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Extracellular Vesicles and Circulating Nucleic Acids

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Extracellular Vesicles and Circulating Nucleic Acids

Conference Report

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Meeting report of the 4th autumn meeting of the German Society of Extracellular Vesicles (GSEV): cutting edge EV research driven by young scientists

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INTRODUCTION

The German Society for Extracellular Vesicles was delighted and excited to conduct its yearly autumn meeting on October 2nd and 3rd 2021 in Freiburg/Breisgau, a charming university town at the foot of the Black Forest. Organized by Irina Nazarenko and her team, it was one of the first in person meetings in Germany since the start of the pandemic in accordance with current coronavirus safety regulations in the beautiful Paulus Saal in the center of Freiburg old town. Although GSEV held one of the last possible in person meetings in March 2020 (IGLD/GSEV Meeting in Frankfurt) as well as conducted a virtual meeting in autumn 2020 (organized by Gregor Fuhrmann, Saarbrücken), it felt like a long time not having

interacted. Consequently, the 150 participants felt a vibrant atmosphere and the air of excitement, having the opportunity to meet again in person and discussing the latest developments in extracellular vesicle (EV) science.

The GSEV autumn meeting traditionally has the intention to actively involve and bring into focus EV scientists at the early career stage and to drive interactions at the current frontiers of EV research. To this end, five keynote lectures of invited internationally renowned EV scientists were combined with 25 presentations of young scientists selected from the abstracts and 9 presentations of technological innovations provided by industrial sponsors. The program was framed by two poster sessions (displaying 27 posters), an industrial exhibition, a social event with Mexican folklore in the evening, and a final round table discussion on the value of the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines and pitfalls of EV research. The best two presentations and three posters were awarded with the ***Margot Zöller Prize*** and ***Peter Altevogt Prize***, respectively. These two renowned researchers, who pioneered the EV research in Germany, enthusiastically sent short encouraging messages to the prize winners and the audience. Thanks to all participants, the organizing team, and the supporting stuff, the meeting provided an enjoyable, lively atmosphere, the perfect prerequisite for productive discussions. In the following, we report on the six scientific sessions and summarize their main scientific content including topics of discussion.

EV ORIGIN AND BIOGENESIS

The scientific program of the meeting started with a session on EV origin and biogenesis chaired by Eva-Maria Krämer-Albers (Mainz, Germany) and Melanie Schwämmle (Freiburg, Germany). The kick-off keynote lecture was given by Graca Raposo (Institut Curie, Paris, France), who took the audience on a journey through the history of EV science, from its roots to the most recent findings of her group on EV functions in development. Raposo[1] pioneered the field in the 1990s by her discovery that B cell-derived EVs carry MHC-class II and present antigens that can activate T cells. This discovery showed for the first time that EVs can deliver signals to other cells and, thus, paved the way for the whole field. More recently, her team found that EVs in the skin mediate bidirectional communication between melanocytes and keratinocytes regulating skin cell differentiation, pigmentation, and homeostasis[2]. Moreover, the team used *Drosophila* as a model to explore EVs shedding from epithelial microvilli *in vivo*. Engaging the protein Prominin, these small EVs apparently bud from microvilli and are loaded with Hedgehog, essentially being involved in the development of the *Drosophila* wing imaginal discs. Finally, Graca took the opportunity to dedicate her presentation to the 80th birthday of Philip Stahl, who together with Harding and in parallel to Johnstone discovered the exosome secretion pathway in maturing reticulocytes[3,4]. Although EV structures had been mentioned before in the literature, these discoveries initiated and mark the beginning of modern EV research.

The session continued with selected presentations from early career researchers. Wolf (Paracelsus Medical University, Salzburg, Austria) presented new work on the protein corona, which appears to form on the surface of EVs according to their biological environment and influences EV target cell interaction and function. EVs isolated from human placental expanded stromal cells (PLX) by tangential flow filtration (TFF) have an intact protein corona, which can be visualized by super-resolution microscopy and transmission electron microscopy, providing pro-angiogenic activities. However, when PLX-EVs were precipitated by ultracentrifugation or size exclusion chromatography, the protein corona was affected and proangiogenic activities were abolished. Intriguingly, the EV protein corona could be reconstituted by adding defined pro-angiogenic corona components, which also restored the ability of these EVs to promote *in vitro* angiogenesis[5]. These findings imply that molecules contributing to the EV corona importantly contribute to EV functions. Furthermore, EV isolation procedures may affect the integrity of the corona and

alter EV functionalities.

Next, Jochen Hernadez Bücher (MPI for Medical Research, Heidelberg, Germany) explored whether cells employ the presentation of ligands on secreted EVs to enhance their signaling properties (described by the term *vesicle-induced receptor sequestration*). Synthetic EVs that were engineered to expose ligands such as FasL and RANK on their surface indeed transmitted their signals more than 100-fold more efficiently than non-EV-bound ligands. Based on diffusion simulations using the immunological synapse as a model, the authors proposed a universal mechanism by which EVs allow for the concentration of ligands promoting local aggregation of membrane receptors facilitating signaling at the EV-cell interface. This could represent a unique structural profit of EV-mediated signaling.

The remaining two scientific presentations of the session focused on the biogenesis of small EVs (sEVs), especially of exosomes within the late endosomal compartment. Kerstin Menck (University Hospital Münster, Germany) outlined the role of the adapter protein CD2AP during different steps of sEV biogenesis and cargo sorting. Interfering with CD2AP expression in a human breast cancer cell line resulted in reduced levels of CD63 and Syntenin in sEVs recovered from the culture supernatant. Moreover, CD2AP overexpression increased CD63 and Syntenin levels in sEVs. Analysis of the endosomal trafficking of CD63 revealed that CD2AP knockdown was accompanied by an accumulation of CD63 in LAMP2-positive compartments, indicating that CD2AP assists CD63 trafficking through this compartment. Furthermore, knockdown of CD2AP diminished the presence of CD63 and Syntenin in large artificial endosomes generated by overexpression of a constitutive active form of the GTPase Rab5 (Rab5-Q79L), while overexpression had the opposite effect. CD2AP is known to directly interact with Alix and thus may act as an adaptor protein for the selection of cargo proteins of exosomes generated in the Syntenin-Alix pathway. Consistently, experimental reduction of Alix expression counteracted the CD2AP functions observed in exosome biogenesis. For her excellent talk at the symposium, Kerstin Menck was awarded with the Margot Zöller Prize.

Barnabas Irmer (University Hospital Münster, Germany) presented a proteomic profiling approach to identify Syntenin-dependent sEV cargoes. Since Syntenin is overexpressed in different types of tumors, Syntenin-dependent modulation of the EV cargo may have functional effects contributing to tumor pathology. sEVs collected from Syntenin-knockout cells revealed a considerable difference in their protein cargo when compared with wild-type EVs, while the cargo of large (l) EVs was similar. Proteins downregulated in Syntenin-deficient sEVs were largely associated with processes of cell-adhesion, extracellular matrix assembly, and cell-cell contacts. Notably, the tumor-antigen-epithelial cell-adhesion molecule (EpCAM) was shown to directly bind to Syntenin by surface plasmon resonance spectroscopy and, consequently, was also lacking in Syntenin-deficient sEVs. Thus, Syntenin levels affect sEV cargo including loading with adhesion receptors, which may modulate the tropism and other signaling functions of tumor cell-derived sEVs.

In summary, the lesson that we may have learnt from this session is that the dynamics and diversity of EVs, which puzzled us from the early days of EV research, is now being unraveled at the molecular and functional level. Deciphering the mechanisms regulating EV cargo composition, whether inside EVs, presented on the membrane, or associated with the surface in form of the corona, are key to understanding EV signaling functions.

The session closed with two talks from industrial sponsors, providing insight into recent technological developments. Siobhan King (ONI/Oxford Nanoimaging Ltd, UK) presented a high-resolution microscopy

platform, a dSTORM device, allowing characterization of EV composition and EV heterogeneity at the single molecule level. She also presented a newly developed software platform providing a set of different analysis and quantification tools. Aquiles Carattino (Dispertech B.V., NL) introduced a system for measurement of both size and scattering intensity of single EVs at a high resolution within the size range of 20-150 nm. The system provides a convenient table-top technology for measuring single EVs as it works label-free, requires only 5 µl of sample volume, and appears to deliver accurate and reproducible results with biological samples.

ENABLING TECHNOLOGIES: REFERENCE MATERIALS, PURIFICATION AND ANALYSIS

The second session focused on innovative technologies allowing EV analyses and preparation and was moderated by Michael W. Pfaffl and Jan Lueddecke. The session was opened by the keynote lecture from Marcella Chiari from the Institute of Chemical and Technological Sciences of the National Research Council of Italy. She presented her recent developments using integrated systems for the isolation and later characterization of EVs from a broad spectrum of matrices or liquid biopsies. Thus far, EVs have prospective potential in the medical field, but there is no consensus about the "best and most integer" isolation method to receive intact and functional EVs. Within two EU-funded HORIZON 2020 projects [INDEX (integrated nanoparticle isolation and detection system for complete on-chip analysis of exosomes) and MARVEL (evolving reversible immunocapture by membrane sensing peptides towards scalable EV isolation)], new separation methods for larger and smaller EVs from cell culture supernatants and complex biofluids were developed by Marcella's group. She described a novel approach for affinity isolation and *in situ* enrichment of EVs from plasma allowing separation of concentrated, pure, and intact EVs. The released EVs were subjected to a multi-parameter interferometric analysis providing information on vesicle size, number, and phenotype. Based on single-stranded DNA tagged antibodies, EVs were captured on the surface of complementary DNA strand-loaded magnetic beads. Following binding of the EVs to the bead surface, they can be released from the beads by DNase I digestion. Using this immunocapture strategy, retained EVs were prepared from complex biological fluids. The conditions were mild, such that they preserved the EVs' integrity and functionality. Furthermore, the captured EVs can be characterized by various methods (nanoparticle tracking analysis, electron microscopy, and flow cytometry). Furthermore, Marcella presented a second new EV capturing method that binds EVs directly to a surface of microchips. This method is based on a membrane sensing peptide that serves as a highly efficient ligand independent of the composition of EV surface proteins. In particular, bradykinin-derived peptide baits were used to capture EVs on biological matrices. Captured EVs were characterized by single particle counting and sizing. Furthermore, their CD9, CD63, and CD81 content was analyzed with fluorescent anti-CD9, anti-CD63. and anti-CD81 antibodies, respectively.

The second talk was presented by Christian Preußer from the Institute for tumor Immunology located at the Philipps University in Marburg. He is head of the newly established and first EV Core Facility in Germany[6]. The scientific goal of the core facility is to interface various scientific research groups working with EVs at the medical campus , unravel EV-mediated communication processes within the tumor microenvironment, and demonstrate their impact on tumor development and progression. Christian presented the actual methodological portfolio and techniques implemented in the last months and the near future. Thus far, various techniques being used in EV research have been optimized and are now successfully used in the facility. Additional technologies considered as powerful tools in EV research will be further included. Christian elaborated on free flow electrophoresis (FFE), a technology being provided by FFE Service (Feldkirchen, Germany), a well-established semi-preparative method to separate and prepare analytes, e.g., by inherent differences of their electric charges. FFE combines a flow driven longitudinal transport of sample material with vertical electrophoresis and allows separation of sample components into

up to 96 different fractions. Protocols are currently optimized for the EV preparation and characterization in a collaborative approach among FFE Service, the core facility in Marburg, and the group of Bernd Giebel in Essen. FFE appears suitable for the purification and characterization of EVs from all types of biofluids or bacteria. Furthermore, the EV Core Facility offers state-of-the-art techniques, such as single vesicle characterization and phenotyping. Through networking with other omics core facilities at the campus, an in-depth and complex molecular characterization of EVs can be offered.

During the remainder of the session, talks were given by young scientists, presenting their PhD or postdoc projects. Yanis Mouloud from the Institute for Transfusion Medicine of the University Hospital Essen introduced a novel, TERT-based immortalization strategy that allows immortalization of human mesenchymal stromal cells (MSCs) for the scaled MSC-EV production. Using this technology, Yanis raised different MSC lines at the clonal level. Connected to their immunomodulatory properties, MSC-EVs are considered as therapeutic agents for many diseases. Previously, his group conducted the world's first MSC-EV application in a human; they successfully treated an otherwise treatment-refractory graft-*vs.*-host disease (GvHD) patient with a self-produced MSC-EV preparation[7] and confirmed the therapeutic potential of MSC-EV products in several animal models. Observing significant variations in the immunomodulatory activity of independent MSC-EV preparations, the clonally expanded immortalized MSCs (ciMSCs) should help avoid product heterogeneities arising from the usage of MSCs of different donors and replicative senescence processes of primary MSCs. Yanis demonstrated that his ciMSC-EV products retain the immunomodulatory potential that the group originally described for EV products of primary MSCs. Currently, ciMSC-EV products are being tested in various disease models. Ideally, in the future, ciMSCs can be used for the scaled-manufacturing of therapeutic *off-the-shelf* MSC-EV products for clinical application.

In the following talk, a new EV isolation technique was presented by Dapi Menglin Chiang working at the Department of Animal Physiology and Immunology, the School of Life Sciences, Technical University of Munich in Freising Weihenstephan. EV isolation is a time-consuming process and is often accompanied with a high amount of lipoprotein contamination. Hence, a fast isolation method with high purity of functional EVs is urgently needed, among others, for various diagnostic and clinical applications. Furthermore, the functional properties of the isolated and contamination free EVs should be maintained. The new EV isolation via EXÖBead® is functionally based on a galectin-glycan recognition. EXÖBead® are magnetic beads coated with galectins that bind EV glycan residues with high affinity. Furthermore, EVs can be easily eluted functionally intact from beads by high concentration of lactose. Isolated EVs showed high abundance of specific EV markers such as CD9, CD63, CD81, and TSG101 in flow cytometry. Compared with other isolation methods, such as size exclusion chromatography (SEC), EXÖBead® isolation EVs resulted in low apolipoprotein A1 contamination. To prove the clinical application, Dapi and his team isolated and analyzed tumor-derived plasma EVs (TDEs) from patients suffering from head and neck squamous cell carcinoma (HNSCC). TDEs are involved in the HNSCC pathogenesis, such as tumor progression and immune evasion. In the clinical samples, HNSCC biomarkers such as EpCAM, PanCK, and PD-L1 were identified on CD45- plasma-derived EVs. Significant difference of TDE immune suppression ability were observed, compared to a classical polyethylene glycol isolation (PEG; ExoQuick®). Furthermore, the EV functionality of the EXÖBead® could be shown in a peripheral blood mononuclear cells activation assay. In summary, the new EXÖBead® isolation is an easy-to-use method with low lipoprotein contamination that maintains the functional abilities of EVs.

The focus of the next presentation, delivered by Josepha Roerig from the Institute of Pharmacy at the Medical Faculty Leipzig, was the uptake and transport of milk-derived EVs across an intestinal barrier model via Caco-2 cells. In recent publications, bovine milk-derived EVs are described as promising oral

drug delivery systems, since they withstand the harsh conditions of the gastrointestinal tract. However, the key factors regarding the milk EV uptake and ability to pass the intestinal barrier is still not understood. The presenter addressed these aspects by *in vitro* standardization approaches. Milk EVs were enriched using differential centrifugation followed by SEC and characterization according to the MISEV 2018 guidelines, using cryo transmission electron microscopy (Cryo-TEM), nanoparticle tracking analysis (NTA), and numerous EV marker proteins. Uptake properties of milk EVs were compared to liposomes in Caco-2 cell culture. Three different EV labeling technologies were benchmarked to obtain reliable and stable EV staining results. Milk EVs outperformed liposomes regarding uptake efficiencies. Furthermore, confocal microscopy revealed the internalization of milk EVs, whereas liposomes rather remained attached on the Caco-2 cell surface. Josepha and coworkers provided the foundation for future investigations of milk EVs for oral drug delivery purposes and for the appropriate comparison with liposomes.

The last talk in this session was given by Hui-Yu Liu working at the Institute of Nanotechnology at Karlsruhe Institute of Technology. Her topic was a new isolation technique using tailored lipid membranes to capture EVs. She presented the use of antibody-functionalized lipid patch arrays for rapid, highly selective and sensitive detection of EVs from complex media. Scanning probe lithography techniques offer unique opportunities for highly localized chemical surface functionalization to bind biomolecules, e.g., tailored supported lipid membranes (SLM). The SLM obtained are single- to multilayer stacks of biomimetic phospholipid membranes that can be tailored for shape and function by the printing process and admixing of functional lipid compounds. This allows, e.g., to alter charge or mechanics of the membranes or to introduce selective binding sites for the capture or presentation of antibodies, e.g., to bind EVs. By exploiting the natural process of vesicle fusion, the use of SLM patch arrays can greatly facilitate the EV detection process and retain the information on the array for downstream analysis.

The session was completed by two industrial talks presenting their latest technologies for EV isolation and characterization. Ingrid Bloß from Particle Metrix GmbH presented results from colocalization experiments using a novel multi-fluorescence NTA device (f-NTA). The fluorescently labeled EVs can now be specifically tracked and will provide a deeper and more specific information, e.g., the numbers of EVs containing CD63, CD81, CD9, and CD41 antigens.

The second industry talk was presented by Stephane Mazlan from Izon Science Europe Ltd. He introduced an automatized process for the EV isolation and single particle characterization via tunable resistive pulse sensing (TRPS). This technology has demonstrated precision in both size and concentration determination where subpopulations in multimodal samples can be accurately portrayed and distinguished. Mazlan reported the current TRPS instrument can measure EVs as small as 30 nm (Exoid, Izon Science) and offers the possibility for automation.

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ASPECTS OF EVS

The session addressed basic questions regarding the nature and purpose of EV release and their role in intercellular communication processes, and how specific cargo loading is regulated. Tumor-released EVs contribute to almost all hallmarks of cancer and the central topic of the session raised their role in cancer initiation and progression. Cutting edge data including their impact on metastasis, central signaling pathways (Toll-like receptor signaling), the extracellular matrix or tumor-associated bystander cells were discussed. Moreover, data on ciliary-associated EVs and their impact on the tumor associated WNT signaling pathway were presented.

The guest speaker Ralf Jacob from the Philipps University Marburg presented the latest results in his ongoing research concerning the EV-associated release of the β-galactoside-binding galectin-3. Galectin-3 is a lectin that contributes to tumor microenvironment immunosuppression, and it regulates diverse functions including cellular homeostasis and cancer biology. Of note, in epithelial cells, the apical transport occurs via different pathways, and galectin-3 was identified as a sorting receptor to recruit cargo molecules into apical transport vesicles. Ralf's group discovered that a late domain motif in the N-terminal domain of galectin-3 directs the recruitment of the lectin into multivesicular bodies for non-classical exosomal secretion into the apical milieu. This highly conserved P(S/T)AP domain motif is required for the interaction with the ESCRT-I factor Tsg101. Tsg101 is critically involved in ESCRT-regulated endosomal membrane remodeling, enabling vesicle formation and finally their release as exosomes. Once at the apical membrane, galectin-3 reenters the cell by endocytosis and traverses the endosomal pathway to be recycled back to the cell surface. This recycling is modulated by the pH gradient between the extracellular milieu and endosomal organelles.

The session went on with Lothar C. Dieterich from the Swiss Federal Institute (ETH) of Technology in Zurich who presented his findings on how pre-metastatic niches are formed in part by melanoma-derived EVs. He showed that melanoma EVs shuttle tumor antigens via the lymphatic vessels to tumor-draining lymph nodes. Here, cross-presentation of the antigens to the antigen-specific $CD8^+$ T cells leads to enhanced apoptosis of the immune cells and thus an immunosuppressive phenotype and tumor progression. Lothar pointed out that the EV-dependent cross-talk of lymph nodes and lymphatic endothelial cells represents a novel pathway contributing to melanoma progression and might serve as a new target for melanoma therapy. For this excellent presentation, Lothar C. Dieterich was awarded with the Margot Zöller Prize.

Next, María Gómez-Serrano from the Institute of Tumor Biology in Marburg presented her newly established *ex vivo* system to analyze EVs of ovarian cancer-associated adipocytes (CAAs). The role of tumor-associated adipocyte-derived EVs in tumor biology is largely enigmatic, which is also due to the lack of established methods to collect and analyze these EVs. To tackle this issue, Gómez-Serrano used primary material, i.e., human mentum, which was cultivated in the presence of ascites, the characteristic tumor microenvironmental fluid of ovarian cancer. During the reprogramming of adipocytes to CAAs in this system, the adipocyte/CAA-released EVs were collected, purified, and characterized. Preliminary data indicate an increase in the number of released EVs, a shift in the average size of EVs, and a change in the percentage of $CD63^+$ EVs comparing adipocyte and CCA-released EVs. Their cargo and modulatory effect on tumor and host cells is currently under investigation.

Primary cilia and cilia-EVs were the focus of the presentation of Ann-Kathrin Volz from the Institute of Molecular Physiology Johannes Gutenberg University in Mainz. Comparing cilia-deficient Bardet-Biedl syndrome (BBS) mutant cells and wild-type cells, she observed an increased release of small EVs by BBS cells and an altered cargo composition. The EVs of BBS mutants were enriched with miRNAs and proteins associated with WNT signaling - a protein family crucial for developmental and disease processes[8]. Of note, knock down of dicer, an enzyme critically involved in miRNA processing, did not show any effect on the WNT-modulating activity of resulting EVs. This suggests that miRNAs are not essential in this pathway, but a protein-mediated mechanism may be involved that will be unraveled in future studies to gain new insights into ciliopathy disease pathogenesis.

Meike J. Saul from the Department of Biology, Technical University of Darmstadt presented her findings on an EV-mediated feedback loop concerning prostaglandin E2 (PGE2). She reported that PGE2 induces the sorting of miR-574-5p into small EVs in two lung cancer cell lines. The miRNA then activates Toll-like

receptors that in turn lead to a decrease of PGE2. In contrast, intracellular miR-574-5p acts oppositely and induces PGE2 synthesis. Thereby, intracellular and extravesicular-derived miR-574-5p regulate the PGE2 protein level in lung adenocarcinoma. Interestingly, this was only observed in adeno- but not in squamous cell carcinoma, indicating a cell-specific response which is probably dependent on the unique tetraspanin composition of the studied cell types.

The next speaker, Venkatesh Sadananda Rao from the Department of Visceral, Thoracic and vascular Surgery, Dresden University of Technology, elucidated the role of extravesicular tissue inhibitor of matrix metalloproteinase-1 (TIMP1) in colorectal cancer (CRC) and colorectal liver metastasis samples during invasion and metastasis. Using 3D extracellular matrix (ECM) remodeling assays, he showed that primary fibroblasts in the presence of CRC-EVs induced ECM remodeling, which was abrogated when TIMP1 was inhibited. This mechanism seems to be clinically highly relevant since the TIMP1 level in the invasive front of liver metastasis tissue samples was positively associated with a poor progression-free survival of the patients. Thus, targeting TIMP1 may represent a novel approach to prevent or attenuate the formation of liver metastasis.

The last speaker of this session was Samantha Lasser from the Skin Cancer Unit located at the German Cancer Research Center (DKFZ) in Heidelberg. She investigated the role of melanoma-derived EVs and with them a group of miRNAs that mediate the conversion of normal human monocytes to myeloid-derived suppressor cells. In a murine system, she analyzed the role of miR-125a on immature myeloid cells. She observed an upregulation of immunosuppressive features such as PD-L1 and factors of the Toll-like receptor family, NF-κB, and JAK-STAT families. To further elucidate the combined effect of EV-associated miR on different Toll-like receptors and finally on antitumor immunity, Lasser plans to use immature myeloid cells from mice with knockouts for specific Toll-like receptors.

In summary, the researchers of this third session presented many different aspects of the formation, promotion, and progression of various malignancies and the important roles EVs seem to play in them.

EV IN CLINICAL DIAGNOSTICS - PERFORMING RELIABLE EV BIOMARKER ANALYSIS

The robust detection and analysis of EVs is an important technical challenge during their development as biomarkers. Enhanced rigor is required to rule out any unwanted byproduct detection and verify that EVs during diseases states are substantially different from those under physiological conditions. Marca Wauben from Utrecht University, Department of Biomolecular Health Science opened the present session on EV biomarker assessment for clinical diagnostics. Marca is a well-known pioneer in the field of single EV analysis by flow cytometry. In her talk, she clearly pointed out that high-resolution evaluation of EVs by flow cytometry is needed and challenging. Interestingly, there are substantial inter-platform and inter-laboratory differences in the study of EVs. That is, two labs may not be able to identify comparable EV populations using comparable instruments. These finding underline the need for standardization of EV isolation protocols and methodologies and are discussed in an international expert consortium, which has already provided a guideline for flow cytometry based-EV analyses[9]. Adding another layer of complexity, Marca also discussed the overlap of EV analysis with lipoprotein particles and other protein complexes from human samples.

This presentation was followed by several talks from young scientists at the PhD and postdoc level. Jerome Nouvel from the Institute of Infection Prevention and Hospital Epidemiology, University of Freiburg presented the validation of a fast protein liquid chromatography for EV isolation from blood specimens. The method was nicely combined with established size exclusion chromatography using alternative gel

separation matrices. It was shown that separation of EVs from serum-derived lipoproteins was feasible using this method. In a subsequent step, this technique was applied to robustly isolate EVs from pancreatic cancer patients. A consecutive analysis of the tumor EVs is still needed, but the fast protein liquid chromatography method was reliably established.

Focusing on a different aspect of EV release, Elmo Neuberger from University of Mainz, Department of Sports Medicine, Prevention & Rehabilitation reported on the influence of physical exercise on DNA being associated with EVs. While it was confirmed that cancer cell EVs could carry DNA as a cargo, it appears less investigated whether under different physiological conditions "freely circulating" DNA is associated with EVs. For the investigation, healthy volunteers underwent treadmill exercise, and their EVs and related DNA were isolated from the volunteer's blood plasma and studied in more detail. For the removal of non-EV-associated, co-purified free circulating DNA, obtained EV samples were treated with DNase. According to the results presented, only 0.12% of the plasma DNA is associated with EVs, approximately a fifth of it being encapsulated as luminal EV cargo. While the amount of EVs as well as total EV-associated DNA increased with exercise, luminal DNA cargo did not increase within the prepared EV fraction. Thus, EVs released under physical stress conditions such as exercise are increasingly decorated with DNA without increasing their luminal DNA cargo[10].

Following this presentation, two postdocs from the Friedrich-Alexander University Clinics Erlangen - **Jan van Deun** and **Jan-Ole Bauer** - presented their joint work on EVs as biomarkers in the detection of tumor and inflammation. van Deun presented that melanoma patients carry protease-harboring EVs which are major players in inflammatory processes. A method was established to rapidly detect the presence of these proteases in a cohort of healthy volunteers and melanoma patients. Protease activity in these samples was studied using FRET-based substrates, which helped to identify 14 proteases predominantly identified in the group of melanoma patients. When a neural network-based classification model was applied, van Deun showed that a < 85% specificity and sensitivity in the identification of tumor patients was achieved. These results provide a sound basis for further biomarker establishment in melanoma patients. In addition to these findings, Bauer was able to correlate EV-associated protease activity to inflammatory effects in a larger group of healthy volunteers. While the majority of these volunteers did not show any elevated levels of protease activity, approximately 30% had higher inflammatory state; this was most likely due to a previous SARS-CoV-2 vaccination. It was concluded that, although EV-protease activity could reflect inflammatory dispositions in patients, a substantial number of healthy volunteers also showed higher protease activity, findings that require larger clinical cohorts in the future.

Maike Saul presented the work of her PhD student Kai Breitwieser (Department of Biology, Technical University Darmstadt) focusing on small EVs and their potential for biomarker development. Although several tetraspanins, including CD63 and CD81, are well established for confirming EV identity, their abundance and distribution seems to vary between EV source and populations. To address this variation, a detailed expression and colocalization study was conducted using a microchip-based detection technique (ExoView®). This method allows for purification free assessment of marker density and relation, and it may potentially be suitable for future EV-biomarker establishment.

This scientific session was closed by Karolina Soroczyńska, a PhD student from the Department of Biochemistry, Medical University of Warsaw (Poland). She is studying endometriosis, a gynecological disorder in the female reproductive system for which clinical therapies are yet lacking. It was previously found that patients showed a higher concentration of two subtypes of arginase enzymes. Her group became subsequently interested in establishing a relationship with small EV release in the patients concerned.

Indeed, she demonstrated the presence of arginase in EV preparations, most of the enzyme recovered on the EV surface. A slight increase in EVs number for affected patients was detected compared to healthy controls. Overall, such arginase-positive EVs may influence endometriosis progression and require future investigations.

Finally, the session was concluded by presentations from the industrial partners. Ben Peacock from NanoFCM presented a nano-flow cytometer, which allows both fluorescence and side scatter detection of small particles, as well as simultaneous sizing and identification of subpopulations. Andrew Malloy from NanoView Biosciences introduced a chip-based system that captures EVs via specific antibodies and allows sizing and fluorescent antibody-based characterization of individual captured Evs. Peter Rhein from Luminex introduced imaging flow cytometer platforms, i.e., the AMNIS image stream and AMNIS cell stream, both allowing high-resolution camera-based detection of objects of different sizes including cells and small EVs.

DRUG DELIVERY AND THERAPEUTIC APPROACHES

At least a proportion of EVs mediate intercellular communication at local and distant sites, likely in a specific manner. Apparently, EVs contain tropisms to selected target cells and tissues. Coupled to their role in physiological and pathological processes, EVs prepared from appropriate cell types now provide the chance to manipulate such processes in a very effective manner. Consequently, EVs have emerged as a promising tool for novel therapeutic interventions. On the one hand, native EVs such as the aforementioned MSC-EVs are considered as novel therapeutic agents for many diseases. On the other hand, coupled to their tropism, EVs are envisioned as natural drug delivery vehicles[11], in which big pharma have become very interested. To use EVs as drug delivery vehicles, strategies need to be developed in which EVs can be manipulated and loaded in very controlled and efficient manners. A pioneer in this area is Prof. Samir el Andaloussi heading a group at the Department of Laboratory Medicine in the Clinical Research Center of the Karolinska Institutet in Stockholm (Sweden). He is also one of the three co-founders of the emerging EV company EVOX Therapeutics Ltd. In his keynote lecture, el Andaloussi introduced in this session, which was chaired by Oskar Staufer and Bernd Giebel, some of the latest results of his group, which develops genetic engineering strategies for EVs permitting loading of therapeutic proteins and RNAs as well as displayed peptides and proteins for targeted delivery to EVs that should serve as biotherapeutics. At first, el Andaloussi described the principles of endogenous EV engineering and the use of various constructs in which luciferase (Luc) is fused to annotated EV proteins/domains for the identification of proteins/domains allowing very effective protein cargo loading to EVs. For the example of tetraspanin-Luc fusion proteins, he demonstrated how such fusion proteins can be used for assessing pharmacodynamics and pharmacokinetics of engineered EVs in mice. Regularly, administered EVs distribute quickly in various tissues and reveal short half-life times, especially in the plasma. Upon introducing engineered forms of a tetraspanin construct containing albumin-binding domains (ABDs) in the second outer loop domain of the given tetraspanin, the half-life time of resulting EVs could be substantially increased; consequently, much higher EV amounts could be delivered to tissues including tumors and lymph nodes. In another engineering strategy, his group introduced an Fc-binding domain into the second outer loop domain of the given tetraspanin and can now load EVs with any given antibody. For the examples of anti-PD-L1 and anti-HER2 antibody-loaded EVs, el Andaloussi demonstrated that such EVs bind to cells expressing the respective antigens and are subsequently taken up by these cells. More precisely, he showed the antibody mediated EV uptake in B16F10 melanoma and SKBR3 breast cancer cells and reported that, following i.v. administration of such EVs, being additionally loaded with chemotherapeutic agents, they specifically target and eliminate subcutaneously implanted melanoma cells, thereby significantly improving the survival rate of the respective mice. The last part of his presentation covered the topic of decoy EVs, EVs that are engineered to

display signal incompetent receptors on the EV surface being used to sequester disease-causing soluble factors in blood. el Andaloussi showed a proof-of-principle experiment in which TNFR or GP130 was displayed on EVs to inhibit inflammation promoting TNF or IL6 signaling, respectively, and how this strategy could be improved upon using N-terminal Syntenin tethered to the respective receptors and by the use of luminal multimerization domains to enhance receptor clustering. Applying this strategy, el Andaloussi's group already successfully used this decoy strategy in three different disease mouse models: in an LPS-induced sepsis model, a TNBS-induced IBD colitis model mimicking Crohn's disease, and an EAE multiple sclerosis model. Importantly, he finally showed that the engineering strategy can be used to generate so-called double decoys where single EVs display both receptors for highly efficient treatment of colitis, surpassing the effect of conventionally used biologics.

Following this outstanding lecture, Aladin Samara of the Felsenstein Medical Research Center of the Rabin Medical Center in Petah Tikva in Israel reported about the concept in using NK-derived EVs for leukemia treatment. The group had prepared small EVs from conditioned media of the NK92MI cell line and explored their killing potential in some *in vitro* experiments. They improved time- and dose-dependent killing activities in all leukemic cell lines tested and observed a substantial reduction of colony formation potentials of patient-derived leukemia cells cultured in the presence of the given EVs. In contrast, these NK-EVs did not affect healthy B cells. The killing effect was correlated with their uptake; labeled NK-EVs were effectively taken up by leukemic cells but not by healthy B cells. Next, intending to develop off-the-shelf NK-EV products for leukemia treatment, Aladin's team wants to test the anti-leukemic potential of the NK-EVs in a xenograft leukemic mouse model.

The third talk in this session was given by Katrin Bedenbender of the Institute for Lung Research of the Philipps-University Marburg. Katrin reported about the impact of bacterial EVs and secreted host factors on the function of endothelial Ribonuclease 1 (RNase1) in sepsis. In addition to their importance for vascular homeostasis, endothelial cells are essential sensors for pathogens, inflammatory mediators and immunity. Once being activated. amongst others, endothelial cells secrete the vessel protective factor RNase1. However, massive and persistent inflammatory conditions frequently result in RNase1 repression, often being associated with vascular pathologies including sepsis progression. In her project, Katrin investigated whether bacterial EVs, so so-called outer membrane vesicles (OMVs), of different bacteria, i.e., *Escherichia coli*, *Klebsiella pneumoniae,* and *Salmonella enterica* from serovar *Typhimurium*, affect the endothelial RNase1 function. She reported that applied OMVs from sepsis sepsis-inducing bacteria in contrast to non-sepsis sepsis-inducing bacteria significantly reduced the activity of RNase1 and promoted endothelial cell activation, as indicated by increased IL-8, CXCL10, ICAM-1, and IL-1β expression. Notably, the LPS-neutralizing antibiotic polymyxin B was found to effectively prevent both, the OMV-induced RNase1 repression as well asnd the endothelial cell activation by the prevention of Toll-like receptor 4 (TLR4) activation. Thus, the current results imply that, via TLR4 activation, OMVs of sepsis sepsis-inducing bacteria promote endothelial cell activation and thereby contribute to the disease progression. Aiming to develop novel therapeutic anti-sepsis strategies, the underlying signallingsignaling cascade should be investigated in more detail.

Finally, two industry talks were given. Vi Tran from FUJIFILM Wako Chemicals Europe GmbH presented the increasing product portfolio the company is providinges for EV research, including products for EV purification and detection. Thomas Bauer-Jazayeri of Fidabio introduced a new flow-induced dispersion analysis device that can be used for EV characterization. The device enables measurements of binding affinity and size (absolute in nm) of biomolecules including EVs in solution, e.g., in storage buffers or their original bioliquids.

MISEV GUIDELINES, CONTROLS, MAIN PITFALLS IN EV RESEARCH: INTRODUCTION AND ROUND TABLE DISCUSSION

The last session of the meeting chaired by Irina Nazarenko started with a presentation by Dirk Strunk (Paracelsus University Salzburg), who started by asking the audience about their handling and respecting of the MISEV guidelines[12]. Then, he reported on a systematic text-mining approach in which his group assessed for the adherence of published EV studies to the MISEV2018 criteria[13]. The analysis revealed that over the years an increasing proportion of EV manuscripts refers to and respects the MISEV criteria. Interestingly, studies including a more elaborated MISEV-guided methodology were cited with higher frequency. Apparently, the MISEV guidelines are successfully helping to improve the quality of EV research. Dirk's presentation was followed by a roundtable discussion on the importance of the MISEV criteria for the progression of the EV field but also about their limitations. The group of invited senior EV researchers and the session chairs provided their expertise of how to apply and deal with these criteria and answered specific questions of the audience regarding the limitations of the field. Condensing this session's take home message, it became apparent that common efforts are required to push the technical limitations in the EV field. EV research in many aspects is still in a developing phase and for its ongoing progress requires a community-driven reflection. Approaches of the international EV community to define guidelines such as MISEV2018 help to sharpen our overall understanding and provide recommendations for everyday research to the profit of overall quality of EV research. However, we also need to be aware that within the progressing EV field these recommendations are continuously evolving, sometimes challenging existing recommendations. Thus, it is an ongoing international goal to dynamically improve and update existing MISEV criteria. Indeed, currently, a new version is being prepared, which is expected to be published in summer 2022. Since limitations and required changes are discussed among EV experts in advance and many GSEV members participate in such discussions, it is always a good idea that scientists entering the EV field discuss with EV experts upfront to avoid some unnecessary pitfalls, which sometimes are hard to recognize within the overwhelming flood of literature about EVs. GSEV is dedicated to providing an interdisciplinary and up-to-date platform allowing the information exchange among its members and is open to all scientists interested in EV research, both within and outside Germany.

POSTER SESSION

Within the poster session, 27 posters were shown and presented mainly by young scientists. It was a huge challenge in selecting the "right" posters for the Peter Altevogt Prize. Their topics of the posters covered the whole area from basic to clinical research and addressed the origin and biogenesis of EVs, the methodology of EV purification and analysis, and the role of EVs as biomarkers and therapeutic agents in (patho-)physiological processes. The poster voting committee, the group of all orally presenting young scientists, finally reached a decision by swarm intelligence and selected three "Best Posters":

Fang Cheng Wong from the University Hospital Dresden won the third prize for her research on the EV-mediated crosstalk between pancreatic cancer and Schwann cells. Pancreatic cancer is characterized by its capacity to invade the neural system, especially Schwann cells. In both murine and human 3D culture models, Fang Cheng Wong showed that the migratory potential of Schwann cells increases after treatment with pancreatic cancer cell-derived EVs but not with EVs from non-cancer cells. The effect was abrogated in the presence of EV uptake inhibitors such as heparin and EIPA, indicating a critical role of EVs for the promotion of pancreatic cancer invasion.

The second prize was awarded to Moshin Shafiq from the University Medical Center Hamburg-Eppendorf for his research on EVs and their role in Alzheimer's disease (AD). He investigated the cellular prion protein (PrPC), a protein abundantly expressed on the cell surface as well as on EVs (exosomal PrCP).

Using wild-type and PrPC-deficient EVs from neuroblastoma cells, Moshin analyzed Aβ fibril formation, a characteristic feature of AD. He detected an altered proteomic and lipidomic profile between the two samples and the strong sequestration of Aβ in wild-type EVs compared to the KO, thus highlighting the crucial role of PrPC-carrying EVs in the pathophysiological processes of AD.

Finally, the first prize was awarded to André Cronemberger Andrade from the Paracelsus Medical University in Salzburg (Austria) for his research on the role of EVs in angiogenesis. Using different purification methods (TFF and ultracentrifugation), he prepared EVs from induced pluripotent stem cells cultured under various hypoxic conditions and analyzed their angiogenesis potential. He showed that hypoxia, in general, led to the stabilization of EV cargoed HIF-1α, while the purification method specifically influenced the enrichment of tetraspanins and Alix in obtained EV preparations. The pro-angiogenic effect was reduced when extended purification protocols were used and correlated with a reduced VEGF content in respective EV samples, again highlighting the impact of the EV preparation method on the activity of obtained EV products.

At this point, the authors like to congratulate all the winners of the Margot Zöller and Peter Altevogt Prizes and thank all speakers, poster presenters, the industry representatives, and all other participants for an outstanding meeting.

CONCLUSION

Overall, the GSEV autumn meeting 2021 provided a highly productive platform for interactions among the participants, especially for the German EV young scientist community. The talk and poster sessions were accompanied by intense discussions, which carried into the coffee breaks and beyond. Advances reported related to the specific molecular and functional characterization of EVs and the refinement of strategies for therapeutic applications of EVs. EV science continues to advance through focused technology development, consistent research guidelines that are accepted across the community, and regular exchanges between basic and applied EV scientists. The establishment of future collaborative research centers with a focus on EV science and the implementation of EV core facilities, such as at the University of Marburg, make a decisive contribution to promoting excellent EV science in Germany and worldwide.

With its next generation of motivated and educated EV researchers, Germany appears to be well equipped for the challenges in this area. The GSEV board on behalf of the whole society thanks all contributors, the sponsors, and the organizing team for their efforts making this meeting a success. We are all looking forward to our next meeting organized together with the IGLD, taking place from March 10th-12th 2022 in Frankfurt am Main. The meeting is completely free of registration fees and the program will address exciting topics, from techniques to EV functions and regulatory affairs. Mark your calendar and see you in March!

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Authors' contributions

All authors contributed to the manuscript equally.

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Extracellular Vesicles and Circulating Nucleic Acids

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Assessing extracellular vesicles from bovine mammary gland epithelial cells cultured in FBS-free medium

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Abstract

Aim: Mammary gland extracellular vesicles (EVs) are found in both human and livestock milk. Our knowledge of the role of EVs in the mammary gland development, breast cancer and mastitis derives mainly from *in vitro* cell culture models. However, a commonly shared limitation is the use of fetal bovine serum (FBS) as a supplement, which naturally contains EVs. For this reason, the purpose of the study was to evaluate novel tools to investigate mammary gland EVs *in vitro* and in a FBS-free system.

Methods: Primary bovine mammary epithelial cells (pbMECs) and a mammary gland alveolar epithelial cell line (MAC-T) were cultured in a chemically defined EV-free medium. To find a reliable EV isolation protocol from a starting cell conditioned medium (10 mL), we compared eight different methodologies by combining ultracentrifugation (UC), chemical precipitation (CP), size exclusion chromatography (SEC), and ultrafiltration (UF).

Results: The medium formula sustained both pbMECs and MAC-T cell growth. Transmission electron microscopy revealed that we obtained EV-like particles in five out of eight protocols. The cleanest samples with the highest number of particles and detectable amounts of RNA were obtained by using UF-SEC-UC.

Conclusion: Our chemically defined, FBS-free medium sustains the growth of both pbMECs and MAC-T and allows the isolation of EVs that are free from any contamination by UF-SEC-UC. In conclusion, we propose a new culture system and EVs isolation protocols for further research on mammary epithelial EVs.

Keywords: Extracellular vesicles, bovine mammary gland epithelial cells, FBS-free medium

INTRODUCTION

Mammary gland extracellular vesicles (EVs) participate in many physiological processes of the mammary gland such as development[1,2] and regulation of epithelial cells polarity[3]. They are likewise involved in pathological conditions, including mastitis[4,5] and breast cancer *in primis*, where EVs are implicated in its onset[6], metastasis[7], and drug resistance[8,9]. Mammary gland EVs are also found in the milk of human[10] and many livestock species such as cattle[11,12], buffalo[13], and goat[14].

Our knowledge of the molecular mechanisms underlying EVs biogenesis, release, uptake, and their effect on recipient cells, derives mainly from *in vitro* cell culture models. Many studies on human cell lines have investigated the role of EVs in mammary gland communication in the context of cancer[9,15,16] or the maintenance of epithelial cells polarity[3]. In mice, EVs play an important role in mammary gland development[3] and involution, and EVs promote the recognition and clearance of apoptotic bodies from macrophages[2].

While most of the published studies focus on humans and mice, only a few studies investigated EVs directly in bovine mammary gland culture models, from primary cells[17] or cell lines[18]. Bovine milk EVs are heterogenous and comprise many EVs subtypes including exosomes (40-100 nm) and microvesicles (100-1000 nm). In addition, milk EVs are biologically active; they are transferred to the newborn and are taken up from intestinal cells[11]. To date, their role is still not known in cell-cell communication within the alveolus of the mammary gland. Besides, the origin of milk EVs is not fully clear, despite recent publications suggesting that they are produced by the milk-secreting epithelial cells, the lactocytes[19,20]. Of note, autocrine and paracrine communication within the bovine mammary gland alveolus is important in all its developmental steps[21], and, in this frame, EVs might also participate in this intense communication.

Cell culture systems allow more options and flexibility to investigate EVs mediated communication as compared to *in vivo* systems. However, they usually share a limitation, namely the use of fetal bovine serum (FBS) as a supplement. FBS naturally contains EVs that can interfere with the experimental setting and outcome[22]; therefore, many studies use commercial or home-made EV-depleted FBS, also in bovine mammary epithelial cells' EVs research[23]. However, the removal of EVs from FBS is never complete[22] and alters the FBS effect on cells, affecting cell growth[24], differentiation[25], and response to pathogens[26,27]. A period of 24-48 h of starvation from FBS affects the culture conditions as well, leading to cell cycle arrest[28] and reducing the background production of cytokines[29], which can cause misinterpretation of cell phenotype and experimental outcome. For these reasons, it is recommended to culture cells directly in FBS-free systems and chemically defined media. Besides the type of medium, it is critical to collect enough EVs for downstream analyses. For this reason, the volume of the starting material usually ranges between 20 and 250 mL[30-32]. Another factor affecting the yield and purity of the EVs sample is the isolation procedure[33].

Few cell lines from the bovine mammary gland exist, with BME-UV1[34] and MAC-T[35] as the most commonly known to date. The use of pure epithelial cell lines prevents contamination from fibroblasts often occurring in primary cultures, which can also be avoided by preplating[36], short trypsinization and

isolating epithelial cells from milk[37]. MAC-T cells are considered a proper model for mammary gland development and lactation[38], as they maintain the capacity to express milk proteins[39] and are responsive to hormonal stimulation[34]. However, cell lines tend to lose the phenotypes of the original tissue[40] and they are set to grow in specific culture conditions, therefore the change of culture conditions can also alter their characteristics.

To gain novel insights on the EVs local communication of the bovine mammary gland, *in vitro* models compiling the guidelines for EVs research are in need. For this reason, in the current study we: (1) developed a chemically defined FBS-free culture that supports the growth of both primary MEC and MAC-T cell line; and (2) compared eight different EVs isolation protocols from only 10 mL of conditioned medium regarding the quantity and size distribution of EVs for further downstream analyses.

METHODS

Primary cells isolation and culture

Mammary glands from lactating cows were collected at a local slaughterhouse postmortem and transported on crushed ice to the lab. Only mammary glands that did not display fibrosis, abnormal cell growth, and signs of mastitis (e.g., redness or hardness) were used to set the cultures. Tissue pieces of ~10 g were pooled from two to four cows each and washed in 70% ethanol, and then cold PBS (Gibco, Thermo Fisher, USA) with antibiotics. Tissues were further minced in ~2 mm^3 pieces and washed six times in cold PBS with antibiotics. They were digested in 0.5 mg/mL collagenase IV (Sigma-Aldrich, USA), 0.5 mg/mL dispase II (Sigma), 5 μg/mL insulin (Sigma), antibiotics, and antimycotics in HBSS (Gibco) buffer for 2 h at 37 °C while gently shaking. The suspension was filtered through a metal mesh to remove larger tissue pieces and then centrifuged at 500 x*g* for 5 min. The pellet was washed twice in PBS and cells were seeded on Nunclon Delta surface dishes (Thermo Fisher) and kept at 37.5 °C 7% CO_2. For the preplating, freshly isolated cells were plated and left for 1 h in the incubator. Then, the medium and cells that were not attached yet were transferred into a new plate. The medium was changed every 2-3 days until 80% confluence; cells were then subpassaged every 3-4 days. Cells at P1 were analyzed at 80% confluency. Cells were kept in DMEM/F12 (Gibco), containing 50 μg/mL gentamicin (Sigma) and 2.5 μg/mL amphotericin B (Sigma), and supplemented with: (1) FBS 10%; or (2) 1:50 B27 (Gibco), 5 μg/mL insulin (Sigma), 5 μg/mL hydrocortisone (Sigma), estradiol (E2) (Sigma), 300 pM progesterone (P4) (Sigma), and 5 ng/mL epidermal growth factor (EGF) (Sigma). The latter medium is referred to as FBS-free medium. FBS-free medium supplements' composition was planned according to the most common supplements used for bovine mammary gland epithelial cells in other studies[41-43], with B27 supplementation partially covering a variety of components of FBS. When plates were coated, rat tail collagen I (Invitrogen, USA) at the final concentration of 6 or 10 μg/cm^2, or laminin (Sigma) at 1 or 2 μg/cm^2 was used. For the growth curve, 6×10^5 cells were seeded on six-well multiwell plates and counted every two days from Day 4 to Day 14. To evaluate the growth, 6×10^5 cells were seeded on six-well multiwell plates and counted every two days from Day 4 to Day 14 using Trypan blue (Sigma) and a Neubauer chamber.

MAC-T cell line

MAC-T cells were kindly provided by Olga Wellnitz from the Vetsuisse Faculty of the University of Bern (Switzerland). Cells were cultured in FBS 10% or FBS-free medium and were passaged every 3-4 days. After two weeks of adaptation in the FBS-free medium, they were lysed in TRIzol (Thermo Fisher) to check the expression of cell type markers (keratin 18, keratin 14, and vimentin). For growth rate evaluation, 1×10^5 cells were plated and counted at 80% confluence for two consecutive passages (referred to P1 and P2). The growth rate was calculated as $\ln(\text{cells } t_0/\text{cells } t_1)/t_1\text{-}t_0$.

RNA isolation and retrotranscription from cells

Once cells reached 80%-90% confluency, they were washed in PBS and lysed in TRIzol (Thermo Fisher), followed by phenol-chloroform RNA isolation. DNA was removed by DNAse I treatment (Sigma). For each sample, 500 ng of total RNA were reverse transcribed using the GoScript Reverse transcription system kit (Promega, USA), following the manufacturer's instructions and cDNA samples were then stored at -20 °C until further use. We followed MIQE guidelines for RNA isolation and retrotranscription[44].

Gene expression analysis

Table 1[45] shows the primer pairs; actin, GAPDH, and histone H3 were used as reference genes, as reported in the literature[37,46]. For the RT-qPCR, the Kappa Mix (Sigma) was used and the primer concentration was 10 μM. The amplification was performed using 500 ng of cDNA and the following amplification program: 95 °C for 3 min (×1), 95 °C for 4 s, 60 °C for 20 s, and 95 °C for 10 min (×40). Relative gene expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method[47]. We followed MIQE guidelines for gene expression analysis[44] and we used as control groups pbMECs grown in FBS-containing medium without preplating, freshly isolated pbMEC on Day 0, and MAC-T grown in FBS-containing medium, respectively. Actin, Histone H3, and GAPDH were used as reference genes, as reported in other publications[46,48].

Extracellular vesicles isolation from conditioned medium

Experimental design

We combined different methods to isolate EVs, testing in total eight protocols, which are schematized in Figure 1. We started from 10 mL of conditioned medium (CM) for all routes except for size exclusion chromatography-ultracentrifugation (SEC-UC) where we started from 0.5 mL.

(1) UC ×2: after the centrifugation at 300 x*g* the supernatant underwent UC at 200,000 x*g* for 70 min. The pellet was resuspended in PBS and spun again at 200,000 x*g*. (2) SEC-UC: after the differential centrifugation at 300 x*g* and 1000 x*g*, 0.5 mL of supernatant were loaded onto a qEV column. Fractions 6-10 were centrifuged at 200,000 x*g* for 70 min. (3) Chemical precipitation (CP): after the differential centrifugation at 300 x*g*, 1000 x*g*, and 12,000 x*g*, the supernatant was precipitated using the miRCURY kit. (4) CP-SEC-UC: after the differential centrifugation at 300 x*g*,1000 x*g*, and 12,000 x*g*, the supernatant was precipitated with the miRCURY kit, the precipitate was loaded on a qEV column, and Fractions 6-10 were spun at 200,000 x*g* for 70 min. (5) UF-SEC-UC: after differential centrifugation, the supernatant was loaded onto an Amicon Tube for ultrafiltration. The concentrate was loaded on a qEV column, and Fractions 6-10 were spun at 120,000 x*g* for 120 min. (6) UC-SEC-CP: after differential centrifugation, the supernatant was centrifuged at 120,000 x*g* for 120 min, the pellet resuspended in 0.5 mL of PBS, and loaded onto qEV column. Fractions 6-10 were precipitated with a miRCURY kit. (7) UC-SEC-UF: after differential centrifugation, the supernatant was centrifuged at 120,000 x*g* for 120 min, and the pellet resuspended in 0.5 mL of PBS and loaded onto qEV column. Fractions 6-10 were loaded on an Amicon Tube for ultrafiltration and the concentrate collected as final EVs suspension. (8) UC-SEC-UC: after differential centrifugation, the supernatant was centrifuged at 120,000 x*g* for 120 min, and the pellet resuspended in 0.5 mL of PBS and loaded onto qEV column. Fractions 6-10 were again spun at 120,000 x*g* for 120 min.

Differential centrifugation of cells derived conditioned medium

The CM from cells cultured in FBS-free conditions was collected at 80%-90% confluency. The CM was differentially centrifuged at 300 x*g*, 1000 x*g*, and 12,000 x*g* for 10 min at 4 °C to remove dead cells, cells debris, and apoptotic bodies, respectively. Immediately, the CM was frozen at -80 °C until the EV isolation.

Ultracentrifugation for cultured cells derived EVs isolation

The CM was thawed on ice and transferred into 13.5 mL Ultra-Clear ultracentrifuge tubes (Beckman

Table 1. List of the designed forward and reverse primer pairs, KRT14 and PRLR are from the work of Finot *et al.*[45] (2018)

Gene	Forward 5` → 3`	Reverse 5` → 3`
Actin	GTCTTCCCGTCCATCGTG	TCTTGCTCTGAGCCTCATCC
Histone H3	ACTGGCTACAAAAGCCGCTC	ACTTGCCTCCTGCAAAGCAC
GAPDH	GGTCACCAGGGCTGCTTTTA	CCAGCATCACCCCACTTGAT
Keratin 18 (KRT18)	ATTTCAGTCTTGGCGACGCT	GCCTCAGTGCCTCAGAACTT
Vimentin (VIM)	CGCTCAAAGGGACTAACGAG	ACGAGCCATCTCTTCCTTCA
Keratin 14 (KRT14)	CAGCCCCTACTTCAAGACCA	AGGTTCAGCTCCGTCTCGTA
Prolactin hormone receptor (PRLR)	CTTGAAAGGAAGCCAAACAGGC	TGGAGAGAATCAACACCGCC
Progesterone receptor (PR)	GGGACTCTCAGTTCACTTTCAA	TTGTCTGAGTACACGGTGGG
Alpha casein CSN1S1	GGAAGCTGAAAGCATTTCGT	GGGCACATCTTCCTTTTGAA
Kappa casein (CSN3)	TGCAATGATGAAGAGTTTTTTCCTAG	GATTGGGATATATTTGGCTATTTTGT

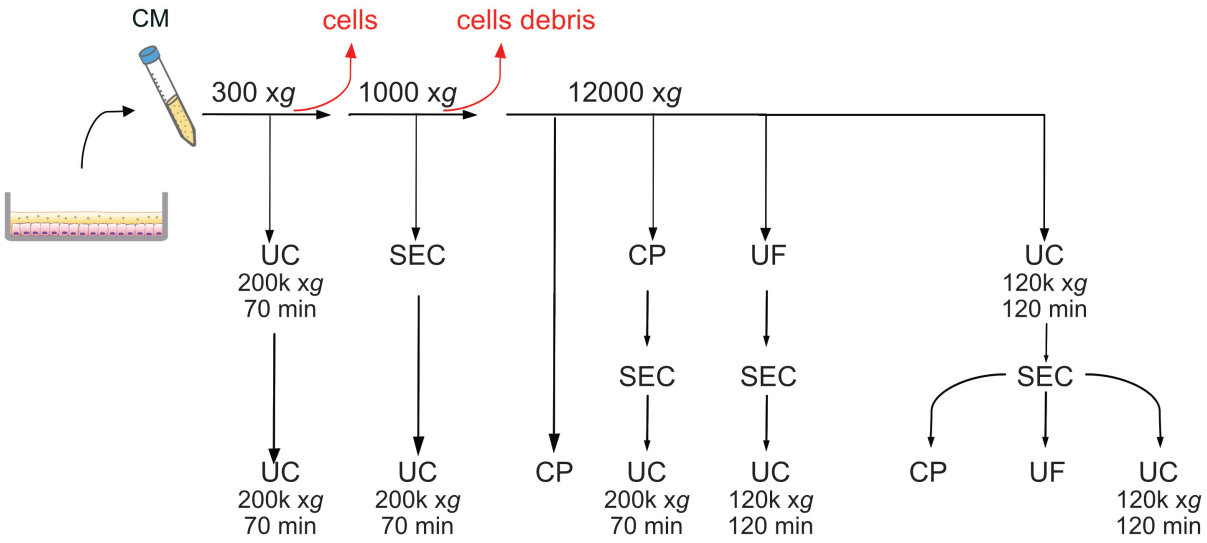

Figure 1. Schematic representation of the eight protocols tested. UC: Ultracentrifugation; UF: ultrafiltration with Amicon tubes; CP: chemical precipitation with miRCURY (Qiagen); SEC: size exclusion chromatography with qEV columns (IZON).

Coulter, USA). Then, they were spun at 200,000 x*g* for 70 min or 120,000 x*g* for 120 min at 4 °C in a Beckman Coulter Ultracentrifuge Optima XE-90, using a Type 50.2 Ti rotor. The pellets were resuspended in 0.22 μm filtered PBS for further ultracentrifugation, or size exclusion chromatography, and stored at -80 °C.

Ultrafiltration for cultured cells derived EVs isolation

To perform ultrafiltration (UF), 10 mL of CM or qEV Fractions 6-10 diluted in PBS were loaded on 15 mL Amicon Tubes with a cut-off of 100 kDa (Merck, Germany) and spun for 1 h RT at 5000 x*g* on a FA-45-6-30 fixed angle rotor. The concentrate was transferred into a new tube and either frozen or loaded onto a qEV column (IZON Science Ltd, New Zealand).

Precipitation with miRCURY (Qiagen) for cultured cells derived EVs isolation

To chemically precipitate the EVs, we used the miRCURY kit (Qiagen, Germany) for cell culture medium, following the manufacturer's instructions. Briefly, the CM after differential centrifugation or from size exclusion chromatography was spun at 3000 x*g* at 4 °C for 5 min to remove debris and cryo-precipitated particles. The supernatant was transferred into a new tube where 0.4 of sample volume of buffer B was

added. After vortexing, the tubes were left on ice for 1 h and then spun at 20 °C at 3200 x*g* for 30 min. The precipitate was resuspended in 100 μL of resuspension buffer and either snap-frozen or loaded onto qEV columns.

Size exclusion chromatography for cultured cells derived EVs isolation

We performed size exclusion chromatography with qEV 70 nm classic columns (IZON). The columns were first equilibrated at room temperature (RT) with 10 mL of 0.22 μm filtered PBS (Gibco), and then 500 μL of CM or EV resuspension from UC or UF was loaded on the column and eluted in filtered PBS. The flow-through was collected in 500 μL fractions and in each fraction the protein concentration was measured by the A280 at the Nanodrop. Fractions 6-10 were pooled together for further concentration.

Milk EVs isolation

The milk EVs were isolated according to the protocol described by Somiya *et al.*[49] with some modifications. Briefly, whole milk was centrifuged at 300 x*g*, 3000 x*g*, and 12,000 x*g* to remove, respectively, cells, fat, and apoptotic bodies. Then, 25 mL of skim milk were heated at 37 °C for 10 min, and 1% of acetic acid was added to precipitate the casein micelles and other proteins. The samples were centrifuged at 10,000 x*g* for 10 min at 4 °C, the supernatant was filtered through 0.22 μm and samples were ultracentrifuged at 210,000 x*g* for 70 min at 4 °C (Optimax 90XE, Beckman Coulter). The pellet was washed with PBS and spun again at 210,000 x*g* for 70 min at 4 °C. Finally, the pellet was resuspended with 500 μL of PBS and centrifuged at 10,000 x*g* for 5 min at 4 °C. The supernatant aliquoted and stored at -80 °C was collected.

Tunable resistive pulse sensing measurements

All measurements were conducted using a qNano Gold (IZON) and NP100 or NP150 polyurethane nanopores (IZON), which detect particles with diameters ranging 50-330 and 70-420 nm, respectively. Filtered PBS (Gibco) was used as an electrolyte buffer and CPC100 (IZON) as calibration particles. Analyses were performed with Izon Control Suite v.3.3.

Transmission electron microscopy

The EVs visualization was performed at the Scientific Center for Optical and Electron Microscopy service of ETH Zurich. Briefly, 3 μL of the vortexed dispersion were placed on glow discharged carbon-coated grids (Quantifoil, D) for 1 min. Negative contrast staining was done in 2% sodium phosphotungstate pH 7.2 for 1 s, followed by a second step for 15 s. Excess moisture was drained with filter paper and the imaging of the air-dried grids was done in a transmission electron microscopy (TEM) Morgagni 268 (Thermo Fisher) operated at 100 kV. For each experimental group, two replicates were analyzed.

Protein isolation and Western blot analysis

The pellet of freshly isolated EVs from 45 mL of medium was immediately lysed in RIPA buffer plus protease inhibitors and then stored at -80 °C. To each sample, 4× Laemmli buffer (Biorad, USA) was added and heated for 10 min at 95 °C. Between 1-3 μg of proteins (for bMEC EVs, milk EVs, and cell lysates) were run on 12% polyacrylamide gel and then total protein was evaluated at the Chemidoc running the stain-free program. Proteins were transferred using a TransTurbo transfer pack (Biorad) with a TurboBlot (Biorad). Membranes were blocked for 1 h in skim milk 5% TBS-Tween buffer (TBST, Bio-Rad, 0.05% Tween 20), and incubated overnight with the primary antibodies diluted in blocking buffer: anti-TSG101 (1:250, PA531260, Thermo scientific), anti-calnexin (1:2000, ab75801, Abcam, UK), and anti-CD9 (1:250, MM2/57, Biorad). After three washes in TBST, membranes were incubated for 1 h at RT with secondary antibodies (Santa Cruz Biotechnology, USA) and StrepTactin-AP Conjugate (Biorad) at the concentration of 1:10,000 and eventually incubated with Clarity Western ECL Substrate (Biorad) for chemiluminescent signal development.

RNA isolation from EVs

Total RNA including microRNA (miRNA) was isolated with the miRNeasy MicroKit (Qiagen, Germany). The length from RNA fragments was evaluated using the Agilent Pico Kit and the Agilent 2100 BioAnalyzer (Agilent Technologies).

EVs RNA retrotranscription and RT-qPCR

For each sample, 1 ng of RNA was reverse transcribed and pre-amplified using TaqMan™ Advanced miRNA assays (Life Technologies), following the manufacturer's instructions. Then, we performed an RT-qPCR using kit TaqMan™ Advanced miRNA assays targeting let-7a-5p (478575_mir assay), miR-200c-3p (mmu482938_mir assay) and miR-223-3p (477983_mir assay). The amplification program was: 95 °C for 30 s (×1), 95 °C for 5 s, and 60 °C for 30 s (×40).

Statistical analysis

To evaluate the gene expression of mammary epithelial markers, we performed the non-parametric Friedman test and Dunn's multiple comparisons tests using GraphPad Prism version 8.2. The differences were considered significant when $P < 0.05$. Unless stated, all values are given as mean ± standard deviation (SD).

RESULTS

Evaluation of cells growth in FBS-free medium

From the isolation (Day 0) to 80% confluency, pbMECs took on average 18.5 ± 4.07 days when grown in FBS-free medium. Cells grew from ~1 × 10^5 on Day 4 to ~1.1 × 10^6 (Day 12) [Figure 2A]. Coating the plates with collagen I or laminin improved neither cell attachment nor growth during the first days [Supplementary Figure 1A]; therefore, we kept plating on uncoated wells. The pbMECs were cultured over three passages in FBS-free medium. MAC-T cells grew in FBS-free medium as well, and the growth rate did not change compared to FBS-containing medium ($P > 0.05$, Table 2).

Evaluation of mammary gland gene markers expression in FBS-free medium

The primary cultures were initially not pure and were composed of a mixed population of fibroblasts (Figure 2B, white arrow) and epithelial cells (Figure 2B, red arrow). We evaluated the epithelial cells' enrichment as indicated by the gene expression ratio between keratin 18 (an epithelial marker) and vimentin (a fibroblastic marker) in pbMECs cultured in: (1) FBS 10%; (2) FBS 10% + preplating; and (3) FBS-free medium. The enrichment of epithelial cells increased 1.7-fold by preplating and 3.4-fold in FBS-free medium [Figure 2C], compared to pbMECs in FBS without preplating. Even if not statistically significant ($P = 0.1377$, FBS-free medium *vs.* FBS 10%), we observed that some cultures reacted to the FBS-free medium by enriching the population in epithelial cells, without the need of preplating. Coating the plate with neither collagen I nor laminin significantly affected the enrichment ($P > 0.05$, Supplementary Figure 1B).

Subsequently, we evaluated the gene expression of cell type and differentiation markers at Day 0 (isolation day), 50% and 80% confluence after day 0, and at 80% confluence after the first sub-passage (P1). The mRNA abundance of the epithelial markers keratin 14 (KRT14) and 18 (KRT18) increased at 80% confluency after the isolation day and at P1, respectively ($P < 0.05$, Figure 2D). On the other hand, the expression of the fibroblastic marker vimentin (VIM) decreased already at 50% confluence after day 0 ($P < 0.05$, Figure 2D). The expression of the functional marker progesterone receptor (PR) significantly decreased already at 50% confluence ($P < 0.05$, Figure 2E), while casein alpha (CSN1S1) and prolactin hormone receptor (PRLR) tended to decrease over time ($P > 0.05$, Figure 2E). Casein kappa (CSN3) did not significantly change, but the trend was rather to increase in one replicate [Figure 2E].

Table 2. Growth rates of MAC-T grown in FBS-containing or FBS-free medium in two consecutive passages

MAC-T cells	Passage 1 (h^{-1})	Passage 2 (h^{-1})
FBS	0.011 ± 0.006	0.023 ± 0.003
FBS-free	0.022 ± 0.015	0.012 ± 0.005

The formula growth rate = ln(cells t0/cells t1)/t1-t0 was used. The time frames from t0 to t1 were between 72 and 216 h. Data are expressed as mean ± SD of three independent experiments. Mann-Whitney test was performed. FBS: Fetal bovine serum.

MAC-T cells cultured in FBS-free medium presented as a homogeneous population and did not morphologically differ from the FBS-containing environment [Supplementary Figure 1C]. Gene expression of KRT14, KRT18 and VIM did not change ($P > 0.05$, Figure 2F), indicating that the FBS-free medium did not alter the cell-type composition.

Evaluation of EVs isolation methods from pbMECs and MAC-T conditioning medium

As shown in Figure 3A, the conditioning FBS-free medium alone did not contain any vesicles, ruling out any contamination of EVs from the medium used. According to TEM, we obtained particles from all routes except from SEC-UC [Figure 3C].

Single or aggregates of cup-shaped particles exhibiting the typical EV morphology in TEM[50-53] were observed following UC ×2, UF-SEC-UC, UC-SEC-UC, UC-SEC-UF, and UC-SEC-UC (Figure 3B and F-I black arrows). The observed structures were heterogeneous in size. We also observed light grey aggregates in CP, CP-SEC-UC, and UC-SEC-CP (Figure 3D, E and G; black asterisk), likely due to lipid aggregates[54,55], while round non-vesicles[56] were observed from CP, CP-SEC-UC, and UF-SEC-UC (Figure 3D and E; white circles). Size and concentration of the isolated particles are indicated in Supplementary Table 1.

Since UF-SEC-UC and UC-SEC-UC had a clean background free of undefined aggregates [Figure 3F and I], and a high number of particle, we selected these two routes to perform tunable resistive pulse sensing (TRPS) analysis and RNA extraction. Figure 4A and B shows at higher magnification the isolated particles. We measured by TRPS the number of particles and their size. We obtained similar numbers of particles [Figure 4C] as well as size distributions [Figure 4D] across replicates ($P > 0.05$, Figure 4C). The last SEC fractions after UF had lower protein concentration than after UC, showing that UF as a first step better cleaned the samples from contaminating proteins [Figure 4E].

When isolating EVs from MAC-T with UF-SEC-UC and UC-SEC-UC, we obtained in both routes cup-shaped particles as shown with TEM [Figure 4F and G]. The TRPS measurement showed that the number of isolated particles [Figure 4H] and size distributions [Figure 4D and I] were similar ($P > 0.05$) between the two protocols.

In pbMEC conditioning medium, the isolated particles were positive for both the EVs markers, TSG101 and CD9, while in MAC-T conditioning medium it was only positive for TSG101 from UF-SEC-UC. All lysates were negative for calnexin, ruling out any intracellular contamination[57] [Figure 4L].

We isolated detectable amounts of RNA from both pbMEC and MAC-T EVs. The length of the isolated RNA molecules is displayed in Figure 5A and B and in Supplementary Figure 3. Specifically, we obtained on average 10.6 ± 9.1 ng from UF-SEC-UC and 8.6 ± 8.2 ng from UC-SEC-UC in pbMECs, while in MAC-T 1.7 ± 1.3 and 2.4 ± 0.6 ng, respectively [Figure 5C]. Finally, the isolated EVs from both pbMECs and MAC-T

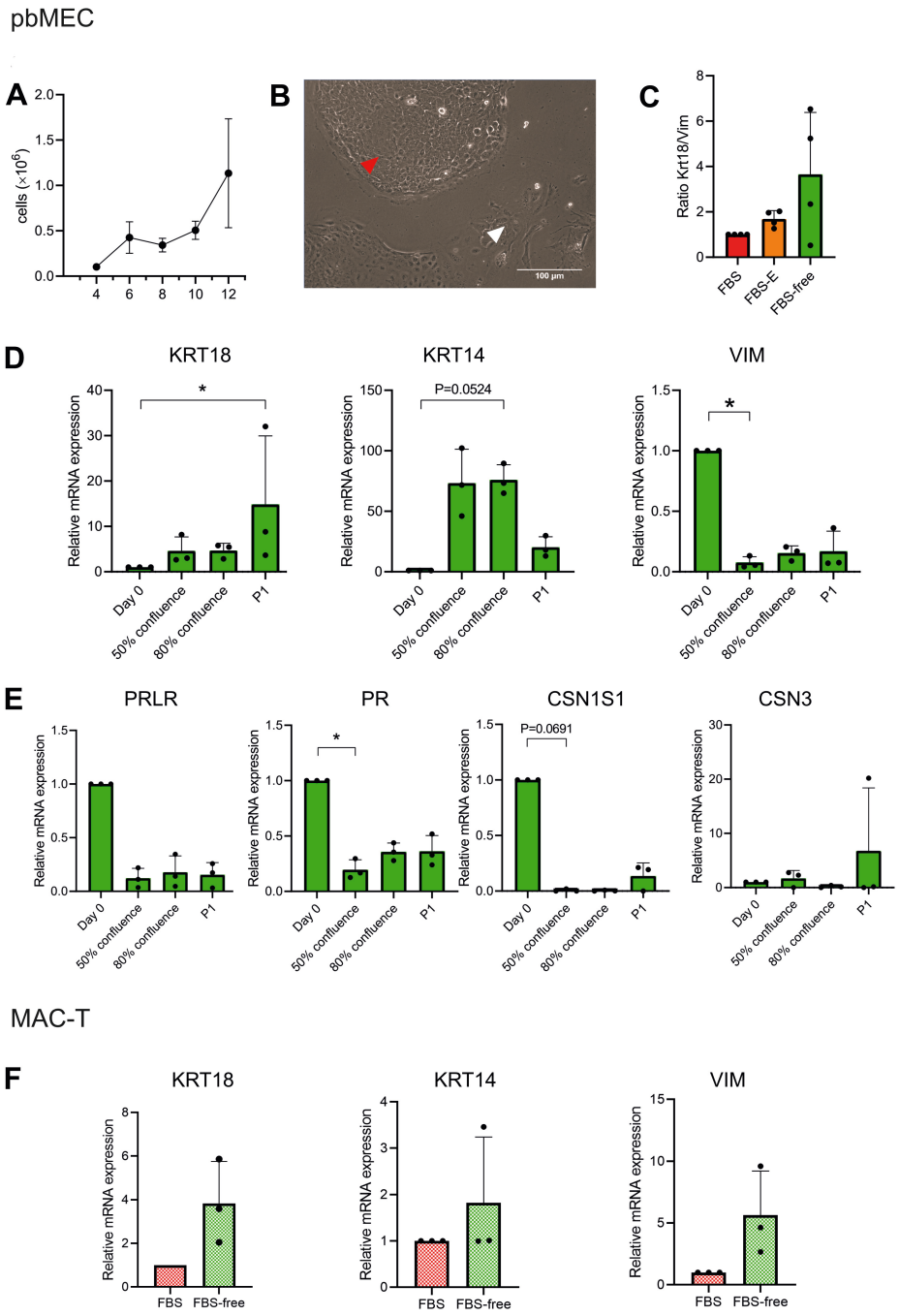

Figure 2. The pbMEC and MAC-T properties in FBS-free medium. (A) Cell count of pbMEC from Day 4 to Day 12 after the isolation from fresh tissue, in six-well multiwell culture dishes. (B) The picture of cultured pbMEC shows the heterogeneous population of epithelial cells (red arrow) and fibroblast-like cells (white arrow). Scale bar: 100 µm. (C) Enrichment of epithelial cells over fibroblasts as means of mRNA expression of keratin 18 (KRT18)/vimentin (VIM) in FBS-containing medium with or without preplating and FBS-free medium. (D, E) mRNA expression in pbMECs of cell-type (D) and differentiation (E) markers on the isolation day (Day 0), at 50% and 80% confluency, and at 80% confluency at the first sub-passage (P1): keratin 18 (KRT18), keratin 14 (KRT14) for epithelial identity, vimentin (VIM) as fibroblastic marker, prolactin hormone receptor (PRLR), progesterone receptor (PR), casein alpha (CSN1S1) and casein kappa (CSN3) coding genes as differentiation markers. (F) Keratin 14 and 18 and vimentin mRNA expression for MAC-T cells cultured in FBS 10% or FBS-free medium. The mRNA expression data are depicted as $2^{-\Delta\Delta Ct}$ [47], values are mean ± SD of four independent replicates, and Kruskal-Wallis and Dunn's multiple comparisons tests (C-E) or Wilcoxon test (F) were performed. The differences were considered significant when $P < 0.05$, (marked with "*" on the graph). pbMEC: Primary bovine mammary epithelial cells; FBS: fetal bovine serum.

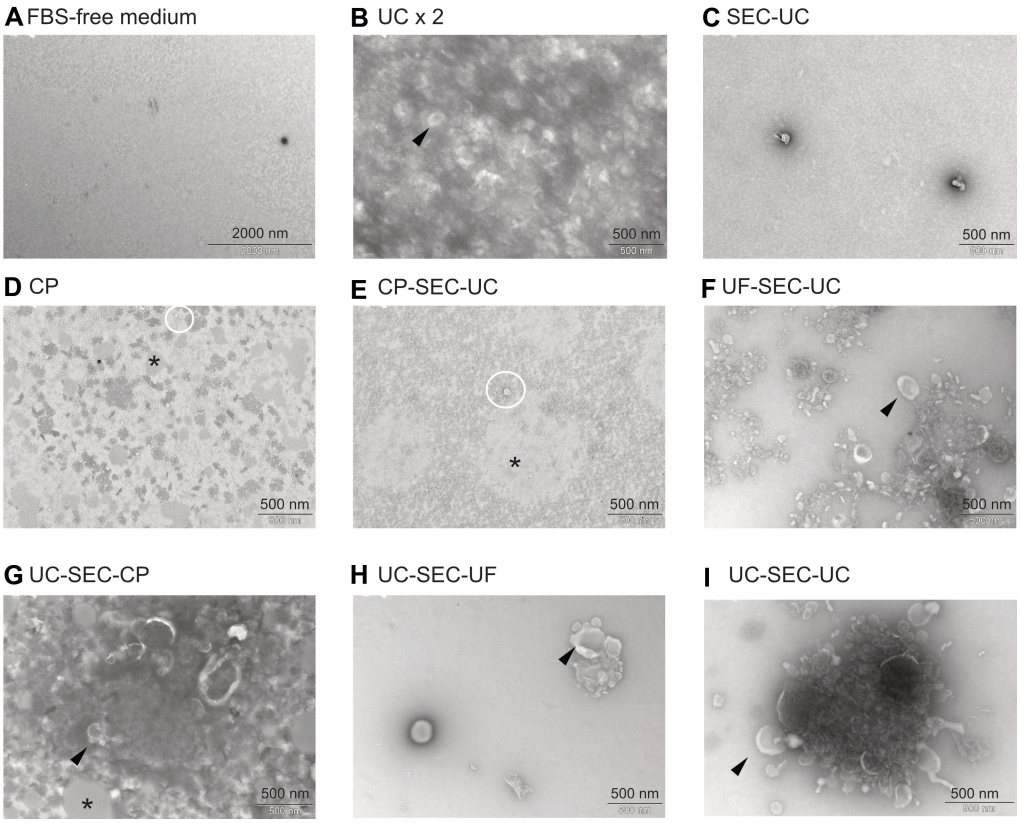

Figure 3. Electron microscopy pictures from FBS-free medium (A) and EVs isolated with UC ×2 (B), SEC-UC (C), CP (D), CP-SEC-UC (E), UF-SEC-UC (F), UC-SEC-CP (G), UC-SEC-UF (H), and UC-SEC-UC (I). Black arrow: cup-shaped particles (vesicles); white circles: non-vesicles particles; black asterisks: undefined aggregates. FBS: Fetal bovine serum; EVs: extracellular vesicles; UC: ultracentrifugation; SEC: size exclusion chromatography; CP: chemical precipitation.

contained the miRNA let7a-5p and miR-200c-3p. The protocol for EV isolation did not affect the amount of detected miRNA [Figure 5D]. On the other hand, we could detect miR-223c-5p only at Cts higher than 35 (not shown in the Figure).

DISCUSSION

In the current study, we established for the first time the culture of primary bovine mammary epithelial cells (pbMECs) and MAC-T cells in FBS-free medium to study EVs from the bovine mammary gland *in vitro*. An important point is that the culture medium is chemically defined, therefore it does not change during the culture and always keeps the same formula, avoiding any possible source of variability given by FBS removal[28,29] or even different batches of FBS.

Our customized FBS-free medium sustained pbMEC growth until confluency and beyond Passage 3, as well as the growth of the MAC-T cell line. The growth rate of MAC-T was lower than in FBS-containing medium. This is in line with previous results, where, however, the culture medium had a different formula[58]. In the primary culture, FBS-free medium promoted the expression of epithelial markers keratin 14 and 18 and the downregulation of the fibroblastic marker vimentin, thereby enriching the population in epithelial cells. In addition, it did not affect the epithelial identity of MAC-T, as keratin 14 and 18 expression levels did not change. Thus, pbMECs and MAC-T cells cultured on FBS-free medium were still relatively close to the primary isolated cells at Day 0 and FBS-containing medium, respectively.

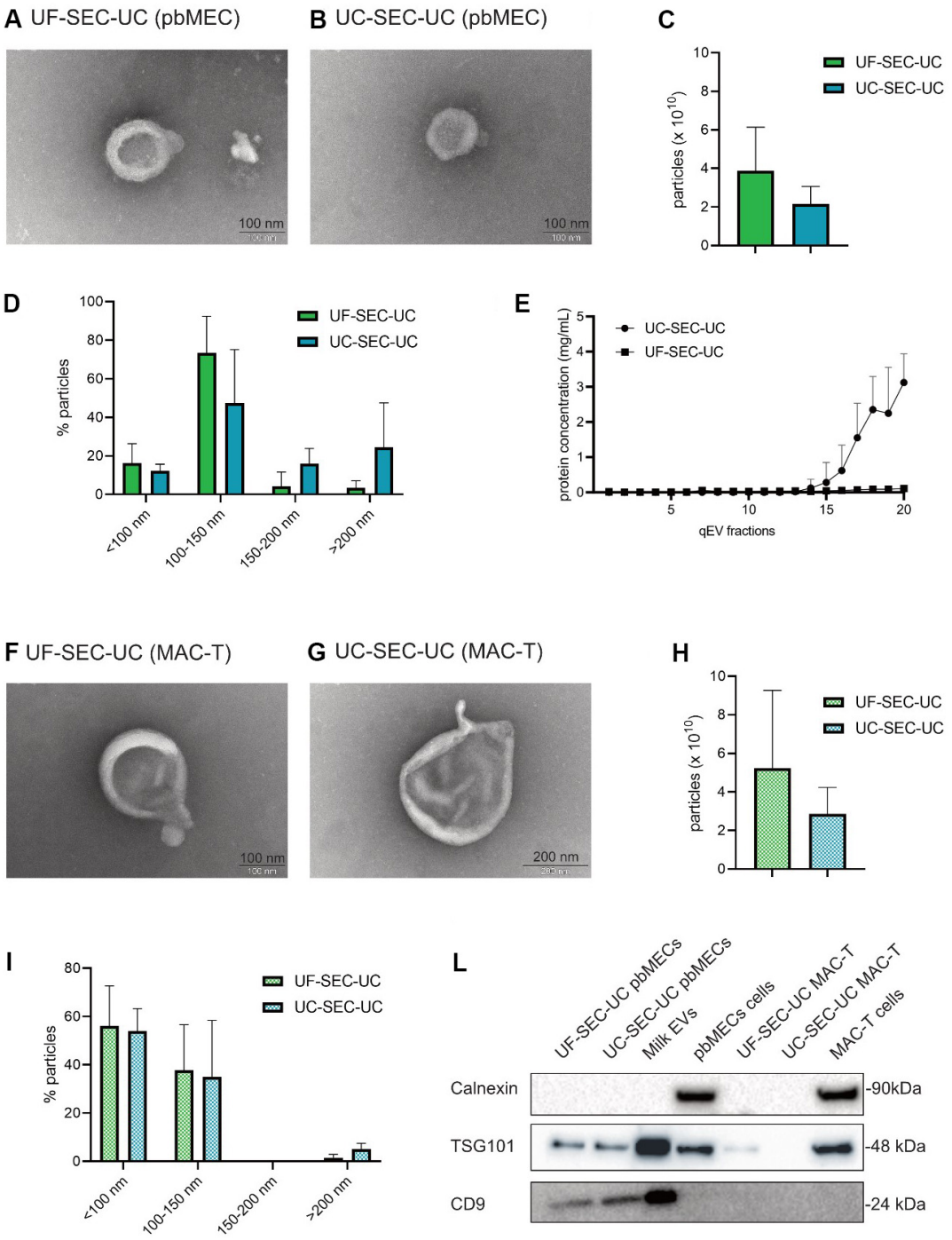

Figure 4. Characterization of the EVs from UF-SEC-UC and UC-SEC-UC in pbMEC and MAC-T (A, B) electron microscopy picture of a vesicle from UF-SEC-UC (A) and UC-SEC-UC (B) on pbMEC conditioned medium. (C, D) Total number of particles per 10 mL of conditioned medium (C) and size ranges (D) from EVs from UF-SEC-UC and UC-SEC-UC in pbMEC conditioned medium. The TRPS was performed with a NP100 nanopore. (E) Protein concentration of the single fractions from SEC from pbMECs conditioning medium. (F, G) Electron microscopy pictures of vesicles from UF-SEC-UC (F) and UC-SEC-UC (G) on MAC-T conditioned medium. (H, I) Total number of particles per 10 mL of conditioned medium (H) and size ranges (I) from EVs from UF-SEC-UC and UC-SEC-UC in pbMEC conditioned medium. The TRPS was performed with a NP100 nanopore. (L) Western blot of pbMECs and MAC-T EVs pellet. Whole cells lysates were used as a positive control for calnexin, while EVs from milk were used as a positive control for EV markers. Full-length blots are presented in Supplementary Figure 2. In (C-E, H, I), values are mean ± SD of three replicates. Wilcoxon test (C, H) was performed. The differences were considered significant when $P < 0.05$. FBS: Fetal bovine serum; EVs: extracellular vesicles; UC: ultracentrifugation; SEC: size exclusion chromatography; pbMEC: primary bovine mammary epithelial cells; UF: ultrafiltration.

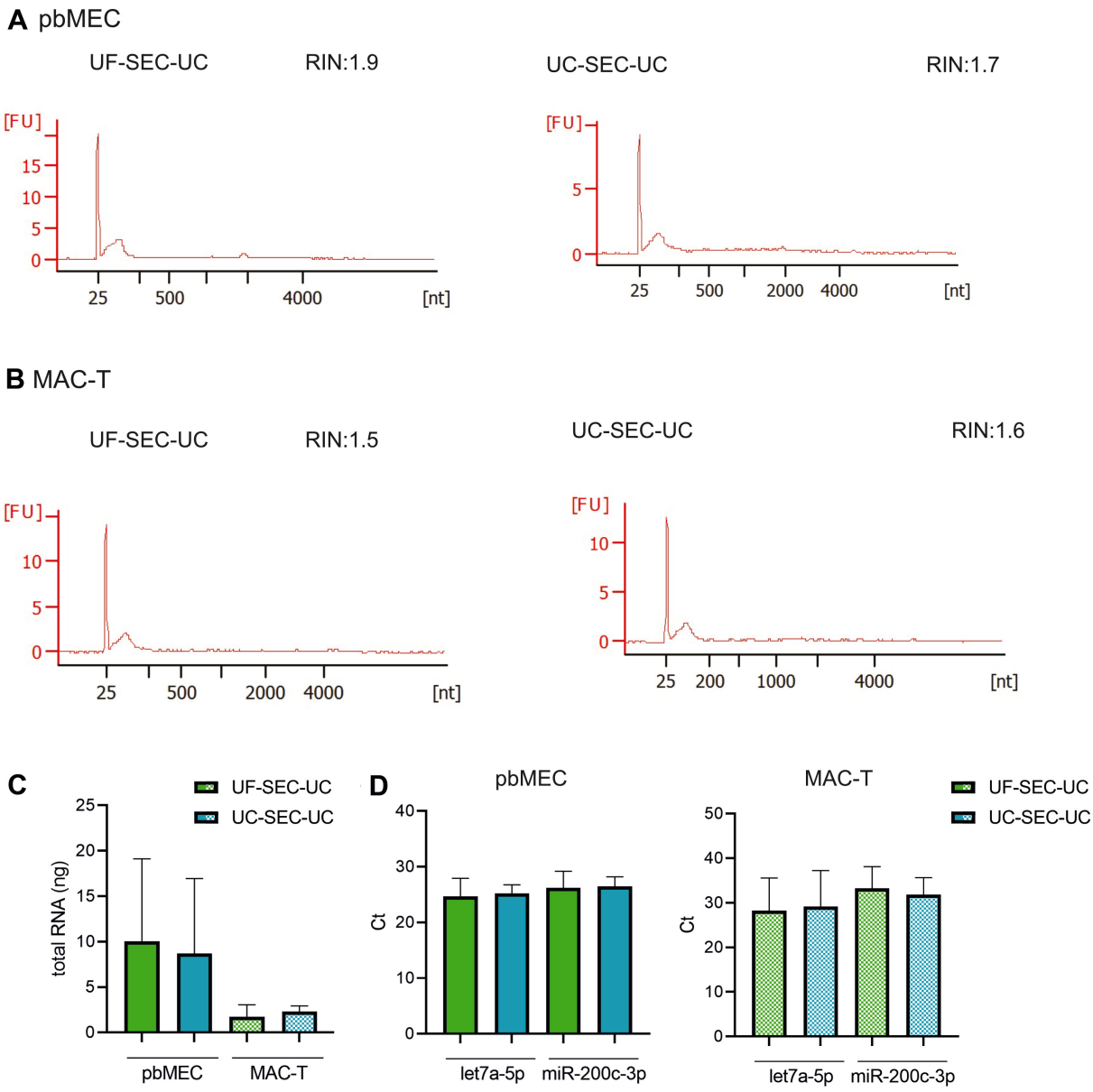

Figure 5. Isolation and characterization of RNA from pbMEC and MAC-T EVs. (A, B) Representative Agilent 2100 Bioanalyzer data from pbMEC (A) or MAC-T (B) RNA from EVs isolated by UF-SEC-UC (right) and UC-SEC-UC (left). (C) Total amount of RNA from pbMECs and MAC-T EVs isolated with UF-SEC-UC or UC-SEC-UC. (D) qPCR of let7a-5p and miR-200c-3p from EVs RNA isolated with either UF-SEC-UC or UC-SEC-UC from pbMEC (left) or MAC-T (right). (C, D) Bars show mean and SD of three independent experiments. EVs: Extracellular vesicles; UC: ultracentrifugation; SEC: size exclusion chromatography; pbMEC: primary bovine mammary epithelial cells; UF: ultrafiltration.

We also observed a downregulation of the differentiation markers PR, PRLR, CSN1S1, and CSN3 already starting at 50% confluency. This trend to de-differentiate might be due to the two-dimensions (2D) culture on plastic dishes, which by itself promotes de-differentiation[42,59]. It has been shown that the growth of pbMECs on three-dimensional (3D) systems supports differentiation[37,42,60]. Thus, further studies should focus on 3D FBS-free culture systems.

The most commonly used techniques to isolate EVs from primary MECs cell cultures or breast cancer lines are UC ×2[6,18,23,61,62] and UF with a cut-off of 100 kDa[17], respectively. By combining these methods, together with size exclusion chromatography and chemical precipitation, we managed to isolate EVs from relatively low amounts (10 mL) of cellular conditioning medium, while in the literature, when stated, the reported starting material is often between 20 and 250 mL[30-32]. The TEM and TRPS measurements excluded any particle contamination in the FBS-free medium, thereby the EVs we observed and analyzed were secreted by the cultured cells. From the eight protocols that we tested, only in one protocol (SEC-UC) we did not isolate any particle after TEM analysis, likely due to the initial low amount of starting material (500 μL). In UC ×2, TEM imaging showed that the matrix was not clean, possibly due to the low number of cleaning steps before the UC. The introduction of more differential centrifugation steps and a SEC step helped to clean the sample likely from proteins in solution and any membranes or content deriving from cell debris and apoptotic bodies, as the CM underwent only centrifugation at 300 x*g*. The miRCURY kit used for CP and CP-SEC-UC instead helped to precipitate vesicles but concomitantly generated many undefined aggregates, as the precipitation itself does not distinguish between the types of macromolecules in solution[33,55]. We obtained a clean sample from UC-SEC-UF, but the particles observed after TEM were few in the whole grid, likely due to the high final volume of the sample (150 μL), collected from the ultrafiltration tube. Both UF-SEC-UC and UC-SEC-UC revealed a better compromise regarding the yield and purity of vesicles. In both pbMEC and MAC-T, the TRPS measurements confirmed the size ranges observed with TEM and were similar between routes, and both gave a consistent number of EVs, on the order of 10^{10} particles from 10 mL of medium, which tended to be higher from UF-SEC-UC. Due to the nanopore size used in the TRPS analysis, we could not detect particles smaller than 50 nm, likely excluding many EVs observed by TEM and therefore underestimating the real concentration of the sample.

Western blot analysis confirmed that the isolated vesicles were actual EV, bearing both CD9 and TSG101, in line with the results obtained by Zhang *et al.*[17] and from studies on milk EVs[19]. We speculate that the faint TSG101 band for MAC-T EVs and absence of CD9 might be due to the low input volume of conditioned medium, as the presence of these two markers in MAC-T exosomes was reported[63]. Importantly, the use of a SEC step to separate secreted proteins from EVs would allow analyzing the cell response, distinguishing the contribution of EVs and secreted proteins. For such purpose, UC-SEC-UC would be more suitable, as the protein-rich fractions are more concentrated, without the initial UF step. On the other hand, if the experimental setup requires the study of EVs only, UF-SEC-UC would be more recommended, as the sample is initially depleted from proteins smaller than 100k kDa.

We were able to extract RNA from both UF-SEC-UC and UC-SEC-UC in pbMEC as well as MAC-T EVs, and the amount of isolated RNA did not differ significantly among conditions. All samples showed the typical profile of exosomal RNA , ruling out any contamination from apoptotic bodies[64]. We also excluded a possible contamination from milk fat globule RNA[65], as the cultured cells expressed very low levels of caseins, indicating low or no milk production. The two miRNAs let-7a-5p and miR-200c-3p were reported as some of the most abundant miRNAs in milk exosomes[66] as well as in cultured pbMEC exosomes[67]. In addition, the no detection of miR-223-3p is also in line with the literature, as this miRNA is not reported among the most abundant[66] and it is only upregulated upon infection[4,5].

In conclusion, we demonstrated that the FBS-free medium culture system is a valid tool to study MEC EVs from both primary cells and the MAC-T cell line. We evaluated and compared different EVs isolation protocols from a relatively low amount of starting cell culture medium (10 mL), and we propose UF-SEC-UC as the preferred method as it yields the highest number of EVs and pure EVs for further downstream analysis in both pbMEC and MAC-T. Our results provide an important reference for further studies that

aim at analyzing MEC EVs in many contexts such as lactation, infection, response to stressors and metabolic challenges.

DECLARATIONS

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Authors' contributions

Conceptualized the experiments, performed the in vitro cultures, EVs isolation, analysed the data and wrote the manuscript: Silvestrelli G

SEU Supervised and financed the project and revised the manuscript: Ulbrich SE

Coordinated and supervised the project and revised the manuscript: Saenz-de-Juano MD

All authors read, edited and approved the final manuscript.

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Extracellular Vesicles and Circulating Nucleic Acids

Editorial

Open Access

Year-end reflections of EVCNA-2021

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Extracellular Vesicles and Circulating Nucleic Acids (*EVCNA*) was conceived in 2019, and the first issue was launched in December 2020, which included a report of presentations, abstracts, and contributed articles from the American Society for Extracellular Microvesicles 2020 meeting. In 2021, the international editorial board of the journal grew to 40 members from 15 countries with 5 Associate Editors. In addition to the Editorial Board comprising of eminent scientists covering many aspects of research in extracellular vesicles and circulating nucleic acids, we have an international junior editorial board of 40 young early career scientists, providing an opportunity for them to participate in editorial duties. In 2021, the journal published 4 quarterly issues with 16 articles, including original research, reviews, commentaries, and conference report. Ten of the articles have been cited 22 times in SCI journals in this one year. Manuscript submissions were thoroughly peer reviewed, and our rejection rate in 2021 was 29%. Our readership in 2021 was diverse spanning more than 15 countries worldwide with more than 28,814 views and 5542 downloads.

In 2021, the editorial team and OAE Publishing Inc., the publisher of *EVCNA*, have partnered with various Extracellular Vesicle and Liquid Biopsy Societies from different countries, including the German Society for Extracellular Vesicles, Italian Society for Extracellular Vesicles, USA, and China CACA TBM Society for Exosomes and Microvesicles. *EVCNA* has also sponsored awards to promote the careers of young investigators and honored a keynote speaker with *CSEMV* & *EVCNA* Best Lectureship Award at the Chinese Academic Conference on tumor markers and the 15th Young Scientists Forum on tumor markers. Prof. Hang Yin of Tsinghua University is the first recipient of *CSEMV* & *EVCNA* Best Lectureship Award.

EVCNA has posted short videos highlighting the work of many authors on the journal website and social media. Those have been well received from the feedback.

Looking forward to 2022, *EVCNA* has at least 6 Special Issues in progress that will be published in the coming year. They cover broad areas of EV research, including the following titles:

"Extracellular Vesicles and Neuroinflammation: Response to Infection, Injury and Aging, Plus Vehicles for Treatment" (Guest Editor: Lynn Pullman); "Exosomes: Diagnostics, Delivery and Therapeutics in Cancer" (Guest Editor: Long Zhang) and "Clinical Use of Circulating Nucleic Acids in Bodily Fluids: Noninvasive Prenatal Testing, Oncology, Transplant Monitoring and Beyond" (Guest Editors: Erik Sistermanns and Peiyong Jiang). It is still possible to contribute an article to any of our Special Issues listed on our website, just let the Guest Editor(s) know. We will also have a Special Issue on the Proceedings of the American Society for Intercellular Communications 2021, highlighting a report of the exciting science presented, abstracts, and contributed articles of work presented at the meeting. Finally, in the coming year, *EVCNA* will be organizing some virtual workshops and mini-symposiums on hot topics of interest to researchers in the EV and circulating nucleic acid field. In particular, we aim to promote translational research focusing on EVs and Nucleic acids as biomarkers and EVs in therapy. EVs and Liquid Biopsy continue to be an exciting field, and there is a lot we still don't know. It is hoped that *EVCNA* will continue to be a platform of choice to share your scientific data, knowledge, and opinions with fellow researchers in this field. For now, this journal has waived APCs, providing a great opportunity for everyone to be able to publish their experimental data or scholarly reviews and commentaries freely.

In 2021, *EVCNA* has enjoyed great support from the scientific community working in this field, and we thank everyone for their contributions. We look ahead to greater success in 2022 and serving the researchers in the field of Extracellular Vesicles and Liquid Biopsy.

Warm Wishes to everyone for Good Health and Success for 2022.

DECLARATIONS

Author's contributions

The author contributed solely to the article.

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Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

AUTHOR INSTRUCTIONS

1. Submission Overview

Before you decide to publish with *Extracellular Vesicles and Circulating Nucleic Acids* (*EVCNA*), please read the following items carefully and make sure that you are well aware of Editorial Policies and the following requirements.

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All submissions are required to be presented clearly and cohesively in good English. Authors whose first language is not English are advised to have their manuscripts checked or edited by a native English speaker before submission to ensure the high quality of expression. A well-organized manuscript in good English would make the peer review even the whole editorial handling more smoothly and efficiently.

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1.5 Work Funded by the National Institutes of Health

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2. Submission Preparation

2.1 Cover Letter

A cover letter is required to be submitted accompanying each manuscript. It should be concise and explain why the study is significant, why it fits the scope of the journal, and why it would be attractive to readers, *etc*.

Here is a guideline of a cover letter for authors' consideration:

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In the second paragraph: concisely explain what was done, the main findings and why they are significant;

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In the fourth paragraph: confirm that the manuscript has not been published elsewhere and not under consideration of any other journal. All authors have approved the manuscript and agreed on its submission to the journal. Journal's specific requirements have been met if any.

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Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
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2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

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Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

2.3.1.3 Abstract

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

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

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The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether the aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

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

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This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

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It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

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Each author is expected to have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data, or the creation of new software used in the work, or have drafted the work or substantively revised it.

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Types	Examples
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Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. *J Urol* 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
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Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. BJOG 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
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Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A*. Forthcoming 2002.

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