupled device camera. Imaging tools commonly used are single-photon emission computed tomography, positron emission tomography, and magnetic resonance imaging. It should be remembered that the labeling compounds have a longer half-life than EVs and continue to be detected by imaging even after EVs are degraded. Bioengineering the EV membranes with inbuilt markers that degrade simultaneously with the EVs will be useful to avoid false-positive findings.

The protein and RNA cargo in the EVs associated with their cell of origin serve as molecular markers for screening a disease and to assess the progression. The assays using highly purified EV samples will provide relevant clinical information. Micro/nanofluidic technology with affinity capture
for precise enrichment of EVs results in EVs that are highly purified compared to the conventional methods that isolate EVs irrespective of the cell of origin.57,98 

Appropriate storage of EVs to maintain the stability of nucleic acids is essential.37,51 If these storage conditions are available, the EVs can most likely be used to diagnose gene mutations.37,51 Previous studies have proven that EVs and EV nucleic acids are stable in multiple environments.37,51,74 The EVs and nucleic acids were relatively more stable at 4 degrees centigrade (up to 168 h and 1 week) compared to room temperature (up to 48 h and 1 day).53,74,98 

More studies to increase the shelf life of EVs in vitro and in vivo will surpass the current limitations to their use.59 Preserving them with curcumin has been shown to increase the stability of EVs and potentiate the efficacy of the cargo.58,99,100 Experimentation on additive substances or bioengineered isotopes to increase the stability can widen the diagnostic opportunities of EVs. Tables 1 and 2.

### Table 1. An Overview of EV Purification Methods, Their Advantages, and Limitations.

| Method                                      | Principle                                                                 | Advantages                                                                 | Limitations                                                                 | References       |
|---------------------------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------|------------------|
| Filtration                                  | Filtration through a membrane based on size                               | Inexpensive                                                                | Lower purity due to protein contamination, efficiency dependent on the quality of membrane | 60, 62, 63, 71,74 |
| Size-exclusion chromatography               | Isolation by gel filtration based on size                                 | Inexpensive, higher purity, no protein aggregates or contamination        | Sample volume limited, cannot be adapted for large volume samples, long run times | 55, 59, 63-65     |
| Ultracentrifugation                         | Separation based on size. Larger EVs are isolated earlier in the cycle  | Commonly used, cost effective, no chemical additives added                | Protein aggregates/clumping                                                  | 55, 59, 63-65    |
| Density gradient ultracentrifugation        | Separation based on density using iodixanol/sucrose gradients            | Pure preparations, no chemical additives added                            | Complex, loss of sample to leaching due to osmotic changes in sucrose concentrations | 33, 56, 64, 65   |
| Ultracentrifugation-linked immunoprecipitation | Purificant using immobilized antibodies directed toward EV markers       | Pure, highly selective, subtype isolation                                | Expensive, high selectivity, contaminated with protein/immunoglobulins, need for pre-processing | 33, 51, 64, 74   |

EVs in the Treatment of Cardiovascular Illnesses

EVs containing miRNAs and proteins perform a multitude of functions in target tissues.101,102 The structure, contents, and profiles of the cargo change with tissue damage to produce tissue regenerating substances.57,103 In heart failure, they mediate communication between cardiomyocytes and fibroblasts.104 For example, MiR-146a-loaded EVs are released by endothelial cells during the development of peripartum cardiomyopathy.105,106 These MiR-146a-loaded EVs serve as biomarkers for diagnosis.105,106 Therefore, they can be used as tissue biomarkers and diagnostic tools in heart failure.104 

EVs are natural carriers of signaling molecules involved in atherosclerosis.104,107 Determining the types and their specific functional properties will be of relevance to discover biomarkers and alter the pathophysiology to control atherosclerosis.

Cardiac progenitor cells (CPCs) and cardiomyocyte derived cells have shown promising results as useful tools in restoring heart function.107 Unlike the EVs secreted from cardiomyocytes that have deleterious effects, EVs produced by CPCs are cardioprotective and mediate cardiogenesis.107-109 They induce angiogenesis to reduce ischemic injury, infarct size, and inhibit apoptosis to prevent undesired remodeling.110,111 However, little is known how the EVs are produced in diseased and non-diseased cardiac tissues and the interaction of the EVs with the surrounding and distant tissues.110 Further studies in these areas would be greatly beneficial to add to the current management of cardiovascular diseases.

In mice, adipocyte-derived EVs in obese mice have been shown to activate differentiation of monocytes into macrophages, inducing insulin resistance.112,113 The same was not seen in lean mice.114 Similarly, MiR and protein profiles of EVs from hypertensive rats were different from the normotensive rats.115,116 Further research to understand these differences can unfold the dynamic nature of EVs, Figure 1.

EVs in the Treatment of Neurological Illnesses

EVs in the neurons are the typical mode of communication between neurons and neuroglial cells.117,118 This interaction is important for the structural and functional integrity of the nervous system.118 A lot of research has been done over the years to understand the molecular mechanisms involving the pathophysiology in neurodegenerative studies like Alzheimer’s disease,119,120 Parkinson’s disease, Huntington’s disease, and other dementia disorders.120-122 But the treatment modalities are still very limited. Research is underway to
Table 2. Clinical and Diagnostic Applications of EVs, Their Benefits, and Limitations.

| Field of use      | Clinical applications                                                                 | Limitations                                                                                                                                  | References |
|-------------------|---------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Diagnostic imaging| Biological contrast media facilitating personalized imaging of specific tissues       | Early degradation of EVs while the markers are still persistent. Circulating markers may result in false-positive findings                | 20, 21, 71, 86, 90, 92, 94, 95 |
| Cardiovascular illnesses | Diagnosis of peripartum cardiomyopathy, heart failure, atherosclerosis. Treatment using EVs derived from cardiac progenitor cells via angiogenesis/ cardiogenesis | Lack of studies that describe the difference in EV properties in diseased and healthy cardiac tissues                                      | 14, 106, 109 |
| Neurological illnesses | Nutrient administration and oxygen supply to the ischemic tissues in stroke. Angiogenesis and neurogenesis in traumatic brain injury observed in rat models. | Further studies to determine their effects on the human nervous system are needed.                                                              | 3, 8, 37, 105, 106, 110, 111, 121, 122, 134, 141, 152 |
| Pulmonary diseases | EVs derived from MSCs can be used for immunomodulation, viral defense, and tissue regeneration | The evidence has been shown in smaller studies with <50 patients. Larger studies are needed to adapt these treatments                      | 129, 134, 138, 141 |

EVs in the Treatment of Lung Diseases Including Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infection

EVs are secreted by the lining of the respiratory tract similar to any other cell in the body and their role in the pathogenesis of lung diseases has been established in previous studies. Recent studies show that EVs and viruses are interdependent on each other for cellular entry and exit owing to their similar physicochemical properties, for example, size and heterogeneous size distribution. Therefore, trials using EVs loaded with antiviral medications to block viral intracellular entry can be explored.

In the past 1 year, mesenchymal stem cell (MSCs) therapy for SARS-CoV-2 infection is being explored in multiple studies. MSCs have unique potential for immunomodulation, antiviral defense, and tissue regeneration. Umbilical cord-derived MSCs have shown promising results in smaller studies. They induce the production of cytokines, paracrine factors that interact with immune cells to stimulate vascular endothelial growth factor, epidermal growth factor, transforming growth factor release mediated tissue regeneration.

The promising tissue regenerative properties of MSC-derived EVs support pulmonary tissue regeneration post coronavirus 2019 (COVID-19) illness.

The limitations with MSC use are shorter half-life, lasting a few minutes in the blood stream. MSCs derived from embryogenic tissue can be mutagenic and tumorigenic. Intravenous administration of MSCs can cause platelet aggregation and thrombogenesis leading to emboli.

EVs derived from MSCs can be administered via intranasal and inhalational route, contributing to lesser immunogenic or thrombogenic side effects. They do not enter the circulation where they can be lost in a few minutes and do not cause thrombus formation. They do not self-replicate like MSCs, therefore are less tumorigenic.

EVs can be administered along with antiviral drugs. Preliminary studies suggest that co-treatment with EVs obtained from mesenchymal cell secretomes is effective in treating COVID-19 patients. Further preclinical data considering safety, ethics, and practicality is needed to adapt this regimen for COVID-19 treatment.

Summary of Clinical Applications

Conclusion and Future Directions

EVs show great promise as diagnostic and therapeutic markers in many diseases. There is a dire need to work on standardization of isolation and storage methods of EVs. This will enable a wider
application of EVs in large-scale treatment trials. Several studies have shown that the current methods available have limitations like contamination of samples, cost issues, instability issues, among others. Ultracentrifugation with immunoprecipitation has been a promising method. If the preprocessing methods are standardized and ways to curb the cost factor are explored. This method can be adapted for therapeutic EV production as it produces highly selective EVs. Further studies are urgently needed to explore these methods which can pave the path for the clinical application of EVs.

The pathological processes in which the EVs are being applied show great benefits with minimal side effects. As EVs are derived from human cells, there is always the benefit of biocompatibility compared to other modalities of treatment. In addition to cardiovascular and neurological diseases, the need of the hour is the treatment of SARS-CoV-2 infection. Though the studies so far for EVs derived from MSCs in COVID illness have been small, there is a great scope as they offer a combination of benefits providing immunomodulatory function, viral defense, and tissue regeneration. This can decrease the severity and duration of COVID-19 illness. Further studies in this area are needed. The regenerative property can also be utilized for cardiac remodeling in post-MI patients, neuroregeneration in stroke patients, thus decreasing the morbidity and mortality.

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ORCID iD
Mujib Ullah https://orcid.org/0000-0003-0168-8700

References
1. Ullah M, Qiao Y, Concepcion W, Thakor AS. Stem cell-derived extracellular vesicles: role in oncogenic processes, bioengineering potential, and technical challenges. Stem Cell Res Ther. 2019;10(1):347.
2. Roefs MT, Sluijter JPG, Vader P. Extracellular vesicle-associated proteins in tissue repair. Trends Cell Biol. 2020;30(12):990-1013.
3. Ko SY, Naora H. Extracellular vesicle membrane-associated proteins: emerging roles in tumor angiogenesis and anti-angiogenesis therapy resistance. Int J Mol Sci. 2020;21(15):5418.
4. Kato T, Fahrmann JF, Hanash SM, Vykoukal J. Extracellular vesicles mediate B cell immune response and are a potential target for cancer therapy. Cells. 2020;9(6):1518.
5. Oggero S, Austin-Williams S, Norling LV. The contrasting role of extracellular vesicles in vascular inflammation and tissue repair. Front Pharmacol. 2019;10:1479. doi:10.3389/fphar.2019.01479

Figure 1. Schematic illustration showing the application of extracellular vesicles in different diseases.
6. Fitzgerald W, Freeman ML, Lederman MM, Vasilieva E, Romero R, Margolis L. A system of cytokines encapsulated in extracellular vesicles. *Sci Rep.* 2018;8(1):8973.

7. Bjørge I, Kim S, Mano J, Kalionis B, Chrzanowski W. Extracellular vesicles, exosomes and shedding vesicles in regenerative medicine—a new paradigm for tissue repair. *Biomater Sci.* 2018;6(1):60-78.

8. Buzas EI, Gyorgy B, Nagy G, Falus A, Gay S. Emerging role of extracellular vesicles in inflammatory diseases. *Nat Rev Rheumatol.* 2014;10(6):356-364.

9. Gutierrez-Vazquez C, Villaroya-Belti C, Mittelbrunn M, Sanchez-Madrid F. Transfer of extracellular vesicles during immune cell-cell interactions. *Immunol Rev.* 2013;251(1):125-142.

10. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev.* 2012;64(3):676-705.

11. Arenaccio C, Chiozziini C, Ferrantelli F, Leone P, Olivetta E, Federico M. Exosomes in therapy: engineering, pharmaco-kinetics and future applications. *Curr Drug Targets.* 2019;20(1):87-95.

12. He C, Zheng S, Luo Y, Wang B. Exosome theranostics: biology and translational medicine. *Theranostics.* 2018;8(1):237-255.

13. Cheng Q, Shi X, Han M, Sambatyan G, Lenz HJ, Zhang Y. Reprogramming exosomes as nanoscale controllers of cellular immunity. *J Am Chem Soc.* 2018;140(48):16413-16417.

14. Liu Q, Piao H, Wang Y, Zheng D, Wang W. Circulating exosomes in cardiovascular disease: novel carriers of biological information. *Biomed Pharmacother.* 2021;135:111148. doi:10.1016/j.biopha.2020.111148

15. Verweij FJ, Revenu C, Arras G, et al. Live tracking of inter-organ communication by endogenous exosomes In vivo. *Dev Cell.* 2019;48(4):573-589. e574.

16. Tkach M, Théry C. Communication by extracellular vesicles: where we are and where we need to go. *Cell.* 2016;164(6):1226-1232.

17. Li I, Nabet BY. Exosomes in the tumor microenvironment as mediators of cancer therapy resistance. *Mol Cancer.* 2019;18(1):32.

18. Jung KO, Jo H, Yu JH, Gambhir SS, Pratt G. Development and MPI tracking of novel hypoxia-targeted theranostic exosomes. *Biomaterials.* 2018;177:139-148. doi:10.1016/j.biomaterials.2018.05.048

19. Ullah M, Kodam SP, Mu Q, Akbar A. Microbubbles versus extracellular vesicles as therapeutic cargo for targeting drug delivery. *ACS Nano.* 2021;15(3):3612-3620.

20. Liu T, Zhu Y, Zhao R, Wei X, Xin X. Visualization of exosomes from mesenchymal stem cells in vivo by magnetic resonance imaging. *Magn Reson Imaging.* 2020;68:75-82. doi:10.1016/j.mri.2020.02.001

21. Betzer O, Barnoy E, Sadan T, et al. Advances in imaging strategies for in vivo tracking of exosomes. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2020;12(2):e1594.

22. Feng R, Ullah M, Chen K, Ali Q, Lin Y, Sun Z. Stem cell-derived extracellular vesicles mitigate age-associated arterial stiffness and hypertension. *J Extracell Vesicles.* 2020;9(1):1783869.

23. Yáñez-Mó M, Siljander PR-M, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles.* 2015;4(1):27066.

24. Andaloussi SE, Mäger I, Breakfield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discovery.* 2013;12(5):347-357.

25. van Niel G, D’Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol.* 2018;19(4):213-228.

26. Shao H, Im H, Castro CM, Breakfield X, Weissleder R, Lee H. New technologies for analysis of extracellular vesicles. *Chem Rev.* 2018;118(4):1917-1950.

27. Ullah M, Akbar A. Clinical relevance of RNA editing to early detection of cancer in human. *Int J Stem Cell Res Ther.* 2020;7(1):066.

28. Hauser P, Wang S, Didenko VV. Apoptotic bodies: selective detection in extracellular vesicles. In: *Signal Transduction Immunohistochemistry.* Springer; 2017: 193-200.

29. Vader P, Breakfield XO, Wood MJ. Extracellular vesicles: emerging targets for cancer therapy. *Trends Mol Med.* 2014;20(7):385-393.

30. Kowal J, Arras G, Colombo M, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A.* 2016;113(8):E968-E977.

31. Atkin-Smith GK, Tixeira R, Paone S, et al. A novel mechanism of generating extracellular vesicles during apoptosis via a beads-on-a-string membrane structure. *Nat Commun.* 2015;6(1):7439.

32. Willms E, Cabanas C, Mager I, Wood MJA, Vader P. Extracellular vesicle heterogeneity: subpopulations, isolation techniques, and diverse functions in cancer progression. *Front Immunol.* 2018;9(738):738.

33. Xu R, Greening DW, Zhu HJ, Takahashi N, Simpson RJ. Extracellular vesicle isolation and characterization: toward clinical application. *J Clin Invest.* 2016;126(4):1152-1162.

34. Ullah M, Akbar A, Yannarelli G. Applications of artificial intelligence in, early detection of cancer, clinical diagnosis and personalized medicine. *Artif Intell Cancer.* 2020;1(2):39-44.

35. Murali VP, Holmes CA. Biomaterial-based extracellular vesicle delivery for therapeutic applications. *Acta Biomater.* 2021;124:88-107. doi:10.1016/j.actbio.2021.01.010

36. Chen S, Zhu X, Huang S. Clinical applications of extracellular vesicle long RNAs. *Crit Rev Clin Lab Sci.* 2020;57(8):508-521. doi:10.1080/0009997x.2020.1751584

37. Ullah M, Ng NN, Concepcion W, Thakor AS. Emerging role of stem cell-derived extracellular microRNAs in age-associated human diseases and in different therapies of longevity. *Ageing Res Rev.* 2020;57:100979.

38. Armstrong JPK, Stevens MM. Strategic design of extracellular vesicle drug delivery systems. *Adv Drug Deliv Rev.* 2018;130:12-16. doi:10.1016/j.addr.2018.06.017

39. Li Z, Zhu X, Huang S. Extracellular vesicle long non-coding RNAs and circular RNAs: biology, functions and applications in cancer. *Cancer Lett.* 2020;489:111-120. doi:10.1016/j.canlet.2020.06.006
40. O’Brien K, Breyne K, Ughetto S, Laurent LC, Breakefield XO. RNA Delivery by extracellular vesicles in mammalian cells and its applications. Nat Rev Mol Cell Biol. 2020;21(10):585-606.

41. Fujita Y, Yoshioka Y, Ochiya T. Extracellular vesicle transfer of cancer pathogenic components. Cancer Sci. 2016;107(4):385-390.

42. Ullah M, Akbar A, Thakor AS. An emerging role of CD9 in stemness and chemoresistance. Oncotarget. 2019;10(40):4000-4001.

43. Sabanovic B, Piva F, Cecati M, Giuliani M. Promising extracellular vesicle-based vaccines against viruses, including SARS-CoV-2. Biology (Basel). 2021, 10(2):94.

44. Liang Y, Duan L, Lu J, Xia J. Engineering exosomes for targeted drug delivery. Theranostics. 2021;11(7):3183-3195.

45. Ullah M, Qian NPM, Yannarelli G, Akbar A. Heat shock protein 20 promotes sirtuin 1-dependent cell proliferation in induced pluripotent stem cells. World J Stem Cells. 2021;13(6):659-669.

46. Akbar A, Pillalamarri N, Jonnakuti S, Ullah M. Artificial intelligence and guidance of medicine in the bubble. Cell Biosci. 2021;11(1):108.

47. Ullah M, Liu DD, Rai S, et al. A novel approach to deliver therapeutic extracellular vesicles directly into the mouse kidney via its arterial blood supply. Cells. 2020;9(4):937.

48. Ullah M, Kuroda Y, Bartosh T, et al. iPSC-derived MSCs from an expandable bank to deliver a prodrug-converting enzyme that limits growth and metastases of human breast cancers. Cell Death Discov. 2017;3(1):1-10.

49. Zhang Y, Bi J, Huang J, Tang Y, Du S, Li P. Exosome: a review of its classification, isolation techniques, storage, diagnostic and targeted therapy applications. Int J Nanomedicine. 2020;15:6917-6934. doi:10.2147/IJN.S264498

50. Ullah M, Akbar A, Ng NN, Concepcion W, Thakor AS. Extracellular vesicle RNA using molecular beacons. World J Stem Cells. 2020;9(37):3435-3450.

51. Zhou X, Xie F, Wang L, et al. The function and clinical application of extracellular vesicles in innate immune regulation. Cell Mol Immunol. 2020;17(4):323-334.

52. Varderidou-Minasian S, Lorenowicz MJ. Mesenchymal stromal/stem cell-derived extracellular vesicles in tissue repair: challenges and opportunities. Theranostics. 2020;10(13):5979-5997.

53. Gandham S, Su X, Wood J, et al. Ivanov AR: technologies and standardization in research on extracellular vesicles. Trends Biotechnol. 2020;38(10):1066-1098.

54. Monguio-Tortajada M, Moron-Font M, Gamez-Valero A, Carreras-Planella L, Borras FE, Franquesa M. Extracellular-vesicle isolation from different biological fluids by size-exclusion chromatography. Curr Protoc Stem Cell Biol. 2019;49(1):e82.

55. Tengattini S. Chromatographic approaches for purification and analytical characterization of extracellular vesicles: recent advancements. Chromatographia. 2019;82(1-2):415-424.

56. Raimondo S, Giavresi G, Lorico A, Alessandro R. Extracellular vesicles as biological shuttles for targeted therapies. Int J Mol Sci. 2019;20(8):1848.

57. Melling GE, Carollo E, Conlon R, Simpson JC. Carter DRF: the challenges and possibilities of extracellular vesicles as therapeutic vehicles. Eur J Pharm Biopharm. 2019;144:50-56. doi:10.1016/j.ejpb.2019.08.009

58. Heath N, Grant L, De Oliveira TM, et al. Rapid isolation and enrichment of extracellular vesicle preparations using anion exchange chromatography. Sci Rep. 2018;8(1):1-12.

59. Buschmann D, Kirchner B, Herrmann S, et al. Evaluation of serum extracellular vesicle isolation methods for profiling miRNAs by next-generation sequencing. J Extracell Vesicles. 2018;7(1):1481321.

60. Witwer KW, Soekmadji C, Hill AF, et al. Updating the MISEV minimal requirements for extracellular vesicle studies: building bridges to reproducibility. In.: Wiley Online Library; 2017.

61. Tian Y, Gong M, Hu Y, et al. Quality and efficiency assessment of six extracellular vesicle isolation methods by nano-flow cytometry. J Extracell Vesicles. 2020, 9(1):1697028.

62. Monguio-Tortajada M, Galvez-Monton C, Bayes-Genis A, Roura S, Borras FE. Extracellular vesicle isolation methods: rising impact of size-exclusion chromatography. Cell Mol Life Sci. 2019;76(12):2369-2382.

63. Corso G, Mäger I, Lee Y, et al. Reproducible and scalable purification of extracellular vesicles using combined bind-elute and size exclusion chromatography. Sci Rep. 2017;7(1):1-10.

64. Bruce TF, Slonecki TJ, Wang L, Huang S, Powell RR, Marcus RK. Exosome isolation and purification via hydrophobic interaction chromatography using a polyester, capillary-channeled polymer fiber phase. Electrophoresis. 2019;40(4):571-581.

65. Crossland RE, Norden J, Bibby LA, Davis J, Dickinson AM. Evaluation of optimal extracellular vesicle small RNA isolation and qRT-PCR normalisation for serum and urine. J Immunol Methods. 2016;429:39-49. doi:10.1016/j.jim.2015.12.011

66. de Oliveira GP Jr, Zigon E, Rogers G, et al. Detection of extracellular vesicle RNA using molecular beacons. iScience. 2020;23(1):100782.

67. Jia S, Zocco D, Samuels ML, et al. Emerging technologies in extracellular vesicle-based molecular diagnostics. Expert Rev Mol Diagn. 2014;14(3):307-321.

68. Ullah M, Akbar A, Ng NN, Concepcion W, Thakor AS. Mesenchymal stem cells confer chemoresistance in breast cancer via a CD9 dependent mechanism. Oncotarget. 2019;10(37):3435-3450.

69. Ohno S, Drummen GP, Kuroda M. Focus on extracellular vesicles: development of extracellular vesicle-based therapeutic systems. Int J Mol Sci. 2016;17(2):172.

70. Chou ST, Chien JC, Lai CP. Imaging extracellular vesicles: current and emerging methods. J Biomed Sci. 2018;25(1):91.

71. Han Y, Jones TW, Dutta S, et al. Overview and update on methods for cargo loading into extracellular vesicles. Processes (Basel). 2021;9:356.

72. Malenica M, Vukomanovic M, Kurtjak M, et al. Perspectives of microscopy methods for morphology characterisation of extracellular vesicles from human biofluids. Biomedicines. 2021;9(6):603.

73. Zhang M, Jin K, Gao L, et al. Methods and technologies for exosome isolation and characterization. Small Methods. 2018;2(9):1800021.
75. Tang TT, Wang B, Lv LL, Liu BC. Extracellular vesicle-based nanotherapeutics: emerging frontiers in anti-inflammatory therapy. Theranostics. 2020;10(18):8111-8129.
76. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. Nat Rev Immunol. 2014;14(3):195-208.
77. Shigemoto-Kuroda T, Oh JY, Kim DK, et al. MSC-derived extracellular vesicles attenuate immune responses in two autoimmune murine models: type 1 diabetes and uveoretinitis. Stem Cell Rep. 2017;8(5):1214-1225.
78. Minciacchi VR, Freeman MR, Di Vizio D. Extracellular vesicles: a review on the applications of fluorescence. J Extracell Vesicles. 2020;9(1):1710020.
79. 80. Raposo G, Stahl PD. Extracellular vesicles: a new communication paradigm? Nat Rev Mol Cell Biol. 2019;20(9):509-510.
81. Beard K, Meaney DF, Issadore D. Clinical applications of extracellular vesicles in the diagnosis and treatment of traumatic brain injury. J Neurotrauma. 2020;37(19):2045-2056.
82. Harting MT, Srivastava AK, Zhaorigetu S, et al. Inflammation-stimulated mesenchymal stromal cell-derived extracellular vesicles attenuate inflammation. Stem Cells. 2018;36(1):79-90.
83. Zinger A, Brozovich A, Pasto A, et al. Bioinspired extracellular vesicles: lessons learned from nature for biomedicine and biotechnology. Nanomaterials. (Basel). 2020;10(11):2172.
84. Riazifar M, Pone EJ, Lotvall J, Zhao W. Stem cell extracellular vesicles: extended messages of regeneration. Annu Rev Pharmacol Toxicol. 2017;57:125-154. doi:10.1146/annurev-pharmtox-061616-030146.
85. Alminana C, Bausersch S. Extracellular vesicles in the oviduct: progress, challenges and implications for the reproductive success. Bioengineering (Basel). 2019;6(2):32.
86. Gupta D, Liang X, Pavlova S, et al. Quantification of extracellular vesicles in vitro and in vivo using sensitive bioluminescence imaging. J Extracell Vesicles. 2020;9(1):1800222.
87. Choi H, Lee DS. Illuminating the physiology of extracellular vesicles. Stem Cell Res Ther. 2016;7(1):55.
88. Gangadaran P, Li XJ, Lee HW, et al. A new bioluminescent reporter system to study the biodistribution of systematically injected tumor-derived bioluminescent extracellular vesicles in mice. Oncotarget. 2017;8(66):109894-109914.
89. Li YJ, Wu JY, Wang JM, Hu XB, Xiang DX. Emerging strategies for labeling and tracking of extracellular vesicles. J Control Release. 2020;328:141-159. doi:10.1016/j.jconrel.2020.08.056.
90. Liebel M, Ortega Arroyo J, Beltran VS, et al. 3D Tracking of extracellular vesicles by holographic fluorescence imaging. Sci Adv. 2020;6(45), eabc2508.
91. Panagopoulos MS, Wark AW, Birch DJS, Gregory CD. Phenotypic analysis of extracellular vesicles: a review on the applications of fluorescence. J Extracell Vesicles. 2020;9(1):1710020.
109. Moghadam AS, Afshari JT, Esmaeili SA, Saburi E, Joneidi Z, Montaziz-Boroujeni AA. Cardioprotective microRNAs: lessons from stem cell-derived exosomal miRNAs to treat cardiovascular disease. Atherosclerosis. 2019;285:1-9. doi:10.1016/j.atherosclerosis.2019.03.016

110. Bian X, Ma K, Zhang C, Fu X. Therapeutic angiogenesis using stem cell-derived extracellular vesicles: an emerging approach for treatment of ischemic diseases. Stem Cell Res Ther. 2019;10(1):1-18.

111. Kholia S, Ranghino A, Garnieri P, et al. Extracellular vesicles as new players in angiogenesis. Vasc Pharmacol. 2016;86:64-70. doi:10.1016/j.vph.2016.03.005

112. Kranendonk ME, Visseren FL, van Balkom BW, et al. Human adipocyte extracellular vesicles in reciprocal signaling between adipocytes and macrophages. Obesity. 2014;22(5):1296-1308.

113. De Silva N, Samblas M, Martinez JA, Milagro FI. Effects of exosomes from LPS-activated macrophages on adipocyte gene expression, differentiation, and insulin-dependent glucose uptake. J Physiol Biochem. 2018;74(4):559-568.

114. Ying W, Rionel M, Bandyopadhyay G, et al. Adipose tissue macrophage-derived exosomal miRNAs can modulate in vivo and in vitro insulin sensitivity. Cell. 2017;171(2):372-384. e312.

115. Otani K, Yokoya M, Kodama T, et al. Plasma exosomes regulate systemic blood pressure in rats. Biochem Biophys Res Commun. 2018;503(2):776-783.

116. Fujioka Y, Otani K, Okada M, Yamawaki H. Plasma small extracellular vesicles in hypertensive rats impair reactivity of isolated blood vessels. J Vet Med Sci. 2020;82(7):897-902.

117. Basso M, Bonetto V. Extracellular vesicles and a novel form of communication in the brain. Front Neurosci. 2016;10:127. doi:10.3389/fnins.2016.00127

118. Budnik V, Ruiz-Canada C, Wendler F. Extracellular vesicles round off communication in the nervous system. Nat Rev Neurosci. 2016;17(3):160-172.

119. Vella LJ, Hill AF, Cheng L. Focus on extracellular vesicles: exosomes and their role in protein trafficking and biomarker potential in Alzheimer’s and Parkinson’s disease. Int J Mol Sci. 2016;17(2):173.

120. Candelario KM, Steindler DA. The role of extracellular vesicles in the progression of neurodegenerative disease and cancer. Trends Mol Med. 2014;20(7):368-374.

121. Coleman BM, Hill AF. Extracellular vesicles—their role in the packaging and spread of misfolded proteins associated with neurodegenerative diseases. In: Seminars in Cell & Developmental Biology: 2015. Elsevier: 89-96.

122. Croese T, Furlan R. Extracellular vesicles in neurodegenerative diseases. Mol Aspects Med. 2018;60:52-61. doi:10.1016/j.mam.2017.11.006

123. Matsumoto J, Stewart T, Banks WA, Zhang J. The transport mechanism of extracellular vesicles at the blood-brain barrier. Curr Pharm Des. 2017;23(40):6206-6214.

124. Ullah M, Liu DD, Rai S, et al. Reversing acute kidney injury using pulsed focused ultrasound and MSC therapy: a role for HSP-mediated PI3K/AKT signaling. Mol Ther Methods Clin Dev. 2020;17:683-694. doi:10.1016/j.omtm.2020.03.023

125. Behfar A, Zingman LV, Hodgson DM, et al. Stem cell differentiation requires a paracrine pathway in the heart. FASEB J. 2002;16(12):1558-1566.

126. Ullah M, Liu DD, Rai S, Concepcion W, Thakor AS. HSP70-mediated NLRP3 inflammasome suppression underlies reversal of acute kidney injury following extracellular vesicle and focused ultrasound combination therapy. Int J Mol Sci. 2020;21(11):4085.

127. Hocking AM, Gibran NS. Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. Exp Cell Res. 2010;316(14):2213-2219.

128. Liu DD, Ullah M, Concepcion W, Dahl JJ, Thakor AS. The role of ultrasound in enhancing mesenchymal stromal cell-based therapies. Stem Cells Transl Med. 2020;9(8):850-866.

129. Guo Q, Yan J, Song T, et al. microRNA-130b-3p contained in MSC-derived EVs promotes lung cancer progression by regulating the FOXO3/NFE2L2/TXNRD1 axis. Mol Ther Oncolytics. 2021;20:132-146. doi:10.1016/j.omto.2020.09.005

130. Mondello S, Thelin EP, Shaw G, et al. Extracellular vesicles: pathogenetic, diagnostic and therapeutic value in traumatic brain injury. Expert Rev Proteomics. 2018;15(5):451-461.

131. Katsuda T, Oki K, Ochiya T. Potential application of extracellular vesicles of human adipose tissue-derived mesenchymal stem cells in Alzheimer’s disease therapeutics. In: Stem Cell Renewal and Cell-Cell Communication. Springer; 2014: 171-181.

132. Katsuda T, Tsuyukiya R, Kosaka N, et al. Human adipose tissue-derived mesenchymal stem cells secrete functional nephrilysin-bound exosomes. Sci Rep. 2013;3(1):1197.

133. Nagano T, Katsurada M, Dokuni R, et al. Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-binding exosomes. Stem Cell Investig. 2015;20(10):2589.

134. Piszczatowska K, Czerwaty K, Cyran AM, et al. The emerging role of small extracellular vesicles in inflammatory airway diseases. Diagnostics (Basel). 2021;11(2):222.

135. Ullah M. The pandemic of novel coronavirus disease 2019 (COVID-19): need for an immediate action. Open Access J Biomed Sci. 2020;2(1):301-302.

136. Ullah M. Novel coronavirus (COVID-19) treatment options. Biomed J Sci Tech Res. 2020;27(3):20872-20874.

137. Börgér V, Weiss DI, Anderson JD, et al. Hill AF: iSEV and focused ultrasound combination therapy. Cyttherap. 2020;22(9):482-485. doi:10.1016/j.jcyt.2020.05.002

138. Chrzanowski W, Kim SY, McClements L. Can stem cells beat COVID-19: advancing stem cell and extracellular vesicles toward mainstream medicine for lung injuries associated with SARS-CoV-2 infections. Front Bioeng Biotechnol. 2020;8:554. doi:10.3389/fbioe.2020.00554

139. Fontaine MJ, Shih H, Schafer R, Pittenger MF. Unraveling the mesenchymal stromal cells’ paracrine immunomodulatory effects. Transfus Med Rev. 2016;30(1):37-43.
140. Zhou Y, Yamamoto Y, Xiao Z, Ochiya T. The immunomodulatory functions of mesenchymal stromal/stem cells mediated via paracrine activity. *J Clin Med*. 2019;8(7):1025.

141. Weiss DJ, Bertoncello I, Borok Z, et al. Stem cells and cell therapies in lung biology and lung diseases. *Proc Am Thorac Soc*. 2011;8(3):223-272.

142. Norozi F, Ahmadzadeh A, Shahrabi S, Vosoughi T, Saki N. Mesenchymal stem cells as a double-edged sword in suppression or progression of solid tumor cells. *Tumor Biol*. 2016;37(9):11679-11689.

143. Li Y-h, Feng L, G-X Zhang, Ma C-g. Intranasal delivery of stem cells as therapy for central nervous system disease. *Exp Mol Pathol*. 2015;98(2):145-151.

144. Galeano C, Qiu Z, Mishra A, et al. The route by which intranasally delivered stem cells enter the central nervous system. *Cell Transplant*. 2018;27(3):501-514.

145. Nijboer CH, Kooijman E, Van Velthoven CT, et al. Intranasal stem cell treatment as a novel therapy for subarachnoid hemorrhage. *Stem Cells Dev*. 2018;27(5):313-325.

146. van Velthoven CT, Kavelaars A, van Bel F, Heijnen CJ. Nasal administration of stem cells: a promising novel route to treat neonatal ischemic brain damage. *Pediatr Res*. 2010;68(5):419-422.

147. Jiang Y, Zhu J, Xu G, Liu X. Intranasal delivery of stem cells to the brain. *Expert Opin Drug Deliv*. 2011;8(5):623-632.

148. Cunnane EM, Weinbaum JS, O’Brien FJ, Vorp DA. Future perspectives on the role of stem cells and extracellular vesicles in vascular tissue regeneration. *Front Cardiovasc Med*. 2018;5:86. doi:10.3389/fcvm.2018.00086

149. Hur YH, Cerione RA, Antonyak MA. Extracellular vesicles and their roles in stem cell biology. *Stem Cells*. 2020;38(4):469-476.

150. Kumar S, Zhi K, Mukherji A, Geth K. Repurposing antiviral protease inhibitors using extracellular vesicles for potential therapy of COVID-19. *Viruses*. 2020;12(5):486.

151. Urciuoli E, Peruzzi B. Inhibiting extracellular vesicle trafficking as antiviral approach to corona virus disease 2019 infection. *Front Pharmacol*. 2020;11:580505. doi:10.3389/fphar.2020.580505

152. Tian J, Casella G, Zhang Y, Rostami A, Li X. Potential roles of extracellular vesicles in the pathophysiology, diagnosis, and treatment of autoimmune diseases. *Int J Biol Sci*. 2020;16(4):620-632.