trillium extracellular vesicles

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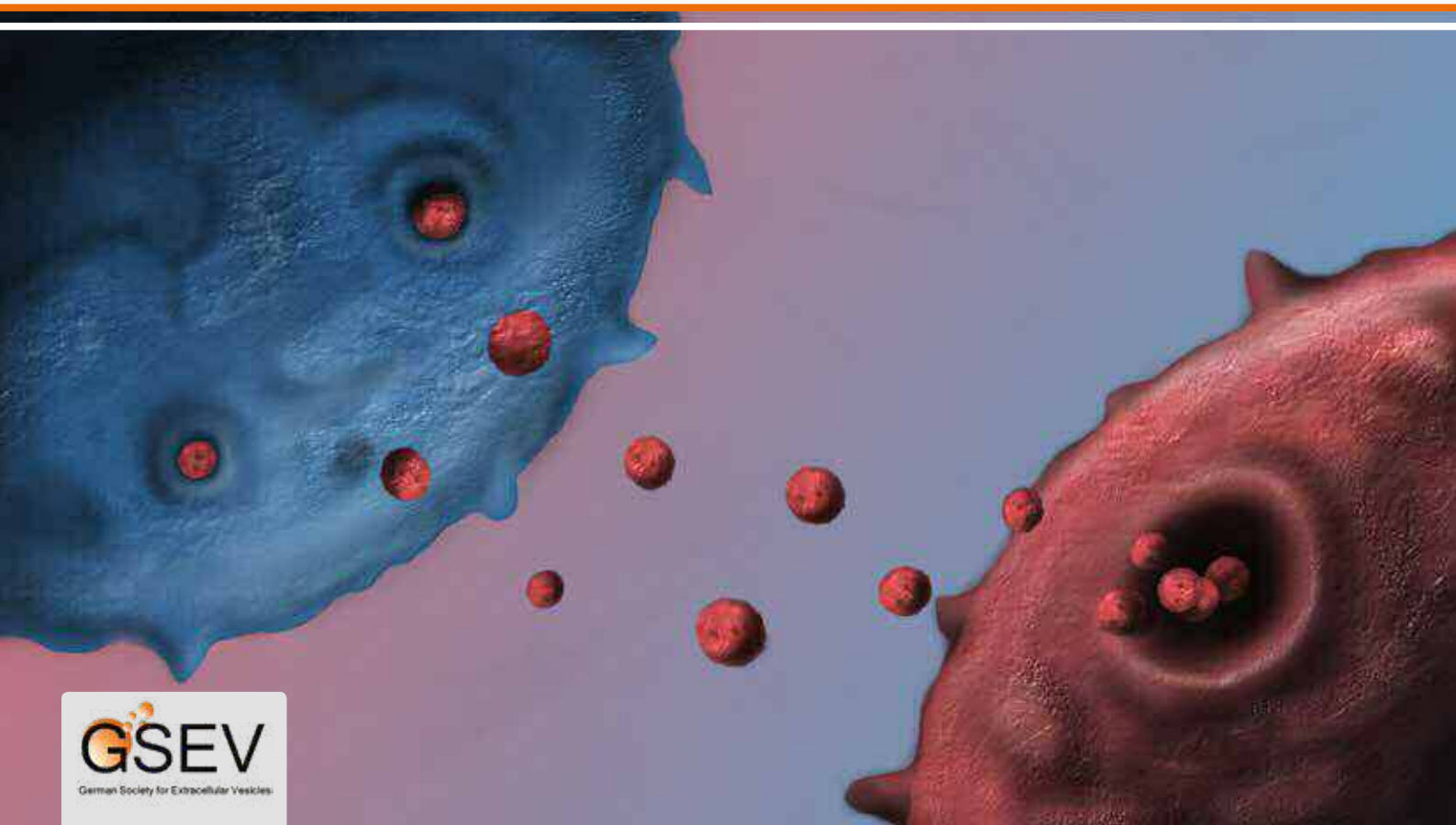
How cells communicate – an introduction to extracellular vesicles

From fundamental understanding to clinical application



GSEV
German Society for Extracellular Vesicles

BIA separations

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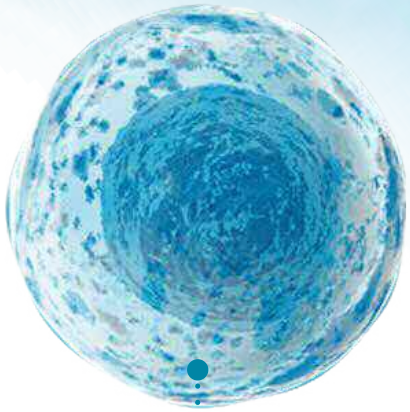

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New topic, new journal, new research

Cells need to communicate with each other to exchange information and fulfil their physiological role. In addition to direct cell-to-cell contact and the exchange of certain soluble factors, there is a third route of information transfer, which has recently received significant attention - extracellular vesicles. These small, nanometer-sized particles are responsible for the exchange of small and large molecules between cells and tissues, and thus affect our body in many ways. In the past years, researchers have asked many fundamental and applied questions to better understand this process. And because many of these questions in this exciting field of research are still open, we decided to publish a new journal - *Trillium Extracellular Vesicles*. As the editor-in-chief of this magazine, I am delighted to introduce you to the topic of extracellular vesicles (EVs).

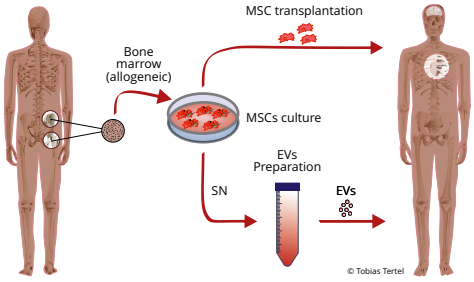
We are the German Society for Extracellular Vesicles (GSEV), a national society founded in 2017 which aims at fostering EV research in Germany and internationally. In this first issue of Trillium Extracellular Vesicles, we have compiled an overview on current EV research. The review articles are ranging from a broad introduction and an overview on current isolation and characterization methods, to the fundamental role of EVs in tumour and developmental biology, the application of EVs as therapeutic tools, and their analyses using advanced flow cytometry methods, and finally the EV role in trans-kingdom communication. In addition, we are providing an overview on GSEV and other national EV societies to further foster transnational collaboration.

This journal brings together several authors from science and clinics, from junior scientists to established researchers, from biologists to physicians. It illustrates very well how diverse the field of EVs is. Our goal is to inspire natural scientists, medical doctors and laboratory physicians for the new world of EVs. New ideas, new projects, new collaborations are to be created and will enable all of us to better understand EVs.

I hope you enjoy reading this journal as much as I enjoyed putting it together. Special thanks go to all authors who have contributed and made the journal to what you are holding in your hands - a vivid introduction to the EV field. I am grateful to all reviewers and members of the Advisory Board, who have helped me with the selection of authors and the revision of all articles. Warm thanks go to Tobias Tertel and Michel Bremer (Essen), who did a perfect job at designing all figures including our first front cover. Finally yet importantly, I am indebted to the Trillium Publisher team, without whom it would have been impossible to put this journal on its feet.

I wish everyone an exciting read and hope to see you again at an upcoming EV symposium!

Gregor Fuhrmann
Editor-in-chief



Extracellular vesicles – a novel exciting research topic

Extracellular vesicles (EVs) appear to be essential parts of an ancient intercellular communication system we just become aware of. According to our current thinking, cells can assemble lipids, proteins and nucleic acids in specific combinations to nano-sized vesicles being released into their extracellular environment. At least, a proportion of such EVs seems to send complex information to neighboring cells as well as to cells in distant tissues. Although the principle may appear new for many of the readers initially, we are pretty aware of cell-derived compositions of lipids, proteins and nucleic acids, which have severe impacts on cells, organisms and even human societies, namely viruses.

Upon studying EVs, we have learned that their share many features with viruses. In this context, it has been considered that at least some of the viruses, e.g. lentiviruses, may represent malignant EVs. Indeed, if EVs represent parts of an ancient communication system, it appears very likely that they have been captured by pathogens. Thinking in this direction it may explain why EV researchers are so much excited about the new research field. If we understand how intercellular signaling mediating EVs are assembled and how they transmit their information to selected target cells, as viruses for example do, EVs may become a very powerful tool in different branches of life sciences.

We obtained evidence that EVs are essentially required to control cell communication in physiological and pathophysiological conditions. As EVs seem to be assembled in cell type specific manners, they provide very promising biomarkers for a variety of diseases. In case we understand their target cell selection mechanism, and become able to alter the information they transmit in a controlled manner, or load EVs with active substances, they would be ideal drug delivery vehicles. If we unravel how EVs influence biological processes, we may use EVs to counteract pathophysiological processes and apply them as therapeutic agents. They might also serve as a novel class of vaccines, or get important tools for many other applications. I hope that this special issue will provide a comprehensive overview over the field of EVs and that it transports some of the enthusiasm that we share within the EV community.

Despite our passion, we need to admit that we are just at the beginning of a very promising research area. There are many hurdles, which we have to deal with. Although we have learned to work with viruses, it is challenging to investigate non-pathologic, biological nanoparticles. They are too small to be detected by light microscopy and so we need special platforms to analyze them in detail. Originally, exosomes, a special class of EVs, were discovered by electron microscopy in 1983. Later on, crude preparation technologies were used to enrich them and to study their biological properties and functions, e.g. differential centrifugation protocols, which in an ultracentrifugation step allow to precipitate small EVs of sizes between 70-150 nm. Many groups have characterized and still characterize the content of ultracentrifuge-pelleted material which for sure contains EVs, but also a huge collection of non-EV associated byproducts.

With the development of new analysis tools and gaining more and more knowledge in the field, we have to learn that many of the previous methods are not specific for EVs and co-prepare or co-analyze nanoparticles of comparable sizes which lack any EV characteristics. Accordingly, and despite the fact that there is an exponentially increasing amount of publications about EVs, the field mainly progresses in recognizing its own limitations and by solving various technical challenges.

Since these challenges are not as effectively reported as strong effects, and since many negative findings remain unpublished, it is hard for interested persons to recognize the true current state-of-the-art in the field. To address these issues, EV societies have been formed in

which experts discuss the progress and challenges in the field and try to summarize it in several position papers, many of them being published in the *Journal for Extracellular Vesicles* (JEV), which is released by the *International Society of Extracellular Vesicles* (ISEV) founded in 2012. One of the recent papers is the report of the *Minimal information for studies of extracellular vesicles 2018* (MISEV2018) which is written by almost 400 authors and comprises more than 40 pages of condensed information.

Thinking that it is of uppermost importance to communicate the current state of the art and understanding that getting an appropriate overview over the field is challenging itself, and many researchers approaching the EV field may not be able to participate on international EV meetings, we have founded the *German Society for Extracellular Vesicles* (GSEV). The GSEV is intended to provide a national platform to support scientists working in Germany to step into the field and provide appropriate discussion platforms for novel as well as experienced EV researcher. For now, the GSEV organizes two meetings a year:

- our Annual Meeting in spring, a shared meeting with the Interdisziplinäre Gruppe für Labor und Durchflusszytometrie (IGLD), Instand, the German Stem Cell Network (GSCN) and the Dachverband für Technologen/-innen und Analytiker/-innen in der Medizin Deutschland (DVA).
- our Autumn Meeting co-organized with other national EV societies from Europe, such as the Austrian Society of Extracellular Vesicles (ASEV) and the UK Society for Extracellular Vesicles

At both meetings, we team-up with specialists in clinical diagnostics, hematologists, transfusion medicals and therapists, to provide a unique platform to promote translational EV research. In addition, EV-related workshops are organized with industry experts in the EV field.

To provide an overview over the field, we have decided to publish and distribute this collection of some aspects and research topics within the EV field, highlighting both, many promising themes but also challenges in the field. We hope that this special EV issue attracts further researchers to actively approach the EV field and provides an interesting reading for anyone who is curious in this exciting field of life sciences.

Bernd Giebel
On behalf of the board of the
German Society for Extracellular Vesicles

German Society for Extracellular Vesicles (GSEV)

Foundation year

2017

Mission

The German Society for Extracellular Vesicles is the national network for scientists and professionals working in the field of extracellular vesicles (EVs). We are promoting EV research on all levels from basic research to application. Our goal is to bring together EV-researchers from Germany and abroad to create a hub for ideas and collaborations, and to support young academics within the field.

We are organising Annual Meetings, which are usually held in spring, and our Autumn Meetings that are often co-organised with other European EV Societies. Through international mobility fellowships GSEV is fostering research collaboration and exchange of ideas with other societies, such as GEIVEX and UKEV. With our working groups on research standards, young scientists and outreach we aim at targeting current challenges in EV-research and enhance scientific visibility.

GSEV
German Society for Extracellular Vesicles

In 2018, we launched an online forum for the scientific exchange of EV researchers across labs to facilitate interactions and encourage discussions about EV research (gsev.proboards.com)

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Twitter: @GSEV10

Austrian Society for Extracellular Vesicles (ASEV)

Foundation year

2016

Mission Statement

The ASEV was founded as a platform for extracellular vesicle research in Austria. The society aims to support and foster basic research as well as translational activities in the field of EVs.

Activities

- The ASEV organizes regular trainings and workshops on established methods for EV isolation and characterization. Our society supports the introduction of novel methods and devices in collaboration with companies in the field.
- The ASEV aims to contribute to method standardization, to provide methodological suggestions, and to support the development of new guidelines where required.
- The ASEV interacts with other national and international scientific societies in the field.

Austrian Society for Extracellular Vesicles
www.asev.at

Regular Meetings

The ASEV organizes an annual meeting open for national and international speakers and participants. On a regular basis, meetings are co-organized with other national and international societies in the field to spread and discuss new findings.

Contact

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French Society of Extracellular Vesicles (FSEV)

Foundation year

2018

Mission

The French Society of Extracellular Vesicles (FSEV) supports all research activities related to the study, characterization, understanding of physiological or pathological roles as well as the therapeutic or non-therapeutic use of extracellular vesicles (EVs). We cover various areas such as cell biology, cancer, virology and regenerative medicine. We involve all living species and their derivatives and include all aspects of vectorization and delivery using advanced EV technologies, imaging and engineering.
FSEV aims to promote and stimulate the progress and dissemination of knowledge in the field of EVs. We hold an annual FSEV symposium, except the year when the ISEV congress is in Europe. We cooperate with other initiatives, such as EMBO workshops or summer schools. FSEV members receive a monthly newsletter with meeting highlights and we communicate through social networks. To support young researchers, FSEV gives awards for oral and poster communications at the national FSEV congress, and will in the near future offer travel grants for international EV events.

FSEV SOCIÉTÉ FRANÇAISE DES VESICULES EXTRACELLULAIRES

Contact

www.fsev.fr
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Grupo Español de investigacion e innovacion en Vesiculas Extracelulares (GEIVEX)

Foundation year

2013

Mission

To promote research and innovation on extracellular vesicles to advance personalized medicine aiming to develop new tools and biomarkers with high social impact that will generate benefits and equity in global health

We promote research and innovation on extracellular vesicles through:

- Bi-annual international conference on EVs
- Practical hands-on workshops
- GEIVEX motility fellowships (Scientific visits of GEIVEX students for 1-3 months in GEIVEX labs)
- GEIVEX international mobility fellowships (scientific visits of one month between members of GEIVEX, UKEV and GSEV Societies)
- Online course in Spanish on EVs
- Promoting GEIVEX participation in other scientific conferences and meetings from other societies

Grupo Español de Investigación en Vesículas Extracelulares GEIVEX

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Società Italiana per le Vesicole Extracellulari (EVIta)

Foundation year

2018

Mission

EVIta promotes basic, clinical and translational research and the activation of an interactive network among Italian researchers in the field of extracellular vesicles. EVIta aims to be a point of reference for the exchange of ideas, information and initiatives in this area.

EVIta was established in Turin, Italy thanks to the support of more than 50 founding members who represent the Italian excellence in the study of extracellular vesicles. Our EVIta symposia bring together Italian researchers in the study of extracellular vesicles in a wide diversity of fields including medicine, biochemistry, physics, veterinary medicine, pharmacology. EVIta provides a wide platform for interactions between research and society. We are setting-up different work-groups, territorial sections and promoting standardization, exchange of idea, workshops in the field of EV research.

Contact

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The Netherlands Society for Extracellular Vesicles (NLSEV)

Foundation year

2018

Mission

The Netherlands Society for Extracellular Vesicles (NLSEV) aims to connect researchers, clinicians, and industries who are active or interested in extracellular vesicles. NLSEV organizes annual one-day scientific meetings, which have until now been attended by about 130 people (junior and senior researchers, technicians, clinicians and sponsors). During these events, highlights in Dutch EV research are presented in selected talks and during poster/networking sessions. Moreover, one or two internationally renowned keynote speakers are invited to these meetings.
We inform the Dutch EV community about upcoming activities of NLSEV, and international conferences related to the EV research field. Our career center page is used to advertise and search for vacancies. Our website also hosts the 'EV Delta' initiative, which is a webpage providing an overview of research performed in Dutch EV labs. The goal of EV Delta is to increase visibility of the different EV labs, to provide insight into the local EV research infrastructure, and thereby stimulate collaboration.

Contact

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The UK Society for Extracellular Vesicles (UKEV)

Foundation year

2013

Mission

UKEV began as an informal network of academics that were working to promote EV research and organising an annual meeting, and was formalised into an official society in 2018. The aim of UKEV is to help facilitate EV research across the UK and beyond. To this end, our goals are to foster collaborations between EV researchers, raise the profile of EV research, provide a focal point for the EV community through scientific conferences such as the annual UKEV Forum, coordinate with other national and international EV societies, and to help share information about EVs
The annual UKEV meeting usually takes place in the second week of December. We also ran our first EV Summer School in July 2019, which was well attended and well received. We hope to host similar training events in future.

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Extracellular vesicles as gold mine for new diagnostic and therapeutic approaches in medicine

Stefan Holdenrieder

Extracellular vesicles (EVs) are a heterogeneous group of subcellular particles shed from cells of origin by diverse mechanisms. They carry specific information and are responsible for efficient intercellular communication that is highly important in many physiological processes as well as for the pathogenesis and progression of several diseases. Their unique properties offer the opportunity to use them also for the delivery of therapeutic drugs. When released into the blood or other bodily fluids they serve as sensitive liquid profiling biomarkers in many dispositions. For future use in diagnostic settings, further efforts are required for better standardization of the methods as well as the analytical, pre-analytical and clinical validation of the markers.

Keywords: *extracellular vesicles, biomarker, liquid biopsy, disease detection, prognosis, pre-analytics, analytical requirements*

From cells to extracellular vesicles

Cellular diagnostics have been the gold standard for diagnostics of many diseases in laboratory medicine and pathology for many years. Absolute and relative count of blood cells and qualitative features like size, morphology, biochemical and genetic characteristics have been and are still the basis for the diagnosis of a vast variety of diseases ranging from anemia, infections, sepsis, leukemia and lymphoma to coagulation disorders. Similarly, histopathological tissue diagnostics in pathology relies on morphological and molecular biological changes of cells in their organ compounds.

In contrast, subcellular particles have been considered as cellular waste. Likewise non-coding regions in the genome were greatly underestimated until the Encode Project revealed that this abundant genetic material is essential for regulation and fine-tuning of genomic transcription, silencing and activation of genomic regions and repair processes [1]. With the advent of new highly sensitive and high-resolution technologies, research interest in these subcellular particles has increased tremendously and has provided fascinating insights into their structure, biology, cellular release and function. Far beyond „just waste“, their key role for the cell-to-cell communication, the maintenance of the physiological balance within the organism and adaptation to external challenges as well as for the pathogenesis and progression of a plenitude of diseases has been recognized [2].

Heterogeneous group of EVs

Extracellular vesicles (EVs) are considered as particles with a lipid bilayer membrane naturally released from cells [3]. They comprise a wide spectrum of heterogeneous subcellular vesicles and vary greatly in size, density, surface composition, biochemical content, mechanism of formation, release from cells and biological functions that can be used for further sub-classification (Figure 1) [3,4].


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In general, EVs range from 50 to more than 1000 nm in size. Small EVs such as exosomes, which range in size between 50 and 150 nm, originate from a special mechanism of formation in the endosomal system, intracellular maturation within multivesicular bodies and secondary release from cells. In contrast, direct budding from the plasma membrane lead to shedding of larger microvesicles of 100-1000 nm size. In addition, other EVs such as ectosomes, apoptotic bodies and oncosomes are released by specific cell types or dying cells [2–4].

EVs are released in different amounts from healthy, stressed, injured and malignant cells, from platelets, stem cells, cells of the immune system and many more [3,5–7]. They differ in their membrane composition in which transmembrane tetraspanins, proteins, lipid rafts, sugars, nucleic acids, MHC complexes, integrins and other cell type specific markers are incorporated [2,3]. These allow them to exert intercellular contacts with neighboring cells in a paracrine way or even targeted interaction with distant cells after release into the blood stream or other bodily fluids in an endocrine way [3,5,7].

EVs carry different types of cargo (Figure 2). Large microvesicles that are shed from the plasma membrane contain molecules that derive from cytosol, mitochondria, golgi apparatus, endoplasmatic reticulum or even organelles themselves. The mechanisms of specific microvesicle loading are under investigation and it has been shown that certain RNA-binding proteins may traffic into MVs leading to a more specific loading with miRNAs [8]. Endosomal exosomes have a specific

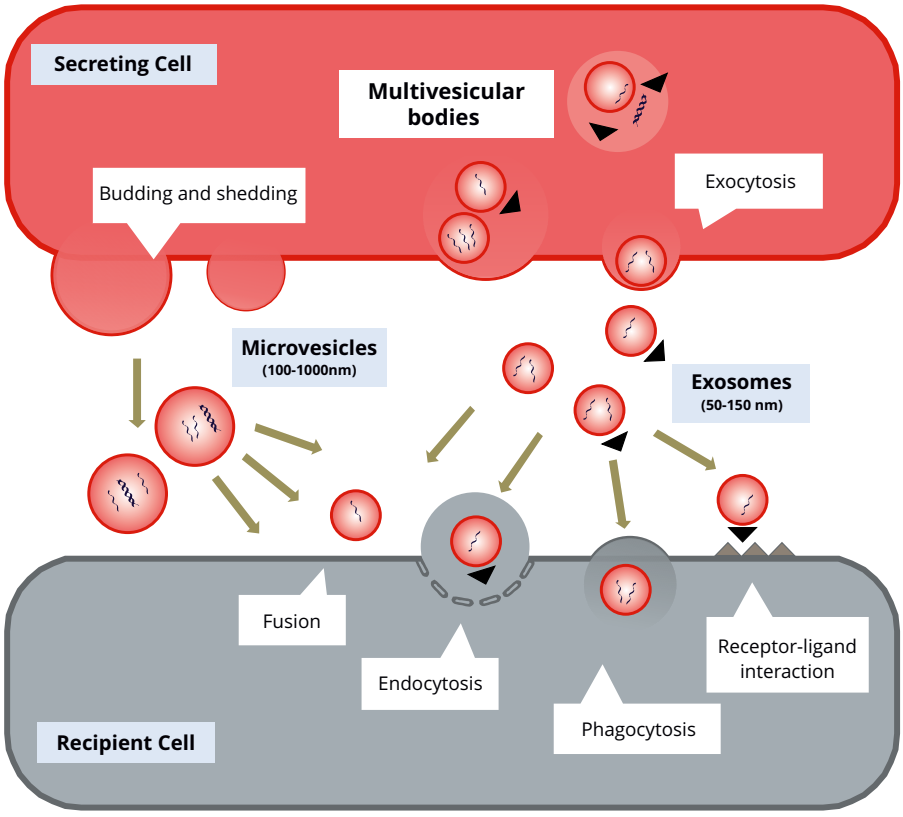

Figure 1: *Biogenesis of different subtypes of extracellular vesicles and their potential interaction mechanisms with target cells (see text for details).*

cargo mechanism that regulates the specific loading of proteins, lipids, enzymes, DNA, diverse forms of RNA (rRNA, tRNA, mRNA, miRNA and other non-coding RNA), that is dependent (or independent) from endosomal sorting complexes required for transport (ESCRTs) or accessory proteins [2,3,9].

A multitude of EV functions and potential therapeutic applications

After specific binding to the surface of the target cell, endocytosis or fusion with the plasma membrane, this cargo may be released in the cytosol of these cells and modulate there the metabolism, immune or other functions [2,3,5,7]. The exact mechanism how this information is recognized and integrated into the cellular processes has to be further elucidated and understood. Research in recent years has shown that exosomes have excellent performance characteristics as carriers of endogenously or artificially loaded nucleic acids as they are less immunogenic and have a high efficiency when compared to other carriers such as liposomes and nanoparticles [10–12].

In addition, there is an exponential interest in cytoplasmic DNA receptors like cGAS-STING and RNA-receptors like RIG-I, their downstream pathways and links with inflammasome activation, cell death induction and stimulation of exosome release with antitumor activity [13–15]. This opens the avenue for therapeutic use of exosomes as vaccines for infectious diseases or even for targeted delivery of nucleic acid drugs in cancer

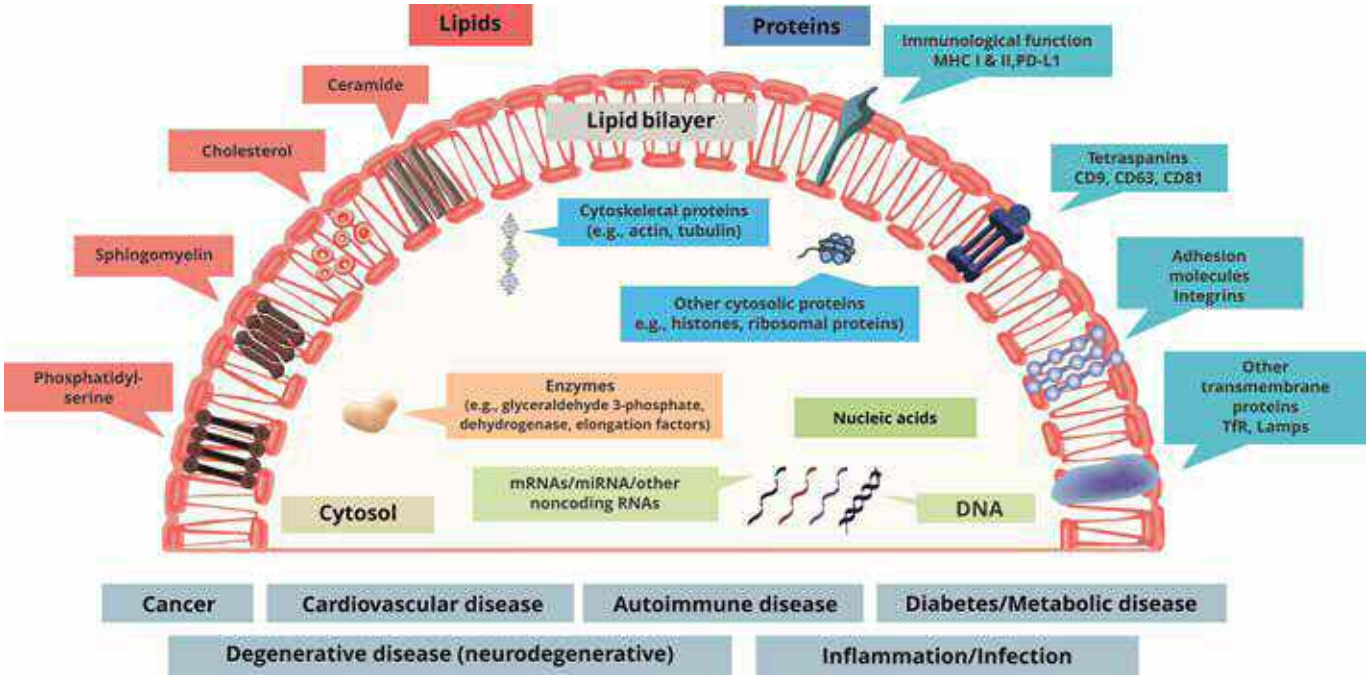

Figure 2: *General composition of extracellular vesicles (EVs) with various types of lipids and membrane proteins. In addition, EVs may carry several types of cargos ranging from nucleic acids to enzymes and other proteins. EVs were shown to be involved in many physiological processes as well as in pathogenesis and progression of different diseases.*

disease or for tissue regeneration in the heart after stroke [5,11,12,14].

Functional studies have shown that EVs are important mediators of pathogenesis and progression of many diseases. In cancer diseases they are able to transfer malignant information to adjacent and healthy cells leading to transformation, progression, invasion and metastasis of the tumor [7,16]. Thereby, processes like promotion of angiogenesis, downregulation and escape from immune response as well as preparation of a metastatic niche are essential steps for tumor expansion. On the other hand, anti-tumor immune activation may also be mediated by EVs [7,17].

In cardiovascular disease, extracellular vesicles play a role in the crosstalk of various cell types like cardiac muscle cells, fibroblasts and endothelial cells. After hypoxic stress and inflammation they are loaded with different pools of protein or RNA, prevent cardiac damages by attenuating apoptosis and promote cardiac repair. In other conditions, cardiomyocyte exosomes promote proliferation and activation of cardiac fibroblasts leading to heart fibrosis and chronic heart failure. After myocardial infarction, exosomes from prestimulated cardiac progenitor cells showed therapeutic potential as they increased cardiac function and reduced infarct size [5].

Also in other acute disease such as stroke and sepsis as well as in aging and degenerative diseases like Alzheimer's and Parkinson's, EVs have shown to play a role in limiting hypoxic damage, fighting infection or promoting chronic disease progression.

Plenty efforts are currently being made to optimize stem cell-derived exosomes for therapeutic use like in the ischemic brain [18,19]. A more detailed overview on EVs derived from stem cells and their therapeutic use is given in article 5 of this issue.

Challenges in EV detection and characterization

As EVs are heterogeneous and much smaller than cells it is quite challenging to detect, isolate and characterize them sensitively and specifically from bodily fluids, tissues or in functional cell culture studies. Nowadays there are plenty of methodical approaches for isolation, enrichment, quantification and EV- and cell-specific classification. All of them have their strengths and limitations as it is discussed in article 2 of this issue. It is always a trade-off between EV yield and purity that can be obtained and results of studies are strictly dependent on the methods used [3,20,21].

Despite the tremendous growth in publication numbers in the EV field in

the recent decade [22], there is still a considerable lack of standardization, data comparability and reproducibility that impairs the better understanding of the biology and function of EVs as well as the translation of results from basic research into patient care [3,20,21]. International and national societies such as the International and the German Society of Extracellular Vesicles (ISEV and GSEV) have been founded to foster the interaction of scientists, the exchange of research experience, and the development of guidelines for the standardized research on EVs in basic and applied science.

One major effort of the international community is the initiative EV Transparent Reporting and Centralizing Knowledgebase (EV-TRACK) that offers a platform for comprehensive, detailed and transparent documentation of EV research and provides an EV-METRIC for adherence to the recommendations [23]. Recently updated ISEV-guidelines on Minimal Information for Studies of EVs (MISEV) [3] is a consensus-based position statement that recommends the appropriate use and precise documentation for methods of EV isolation (separation), enrichment (concentration), quantification and structural and functional characterization. In addition, it clarifies the validation of new EV-associated components and gives assistance for the correct performance of functional studies [3].

As it is difficult to reconstruct the biogenesis of already released EVs they have to be described and classified by size, density, biochemical composition, description of conditions or cells of origin. Therefore, a minimum number of transmembrane/lipid-bound and cytosolic proteins as well as negative protein markers have to be determined to clearly identify EV subclasses and to exclude contamination by exomeres, lipoproteins or other large and abundant proteins which do not exhibit the EV typical lipid bilayer membrane. For functional studies, the topology of a marker outside (secreted) or inside (luminal) the EVs has to be investigated, too. The use of multiple technologies, standards and appropriate controls will strengthen the findings [3].

EVs as biomarkers in various diseases

Beyond pure quantification, specific EV surface markers and the protein, RNA or miRNA pattern of their cargo provide a rich source of information about the cells of origin and EV function [2,5,7,9,21]. If they are released into body fluids such as blood plasma, urine, cerebrospinal fluid, effusions, saliva *etc* they may serve as sensitive new disease biomarkers as these EV markers are enriched in this specific compartment and reflect the biochemical status of the cells they derive from even if they are far distant.

As in many disease states, thousands of EVs are released from a single cell and they are abundantly found in tissue samples. Thereby, EVs serve an as ideal liquid profiling / liquid biopsy biomarker class outperforming other candidates such as circulating tumor cells (CTCs) or circulating tumor DNA (ctDNA) in terms of sensitivity because they are more rarely detected in patients' blood. In addition, differentiated analysis of EVs can possibly overcome the difficulties in detecting the heterogeneity of molecular composition and function of cancer cells [7,9,16]. Finally, EVs provide information not only of the diseased cells themselves but also of the response of the host toward these pathologic conditions, e.g. by differentiating of immune suppressive cancer EVs and immune activating EVs of healthy cells [17]. Similarly, complex interactions like in thrombogenesis may be mirrored by EVs deriving from endothelial, immune, cardiac cells and platelets. Therefore, comprehensive analysis of EV surface markers or complete protein and RNA profile by omics technologies will provide important insights [5,6,9,21].

In cancer diseases, EV levels are considerably increased and can be identified by their surface markers, protein, miRNA and lncRNA patterns. They may be used for diagnosis, estimation of prognosis and disease monitoring as they correlate with therapy response (reviewed in [7,16]). In addition, exosomal PD-L1 was described to contribute to immunosuppression and to be associated with anti-PD-1 response [24].

Likewise in cardiovascular diseases, cardio-specific miRNAs have been found in plasma EVs e.g. after acute myocardial infarction [5]. However, analysis is laborious and time consuming when compared to modern high-sensitive troponin assays limiting its diagnostic value. However, EV-markers may be relevant for estimating prognosis and elucidating the interplay of different cell types.

In sepsis and septic shock, miRNAs are described to be differentially regulated in diverse compartments such as blood cells, serum and exosomes. While

plenty of investigated miRNAs were down- and up-regulated, several of them were only changed in serum and/or exosomes [25]. This indicates that for estimation of diagnosis or prognosis the complex regulative network of miRNA markers in diverse compartments will have to be taken into account.

Remarkably, EVs are also released into the circulatory system in response to physiological changes, such as incremental cycling exercises. Highest EV marker levels were observed at maximum exhaustion with different subtypes deriving from platelets, endothelial cells and leukocytes contributing to the exercise-associated adaptive systemic signaling [26].

While many EV biomarker studies show promising results, many more studies are needed to explore the full potential of the wealth of biomarker candidates in this new compartment for relevant clinical questions.

Analytical requirements for the use of EVs as diagnostic biomarkers

Adherence to guidelines and appropriate documentation is only a very first step to develop robust and reliable biomarkers. As many laboratories are accredited according to ISO 15189 [20] or work according to similar national regulations such as the guideline of the "Bundesärztekammer" (RiliBÄK) in Germany [27], there are strict requirements for the implementation of new markers and methods that are to be used for patient care. By 2022, the new EU In-Vitro Diagnostic Medical Device Regulation (IVDR) will set the stage for medical laboratories on a European level.

All of them require the comprehensive validation of new methods used in medical diagnostics by either manufacturers of a commercial product (followed by a verification by the user) or the medical laboratory itself if the method is an in-house development. This includes high-quality standardization of the methods used, followed by detailed analytical, pre-analytical and clinical validation [20,27].

Analytical evaluation comprises the analytical sensitivity and specificity, accuracy, precision, linearity, measuring range, sample stability and diversity and the uncertainty of measurement [20]. Although this work is less enticing than basic research, it is fundamental for later accurate, reliable and valid use of the methods in patient care and correct interpretation of the results. Particularly the establishment of reference material and implementation of external quality assessment (EQA) schemes – as currently done by certain labs – is essential for quality assurance over methods, users and laboratories [28].

Pre-analytical evaluation considers on the one hand the status of the blood donor like age, sex, body-mass index, pre/post-prandial status, exercise level, time of day of collection, current diseases, medication etc. [3,20]. On the other hand, it takes into account all features that affect the sample from blood drawing until later analysis that could affect the measured marker levels, e.g. type of material, container, volume, anticoagulant, time to processing, mode of transport, temperature, exact centrifugation protocols, depletion of platelets and lipoproteins, short- and long-term storage conditions, deep-freezing, thawing, etc. [3,20,29]. Ideally, additional measures to quantify interfering factors such as hemolysis (as in the HIL index), platelet or neutrophil activation that may lead to additional in-vitro release of exosomes as well as serial venipunctures in individuals to test the biological variation are recommended. In the future, exosome-preserving tubes (as already available for cfDNA and RNA) and high-quality standards and documentation of pre-analytics (e.g. by SPREC codes) provided by hospital-integrated biobanks may facilitate the pre-analytic procedures. Although cumbersome, all these issues are pivotal for any downstream exosome analysis.

Clinical requirements for the use of EVs as diagnostic biomarkers

Clinical evaluation finally verifies the ability of a method to answer clinical questions, such as (i) the discrimination of diseased from non-diseased persons (diagnosis), (ii) the estimation of the outcome of a diseased person or the risk of disease recurrence (prognosis), (iii) the response to a specific treatment (prediction), (iv) or the serial monitoring of a disease course for early detection of therapy response. For all indications, the methods have to fulfil meaningful user-required specifications [30].

For diagnostic purposes, clinical sensitivity indicates the proportion of correctly identified positive results, while specificity describes the proportion of correctly identified negative results. The overall diagnostic performance is best shown by the complete profile of sensitivity and specificity using receiver operating characteristic (ROC) curves that are able to identify

clinically relevant cutoff values to answer clinical questions with an acceptable error rate (e.g. at 95% specificity). However, for clinicians the positive (PPV) and negative predictive values (NPV) may be even more informative as they indicate the probability of disease if the value is positive (PPV) or the absence of disease if the value is negative (NPV). Although these items seem to be sophisticated, they are paramount for the interpretation of any application in patient care. Accordingly, studies with large number of patients and controls in defined conditions have to be performed followed by validation studies in an independent set of samples from another patient cohort [29,30].

Acceptance of EVs as clinical biomarkers

With all analytical, pre-analytical and clinical requirements met, it should not be forgotten that there are some essential points that are extremely relevant for the acceptance in the clinical routine application (Table 1). Among others, markers and methods have to:

- answer clinical questions precisely and improve the workflow of the clinicians
- be very robust (low pre-analytic and biological variability), reliable and valid
- be quantifiable and highly quality controlled (internal and external systems)
- be fast and easy to perform (depending on the information and the clinical need)
- be inexpensive and reimbursable

For future wide spread applications they should, in addition,

- be flexible (for adaptation of new parameters)
- be scalable (for high throughput automation)
- be portable (for point-of care diagnostics)
- be miniaturizable (for mobile and smart devices)

Conclusion

It is obvious that EVs open a wide avenue for future research in cell biology, function and intercellular communication in health and disease. In addition, their properties are ideal for the use of EVs for drug delivery and vaccination. Moreover, EVs provide a fascinating platform for future liquid profiling diagnostics in the blood and bodily fluids that could revolutionize our understanding of both disease and host response. Even if some way has to be made before EVs reach patient care, it is now the time of exploring the EV goldmine right ahead of us.

Table 1:
Requirements to develop EVs as clinical biomarkers

1. Analytical performance
Standardization of methods, calibration, analytical sensitivity and specificity, accuracy, precision, linearity, measuring range, quality controls
2. Preanalytical performance
Biological variability within the donor, standardized blood collection and sample handling, materials, tubes, stability against influencing factors (temperature, time etc.)
3. Clinical performance
Clinically meaningful EV markers, reference ranges, high clinical sensitivity and specificity, PPV and NPV, interpretable individual changes, dynamics
4. Routine suitablility
Clinician: fast, reliable, inexpensive; Laboratory: QC, easy/simple, robust
5. Future options
Flexible, portable, scalable

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Isolation and characterization of extracellular vesicles

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Research into extracellular vesicles (EVs) gained significant traction in the past decade and EVs have been investigated in a wide variety of studies ranging from basic biology to diagnostic and therapeutic applications. Since EVs are secreted by most, if not all, eukaryotic and prokaryotic cells, they have been detected in body fluids as diverse as blood, urine and saliva as well as in cell culture media. In this chapter, we will provide an overview of EV isolation and characterization strategies and highlight their advantages and disadvantages.

Keywords: *extracellular vesicle isolation, purification, characterization, exosome*

EV isolation: finding the needle in the haystack

The fundamental goal of a successful isolation method entails both the efficient enrichment of EVs from a given biofluid and the depletion of non-EV material, yielding preparations of high purity without significant loss of EVs (Figure 1). To this end, some approaches utilize principles that tackle general EV characteristics including size, density or charge, while others hone in on more specific attributes such as marker protein expression. Although enriching EVs itself is not trivial, separating them from contaminants such as protein complexes, aggregates and lipoproteins, which oftentimes share biophysical properties with EVs, is still a major challenge in the field. Each biofluid presents its own difficulties, ranging from high protein content (e.g. milk) to low EV concentration (e.g. urine) or lipoproteins that outnumber EVs by an order of magnitude (e.g. plasma).

Sedimentation-based methods

The most conventional method of EV isolation, which is still considered to be the gold standard by many, is differential ultracentrifugation (dUC) [1]. During multiple rounds of centrifugation with increasing speed, biofluids are initially depleted of large components before pelleting EVs at speeds of at least 100,000 g. Given sufficient run time, dUC is an effective enrichment method, but the resulting pellet will invariably contain non-EV contaminants that co-sediment alongside EVs and centrifugal force can lead to EV aggregation and damage.

Since EV preparations derived from sedimentation oftentimes do not display adequate purity for downstream assays, dUC can be combined with density gradient centrifugation (DGC). In this approach, the EV-containing ultracentrifugation pellet is resuspended and further fractionated by floatation into a density gradient. During centrifugation of the gradient, which is commonly prepared using sucrose or iodixanol, EVs will migrate until reaching a position where their density matches that of the density matrix. Individual density fractions are collected, concentrated by ultracentrifugation or filtration and utilized for downstream experiments. While there are various DGC protocols, including continuous or stepped gradients as well as top-down and bottom-up floatation, the underlying principle of density-based fractionation is identical. Even though

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the combination of dUC with DGC provides orthogonal separation based on sedimentation and density, it does not achieve absolute EV purity, since densities of common contaminants such as high-density lipoproteins overlap with those of EVs [2].

Filtration-based methods

Filtration-based isolation primarily depends on size and molecular weight differences between EVs, proteins and other components present in biofluids. Ultrafiltration is a size-based technique that utilizes membrane filters with a defined molecular weight or size exclusion limit to separate EVs from biofluids and potential contaminants. Transmembrane pressures that force the suspension through the membrane filters can be generated by pumps or centrifugation. Sequential filtration is often performed with filters that decrease in their pore size to first eliminate larger structures and then smaller compounds from the biofluid before EVs will be captured. Tangential flow filtration (TFF) has emerged to retain and thus concentrate EVs by concurrently decreasing the sample volume. In contrast to traditional ultrafiltration, TFF enables tangential fluid flow across the surface of a hollow fiber membrane, which prevents clogging of filters. Another common size-based separation technique is size-exclusion chromatography (SEC), also known as gel filtration. In SEC, EV samples are loaded on a column prefilled with a porous stationary phase (e.g. sepharose). Small particles can enter the pores of the stationary phase, leading to increased retention time and delayed elution compared to larger particles that are excluded from entering the pores. Using specialized columns, this method allows the separation of EV size-defined subtypes as individual populations.

Affinity-based methods

Continued advances in characterizing the molecular composition of EVs also opened new avenues for their isolation. Once specific proteins, lipids or carbohydrates on the EV surface are identified, they can be targeted by appropriate capturing receptors immobilized on beads or membranes. During this affinity-based isolation, EVs are purified by selective receptor-ligand interaction, potentially resulting in high specificity and purity.

Table 1: Comparative overview of commonly used EV isolation methods.

Method	Principle	Advantages	Disadvantages
dUC	Sequential separation of EVs and other biofluid components based on their sedimentation rate and density	High EV yield, established protocols, medium to large sample volumes, scalable	Time-intensive, high equipment cost, low portability, low purity, limited parallel sample processing, potential EV damage
DGC		Improved purity compared to dUC, lends itself to combination with other method	Labor- and time-intensive, low yield, low throughput
Precipitation	Solubility of EVs is altered by water-excluding polymers or manipulation of EV surface charge	Quick and simple, large sample volumes, high yield, small to large sample volumes, low cost, scalable, parallel sample processing	Very unspecific, co-precipitation of non-EV contaminants, precipitation reagent needs to be removed (for some applications)
Affinity	Interaction between specific EV surface molecules and capture antibodies	High purity, enables subtyping of EV populations, parallel sample processing, commercial kits and self-made protocols	Biased for specific populations, harsh or no elution, high reagent cost, limited scalability, small sample volumes
(Ultra)filtration	EV isolation is exclusively based on the size differences between EVs and other particulate constituents	Quick and simple, portable, low to moderate costs, small to large sample volumes, parallel sample processing, lends itself to combination with other method	EV loss on membrane, potential clogging and EV trapping, shear stress
SEC		Moderate to high purity, low physical forces preserve EV integrity, availability of commercial kits and self-made devices	Low to moderate equipment cost, small to moderate sample volumes, fractions might be sample-dependent
Microfluidics	Microscale isolation based on a variety of EV properties including immunoaffinity, size, and density	Isolation from minimal volumes, modifiable, portable, fast, easy automation and integration	Lack of standardization and validation, low to moderate throughput, expensive

DGC: density gradient centrifugation; dUC: differential ultracentrifugation; SEC: size-exclusion chromatography

Table 2: Comparative overview of commonly used EV characterization methods.

Analyte	Method	Advantages	Disadvantages
Particle	DLS	Analysis under physiological conditions	Limited specificity, low resolution of polydisperse samples
	NTA	Improved size resolution, analysis of EV charge	Limited specificity, struggles with fluorescence
	TEM	Visualizes EV morphology and potential contaminants	Dedicated equipment, toxic chemicals, morphological artifacts due to sample preparation
	Cryo-EM	No artifacts due to fixation and dehydration,native morphology, stain-free visualization	Dedicated equipment, freezing artifacts, low signal-to-noise ratio, cumbersome sample preparation and handling
	Immuno-EM	Visualizes EV morphology and specific proteins	Dedicated equipment, cumbersome sample preparation, toxic chemicals, reliance on high-quality antibodies
	AFM	Works in air, vacuum, and liquids, 3D imaging, convenient sample preparation	Dedicated equipment, data tip-dependent, tip can damage the sample
	FCM	Rapid assessment of sizes, counts and cluster identification, potential for single-EV analysis, multiparametric, sorting can be implemented	Limited sensitivity and resolution, bead-based approaches are only semi-quantitative, expensive equipment and specialized operators
Protein	WB	Low cost, established protocols, simple readout	Low throughput,reliance on specific antibodies, lack of widely applicable EV protein loading controls
	Mass spectrometry	High sensitivity and resolution, quantitative methods are available, identification of PTMs, simultaneous detection of multiple proteins, adjustable to various research questions	Time-consuming, expensive instrumentation, requires very clean sample preparation, high risk of contamination, complex data analysis, sample and setting-dependent limitations
	ELISA	Established protocols, availability of commercial kits and self-made protocols, direct and indirect detection, simple readout	Immunoreactivity is antibody-dependent, cross-reactivity
Nucleic acids	(RT)-(q)PCR dPCR	High sensitivity, relatively cheap, simple workflows, established protocols	A priori knowledge is required, expensive (dPCR), lack of widely applicable EV reference sequences
	NGS	High throughput, *de novo* detection of novel transcripts, high resolution, detection of sequence variants	Expensive, laborious sample preparation, dedicated equipment, complex data analysis
	Northern/ Southern blot	High specificity, identification of RNA/DNA sequence lengths and alternative splicing products	Lower sensitivity compared to newer methods (e.g. PCR), requires large amounts of sample, limited number of sequences can be analyzed in parallel, time-consuming, toxic chemicals
	Microarray	Established protocols and analysis pipelines, simultaneous analysis of multiple genes	Restricted to pre-defined sequences, hybridization-dependent, low signal-to-noise ratio

AFM: atomic force microscopy; DLS: dynamic light scattering; dPCR: digital PCR; ELISA: enzyme-linked immunosorbent assay; FCM: flow cytometry; NTA: Nanoparticle Tracking Analysis; NGS: Next-Generation Sequencing; qPCR: quantitative polymerase chain reaction; PTM: post translational modification; TEM: transmission electron microscopy; WB: Western blot

Some of the first affinity-based isolation methods focused on common EV-associated tetraspanins such as CD63, CD81 or CD9 [3]. Since these transmembrane proteins display readily accessible domains on the EV surface, they lend themselves to capture by specific antibodies. Common immunoaffinity-based isolation protocols utilize a mix of antibodies to capture a wide range of EVs that display these markers. An alternative approach, which is of significant interest for diagnostic applications, might target surface proteins or glycan structures that are only expressed in specific tissues. Alternatively, a robust, disease-specific marker that is exclusively presented on EVs from the affected tissue, potentially allows specific capture of relevant EV subpopulations [4].

The interaction with surface targets is not limited to antibodies: successful isolation has been reported based on using lectins, phosphatidylserine binding pro-

teins and synthetic peptides to capture EVs. Less specific approaches include isolation based on affinity to charged membranes or capturing EVs using heparins, which bind to several EV surface proteins.

As any other method, affinity-based isolation introduces bias since it very likely only captures a subset of all EVs in a given sample. While their selectivity potentially results in favorable purity and signal-to-noise ratio, the dependency on high-affinity antibodies, substantial reagent costs and inability to elute captured EVs limit many of the affinity-based isolation methods available today.

Precipitation-based methods

Originally, the precipitation technique was established to separate and concentrate viruses. Since viruses and EVs share biophysical properties, precipitation techniques have been adapted for the enrichment of EVs. In general, EV precipitation relies on manipulating their solubility and dispersibility in aqueous suspensions. The most common precipitation technique is the polymer-based precipitation. Hydrophilic polymers such as polyethylene glycol (PEG) tie up water molecules, which leads to the concentration of EVs until solubility is exceeded and precipitation occurs. Since EVs are negatively charged, polymer solutions can be supplemented with positively charged protamine sulphate in charge-based precipitation approaches or used in combination with dextran in a two-phase-system. 'Salting-out' allows immediate EV precipitation due to EV charge neutralization with acetate ions. After the precipitation process is completed, EVs can be pelleted by low-speed centrifugation. In addition, indirect EV precipitation approaches have been developed that first precipitate soluble proteins present in biofluids with organic solvents. After centrifugation, EVs can be isolated from the pre-cleared supernatant by the method of choice.

Fluidics-based methods

Microfluidic platforms for EV isolation from particularly small sample volumes attracted the attention of researchers and clinicians in the last years. In general, these miniaturized tools follow the same principles as large-scale isolation methods. For example, EV-targeting antibodies can be immobilized on solid surfaces of microfluidic devices. Oligonucleotide and peptide aptamers offer an alternative to antibodies and both allow affinity capturing and produce a reporter signal upon EV binding. Membrane-based filtration approaches and viscoelastic flow sorting have been also implemented in microfluidic isolation procedures. Alternative microscale systems are nanotraps that consist of nanowires in 3D-networks that bind to and capture EVs from various biofluids. Other devices have implemented innovative sorting mechanisms such as acoustic trapping (e.g. by ultrasound) or electrophoretic and electromagnetic manipulation for EV isolation [5].

Asymmetric-flow field-flow fractionation (AF4) is a hydrodynamic method at microscale level that separates EVs based on their differences in size and diffusivity [6]. Samples are applied to a miniature fluidic channel, in which two orthogonal flows control EV separation. A laminar parabolic flow (i.e. fastest flow in the center, slowest flow at the walls) runs along the channel axis and transports the sample towards the detector. The second flow is perpendicular to the laminar flow and vertically distributes particles against the chamber wall. Smaller particles dif-

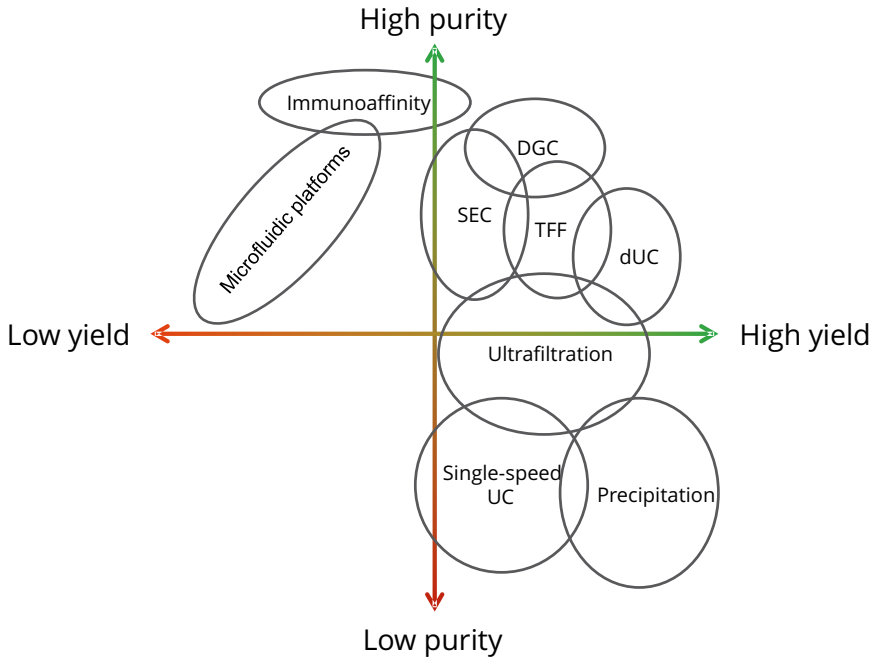

Figure 1: *Schematic illustration of commonly used EV isolation methods. Different EV isolation methods are arranged on a 2D landscape that classifies yield (x-axis) and purity (y-axis). Note that the degree of recovery and specificity of a particular method might fluctuate depending on the biofluid, experience of the operator, and distinct variations in equipment and protocols. DGC: density gradient centrifugation; dUC: differential ultracentrifugation; SEC: size-exclusion chromatography; TFF: tangential flow filtration UC: ultracentrifugation.*

fuse further from the flow chamber wall and are eluted earlier than larger ones. AF4 can be integrated with various types of detectors depending on the information required.

EV characterization: defining the needle

Analytical characterization of EV preparations is a multi-step process that serves several purposes. First, it is important to detect EVs in order to verify that a successful isolation has been performed. Next, quantifying EVs and characterizing the molecular composition of EV preparations are equally important and should include the analysis of both EV-enriched markers and contaminants. While many established analytical methods can be applied to the EV field, novel tools that fit more specific needs are also in development.

Particle level

There are several methods to assess EV preparations on a particle level, which usually includes quantity, size and morphology. One of the oldest methods to characterize particles in suspension is dynamic light scattering (DLS), which relies on illuminating samples with a monochromatic light source and analyzing light scattered by particles to calculate their size. While still widely used, DLS only captures bulk signal from all particles and therefore struggles with polydisperse samples. This limitation was partly overcome by Nanoparticle Tracking Analysis (NTA) techniques, which also record scattered light but track each particle individually. Based on the Brownian motion reflected in scatter patterns, particle sizes can be calculated using the Stokes-Einstein equation. NTA is therefore capable of both counting particles and providing more granular size distributions than DLS. One of the biggest challenges of NTA is that current platforms are unable to distinguish between EVs and non-EV particles of similar size. As EV preparations are commonly contaminated with protein aggregates, lipoproteins and other nanoparticles, NTA-based analysis of low-purity samples is bound to overestimate EV concentrations [7]. Fluorescence-enabled NTA, which tracks scattered laser light at a specific emission wavelength, was recently developed to overcome this shortcoming. Using lipophilic dyes or fluorochrome-conjugated antibodies, NTA's specificity can be narrowed to particles surrounded by lipid membranes or decorated with marker proteins, respectively [8, 9]. Despite still facing technical challenges, this approach has enormous potential upsides and could significantly enhance detection of EVs and individual subpopulations. An alternative, non-optical, method is Tunable Resistive Pulse Sensing (TRPS), in which EVs flow through a nanopore, and changes in ionic current are used to compute particle sizes and concentration.

Flow cytometry (FCM) has emerged as another method that allows the detection and characterization of thousands of EVs in suspension. However, most conventional flow cytometers are not yet capable of detecting EVs with diameters of less than 300 nm due to limited sensitivity and resolution. To overcome this obstacle, bead-based (e.g. beads coated with antibodies against EV surface proteins) procedures were designed to artificially enrich EVs and increase their fluorescent signal. In addition, bead-free protocols that take advantage of fluorescent-labeling strategies were established to perform multiparametric characterization of EVs and to assess EV heterogeneity. Double labeling with protein- and lipid-specific dyes also enables the discrimination of EVs and common contaminants. However, technological progress accelerated the development of next-generation flow cytometers that allow label-free analysis of EV sizes and concentrations via the scatter channel. By combining the capabilities of flow cytometry with high resolution imaging at the single-particle level, imaging flow cytometry (IFCM) facilitates precise EV characterization and phenotyping [10, 11].

Microscopy-based techniques are frequently used to visualize the morphology of single EVs. Accurate EV imaging can uncover the shapes, structure and size of vesicles as well as other non-vesicular components [12]. Although there are various imaging technologies, resolution limits of each method have to be considered. As a result of sample preparation preceding EV imaging, not all visualization tools can reveal the natural EV morphology. The most prominent visualization technique is electron microscopy (EM). Transmission electron microscopy (TEM) is a standard imaging method that provides structural information about EVs and background contamination in EV preparations. TEM analysis requires extensive sample preparation, which can lead to dehydration of EVs and morphological artefacts (e.g. cup-shaped appearance of vesicles). Cryo-electron microscopy (cyro-EM) allows preserving the native spherical shape since the EV samples remain in their native hydrated state. Immunogold labeling combined with EM analysis (immuno-EM) is a powerful labeling strategy to localize specific pro-

teins in EV preparations. In immuno-EM, gold probes serve as reporter molecules that are conjugated to specific antibodies. Topographic information about EV surfaces can be obtained by scanning electron microscopy (SEM). Atomic force microscopy (AFM) is another method to gather information about the three-dimensional shape (i.e. topography) of isolated EVs. AFM can be performed with vesicles immobilized on distinct surfaces, or operated in liquid, which preserves the physical EV properties. While advanced EV labeling techniques and high-resolution imaging technologies are in development, the molecular composition of EVs remains to be comprehensively characterized.

Molecular level

Many previously established techniques have been used to characterize EVs on a molecular level and revealed the presence of common EV cargo molecules present in almost all isolated EVs as well as tissue-specific signatures present in EVs derived from distinct tissues [13]. Particular interest has been paid to vesicular proteins, which can be used to both substantiate EVs and assess levels of contamination.

Over the past three decades, thousands of proteins have been found in and on the surface of EVs. Deciphering the EV protein composition is interesting for three reasons. First, proteomic studies on EVs provide clues about the molecular mechanisms involved in EV biogenesis and cargo sorting. Second, membrane-bound and cytosolic proteins present or enriched in almost all EVs or specific EV subtypes, as well as proteins not expected to be present in EVs, are identified. It is therefore recommended to analyzed vesicle preparations to demonstrate the presence of EVs and evaluate the depletion of common contaminants [14]. Third, large-scale proteomics datasets suggest EV protein biomarkers for certain diseases that might be relevant from a clinical and biological perspective. Since EVs are usually isolated in small amounts, sensitive methods are required to analyze their protein composition. After protein digestion and sample preparation, EV peptides can be subjected to mass spectrometry, which allows the detection, identification, characterization, and quantification of EV proteins. Additional, mass spectrometry can also be used for the analysis of posttranslational modifications (e.g. phosphorylation, acetylation, glycosylation) and other EV-associated molecules (e.g. lipids and metabolites) [15]. Western blot analysis is another very common approach to analyze the protein composition of EVs. In this method, proteins are separated according to their molecular weight by electrophoresis (e.g. SDS-PAGE) and then transferred onto a membrane to allow antibody-mediated identification of specific proteins. Other antibody-dependent protein methods are for example enzyme-linked immunosorbent assay (ELISA), and immunoprecipitation. Since proteins, particularly lipoproteins, are highly abundant in some biofluids, protein assays should also be used to identify potential contamination in the preparation.

Nucleic acids, including DNA, mRNA and non-coding RNA such as microRNA, are amongst the most frequently studied EV components. Depending on the respective scientific question, experiments might include characterizing complete nucleic acid profiles or quantifying levels of individual species. Facilitated by high-throughput Next-Generation Sequencing (NGS) approaches, DNA and RNA in EVs can be deciphered at single-nucleotide resolution without a priori sequence knowledge. Alternatively, specific sequences that are of interest can be detected and quantified using quantitative PCR (qPCR), RT-qPCR or digital PCR. Combining analytical assays with upstream nuclease and/or proteinase digestion steps is a common way of determining if nucleic acids are genuinely encapsulated in EVs, i.e. protected from nucleases, bound to their surface or associated with non-EV protein carriers [16].

The quest for standardization and reproducibility

The need for standardized EV isolation and characterization methods became painfully clear in 2017, when an international consortium published a meta-analysis of over 1,000 studies from various areas of EV research [17]. Some of the most noteworthy findings of the EV-TRACK report included the heterogeneity of methods used in the EV field – including a total of 1,038 individual EV isolation protocols – and the poor reporting of experimental details in publications. Driven by the need for transparent methods and rigorous EV characterization, EV-TRACK has since morphed into both a coaching tool for the community and an extensive repository of methodological data. Uploading experimental details and reporting the resulting quality score (EV-METRIC) in publications is encouraged to enhance transparency and reproducibility in the quickly growing community. Additionally, EV-TRACK now aggregates biological information on EVs from over 2,000 experiments and is

thus a valuable resource in the quest to better understand EV heterogeneity, quality and physiology.

As another important effort to improve EV experiments, the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines, was published by the International Society for Extracellular Vesicles (ISEV) in 2014 [18] and updated in 2018 [14]. These guidelines provide practical recommendations for various areas of EV research, ranging from terminology and sample processing to EV characterization and functional studies.

Both the rapid growth of the EV field and the widespread use of high-throughput characterization techniques have led to an accumulation of large amounts of EV profiling data. In addition to established repositories for nucleic acid and protein sequences, there are now dedicated data bases for EV-associated molecules. ExoCarta [19], EVpedia [20] and Vesiclepedia [21] provide searchable information on EV analytes from dozens of species and hundreds of studies.

Conclusion

While presenting intriguing opportunities for both basic biology and translational application, the study of EVs is fraught with obstacles. Despite the abundance of available isolation methods, universally applicable and standardized protocols remain elusive. Each method must be carefully considered regarding its suitability for a given experiment, which depends on various factors including the type and starting volume of biofluid, desired level of purity, downstream assays, experience of operators and the respective scientific questions. Owing to the growing recognition of EV heterogeneity and the need to not only separate EVs from contaminants but also fractionate EV subpopulations, linear combinations of isolation methods are increasingly utilized. Depending on the respective experiment and the required degree of purity, this might mean combining enrichment and purification methods or integrating orthogonal methods that target different EV characteristics. As demonstrated in a variety of research articles, each method is inherently biased towards specific EV populations, which makes thorough characterization of resulting isolates essential for any EV workflow. Using different methods to isolate EVs from the same biological sample was shown to qualitatively and quantitatively alter the composition of resulting vesicle preparations [22-24]. The diversity of EV isolation protocols used by different researchers therefore considerably complicates comparison of the results obtained by individual laboratories, emphasizing the need of standardization.

Similarly, a multitude of analytical methods has been used to study EVs on the macro and micro level. Assays based on light scattering, TRPS, flow cytometry and imaging allow characterization of individual particles while high-throughput technologies such as NGS and mass spectrometry significantly contribute to deciphering their molecular composition. Despite the availability of valuable analytical tools, each method brings about its unique limitations, which impedes progress towards more fully understanding vesicle biology. Among other pressing questions, the identification and separation of vesicle subclasses, as well as potentially specific cargo sorting remain two of the most studied areas in the EV field. Even though proteins and nucleic acids share the spotlight in EV profiling studies, we would be remiss not to mention other analytes such as lipids and metabolites that also deserve attention. In addition to the most important methods discussed here, further techniques including super-resolution microscopy, surface plasmon resonance spectroscopy, Raman spectroscopy and single particle interferometric reflectance imaging are successfully used to study EVs. Detection and sensitivity limits of established methods are, however, not always compatible with the EV field's requirements, which prompted the development of dedicated instrumentation for nanosized vesicles.

Driven by technological advances and a growing understanding of EV biology, the enthusiasm about EVs seems unabated. The past decade of EV research was characterized by explosive growth, and EVs made their way into virtually all areas of life science, ranging from mental disorders to infertility research and ocular diseases. At the same time, it featured a remarkable learning curve for the community, which was confronted with the limitations and challenges of this young and rapidly expanding discipline and in turn quickly implemented measures to boost experimental rigor, increase transparency and, ultimately, enhance the validity of EV studies across the board. While it is too early to tell if the EV-TRACK and MISEV initiatives will leave a lasting impact on the field, their exponential citation rates and the fact that publications citing the guidelines consistently score higher EV-METRICs are promising harbingers of their success [25]. Given a wide adoption of good experimental and reporting practice, EV research is bound to flourish, paving the

way for novel insights into basic biology as well as clinical and biotechnological applications.

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Norwegian Society for Extracellular Vesicles (NOR-EV)

Foundation year

2019

Mission

The Norwegian Society for Extracellular Vesicles (NOR-EV) was founded by Alicia Llorente, Reidun Øvstebø and Kari Bente Foss Haug, all based at Oslo University Hospital, Norway. The society's goal is to be a network for scientists, consultants and students interested in extracellular vesicles, to raise awareness about this research topic in Norway and to promote communication between their members and other relevant national and international scientific communities. NOR-EV will maintain a webpage and organize activities such as courses, conferences and information meeting in order to disseminate the work of its members and establish links with national and international actors.

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Tumor cell communication through EVs: new challenges and opportunities

Cecile L. Maire and Franz L. Ricklefs

Extracellular vesicles (EVs) are small, heterogeneous, lipid-bilayer particles that are potent vehicles of intercellular communication, contributing to the interaction of tumor cells with the microenvironment and promoting tumor growth. Furthermore, EVs have gained substantial interest due to their potential utility for liquid biopsy approaches in cancer.

Keywords: *extracellular vesicle, cancer, biomarker, liquid biopsy*

Physiological roles of EVs

Extracellular vesicles (EVs) play an important role in intercellular communication in and between different tissues of an organism [1, 2]. EVs include exosomes and microvesicles. Exosomes are membrane vesicles measuring 60-150 nm that are released into the extracellular space by multivesicular bodies (MVB) after fusion with the plasma membrane. In contrast, microvesicles (ectosomes) budd from the plasma membrane. Both vesicular entities are loaded in different ways in the cell and thus probably have different biological functions. EVs carry complex biological information consisting of DNA, RNA, proteins and lipids that can alter the phenotype of the recipient cell at several levels. The recipient cells can be affected via direct receptor binding, the fusion of their membrane with the EVs membrane and then the release of their encapsulated molecules (transcription factors, oncogenes, miRNA (miR) and long non-coding RNA) [1, 2] [Figure1]. Moreover, the lipid membrane of EVs surrounds and protects their content from degradation and thus allows their physiological and pathological information to be send over a long distance. In this way, EVs are involved in every aspect of the physiology of the body.

In the past, EVs were thought to embody the cellular waste removal system, which is why EVs were commonly named the "cellular trash system", underestimating their physiological or pathological functions. However, EVs have been implicated in cell-cell communication and have been observed to transfer functional nucleic acid and proteins between cells. In normal physiology one major aspect of EVs seems to take place in the interaction with the immune system. Their immunogeneic properties range from their involvement in antigen presentation enabling EVs to induce a T helper cell response[3] as well as their capacity to activate cytotoxic T cells[4] during infection.

EVs in cancer

Despite their multifaceted roles in normal physiology, EVs maintain and influence essential processes in the pathogenesis of various diseases, and their role in tumor biology is under thorough investigation. It seems, that during oncogenesis, cells increase the production of EVs, probably due to their increased metabolism. The release of EVs by tumor

cells is thought to play a major role in facilitating signalling to surrounding tumor cells and to distant sites via blood or other biological fluids. Tumor cell EVs (tEVs) are involved in the establishment of all hallmarks of cancer including tumor growth [5], affect the tumor's immune escape by modulating T cell activation [6] build pre-metastatic niches [7] remodel the extracellular matrix [8] and promote angiogenesis [9] [Figure2].

Tumor growth

Characterization of the genome and RNA transcriptome revealed that tumor cell subpopulations with different genomic and transcriptomic subtypes can coexist and have emerged from a distant related precursor, most likely a plastic stem-like cell. This intratumoral heterogeneity provides the fuel for treatment resistance and allows cancer to be a dynamic disease [5, 10]. Heterogeneous tumor cells can transfer tEVs to other tumor cell subtypes enhancing their pro-tumorigenic behaviour. Furthermore, analyses of the Cancer Genome Atlas (TCGA) revealed that EV-related signatures are associated with decreased patient outcomes which may be propagated throughout the tumor via EV communication [5].

Immune system

Novel strategies that aim to prime the patient's immune system against cancerous tissue have recently gained momentum in the clinic. There are many reports demonstrating that immune cells can be regulated by tEVs enabling an immune evasion by the tumor. Raposo and coworkers demonstrated first the immunoregulatory potential of EVs, as they showed that EVs secreted by B cells can carry the major histocompatibility complex (MHC-II) [11]. Many more studied followed, leading to an active area of investigation in the field of EV-based immunomodulation, especially in the cancer biology. An emerging general concept is that EVs from healthy immune cells can stimulate the immune system and induce an anti-tumor effect, while tEVs most commonly provoke an inhibition of the immune system. For instance, EVs from antigen presenting cells (APC) can active T-cells and maintain their activation. tEVs on the other hand have been shown to suppress the activity of T-cells [6], natural killer cells [12] and enhance the activity of myeloid-derived suppressor cells

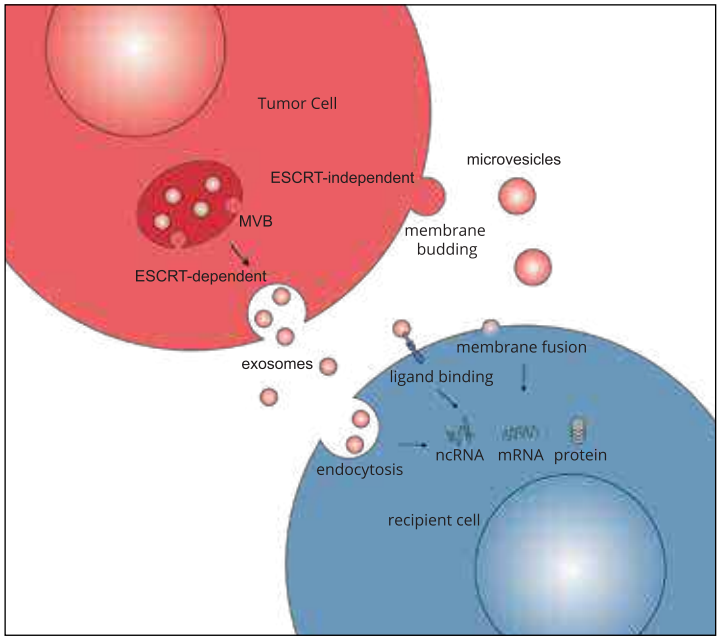

Figure 1: EV biogenesis and their interaction with recipient cells. *Exosomes are produced from multivesicular bodies (MVBs) in a process coordinated by the endosomal sorting complex required for transport (ESCRT) machinery. Components of tumor cells are integrated in EVs, including proteins, RNAs and DNA. EVs either fuse with the plasma membrane or are endocytosed by recipient cells, releasing their content with the potential to reprogram the recipient cell.*

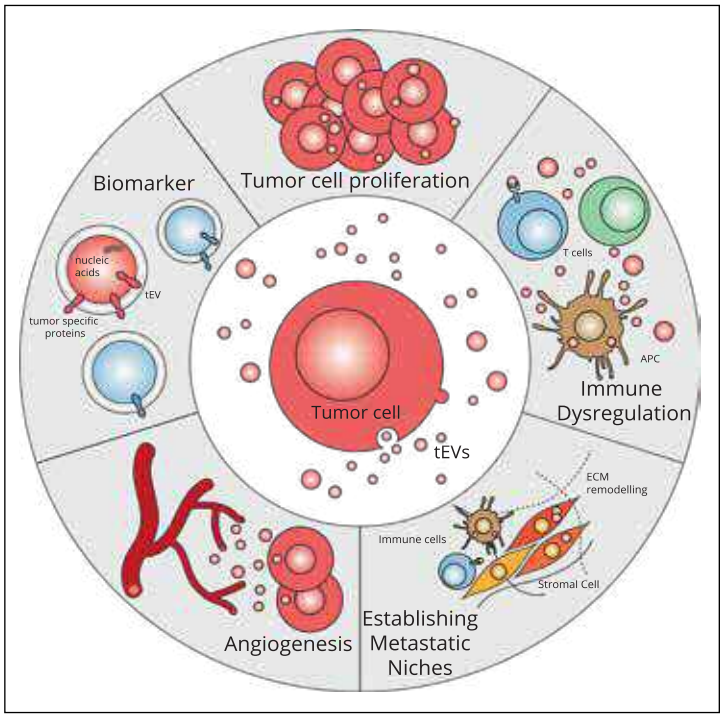

Figure 2: Exhibit of tumor cell EVs pathophysiological roles. *Tumor cell EVs (tEVs) maintain and influence essential processes in cancer biology. tEVs can I) cause tumor cell proliferation and maintain intratumoral heterogeneity, II) cause immune dysregulation and promote tumor immune escape, III) are responsible for the formation of pre-metastatic niches, IV) can cause angiogenesis to secure increased nutrition supply, V) release tumor specific proteins and nucleic acids in diverse body fluids that can be used for "liquid biopsy" approaches. Additional EV-mediated processes are present (a.e. convection of drug resistance, interference with immunotherapy approaches, metabolic changes) tEVs = tumor cell EVs, ECM = extracellular matrix, APC = antigen presenting cells*

[13]. Regarding therapy approaches, in vivo data using dendritic cell (DC) EVs demonstrated that DC EVs could be used to induce an antigen specific immune response. The findings stimulated the investigation of DC EVs as an autologous cancer vaccine [14]. In this regard, early mouse studies showed good response rates [15] that lead to phase I trials in humans for patients with non-small lung cancer and melanoma. In some patients DC EVs promoted disease stabilisation, yet the utility of DC EVs for immunotherapy approaches needs to be established in future trials [16].

Metastatic niches

While expending in the primary organ, tumor cells regularly shed tEVs in the circulation. Although EVs cannot survive long in the blood stream, they can protect and deliver their cargo to numerous healthy cells and thereof disturb the delicate balance of specific microenvironment. In melanoma, tEVs expressing Met tyrosine kinase can be found in the bone marrow which provoke the bone marrow stem cells relocation to the lung where they differentiate into blood vessels creating a perfect environment for the colonization of melanoma CTCs [7]. Emergence of pre-metastatic niches can also result from the accumulation of tEVs at a specific site where the interaction of integrins transported by tEVs modify the extracellular matrix (ECM) and the release of growth factors when taken up by nearby healthy cells [8]. tEVs from colorectal cancer can also increase liver macrophages infiltration and polarization to induce a pro-inflammatory phenotype through miR-21 and the release of pro-inflammatory cytokines and chemokines to remodel the ECM for CTCs arrival [17]. Recent investigation in a mouse model of breast cancer showed that tEVs secreted by resistant cells post chemotherapy carry Annexin VI which allows them to accumulate into the lungs where they are taken up by monocytes and endothelial cells leading to increase angiogenesis and formation of pre-metastatic niche post therapy [18]. Therefore, the capacity of tEVs to migrate and accumulate at specific sites favours the development of pre-metastatic niches that precede the arrival of CTC and the emergence of metastases.

Angiogenesis

A hallmark of cancer is the induction of angiogenesis to secure the supply of nutrition for tumor cell growth and its increased metabolism. tEVs have been shown to act as key regulators of tumor vascularization via transfer of pro-angiogenic molecules from tumor cells to endothelial cells [9]. In various cancers, tEVs have been shown to promote endothelial cell migration, growth and tube formation. This effect was even more pronounced by tEVs produced from hypoxic tumor cells [9], underlining that tumor cells can alter the cargo of tEVs for their specific needs. More recently, Chen et al. demonstrated that tEVs from breast cancer cells can promote aerobic glycolysis by the transmission of a myeloid specific HIF1a-stabilizing long noncoding RNA to macrophages inducing a feedback loop and lactate release in tumor cells [19].

EVs as biomarkers in cancer

Besides their implication in tumor promotion and maintenance, tEVs are released into the bloodstream and distributed throughout the body. tEVs end up in almost all body fluids and organs, including blood, saliva, urine, cerebrospinal fluid, bile, breast milk and stool [20]. It is now widely accepted that the molecular cargo of tEVs is representative of the secreting cell and that these molecules are protected from fragmentation and degradation making circulating tEVs ideal for defining subgroups, stratifying patients, and monitoring therapy by "liquid biopsy " [21]. For example, one of the first reports by Skog et al. reported that serum EVs from patients with malignant glioma carry the mutant tumor-specific EGFRvIII protein and contain transcripts coding for this mutant tumor-specific variants [22, 23]. In addition, EVs in CSF and serum may contain mutant IDH1 transcripts [24]. This phenomenon has been demonstrated in multiple cancer types as well as divergent molecular entities, namely miRNA, mRNA and lncRNA as well as DNA [20]. As biomarkers, serum and plasma EVs are indeed even better suited than circulating tumor cells (CTCs) because they reflect the heterogeneous tumor composition and thus the tumor as a whole better than individual CTCs [25]. In total, there are multiple studies highlighting the potential of EVs being a rich and readily accessible source of cancer biomarkers [21].

Yet, it needs to be mentioned, whilst tEVs represent a promising class of circulating biomarker, that current challenges need to be addressed and hopefully solved within the upcoming years. One major challenge for the filed remains the lack of standardisation of protocols for EV isolation, enrichment and characterisation as well as documentation. To address these challenges efforts are being made to fa-

cilitate inter-study methodological comparisons and to develop study guidelines as well as reporting in EV research. A couple of examples are: minimal informations for studies on EVs (MISEV) guideline 2019; EV-METRIC and EV-TRACK (http://evtrack.org).

Conclusion

It is now widely accepted that EVs participate in numerous pathological and physiological mechanisms, and as such they have been intensively studied over the past decade. There is a growing body of evidence that tEVs carry tumor-representative cargo and that they facilitate tumor growth maintenance and dissemination. However, since EVs display a heterogeneous biological entity, the complexity and challenges associated with EV research remains, which needs to be addressed since tEVs are promising candidates for liquid biopsy approaches.

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Extracellular Vesicles – developmental messengers of tissue crosstalk

Leonie Witte and Julia Christina Gross

Extracellular vesicles are membrane particles secreted from cells into all body fluids. They convey signals that induce long-term changes in cellular behavior. During development, EV secretion and the specific loading of signalling factors in EVs contributes to organ development and tissue differentiation. Different biomolecules such as proteins, lipids and nucleic acids transmit these signals and the content, size, and membrane composition of EVs are highly dynamic and depend on the cellular source, state, and environmental conditions.
Here we review current in vivo studies in different model organisms regarding extracellular vesicles and their physiological role in the formation of cell types, tissues and refined body structures.

Keywords: *cell-cell communication, exosomes, developmental signaling, morphogen signaling, model organisms*

Introduction

In development, multicellular organisms face the challenge of building a body from scratch. This embryonic self-organisation is regulated in space and time and by generation and decay of signals and forces. Cellular communication depends on a common code understood by transmitting and receiving cells and the successful transmission in the presence of noise. Recently, Extracellular vesicles (EV) have emerged as membranous signal carriers that fulfill several of these requirements and represent a cellular communication system that allows individual cells to integrate into an organism and function as information units for an individual cell or a cell population. Studies in different model organisms, i.e. *Drosophila*, zebrafish or mouse reveal their universal role as signal carriers at the organ or systemic levels during development and homeostasis.

As the contribution of EV to tumour progression, e.g. growth, vascularisation, immune invasion and metastasis is recognised more and more, a deeper insight into their biology in health is crucial. In this review we will focus on different *in vivo* studies to date that elucidate the function of EV under physiological conditions. In general, we here discriminate three types of signalling principles and compare them to human ways of communication: (1) Transfer of EV to recipient cells, (2) EV-bound signal crosstalk and (3) Systemic role of EV-bound signals.

The telegram – EVs for unidirectional signal transfer

The most direct way of communication is the transmission of information from a donor to one or more recipients. For intercellular communication, this translates to the delivery of a messaging molecule from one cell to another or to multiple receiving cells. In a developmental context, this is commonly employed when signalling molecules are produced and secreted from a distinct population of source cells in order to spread across the extracellular space and induce target cell responses depending on their absolute or relative concentration. Such morphogen gradients govern the patterning and growth of multiple

tissues during development of vertebrates and invertebrates and can be used to study how these molecular messages are sent in a manner that is suitable to orchestrate the development of complex tissues and organs. In order to coordinate cell behaviour and growth on a tissue level, morphogen signalling has adapted to encode information on multiple levels and provides highly context-specific information in a space-, time- and dose-dependent manner.

This high degree of regulation requires tight control over morphogen secretion, their extracellular spread and target cell activation. The release of morphogens on extracellular vesicles is one way how cells secure this spatiotemporal regulation and fine-tune signal strength distribution for long-range cell communication. In *Drosophila*, EV-mediated spread of Wingless (Wg), the main *Drosophila* Wnt protein, and proteins of the Hedgehog (Hh) morphogen family have been found to contribute to gradient formation across the wing imaginal disc (WID) epithelium during wing development [1]. The lipid-modifications of both proteins render them hydrophobic, a property that limits their (uncontrolled) extracellular dispersion and raises the need for diffusible carriers. Wg was found in distinct punctae colocalising with membranous glycosyl phosphatidylinositol-GFP and the human exosomal marker-construct CD63-GFP in the extracellular space of WIDs [2]. *In vitro* studies confirmed the presence of signalling active Wg on exosomes from cultured *Drosophila* S2 cells [3], [4]. Exosome secretion, extracellular levels of Wg and Wg target gene activation were controlled by the SNARE protein Ykt6 [3].

In mammals, secretion and extracellular spread of Wnt proteins on EVs plays an important role during sperm development. Spermatozoa undergo a week-long maturation process that involves changes in protein composition and subcellular localisation and is necessary for their mobility and consequently mouse fertility [5]. This crucial maturation step occurs during their transport along the epididymis, a coiled tubular system that connects the testis and the vas deferens. In mice, this process heavily relies on post-transcriptional Wnt signalling [6]. In order to deliver the required Wnt signal, Wnt ligands are packaged into exosomes and secreted from epididymal cells into the epididymal lumen [6]. These Wnt-bearing exosomes in the luminal fluid are signalling active and induce WNT/STOP signalling in the transiting spermatozoa population and thus promote sperm maturation and fertility. Here as well, EVs are used as a shuttle for the delivery of a required developmental stimulus to a target cell population. It is not clear yet, whether EV are merely the carrier for hydrophobic Wnts or whether additional EV signals, such as RNAs, are contributing to an overall differentiation programme transferred by EVs.

Similarly to Wg, Hh is secreted on EVs from its *Drosophila* WID source cells, where it colocalises with CD63-GFP as well as MVB markers [7], [8]. Interference with ESCRT-mediated ILV formation and EV secretion impaired extracellular Hh transport and long-range target gene induction [8], [9]. Similarly to Wg, *in vitro* EV-bound Hh only contributes a fraction of the overall signalling capacity [8].

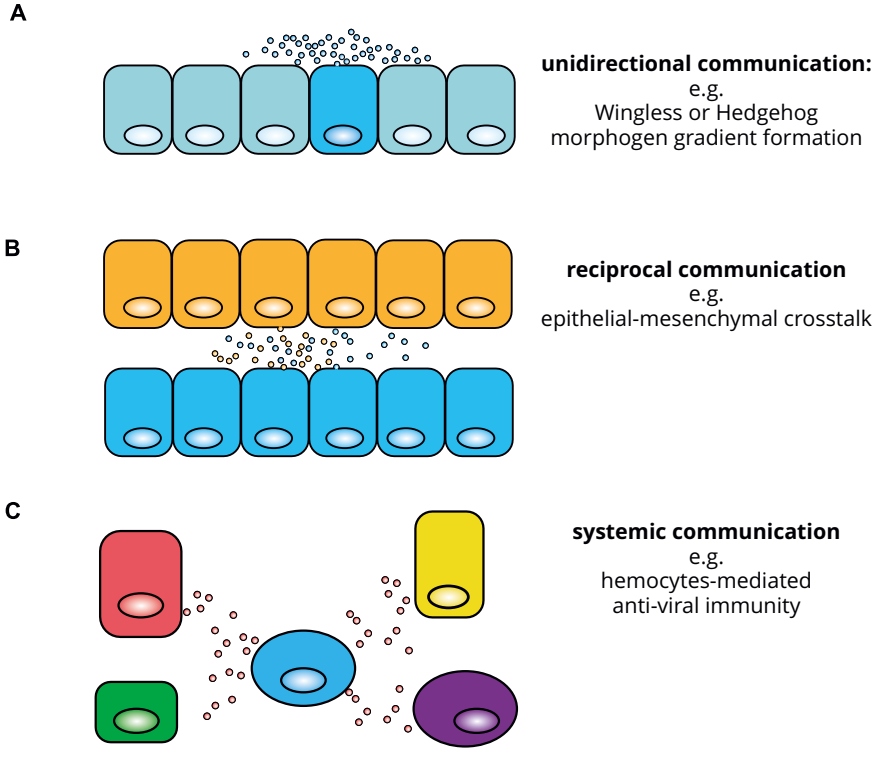

Figure 1: Mechanisms of EV-mediated tissue crosstalk in development. *During development, EV-mediated signal transfer can follow different basic signalling principles: (A) Unidirectional EV transfer from source cells to recipient cells, (B) Reciprocal exchange of EV-bound signals between different cell populations (C) Systemic EV-mediated communication between cells and tissues after EV distribution throughout the organism.*

The long-range transport of Wg and Hh is especially interesting, as both morphogens undergo post-translational lipidation that negatively affects their solubility and hence their ability for free diffusion [1]. Packaging these morphogens on vesicle carriers however does not only enhance their signal range by increasing their extracellular mobility, it also adds additional levels of regulatory control over their extracellular amounts by allowing context-dependent endosomal routing, sorting and secretion. In line with this idea, Hh-containing MVBs and EVs have been shown to travel along cytonemes, dynamic signalling filopodia that spread out from the basal side of source cells, to even further increase the local and temporal precision of Hh delivery to its dedicated target cells [7], [10]. Although a similar mechanism has been proposed for Wg signal transduction, cytoneme-based secretory Wg transport in WID has not yet been demonstrated. In addition to EVs, also lipoprotein particles have been described as vehicles for long-range signal transduction of both Wg and Hh in WID [11]. This apparent diversity of extracellular trafficking routes indicates that secretory cargo shuttling is highly context dependent and highlights how much energy cells invest in order to gain control over extracellular morphogen levels and their precise timely and spatial delivery. The secretion of Hh and Wg from their source cells on EVs and their subsequent spread across the extracellular space for the delivery to target cells represents a prime example of how EVs can act as carriers for the unidirectional transfer of signalling molecules. While this one-sided molecule transfer is sufficient for the guidance of many developmental processes, more complex developmental challenges require reciprocal exchange of information. Many examples exist in which EVs act as vehicles for the exchange of information between one cell group and another cell population.

The phone call – EVs for reciprocal intercellular communication

For development of complex tissue structures, cells of different origin and determination need to exchange signalling information with one another in order to coordinate the joint development into tissues or organs. Bidirectional exchange of signalling molecules can be mediated

by secretion of EVs and their reciprocal uptake by the respective other cell population. Such EV-bound crosstalk occurs between the surface epithelium and underlying mesenchyme during organogenesis and is necessary for the coordinated development of different organs, such as skin, mammary glands, lung and kidney.

During tooth development, EVs secreted from both epithelium and mesenchyme cells diffuse through the basement membrane and are then preferentially taken up by cells of the respective other tissue type. After uptake they reciprocally induce cell differentiation and matrix synthesis: miRNAs transferred by epithelial EVs stimulate mesenchyme cells to undergo mineralisation while mesenchymal EVs induce the production of basement membrane compounds in epithelial cells [12]. But EV transport much more than single signalling molecules – they transfer a detailed description of the sending cells' content. This integration of context-dependent developmental requirements allows cells to adjust to changing environmental needs and is necessary to coordinate their growth into functional tissues.

To facilitate directed EV exchange between non-adjacent cells, cell protrusions or filopodia have been found to function as signalling highways, enabling directed bidirectional cargo transport to and from the corresponding signalling partner. During early *Xenopus* development, the specific distribution of morphogens is necessary for the induction of a first dorso-anterior specific gene expression pattern. This process is guided by the precise localisation and reception of maternal morphogens during blastocoel expansion and makes use of filopodia spread from the basolateral side of the *Xenopus* blastocoel to connect blastomeres over multiple cell diameters [13]. EVs are shed from the tips of such filopodia to allow active delivery of morphogens, receptors and cytoplasmic proteins to surrounding blastomeres. At the same time, EVs are actively taken up at the filopodia bases themselves, turning them into signalling hubs that facilitate an active exchange of proteins and cytoplasmic content [13].

Other examples for reciprocal communication are found in the nervous systems, when neurons and glia cells interact during neural circuit development and synaptic plasticity. In response to neuronal signals oligodendrocytes secrete exosomes. These enter neurons to make their cargo functionally available to the neuronal metabolism and improve axonal growth and survival [14]. This also applies to regenerative capacities of neurons in the CNS, that are improved by fibroblast–derived exosomes inducing Wnt signalling [15]. Moreover, synapse maintenance and plasticity is regulated by the exchange of signals between neighboring neurons. The release of the transmembrane protein PRR7 on exosomes in an activity-dependent manner and the uptake of those exosomes into neighboring neurons reduces excitatory synapse numbers by blocking exosomal Wnt secretion and signalling in the recipient cells [16].

The twitter message – EVs for systemic communication

EVs can be found in virtually any body fluid, ranging from blood plasma over urine to even breast milk [17] . Hence, signalling information in the form of proteins, lipids and nucleic acids carried on EVs can be distributed systemically throughout the whole body and is available to an enormous audience of potential target cells. At the same time, EV-based communication is eligible to any cell type that contacts extracellular space and blood stream and basically all cell types and tissues have been found to secrete EVs [17]. The resulting heterogeneity of vesicles of individual origin, cargo and purpose travelling in the blood stream is overwhelming. This sheer amount of information and signalling complexity turns understanding the function and biology of individual EVs into a challenge. However, *in vivo* developmental model systems can provide means to study systemic EV-mediated intercellular communication, as they allow the direct visualisation and live imaging of EV secretion, travel and uptake. For example, translucent zebrafish embryos can be used to visualize fluorescently labelled exosomes *in vivo*. Especially the expression of a CD63-pHlourin construct that links the exosomal marker CD63 with pH-dependent GFP has proven useful to visualize EV secretion events from single cells [18]. Expression of a CD63-pHlourin reporter in zebrafish embryos allows the tracking of individual EVs from their site of secretion at the yolk syncytial layer throughout the blood stream until uptake into their target cells, macrophages and epithelial cells at the caudal vein plexus [19]. Interference with exosome biogenesis in the syncytial yolk sac affected growth of the caudal vein plexus, underlining the biological relevance of this systemic EV transfer. In addition to contributing to our understanding of EV biogenesis and function during development, such *in vivo* model systems are used to study the secretion

and fate of EVs during disease conditions. Using a zebrafish melanoma model, it was possible to track tumour-derived EVs throughout the zebrafish embryo and identify patrolling macrophages and endothelial cells as their major recipient cell population. EV uptake and subsequent activation of macrophages was furthermore found to stimulate metastatic outgrowth, demonstrating the functional relevance of systemic cross-tissue EV exchange for cancer progression [20].

Another example for a systemic role of EVs was recently discovered in the immune system of *Drosophila* fruit flies. In invertebrates, antiviral immunity is based on a nucleic-acid-based, sequence-specific mechanism (RNA interference). Macrophage-like circulating immune cells, called haemocytes, amplify and spread antiviral signals by taking up viral RNA from infected cells and copy viral cDNA templates to generate small viral RNAs. These vsRNAs are packaged and secreted into EVs. Systemic spread of those conveys antiviral activity and virus-specific immunity for all up-taking cells. This immunity lasted several weeks after clearance of the virus and therefore represents an adaptive immune system not previously anticipated in flies [21].

Conclusion

As demonstrated by these examples, EVs play an important role in tissue crosstalk under physiological conditions. The context specificity of EVs, the signals exchanged and induced changes in cellular behaviours are beginning to emerge from these *in vivo* studies. In the way they package and combine information from multiple sources, and similar to viruses, EV seem to function as small programs that are sent out to coordinate and modulate cell behavior at a tissue or organismal level. In light of the potential clinical applications of these messengers, these and further *in vivo* model systems should be the focus of more detailed studies in development and homeostasis.

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From mesenchymal stem cells and stromal cells
From bench to bedside

Bernd Giebel, Verena Börger, Mario Gimona and Eva Rohde

Human mesenchymal stem/stromal cells (MSCs) represent a promising tool in regenerative medicine. Until now, almost one thousand NIH-registered clinical trials investigated their immunomodulatory and pro-regenerative therapeutic potential in various diseases. Despite controversial reports regarding the efficacy of MSC-treatments, MSCs appear to exert their beneficial effects in a paracrine manner rather than by cell replacement. In this context, extracellular vesicles (EVs), such as exosomes and microvesicles, seem to induce the MSCs' therapeutic effects. Here, we briefly illustrate the potential of MSC-EVs as therapeutic agent of the future.

Keywords: *MSCs, stem cell therapy, extracellular vesicles, exosomes, immunomodulation, cell-free therapy*

Stem cells in regenerative medicine

With the beginning of the new millennium, great hopes were placed in the rapidly developing field of stem cell research. It was and is the goal to develop stem cell therapies that help to successfully treat a wide range of degenerative diseases as well as more acute diseases such as stroke or myocardial infarction. Conceptually, it was assumed that injected stem cells or their descendants migrate into affected tissues and replace lost cell types via transdifferentiation, thus alleviating disease associated symptoms. At that time, human embryonic stem cells came into focus of scientific interest. However, due to their high teratogenic potential and ethical explosiveness they could not be used for stem cell therapies. Instead, various somatic stem cell types were considered and used as therapeutic agents.

MSCs

Especially fibroblastoid cells that can easily be raised from bone marrow and other tissues (including fat and umbilical cord) and that were initially described by Friedenstein and colleagues in the 1960s [1], became the therapeutic cell source of choice in a still increasing number of clinical trials. Such cells display high proliferation potential and lack teratogenic potential. Since these cells were able to differentiate into adipogenic, chondrogenic and osteogenic cell types, (which are considered as mesodermal derivatives), they were initially referred to as mesenchymal stem cells (MSCs) [2]. Relying on their therapeutic potential and the fact that their use in animal models produced no recognizable side effects [3], MSCs were then also used successfully in initial therapeutic trials. The question quickly arose as to how the immune system responds to the application of donor MSCs. While it was initially thought that MSCs are rejected in principle, it has been shown that MSCs can modulate the activity of different types of immune cells in patients. They very efficiently suppress immune effector responses and propagate

regulatory immune responses, that is, they switch the immune system from the defense to the tolerance state [4-6]. In addition to the regenerative potential of MSCs, their immunotherapeutic activity has been tested in the clinic [7]. To date, nearly 1,000 clinical trials have been registered at the National Institute of Health (NIH) in, which MSCs had been or will be used to treat a wide variety of different diseases (www.Clinicaltrials.gov).

Although the outcome of several clinical studies appears controversial, many studies show therapeutic effects of applied MSCs in at least some patients. Ongoing studies that investigated the bio-distribution of injected or infused MSCs *in vivo* have shown that most of the cells end up in the lungs and only occasionally are found in the region of the intended target tissue. Attempts to clarify whether the cells need to migrate into affected tissues to achieve their therapeutic functions demonstrated that in most cases MSCs act in a paracrine rather than a cellular manner [8, 9]. The differentiation potential of MSCs, which sometimes was regarded as pluripotent, has also been questioned experimentally. Consequently, today, many scientists question the stem cell character of MSCs. To keep the abbreviation MSC, these cells are now increasingly referred to as *mesenchymal stromal cells*; also the term *medical signaling cells* has been suggested by a leading MSC researcher [10].

Whatever MSCs may be called in the future, many scientists have tried to identify the active therapeutic substance(s) that they release into their environment. In 2009, at the example of an acute renal damage model and in 2010, at the example of a myocardial infarction model, and by using different preparation methods, two groups demonstrated that the active component is located in fractions of processed culture supernatant that contain high concentrations of vesicular structures. At that time these vesicles were called microvesicles or exosomes, respectively; today, one would correctly refer to them as extracellular vesicles (EVs) [11, 12].

Extracellular vesicles

As described in article 1 of this issue, EVs are entities that mediate intercellular communication over long distances, EV are delivered by different cells and are detectable in all bodily fluids [13]. They are composed of a heterogeneous collection of lipids, proteins and RNAs. As non-self-replicating units that according to their small size (70-150 nm) can be sterilized by filtration, small EVs have in principle significant advantages over cells for therapeutic applications.

MSC-EVs

Indeed, EVs derived from MSC culture supernatants have been already successfully used in an individual treatment attempt of a steroid refractory graft-versus-host disease (GvHD) patient and in a clinical trial to treat chronic kidney disease [14, 15]. In both settings promising therapeutic effects have been observed without any reported or detected side effects. More recently, a first-in-man approach of topical MSC-EV administration has been performed to improve the implantation-induced injury that occurs during the surgical procedure of cochlea electrode insertion. Like in the other two applications no adverse reactions were recorded, however, the hearing capability of the treated patient was significantly improved (Warnecke, Gimona, Rohde et al., in preparation).

Irrespective of how MSC-EVs have assisted in improving the symptoms in aforementioned patients, their therapeutic potential is also documented in an increasing amount of different preclinical models. In addition to the initial reports on the therapeutic potential of MSC-EVs in the acute renal damage and cardiac infarction models, positive effects of MSC-EVs were confirmed in acute and chronic renal

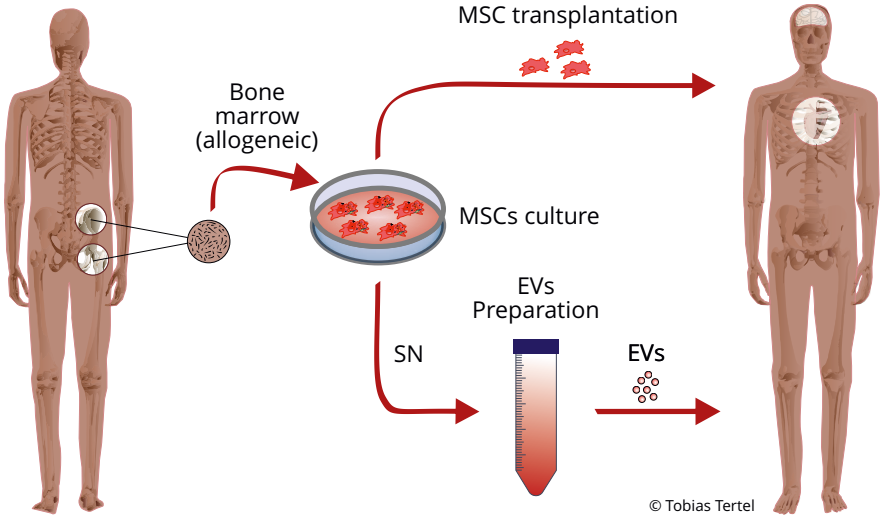

Figure 1: *MSCs can be efficiently generated from bone marrow aspirates. Traditionally, expanded MSCs are used as cellular therapeutics. An increasing number of studies have reported that EVs prepared from culture supernatants (SN) of MSCs exert comparable therapeutic effects than the cells themselves. Accordingly, an increasing number of research groups and companies aim to translate MSC-EVs into the clinics to be used as therapeutic agents in regenerative medicine and immune therapy in the future.*

damage models by independent groups [16-18]. Positive effects have also been reported on liver, lung and muscle regeneration [19-21]. Furthermore, MSC-EVs were found to promote blood circulation in a rat model of critical limb ischemia, the healing of skin burns, and the survival of allogeneic skin grafts [22-24]. Within the nervous system, positive effects were observed on ischemic stroke symptoms in rat and mouse models as well as on the regeneration of sciatic nerves in rats [25-27].

MSC-EVs can act via different mechanisms to improve the symptoms of respective diseases. Their exact modes of action have not been unraveled, yet. However, it appears that their capability to modulate immune responses and switch the immune system from the acute inflammatory into its regulatory state, i.e. to switch from defense to tolerance, is one of their key-functions [28, 29]. An important aspect in translating MSC-EVs into the clinic is the consideration that individual MSC-EV preparation may vary in their therapeutic effectiveness. To this end, MSCs have already been recognized as a heterogeneous cell entity. Independent of their origin, MSCs from given sources can differ in size and in the expression level of *bona fide* MSC cell surface antigens [30, 31]. Accordingly, it can be assumed that there are different MSC subtypes, which very likely differ in their therapeutic potentials. In this context, we are not aware of any generally accepted criteria to discriminate different MSC subtypes. In line with the postulated functional heterogeneity of MSCs many groups reported positive clinical effects following MSC administration, however, there are also several reports which could not reproduce observed effects [32]. Indeed, a phase III clinical trial in which GvHD patients were treated with MSCs ("Osiris Study", NCT00366145), failed to show efficacy [33]. Thus, to avoid such drawbacks in the MSC-EV field it is mandatory to define criteria to discriminate therapeutic active from less active and nonactive MSC-EV preparations. For now, it is one of the mayor challenges of the field to set up functional assays which reflect the therapeutic potency of MSC-EV preparations.

Furthermore, MSC-EVs need to be manufactured under GMP-compliant conditions, preferably in a scalable manner. Due to the novelty of the field, however, there are still some technical hurdles. So far, there are no standardized procedures to prepare EVs in larger quantities. Furthermore, there is a lack of qualified techniques to study EVs at the single-particle level similar to cells using flow cytometry. As device manufacturers and the pharmaceutical industry become increasingly aware of the potential of the EV field, we expect that preparative and analytical methods will improve significantly in the coming years. Also, the regulatory requirements, which for now have been formulated by only some national regulatory authorities, might get harmonized. We are involved in international activities to promote MSC-EVs and other EV products effectively into the clinics, and have published a number of manuscripts discussing the current state of the art in more details as well as strategies to address challenges in the translational field [34-36].

Conclusion

MSCs have been and are widely used in regenerative and immunotherapies. Recent findings suggest that their therapeutic effect is mediated, at least in part, by EVs. Because EVs can be sterilized by filtration and do not replicate themselves, EV therapies offer significant advantages over cell therapies. It appears that MSC-EVs mediate immunosuppressive functions and promote angiogenesis; direct influences on somatic stem cells can be assumed. Even though no side effects have been described so far in the preclinical models or in the individual therapeutic treatment's attempts, safety evaluation in early clinical trials are needed to find or exclude pro-tumorigenic, immunologic or other potentially harmful adverse events. The future will show whether MSC-EVs can affirm themselves as safe and potent cell-free therapeutics.

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Analysis of extracellular vesicles by flow cytometry – basics, limitations and prospects

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Flow cytometry is a well-established technique that classically is used to detect cells and quantify related parameters on the cellular surface, e. g. the expression of surface protein markers. Within the last few years, there also have been considerable advances of using flow cytometry to detect extracellular vesicles (EVs) in the size-range of exosomes and quantify their concentration and surface marker expression in EV-containing samples such as cell culture supernatant or biological fluids. Of note, such analyses of EVs within a size range well beyond <300 nm are still challenging and require a suitable instrumentation and a certain level of expertise to make sure essential controls are included and to ensure that resulting datasets can be interpreted appropriately. After giving a brief, basic introduction into flow cytometry which is essential especially for non-experienced readers in order to understand submicron particle flow cytometry data, we will give a concise overview about current possibilities and methods available in the field.

Keywords: *Flow cytometry, Extracellular vesicle, Surface markers, Exosome, Microvesicle*

The essential basics of flow cytometry

For a better understanding we first want to illustrate the principles of flow cytometry (FCM). Flow cytometry has evolved over the last few decades and nowadays is a well-established technique that determines events based on their physical and/or chemical characteristics. Samples containing single events (classically cells) are injected into a flow cytometer and pass one or more laser beams in a fluid stream. The resulting light signals are then detected, quantified and displayed in a software after appropriate processing. Typically, depending on the type of flow cytometer used, between 10,000 and 40,000 events per second can be analyzed.

Classically, flow cytometry is used to measure cells and analyze different parameters such as surface protein expression, cell cycle or viability, but within recent years instruments and methods have been developed which also allow for the detection of much smaller particles such as extracellular vesicles (EVs) or viruses [reviewed in [1, 2]]. Flow cytometry was initially developed in the 1960s and especially after fluorochrome-conjugated antibodies have become available, it has been used for numerous applications for basic biological and especially immunological and hematological research. Three decades later, it reached clinical breakthrough with HIV and leukemia/lymphoma diagnostics becoming mainstream. Today, flow cytometry has become indispensable in research and clinical diagnostics and it is one of the most impressive and versatile techniques for characterizing single cells in a high-throughput, multi-parametric manner [reviewed in [3]].

For the sake of simplicity and for better understanding, we here first describe the basic principle of the technique of flow cytometry on cells [4]. As we will see

later, the preliminary preparative and analytical methods suitable for analyzing extracellular vesicles are fundamentally different from that used for cells. The light signals generated by cells passing lasers lead to light scattering around the cell and also to the excitation of fluorescent dyes (fluorochromes), which then emit a corresponding light signal. As already mentioned, flow cytometric analyses of cells can be performed to collect a variety of different information, the most prevailing information being if a surface protein is present on the cellular surface or not, by using a fluorochrome-conjugated antibody specifically raised against that surface protein. Furthermore, cells can be also classified based on differences of how they scatter light. Blood cells, for example, can roughly be divided into three basic groups (lymphocytes, monocytes, and granulocytes) merely based on how they scatter light, since these cell types have different sizes and a different granularity (Fig. 1A). If these cells are then additionally labelled with antibodies which are directed against certain proteins on the cellular surface, it becomes possible to further identify subtypes within those groups, such as B cells (CD19 positive) versus T cells (CD3 positive) within the lymphocyte population (Fig. 1B). The prerequisite is that the antibodies used for this are conjugated with different fluorochromes which can be distinguished by the instrument used. Overall, the determination of various cell populations is facilitated through the usage of different fluorochromes, which give different light properties after laser light excitation. Thus, in our example, if antibodies recognizing a surface protein specifically expressed on T lymphocytes (CD3) are labelled with a fluorochrome with distinct

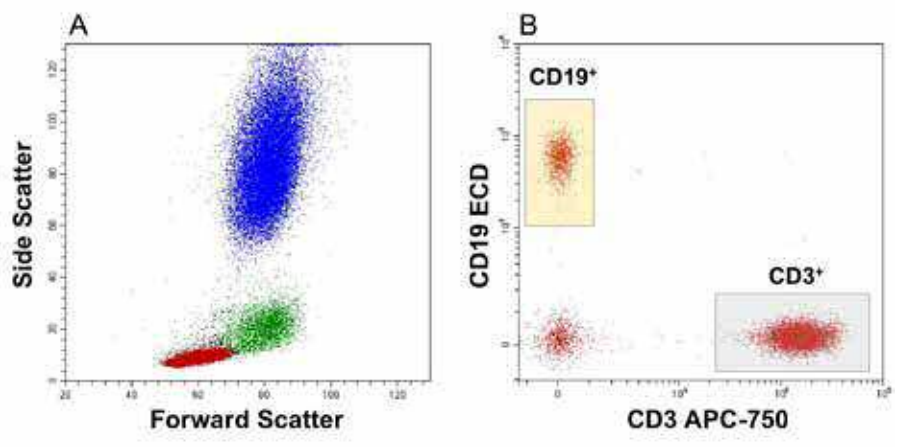

Figure 1: *Whole blood was stained with APC-750 labelled anti-CD3 antibodies specifically staining T-cells and ECD-labelled anti-CD19 antibodies specifically staining B-cells. Subsequently red blood cells were lysed and remaining leukocytes were analyzed using scatter parameters (A). For cellular analysis, forward scatter indicates size and side scatter describes the granularity of the cells. Populations of lymphocytes (red), monocytes (green) and neutrophils (blue) can be distinguished based on light scattering properties. (B) Afterwards the lymphocyte population was analyzed for their surface expression of CD3 (T-cells, grey box) and CD19 (B-cells, yellow box) by using APC-750 or ECD conjugated antibodies, respectively. These two different fluorochromes result in emission of light of a different wavelength and color following laser excitation.*

spectral properties than antibodies being used to recognize a B cell-specific surface marker (CD19), both cell populations can be accurately identified and quantified in the same sample (Fig. 1B).

The use of lasers of different wavelengths and the availability of a very large number of fluorochromes have increased the combinatorial options being available to label and identify different cellular subsets within one sample over time. Nowadays, 30 or even more parameters can be captured simultaneously from one cell with more advanced instruments allowing such high-dimensional analyses [5].

A brief introduction into EV flow cytometry

As mentioned above, flow cytometry can be performed in a relatively simple way with cells. However, it becomes much more difficult when EVs are sought to be analyzed due to various reasons. Cells can be as small as 2-4 micrometers (such as platelets), and they also can have diameters of 20 µm (e.g. monocytes) or even be bigger. Extracellular vesicles, however, are much smaller and subsequently much more challenging to analyze by flow cytometry. The diameter of an exosome-sized EV (~100 nm) is about 100-fold smaller than a 10 µm-sized leukocyte (Fig. 2). This means that the surface area of a small EV is about 10,000 times smaller and its volume approximately 1 million times smaller than that of a typical cell [6]. As already mentioned, flow cytometry "only" detects the light properties of

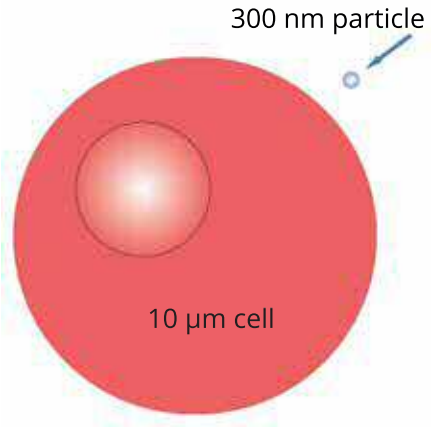

Figure 2: *This figure illustrates the size ratio between a 10µm sized cell and a 300nm small particle. It impressively underlines which special requirements in terms of sensitivity flow cytometers have to meet in order to be able to detect these smallest particles.*

events, and thus it is clear that very small particles with diameters beyond the wavelength of visible light (~380-700 nm) are very challenging to detect [7]. Those events scatter only very small amounts of light compared to cells, and only few antibodies can bind to a single EV, meaning that that the sensitivity of the method required to detect such small amounts of light will require methods or instruments with very high sensitivity. It is therefore also understandable that older, conventional flow cytometers, which have lower photosensitivity due to their hardware specifications, can only recognize significantly fewer extracellular vesicles with a bias towards brighter or bigger events than newer devices that are significantly more sensitive. A comparison between various conventional flow cytometers published in 2018 demonstrated that only 21 out of 46 flow cytometers tested could detect EVs in a range between 600 - 1,200 nm and not smaller [8]. Therefore only few instrument are sensitive enough to detect particles below sizes of around 300 nm [9] and thus would only be suitable to analyze a subfraction of the submicron particles in a given sample. Consequently smaller events would be just missed and would not be detected above background levels in that case. In worst case, one would just detect the 'tip of the iceberg' and miss all other EVs that are just below the limit of detection when using conventional and rather insensitive instruments.

Sometimes EVs can come in swarms

Another critical factor is the design type of the flow cytometer and the inherent increased likeliness of EVs 'coming in swarms' which can lead to data misinterpretation as you will read below. Depending on the flow cytometer being used, the generated laser beam can have a height of up to a few micrometers. If cells of a size of about 10 µm pass the laser beam, it is relatively likely that only one cell passes the laser beam during laser excitation, and thus will be recognized as a single cell. For EVs this is much different. They have a size that is far below the height of the laser beam. Consequently, several particles or EVs can pass the laser beam at the same time especially when the sample is relatively high concentrated, but the computer does not recognize the incoming signal as a signal of several particles, but instead interprets the signal as one single 'event'. This effect also called coincidence and in severe cases 'swarm effect' [10, 11]. The swarm effect is thus not only responsible for not correctly reproducing the exact concentration of EVs per unit volume, but also for detecting signals received from individual EVs. Several events with different fluorescence characteristics are not detected as individual events and therefore fluorescence signals of all particles measured as one event are cumulatively quantified, resulting in higher fluorescence intensities. Thus, suddenly particles in a sample containing distinct populations of EVs with exclusive marker expression can be detected as double positive events which actually do not exist. However, coincidence or swarm detection can be avoided by relatively simple means, namely by measuring unknown samples in serial dilutions. If the dilution factors lead to the expected reduced number of measured EVs, the risk for coincidence or swarm detection is minimized and one can make sure that samples are analyzed while being in a linear range of signal detection for the respective sample type

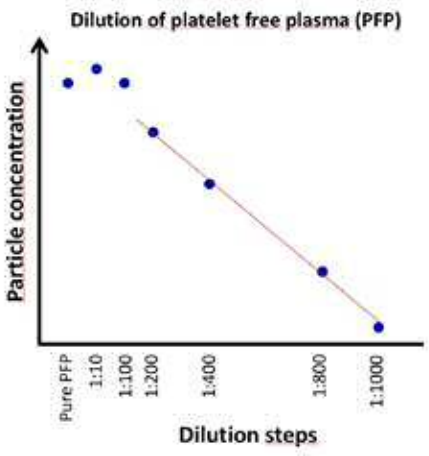

Figure 3: *Samples were measured for 1 minute. The total amount of detected particles was calculated. As can be seen the amount of particles are identical between pure PFP and 1:100 dilution which clearly indicates coincident events or swarm detection. Further dilution from 1:200 up to 1:1000 shows the number of particles in a linear range corresponding to the dilution steps.*

(Fig. 3). Thus, samples should always be measured at the lowest concentration possible. In addition, to avoid coincident events and to improve instrument sensitivity, EV measurements should generally be performed at the lowest possible measurement/flow speed. Higher measurement speeds often also increase the diameter of the core stream or sample stream which may increase the likeliness of coincidence detection to happen [10]. When using imaging flow cytometry (IFCM) for EV analysis different options how to process the images and quantify the number of distinguishable dots per event/image allows for additional accuracy to quantify coincidence or only include true single events in the EV quantification [9, 11].

Higher sensitivity also comes with higher background

Another source of potential errors that can lead to data misinterpretation can come from fluorescently labelled antibodies when using them to detect EV

surface markers. When staining cells with antibodies, it's relatively easy to detect signals since in most cases quite some antigens of a certain surface protein are available on the surface of one cell, and it's relatively easy to wash away unbound antibodies by slow-speed centrifugation. Also, protocols for measurement of cells make it quite easy to exclude small particles or 'background' from the analysis just by setting a region to define the main cell population, or a threshold. When antibodies, however, are sought to be used to detect surface markers on EVs, this can lead to certain limitations. First of all, the composition of surface proteins present on the surface of EVs often resembles the surface protein signature of the cells releasing them. For example, EVs derived from platelets carry platelet-related surface markers such as CD41, and EVs released from red blood cells also carry erythrocyte-specific surface markers such as CD235a or GlycophorinA (Fig. 4). However, because the surface of EVs is significantly smaller than the surface of cells, significantly fewer antibodies can be bound to the surface of a single vesicle. This, in turn, means that the fluorescence detection of the instrument used has to be sensitive enough to detect the signals of only few fluorochromes, or relatively few antibodies bound to a single EV. As mentioned before, several commercially available flow cytometers are sensitive enough to achieve this [9, 12-15].

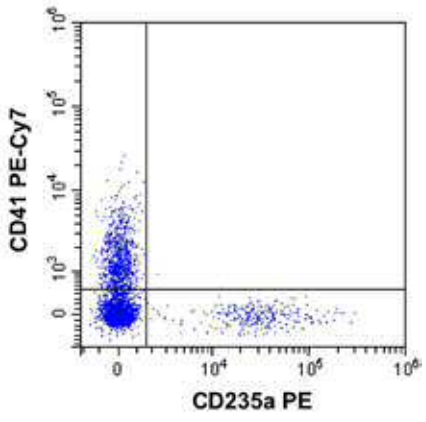

Figure 4: *Platelet free plasma (PFP) was stained with AlexaFluor647-labelled Lactadherin as well as with PE-Cy7-labelled anti-CD41 antibodies recognizing platelet-derived EVs and PE-labelled anti-CD235a antibodies specifically recognizing red blood cell derived EVs. Triggering signal was set according to the Lactadherin negative control sample (Data not shown). Lactadherin positive events were gated and subsequently analysed for CD41 and CD235a surface expression. PE and PE-Cy7 are two different fluorochromes which result in distinguishable emitted light after laser excitation.*

However, this increased sensitivity and therefore the really low detection limit leads to another potential pitfall for such antibody-based assays: Commercially available fluorescence-conjugated antibodies presently are not validated for EV flow cytometry, and different groups have reported that the usage of such antibodies can lead to a considerable amount of background which is probably related to protein complexes or antibody aggregates [9, 16, 17]. To account for this, it is therefore essentially required to include so-called 'buffer controls' containing the antibodies in PBS at the same concentrations as in the sample of interest (but without any EVs), and it is also strongly recommended to centrifuge or filter antibody solutions before usage to reduce this kind of background. Other recommendations for example comprise a detergent lysis control to demonstrate that actual membranous vesicles are measured [9, 18-20].

Does size matter?

The brief answer to this question is definitely yes. Firstly, differences in size can relate to different EV subsets with distinct functions, e.g. small EVs like exosomes or small microvesicles might be more relevant for a therapeutic or diagnostic approach than bigger EVs. Secondly, knowing the accurate size of EVs in a sample or the size range a certain method can measure can be very important to make appropriate conclusions and to compare data between labs or instruments. EV size nowadays in most cases is measured by a method called Nanoparticle Tracking Analysis (NTA) which is based on the detection of scattered light of particles and their movement in solution [21]. In the last decade, there also have been several studies using polystyrene or silicate beads to set size gates for EV flow cytometry experiments [22]. Meanwhile it is well known in the field that a proper size determination by simply using such beads as reference material is not valid since the refractive index, which describes the light scattering properties of a particle, drastically differs depending on the material and thus is very much different between a polystyrene (PS) bead and a biological vesicle. For example, Chandler et al. demonstrated that – in terms of their light scattering properties - 0.4 µm PS beads reflect EVs with a size >1µm [23]. Therefore, currently available beads have a refractive index which, depending on the type of beads used, is more or less far away from the refractive index of biological material, making accurate sizing almost impossible. Of note, two recent studies have described how measured scatter signals can be converted to absolute size by using appropriate scatter calibration standards and modelling based on Mie theory [13, 24]. Another group recently showed that viruses might represent another promising biological reference material for EV size calibration [22]. Taken together, these works demonstrate that it is generally possible to combine multiparameter fluo-

rescence detection with absolute sizing for single EVs by flow cytometry, which overall underlines the promising potential of EV flow cytometry.

In addition, calibration of fluorescence intensities also has come up relatively recently in the EV field and is required to achieve a generally great comparability between results from different labs or generated with different methods or instruments [9, 14]. In relation to that, it has become clear that the field highly desires more suitable or improved reference and calibration materials such as beads made from more EV-like materials or biological reference materials [9, 25, 26], since most materials being used nowadays were designed and produced for cell-based flow cytometry assays. In a recent publication for example, we have used GFP-tagged EVs as biological reference material to optimize a method called imaging flow cytometry for the accurate analysis of single fluorescent EVs [9]. Nowadays, there is consensus in the field that EV flow experiments require detailed reporting of all steps and parameters, calibrated instruments, and the inclusion of all recommended controls.

Pitfalls and limitations all over? Still, it's worth it!

Despite all the aforementioned pitfalls and limitations, and despite all required controls, validations and calibrations, why would someone aim to use flow cytometry for EV analysis? The answer is relatively simple: Because the prospects, promises and the potential of EV flow cytometry are worth it. The most important advantage of flow cytometry lies in high throughput single EV analysis and in the possibility of simultaneous detection of different antigens on the surface of thousands of single particles in a relatively short time. In contrast, other methods often only offer bulk analysis or are not very quantitative. Thus, EV FCM makes it possible to rapidly detect co-expressing markers and quantify both the frequency of such an EV population volumetrically and their brightness in terms of absolute units of fluorescence. For illustration, the following approach has been adopted as a standard method in the core facility in Vienna: Some of the cell membrane-derived EVs are endowed with a protein on the cell surface called phosphatidylserine (PS) [27]. PS can be detected in relatively simple ways by flow cytometry by using fluorescent probes. However, as already mentioned, the surface of EVs is relatively small and enough expressed fluorescence labelled PS molecules must be excited, so that an appropriately measurable signal occurs. If this signal exceeds a certain threshold and thus lies within the detection range, this signal is used as a primary input signal for the detection of all other bound antibodies (Fig. 4). But even with this standardized method we have to compromise for now. On the one hand we need the mentioned signal above the detection limit for the method and instrument we use, on the other hand we use this method to capture only a part of the EVs. Based on previous reports, about 70% of all EVs do not carry PS on their surface and therefore cannot be detected by the method described as such [27]. Obviously, these PS negative EVs can be just as important and should be included in this analysis ideally. Ongoing approaches attempt to include these PS-negative EVs by using dyes that are either incorporated into the particle membrane or absorbed by the particles. The absorbed, initially non-fluorescent substances are then converted into a fluorescent dye via active enzymatic processes within the vesicle. Unfortunately there is no "universal" dye for EV staining available yet and therefore also the usage of such dyes has limitations. However regardless of the limitations, the combination of such dyes and the PS might allow the determination of a larger amount of particles. This example demonstrates that generally assays can already be used even despite limitations, but there are always ideas and potential solutions to improve them.

Conclusion

In summary, several studies using high-resolution flow cytometers have demonstrated proof-of-concept that flow cytometry facilitates the analysis of subsets of extracellular vesicles also down to the size-range of exosomes [9, 12, 14, 15], and also the separation of subsets by flow cytometry based sorting [12, 15] within recent years. The development of more suitable instruments with more sensitive detectors, lower background noise and brighter, more clean and more specific probes will surely further improve such methods in the near future. Despite these advances for flow cytometry based single EV analysis methods, a valid alternative for labs currently lacking access to suitable high resolution instruments could be bead-based flow cytometry assays which are suitable to collect information about the surface markers being expressed on bulk EVs of interest [28, 29]. Also, next generation reference materials including biological reference materials [9, 25, 30, 31] and refined methods for instrument calibration [9, 13, 24] will collectively

contribute to the development of standardized assays which ultimately also will suit the requirement for clinical or diagnostic procedures to detect relevant EV based biomarker for various diseases. Finally, several international societies publish both position papers and framework papers, which are regularly updated and can alert interested people to the ongoing process of EV research [18, 19].

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Extracellular vesicles in plant host-microbe interaction

Constance Tisserant and Arne Weiberg

Extracellular vesicles (EVs) are secreted lipid bilayer membrane particles that are increasingly drawing attention due to their potential role in intercellular communication. EVs have been mainly reported in mammalian systems, but are also found in non-mammalian classes, such as Archeae, bacteria, fungi, oomycetes, protozoa, invertebrates and plants. Over the last decade, EV research on mammalian systems has been massively advanced driven by the interests and applications of the biomedical field, while research on non-mammalian EVs that aims to understand the biological origins and functions of EVs remains rather descriptive and premature. Nevertheless, recent pioneering works resulted in novel concepts that place EVs carrying regulatory small RNAs as central players in inter-species and cross-kingdom communication with emphasis on host-pathogen, host-parasite and host-microbiome interactions. EVs transport small RNAs from microbe/pathogen/parasite to animal or plant hosts, and vice versa, which results in the manipulation of host immunity or microbial virulence, respectively. This article highlights some of the latest discoveries regarding EV-mediated communication across species and kingdoms with a special focus on plants and their interacting microbes.

Keywords: *Extracellular vesicles, outermembrane vesicles, bacteria, fungi, plants, inter-species, cross-kingdom, small RNAs*

Introduction

In 2015, a satellite workshop at the International Symposium on Extracellular Vesicles (ISEV) in Sao Paulo, Brazil, emphasized on the potential significance of EVs in cross-kingdom communication in hosts, microbes and parasites (1). As an important conclusion of this meeting, the necessity to develop robust protocols for EV isolation and the identification of suitable biomarkers for the different types of EVs was pointed out, in order to advance the study of specific roles of EVs in host-pathogen interactions. In August 2018, around 50 scientists from all over the world came together for a mini-symposium on EVs in inter-organismal communication in Munich, at the Ludwig-Maximilians University. The scientific discussions included new findings of EVs discovered in plant, fungal, oomycete, nematode and bacterial systems, as well as exchange of current opinions on how to integrate such discoveries into the current concepts of EVs in inter-species and cross-kingdom communication (2). As two important goals for the future, the participants defined a better understanding of EV biology across kingdoms, and the extension of our knowledge on how EVs participate in plant immunity and in plant-pathogen interaction.

Plants recognise by pattern recognition receptors (PRRs) pathogen molecular patterns located at the cell periphery, which activate cellular immune signalling. Host-adapted plant pathogens secrete small peptides, called effectors that

interact with, and sometimes enter, plant cells to suppress host immunity. In defence, plants evolve nucleotide-binding domain leucine-rich repeat containing (NLR) receptors that perceive pathogen effectors and mount an effector-triggered immunity. To escape recognition, pathogens co-evolve their effector repertoires, leading to a continual molecular arms race between pathogens and host plants (3). During plant-pathogen interactions, pathogen effectors and plant antimicrobial molecules must be transported through the extracellular space between plant and pathogen cells, without getting degraded or diluted, to be functional. Extracellular vesicles (EVs) fit perfectly to take over this function (Figure 1). Indeed, EVs mediate intra-organismal cell-to-cell communication in mammals with impact on disease progression, cancer development as well as on immune activation (4). Important to note, the biogenesis of EVs is conserved over the entire tree of life including Archaea, bacteria, fungi, oomycetes, protozoa, animals, and plants. And this fact clearly shows on the one hand the ubiquitous prevalence of EVs in biology, and on the other hand the possibility that EVs serve as communication channel between hosts and microbes with beneficial or pathogenic outcomes (5-8). Here, we discuss the state-of-the-art regarding potential roles of EVs for host-pathogen interaction by highlighting significant achievements and by summarizing recent discussions on conceptual open questions, for instance: Where do EVs come from? What molecules do EVs transport? How are cargo molecules loaded into EVs? How do EVs pass across cellular barriers and extracellular matrices? What is the biological function of EVs in host-pathogen interactions?

Where do EVs come from?

EVs are lipid bilayer particles that are synthesized and released by cells via unconventional secretion pathways (9). In mammals, exosome-like EVs derive from intracellular multivesicular bodies (MVB) that fuse to the cytoplasm membrane and release EVs into the periplasm and the extracellular lumen. A recent study also links EVs of the model plant *Arabidopsis thaliana* to MVBs (10), indicating that biogenesis of EVs seems to be well conserved in plants and animals. Moreover, tetraspanins that are EV transmembrane spanning proteins in mammalian exosome-like EVs are also detected in Arabidopsis EVs (10), suggesting

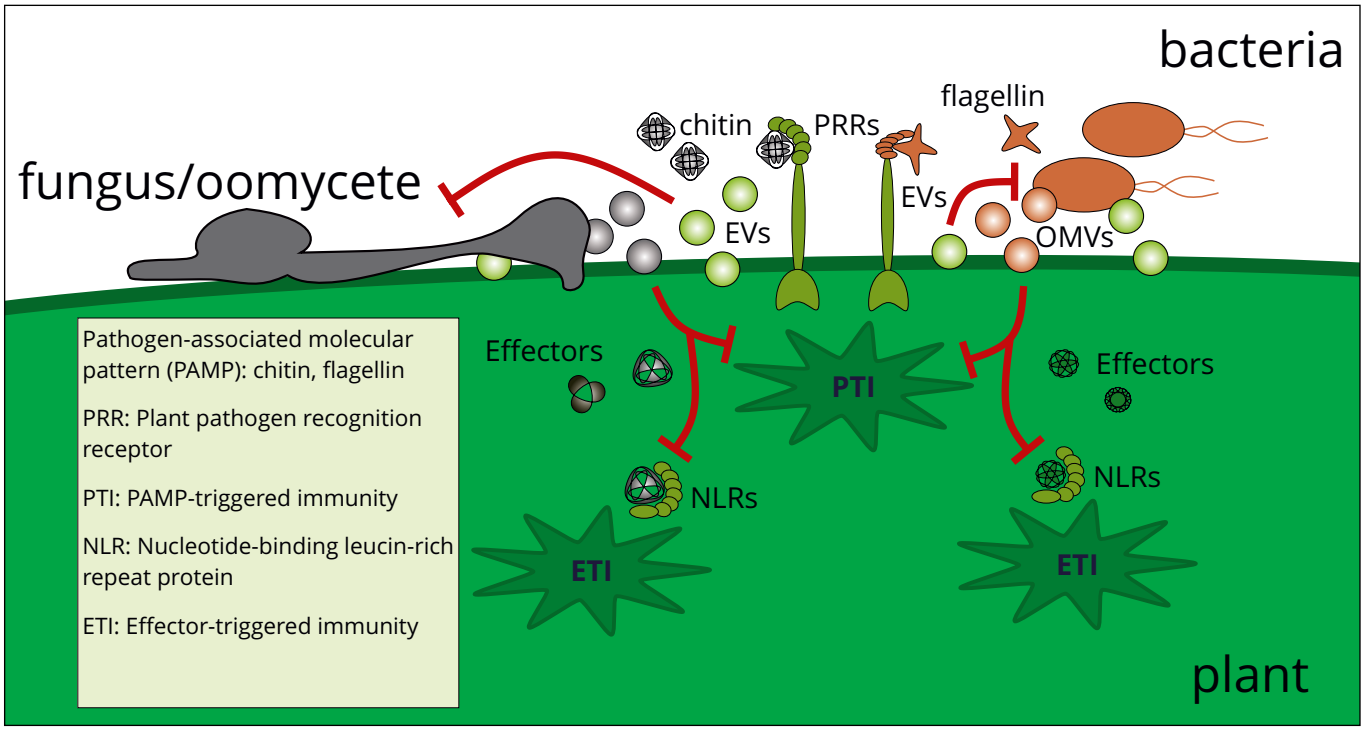

Figure 1: *The potential roles of EVs in plant immunity and pathogen virulence. Upon pathogen attack, plants activate two layers of immunity. First, conserved pathogen-associated molecular patterns (PAMPs), such as fungal chitin and bacterial flagellin, are perceived by cell membrane-spanning pattern recognition receptors (PRRs) that mediate activation of PAMP-triggered immunity (PTI). As part of the defence response, plants release EVs containing antimicrobial substances. Fungal/oomycete and bacterial pathogens secrete plant cell-damaging enzymes and deliver effector molecules into plant cells to suppress plant immunity for infection. Pathogen-released EVs or outer membrane vesicles (OMVs) might deliver plant cell-damaging enzymes and effectors. Plants can recognise pathogen effectors via intracellular receptor proteins (NLRs) and mount effector-triggered immunity (ETI) including release of plant antimicrobial EVs. Pathogen effectors and other EV cargo molecules might counteract PTI and ETI to succeed infection.*

that plant EVs are true exosomes. Another study reported on the syntaxin PEN1 being enriched in Arabidopsis EVs. PEN1 is a known factor of intracellular membrane trafficking and endo/exocytosis, and has been described to participate in plant immune response (11).

In microbes, biogenesis of EVs is inadequately understood. Fungi and oomycetes form MVBs, suggesting that exosome-like EVs may also exist in eukaryote microbes (12). Since bacteria do not possess endomembrane organelles, EV release is considered to appear via outer-membrane budding, thus EVs are often called outer-membrane vesicles (OMVs) (13). Genetic manipulation strategies (e.g. gene knockout) in microbes have not delivered any EV null mutant strain, which could genetically link secretion pathways to EV biogenesis and release, but mutants only exhibited moderate, quantitative differences in EV number or size (14, 15). In fact, EV populations are rather heterogeneous in size, concentration and cargo composition indicating that different secretion pathways of EVs might be in place. And this offers an explanation why a single gene mutation does not consequently lead to a "zero EV" phenotype. To deepen our understanding of EV biogenesis and its connection to the secretory pathways, attempts that combine genetics with biochemical inhibitors that specifically block certain steps in a secretion process are definitely worth trying.

What is transported in EVs?

Over the last years, mass spectrometry and high-throughput sequencing analyses allowed identification of several proteins and nucleic acids, mainly messenger RNAs (mRNAs) and non-coding small RNAs (sRNAs), as EV cargoes. For instance, comparative analysis of EV proteomes from different fungal species revealed common sets of molecular function or biological process, such as cell wall modelling, indicating a conserved role of EVs in fungi (16). Frequently, known virulence-associated proteins were described in EVs isolated from pathogenic bacteria and fungi, suggesting EVs as translocation vehicles for virulence factors towards the host (16, 17). Moreover, antimicrobial proteins have been determined in plant EVs upon bacterial infection (11). Beside proteins, diverse RNA species in bacterial, fungal and plant EVs and have been detected and analysed by next generation sequencing. In particular, gene-regulatory RNAs were discovered and are suggested to mediate gene regulation in cells and organisms that incorporate EV-RNAs during host-pathogen interaction (11, 18). Other types of RNAs were also discovered in EVs, such as mRNAs or long non-coding RNAs that might be functional in host-microbe communication, too. Nevertheless, the importance of EV proteins and RNAs in host-pathogen interactions needs to be explored in greater detail.

EVs in host-pathogen interactions

To test the function of pathogen EVs for infection, host cells and tissues are often treated with pathogen EV extracts in culture. Fluorescence labelling of EVs may allow visualization of EV-uptake by host cells or tissues. Several immunomodulatory effects were found in different mammalian host cell types upon pathogen EV treatment. For instance, EVs of human pathogenic fungi and bacteria can stimulate or repress host immunity by redirecting immunity-related factors, such as cytokines, in macrophages and other immunity-related cell types (5, 6). These examples indicate that pathogens use EVs as virulence factors, while host cells have evolved recognition of pathogen EVs for immune activation. However, from the basic research point of view, *in-vitro* culture assays provide only limited insights into how important EVs are during host-pathogen interaction at the organismal level. To further address this open question, EVs should be studied directly at the host-pathogen interface, with blocking their production or activity during infection to reveal their real biological significance.

EV-mediated delivery of small RNAs (sRNAs) that trigger inter-species or even cross-kingdom RNA interference (ckRNAi) has been documented in animal-parasite and plant-fungal interactions (19). For example, EVs of parasitic nematodes carry conserved microRNAs that mediate type-II immune suppression in mice (20). Moreover, aggressive fungal plant pathogens, such as *Botrytis cinerea*, deliver sRNAs into host plant cells that hijack the plant RNAi pathway to silence host immunity genes for infection (21). However, whether fungal sRNAs are transported into plants via EVs needs to be demonstrated. This phenomenon of ckRNAi is bi-directional, because plants transfer small interfering RNAs (siRNAs) into attacking fungal pathogens, too, that suppress fungal virulence genes. Hereby, plant siRNAs are transported into infecting pathogens by exosome-like EVs (9). Plant host-induced gene silencing (HIGS) reflects yet another RNAi-based communication between plants and pathogens

and is a tool to engineer crop plant resistance against notorious plant pathogens/pests (22). HIGS has been employed even before the discovery of natural ckRNAi, inspired by environmental RNAi found in nematodes (23). How important EVs are for the delivery of plant HIGS RNAs into pathogens/pests is not known, yet. Taken together, these observations suggest that EVs are a central player in inter-species and cross-kingdom RNA communication (Figure 2).

An interesting new direction of EV-mediated communication during infection is reported at the microbial population level. EVs play a functional role in the "division of labour" phenomenon observed in *Cryptococcus neoformans var. gattii* that is critical to activate infectivity. In this regard, EVs are a communication channel between phagocytosed cryptococcal individuals and the extracellular Cryptococcus population (24). Moreover, recent reports indicate that microbiota-derived vesicles might be protective against diseases in the gut system (25). Furthermore, diet-consumed plant-derived exosome-like EVs (sometimes called dietary EVs) might be taken up by the gut microbiota with effects on the host well-being (26). These latest observations open new perspectives onto EVs as pluripotent multi-organismal communication media to direct microbial organisation connected to plant and animal host fitness.

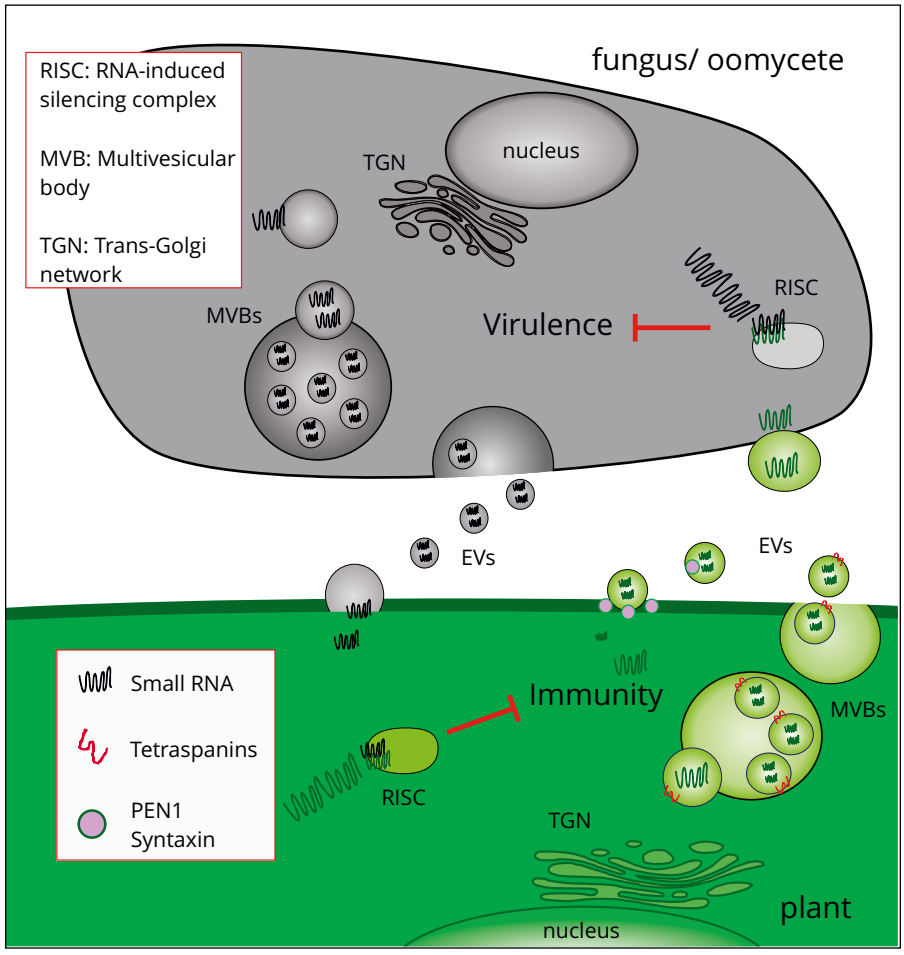

Figure 2: *The role of EVs in cross-kingdom RNA communication. During infection, eukaryotic pathogens including fungi/oomycetes secrete EVs loaded with small regulatory RNAs. Fungal/oomycete/plant EVs are likely secreted from the Trans-Golgi network (TGN) via multivesicular bodies (MVBs). Secreted small RNAs of fungal plant pathogens can enter plant cells and hijack the host RNA-induced silencing complex (RISC) to suppress important immunity genes. As part of the host defence, plants release antimicrobial EVs containing small RNAs. Antifungal EVs contain tetraspanins integrated into the vesicle membrane. Other types of antimicrobial plant-secreted EVs include the syntaxin PEN1 that also accumulates at the local cell sites of fungal and oomycete infection. Antifungal exosome-like EVs carry plant siRNAs that can induce gene suppression of virulence genes in the attacking fungi/oomycetes.*

How do EVs pass cellular barriers?

Cellular release of EVs by bacterial, fungal and plant cells raise the question of how do lipid membrane particles of 50-500 nanometres in diameter pass complex cellular barriers, such as the periplasmic matrix and the cell wall. Is the plasticity of the cell wall way higher than formerly expected? The shape and size of EVs might be flexible as well, thus, EVs might fit through cell wall pores and channels despite the EV size (27). This is supported by observations made by scanning EM showing the transfer of AmBisome® particles, a commercial antifungal liposome formulation, through the fungal cell wall (28). EM tomography at the fungal-plant interface revealed multiple morphologies of extracellular lipid formations, from round-shaped individual particles to interconnected tube-like membrane structures and networks (29, 30). Cell wall remodelling enzymes that have been identified as part of the EV cargo in fungi might break down local cell wall structures to facilitate passage of EVs in particular through these tight matrices. Finally, plant pathogens, such as *B. cinerea*, release cell wall-degrading enzymes during host colonization, which soften the plant cell wall and thus might promote fungal EV entry into plant cells. Other types of plant-interacting microbes can form intimate cell/cell contacts with

accommodating host plants, at which cell walls are largely removed, such as the haustorium of pathogens, the arbuscle of plant-mycorrhizal fungi, or the nodule of nitrogen-fixing bacteria. These interfaces enable extensive bi-directional molecular exchanges, possibly through microbial and plant EVs.

Conclusion

EVs attract growing attention in the field of molecular plant-microbe interactions. The transport of pathogenic proteins and sRNAs into host plant cells by EVs offers an answer to a longstanding question regarding the mechanism of effector delivery. Moreover, EVs are presently secreted by all types of living cells, pathogenic or not, and EV-transmitted regulatory RNAs can serve as a unique message understood by all life forms, as it largely relies "only" on nucleobase pairing (18).

In order to advance our understanding in the biological function of EVs in host-microbe interactions, the development of reliable protocols and standards for EV purification, visualization, and identification via proper EV biomarkers as well as the analysis of their molecular composition are required. Only then, investigation of EVs in situ will become a key to decode the communication code of EVs between cells to achieve accommodation, infection, or immunity that will extend our views on EVs in host-microbe interactions.

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Advancing extracellular vesicle research globally. Our vision is to be the leading advocate and guide of extracellular vesicle research and to advance the understanding of extracellular vesicle biology.

Activities:

- The Journal of Extracellular Vesicles, the leading journal for EV research. More than 300 articles have been published in JEV as of 2019, and the journal recently received its first journal impact factor of 11.0.
- ISEV organizes various initiatives such as Education Days and massive online open courses (MOOCs). See Basics of Extracellular Vesicles and their role in health and disease www.coursera.org/learn/extracellular-vesicles
- ISEV leads rigor and standardization efforts by holding workshops and through publication of position papers and guidelines, including the recent MISEV guidelines.
- ISEV maintains a strong social media and conducts regular email campaigns to provide a space for interactions between scientists.
- Contacts with other EV related societies are cultivated, for example, with a "Meet the National Societies" event at the annual ISEV meeting.

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- ISEV holds an annual meeting, rotating between the three geographical chapters: Asia/Pacific, Europe/Africa, and the Americas.
- Two or more workshops on specific themes are held each year, with approximately 50 attendees chosen from applications.
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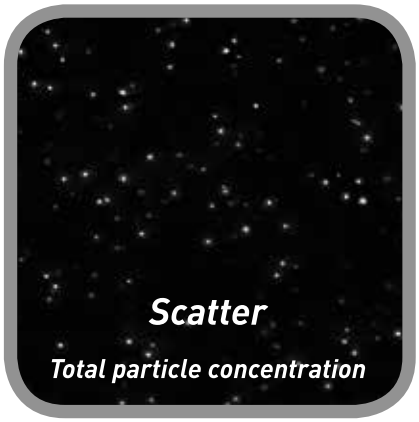

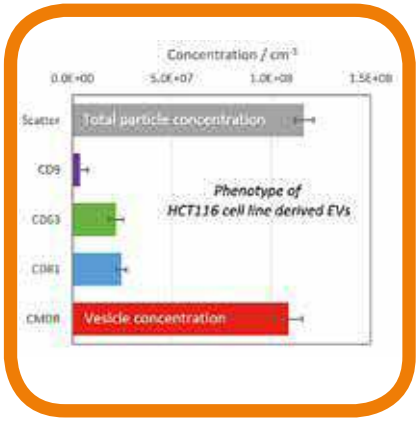

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