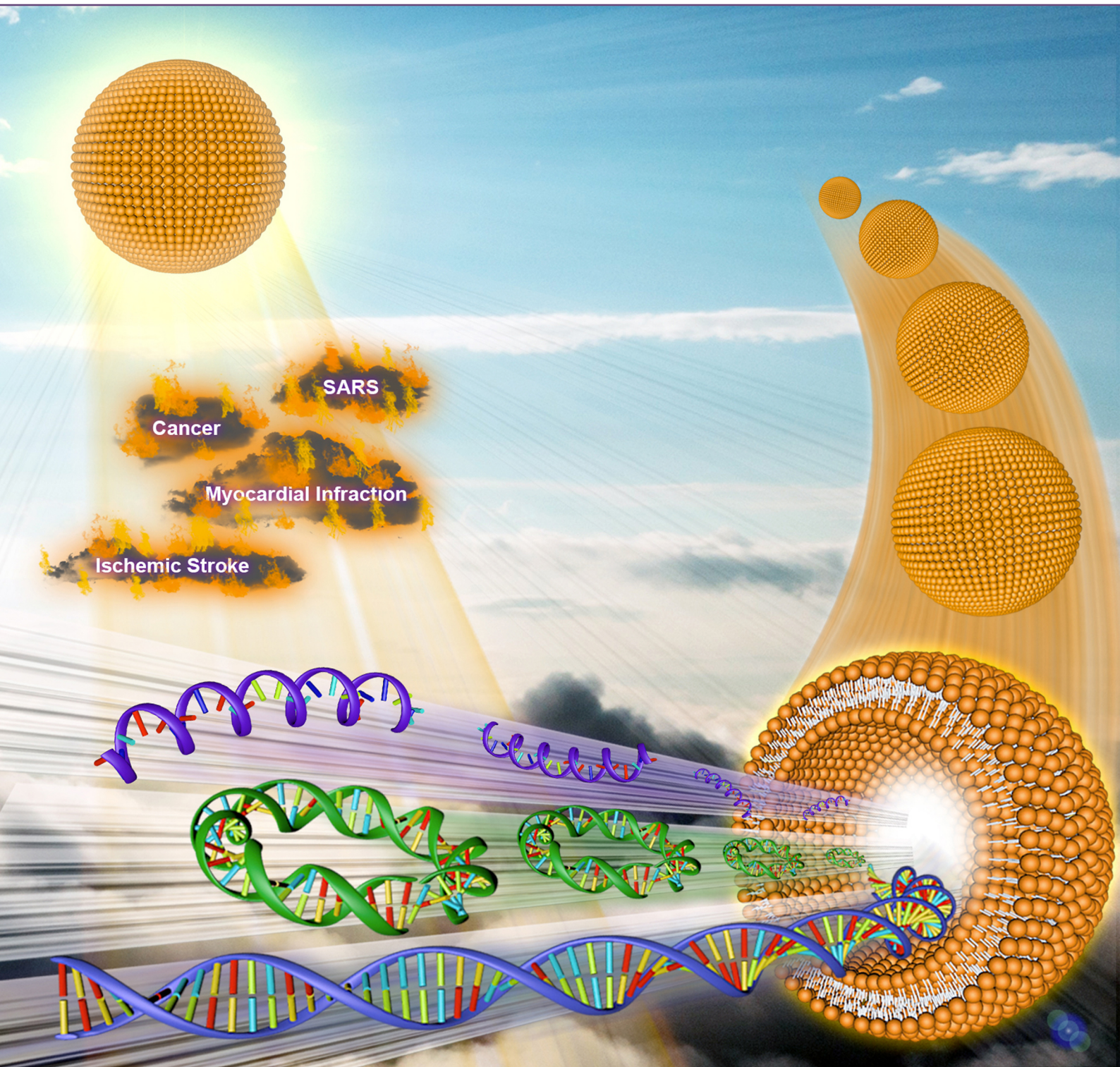


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Editorial

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Acknowledgement to reviewers of *Extracellular Vesicles and Circulating Nucleic Acids* in 2021

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Table 1. The reviewers in 2021

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Commentary

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Liquid biopsy of extracellular vesicle biomarkers for prostate cancer personalized treatment decision

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Abstract

Liquid biopsy of tumor-derived extracellular vesicles (EVs) has great potential as a biomarker source for prostate cancer (CaP) early diagnosis and predicting the stages of cancer. The contents of EVs play an important role in intercellular communication and have specific expression in blood and urine samples from CaP patients. Powered by high-throughput, next-generation sequencing and proteomic technologies, novel EV biomarkers are easily detected in a non-invasive manner in different stages of CaP patients. These identified potential biomarkers can be further validated with a large sample size, machine learning model, and other different methods to improve the sensitivity and specificity of CaP diagnosis. The EV-based liquid biopsy is a novel and less-invasive alternative to surgical biopsies which would enable clinicians to potentially discover a whole picture of tumor through a simple blood or urine sample. In summary, this approach holds promise for developing personalized medicine to guide treatment decisions precisely for CaP patients.

Keywords: Prostate cancer, liquid biopsy, extracellular vesicle, biomarker, early diagnosis, risk prediction



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Prostate cancer (CaP) is one of the most common malignancies in male in Western countries. With economic development and lifestyle changes, the incidence and mortality rate of CaP among Asian males have been rising rapidly^[1,2]. In China, CaP is the leading male genitourinary malignant tumor, and its incidence is now higher than bladder cancer^[3]. In 2020, CaP caused more than 50,000 deaths in China, which were nearly twice as high as in the United States^[4]. The survival of CaP patients is highly dependent on tumor stage and risk classification. It was reported that low-risk and localized CaP achieved a favorable prognosis by long-term close monitoring, while metastatic patients had a median survival of 30 months^[5]. The treatment options for different risk classifications are also unique. The principal treatment for clinically low-risk CaP is radical treatment, such as prostatectomy and radiotherapy. In addition to radical treatment, comprehensive treatment such as radiotherapy, chemotherapy, and endocrine therapy is required for patients with high-risk CaP. Therefore, CaP early diagnosis and grading are crucial to initiate proper treatment, improve patients' outcomes, and prolong survival.

Currently, prostate-specific antigen (PSA) is the most used diagnostic biomarker for CaP diagnosis in the clinic. The widespread use of PSA-testing has increased the diagnostic rate of CaP, but it is also accompanied by a high false-positive rate of CaP and overtreatment of indolent tumors due to the poor accuracy^[6]. PSA cannot differentiate among benign prostate changes, indolent cancers (unlikely to cause significant symptoms), and early versus advanced stages of CaP. It often leads to 20% to 42% over-diagnosis and over-treatment, which may cause a patient more harmful than good^[7]. The Prostate Cancer Prevention Trial showed that 14.9% of prostate tumors in men with PSA levels lower than 4.0 ng/mL had Gleason scores of seven or higher^[8].

Also, due to the limitations of its low specific and low sensitive character, early detection and real-time monitoring of tumor progression cannot be achieved^[9]. Needle biopsy is the gold standard for CaP diagnosis. However, tumor biopsy has several limitations in clinical application, including the pain associated with the invasiveness of the procedure, the significant risk of hemorrhage and urinary retention, and the risk of false-negative results due to tumor heterogeneity. Magnetic resonance imaging (MRI) has somewhat improved selection for biopsy but the utility is still limited by occasional false negatives in 5%-15% of Prostate Imaging-Reporting And Data System (PIRADS) and false positives in 40%-60% of PIRADS scores 3-5 (equivocal or positive MRIs)^[10]. There is thus an unmet need to develop tools to noninvasively detect CaP at an early stage with high sensitivity and specificity and to improve individualized treatment.

Liquid biopsy refers to the technology that makes full use of body fluids minimally or non-invasively obtained, such as blood, urine, or saliva, and it is receiving great attention as a novel diagnostic tool to access response to clinically and biologically relevant information^[11]. EV-based liquid biopsy can be integrated to maximize insights into tumor status especially in view of dissecting tumor heterogeneity. Liquid biopsy components include circulating tumor cell, circulating tumor DNA, and/or extracellular vesicles (EVs)^[12,13]. Compared with other types of liquid biopsy, the use of EVs such as exosomes may offer unique advantages. Firstly, exosomes are highly abundant in most biological fluids, such as blood plasma, where one can detect 10^{8-13} exosome particles/mL^[14]. Secondly, tumor-derived exosomes have specific biomarkers distinguished from exosomes from normal tissues, which can be used for cancer diagnosis and prognosis^[15]. Furthermore, exosomes have strong stability, which means they can be stored at -80 °C for several months or even years^[16]. Thus, exosomes are emerging as a newly attractive biomarker of liquid biopsy for non-invasive cancer diagnosis. EVs are potent and clinically valuable tools for CaP early diagnosis and prognosis as they are highly representative of their cell of origin^[17].

It was reported that the differential expression of EV contents in nucleic acids (i.e., DNA, mRNA, and non-coding RNAs), proteins, and lipids may create favorable conditions for CaP invasion and metastasis^[18-22]. We have recently summarized the emerging role of EVs in liquid biopsy for monitoring CaP invasion and metastasis^[23]. Current high-throughput technologies for genomic, transcriptomic, and proteomic analysis are driving ground-breaking discoveries in the field of new EV biomarkers. While most of the current CaP EV studies have been focused on the mechanism of metastasis and progression and as well as between cell-cell communications^[24,25], only a few reliable EV biomarkers for the risk classification of CaP were clinically applied.

In this commentary, we have to emphasize a very important milestone study, highlighting the clinical importance of EV biomarkers in CaP stratification, which is the discovery of a novel urine EV gene expression assay [the ExoDx Prostate IntelliScore (EPI) urine exosome assay] to differentiate high-grade CaP from low-grade CaP and avoid unnecessary biopsies^[26]. This test has been approved by the United States Food and Drug Administration with the “Breakthrough Device” designation. The EPI test is a urine EV gene expression assay that does not require pre-collection digital rectal exam and relies on the isolation and analysis of urinary EVs. In the EPI test, first catch urine samples (25-50 mL) are collected and EVs are isolated by a proprietary ultrafiltration centrifugation technique. After extracting exosomal RNAs, the RNA copy numbers from three genes (i.e., ERG, PCA3, and SPDEF) are determined by RT-qPCR. EPI is a noninvasive, easy-to-use, urine EV-RNA assay that has been validated across three independent prospective multicenter clinical trials with 1212 subjects^[27]. The test can discriminate high-grade (\geq GG2) from low-grade (GG1) cancer and benign disease. EPI effectively guides the biopsy-decision process independent of PSA and other standard-of-care factors. The absence of clinical variables in the EPI algorithm represents an important differentiator from other assays predicting high-grade CaP, including 4K score test (OPKO Diagnostics, Miami, FL) and SelectMDX (MDx Health, Irvine, CA). As EPI performance is based on gene expression only, this assay is more accurate than existing risk assessment methods such as clinical features. There is an option for the urologist to introduce other parameters, such as obesity status, underlying genetics, and race, for developing a more personalized risk assignment at both initial and or repeat biopsy time-points. Among urologists, 68% reported that the EPI test influenced their biopsy decision with respect to selecting the right patients to biopsy at the right time, thereby improving their ability to identify clinically significant disease and reduce biopsies when the test was negative^[28]. Although the EPI test has achieved some satisfactory results, future studies need to incorporate it in determining the use of MRI and inclusion of the EPI-risk score into an algorithm that includes the PIRADS designation and other clinical variables. More clinical trials still need to further confirm its clinical value. In addition to the EPI test, early diagnosis or efficient prognostic EV biomarkers are warranted for improving risk stratification, personalized postoperative adjuvant therapy, and prognostication of CaP patients for clinical translation in the future.

Although the analysis of EV contents is promising in the early diagnosis and progression grading of CaP, the application of the EV-based liquid biopsy in the clinic is still facing challenges. Due to the heterogenous nature of human samples and complications in isolation, it is ideal to optimize the isolation technique to obtain relatively homogeneous EV cargoes with reproducible, high-yielding and throughput, and scale-up capability. The contaminations in blood (e.g., high-abundance blood proteins and apolipoproteins) and urine (e.g., mucoprotein, also called as the Tamm-Horsfall protein) need to be removed before genomic and proteomic analysis.

EVs act as cellular messengers and have been shown to transfer proteins and nucleic acids between tumor cells that influence tumor initiation, proliferation, progression, and metastasis^[29-31]. An increasing number of studies have screened candidate biomarkers from body fluids that may be used to diagnose CaP. Due to the

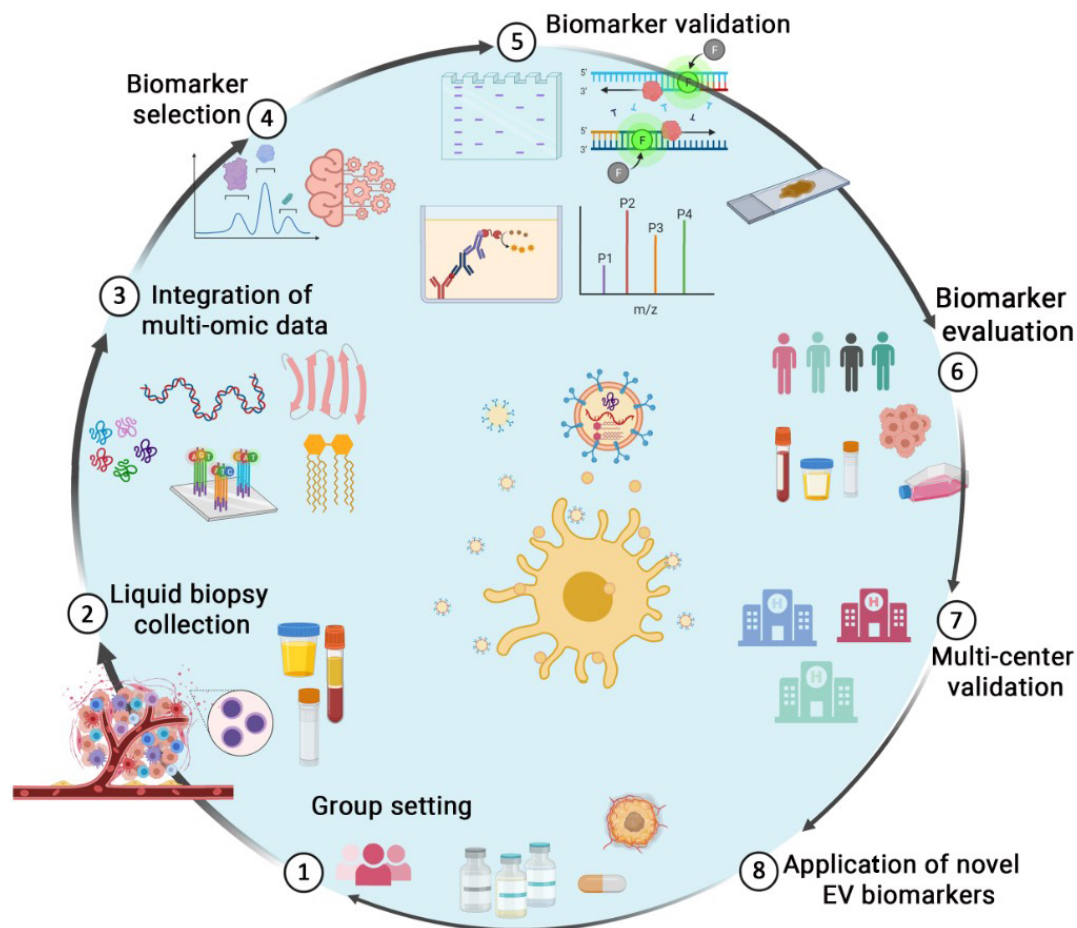


Figure 1. Overview of EV biomarker discovery and validation for CaP personalized treatment decision. Step 1. Group different stages of CaP patients and healthy control subjects. Step 2. Collect human samples including blood, urine, and semen. Step 3. Integrate multi-omic technology including genomics, transcriptomics, proteomics and lipidomics for profiling. Step 4. Select candidate biomarkers by machine learning and establish machine learning models. Step 5. Validate selected biomarkers by different methods such as WB, qRT-PCR, ELISA, PRM, and immunofluorescence in samples such as blood, urine, tissue, and cultured cell lines. Step 6. Evaluate selected biomarkers in a large set of independent patient samples. Step 7. Multi-center EV biomarker diagnosis performance verification. Step 8. Novel EV biomarkers for early diagnosis and patient grading, and for precision medicine. ELISA: Enzyme-linked immunosorbent assay; CaP: prostate cancer; EV: extracellular vesicle; qRT-PCR: quantitative reverse transcription PCR; PRM: parallel reaction monitoring; WB: western blot.

heterogeneity and variety of genetic backgrounds of patients, the lack of data from large-scale samples for the validation of CaP biomarkers is the main deterrent to translating the potential EV biomarkers from bench to the bedside. In order to discover specific and sensitive biomarkers of tumors in diagnosis and screening, more multidisciplinary technologies and collaborations are highly expected in the near future.

The advances in high-throughput and modern omics technologies such as genomics, transcriptomics and proteomics have greatly promoted EV biomarker research in the recent decade. Machine learning has been applied to integrate multi-omics sequencing data, which are still generated at ever-growing rates and scales. Machine learning can lead to a high-quality performance for liquid biopsy-based diagnosis for multiple human cancers^[15] and holds promise for EV-based CaP liquid biopsy. Comprehensive multi-omics data analysis with machine learning has been a frontier in cancer genomics^[32] and should be performed in CaP EV biomarker research in the future [Figure 1].

Oncogenesis is driven by a complex and intricately controlled gene expression programming related to molecular-level variation in many genes. Due to the high heterogeneity and intricacy, a single biomarker does not fully characterize tumor properties, and more accurate predictions using multi-parameter markers are required^[33,34]. A set of candidate biomarkers can be evaluated for their differences in abundance between patients and normal controls, resulting in more robust predictive and diagnostic capacities^[35]. For CaP EV-based biomarker research, a comprehensive pipeline for discovery and validation is shown in [Figure 1](#).

Most importantly, these putative EV biomarkers need further validation independently at multiple levels, such as blood, urine, tumor tissue, and cell lines, to increase their specificity and sensitivity before clinical application. During this stage, the sensitivity and specificity of the selected EV markers need to be compared with the current PSA test, MRI imaging, and tissue biopsy. These markers need to ensure they are disease-specific, rather than trial-dependent. Assessing multi-center EV biomarker diagnosis performance in CaP is necessary to further evaluate the clinical value of these biomarker set prior to its widespread use. These EV biomarkers can be used to diagnose CaP and predict the stages of cancer and the tumor biological activity. This EV-based liquid biopsy can guide clinicians in choosing the best treatment methods for an individual CaP patient and significantly improve their prognosis.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception, design, writing, and editing of the paper, and performed literature searches and interpretation: Hand M, Pang B, Zhou C, Li X, Wang Q, Jiang J, Li Y

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Consent for publication

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Commentary

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Considerations before the application of 5-hydroxymethylation levels of long non-coding RNAs for non-invasive cancer diagnosis

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Abstract

Previous studies have suggested that aberrant 5-hydroxymethylcytosines (5hmC) modifications are related to cancer pathobiology. Genome-wide profiling 5hmC in circulating cell-free DNA (cfDNA) using the highly sensitive chemical labeling-based 5hmC-Seal technique has been demonstrated to have the potential to be a robust epigenomic tool for cancer biomarker discovery. Prior studies have mostly focused on cfDNA-derived 5hmC-Seal data summarized in well-annotated genic features (e.g., gene bodies) or unbiased bins. Zhou *et al.* recently proposed long non-coding RNAs (lncRNAs) as an alternative molecular target for biomarker discovery using publicly available 5hmC-Seal data. Considering its potential clinical impact, we would like to comment on Zhou *et al.* and advocate more serious consideration of critical issues such as the availability of clinical information and technical variables, especially when performing secondary analysis using publicly available data, with the aim of improving data transparency and translatability.

Keywords: 5-Hydroxymethylcytosine, long non-coding RNA, cell-free DNA, cancer biomarker

The 5-hydroxymethylcytosine (5hmC) is an emerging epigenetic marker that reflects gene activation



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status^[1]. Previous studies have suggested that aberrant 5hmC modifications are related to cancer pathobiology. Genome-wide profiling of 5hmC in circulating cell-free DNA (cfDNA) using the 5hmC-Seal technique^[2], a highly sensitive chemical labeling approach suitable for a very limited amount of clinical biospecimens (e.g., 1-2 ng of cfDNA from a few mL of plasma) has been demonstrated by our team and other groups to be a robust epigenomic tool for cancer biomarker discovery with the goal of achieving non-invasive cancer diagnosis and prognosis^[3-6].

Analytically, although our previous studies mostly focused on the 5hmC-Seal profiles summarized in well-annotated genic features (e.g., gene bodies) or unbiased bins, recently, we started exploring the possibility of integrating 5hmC profiles summarized for long non-coding RNAs (lncRNAs) and repetitive elements to improve biomarker discovery using glioblastoma (GBM) as an example^[3]. Specifically, in the cell, lncRNAs are known to regulate gene expressions at both transcriptional and post-transcriptional levels, and play important and heterogeneous regulatory roles in nearly all cellular and biological processes, including transcriptions, translation, and nuclear trafficking, as well as tumorigenesis and therapy resistance^[7]. In GBM, dysregulation of lncRNAs can contribute to the epithelial-mesenchymal transition, therefore promoting cancer metastasis^[8]. In addition, a recent study reported a positive association between 5hmC and lncRNA transcription in colorectal cancer, indicating the regulatory role of 5hmC on lncRNA expression^[9]. Given its tissue-specificity and roles in tumor initiation, progression and resistance to therapy, lncRNAs remain to be promising markers for cancer diagnosis and prognosis.

Specifically, we read with interest that a recent study published by Zhou *et al.*^[10] described the development of plasma-derived 5hmC-lncRNA diagnostic score (5hLD-score) for cancer diagnosis and surveillance using publicly available 5hmC data. The proposed 5hLD-score was shown the capability of distinguishing tumors from healthy controls in their training and internal validation cohorts. Further validation showed the 5hLD-score achieved area under the curve (AUC) of 0.85, 0.89, and 0.77 in a non-small cell lung cancer cohort, an esophageal cancer cohort, and a hepatocellular carcinoma (HCC) cohort, respectively. The authors identified an association between the 5hLD-score and the progression of liver cancer in the HCC cohort, as well as the capability to identify the origin and location of tumors. This study further supported the clinical potential of 5hmC levels in lncRNAs for cancer early detection and progression monitoring. However, we would like to comment on a few important issues of Zhou *et al.* and advocate that there are several critical issues that need to be taken into consideration in order to make an informed conclusion of the current status of applying 5hmC levels in lncRNAs as a marker for cancer diagnosis and prognosis, especially when such a conclusion was drawn from performing secondary data analysis using public data.

Firstly, during statistical modeling, differential 5hmC modifications should be identified in the training set solely. Instead, Zhou *et al.* used the whole Li's cohort (training and internal validation set combined) to perform the differential analysis. This procedure would have caused data leakage, which introduced the knowledge of the validation set into the modeling process, and could have led to model overfitting in the validation set. Therefore, the observed differences in terms of the AUCs between the internal validation set and the independent validation set presented by Zhou *et al.* could be due to data leakage and model overfitting, which should be evaluated using appropriate tests such as the Delong test^[11].

Secondly, when using the 5hmC profiles generated from different platforms/protocols, sequencing length, depth, or platform information should be taken into considerations. Regarding these potential technical biases, Zhou *et al.* did not take them into considerations in their analysis. To our best knowledge, the Li's cohort^[6] was sequenced with 150 base-pair (bp) paired-end library, while the Cai's liver cancer cohort^[5] was sequenced with 38 bp paired-end library. In addition, the publicly available 5hmC data were generated at

different times and core facilities. Those unaccounted factors, taken together, could cause substantial batch effects, with the likelihood of leading to misinterpretation of the results.

Thirdly, clinical variables, such as age, gender, tumor stages, place of residence, and lifestyle, have been established as potential confounders in epigenetic studies. These variables (known or hidden) contribute to the epigenetic differences between cases and controls. Not appropriately adjusting for these confounding variables could lead to biased interpretation of results. For examples, in figure 5, Zhou *et al.*^[10] argued that the 5hLD-scores were associated with liver cancer progression. However, this finding could be confounded by patient's age, as the liver cancer patients were much older than patients with hepatitis B infection history in the Cai cohort^[4].

Finally, unlike mRNAs with protein-coding potential or microRNAs with high sequence conservations, lncRNAs possessing unique features such as lower transcription rate, reduced stability and lower expression levels can pose analytic constraints in the characterization and annotation of lncRNAs^[12]. For example, the GENCODE^[13] lncRNAs were identified from RNA-Seq data and algorithm not optimized for the full exploitation and annotation for non-polyA lncRNA transcripts or functional lncRNAs with relatively lower expression. Furthermore, given the relatively lower expression of lncRNA in non-brain tissue types, the signal to noise ratios of 5hmC mapping over lncRNA regions on cfDNA are expected to be even lower in non-brain cancer patients included at least in theory. However, Zhou *et al.* did not provide any evaluation of the expression levels or tissue-specificity of these lncRNAs before proceeding to the marker discovery phase. As a result, the 5hmC profiles of lncRNAs in the current study could have been subjected to random noise due to low abundance. Last but not least, we observed synergistic effects between the 5hmC of lncRNAs and other genomic feature types (i.e., gene body, repetitive elements and histone marks) in our GBM study^[3], it would be interesting if future studies could incorporate other genomic feature types and compare the performance by feature type, separately and integratively.

In conclusion, in our opinion, the 5hmC levels of lncRNAs could be a promising biomarker for cancer diagnosis and monitoring, though future large studies of individuals with more comprehensive clinical, pathological, and epidemiological information, as well as the application of more robust data analysis plans (e.g., consideration of hidden variables) will help improve data transparency and provide more insights into the translatability of these molecular targets.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception: Zhang Z, Zhang W

Drafted the manuscript: Zhang Z, Zeng C, Zhang W

Approved the final version: Zhang Z, Zeng C, Zhang W

Availability of data and materials

Not applicable.

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Conflicts of interest

Zhang W has been an advisor to Epican Genetech, which has a license of the 5hmC-Seal technique from the University of Chicago for clinical application. Other authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Nucleic acid functionalized extracellular vesicles as promising therapeutic systems for nanomedicine

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Abstract

Extracellular vesicles (EVs), as natural carriers, are regarded as a new star in nanomedicine due to their excellent biocompatibility, fascinating physicochemical properties, and unique biological regulatory functions. However, there are still some challenges to using natural EVs, including poor targeting ability and the clearance from circulation, which may limit their further development and clinical use. Nucleic acid has the functions of programmability, targeting, gene therapy, and immune regulation. Owing to the engineering design and modification by integrating functional nucleic acid, EVs offer excellent performances as a therapeutic system *in vivo*. This review briefly introduces the function and mechanism of nucleic acid in the diagnosis and treatment of diseases. Then, the strategies of nucleic acid-functionalized EVs are summarized and the latest progress of nucleic acid-functionalized EVs in nanomedicine is highlighted. Finally, the challenges and prospects of nucleic acid-functionalized EVs as a promising diagnostic system are proposed.



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Keywords: Extracellular vesicles, nucleic acid, nanomedicine, mRNA vaccine, aptamer

INTRODUCTION

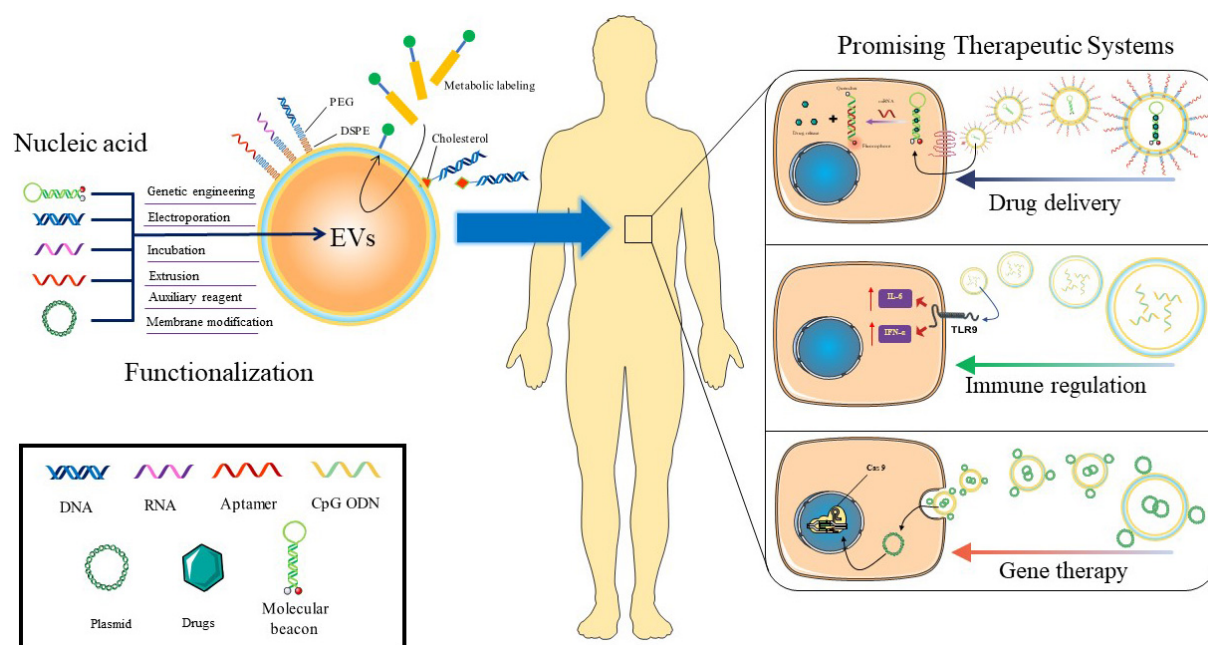
Extracellular vesicles (EVs) are natural nano-carriers produced by living cells for intercellular communication^[1-3]. EVs can be classified as exosomes, microvesicles, or apoptotic bodies according to their biogenesis type and particle size^[4]. Exosomes and microvesicles are the most widely studied, thus “EV” is commonly used to refer to these two subgroups^[5]. Exosomes, with particle sizes ranging from 30 to 150 nm, are formed when multivesicular bodies fuse with the cell membrane and release the vesicles inside^[6]. Microvesicles, with particle sizes of 50-1000 nm, are formed by cell membrane bubbling^[6]. Due to the limitation of the separation method, exosomes and microvesicles are difficult to separate in the range of 30-200 nm, which are commonly referred to collectively as small EVs. The EVs summarized in this paper mainly refer to small EVs, including exosomes and microvesicles. Due to their good biocompatibility, low immunogenicity, excellent extensibility, and unique biological regulatory function, EVs have attracted wide attention in the field of nanomedicine and are considered as a new star in nanomedicine^[7-9].

EVs have been developed as a delivery carrier of drugs or contrast agents, showing great application potential in the field of disease diagnosis and treatment^[5,8,9]. However, native EVs have difficulty meeting the functional requirements of the complex physiological environment; therefore, necessary engineering design and modification can significantly improve the performance of EVs as a therapeutic system. As biological macromolecules, nucleic acid has unique biological functions and has been widely used in the field of nanomedicine^[10-13]. RNA interference, antisense oligonucleotides, and cluster regularly spaced short palindromic repeats-associated protein 9 (CRISPR/Cas9) system can downregulate, enhance, or correct gene expression and have wide application potential in gene therapy research^[14-19]. Nevertheless, these promising therapies are severely limited by inefficient biological distribution and sensitivity to degradation. The development of intracellular delivery carriers can effectively overcome the above limitations of nucleic acid therapy. EVs are natural carriers of information, matter, and energy exchange between cells, involving molecular transport between cells. Functional genetic components such as DNA, mRNA, and ncRNA loaded by EVs can be transported to target cells to perform the function of gene expression regulation. This suggests that EVs are a good nucleic acid delivery carrier. The combination of nucleic acid and EVs makes up for their shortcomings and is expected to provide a promising diagnosis and treatment system for nanomedicine. In addition, nucleic acids also have targeting (aptamer), programmability, drug loading, and immunomodulatory functions^[20], which will greatly improve the application prospects of EVs.

This review briefly summarizes the function and mechanism of nucleic acid in diagnosis and treatment and preliminarily clarifies the necessity and advantages of nucleic acid-functionalized EVs. This review provides a basic understanding of this field by highlighting the engineering strategies and representative progress (Scheme 1). Finally, the challenges and future development of nucleic acid-functionalized EVs are proposed.

FUNCTION AND MECHANISM OF NUCLEIC ACID IN NANOMEDICINE

As biological macromolecules, nucleic acid has unique biological functions and has been widely used in the field of nanomedicine^[21-23]. Among them, the most common functions are targeting, programming, gene expression regulation, and immune regulation. This section briefly introduces the functions and mechanisms of nucleic acid [Figure 1]. Related studies on the use of nucleic acid in the biomedical field can also be found in earlier literature^[24,25].



Scheme 1. Nucleic acid-functionalized EVs are promising therapeutic systems for nanomedicine. As a functional macromolecule, nucleic acid has the capabilities of targeting, self-assembly, drug loading, gene editing, and immune regulation. By combining nucleic acid with EVs, EVs acquire the functional properties of nucleic acid, thus showing unprecedented application potential in drug delivery, immune regulation, and gene therapy, which are expected to provide a promising therapeutic system. EVs: Extracellular vesicles.

Targeting ability

Aptamers are oligonucleotide sequences with specific affinity activity screened by the systematic evolution of ligands by exponential enrichment technique. The obtained oligonucleotide sequences with specific recognition and affinity for proteins, bacteria, cells, and other target molecules are also called chemical antibodies^[26,27]. Aptamers can be used as drugs themselves or combined with drugs, siRNA, and nanoparticles to form targeted drug delivery systems, which can target specific tumor cells, reduce toxicity to normal cells, significantly reduce drug dosage, and improve efficacy^[28-31]. Aptamers have become valuable affinity probes in biochemistry research, disease diagnosis, and treatment. Recently, Wang *et al.*^[32] developed a DNA adapter with excellent targeting properties and unique functional versatility that can be used for biomarker detection, medical molecular imaging, and therapeutic targeted drug delivery. In another study, Liu *et al.*^[33] developed a fluorescent probe based on DNA aptamer for specific molecular typing of mammary neoplasms. Aptamers for new targets are being screened, providing new options for targeted therapy.

The aptamer, as a specific recognition element, has the advantages of simple synthesis, easy modification, biodegradability, and low toxicity, and it has aroused wide attention in both basic and clinical research^[28]. In particular, some aptamers for surface biomarkers of cancer have been screened out and used in the design of targeted delivery systems for cancer^[34-36]. With the development of technology, aptamers can be modified by various functional groups, which will further expand the application prospects of aptamers.

Drug delivery carriers

Nucleic acids have the property of self-assembly, and they can be assembled into a double helix structure through complementary pairs of bases, or complex structures such as G-quadruplets can be constructed through complementary pairs of bases^[37,38]. DNA origami technology uses the folding and self-assembly of nucleic acids such as DNA and RNA to form complex structures^[39,40]. DNA origami technology can

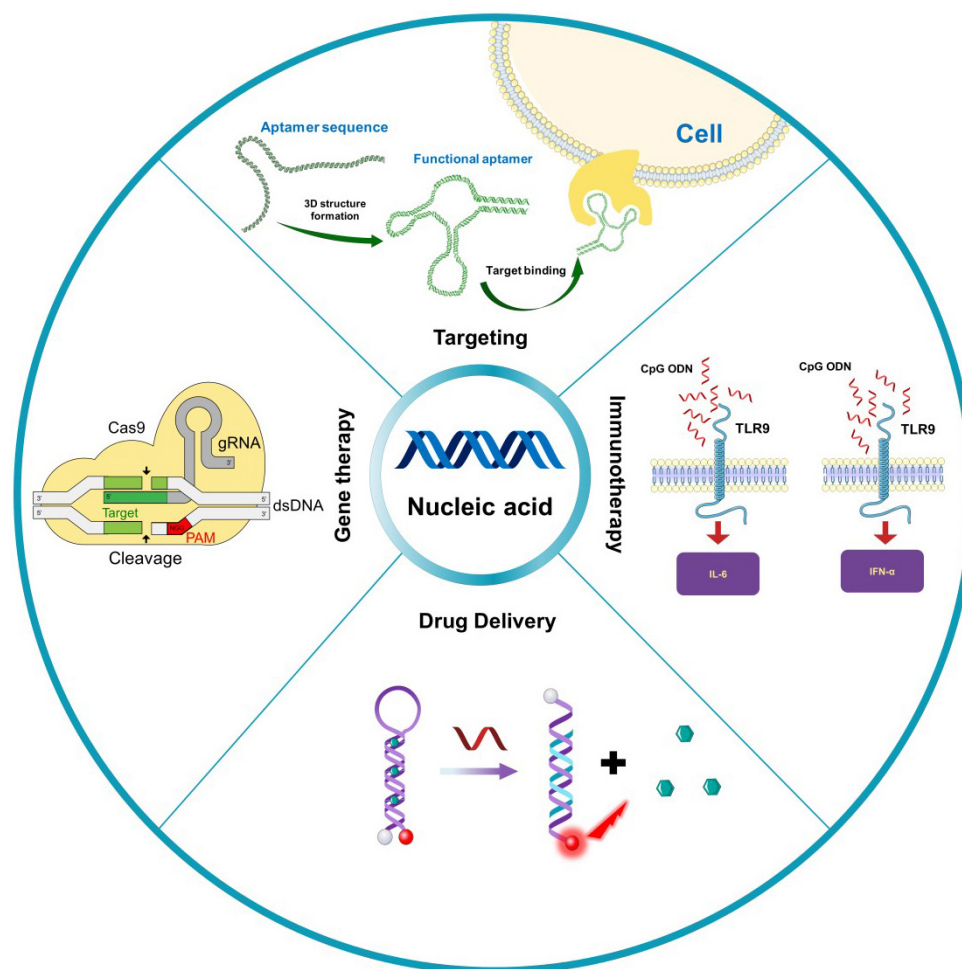


Figure 1. Function and mechanism of nucleic acid in nanomedicine. Nucleic acid has the characteristics of targeting (aptamer), immune regulation (CpG ODN), drug delivery (molecular beacon or DNA origami), and gene editing (CRISPR/Cas9). CRISPR/Cas9: Cluster regularly spaced short palindromic repeats-associated protein 9.

synthesize homogeneous nanostructures with sizes between 50 and 400 nm that can be used as drug delivery materials to enhance drug delivery and survival in malignant environments^[40-43]. DNA origami technology can also design dynamic, multi-stimulus responsive nanostructures to achieve controlled release of drugs^[44,45]. Jiang *et al.*^[46] used DNA origami to deliver adriamycin into the body. They found that drug-loaded DNA triangle origami showed a strong tumor treatment effect, and no systemic side effects were observed when treating human MDA-MB-231 breast tumor cells^[47]. As an effective and biocompatible drug carrier, DNA origami has great potential in tumor therapy^[41,48-53].

In addition to DNA origami, there are other types of nucleic acid drug carriers. Molecular beacons are fluorescently labeled stem-loop oligonucleotide chains capable of loading and transporting doxorubicin^[54]. The advantage of molecular beacons as drug carriers is that drug release requires conditions to trigger the destruction of nucleic acid secondary structure, and the drug release process can be monitored in real time by fluorescence signal. For example, Ma *et al.*^[55] reported a drug delivery system based on molecular beacon for detecting telomerase activity and telomerase triggered drug release in living cells. This provides a feasible strategy for conditionally controlled release and treatment monitoring. DNA hydrogel is a new kind of important DNA material, which is a three-dimensional polymer network constructed by DNA as a

structural element^[56]. It has been used extensively to develop drug delivery systems (DDS) because of its advantages of high water content, large drug loading space, and good biocompatibility^[57]. To sum up, the nucleic acid drug carrier has the advantage of being programmable, showing great application potential in the construction of DDS.

However, naked nucleic acid nanostructures have relatively high electrical charges, which may influence their behavior in blood circulation and scavenging. Additionally, the DNA nanostructure has a potential immune risk, being it easy to trigger the body's inflammatory response. Polymer coating protects nucleic acid drug carriers from overexposure and has been shown to improve structural integrity and circulatory stability as well as to attenuate immune stimulation.

Gene therapy

Gene therapy, as an indispensable tool in biomedical research, has shown potential to treat a variety of diseases, including single-gene inherited diseases, cancer, cardiovascular disease, diabetes, infectious diseases, and inflammatory diseases, which has profoundly influenced the development of medicine. Gene therapy is the treatment of diseases by introducing genetic material into cells and editing genes that produce defective proteins or interfering with gene expression^[58]. Nucleic acids are the main tools of gene therapy, such as DNA and mRNA molecules for gene overexpression and small RNA molecules such as siRNA, miRNA, and antisense oligonucleotides for gene knockdown^[59]. For example, Kusano *et al.*^[60] reported the potential therapeutic effect of intramuscular sonic hedgehog gene transfer on myocardial injury repair. In recent years, gene editing strategies based on the CRISPR/Cas9 system have been applied to the treatment of genetic diseases. The CRISPR/Cas9 system needs to guide nucleic acid sequence to control gene editing sites and is also a representative of nucleic acid participation in gene therapy^[61]. The biggest limitation of gene therapy is the efficient delivery of gene regulatory systems to cells. Nucleic acid in its natural form is not easily absorbed by cells and is easily degraded and removed, so carriers are needed to deliver nucleic acid into cells. Although viral vectors such as adenoviruses, lentiviruses, and retrovirus show advantages in transfection rates and life-long expression, insertional mutations and other persistent side effects make clinical use difficult. EVs have nucleic acid and protein delivery functions and are potential gene therapy vectors.

Immune regulation

Nucleic acid has the potential for immune regulation. In the process of biological evolution, higher organisms have evolved the mechanism of recognizing microbial nucleic acid sequences through pattern recognition receptors, thus activating anti-infection immunity^[62]. This provides the basis for the immune regulation of nucleic acids. CpG oligodeoxynucleotide (CpG ODN) is a commonly used immune adjuvant that can effectively trigger a mammalian immune response through toll-like receptor 9 (TLR9) signaling and has been used as an immune adjuvant against infection and tumor^[63-70]. In addition, poly I: C, PolyA: U, *etc.* may enhance the activity of nucleotide kinases and participate in immune regulation^[66,71].

In addition to the oligo nucleic acid chain, immune-gene therapy is another important way of nucleic acid immune regulation. It works by introducing genes that promote immune activation into the body's cells. There are two cases. The first is the introduction and expression of cytokine genes to enhance the body's immunity^[72,73]. This method has broad spectrum and non-specificity. The other is a process that stimulates specific immunity by introducing specific epitope genes into the body. The method is also known as a nucleic acid vaccine (NAV). NAV aims to introduce the gene sequence encoding specific antigen protein into animal somatic cells, synthesize antigen by using the protein expression system of animal itself, and induce the animal body to produce acquired immunity for the purpose of preventing and treating diseases. DNA vaccines are also known as naked vaccines, so named because they do not require any chemical

vectors^[74]. After the DNA vaccine is introduced into the host, it is taken up by cells (tissue cells, dendritic cells, or other antigen-presenting cells), and the antigen protein is expressed by using the protein synthesis system of the cells, which stimulates the host to produce cellular and humoral immunity through a series of cascading processes^[75,76]. Compared with traditional inactivated vaccine, DNA vaccine has the following advantages: (1) enhanced immune protection; (2) sequence design can be used to modify antigen determinants or prepare polyvalent vaccines; and (3) producing a safe and durable immune response that does not require multiple immunizations. However, DNA vaccines are potentially dangerous: (1) Continuous expression of foreign antigens may have adverse consequences. Long-term expression of exogenous antigen by plasmids may lead to immune tolerance or anesthesia. (2) After being injected into the body, foreign DNA may be integrated into the host genome to inactivate or activate the tumor suppressor genes of the host cells and transform the host cells into cancer cells, which may be the worthiest of in-depth study among many safety issues of the nucleic acid vaccine.

mRNA vaccines can trigger a specific immune response by introducing mRNA encoding specific antigens into the body and using the protein synthesis mechanism of the host cell to produce antigens. Compared with traditional vaccines, mRNA vaccines are simpler to produce, faster to develop, do not require cell culture, have lower cost^[77], and are more immunogenic in expressing conformation stable proteins or exposing key antigen sites^[78-82]. Even when compared with DNA vaccines, there are significant advantages. mRNA vaccines do not need to enter the nucleus, so they do not carry the risk of integration into the host genome^[83]. However, two challenges must be overcome before mRNA vaccines work. The first challenge is the design and synthesis of mRNA. The high expression, specificity, and immunogenicity of kernel mRNA are important to the success of vaccines. In addition, mRNA also requires special design and modification to improve its stability. Another important challenge is the construction of a delivery carrier. A carrier with targeted properties can improve the enrichment of mRNA in the target cell, which is conducive to the efficient expression of the antigen. In this process, the lysosomal escape ability of the carrier is equally important for protecting mRNA from degradation. It is exciting that the approval of two coronavirus disease 2019 (COVID-19) mRNA vaccines (mRNA-1273 and BNT162b2) promote the development of mRNA vaccine technology. Clinical trials have shown that the two-dose regimen of BNT162b2 provides 95% protection against COVID-19 in humans over 16 years of age. Median safety over two months was same as other vaccines^[84]. However, mRNA vaccines also have some problems to be solved, such as poor stability of the mRNA itself, low cell entry efficiency, and low translation efficiency^[85]. The development of intracellular delivery carriers with nucleic acid protection has become a research focus in this field^[86-88].

ENGINEERING STRATEGIES FOR NUCLEIC ACID-FUNCTIONALIZED EXTRACELLULAR VESICLES

Nucleic acid has developed into an important functional subassembly for the modification and functionalization of drug delivery carriers due to its unique physiological and biochemical properties. EVs, a rising star in drug delivery, has also sparked with nucleic acid subassembly. Therefore, it is important to know the strategy of nucleic acid functionalization of EVs. The current engineering strategies of EVs with nucleic acid can be divided into two types: membrane modification and encapsulation. Each type contains several fabrication approaches. Commonly used fabrication approaches and their merits and demerits are summarized in [Table 1](#).

Membrane modification strategies

By modifying specific chemical groups, the coupling between nucleic acid and EVs can be efficiently realized [[Figure 2A](#)]. Hydrophobic molecules such as 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE) can be inserted into the phospholipid bilayer of EVs. Nucleic acid molecules can be anchored to the

Table 1. Comparison of different engineering strategies for nucleic acid-functionalized EVs

Approach	Components	Merits	Demerits	Ref.
Parental cell treatment	mRNA, SgRNA	High loading efficiency, no damage to EVs	The operation is difficult and the process complex	[89-91]
Incubation	siRNA	Facile method and easy to operate	Low load efficiency	[92]
Membrane modification	Aptamer, DNA hinge, CpG ODN, molecular beacon	Simple operation, high load efficiency	Nucleic acids are exposed to the surface and have no protective effect	[93-95]
Extrusion	siRNA	High load efficiency	Complex preprocessing	[96]
Electroporation	CpG ODN, siRNA, molecular beacon	Simple operation, high load efficiency	The formation of pores in EVs may cause irreversible damage	[94,97,98]
Sonication	miRNA, siRNA	Simple operation	Structural failure and low load efficiency for macromolecules	[99,100]
Streptolysin O	DNA junction, molecular beacon	Simple operation	The integrity of EVs may be impaired	[101,102]
Liposome	Plasmid, small RNA	Simple operation, high load efficiency	Particle size becomes larger, and EVs aggregates	[103,104]

EVs: Extracellular vesicles; CpG ODN: CpG oligodeoxynucleotides.

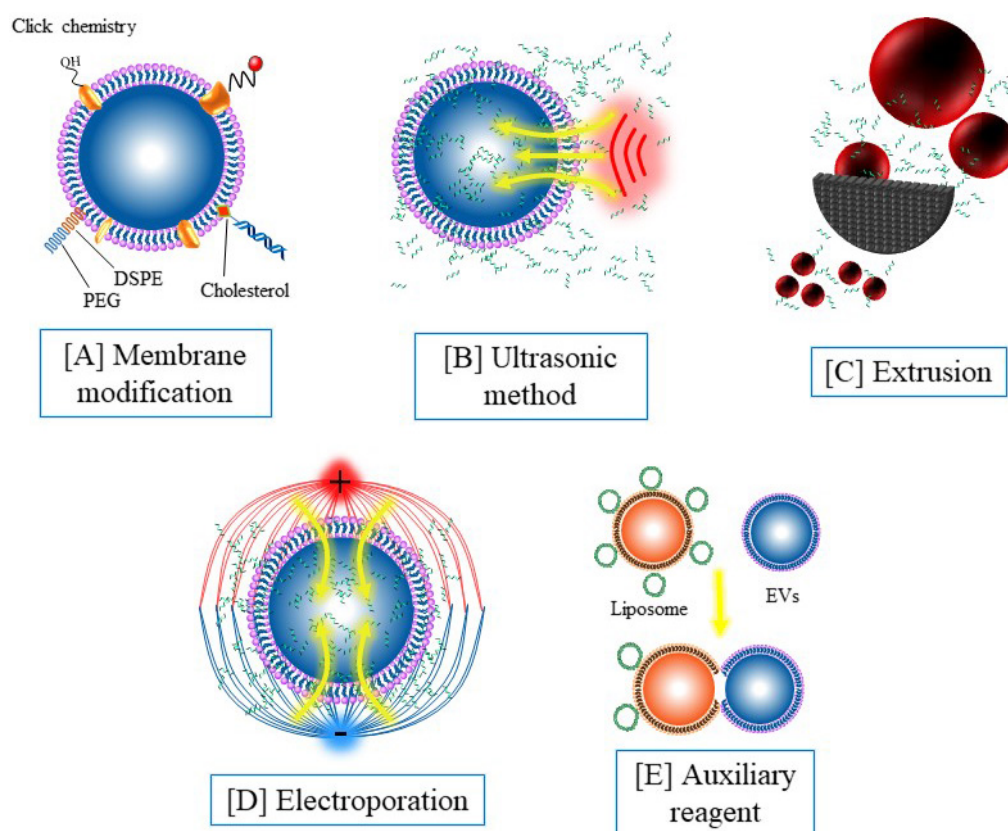


Figure 2. Engineering strategies for nucleic acid-functionalized extracellular vesicles: (A) membrane modification by using 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine insertion, click chemistry, and covalent modification; (B) ultrasonic oscillations mediate nucleic acid loading; (C) nucleic acid loading mediated by extrusion; (D) nucleic acid loading mediated by electroporation; and (E) nucleic acid loading mediated by an auxiliary reagent.

surface of the vesicles by nucleic acid coupling DSPE^[93]. Our previous study found that fresh EVs are rich in sulfhydryl groups, and nucleic acid can be conjugated with EVs by modifying the maleimide group with nucleic acid^[94]. This method is mild and specific and has a wide application prospect. In recent years, click

chemical modification based on glucose metabolism chemistry has been introduced into the engineering of EVs^[105-107], which also provides a promising approach for nucleic acid modification. The membrane modification strategies can anchor nucleic acid to the surface of the vesicles, thus endowing the EVs with targeted recognition and other functions. However, these loading methods leave the nucleic acid exposed to the outside of the EVs and cannot obtain the protection of the EVs.

Encapsulation strategies

In addition to surface modifications, nucleic acid components can also be encased inside EVs [Figure 2B-E]. Parental cell treatment is an early method used to introduce nucleic acids into EVs. Although this method has high efficiency and simple follow-up operation, the preprocessing such as plasmid construction is still tedious and time-consuming. Electroporation is a transfection method that uses electrical pulses to create temporary holes in the plasma membrane to drive charged molecules in by establishing an electric potential in the membrane. Electroporation is an effective nucleic acid loading method and has been widely used in EVs for nucleic acid loading. In our previous work, molecular beacons were loaded into EVs through electroporation with a transfection efficiency at about 60%^[94]. It has also been reported that incubation, extrusion, and sonication can induce nucleic acid to enter EVs. However, these methods are widely used in small molecule loading, but not widely used in nucleic acid loading due to their low efficiency for macromolecules. In recent years, the nucleic acid loading method using streptolysin O and liposome is a potential alternative to electroporation^[101]. In contrast to membrane modification, encapsulation strategies can isolate nucleic acid from the external environment, avoiding premature exposure and degradation of nucleic acid.

APPLICATION OF NUCLEIC ACID FUNCTIONALIZED EXTRACELLULAR VESICLES IN BIOMEDICINE

Nucleic acid-functionalized EVs have attracted extensive attention in biomedicine for their outstanding advantages. This section briefly highlights the current representative progress of nucleic acid-functionalized EVs to provide a preliminary understanding for interested researchers. The specific contents are summarized in Table 2.

Nucleic acid-functionalized extracellular vesicles for targeted drug delivery

EVs have shown fascinating interest in the field of drug delivery and are regarded as promising for the next generation of nanomedicine. However, how to improve active targeting is an important problem for EVs. Aptamers can specifically recognize and bind to targets, showing great application potential in the construction of targeted drug delivery systems. Wan *et al.*^[93] reported targeting exosomes with aptamers carrying paclitaxel, a common anticancer drug in clinical practice. They covalently linked the AS1411 aptamer with cholesterol-PEG and subsequently grafted it onto mouse DC membranes. Then, modified DCs are mechanically extruded to create aptamer-guided nanovesicles. By using this extruding method, $\sim 3 \times 10^{10}$ targeted nanovesicles were obtained from approximately 1×10^7 cells within 1 h. Chole-PEG2000 was selected because of its amphiphilic and relatively rigid properties, which could stabilize nanovesicles by hydrophobic effect on the lipid bilayer. Strategies for preparing DSPE-aptamers may be used to mass produce targeted exosomes secreted by immune cells for cancer treatment. The approach is considered safer than cell-based immunotherapies because the vesicles have lost their ability to expand^[93].

Guo's team reprogrammed exosomes using aptamer localization on the surface of exosomes to guide siRNA/miRNA cargo for targeted delivery and cancer treatment^[115]. The authors designed a nanostructure with a three-way connection to make the ligands locate onto the interface of EVs. Placing membrane-anchored cholesterol at the tail of the three-way connection causes RNA aptamers or folic acid to appear on

Table 2. An overview of nucleic acid-functionalized EVs in nanomedicine.

NA type	Function	EV origin	Loading strategy	Composition	Disease target	Outcomes	Ref.
ncRNA	Regulate gene expression	MSCs	Parent cell treatment	Exogenous miR-let7c	Renal fibrosis	miR-let7c-MSC therapy attenuated kidney injury	[108]
	Regulate gene expression	HEK 293T	Parent cell treatment	circRNA	Depressive-like behaviors	Efficiently delivered circDYM to the brain and alleviated CUS-induced depressive-like behaviors	[109]
	Regulate gene expression	DCs	Electroporation	miR-let7	Breast cancer	Selectively targeted tumor tissues in tumor-bearing mice and inhibited tumor growth	[110]
	Regulate gene expression	MDA-MB231	Parent cell treatment	miRNA and siRNA	NA	Significantly reduced its therapeutic dose	[111]
	Interfering gene expression	Normal human fore-skin fibroblast	Electroporation	Alexa Fluor 647-tagged siRNA	KRAS ^{G12D}	Suppressed cancer in multiple mouse models of pancreatic cancer and significantly increased overall survival	[98]
siRNA	Interfering gene expression	Neuro2A cells or DCs	Co-incubation	Cholesterol-conjugated siRNAs	Human antigen R	Facilitated concentration-dependent silencing of human antigen R	[112]
	Interfering gene expression	Umbilical-cord-derived mesenchymal stem cells	Co-incubation	Hydrophobically modified siRNA	Huntingtin mRNA	Significant bilateral silencing of up to 35% of Huntingtin mRNA	[113]
	Interfering gene expression	HEK293T cells	Co-incubation	siRNA	Survivin gene	Significantly suppressed KB cell-derived cancer	[114]
	Interfering gene expression	HEK293T cells	Transfection	siRNA with 3WJ-folate arrow	Survivin gene	Suppressed tumor growth in three animal models	[115]
	Interfering gene expression	Mouse embryonic fibroblasts	Electroporation	mRNA	Phosphatase and tensin homologue-deficient glioma	RNA-containing exosomes restored tumor-suppressor function, enhanced inhibition of tumor growth, and increased survival	[116]
mRNA	Coding protein	HEK293	Parent cell treatment	mRNA	Cerebral ischemia	Reduced inflammation and promoted cell survival	[90]
	Gene therapy	HEK293T cells	Transfection	Plasmid/DNA aptamers Pgc1 α and Il-10 mRNA	PGC1 α	Delivery of Pgc1 α or Il-10 mRNA efficiently induced white adipocyte browning and alleviated IBD, respectively	[117]
	Immunotherapy	HEK293T cells	Transfection	Plasmid encoding Gag-OVA	CD4 ⁺ and CD8 ⁺ T-cell	Facilitated antigen cross-presentation and improved induced immunity	[118]
	Gene therapy	4T1 cells	Transfection	Minicircle DNA	TK-NTR	Mediated gene transfer that enables effective prodrug conversion and tumor cell death	[119]
	Drug delivery	RAW264.7	Electroporation	miR-21 molecular beacon	4T1	Realized a specific microRNA-responding delivery system for visual therapy of tumors	[94]
DNA	Cancer target	RAW264.7	Membrane modification	AS1411 aptamer	MDA-MB231	Caused remarkable tumor tissue damage and reduced the percentage of proliferating Ki67-positive tumor cells	[93]
	Gene editing	SKOV3	Electroporation	DNA plasmid	SKOV3	Suppressed expression of poly(ADP-ribose) polymerase-1 (PARP-1), resulting in the induction of apoptosis in ovarian cancer	[91]

Gene editing	HEK293FT	Liposome	DNA plasmid	MSCs	Endocytosed MSCs and expressed the encapsulated genes in the MSCs	[103]
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EVs: Extracellular vesicles; MSCs: mesenchymal stem cells; DCs: dendritic cells.

the outer surface of the EVs. Instead, placing cholesterol at the three-way arrow resulted in partial loading of RNA nanoparticles into vesicles. As a result, RNA nanostructures are directionally attached to the lipid bilayer membrane of EV, and the target ligand decorates the outer surface of EVs. This directionally engineered ligand showed that the engineered EVs can deliver siRNA to target cells specifically and realize effective blocking of tumor growth^[115]. Recently, sgC8, an aptamer of membrane-bound protein tyrosine kinase 7, has been coupled to diacyl-lipids via PEG ligands in therapy platforms^[120]. The immature dendritic cell-derived EVs are loaded with doxorubicin through electroporation, and then the EVs are functionalized by surface-targeting ligands through the hydrophobic effect^[120]. This sgC8-guided exosome exhibits selective and dose-dependent cytotoxicity to human leukemia cells. In terms of the mechanism of cell internalization, studies have shown that clathrin-mediated endocytosis plays a major role in sgC8 aptamer-mediated endocytosis of various endocytosis pathways. These results suggest that targeted ligands themselves may influence exosome interactions with target cells^[120]. Nevertheless, whether other ligand-target pairs affect EV internalization by different cancer cells remains to be determined.

Nucleic acid-functionalized extracellular vesicles for gene therapy

Gene therapy is regarded as a possible cure to eradicate cancer and genetic diseases. The CRISPR/Cas9 system is a new gene editing tool and designed to work as a Cas9 nuclease single guide RNA (sgRNA) complex which has been widely used in life science. Recognizing the complementary 20-nucleotide genome sequence by sgRNA, Cas9 nuclease cleaves the double-stranded DNA and destroys three bases upstream of the adjacent motif of the target gene, leading to gene deletion, insertion, and mutation through error-prone non-homologous end linking or precise homologous directed repair. Although the CRISPR/Cas9 system is considered a promising gene therapy strategy, one key hurdle remains: the lack of a safe and effective way to transport the CRISPR/Cas9 system in the body. In recent years, EVs have been widely studied as promising drug delivery carriers, but their encapsulation efficiency of large nucleic acids is low. Lin *et al.*^[103] developed a hybrid method of exosomes and liposomes by simple incubation method. The synthesized hybrid nanoparticles effectively encapsulate the CRISPR-Cas9 plasmid, similar to liposomes. Further experiments showed that the synthesized hybrid nanoparticles could be incorporated into mesenchymal stem cells (MSCs) to express encapsulated genes that could not be transfected by liposomes alone. In another study, Kim *et al.*^[91] achieved tumor-targeted gene editing using tumor-derived EVs loaded with CRISPR/Cas9 plasmid by electroporation. These studies provide a new method for delivering the CRISPR/Cas9 system *in vivo*, which is expected to enable precise gene editing *in vivo* and be used in the treatment of cancer and other genetic diseases.

In addition to gene editing systems, gene therapy can also be achieved by regulating gene expression. Liu's team^[95] used molecular beacons to silence the *miR-21* gene, thus enabling EV-mediated gene therapy. In earlier studies, the Kalluri group^[96] achieved targeted gene therapy for pancreatic cancer by using exosomes from normal fibroblast-like mesenchymal cells carrying interference sequences targeting oncogenic KrasG12D. Recently, the study entered a phase I

clinical trial (ClinicalTrials.gov, Identifier: NCT03608631). Non-coding RNAs (ncRNAs) are natural tools for gene expression regulation and are also loaded into EVs for intracellular delivery and gene therapy^[108,109,111,121]. Nucleic acid-functionalized EVs also show good application potential in tissue repair. Mathiyalagan *et al.*^[121] reported that EVs derived from CD34⁺ stem cells can target recipient cells and transfer miRNA precursors to regulate gene expression. In another study, Guo *et al.*^[122] used MSC-derived exosomes loaded with phosphatase and tensin siRNA for spinal injury repair. MSC-derived exosomes have been reported to have a protective effect in many diseases such as myocardial infarction^[9,123], bone defects^[124], and kidney diseases^[125] and can play a synergistic role with siRNA in tissue repair. These studies confirmed that EVs, as small RNA delivery carriers, have good potential in gene therapy. EVs have also been used to deliver large RNA. Yang *et al.*^[116] developed a technique for mass production of mRNA-encapsulating EVs through a homemade electroporation device. A new study found that both nerve growth factor mRNA and protein delivered via EVs can effectively treat ischemic brain injury^[90]. This will further promote the application of nucleic acid-functionalized EVs in the biomedical field.

Nucleic acid-functionalized extracellular vesicles used in immunotherapy

Immunotherapy has made remarkable achievements in clinical trials of malignant tumors, which brings new hope for tumor treatment. However, the suppressive state of the tumor immune microenvironment greatly limits the effect of immunotherapy. Therefore, regulating the immune state of the tumor microenvironment is of great significance to improve the effect of immunotherapy. CpG ODN can activate DCs and macrophages through TLR9, thus improving antigen presentation and immune activation effect. Yu *et al.*^[97] prepared exosomes from different origins and compared their physicochemical properties and delivery efficiency to verify whether EVs can effectively deliver immune-stimulating molecules to lymph nodes. It was found that EV encapsulation greatly increased the amount of internalization of immunomodulatory molecules, which induced higher tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6) expression than free monophosphoryl lipid A (MPLA) and free CpG ODN. After subcutaneously loading CpG and MPLA exosomes, the expression of cytokines interferon- γ (IFN- γ) and TNF- α increased, and T cells were activated. This suggests that the delivery of immune adjuvants by extracellular vesicles is a potential immunotherapy strategy.

The nucleic acid vaccine is a new immunotherapy method. The intracellular delivery of nucleic acid and antigen expression can be effectively realized by loading the DNA or mRNA encoding antigen into EVs. In a preprint, Tsai *et al.*^[126] used exosome-mediated mRNA delivery as a severe acute respiratory syndrome coronavirus (2SARS-CoV-2) vaccine. The results show that the vaccine triggered long-term antiviral immune responses include cellular and humoral immunity, suggesting that exosome-based mRNA formulations represent a previously untapped platform for combating coronavirus disease 2019 (COVID-19). Recently, Allele Biotechnology and Pharmaceutical^[75] announced that they have designed an induced pluripotent stem cell (iPSC) line carrying genes encoding multiple SARS-COV-2 antigen. This iPSC line can release large amounts of EVs that carry viral mRNA and proteins. Alleles indicated that the engineered cell line conquers two problems: (1) vaccines containing multiple antigens may have better performance than vaccines containing single mRNAs, such as Pfizer/biotech and Moderna vaccines; and (2) while the Pfizer/BioNTech vaccines need to be stored at -80 °C, iPSC-derived EVs prevent messenger RNA degradation, making RNA remain intact for several months at 4 °C.

CHALLENGES AND PROSPECTS

Nucleic acid-functionalized EVs show great application prospects in the biomedical field. It enables EVs to be a promising candidate in the hot areas of targeted drug delivery, gene therapy, and immunotherapy. However, some challenges to using nucleic acid-functionalized EVs remain. Firstly, the lack of research

methods on EVs has greatly hindered the development of nucleic acid-functionalized vesicles. The low natural production rate of EVs greatly affects mass production. At the same time, EVs are heterogeneous, and it is difficult to obtain high purity homogeneity subgroups by existing isolation techniques. Although purified EVs can be isolated from cell lines secreting EVs, these EVs have immunogenic and carcinogenic potential. This greatly impedes downstream modification, quality evaluation, and clinical application. Secondly, RNA-based nucleic acid functionalization is affected by the lack of stability of RNA, which is easily destroyed and leads to the failure of functionalization. In addition to the above outstanding problems, nucleic acid-functionalized EVs are also faced with the lack of modification methods, the dilemma of selection of EVs, and the difficulty of clinical transformation. Nevertheless, nucleic acid-functionalized EVs provide a new tool for biomedicine with great potential and application prospects.

Reviewing the latest research progress, we speculate that nucleic acid-functionalized EVs will become a hot research area in the future. We boldly forecast its future research direction. The multi-functional diagnosis and treatment platform based on the programmable characteristics of the nucleic acid will realize personalized and precise treatment. Nucleic acid has programmable performance and can achieve intelligence and multi-function through sequence design. Nucleic acid-functionalized EVs enable the EVs to acquire intelligent characteristics such as stimulus response, intelligent controlled release, and therapeutic feedback. It promises to provide new strategies for personalization and precision medicine. A gene-editing system based on EVs is expected to achieve precise and efficient gene therapy. CRISPR, a gene-editing system, has made significant progress at the cellular level, showing satisfactory gene editing efficiency. However, *in vivo* gene editing is still hampered by the lack of delivery vectors. EVs are natural delivery carriers of bioactive molecules and have the ability to allow bioactive molecules to escape from lysosomes. Recent studies have found that EVs have tissue targeting ability such as homologous targeting. A gene-editing system developed by EVs is expected to achieve accurate and efficient gene editing *in vivo*.

DECLARATIONS

Authors' contributions

Responsible for conceptualization: Fan Z, Wang L

Writing the manuscript: Liu C, He D

Responsible for the visualization: He D

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Responsible for financial support: Fan Z, Liu C, Wang L

Responsible for the revision: He D, Zhong Z, He Q, Liu C

All authors read and approved the final manuscript.

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Free flow electrophoresis allows quick and reproducible preparation of extracellular vesicles from conditioned cell culture media

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Abstract

Aim: Despite intensive research during the last decade, it remains challenging to prepare extracellular vesicles (EVs) of high purity, especially from primary body liquids or protein-rich conditioned media. For now, time-consuming combinations of at least two orthogonal methods, e.g., density and size separation, are required to enrich EVs to high purity, often at the expense of processing time. Therefore, novel technologies are required that allow EV preparation in acceptable time intervals and to fair purities. Free-flow electrophoresis (FFE) constitutes a well-established semi-preparative method to separate and prepare analytes, e.g., by inherent differences in their electric charges. FFE combines a flow-driven longitudinal transport of sample material with vertical electrophoresis and allows the separation of sample components into up to 96 different fractions. It was our aim to evaluate the potential of FFE for the separation of EVs from other sample components of EV-containing protein-rich conditioned cell culture media.



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Methods: Exemplarily, conditioned media of mesenchymal stem/stromal cells raised in the presence of EV-containing 10% human platelet lysate were processed. We analyzed the obtained fractions by different technologies, including imaging flow cytometry, western blot and nanoparticle tracking analysis.

Results: We demonstrate that FFE quickly and reproducibly separates EVs from a huge proportion of molecules included in the original sample.

Conclusion: Our results qualify FFE as a feasible, quick and reproducible technology for the preparation of *bona fide* EVs.

Keywords: Extracellular vesicles, exosomes, mesenchymal stem cells, mesenchymal stromal cells MSCs, MSC-EVs, free-flow electrophoresis

INTRODUCTION

Virtually all cells release different types of membrane-surrounded nano- and micron-sized particles into their extracellular environment. Depending on their subcellular origin, these extracellular vesicles (EVs) are discriminated into different subtypes^[1]. The most prominent EV types are exosomes (70-150 nm) arising from the endosomal system, microvesicles (100-1000 nm) budding from the plasma membrane, and apoptotic bodies, membrane-surrounded large fragments of dying cells (up to several micrometers)^[2]. Apparently, EVs are assembled in cell type specific manners, and a proportion of them mediates complex interactions at local and distant sites in both healthy and pathological conditions^[3]. To unravel their functions, it is a common strategy to prepare EVs and analyze their molecular content in larger detail, e.g., by proteome or RNA profiling. Traditionally, differential centrifugation-based methods are used for the enrichment of small (exosome-)sized EVs^[4]. More recently, size exclusion technologies have become popular, originally introduced for EV preparation many years ago^[5-7]. Lipoproteins and protein aggregates are difficult to remove with any of the given technologies, particularly when it comes to primary body liquids, serum- or human platelet lysate (hPL)-containing media. To obtain relatively pure EV samples, the method of density gradient centrifugation has been combined with size exclusion chromatography to separate EVs from most of the lipoproteins and protein aggregates^[8,9]. Despite the high purity of the obtained EVs, the recovery is relatively low and the whole procedure is very time-consuming. Thus, the EV field urgently requires novel methods allowing preparation of EVs with improved purities and recoveries in a quick and reproducible manner.

Free-flow electrophoresis (FFE) is a matrix-free, well-established method for the separation of a wide variety of charged or chargeable analytes [Figure 1A]. It has been used successfully for the separation and preparation of cells; proteins in cell lysates and plasma; enzymes from extracts of bacteria, micro-organisms and mammalian cell line cells; and the preparation of cellular organelles such as peroxisomes^[10-13]. The central unit of FFE is a separation chamber, mainly composed of a separation plate and a 0.2 mm distanced front plate including a longitudinally arranged anode and cathode [Figure 1B]. Following assembly, buffers with defined pH values are loaded into the vertically arranged separation buffer inlets at the lower edge of the separation chamber. The buffers are continuously transported along the longitudinal axis of the separation chamber by a constant laminar flow, forming concrete longitudinal buffer lanes. Likewise, the sample to be separated is applied at a concrete vertical position at the front end of the horizontal lane and transported by the laminar flow together with the respective buffer along the longitudinal axis [Figure 1]. Typically, buffer application schemes are designed such that the buffer with the lowest pH is closest to the anode and that pH values of the buffers gradually increase towards the cathode. Driven by a vertical electric field and depending on their electric charge, sample components migrate vertically through different buffer

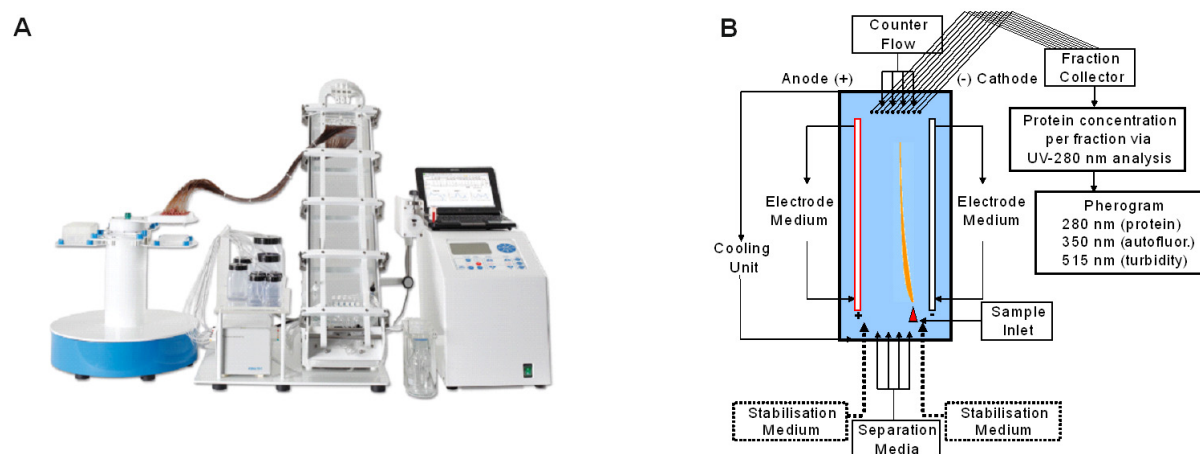


Figure 1. Free flow electrophoresis (FEE) and its principle. (A) Image of the FFE device developed by FFE Service. (B) Principle of the FFE device, as described in detail in the text: analytes loaded into the sample inlet are transported by a longitudinal flow and separated in a vertical electric field (see orange line as an example for one analyte). The migration speed of given analytes within the electric field is related to their isoelectric points and depends on the pH values of separation buffers within the separation area. To avoid analytes getting into contact with either electrode, specific stabilization buffers that regularly feature higher pH values protect the electrodes. At the end of the separation chamber driven by a counterflow, separated analytes are collected in 96 different pores connected with hoses to a fraction collector loading samples into 96-well plates. By spectral analyses at different wave lengths, pherograms of the fractioned samples are recorded.

zones. The migration speed and distance of each sample component depend on its charge density and/or isoelectric point (pI). Components with higher negative charge densities or lower pIs migrate more quickly towards the anode than those with lower charge densities and higher pIs. Thus, the higher is the charge density or the lower is the pI, the quicker the analytes approach the anode. Still being transported by the horizontal laminar flow, separated sample components continuously migrate towards the top of the separation chamber where they are collected by a collector unit in up to 96 different vertically arranged fractions. For the initial analysis, obtained fractions are usually analyzed in a microtiter plate reader for their light absorption and emission capabilities at different wavelengths. Absorption at 280 nm reflects the protein contents of the fraction, emission at 350 nm the autofluorescence of ingredients illuminated at 280 nm, and absorption at 515 nm the turbidity caused by solid ingredients such as protein aggregates, EVs, and other particles and solid compounds.

Aiming to assess the feasibility and reproducibility of FFE for the preparation of EVs from complex fluids, we decided to use 48 h conditioned media (CM) from human bone marrow-derived MSCs grown in 10% hPL supplemented, non-EV-depleted media. Coupled with our interest in translating MSC-EVs into the clinics^[14,15], we routinely characterize obtained EV preparations by applying standard EV characterization technologies as recommended by the *minimal information for studies of extracellular vesicles* 2018 (MISEV2018) guidelines^[16]. Furthermore, we previously optimized imaging flow cytometry (IFCM) protocols for the characterization of EVs at the single-EV level and have started to analyze EV contents in different biofluids including EV preparations obtained with different protocols by IFCM in addition^[17–21].

Here, using as an example one of our standard MSC-EV preparations^[14,22] and MSC-CMs, we developed and optimized an FFE protocol with separation buffers of different pH values for the reproducible preparation of respective EVs.

METHODS

Preparation of MSC-CMs

MSCs were raised from samples of healthy bone marrow donors following informed consent according to the Declaration of Helsinki, exactly as described before^[14,23]. Their usage was approved by the ethics committee of the University of Duisburg-Essen (12-5295-BO). Briefly, obtained MSCs were expanded in DMEM low glucose (PAN Biotech, Aidenbach, Germany) supplemented with 10% hPL^[24,25], 100 U/mL penicillin-streptomycin-glutamine (Thermo Fisher Scientific, Darmstadt, Germany), and 5 IU/mL heparin (Ratiopharm, Ulm, Germany) at 37 °C in a humidified 5% CO₂ atmosphere. Upon reaching 50% confluency, CMs were exchanged every 48 h until MSCs reached a density of 80%-90% confluency. After harvesting, CMs were spun at 2000 g for 15 min in a 5810R centrifuge (rotor A-4-81, Eppendorf, Hamburg, Germany) to remove residual cells and larger particles. Thereafter, CMs were stored at -20 °C until usage. MSC characteristics and absence of mycoplasma infections were documented in regular intervals, exactly as described before^[14,23]. The MSC-CMs used in this study were obtained from approximately 6×10^6 cells. For one of the MSCs, we used 24 h medium exchange intervals for all other 48 h intervals.

Preparation of MSC-EVs

The MSC-EV preparation used in this study (MSC-EVs 31.2) was comprehensively characterized before, and it has been tested for its therapeutic activity in an ischemic stroke model^[26]. Briefly, MSC-EVs 31.2 were prepared from 48 h CM of 4.3×10^8 cells (4.5 L MSC-CM) applying our reported PEG/UC procedure^[14,22]. The EV preparation was characterized by nanoparticle tracking analysis (NTA) and Western blot. The average particle sizes were 108.2 nm; the particle concentration was 2.8×10^{11} particles per mL; and the protein concentration was 7.7 mg protein/mL. The following administration into ischemic stroke mice, samples of this MSC-EV preparation mediated neuroprotective effects^[26].

Free flow electrophoresis

FFE experiments were performed on FFE NextGen systems (FFE Service GmbH, Feldkirchen, Germany), equipped with nine inlets for the loading of the separation buffers at the front and three inlets for the counterflow buffers at the end of the separation chamber. All separations were performed at 10 °C, and 500 mm × 100 mm separation chambers were used with 0.2 mm gap width, covered with transparent plastic film.

The following FFE workflow was used [Supplementary Figure 1]. Before loading the separation chambers with the different buffers air bubble-free, the appropriate assembly of the FFE device was confirmed by running a routine program checking for the tightness of the device and the homogeneity of the laminar fluid stream within the whole chamber. To this end, a so-called stripe test was performed, in which the nine inlets at the front of the separation chamber were fed alternating with clear and pink colored water. Without applying an electric field, it was proven visually and by spectral analysis that, under laminar flow, aqueous lanes remain straight without intermingling with their neighbor lanes [Supplementary Figure 2A]. Next, the separation chamber was loaded with the separation buffers of choice flanked by the anode and cathode stabilization buffers as provided below. Depending on the detailed separation protocol, variable areas of the separation chamber were chosen for the sample fractioning, i.e., by selecting the numbers of buffer inlets to be filled with separation buffers at the front of the separation chamber. To control the electrophoretic separation performance, a mixture of different colored dyes with different pIs (range of pI 4-8) was administered into the loading inlet, applying a laminar flow of 201 mL/h and an electric voltage of 1000 V regularly, resulting in a current of approximately 190 mA [Supplementary Figure 2B]. The accuracy of the electrophoresis-driven separation process was controlled visually. Following appropriate separation of the colored dye mix, the EV-containing samples were loaded into specific sample inlets with a flow of 7.5 mL/h. Simultaneously, the laminar flow samples were separated by electrophoresis (1000 V, ~190 mA). Upon

applying a counterflow at the end of the separation chamber, typically with a flow rate of 195 mL/h, sample fractions were collected via a collection unit installed at the end of the separation chamber allowing the collection of up to 96 different fractions in 96-well microtiter plates, typically as 150 μ L aliquots per well. Depending on the protocol and continuous sample loading, separation time and the corresponding volume of each obtained fraction can be increased. Within 27 min, up to approximately 1.8 mL per sample fraction can be obtained, which in our experimental series were collected in 96-well polypropylene deep-well plates with loading capacities of 2 mL per well (polypropylene, Protein LoBind, Eppendorf, Hamburg, Germany). Typically, if larger (scaled) sample fractions are collected in deep-well plates, conventional microtiter plates are filled with 150 μ L aliquots per sample fraction immediately before (pre-scaled sampling) and after loading of the deep well plates (post-scaled sampling). Analyses of the fractions collected in the microtiter plates before and following the scaled sampling retrospectively allow evaluation of the stability of the separation process.

Spectroscopic analyses of the collected sample fractions, typically in microtiter plates, for each of the fractionated samples were performed in a microplate reader (Tecan M200, Tecan, Männedorf, Switzerland) equipped with UV-Vis and fluorescence spectroscopy detectors and I-control 1.8 software (Tecan). Briefly, the protein content of the sample fractions was analyzed by absorption of UV light at 280 nm excitation, the autofluorescence of 280 nm illuminated fractions at 350 nm emission, and their turbidity by absorption at 515 nm extinction; 10 nm bandwidth and a photomultiplier gain setting of 80 were used. The light absorbance values of the different sample fractions were plotted in pherograms across all sample fractions. Deep-well plates loaded with up to 96 different sample fractions were stored at -80 °C until further processing. pH analyses of the collected samples were performed in an automatized manner with a Tecan MSP9259 microplate robotic system (Tecan) equipped with a WTW inoLab pH730 pH-meter (Weilheim, Germany) and “gemini for miniPrep” software.

For the initial experiments, we used an MSC-EV preparation that had been comprehensively characterized before^[26]. For the fractioning of this sample, an interval zone electrophoresis protocol was used (longitudinal transport occurs before and after but not during electrophoresis), and the FFE separation chamber was loaded via five inlets with five separation buffers (10 mM Tris-acetate) of different pH values (pH 4.8, 5.4, 6.4, 7.4, and 8.4). The electrode stabilization buffer, 150 mM Tris-acetate pH 8.3, was loaded into two inlets in juxtaposition of the anode and one inlet to the cathode [Figure 2A]. The counterflow buffer was a 250 mM mannitol solution. The sample fractioning time was adjusted to 6 min with constant electrophoresis.

For the separation of the MSC-CMs a continuous zone electrophoresis process was used (longitudinal transport and electrophoresis occur simultaneously). Here, three separation buffers were loaded via three separate loading inlets. Buffer 1 was 15 mM Tris-HCl adjusted with e-aminocaproic acid (EACA) to pH 4.5; Buffer 2 was 5 mM Tris, titrated with acetic acid to pH 4.5, and Buffer 3 was 15 mM Tris, titrated with acetic acid to pH 6.0. The separation buffers were flanked by the anode and cathode stabilization buffers (170 mM Tris, 130 mM acetic acid, pH 7.3) [Figure 3A]. In addition, 300 mM bisamino-trismethan (BisTRIS) was used as a counterflow buffer applied via the three counterflow buffer inlets at the top end of the separation chamber.

Dot blot

Optitran Whatman BA-S83/0.45 μ m nitrocellulose membranes (Whatman GmbH, Dassel, Germany) were cut and placed into the Dot blot chamber (96-well Bio-Dot®, BioRad, Feldkirchen, Germany). Then, 200 μ L of each FFE separated fraction were transferred to 1 of the 96 wells of the Dot blot chamber. Next, 15 μ g of

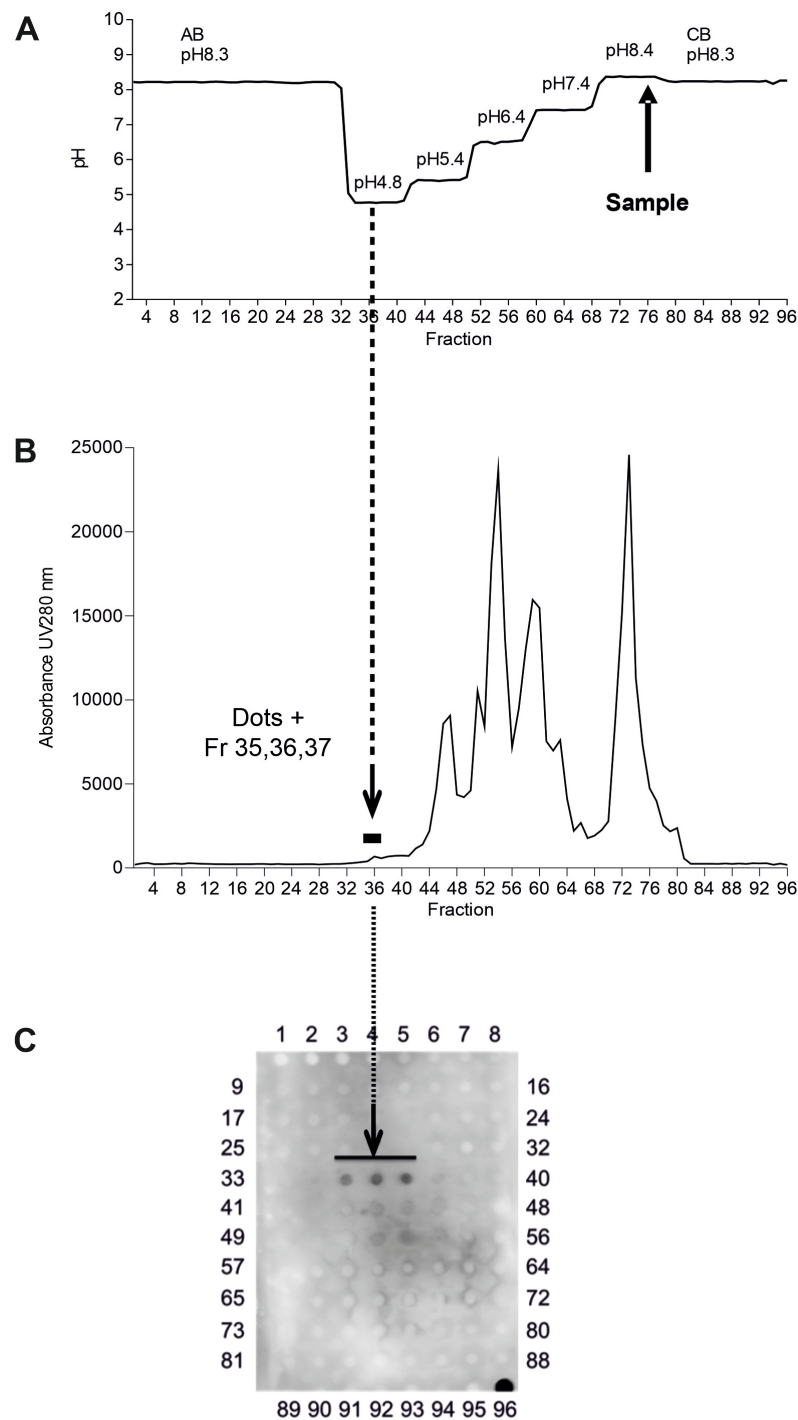


Figure 2. Free flow electrophoresis (FEE) effectively separates components of MSC-EV preparations. (A) A five-step pH-profile (pH 4.8, 5.4, 6.4, 7.4, and 8.4) was used for the fractioning of a 133 μ L sample of a well-characterized MSC-EV preparation that was applied at the vertical position corresponding to that of the collected Fraction 78. An interval zone electrophoresis was performed at 1000 V for 6 min in a 6 cm broad separation area. (B) A pherogram of the protein content within the different fractions. (C) The results of dot blot analysis of all 96 obtained fractions using a mixture of anti-CD9 (VJ1) and anti-Syntenin (EPR8102) antibodies. As a control, at Position 96, 15 μ g of a PEG precipitated MSC-EV sample were applied. Fractions delivering positive dot blot signals (Fractions 35-37) are labeled by arrows and the black line in (C).

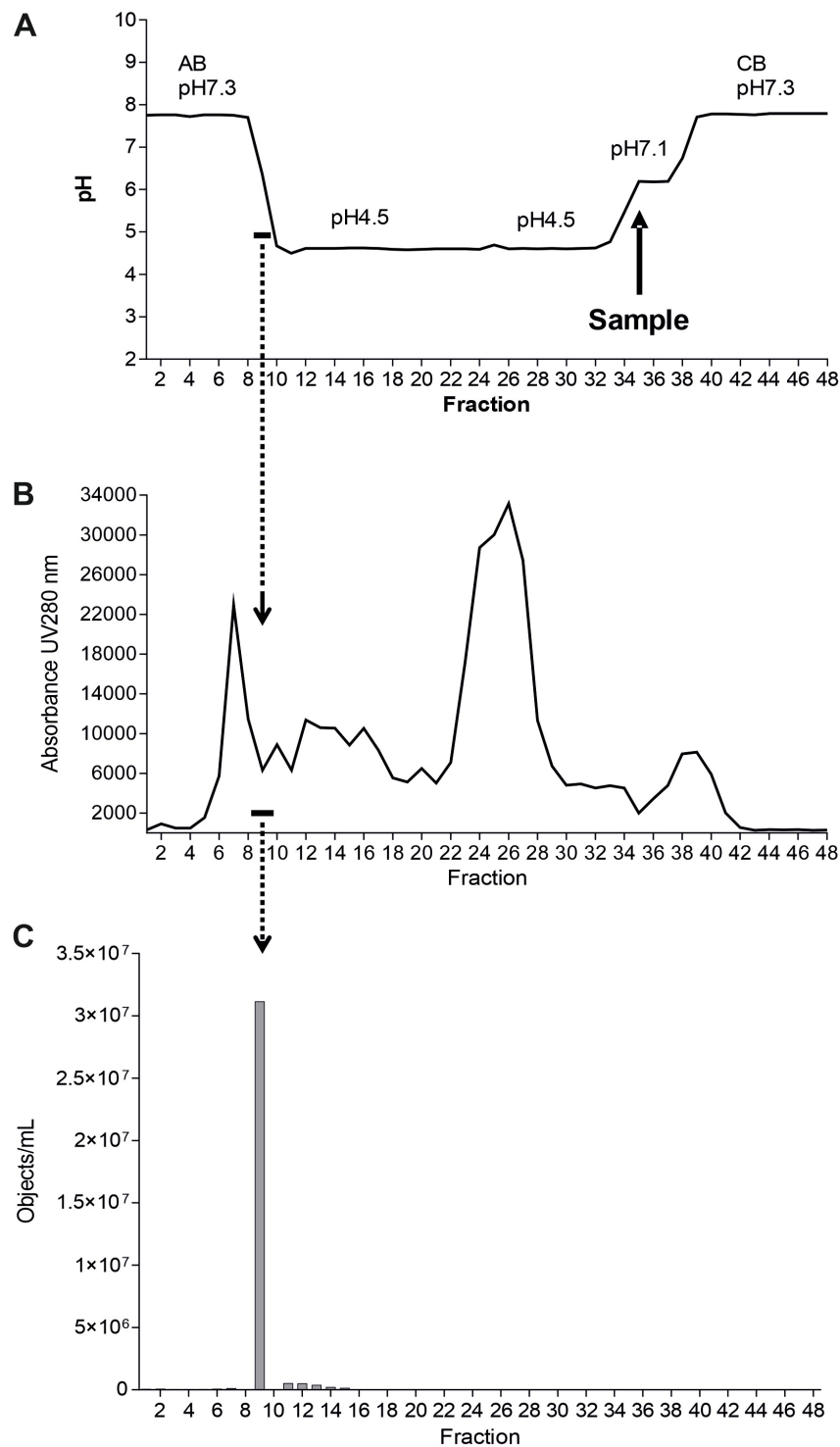


Figure 3. Imaging flow cytometry (IFCM) recovers CD9⁺ objects mainly in one free flow electrophoresis (FEE) fraction. (A) Following optimization, a three-step pH profile (pH 4.5, 4.5, and 7.1) was set up for the fractioning of 3.125 mL of a given MSC-CM. The sample was applied at the vertical position corresponding to that of the collected Fraction 35. The FFE process of continuous zone electrophoresis was performed at 1000 V for 3.5 min in a 3 cm broad separation area. (B) A pherogram of the protein content within the different fractions. (C) The results of IFCM analyses of 25 μ L aliquots of obtained fractions that had been stained with anti-CD9 antibodies. The fraction containing detectable CD9⁺ objects (Fraction 9) is labeled by arrows and the black line in (B).

an unfractionated PEG/UC MSC-EV preparation were diluted to final volumes of 200 μ L and applied as positive control. Applying a water jet vacuum pump, liquid phases of the loaded samples were sucked through the membrane. Thereafter, the blot aperture was disassembled and the membrane air-dried for 1-2 min. Subsequently, the membrane was blocked under low agitation in 5% skim milk powder solution (TBST, Sigma Aldrich, Taufkirchen, Germany) for 30 min at room temperature. To label exosomal antigens, mouse anti-CD9 antibodies (VJ1, 1:1000, kindly provided by Francisco Sánchez-Madrid) and rabbit anti-Syntenin antibodies (EPR8102, 1:1000, 1 h, RT, Abcam, Cambridge, United Kingdom, ab133267) were applied in TBS-0.1% Tween-20 (TBS-T) containing 5% (w/v) skim milk powder (Sigma-Aldrich) for 1 h at room temperature. Following incubation, membranes were rinsed and washed three times for 5 min and once for 10 min in TBS-T. For the detection, membranes were incubated with peroxidase AffiniPure F(ab')₂ fragment donkey anti-mouse IgG (1:10,000, polyclonal 715-036-150; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or peroxidase-AffiniPure F(ab')₂ fragment donkey anti-rabbit IgG (1:10,000, polyclonal 711-036-152; Jackson ImmunoResearch Laboratories) for 1 h and rinsed three times for 5 s and once for 10 min in TBS-T. SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Darmstadt, Germany) was used as a chemiluminescent detection substrate. Obtained signals were documented with the Fusion FX7 detection system (Vilber Lourmat, Eberhardzell, Germany).

Imaging flow cytometry

IFCM analyses were performed on the AMNIS ImageStreamX Mark II Flow Cytometer (AMNIS/Luminex, Seattle, WA, USA), as described previously^[19-21]. For staining, samples were incubated with CD9-PE (1:50, MEM61, Exbio) for 1 h at room temperature. All controls recommended by the MIFlowCyt-EV guidelines for flow cytometric EV analysis^[27] were performed, exactly as described previously^[20]. After dilution with PBS, samples were measured using the integrated auto-sampler for 96-well U-bottom plates. Acquisition time was 5 min per well. Data were acquired at 60 \times magnification, low flow rate (0.3795 ± 0.0003 μ L/min), and with removed beads option deactivated. Analysis was performed as described previously using IDEAS software version 6.2^[19].

Nanoparticle tracking analysis

Samples were analyzed for particle size and concentration on a ZetaView™ PMX-120 BASIC platform (ParticleMetrix, Meerbusch, Germany). The machine was calibrated using a polystyrene bead standard (100 nm, Thermo Fisher Scientific). Samples were loaded and recorded at all 11 positions, with 5 repetitions. Further settings included: sensitivity 75, shutter 75, minimum brightness 20, minimum size 5, and maximum size 200. Each sample was measured three times. The videos were analyzed with the ZetaView Analyze program (Version 8.03.08.02); the median value (X_{50}) for size was used for data analysis.

Protein concentration analysis

The protein content of selected samples was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Protein analysis was performed in 96-well plates according to the recommendations of the manufacturer.

Chloroform-methanol precipitation

The FFE fractions were too diluted for direct application to Western blots. Consequently, samples were concentrated by chloroform-methanol precipitation. Briefly, 800 μ L methanol was added per 200 μ L sample volume. After mixing, 200 μ L chloroform and 600 μ L ddH₂O were added. After repeated mixing, samples were centrifuged for 3 min at 14,000 g. Following protein concentration in the interphase, the upper aqueous phase was removed, and 800 μ L methanol was added. Precipitated proteins were sedimented by centrifuging the samples for 5 min at 14,000 g. Supernatants were removed and pellets were air-dried for approximately 5 min. Dried pellets were resuspended in 40 μ L non-reducing Laemmli buffer.

Western blot

Western blot was performed as described previously^[22]. Briefly, samples were separated on SDS-PAGE gels (12% tris-glycine/bis-acrylamide). Separated proteins were transferred to a polyvinylidene fluoride membrane (PVDF; Millipore, Darmstadt, Germany) by Wet Blot (Mini Trans-Blot® Cell, BioRad, Feldkirchen). TBS-T containing 5% (w/v) skim milk powder (Sigma) was used to block the PVDF membranes. The following antibodies were used to detect defined proteins: mouse anti-human CD9 antibody (TEA3, 1:1,000, 4 °C overnight, kindly provided by Francisco Sánchez-Madrid) and rabbit anti-human Syntenin antibody (1:1000, EPR8102, Abcam). All antibodies were diluted in TBS-T containing 5% (w/v) skim milk powder. Subsequently, membranes were washed four times (3× 10 s, 1× 10 min) in TBS-T and counterstained for 1 h at room temperature with Peroxidase-AffiniPure F(ab')₂ Fragment donkey anti-mouse IgG (1:10,000; Jackson ImmunoResearch Laboratories) and peroxidase-AffiniPure F(ab')₂ Fragment donkey anti-rabbit IgG (1:10,000; Jackson ImmunoResearch Laboratories). SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) was used as a chemiluminescent detection substrate.

Transmission electron microscopy sample preparation

Transmission electron microscopy (TEM) was performed as described previously^[26]. Briefly, 10 µL droplets of EV-containing samples were placed onto 200 mesh copper grids covered with carbon-coated formvar films (Plano GmbH, Wetzlar, Germany) for 5 min to allow the EVs to adhere to film surfaces. All following steps, contrasting, and fixation with 2% uranyl acetate were performed by placing the grids on droplets of different solutions. The incubations were conducted at room temperature.

Images were acquired using a JEOL JEM 1400Plus (JEOL Ltd., Tokyo, Japan), operating at 120 kV and equipped with a 4096 × 4096 pixels CMOS camera (TemCam-F416, TVIPS, Gauting, Germany). Image acquisition software EMMENU (Version 4.09.83) was used for taking 16-bit images. Image post-processing was carried out using ImageJ (Version 1.52b).

RESULTS

FFE allows separation of protein and EV contents from PEG prepared MSC-EV samples

Aiming to test the suitability of FFE for the fractionation of complex EV-containing samples, we started our experimental series with MSC-EV samples routinely prepared in our lab using a combined PEG/UC preparation method^[14,22]. First, 133 µL of a well-characterized MSC-EV preparation (MSC-EV 31.2) that had been administered to ischemic stroke mice^[26] were fractionated within 6 min with a free-flow interval zone electrophoresis protocol using five different pH zones (pH 4.8, 5.4, 6.4, 7.4, and 8.4) flanked by anode and cathode stabilization buffers (pH 8.3) [Figure 2A]. In total, 96 different fractions (1230 µL each) were collected from the separation area, including the regions with the anode and cathode stabilization buffers. Analyses of the protein content (UV280 nm) of different fractions revealed that most of the protein was recovered in Fractions 44-80 (absorbance at 280 nm > 1500) [Figure 2B]. To identify the EV-containing fractions, a dot plot immunostaining was performed. Coupled with the usage of hPL supplemented media, the most abundant EV population in our MSC-EV samples is the CD9⁺CD81⁻ EV population^[21], expectedly also being positive for the EV marker Syntenin^[17]. Consequently, we decided to focus on anti-CD9 and anti-Syntenin analyses. Accordingly, 200 µL of each obtained FFE fraction was applied and probed with a mixture of anti-CD9 and anti-Syntenin antibodies. Positive signals were detected in Fractions 35-37, which were derived from the pH 4.8 zone, adjacent to the anode stabilization buffer [Figure 2C]. Since these fractions had a rather low protein content, we concluded that fractionation by FFE separates protein impurities in EV preparations from their EV content.

FFE allows separation of CD9⁺ objects and protein contents from MSC-CMs

Intending to use FFE for the preparation of EVs from CMs, we decided to next prepare EVs from MSC-CMs. An MSC-CM (CM1) was used for this experiment that had been cleared from cells and larger particles and had a protein concentration of 4.39 mg/mL, according to NTA average particle sizes of 116.2 ± 8.1 nm and a particle concentration of 2.50×10^{10} particles/mL. According to IFCM, the content of CD9⁺ objects was 3.14×10^8 objects/mL MSC-CM, resulting in purity indexes of 5.69×10^9 particles/mg protein and 7.14×10^7 CD9⁺ objects/mg protein, respectively [Table 1].

To decrease the processing time, the separation protocol was optimized and simplified. Instead of five buffers with different pH values, three different buffers were chosen for the separation area: a high conductivity buffer with pH 4.5, a low conductivity buffer of pH 4.5, and a buffer with pH 7.1. These buffers were flanked by anode and cathode stabilization buffers with pH 7.3; the separation area was reduced to half of the separation chamber [Figure 3A]. Applying the optimized protocol, 3.125 mL of MSC-CM were processed in 25 min. In total, 48 different fractions were collected, each with a volume of 1.8 mL. In addition, for the spectroscopic evaluation of the stability of the separation process, 150 mL aliquots of all fractions were collected before and after the scaled sampling process. The obtained pherograms revealed that most of the protein content was recovered in Fractions 7-8 and 23-27 [Figure 3B]. Upon testing 200 µL of each of the obtained samples in the dot plot procedure, expectedly, we failed to detect any EV proteins due to the much lower EV concentration within the starting material. Since, according to our experience, IFCM is more sensitive than Western or Dot blot, we decided to analyze relevant fractions by IFCM. Preliminary IFCM analyses performed during the FFE protocol establishment period qualified anti-CD9 labeling as a robust and sufficiently sensitive method for the identification of EV-containing samples. These analyses demonstrated that CD9⁺ objects, assumedly CD9⁺ EVs, were exclusively recovered in up to three fractions, all within the range of Fractions 7-10. Consequently, following the processing of MSC-CM with the optimized protocol, we focused our anti-CD9 IFCM analyses on Fractions 6-11. In our proof-of-principle run, Fractions 6-8, 10, and 11 hardly contained any CD9⁺ objects. In contrast, Fraction 9 contained more than 3.12×10^7 CD9⁺ objects per mL [Figure 3C, Supplementary Figure 3, Table 2]. As before, the fraction containing most CD9⁺ objects had a relatively low protein content, resulting in a purity of 9.24×10^7 objects/mg protein [Table 2]. These results imply that FFE might also be a suitable method for preparing EVs from MSC-CMs to fair purities.

FFE allows preparation of bona fide EVs

Although our previous results qualified IFCM as a second-generation EV analysis method for the detection and characterization of EVs including small EVs at the single-EV level^[17,19-21], we also characterized Fractions 7-10 by NTA and Western blot (WB) and determined their protein content in addition to the spectral analysis by BCA [Tables 1 and 2]. Consistent with the number of CD9⁺ objects, the highest particle counts, 1.5×10^{10} particles per mL as determined by NTA, were found in Fraction 9, which as a consequence thereof was also analyzed by TEM. Fractions 7, 8, and 10 contained clearly fewer particles [Table 2]. The average sizes of the recorded particles were 127.3 ± 7.9 (Fraction 7), 146.6 ± 13.9 (Fraction 8), 131.4 ± 6.2 (Fraction 9), and 134.8 ± 8.3 nm (Fraction 10) [Figure 4A]. Due to the low protein concentration of the obtained FFE fractions, the protein content was concentrated by chloroform-methanol precipitation (200 µL aliquots each) and analyzed by WB. Signals for CD9 were obtained within the lanes of Fractions 8-10, with the strongest band in the lane of Fraction 9 [Figure 4B]. Unfortunately, most likely due to the low protein concentration of the prepared samples, other EV specific proteins were not successfully detected in Western blots (data not shown). TEM analyses revealed objects with a vesicular appearance in Fraction 9 [Figure 4C]. Altogether, these data imply that FFE Fraction 9 contains *bona fide* EVs. Thus, FFE can also quickly prepare EVs from cell culture supernatants.

Table 1. Protein, IFCM and NTA data of MSC-CM

	Prot conc	CD9 ⁺ obj conc	Obj purity index	Total CD9 ⁺ obj	Particle size	Particle conc	Part purity index	Total particles
Method	BCA	IFCM	IFCM/BCA	IFCM × Vol	NTA	NTA	NTA/BCA	NTA × Vol
	[mg/mL]	[obj/mL]	[obj/mg]	[obj/3.125 mL]	[nm]	[particle/mL]	[particle/mg]	[part/3.125 mL]
CM1	4.39	3.14E+08	7.14E+07	9.81E+08	115.1	2.50E+10	5.69E+09	7.81E+10
CM2	4.76	1.85E+08	3.87E+07	5.77E+08	113.0	2.20E+10	4.62E+09	6.88E+10
CM3	4.64	2.84E+08	6.12E+07	8.88E+08	118.0	3.10E+10	6.68E+09	9.69E+10
CM4	4.57	2.36E+08	5.17E+07	7.39E+08	115.1	2.20E+10	4.81E+09	6.88E+10
CM5	4.80	2.46E+08	5.13E+07	7.70E+08	117.3	2.60E+10	5.41E+09	8.13E+10

IFCM: Imaging flow cytometry; NTA: nanoparticle tracking analysis; BCA: bicinchoninic acid.

FFE-is highly reproducible and robust for protein fractioning

After gaining evidence that FFE allows the preparation of *bona fide* EVs from MSC-CM, we explored the reproducibility of the method. To this end, in addition to the previous MSC-CM (CM1), we included CMs from four additional MSC stocks (CM2-5). All MSC-CMs revealed protein concentrations between 4.3 and 4.8 mg/mL, their average particle concentration varied between 2.2 and 3.1×10^{10} particles/mL, and their number of CD9⁺ objects between 1.85 and 3.14×10^8 per mL [Table 1]. The purity indexes varied between 4.64 and 6.68×10^9 particle/mg protein and between 3.87 and 7.14×10^7 CD9⁺ objects/mg protein [Table 1].

Applying the former FFE protocol, fractioned aliquots of the five different MSC-CMs were fractioned on three different days, with two independent runs per MSC-CMs and day. As an initial analysis, the protein content of all 48 obtained fractions of each individual run was determined spectroscopically, measuring the light absorption of each individual fraction at 280 nm. Upon comparing the obtained pherograms, we observed a high degree of reproducibility of the method. All pherograms obtained from MSC-CMs that had been fractioned on the same day were almost identical [Figure 5A], indicating extreme robustness and reproducibility of the process, at least for intra-day performances. Pherograms obtained from identical MSC-CMs that were fractioned on different days were very similar to each other as well, but they showed some minor differences [Figure 5B]. According to our experience, these slight differences in the day-to-day performance are due to minor, unavoidable variations in setting up the FFE aperture. Overall, however, in terms of protein fractioning, our results demonstrate FFE is highly reproducible and robust.

FFE reproducible allows preparation of EVs from MSC-CM

After learning FFE reproducibly fractions different MSC-CMs regarding their protein concentration, we next investigated Fractions 7-10 for their CD9⁺ object and particle content by IFCM and NTA, respectively. Furthermore, samples of these fractions were investigated by anti-CD9 Western blots. The IFCM data reveal that consistently the majorities of CD9⁺ objects were recovered in Fraction 9 in all cases, with the exception of MSC-CM4, where minor proportions of CD9⁺ objects were also recovered in Fraction 10 but never in Fraction 7 or 8 [Figure 6A, Table 2]. In good agreement, NTA also detected the highest particle content in all Fraction 9 samples (between 7.9×10^9 and 1.2×10^{10} particle/mL and 1.19 and 3.12×10^7 CD9⁺ objects/mL) followed by Fraction 10 in all cases [Figure 6B, Table 2]. According to the purity indices expressed as particles per mg protein, Fraction 9 contained between 4.54 and 7.82 times more particles per mg protein than the original CMs. The average sizes of the recorded particles in Fraction 9 were between 110.1 and 133.1 nm [Table 2]. Following chloroform-methanol precipitation of the samples, the data were further substantiated by results of Western blots, which also showed the highest anti-CD9 signal intensities in all Fraction 9 samples [Figure 6C]. Despite the fact that, assumedly due to issues in chloroform-methanol precipitation, the CD9 band intensity of the Fraction 9 of MSC-CM5 is somehow weaker than that of the

Table 2. Protein, IFCM and NTA data of obtained FFE Fractions 7-10

FFE-Fraction		Prot conc	CD9 ⁺ obj conc	Obj purity index	Total CD9 ⁺ obj	Particle size	Particle conc	Part purity index	Ttotal particles	Recovery	Recovery
Method	BCA	IFCM	IFCM/BCA	IFCM × Vol	NTA	NTA	NTA/BCA	NTA × Vol	CD9 ⁺ obj	Particles	
	[mg/mL]	[obj/mL]	[obj/mg]	[obj/1.8 mL]	[nm]	[particle/mL]	[particle/mg]	[part/1.8 mL]			
EV (CM1)	7	0.046	1.22E+05	2.63E+06	2.19E+05	127.3	9.80E+08	2.12E+10	1.76E+09	0.02%	2.26%
	8	0.209	0.00E+00	0.00E+00	0.00E+00	146.6	2.40E+09	1.15E+10	4.32E+09	0.00%	5.53%
EV (CM2)	9	0.337	3.12E+07	9.24E+07	5.61E+07	131.4	1.50E+10	4.45E+10	2.70E+10	5.72%	34.56%
	10	0.118	6.36E+03	5.40E+04	1.14E+04	134.8	4.00E+09	3.40E+10	7.20E+09	0.00%	9.22%
EV (CM3)	7	0.152	9.94E+04	6.55E+05	1.79E+05	134.9	5.20E+08	3.43E+09	9.36E+08	0.03%	1.36%
	8	0.186	0.00E+00	0.00E+00	0.00E+00	133.8	1.20E+09	6.47E+09	2.16E+09	0.00%	3.14%
EV (CM4)	9	0.377	1.19E+07	3.15E+07	2.14E+07	117.2	7.90E+09	2.10E+10	1.42E+10	3.71%	20.68%
	10	0.270	3.22E+06	1.19E+07	5.80E+06	113.3	4.40E+09	1.63E+10	7.92E+09	1.01%	11.52%
EV (CM5)	7	0.176	0.00E+00	0.00E+00	0.00E+00	123.9	6.40E+08	3.63E+09	1.15E+09	0.00%	1.19%
	8	0.203	0.00E+00	0.00E+00	0.00E+00	116.0	6.10E+08	3.00E+09	1.10E+09	0.00%	1.13%
EV (CM6)	9	0.360	2.27E+07	6.31E+07	4.09E+07	110.1	1.40E+10	3.89E+10	2.52E+10	4.61%	26.01%
	10	0.102	2.73E+06	2.67E+07	4.91E+06	135.8	5.10E+09	5.00E+10	9.18E+09	0.55%	9.48%
EV (CM4)	7	0.155	4.45E+04	2.87E+05	8.01E+04	139.4	3.00E+08	1.94E+09	5.40E+08	0.01%	0.79%
	8	0.180	1.06E+03	5.88E+03	1.90E+03	144.8	1.50E+09	8.34E+09	2.70E+09	0.00%	3.93%
EV (CM5)	9	0.417	1.37E+07	3.29E+07	2.47E+07	116.7	9.20E+09	2.21E+10	1.66E+10	3.34%	24.09%
	10	0.214	3.87E+06	1.81E+07	6.97E+06	119.0	4.00E+09	1.87E+10	7.20E+09	0.94%	10.47%
EV (CM5)	7	0.193	4.23E+04	2.20E+05	7.61E+04	170.6	3.80E+08	1.97E+09	6.84E+08	0.01%	0.84%
	8	0.247	0.00E+00	0.00E+00	0.00E+00	148.8	1.90E+09	7.68E+09	3.42E+09	0.00%	4.21%
EV (CM5)	9	0.394	1.74E+07	4.42E+07	3.13E+07	133.1	1.20E+10	3.05E+10	2.16E+10	4.07%	26.58%
	10	0.165	2.52E+06	1.53E+07	4.54E+06	140.1	5.10E+09	3.09E+10	9.18E+09	0.59%	11.30%

IFCM: Imaging flow cytometry; NTA: nanoparticle tracking analysis; BCA: bicinchoninic acid; FFE: free flow electrophoresis; EV: extracellular vesicle.

other Fraction 9 samples, altogether, the data demonstrate that CD9⁺ EVs can specifically be recovered in Fraction 9. Thus, FFE allows reproducible enrichment of EVs from MSC-CMs.

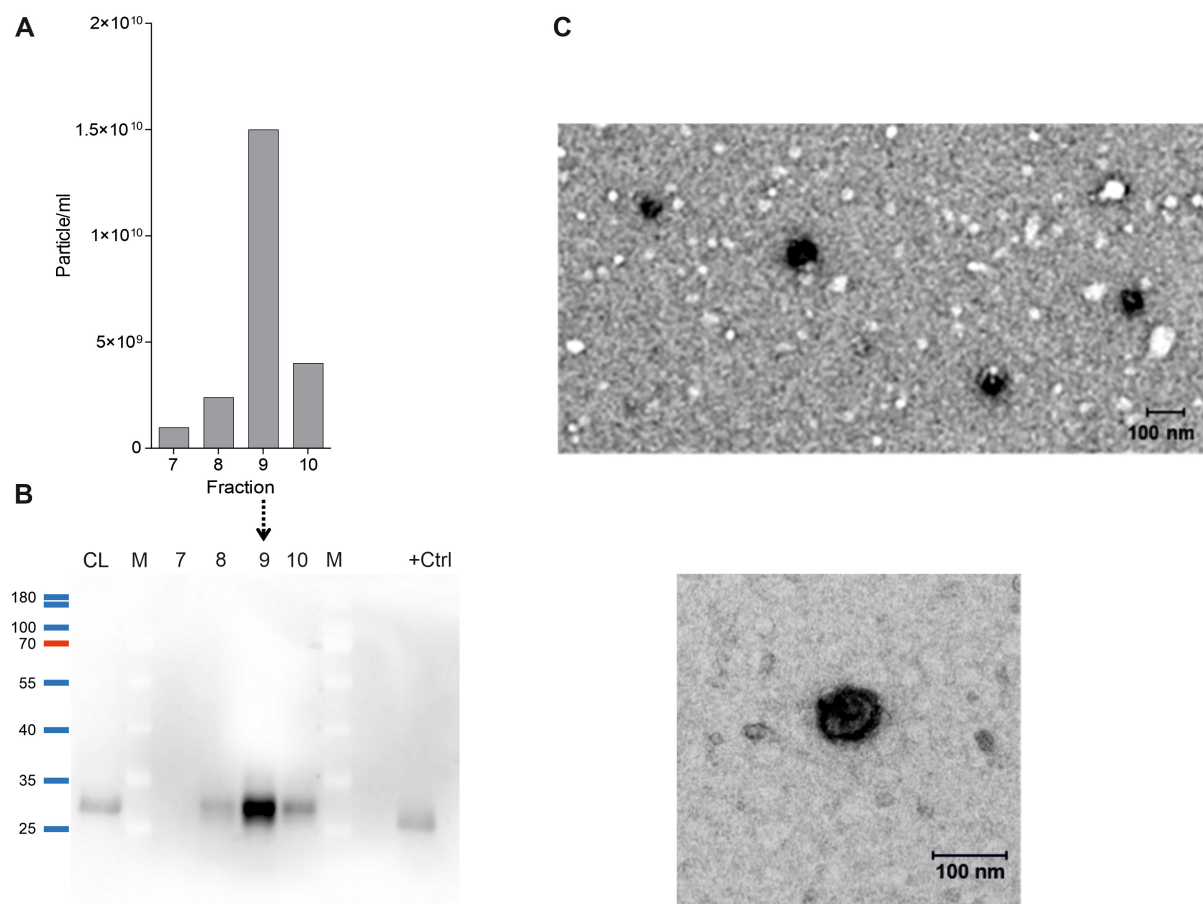


Figure 4. Free flow electrophoresis (FFE) allows preparation of *bona fide* extracellular vesicles (EVs). FFE fractions considered to contain the majority of EVs were analyzed by nanoparticle tracking analysis (NTA), Western blot (WB), and Transmission electron microscopy (TEM). (A) The results of NTA analyses depicted as particles per mL and mean particle size (131.4 nm) of Fractions 7-10 of the FFE fractionated MSC-CM (for details, see CM1 in Table 1). (B) Anti-CD9 Western blot of the same fractions depicted in (A). (C) TEM images of detectable Fraction 9 components following uranylacetate fixation: (top) 10,000× (1.189 nm/px) magnification (120 kV); and (bottom) 50,000× (0.240 nm/px) magnification.

DISCUSSION

Small EVs, especially exosomes, were discovered almost 40 years ago, and the first report on their functional impacts was reported in 1996^[28-30]. However, EV preparation remains challenging, and, to our best knowledge, no technologies have been described allowing the preparation of reasonably pure EVs in short time intervals. Here, we evaluated the suitability of FFE for the fractioning of EVs from a preclinically tested MSC-EV sample and the preparation from MSC-CMs. We demonstrated that CD9⁺ EVs from given EV preparations or MSC-CMs are very reproducibly recovered in discrete fractions, specifically in 3 out of 96 or 48 fractions, respectively, while most of the protein is recovered in other fractions. Thus, FFE allows quick and reproducible separation of EVs from a huge proportion of other molecules and compounds included in the original EV-containing samples. Depending on the EV starting concentration and the experimental needs, a continuous separation procedure can be performed, as demonstrated here by preparing EVs from more than 3 mL of MSC-CMs within 25 min. Since the application zone is much broader than that of each collected fraction and electrophoresis results in isoelectric focusing, analytes can be slightly concentrated compared to the starting samples. However, even though FFE can be used for EV preparation, the preparation remains low scale and is not necessarily quantitative. For example, after the application of

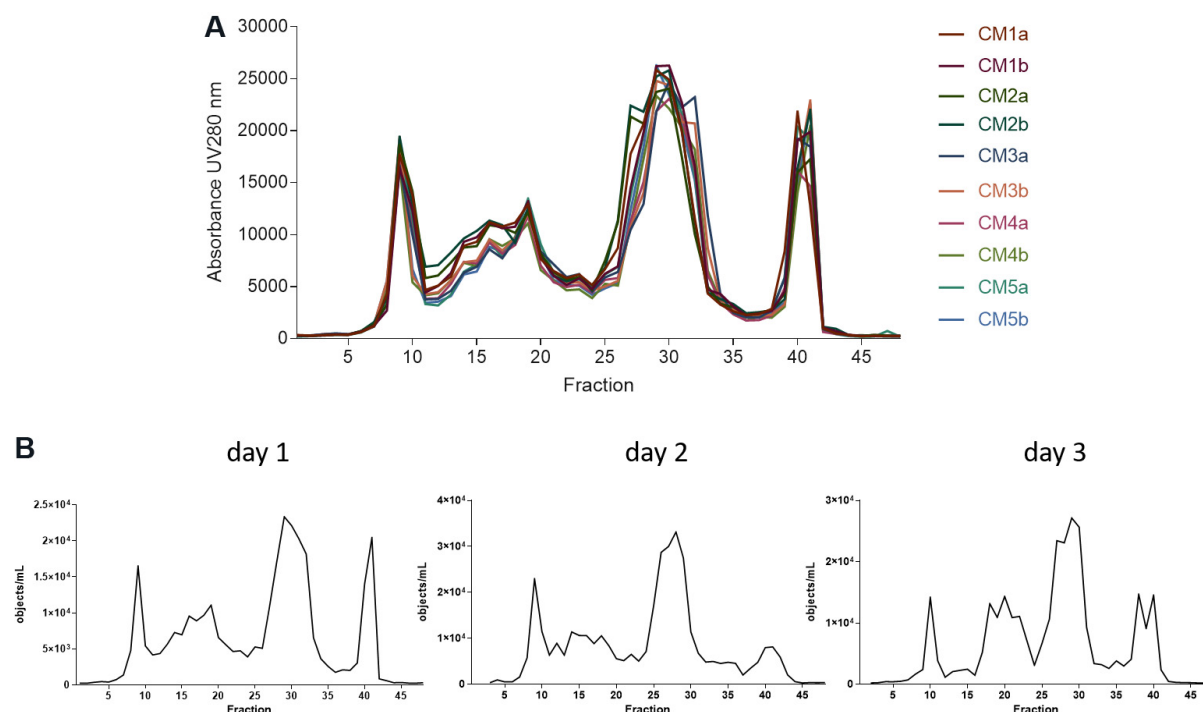


Figure 5. Free flow electrophoresis (FEE) fractionation is highly reproducible and robust. (A) Pherograms of obtained fractions of five different MSC-CMs (CM1-5) all of which were processed two independent times on the same day (a, b). (B) Pherograms of fractions obtained of MSC-CM1 that were fractionated on three different days. FFE conditions were applied as indicated in Figure 3.

samples, the performance of the separation process should be stabilized before beginning with the collection of the different fractions. Comparably, the collection period should be finished before the last sample components reach the collector unit, resulting in the additional loss of sample material. However, FFE provides several new and beneficial options for EV research. The pherograms of the obtained fractions, for example, reveal information about the complexity of the initially applied EV samples. As long as proteins are recovered in other non-EV FFE fractions, they can be considered as byproducts or impurities. In terms of EV-based therapeutics, it is important to understand that byproducts may contribute to the therapeutic effect of the EV product and can be tolerated as long as they neither negatively affect the product's function nor cause any side effects and as long as the product assembly is reproducible in independent batches of the same EV-product type^[31]. To demonstrate the reproducibility of the molecular composition of obtained EV samples, we previously discussed the need for a fingerprinting method for such products, especially when their clinical application is considered^[32]. Due to its high reproducibility, we understand FFE as a very potent method for generating reliable and reproducible fingerprints of obtained EV products and envision its huge potential for quality control of EV-based therapeutics.

Furthermore, combined with imaging flow cytometry, which can be performed in an automatized manner in the 96-well format and which we optimized for single EV analyses^[19-21], EV-containing fractions can quickly be identified and used for different down-stream analyses, as exemplarily shown for NTA, Western blot, and TEM.

As exemplified in this manuscript, different separation profiles can be applied for the separation of EVs from proteins and other sample components. We may not have established the perfect EV preparation protocol for MSC-EV or MSC-CM samples here. Indeed, proteomic profiling of obtained EV-containing

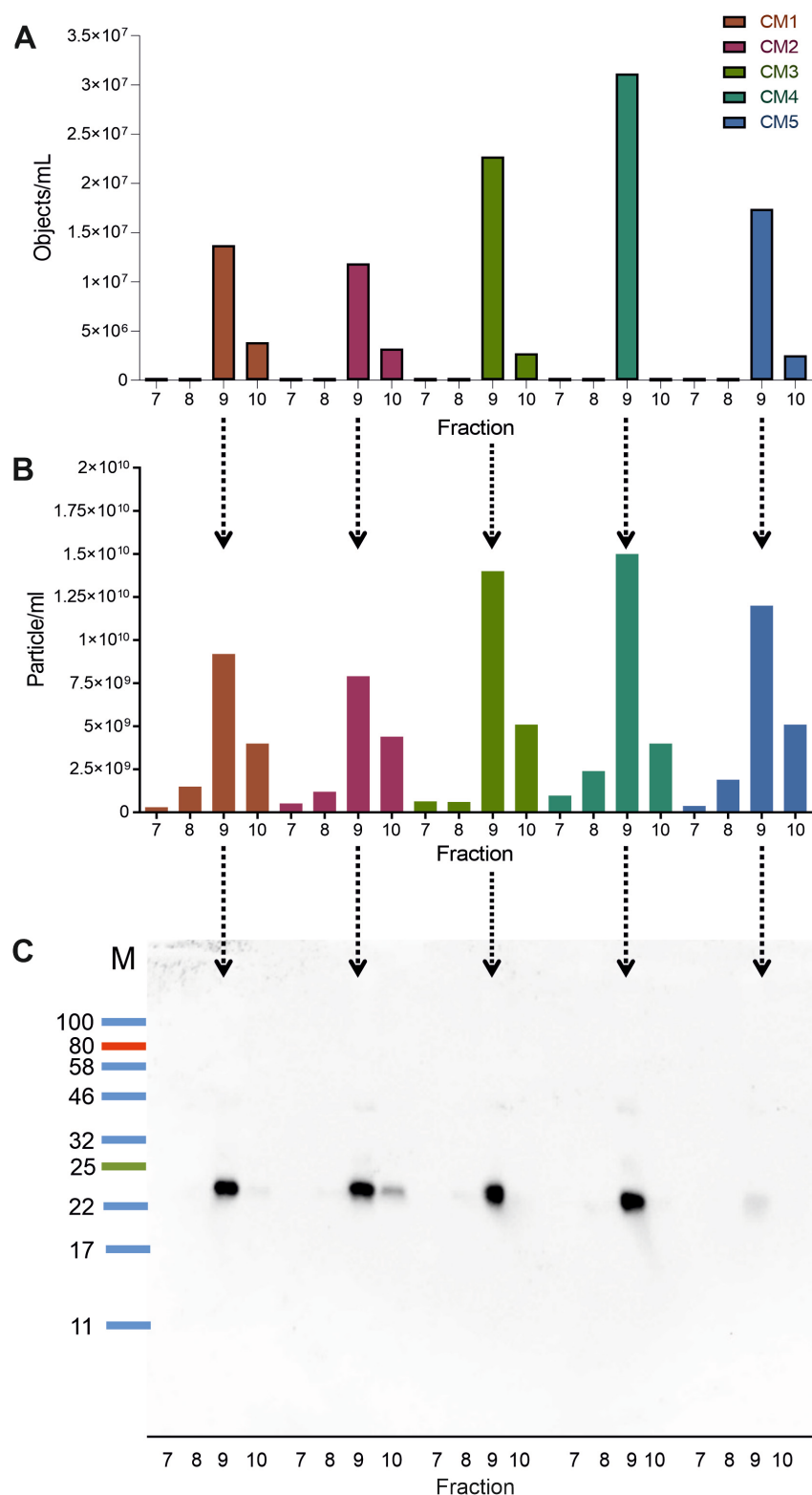


Figure 6. Free flow electrophoresis (FEE) is reproducible, allowing preparation of extracellular vesicles (EVs) from MSC-CMs. Five MSC-CMs were fractionated on the same day by FFE applying the protocol indicated in Figure 4. To evaluate the reproducibility of the EV preparation, obtained Fractions 7-10 were analyzed: (A) following anti-CD9 labeling by imaging flow cytometry; (B) by nanoparticle tracking analysis; and (C) following chloroform-methanol precipitation by anti-CD9 Western blot (exposure time 270 s).

fractions still recovered impurities in our CD9⁺ EV fractions (data not shown). However, by using different separation buffers, the separation capabilities can be significantly influenced and possibly improved much further, such that FFE will finally also allow separation of different EV subtypes. Indeed, in ongoing projects, in which we are investigating the usefulness of FFE for the preparative separation of EVs from other plasma sample components, we can recover different EV subtypes in different FFE fractions and are currently unraveling this EV heterogeneity in more detail (data not shown). This series of preliminary results implies that different EV types differ regarding their ionic strengths or pIs, respectively, likely providing another level of complexity to the EV field.

In summary, we demonstrated that FFE is a fast and efficient method to separate EVs from a huge proportion of other sample components included in different EV-containing liquids. Our results reflect the high reproducibility of the FFE-based sample separation and qualify FFE as an ideal device for the molecular fingerprinting of obtained EV products.

DECLARATIONS

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Authors' contributions

Designed the experiments: Staubach S, Weber G, Giebel B

Performed the FFE separation: Weber G

Most of the characterization of the obtained FFE samples: Staubach S

Supported the IFCM analyses: Tertel T

Performed the TEM analyses: Walkenfort B

Critically discussed data: Staubach S, Tertel T, Walkenfort B, Buschmann D, Pfaffl MW, Weber G, Giebel B

Wrote the manuscript: Staubach S, Giebel B

All authors approved the manuscript.

Availability of data and materials

Additional data is provided within the supplements.

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Conflicts of interest

Weber G is CEO and Founder of FFE Service GmbH. Giebel B is a scientific advisory board member of Innovex Therapeutics SL and Mursla Ltd. and a founding director of Exosla Ltd. Other authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The samples of healthy bone marrow donors was approved by the ethics committee of the University of Duisburg-Essen (12-5295-BO).

Consent for publication

Not applicable.

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Original Article

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Genome-wide analysis reflects novel 5-hydroxymethylcytosines implicated in diabetic nephropathy and the biomarker potential

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Abstract

Aim: Diabetic nephropathy (DN) has become the most common cause of end-stage renal disease in most countries for patients with type 2 diabetes (T2D). Elucidating novel epigenetic contributors to DN can not only enhance our understanding of this complex disorder but also lay the foundation for developing more effective monitoring tools and preventive interventions in the future, thus contributing to our ultimate goal of improving patient care.

Methods: 5-hydroxymethylcytosines (5hmC)-Seal, a highly selective chemical labeling technique, was used to profile genome-wide 5hmC, a stable cytosine modification type marking gene activation, in circulating cell-free DNA (cfDNA) samples from a cohort of patients recruited at Zhongnan Hospital, including T2D patients with nephropathy (DN, $n = 12$), T2D patients with non-DN vascular complications (non-DN, $n = 29$), and T2D patients without any complication (controls, $n = 14$). Differential analysis was performed to find DN-associated 5hmC



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features, followed by the exploration of biomarker potential of 5hmC in cfDNA for DN using a machine learning approach.

Results: Genome-wide analyses of 5hmC in cfDNA detected 427 and 336 differential 5hmC modifications associated with DN, compared with non-DN individuals and controls, and suggested relevant pathways such as NOD-like receptor signaling pathway and tyrosine metabolism. Our exploration using a machine learning approach revealed an exploratory model comprised of ten 5hmC genes showing the possibility to distinguish DN from non-DN individuals or controls.

Conclusion: Genome-wide analysis suggests the possibility of exploiting novel 5hmC in patient-derived cfDNA as a non-invasive tool for monitoring DN in high-risk T2D patients in the future.

Keywords: Type 2 diabetes, nephropathy, epigenetics, 5-hydroxymethylcytosine, cfDNA

INTRODUCTION

Diabetic nephropathy (DN) is one of the most common complications of type 2 diabetes (T2D) and a leading cause of end-stage renal disease globally^[1]. Approximately 20-40% of T2D patients will ultimately develop nephropathic diseases, thus posing a significant risk for T2D patients^[2]. Early detection and preventive intervention of DN has been limited due largely to the lack of a comprehensive understanding of its complex pathogenesis and effective biomarkers. Notably, conventional clinical markers to evaluate renal functions of DN, including serum creatinine, estimated glomerular filtration rate (eGFR), and urinary albumin, can be influenced by many factors^[3]. Pathologically, the “gold standard” to diagnose DN has been percutaneous renal biopsy. However, various complications can be caused by the procedure, such as bleeding, pain, and infection^[4]. Therefore, investigation of novel molecular contributors implicated in DN would not only enhance our understanding of this disease but also provide opportunities to develop more effective diagnostic and preventive approaches. Of particular interest to us are novel epigenetic modifications revealed in circulating cell-free DNA (cfDNA), a clinically convenient liquid biopsy, which may reflect systematic changes in the body during pathogenesis^[5].

Particularly, epigenetic modifications are gene regulatory elements that sit between phenotypes and genotypes^[3]. The most-investigated epigenetic modification is DNA methylation, i.e., 5-methylcytosine (5mC), which has been implicated in normal physiological processes and pathogenesis. The regulation of DNA methylation *in vivo* is a dynamic process. The ten-eleven translocation enzymes can oxidize 5mC into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine, and 5-carboxylcytosine under an active demethylation process^[6]. Unlike other demethylated products of 5mC, 5hmC is relatively abundant and biochemically stable in the human genome. Previous studies have confirmed that the 5hmC modifications show a distinct genomic distribution and gene regulatory role from 5mC^[7] and have been implicated in a variety of diseases. Notably, recent studies have begun to demonstrate an association of altered 5hmC with diabetes-related conditions such as hyperglycemia^[8].

Technically, the widely used bisulfite conversion-based epigenomic profiling techniques, although offering opportunities of profiling genome-wide cytosine modifications, cannot distinguish 5hmC from 5mC^[9]. Therefore, to investigate whether the 5hmC modifications are implicated in DN, we utilized the 5hmC-Seal technique^[10], a highly sensitive chemical labeling technique for genome-wide profiling of 5hmC, and next-generation sequencing (NGS), in cfDNA samples derived from a cohort of T2D patients with and without nephropathy. The 5hmC-Seal technique has been systematically validated using spike-in controls and serial DNA inputs by our team and other groups as a reliable approach for biomarker discovery^[9,11-15] using limited

clinical biospecimens, e.g., as low as a few nanograms of cfDNA that can be conveniently isolated from 1-2 mL of plasma^[11]. Therefore, the 5hmC-Seal technique has a technical advantage, especially suitable for future clinical implementation of cfDNA-based non-invasive tools for disease diagnosis, prognosis, and surveillance. Furthermore, our previous genome-wide analyses of 5hmC in cfDNA suggested a link between altered 5hmC and T2D-associated vascular complications in general^[16]. For example, the 5hmC-based signatures in cfDNA were shown to have the potential to distinguish T2D patients with multiple vascular complications from those with single vascular complications^[16], as well as between T2D patients who developed diabetic retinopathy and those who did not^[17]. However, whether there are specific 5hmC changes implicated in DN has not been investigated yet.

Specifically, in the current study [Figure 1], we profiled genome-wide 5hmC in cfDNA samples derived from a cohort of 55 patients with T2D using the 5hmC-Seal technique and NGS. Differential analysis was performed to identify DN-associated modified genes as well as involved pathways. To investigate the feasibility of future biomarker studies targeting 5hmC for DN, we also explored the distinguishing capacity of 5hmC for DN by summarizing the genome-wide 5hmC profiles through feature selection using a machine-learning approach. Findings from this study enhance our understanding of DN-associated epigenetic changes and involved pathways, and provide the foundation for developing more effective and non-invasive tools for DN monitoring and preventive intervention in the future.

METHODS

Study populations

In total, 55 patients with T2D, including 12 patients with DN, 29 patients with non-DN complications (i.e., macrovascular complications, neuropathy, and retinopathy), and 14 sex- and age-matched T2D controls without complications, were recruited at Zhongnan Hospital of Wuhan University, China. Patients were diagnosed according to the 2017 Standards of Medical Care in Diabetes of the American Diabetes Association^[18]. All study participants were excluded for other kidney diseases. Clinical variables were collected from the medical records following a standard protocol. Fasting plasma samples (~ 2 mL/patient) were collected the next morning after hospital admission. This study was approved by the Medical Ethics Committee of Zhongnan Hospital of Wuhan University (2019069). Informed consent was obtained from each participant.

Laboratory measurements

Laboratory measurements were performed at Zhongnan Hospital for the current study. Kidney function parameters (creatinine, urea nitrogen, uric acid, and eGFR)^[19] and serum glucose were examined by the AU5800 Chemistry Analyzer (Beckman). The HA-8160 Glycohemoglobin Analyzer was used to measure blood glycated hemoglobin (HbA1c). Serum insulin was assayed by the i4000SR Immunology Analyzer (Abbott Laboratories).

Preparation of cfDNA samples, 5hmC-Seal assay, and data processing

Details about the preparation of circulating cfDNA samples, 5hmC-Seal library construction, sequencing, and data processing are described in our previous publications^[10,11,20]. Briefly, plasma samples were separated and stored at - 80 °C after centrifuging twice at 1350 × g for 12 min and 13,500 × g for 5 min. cfDNA was extracted from the plasma using the Circulating Nucleic Acid Kit (Qiagen) and the concentration of cfDNA was examined using the Qubit High Sensitivity dsDNA Assay (Invitrogen) according to the manufacturers' instructions. The 5hmC-Seal library construction and NGS were performed at the Innovation Center for Genomics, Peking University (Beijing, China). Briefly, each cfDNA sample was first prepared and ligated with adaptors. Next, the T4 bacteriophage enzyme β-glucosyltransferase was used to transfer an engineered glucose moiety containing an azide-group to 5hmC in duplex DNA. A biotin tag was then installed onto the

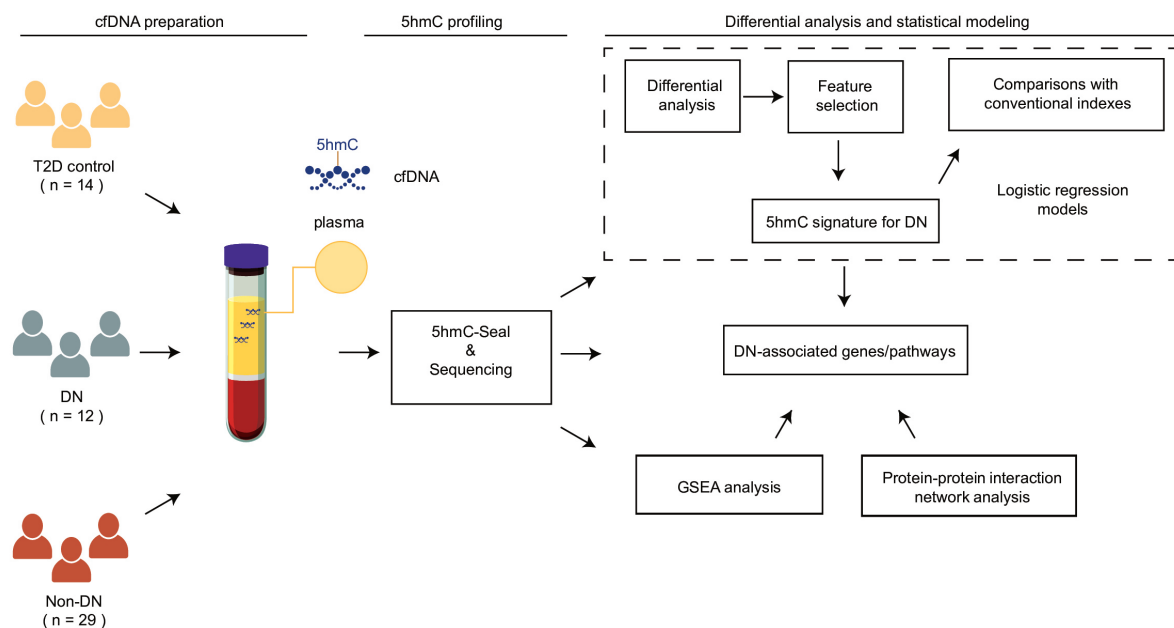


Figure 1. Study design. In total, 55 patients with type 2 diabetes (T2D), including 12 patients with diabetic nephropathy (DN), 29 patients with non-DN complications (Non-DN), and 14 controls (CTRL), were profiled for genome-wide 5-hydroxymethylcytosines (5hmC) using the 5hmC-Seal technique and next-generation sequencing, followed by differential analysis, gene set enrichment analysis (GSEA), protein-protein interaction network analysis, feature selection, and modeling to inform biological insights and evaluate biomarker potential.

azide group using Click chemistry, followed by capturing of 5hmC-containing DNA fragments using avidin beads. The 5hmC-Seal library for each cfDNA sample was then constructed through PCR amplification, followed by paired-end sequencing (PE39) with the Illumina NextSeq 500 platform. On average, ~ 23 million unique reads per cfDNA sample were obtained from NGS. According to our previous studies^[11-13,16,17], 5hmC profiles are more abundant in gene bodies and exonic regions relative to their flanking regions and depleted at the transcription start sites. Therefore, well-annotated gene bodies provided by GENCODE (hg19)^[21] were our primary targets to summarize the 5hmC-Seal data by counting the sequencing reads using feature Counts^[22]. The principal components analysis (PCA) was conducted to explore the potential confounding factors in global 5hmC data. The kidney-derived histone modification marks for enhancers, i.e., H3K4me3 and H3K27ac, were obtained from the Roadmap Epigenomics Project^[23] to help provide biological insights.

Identifying DN-associated 5hmC signature in cfDNA

Multivariable logistic regression models were used to identify gene bodies containing differential 5hmC levels (i.e., normalized read counts) between DN patients and T2D controls, as well as between DN and non-DN patients. Although not the focus of the current study, we also performed differential analysis between T2D controls and patients with non-DN complications for comparison. Adjusted covariates included age and sex. To evaluate potential protein-protein interaction (PPI) networks, those genes showing a trend of differential modifications (Wald test $P < 0.01$ and fold change $> 10\%$) between diagnosis classes, e.g., DN vs. controls, were supplied to the *stringApp* from Cytoscape^[24,25] with the default parameters based on the STRING database (confidence score > 0.8 and maximum additional interactor = 50) with linker genes allowed^[26]. Hubs of the PPI networks were estimated based on the measurement of betweenness centrality, which represents the magnitude of influence a component gene has over the flow of information in a gene network^[24]. Moreover, because of the limited sample size, instead of evaluating pathways among individual genes, Gene Set Enrichment Analysis (GSEA)^[27] was used to explore the functional relevance of

canonical pathways in the whole-genome 5hmC data between diagnosis classes, e.g., DN vs. controls, using the clusterProfiler tool(v4.0)^[28]. Specifically, over-/under-represented pathways maintained in the Kyoto Encyclopedia of Genes and Genomes (KEGG)^[29] database (≥ 15 genes and false discovery rate $< 5\%$) and normalized enrichment score were obtained from GSEA.

Summarization of a 5hmC-based epigenetic score for DN

To evaluate whether a cfDNA-based score with potential diagnostic value could be summarized from the genome-wide 5hmC data, those genes that showed a trend of differential 5hmC between DN and controls or DN and non-DN complications, but not between non-DN complications and controls, were further selected to explore a signature panel by applying the elastic net regularization^[30] on the multivariable logistic regression models. To improve modeling efficiency, we filtered out most uninformative gene bodies (i.e., $P > 0.05$) before feature selection. Component genes of the exploratory model were selected if they were consistently present (100%) in 100 iterations using repeated two-fold cross-validation to differentiate between DN and controls. A weighted score to summarize the genome-wide 5hmC for each individual was computed as follows:

$$score = \sum_{i=1}^n (\beta_i \times G_i)$$

where G_i represents the normalized read counts of the i th gene body and β_i represents its regression coefficient, following our previous publications^[11,12,16,17]. The area under the receiver operator characteristic curve (AUROC) was used to demonstrate model performance. The optimal score cutoffs for the AUROCs were determined by the score that maximized the Youden index, and the corresponding sensitivity and specificity were estimated.

Comparison between the 5hmC-based score for DN with conventional clinical variables or risk factors

To compare the performance of the 5hmC-based scores for DN relative to various clinical variables, univariable logistic regression models for available clinical variables were examined as follows:

$$f(Y_i) = \frac{e^{\beta_0 + \beta_i X_i}}{1 + e^{\beta_0 + \beta_i X_i}}$$

where Y_i represents binary diagnosis classes (i.e., DN vs. non-DN/controls or DN vs. non-DN). X_i represents age, sex, or each of the clinical variables body mass index (BMI), smoking history, drinking history, glucose, HbA1c, insulin, creatinine, uric acid, urea nitrogen and eGFR. The predicted probabilities of the univariable logistic regression models were used for assessing classification performance, i.e., DN vs. non-DN/controls or DN vs. non-DN, via the AUROC. Sensitivity and specificity at the cutoff that maximized the Youden index were estimated for each variable.

RESULTS

Clinical and demographic characteristics of the study participants

Table 1 shows the clinical and demographic characteristics of the 55 study participants. Overall, there were no significant differences regarding major demographic and clinical variables between patient groups. There

Table 1. Demographical and clinical characteristics of the study participants

Clinical variable	T2D control (n = 14)	DN (n = 12)	Non-DN (n = 29)	p ^A	p ^B	p ^C
Age (year)	47.1 ± 9.7	56.8 ± 14.5	57.7 ± 8.1	0.08 ^a	0 ^a	0.71 ^a
Sex (male/female)	10/4	9/3	13/16	0.52 ^b	0.14 ^b	0.25 ^b
BMI (kg/m ²)	24.9 ± 3.7	25.4 ± 2.3	25.7 ± 4.1	0.49 ^a	0.62 ^a	0.99 ^a
Smoking (Yes/No)	6/8	7/5	5/24	0.69 ^b	0.15 ^b	0.02 ^b
Drinking (Yes/No)	2/12	4/8	4/25	0.5 ^b	1 ^b	0.32 ^b
T2D duration (year)	3.7 ± 3.9	4.5 ± 6.4	6.9 ± 6.3	0.7 ^a	0.14 ^a	0.08 ^a
SBP (mmHg)	123.6 ± 9.4	135.4 ± 20.2	133.9 ± 19.2	0.17 ^a	0.09 ^a	0.82 ^a
DBP (mmHg)	78.3 ± 7.9	82.1 ± 10.6	77.8 ± 10.6	0.28 ^a	0.47 ^a	0.13 ^a
Glucose (mmol/L)	11.5 ± 3.3	9.5 ± 3.5	8.5 ± 3.1	0.13 ^a	0.01 ^a	0.46 ^a
HbA1c (%)	9.1 ± 1.8	7.9 ± 2.2	8.4 ± 1.9	0.17 ^a	0.33 ^a	0.42 ^a
Insulin (μU/mL)	10.3 ± 6.0	7.4 ± 7.1	9.5 ± 7.0	0.28 ^a	0.66 ^a	0.28 ^a
Kidney Function:						
Urea nitrogen (mmol/L)	5.8 ± 1.3	5.7 ± 1.8	5.9 ± 1.7	0.78 ^a	0.83 ^a	0.69 ^a
Creatinine (μmol/L)	60.3 ± 13.9	78.8 ± 31	63.1 ± 18.2	0.15 ^a	0.86 ^a	0.14 ^a
Uric acid (μmol/L)	283.6 ± 51.9	319.7 ± 99.1	291.1 ± 81.9	0.4 ^a	0.93 ^a	0.51 ^a
eGFR (mL/min/1.73 m ²)	111.6 ± 12.2	89.2 ± 28.4	98.5 ± 13.4	0.02 ^a	0.01 ^a	0.24 ^a
Medication (Yes/No)						
Insulin treatment	6/8	3/9	15/13	0.59 ^b	0.74 ^b	0.19 ^b
Oral glucose-lowering medicine	6/8	6/6	15/13	1 ^b	0.74 ^b	1 ^b

^AComparison between T2D control (CTRL) and Nephropathy (DN); ^Bcomparison between T2D control (CTRL) and patients with non-nephropathy complications (Non-DN); ^Ccomparison between DN and Non-DN; ^a Wilcoxon test; ^bchi-square test. DN: diabetic nephropathy; BMI: body mass index; T2D: Type 2 diabetes; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: glycated hemoglobin; eGFR: estimated glomerular filtration rate.

were comparable distributions of potential confounders for epigenetic modifications between patient groups, such as baseline BMI and sex ($P > 0.05$). Notably, differences in age at the time of blood collection were observed between T2D controls and DN patients. Therefore, age was used as a covariate in downstream differential analysis when comparing between diagnosis groups (e.g., DN vs. controls). Moreover, in total, 24 patients used insulin treatment and 27 patients used oral glucose-lowering medications, showing no significant disparity regarding medication treatment between different diagnosis groups ($P > 0.05$).

Overview of the genome-wide 5hmC profiles in cfDNA

Consistent with our observations in the cfDNA samples from other studies^[13,15], the distribution of genome-wide 5hmC was also more abundant in gene bodies and exonic regions relative to their flanking regions and the transcription start sites [Supplementary Figure 1A], supporting our focus on gene bodies in this proof-of-concept study. Moreover, PCA demonstrated no significant correlations between 5hmC and potential confounders, including sex and age [Supplementary Figure 1B and C]. In addition, we observed a trend of increased genome-wide 5hmC modification levels on kidney-derived enhancer marks: H3K4me1, across controls, patients with non-DN complications, and patients with DN (P -trend = 0.049).

Differentially modified genes associated with DN and the PPI network analysis

In total, 336 genes were detected to show a trend of differential modification between T2D controls and patients with DN (Wald test $P < 0.05$), among which 271 genes had a fold change of at least 10% (Supplementary Table 1 and Supplementary Figure 1D). In comparison, 427 genes were found to be differentially modified between patients with DN and patients with non-DN complications (Wald test $P < 0.05$), among which 250 genes had a fold change of at least 10%, indicating the presence of 5hmC signatures

specific to DN complications [Figure 2A, Supplementary Table 1, and Supplementary Figure 1E]. Genes with a more stringent cutoff (Wald test $P < 0.01$ and fold change $> 10\%$) were further evaluated for PPI networks to explore potential biological connections with relevant functions. Notably, the PPI network analysis implicated those genes showing differential modifications between DN and T2D controls in hub genes relevant to kidney diseases [Supplementary Figure 2]. For example, SMARCA5, a member of the SWI/SNF-related matrix-associated action-dependent regulator of chromatin subfamily A as well as a differentially modified gene, was found to be connected with biomarkers for diabetic kidney disease^[31]. *RUVBL1*, which encodes RuvB like AAA ATPase 1, is connected with certain differential genes (e.g., *COMMD2* and *GPS1*), and its deletion could lead to acute kidney injury in mice^[32]. In contrast, the PPI network analysis based on a list of genes showing differential 5hmC between DN and non-DN patients also identified connections with hub genes implicated in DN [Supplementary Figure 3]. For example, signal transducer and activator of transcription 1 (STAT1) are connected with certain differential genes (e.g., *MX1*, *IFI44L*, and *IFIH1*), and its activation was shown to cause cell apoptosis and renal fibrosis, thus being implicated in DN^[33].

GSEA implicating pathways differentially modified in patients with DN

The GSEA results reveal over- or under-representation of certain canonical pathways in patients with DN relative to controls, such as the NOD-like receptor signaling pathway, neuroactive ligand-receptor interaction, platelet activation, tyrosine metabolism, and necroptosis [Figure 2B and Supplementary Table 2]. Several core genes that contributed to the over- or under-representation of these pathways were also differentially modified between DN and T2D controls, including *CXCL1* and *PKN2* in the NOD-like receptor signaling pathway; *PYY*, *GRM*, *EDN2*, *GCGR*, and *MLN* in neuroactive ligand-receptor interaction; and *IL1B* in necroptosis [Supplementary Table 2]. In addition, the GSEA results between DN patients and patients with non-DN complications indicate significant over- or under-representation of KEGG pathways such as tyrosine metabolism, olfactory transduction, and signaling pathways regulating pluripotency of stem cells [Figure 2B and Supplementary Table 2], although they are not differentially modified at the single-gene level, likely due to the small sample size. Interestingly, several over-represented pathways between DN and controls/non-DN patients are known to be associated with DN or kidney-related diseases, such as the NOD-like receptor signaling pathway and tyrosine metabolism^[34, 35].

Summarization of 5hmC-based epigenetic score for DN

An exploratory model comprised of ten genes (i.e., *UQCRFS1*, *VAR2*, *WFOX*, *CSPG4*, *TMCO4*, *SLC38A3*, *RPL36*, *CTD.2116N17.1*, *MATN4*, and *CABP7*) was identified using the elastic net regularization and multivariable logistic regression models for distinguishing DN from T2D controls [Figure 2C]. Of note, the 5hmC scores were significantly different between patients with DN and controls, as well as between patients with DN and those with non-DN complications [Figure 2D] (t -test, $P < 0.01$). When using the 5hmC score as the only predictor, the AUROC results show 100% sensitivity and 97% specificity to classify DN and non-DN complications, in addition to the performance of distinguishing patients with DN from controls [Table 2].

We compared the sensitivity and specificity of various clinical variables in our cohort for distinguishing DN from T2D controls or patients with non-DN complications [Table 2]. Notably, logistic regression results indicate that the 5hmC scores in general outperformed age, sex, and various conventional clinical variables, including clinical variables of kidney functions, featuring greater AUROCs and higher sensitivity/specificity [Table 2]. For example, the 5hmC scores significantly outperformed the eGFR in differentiating between patients with DN and controls (AUROC, 100% vs. 78%) as well as between DN and non-DN complications (AUROC, 98% vs. 62%).

Table 2. Biomarker potential of the 5hmC model and comparisons with clinical variables

Clinical variable/model	T2D control vs. DN			Non-DN vs. DN		
	Sensitivity	Specificity	AUROC	Sensitivity	Specificity	AUROC
Age (year)	0.83	0.50	0.70	0.42	0.83	0.54
Sex (male/female)	0.75	0.29	0.52	0.75	0.55	0.65
BMI (kg/m ²)	1.00	0.36	0.58	1.00	0.24	0.50
Smoking (yes/no)	0.58	0.57	0.58	0.58	0.83	0.71
Drinking (yes/no)	0.33	0.86	0.60	0.33	0.86	0.60
Glucose (mmol/L)	0.73	0.64	0.68	0.55	0.68	0.58
HbA1c (%)	0.55	0.86	0.67	0.55	0.85	0.59
Insulin (μU/mL)	0.83	0.62	0.69	0.67	0.71	0.66
Creatinine (μmol/L)	0.36	1.00	0.68	0.36	0.93	0.65
Uric acid (μmol/L)	0.64	0.71	0.60	0.73	0.48	0.57
Urea nitrogen (mmol/L)	0.45	0.86	0.54	0.45	0.72	0.54
eGFR (mL/min/1.73 m ²)	0.64	1.00	0.78	0.64	0.79	0.62
5hmC model	1.00	1.00	1.00	1.00	0.97	0.98

T2D: Type 2 diabetes; DN: diabetic nephropathy; AUROC: the area under the receiver operator characteristic curve. BMI: body mass index; HbA1c: glycated hemoglobin; eGFR: estimated glomerular filtration rate.

DISCUSSION

Enhancing our understanding of the molecular contributors to DN pathogenesis would provide opportunities for developing more effective clinical tools to prevent and manage this complication. Equipped with the highly sensitive 5hmC-Seal technique, we sought to investigate DN-associated 5hmC in patient-derived cfDNA using a cohort of T2D patients with and without DN. Genome-wide analysis of 5hmC indicated there existed differential 5hmC modifications and over-/under-represented pathways in cfDNA that provided links between 5hmC signatures for DN and relevant pathways/genes. Besides previously implicated pathways and genes in DN or kidney disease, such as the NOD-like receptor signaling pathway and *CXCL1* of the inflammasome family^[34,36], interestingly, our identified DN-associated 5hmC signatures were also shown to be connected with PPI hubs relevant to kidney disease and the pathogenesis of DN^[32,33], thus reflecting the DN relevance of the 5hmC profiles in patient-derived cfDNA. Additionally, certain significant pathways such as Fc gamma R-mediated phagocytosis and natural killer cell-mediated cytotoxicity were found to be enriched in those genes dysregulated in DN from a meta-analysis of mouse microarray data^[37], lending further support for the existence of biological links between the 5hmC landscape reflected in DN patient-derived cfDNA and the underlying pathogenesis.

One important question about the cfDNA-based methods is whether the patient-derived cfDNA samples reflect the target tissue. Our genome-wide scan examining co-localization of the 5hmC-Seal reads and kidney-derived enhancer markers demonstrated a trend of increased modification levels between T2D controls and DN patients, suggesting the contribution of the target tissue (i.e., kidney) to the 5hmC profiles in DN patients. The current tissue-derived histone modifications, however, included only two individuals from the Roadmap Epigenomics Project; with the availability of more reference epigenomes in the future, a more comprehensive evaluation would provide more insights into the relative proportions of cfDNA sources in patients with DN.

Considering that cfDNA could reflect the systematic and dynamic physiological condition of the patient, our findings targeting novel epigenetic information in cfDNA could provide the foundation for developing a convenient clinical tool for the care of T2D patients. Therefore, besides differential analysis, we also

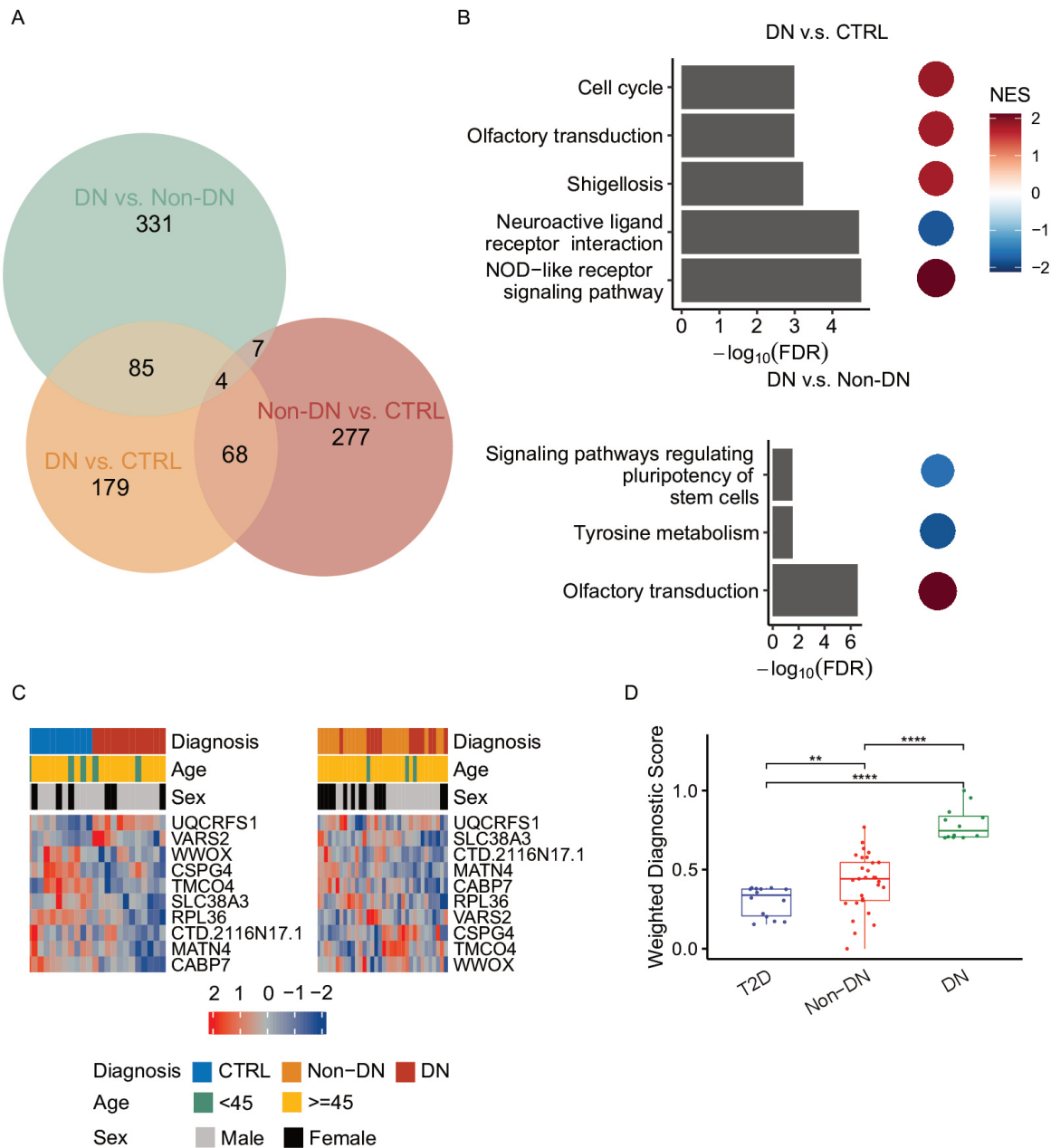


Figure 2. Novel 5hmC modifications implicated in diabetic nephropathy. Genome-wide analysis of the 5hmC-Seal data in patient-derived cfDNA reflected novel epigenetic modifications implicated in diabetic nephropathy (DN). (A) The Venn diagram shows differentially modified gene bodies specific to DN. (B) KEGG pathways significantly over-/under-represented in DN patients relative to either controls (CTRL) or non-DN patients (Non-DN) were identified from the GSEA. (C) The exploratory model comprised of ten component genes could distinguish DN from CTRL, as well as DN from Non-DN. (D) The 5hmC scores computed with the ten-gene exploratory model for DN were significantly different between DN and CTRL/Non-DN. Statistical significance (t -test): ns, $P > 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$. KEGG: Kyoto Encyclopedia of Genes and Genomes; GSEA: Gene set enrichment analysis; NES: Normalized enrichment score.

sought to evaluate the possibility of summarizing the genome-wide 5hmC profiles in cfDNA into an epigenetic score with biomarker potential. Particularly, findings from the feature selection based on machine learning and modeling in the current study provided promising results for the future development of cfDNA-based diagnostic or monitoring tools for DN. In particular, although limited by the sample size,

the 5hmC-based exploratory model for DN showed a consistent trend of outperformance over various conventional clinical indices for diabetic complications, especially those related to kidney functions, thus supporting the potential advantage of utilizing the 5hmC features in cfDNA as a novel biomarker for DN. Moreover, because the 5hmC-Seal technique can provide the genome-wide distribution of 5hmC in a single test, it is possible to integrate the DN-associated model with models for other diabetic complications to develop a comprehensive tool for routine care of patients with T2D in the future.

We are aware of several limitations in the current study. Firstly, the sample size is relatively small. Although our primary goal was to demonstrate the relevance of 5hmC to DN and the feasibility of using novel epigenetics and non-invasive liquid biopsy to develop management tools for DN in the future, future larger scale investigations studies will be necessary to provide a more comprehensive picture of the epigenetic landscape of DN or DN-associated pathways. Secondly, also limited by the current sample size, our modeling of 5hmC for their biomarker value was preliminary using a single cohort. Although testing using patients with and without DN helped us evaluate the biological relevance of the identified 5hmC features, future investigations involving more independent samples for both training and independent validation will be necessary to develop a clinically useful model based on 5hmC in cfDNA. Thirdly, the current study only focused on the 5hmC modification over genic regions; because the functional relevance of genic regions was better annotated and established, it would be interesting to extend the 5hmC analysis to other genomic regions, such as long non-coding RNA^[38] and enhancer markers as well as co-regulation analysis between 5hmC and gene expression in the future. Finally, future studies that expand to other populations and ethnic backgrounds will provide insights into any population-specific epigenetic modifications associated with DN, because of the long-appreciated baseline differences in epigenetic modifications across human populations^[39].

In conclusion, novel 5hmC modifications detected in patient-derived cfDNA samples were found to be implicated in DN. The 5hmC-Seal technique implemented with cfDNA holds promise for the future development of a non-invasive, clinically convenient tool for early detection of DN in high-risk T2D patients, thus contributing to the ultimate goal of improving clinical outcomes through personalized preventive intervention and/or treatment.

DECLARATIONS

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Authors' contributions

Sample and data collection, clinical interpretation: Yang Y, Yang K
Bioinformatics and data analysis: Zeng C, Xu S, Zhang Z, Cai Q, Zhang W
Funding: Liu SM, He C, Zhang W
Conceptualization: He C, Zhang W, Liu SM
Supervision: Zhang W, Liu SM
Drafting and approval of manuscript: all authors

Availability of data and materials

The 5hmC-Seal sequencing data and related metadata have been deposited into the NCBI Gene Expression Omnibus database (Accession No. GSE186021).

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Conflicts of interest

CH was the scientific founder of Epican Genetech, which obtained a license from the University of Chicago to develop the 5hmC-Seal technique for clinical applications. WZ was an advisor of Epican Genetech and he received research support from the company.

Ethical approval and consent to participate

This study was approved by the Medical Ethics Committee of Zhongnan Hospital of Wuhan University (2019069). Informed consent was obtained from each study participant.

Consent for publication

Not applicable.

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Obituary

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Obituary for Prof. Dr. Johng Sik Rhim

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With deep sadness, we announce that our Editorial Board member Johng Sik Rhim passed away peacefully on March 5, 2022, in Bethesda, MD, USA.

Dr. Rhim is a pioneer in human cell transformation whose career has been suffused with six decades of contributions to the field of oncology and cancer research. His interest in medicine developed after a grueling six-year battle with septic arthritis, beginning when he was just three years old, and he earned a Doctor of Medicine from Seoul National University College of Medicine in 1957. Dr. Rhim completed a medical internship at Seoul National University Hospital through the beginning of 1958, and relocated to the United States to accept a two-year research fellowship at Dr. Albert B. Sabin's laboratory at the Children's Hospital Research Foundation later that year.

Throughout the early 1960s, Dr. Rhim would pursue research fellowships and associate positions with Baylor University College of Medicine, the Graduate School of Public Health at the University of Pittsburgh, and Louisiana State University School of Medicine. In 1964, he joined the National Institute of Allergy and Infectious Diseases at the National Institutes of Health as a visiting scientist. Dr. Rhim subsequently departed in 1966 to become the project director for Cancer Research at Microbiological Associates of Bethesda, Maryland.



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In 1978, Dr. Rhim was named as a senior investigator at the National Cancer Institute, a part of the National Institutes of Health. He would remain active with the National Cancer Institute until 1998, whereupon he accepted his current role as the Associate Director, Center for Prostate Disease Research, Research Professor Emeritus, Department of Surgery, at the Uniformed Services University of the Health Sciences. Dr. Rhim's research has focused on the development of human cell culture systems for use in cancer studies, which have allowed for new in-depth analysis of the mechanisms of action of therapies and carcinogens. He holds many patents for processes related to cell transformation, and looks forward to furthering advances in patient-derived cell models based on his early models. As a pioneer in human cell modeling and cultures, Dr. Rhim has contributed more than 300 articles and maintained involvement with numerous professional societies, including the American Medical Association, the American Association for the Advancement of Science, the Society of Experimental Biology and Medicine, and the International Association of Leukemia Research^[1].

Johng Sik Rhim was Editorial Board member of *Extracellular Vesicles and Circulating Nucleic Acids*. He supported the peer-review for *EVCNA*, and we highly appreciate his support to *EVCNA* at its early stage.

DECLARATIONS

Acknowledgments

Dr. Rhim's profile was reproduced from "Marquis Who's Who in the World" in August 2020 with permission.

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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1. Marquis Who's Who Honors Johng Sik Rhim with Inclusion in Who's Who in the World. Available from: <https://www.24-7pressrelease.com/press-release/475065/marquis-whos-who-honors-joh> [Last accessed on 24 March 2022].

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Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
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The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

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The abstract should be a single paragraph with word limitation and specific structure requirements (for more details please refer to Types of Manuscripts). It usually describes the main objective(s) of the study, explains how the study was done, including any model organisms used, without methodological detail, and summarizes the most important results and their significance. The abstract must be an objective representation of the study: it is not allowed to contain results which are not presented and substantiated in the manuscript or exaggerate the main conclusions. Citations should not be included in the abstract.

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Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

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Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

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