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Obstacles and opportunities in the functional analysis of extracellular vesicle RNA – an ISEV position paper

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ABSTRACT

The release of RNA-containing extracellular vesicles (EV) into the extracellular milieu has been demonstrated in a multitude of different *in vitro* cell systems and in a variety of body fluids. RNA-containing EV are in the limelight for their capacity to communicate genetically encoded messages to other cells, their suitability as candidate biomarkers for diseases, and their use as therapeutic agents. Although EV-RNA has attracted enormous interest from basic researchers, clinicians, and industry, we currently have limited knowledge on which mechanisms drive and regulate RNA incorporation into EV and on how RNA-encoded messages affect signalling processes in EV-targeted cells. Moreover, EV-RNA research faces various technical challenges, such as standardisation of EV isolation methods, optimisation of methodologies to isolate and characterise minute quantities of RNA found in EV, and development of approaches to demonstrate functional transfer of EV-RNA *in vivo*. These topics were discussed at the 2015 EV-RNA workshop of the International Society for Extracellular Vesicles. This position paper was written by the participants of the workshop not only to give an overview of the current state of knowledge in the field, but also to clarify that our incomplete knowledge – of the nature of EV(-RNA)s and of how to effectively and reliably study them – currently prohibits the implementation of gold standards in EV-RNA research. In addition, this paper creates awareness of possibilities and limitations of currently used strategies to investigate EV-RNA and calls for caution in interpretation of the obtained data.

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Introduction

Extracellular vesicles (EVs), including exosomes and microvesicles, are released into the extracellular space by many cell types. EVs carry a repertoire of bioactive molecules, including proteins, nucleic acids, lipids and carbohydrates.[1] Their role has been repeatedly highlighted in cell-to-cell communication and lately they have been implicated in the progression of several diseases, including neurodegenerative, cardiovascular and infectious diseases as well as cancer.[2–6] EVs are present in various body fluids and since the molecular content of EVs reflects the type and activation status of their parent cell, they are regarded as potent biomarkers for disease.[5,7,8] Additionally, EVs are being explored as delivery vehicles for therapeutic purposes. In the last 10 years it has become established that EVs contain RNA molecules, and thereby represent a vehicle through which cells may transfer genetically encoded messages to other cells. Although other extracellular carriers of RNA also exist, the purpose of this article is to highlight particularities in the study of EV-associated RNA. Current research in the EV field aims to characterise the RNA content of EVs and the details of its delivery *in vitro* and *in vivo*. Although this field has attracted enormous interest spanning basic research, clinics, and industry, understanding of many aspects of the formation and function of RNA-containing EVs remains elusive. A lack of standardisation with regard to EV purification and characterisation of their molecular contents, as well as technical difficulties in unequivocally demonstrating that EV-RNA is a causative agent in EV-mediated effects on target cells, are among the present challenges to this field.

Following up on the first workshop organised by the International Society for Extracellular Vesicles (ISEV) on "Extracellular Vesicle RNA" (New York, 2012),[9] the society recently organised a second workshop to address the question: "EV-associated RNA: is there a purpose?". This workshop, held in September 2015 in Utrecht, the Netherlands, brought together 70 international experts in the field – including principal investigators, postdocs, and PhD students as well as representatives from industry – to share knowledge and technical expertise on how to address the nature and function of EV-associated RNA.

Below, we report on the topics that were addressed during the workshop and substantiate them with references to recent literature. We raise awareness of various factors affecting EV-RNA characterisation (e.g. EV purity and biases in RNA sequencing methodologies), discuss the heterogeneity in RNA content of EV, describe both active and passive RNA sorting processes which have been suggested to determine the RNA content of EVs, and conclude that providing undisputable evidence that RNA mediates EV-induced effects remains difficult with currently available methodologies. In reporting this discussion we provide researchers from both inside and outside of the EV community with a critical overview of the current status of the EV-RNA research field and an outlook to future challenges.

1. Purification of EVs prior to EV-RNA isolation

1.1. New insights in the heterogeneity of EV and their RNA content

At the time of the previous ISEV workshop on EV-RNA in 2012, the research field had already recognised the heterogeneous nature of vesicles present in the extracellular environment.[10] The typically presented classification divided EVs into two subtypes: EVs with diameters ≤100–150 nm and buoyant densities of 1.11–1.19 g ml^{-1} that are formed inside multivesicular bodies (MVBs) were defined as exosomes, while EVs with diameters ranging up to 1000 nm which presumably bud from the plasma membrane were variably called ectosomes, microvesicles, microparticles, or large oncosomes. The exact definitions varied widely between publications and overlap in sizes between these two categories was not generally commented on.

It is clear that these categories represent an oversimplification. For instance, EVs formed directly at the plasma membrane can share several biophysical properties with EVs formed in MVBs, such as size, isolation by high-speed ultracentrifugation, and floatation in density gradients at the expected 1.11–1.19 g ml^{-1} position.[11] Thus, EVs in the small size range likely represent vesicles heterogeneous in origin, with an unknown portion coming from MVBs. The definition of larger EVs is even less precise, and these vesicles comprise a wide range of membrane-enclosed entities. Indeed, it is not yet clear how to divide EVs into their relevant subtypes, or even how many functionally distinct subtypes there may be.

Several laboratories have now started characterising the protein composition of subtypes of EVs. EV subtypes have been isolated by a number of means, including recovery at different centrifugation speeds, through different filters, at slightly different positions in density gradients, via immuno-isolation by different surface molecules, or by flow cytometric sorting.[12–20]

Extensive comparison of these results (obtained with EVs from different cellular sources) has not yet been performed, but the available data already indicate that some proteins classically regarded as "exosome

markers" are in fact present in all different EV types (e.g. heat shock proteins, flotillins and major histocompatibility complex molecules). Such intracellular or membrane-associated proteins can therefore be used as EV markers, but will not define the nature of the EV subtype analysed. Furthermore, within the "exosome" population, subsets could be defined based on combinations of protein markers which colocalise or are co-depleted in vesicles enriched in endosomal proteins vs. plasma membrane proteins.[12,19] Given the difficulty of separating subtypes of EVs with ultracentrifugation, Thery's group has recently chosen to refer to vesicles sedimenting at 100,000 g as "small EVs" (sEVs) rather than exosomes, those pelleting at intermediate speed (lower than 20,000 g) as "medium EVs" (mEVs, including microvesicles, ectosomes) and those pelleting at low-speed (e.g. 2000 g) as "large EVs" (lEVs, including large fragments of the releasing cell and large apoptotic bodies). EVs of small size are also enriched in many studies by the use of filters of small pore size (100 or 220 nm). These definitions are perhaps less biologically meaningful but far more experimentally tractable than the previous exosome/microvesicle definitions, since EV size, often determined by nanoparticle tracking analysis or electron microscopy, is frequently reported in EV studies. Until we have stringent and robust methods for separation and characterisation of MVB vs. plasma membrane-derived EVs, the proposed nomenclature could increase clarity of discussion and ease of cross-referencing between studies. However, consensus has not been reached on this issue and the current nomenclature is therefore maintained until further notice.

It is still unknown whether all EVs contain RNA and how diverse the RNA content of different EV subpopulations may be. Various studies indicate that the RNA content of EVs varies among cell types and among EV subpopulations. For example, miR-145 is present at very low levels in HepG2 cell-derived large EVs, whereas the same miRNA is present at significant levels in both large and small EVs derived from A549 cells.[21] In immune cells, the miRNA content of EVs was shown to differ by immune cell type when comparing EVs from B- or T-cell lines and primary dendritic cells (DC).[22] Another remarkable example of EV-RNA heterogeneity is the sex difference observed in the miRNA content of urinary EVs.[23] With regard to the RNA content of different EV subpopulations, it was shown that EV populations that separated into different fractions based on pelleting at different g-forces differed in RNA content.[24] Even EVs sedimenting at the same g-force are heterogeneous in nature and may be further separated based on differences in migration velocity in density gradients; recent data indicate that EV subpopulations isolated based on this parameter differ in both protein and RNA content.[20] In a recent study, the copy number of a given miRNA molecule was suggested to be on average lower than one per vesicle/particle in an EV sample.[25] If we assume that all of the detected miRNA species were indeed EV-associated and that EV quantifications were accurate, one explanation for these data is that specific miRNA sequences could be restricted to specific subtypes of EVs. This scenario would be consistent with a high specificity in delivery of RNA molecules to target cells (see also section 4.1.2).

The presence of extracellular RNA circulating in non-EV-associated forms, for instance in large protein (e.g. Argonaute 2 (AGO2)) or lipoprotein complexes, adds another layer of complexity to the analysis of EV-RNA. These complexes have been shown to co-isolate with EVs during common isolation procedures such as ultracentrifugation [26] (see section 1.3.1). Thus it is possible that, of the numerous types of nucleic acids described "in EVs" in the existing literature, some are contained within specific subtypes of EVs and some are perhaps not present in EVs at all but exclusively in other carriers which co-isolate. A particular point of concern is the potential carryover of extracellular RNA originating from foetal bovine serum used in cell culture media, which, if not effectively removed, affects the analysis of EV-RNA released by the cell of interest.[27,28] This urges the need for including control isolates from non-conditioned culture medium in the RNA analysis.

1.2. Update on EV purification methods and effects on EV-RNA analysis

Participants of the meeting expressed their concern about the enduring lack of standardisation with regard to the collection, storage, and processing of EV-containing fluids, and the large diversity of methods used for EV isolation. Each of these factors influences not only the type and number of EV isolated, but also the level of contamination with non-EV-associated RNA in the obtained EV preparation.

Though differential centrifugation remains the most commonly used method of EV isolation, several different techniques have risen in prominence since the 2012 meeting on EV-RNA (Figure 1). Pros and cons of commonly used EV isolation techniques have been reviewed more elaborately elsewhere (see for example [29,30]), but are briefly summarised below. Differential centrifugation can be followed by density gradient ultracentrifugation to separate low-density EVs from high-density protein aggregates that often contaminate

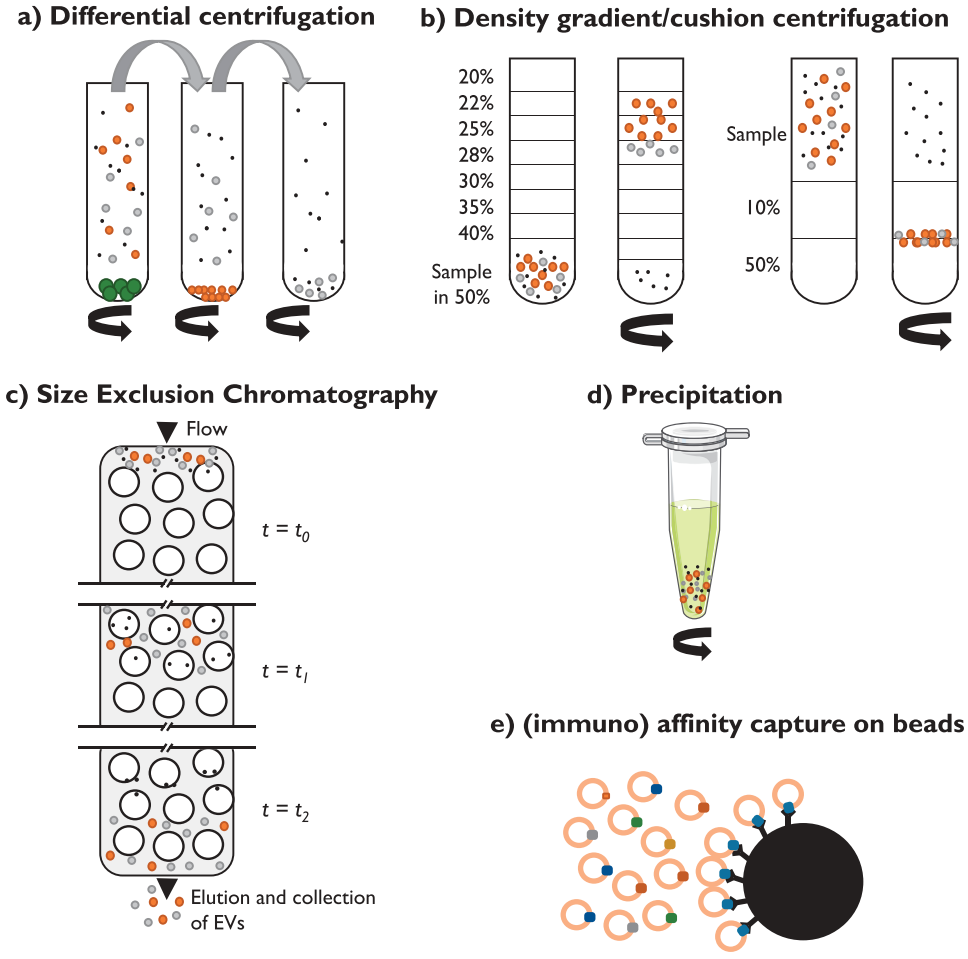

Figure 1. Schematic illustration of commonly used EV isolation techniques. (a) Differential centrifugation is the sequential pelleting of particles with decreasing sedimentation coefficients. Typically 2000 g is used to pellet large EVs, 10,000–20,000 g to pellet middle-sized EVs (green), and finally ~100,000 g to pellet the smallest EVs (different EV subpopulations are indicated in grey and orange). At these high g-forces, complexes of soluble proteins (black dots) may also sediment. (b) Lipids have a density that is approximately 1 g cm^{-3}, while proteins and RNA have a higher density (>1.3 g cm^{-3}). Therefore density gradients can be used to separate subpopulations of EVs with different ratio of lipids, RNA, and proteins. Moreover, these gradients can be used to purify vesicles away from soluble proteins, RNA, and protein–RNA complexes as the latter structures will not float at the same density as the lipid containing EVs. (c) Size exclusion chromatography separates particles based on their size, by trapping the smaller molecules (such as proteins and protein complexes) in the pores. The larger molecules (such as EVs) are too large to enter the pores and will elute first. (d) Precipitation of EV from cell culture medium or body fluids is based on volume-excluding polymers such as polyethylene glycol (PEG) with which biological materials such as proteins and EVs are precipitated from the solution. (e) (Immuno-)affinity capture isolates vesicles using beads coated with antibodies or proteins (such as heparin) with affinity for an EV transmembrane protein. Vesicles displaying the protein of interest will bind to the beads and can thereby be isolated from the vesicle-containing solution.

EV ultracentrifugation pellets. Size exclusion chromatography (SEC) is now a more widely used technique for EV isolation. It can be used for low volume samples and allows separation of EV from the bulk of soluble proteins. However, since separation is purely based on particle size, contaminating particles in the EV size range such as (lipo)protein complexes may be co-isolated. Immuno-affinity capture presents an alternative method for EV isolation. The method can yield pure EV subpopulations, but is highly influenced by both the choice of affinity reagent and the ligand density on different EV types. In addition, various commercial kits that make use of volume-excluding polymers such as polyethylene glycol (PEG) are currently available for rapid EV isolation from culture media or body fluids. However, such polymers co-precipitate protein (complexes) that contaminate EV isolates. It was highlighted during the meeting that the different EV isolation techniques are based on different principles and will therefore enrich for different subpopulations of vesicles. In addition, these methods co-isolate contaminants (e.g. protein complexes and lipoproteins) to

different degrees. Combinations of techniques, such as density gradient centrifugation followed by size exclusion or immuno-affinity capture, are being used more frequently. Moreover, comparative studies on different techniques including SEC for isolation of pure EV populations have been published.[31] There are few data available on the impact of different EV isolation methods on EV-RNA yield and purity.[32,33] In one of these studies, RNA was obtained from EVs isolated by ultracentrifugation, density gradient centrifugation, and two commercially available precipitation-based kits.[32] Although three to eight times less protein and fewer particles were detected compared to the kit-based EV isolation, density gradient-based isolation of EV yielded the purest EV population, as assessed by immunogold electron microscopy and Western blots. Importantly, with some commercial kits 100-fold more RNA could be isolated compared to the density gradient method, albeit the technical reproducibility of the kit-based isolations was often low. This study highlights the trade-off of yield vs. purity: it is clear that although some isolation techniques give higher yields of RNA, it comes at the cost of lower purity, which can affect conclusions drawn from RNA analysis. Which isolation method is optimal and which impurities are acceptable depends on the research question and downstream analysis; in the discovery phase of EV-RNA biomarkers, when association of disease markers with EV still needs confirmation, contamination of EV isolates may lead to erroneous conclusions. Also studies to unravel the role of EV-RNA in (patho)physiological processes require pure EV populations. However, when detecting established EV-RNA-based biomarkers in low volume patient samples, increased protein/lipid/RNA yields at the cost of lower EV purity may be acceptable. In addition, preferences for specific EV isolation methods will depend on the type and volume of the starting material, the number of samples to be analysed, and the logistical setting and laboratory infrastructure.

At this stage, the EV-RNA community would certainly benefit from more comparative studies on the effects of EV isolation strategies on EV-RNA yield and identity, and from studies that critically evaluate the potential use of kit-based assays for clinical applications.

1.3. Isolation of EVs from different sources prior to EV-RNA isolation and characterisation

EVs are isolated from a variety of different sources, including body fluids with highly variable composition (e.g. plasma, serum, milk, urine, nasal washes, cerebrospinal fluid and saliva), cell culture media of cell lines and primary cells, and tissues or tumours. Additionally, EVs from various different species are being investigated, ranging from humans to microbes. It is therefore difficult to provide general recommendations for EV isolation and characterisation. For several body fluids, considerations and recommendations were provided after the previous EV-RNA meeting in 2012.[34] Although these are still valid, we have extended our knowledge on the complexity of body fluids and how this affects EV isolation. Although no gold standards can be provided yet for isolation of EV from the different fluids, recent data progressed our understanding of the nature of contaminants in EV isolates from different body fluids and of pre-analytic variables that affect the type or purity of isolated EV (see for example [35–40]). The non-EV contaminants found in EV preparations differ substantially between body fluids; EVs from certain fluids (e.g. nasal fluid, saliva, milk and urine) can contain bacteria-derived material. Other fluids may contain substantial amounts of biofluid-specific contaminants, such as Tamm–Horsfall glycoprotein in urine or glycosaminoglycans/proteoglycans in synovial fluid samples. Standardised and optimised pre-analytical conditions for EV isolation should therefore be carefully determined for each of the biological fluids separately.

Blood plasma is the most commonly used source of EVs in EV-RNA analysis studies. It represents a very complex fluid from which EV isolation remains challenging. Below, we highlight recent developments in research on EVs in plasma to exemplify the challenges we face when performing EV-RNA analysis in body fluids.

1.3.1. EV-RNA isolation from blood plasma

Both serum and plasma are used for EV research and biomarker discovery. Serum contains high numbers of EV released by platelets in response to coagulation. Although platelet-derived EV may be considered as biomarkers for a variety of pathological processes,[41] there is limited knowledge on differences in the RNA content of EV isolated from serum and plasma samples obtained from the same donor.[42] Plasma mainly contains EV originally present in circulating blood and is therefore the preferred source when studying (patho)physiological functions of EV. Anticoagulation of blood samples has a major impact on the number and composition of isolated EVs because the efficiency of this process affects the number of platelet-derived EVs in plasma preparations. The recommendation of the International Society on Thrombosis and Haemostasis is the use of citrated platelet free plasma [43] for EV isolation and analysis. However, acid-

citrate dextrose (ACD) has been shown to be superior to citrate with respect to preventing *in vitro* generation of platelet EVs within the blood collection tube, and is compatible with downstream RNA analysis.[44] Thus, using ACD as anticoagulant ensures isolation of EVs that are present in circulation *in vivo* (and not those released *in vitro* by platelets in the blood collection tube).

Protein complexes may co-purify with EVs from blood plasma and may also mimic EVs during enumeration of vesicles.[26,45] These protein complexes include RNA-binding proteins such as AGO proteins,[26,46] which form complexes with miRNAs. Importantly, lipoproteins can also contaminate blood-derived EV preparations. Both LDL and HDL were shown to transport miRNA,[47] which may be co-isolated with EV-associated RNA. In addition, EV-sized chylomicrons are present in platelet-free blood plasma samples, and can confound EV enumeration, most prominently in the postprandial state.[39] Postprandial state also affects the levels of HDL particles that co-purify with EVs.[48] HDL cannot be discriminated from EVs based on buoyant density (1.06–1.20 g cm^{-3}), but may in theory be separated from EV by SEC or ultracentrifugation because of their much smaller size (10 nm). Other lipoproteins such as VLDL and chylomicrons may be more effectively removed using a density gradient as they have a density <1.06 g cm^{-3}, but are similar in size to EV (≥60 nm).

SEC was shown to allow separation of EV from contaminating proteins and HDL present in platelet concentrates.[49] However, a more recent study proposes that EV-mimicking LDL particles are present in blood plasma at almost one order of magnitude higher concentration than EVs and suggests that they cannot be fully removed from EV preparations by any of the known EV isolation and purification methods.[39] As a result, detection of blood plasma-derived EVs based on particle counts might strongly overestimate EV numbers, and proteomic or nucleic acid analysis of these EV preparations may contain significant contamination from non-EV sources.

1.4. The importance of knowledge exchange and correct reporting

The participants stressed the importance of setting up a forum on which key issues with regard to best practice for fluid collection, storage, processing, and for EV isolation methodologies can be discussed for each individual fluid. Arising from discussions at the Utrecht EV-RNA workshop, an initiative to meet this need was taken at the ISEV meeting in Rotterdam 2016, where the "Experts Meet" sessions were introduced. In each of these sessions, researchers with hands-on expertise on working with particular body fluids (blood, milk, urine) met and discussed recent developments. This may in the future lead to renewed and refined guidelines and also could fuel collaborative research in which several labs analyse the same samples to further develop standardised protocols. Ideally, researchers should engage with biobanks to ensure that collection of new samples will occur using the best possible protocols for collection and storage of body fluids. It was also highlighted during the meeting that methods sections of EV publications usually contain too few details to be able to reproduce the obtained results. Currently there is a strong need to develop tailored checklists for descriptions of collection methods, storage conditions, and EV purification methods, which will improve best practices and reproducibility of published results.

2. Analysis of the quantity and diversity of EV-RNA

Several different types of small and long RNAs have been identified in EVs (reviewed in [50]). The EV isolation method of choice determines the yield and purity of EV preparations, and as a consequence, the quantity and quality of EV-RNA.[32,51] Measuring the quantity and integrity of EV-associated RNA is challenging due to low RNA quantities and a lack of standards, such as those established for cell RNA. Below, we address topics discussed at the workshop concerning quantification of EV-RNA and reliable assessment of the nature of EV-associated RNAs.

2.1. Assessing EV-RNA quantity

The study of EV-RNA poses challenges both shared with and distinct from the study of cellular RNA. Many of these stem from the fact that researchers studying EV-RNA are typically working with very small quantities of RNA relative to quantities found in cells; this is generally true for EVs from *in vitro* cell cultures but especially pertinent for those harvested from patient or animal samples, where large sample volumes may be difficult to obtain. Even the quantification of these small amounts of RNA can be non-trivial. In contrast to cellular RNA, in which intact ribosomal RNA dominates the pool of RNA and detection signal, EV samples are mostly devoid of intact large and small ribosomal RNA subunits. As a result, the required RNA quantity for specific analysis methods (e.g. sequencing, microarrays or quantitative reverse transcription polymerase chain reaction (RT-qPCR)) does

Table 1. Suitability of RNA detection methods for quantification of EV-RNA.

Method	Lower detection limit	RNA vs. DNA specific?	Remarks
Nanodrop spectrophotometer family (Nanodrop, Thermo Fisher Scientific)	3 μg μl^{-1} to 2 ng μl^{-1} range for microliter volumes of RNA	No	Not generally suited for measuring EV-RNA due to high lower limit for detection.
Qubit RNA HS (high sensitivity) assay (Thermo Fisher Scientific)	>0.2 ng μl^{-1} (initial sample concentration if using the maximum volume for the kit, 20 μl of sample)	Yes	Not generally suited for measuring EV-RNA due to high lower limit for detection.
Bioanalyzer Pico chip (Agilent Technologies)	50 pg μl^{-1}	No	Most sensitive quantification method for total RNA, but prone to error. Most relevant for assessing total RNA content and length distribution.
Bioanalyzer small RNA chip (Agilent Technologies)	50 pg μl^{-1} of purified miRNA or 10 ng μl^{-1} of total (cell) RNA in size range of 6–150 nt	No	Similar properties as Pico chip. Useful for resolving miRNA from tRNA and other small RNA species.
Quant-iT RiboGreen RNA Assay kit (Thermo Fisher Scientific)	Detection range of 1–200 ng (sample diluted to 1 ml)	No	Less sensitive to contaminants, such as protein and phenol chloroform.
Quantitative reverse transcription polymerase chain reaction (RT-qPCR)	1 fg (~2500 copies for mRNA) of a particular transcript	No	Most sensitive quantification method overall but does not analyse total RNA, must select primers specific to target transcript(s) and validate to check for off-target amplification.

not necessarily match respective recommendations for cellular RNA samples. RNA quantification methods have recently been compared and evaluated by Aranda et al. [52]. In this section we review RNA quantification methods discussed at the workshop and comment on their suitability for use in EV-RNA studies (summarised in Table 1).

The Nanodrop spectrophotometer family (Nanodrop 1000, 2000, or 2000c; Thermo Fisher Scientific, Wilmington, USA) measures microliter volumes of RNA based on UV-absorbance that is accurate in the range of 3 μg μl^{-1} to 2 ng μl^{-1}. As RNA obtained from EV preparations is typically present in less than 2 ng μl^{-1} concentration, Nanodrop is not a suitable method for measuring EV-RNA unless working with a highly concentrated sample.

The Qubit RNA HS (high sensitivity) assay (Thermo Fisher Scientific) is highly specific for RNA but has a limit of >0.2 ng μl^{-1} when using the maximum volume for the kit (20 μl of sample). Therefore, it is not convenient for measuring EV-RNA unless using a large volume of sample or a relatively concentrated sample. However, it was shown that with the addition of spike-ins to bring sample RNA concentration above the minimum, the Qubit RNA HS assay is able to quantify small amounts of RNA with high specificity, down five-fold (to 1 ng) from the assay's previous lower detection limit.[53] This technique may be particularly useful if it is necessary to measure RNA in the presence of DNA contamination.

With a lower detection limit of 50 pg μl^{-1}, the Bioanalyzer Pico chip (Agilent Technologies, Foster city, USA) is one of the most sensitive RNA quantification methods currently available. Notably, it requires only 1 μl of sample and gives electrophoresis-like length profiles which are useful for estimating the size distribution of RNA in EV samples. However, the chip and the software based on the RIN algorithm are designed to assess quality based on the large ribosomal subunits, which are not present at the same level in EVs. It should also be noted that peaks of mRNAs and long RNAs are less well discerned, as they are distributed over a greater range of transcript lengths. The small RNA chip (Agilent Technologies) may therefore be more relevant for assessing EV-associated RNA content and length. It has been reported that RNA quantification by chip-based systems is in general error prone.[54] In both Pico and small RNA chips the RNA concentration is determined relative to a supplied RNA ladder and internal marker peaks, which can show variability between measurements if the chip is not prepared meticulously. Occasionally, aggregates in the RNA dye can cause peaks in the electrophoresis profiles, leading to quantification errors. Furthermore, the Pico assay is sensitive to differences in salt concentration, which can vary between different RNA isolation kits and can also be affected by DNase treatment. The Nano chip is less sensitive to salt but has a quantitative detection limit of 25 ng μl^{-1} (qualitative lower limit 5 ng μl^{-1}). In addition, contaminating DNA in EV-RNA isolates is detected in any Bioanalyzer 2100 chip, as their detection strategy employs a dye that is not specific to RNA. Despite these caveats, the Bioanalyzer remains a popular method to quantify EV-RNA, particularly when it is desirable to obtain a size profile of RNA present in the sample.

When access to specialised RNA quantitation equipment is restricted, an alternative is to use the Quant-iT RiboGreen RNA Assay kit (Thermo Fisher Scientific). This assay is based on a nucleic acid-specific fluorescent dye that can be used to quantify RNA with a linear detection range of 1–200 ng using any standard

fluorescence microplate reader. This assay is sensitive to DNA contamination, but less sensitive to protein and phenol chloroform. The use of a standard curve that can be adjusted for low input RNA and the custom of running samples in triplicate improve suitability for low input samples.

Finally, RT-qPCR was used by various participants of the workshop to measure transcript abundances in EV-RNA preparations. This technique quantifies levels of a particular nucleic acid transcript in a sample by measuring its increase in concentration over time (using fluorescent nucleotides or a fluorescent probe) when subjected to exponential amplification by PCR.[55] Although this method does not directly measure total RNA, it is extremely sensitive, able to detect 1 fg or ~2500 copies of a given transcript in an optimised system.[56] Given the often material-limited nature of EV-RNA research, measuring a panel of individual transcripts by RT-qPCR as a proxy for total RNA content may be more experimentally feasible than any other quantification method (see section 2.4 on normalization strategies and reference transcripts for further discussion). RT-qPCR is sensitive to DNA contamination, though this can be minimised by good experimental practice, such as running a gel to verify a single amplicon of the expected size and designing primers over exon-exon junctions in the case of mRNA.

In summary, sensitive techniques such as Agilent Bioanalyzer pico chip and the Quant-iT RiboGreen RNA Assay are far more suitable for EV-RNA quantification than the Nanodrop. Detection of the levels of particular transcripts by highly sensitive RT-qPCR may be used as a proxy for total RNA quantity in samples containing a very low amount of RNA. Most techniques, with the exception of the Qubit RNA HS Assay, are also sensitive to DNA contamination. We therefore recommend pre-treatment of samples with DNase for accurate RNA quantitation (see section 2.2.1.2 for further discussion).

2.2. Assessing EV-RNA quality

Isolation of intact (non-degraded) RNA is of great importance in quantitative gene expression profiling experiments. RNA may be degraded in many ways: by enzymes, namely ribonucleases (RNases), which are both ubiquitous and extremely stable; by mechanical stress introduced by freezing, thawing or centrifugation; by base-catalysed hydrolysis; by heat, especially in the presence of divalent cations; and by UV damage. Exposure to any of these agents can cause RNA damage and influence the results obtained by downstream quantitative applications.[57] This risk becomes more pertinent when working with small quantities of RNA, as it is more likely to become fragmented over the course of many handling steps required for the isolation procedure. However, it should also be taken into account that EVs may contain processed fragments of longer RNAs that are biologically relevant. In addition to RNA integrity, another important quality measure is the purity of RNA. The following sections will deal with assessment of EV-RNA purity and integrity. Methods and considerations for these experiments are summarised in Table 2.

2.2.1. Experimental artefacts and contaminants affecting EV-RNA analysis

2.2.1.1. Non-EV associated RNA and lab-derived contaminations. A major source of contamination is the presence of other RNA-containing structures in EV samples. Potential contaminants include

Table 2. Methods for determining EV-RNA purity and integrity.

Method	Use	Pros	Cons
Agilent Bioanalyzer chips	Integrity	• Small volume required • Highly sensitive • Total length profile of RNA	• Not suited for assessing small RNA integrity • Assessment based on intact 18S/28S rRNAs generally depleted from EVs • Sensitive to contaminants such as DNA
Next generation sequencing	Integrity & purity	• Detects fragmentation, for example as 3′ bias in mRNA reads after poly-A selection • Detects presence of foreign genetic material (e.g. derived from foetal bovine serum)	• Erroneous assessment of fragments in the case of highly modified RNA types • Long reads (i.e. PacBio) most useful but require lots of material
RT-PCR and derivatives (i.e. 5′/3′ RACE)	Integrity	• Robust and sensitive, can map exact sites of fragmentation	• Analysis of single transcripts only
Northern blot	Integrity	• Robust and sensitive • Simultaneous detection of full length and fragmented stretches of the same RNA	• Analysis of single transcripts only • Time-consuming
Proteinase-nuclease protection assay	Purity	• Rigorously determine that RNA is present in EV lumen	• Leftover nucleases may still be active at point of vesicle lysis
Blank run	Purity	• Test kits and reagents for nucleic acid contamination	—
Picogreen	Purity	• Test for presence of dsDNA	• Not DNA-specific in samples with RNA concentrations over 130 ng ml^{-1}

ribonucleoprotein complexes (RNPs), viral particles, and lipoproteins (HDL and LDL), which may originate either from the EV source or from foetal bovine serum used in cell culture media.[27,28] Steps taken to increase stringency of the isolation protocol, for example washing and re-pelleting EVs after centrifugation, may decrease contamination but cannot fully eliminate it, since contaminating particles present in the first pellet may re-pellet together with EVs (see references in section 1.3.1 for details). RNPs may also in theory become non-specifically associated with the EV surface, especially after high centrifugal force is applied to the sample. To rigorously distinguish between RNA encapsulated within EVs from RNA outside EVs, it is critical to treat EV samples with proteinase and RNase to disrupt ribonucleoproteins exterior to vesicles (see section 3.2.3 for more details). Low RNA inputs can also amplify the effects of lab-derived contamination, especially when performing high throughput sequencing. This was shown both for DNA [25] and RNA [58] contamination, coming from various sources including commercial nucleic acid extraction kits and sample cross-contamination. A blank run can be performed (e.g. sequencing a pure buffer sample processed similar to the EV-RNA samples) to control for these possibilities, but in general researchers should be meticulous when working with EV-RNA and aware of the potential pitfalls.

2.2.1.2. Are DNA and rRNA naturally associated with EVs? The question of which RNA components should be considered as "true" EV-RNA and which as impurities or contaminants has been extensively addressed during the workshop. The presence of both DNA and rRNA in EVs, for example, is disputed. Extracellular DNA is known to be present in various biological fluids (e.g. plasma and urine) and in culture medium as a result of necrosis/apoptosis or active cellular secretion processes,[59] but it is not clear if it is also present inside EVs. In order to determine if DNA present in an EV preparation is truly encapsulated in vesicles, researchers should perform proteinase and DNase treatments prior to vesicle lysis (compared with DNase treatment post-lysis) and read out DNA to demonstrate protection or lack thereof.[60]

If DNA is not the intended object of study, the presence of DNA in EV-RNA preparations can interfere with downstream analysis. It was mentioned above that ssDNA and dsDNA could interfere with RNA signals in the Bioanalyzer small RNA and RNA pico chips as well as in RT-PCR. Although RNA extraction kits usually give very low DNA contamination,[61] many EV-RNA researchers indicated that they regularly experience DNA contamination in EV-RNA preparations. It is advisable to test the RNA extraction kits with or without DNase treatment according to the desired downstream applications. Especially for downstream deep sequencing analysis, it is important to treat EV-RNA samples with DNase. The most suitable kit according to workshop attendants was the Ambion Turbo-DNA free kit (Thermo Fisher Scientific). It effectively eliminates most DNA, and RNA can subsequently be cleaned up from the enzyme and buffer with beads, which is useful for very low RNA input samples and ease of handling. Picogreen was suggested as an assay for detecting DNA contamination, as it is sensitive to dsDNA down to 250 pg ml^{-1} and selective for dsDNA over RNA for RNA concentrations less than 130 ng ml^{-1}.[62] Alternatively, in deep sequencing protocols based on poly-A enrichment, presence of genomic DNA could be assessed by the percentage of intronic reads, though this may not hold true for other protocols.[63]

During the round-table discussions, attendants of the workshop also discussed the origin of DNA and rRNA in EV-RNA preparations. It was raised that large DNA fragments (>3 kb) could elute in EV-containing fractions obtained by SEC, either due to non-specific binding to EV or due to similarity in size and molecular weight. Similarly, large DNA fragments could pellet at high g-force if they have sufficiently high molecular weight, or become associated with EVs during centrifugation. These considerations may also apply to ribosomes present in extracellular fluids and explain the variable presence of rRNA in EVs in the literature. Ultimately, demonstration of protection from nucleases after proteinase treatment is the only way to assert that a given nucleic acid species is encapsulated in a lipid membrane structure and not adhered to it or simply co-isolating.

2.2.1.3. RNA integrity and RNA fragments. Several methods exist to measure RNA integrity. In most cases, research groups assess total RNA quality using Agilent Bioanalyzer chips. The standard metric for cellular RNA quality, the RNA integrity number (RIN), determined using Agilent Bioanalyzer chips, corresponds to the presence and profile of intact 18S and 28S ribosomal RNA subunits. However, ribosomal RNAs are generally depleted from EVs, making this approach ineffective as quality control for EV-RNA. Next-generation sequencing technologies allowing single-molecule RNA sequencing (e.g. Pacific Bio) may be useful to assess the overall integrity of long EV-RNA; however, the large quantity of RNA starting material required for these technologies currently impedes their

application to analysing EV-RNA. Alternatively, more classical techniques such as RT-PCR and derivatives (e.g. 5′/3′ RACE) allow the determination of integrity for a selected set of RNAs, which could be used as quality control markers to validate an EV preparation technique or batch.[64,65] For example, "housekeeping" mRNAs or miRNAs at a range of levels of expression could serve this purpose.

An interesting question rising in the field is whether the RNA associated with EVs is fully intact, fragmented, or specifically processed. There is an increasing body of evidence supporting the involvement of ncRNA fragments in many biological processes, including gene regulation.[66–69] Small RNA-seq analysis has indicated that EVs are associated with various fragments derived from mRNAs and ncRNAs, including rRNA, tRNA (also called tRFs, tRNA-derived RNA fragments), YRNA, snRNA, snoRNA, lncRNA and vault RNA.[70–74] Many of these fragments even showed pronounced enrichment in the EVs compared to their parental cells. The degree of fragmentation of particular RNAs may also depend on the protection by encapsulation in the vesicle membrane; for example, in nematode-derived EVs, full length YRNAs were found exclusively inside EVs whereas fragments were found outside EVs.[75]

Currently, there is no definitive proof indicating whether the RNA fragments found in EVs are formed by specific processing steps or whether these are artefacts induced by handling during the EV-isolation procedure. Specific RNA cleavage may occur in the parental cell cytoplasm, prior to enclosure of such fragments into EVs; alternatively, RNA fragments may be generated due to processing inside the EVs, as part of an extracellular maturation process. For example, EVs containing the enzyme Dicer were reported to perform cell-independent miRNA biogenesis by cleaving pre-miRNAs shuttled into the vesicles.[76] This remains controversial, as some attendants of the meeting were unable to detect Dicer or other main components of the RNA interference machinery in the vesicle fractions of sucrose gradient-based purifications, and thus this particular pathway remains to be explored in more detail.

Artefactual EV-RNA fragments could arise due to non-specific degradation of ncRNAs after sample collection or technical inability to amplify the full-length precursors. Common reverse transcriptase enzymes used in RNA-sequencing library preparation protocols are unable to read through highly modified or structured RNAs such as tRNAs, and fall off, producing what looks in the analysis like a fragment. However, a recent protocol based on RNA pretreatment with *E.coli* AlkB (which demethylates tRNAs and removes most of the so-called "hard-stop" modifications responsible for reverse transcriptase fall-off) showed a sharp increase rather than a decrease in tRNA-derived fragments,[77] which implies that the presence of these fragments is not necessarily artefactual. During the meeting it was discussed that sequencing of EV-RNA using a thermostable group II intron reverse transcriptase [78] may also improve the efficiency with which full-length tRNA can be sequenced. In addition, Northern blotting can be used to distinguish full length and fragmented stretches of the same RNA, since this technique is not susceptible to the above mentioned biases. Using this method, defined and biologically relevant fragmentation of YRNA was demonstrated by applying oligo probes targeting the putative fragment(s) or full length RNA.[79,80] Additionally, RT-PCR using various placements of primers along the transcript can be used to map the RNA fragmentation state. This has for example been applied for detection of a fragmented form of 7SL RNA in HIV-1 virus-like particles, using a combination of S1 nuclease mapping and analysis of RT-PCR amplicons with primers located inside or outside the fragment.[81]

2.3. Biases in RNA isolation and high-throughput RNA sequencing

Due to the enrichment of small RNAs in EV, recent sequencing studies in the field have largely focused on the assessment of miRNAs and other small non-coding RNAs. Though the discussions at the workshop focused on biases specific to deep sequencing experiments, it is worth emphasising that for all experiments analysing quantitative gene expression, the RNA extraction strategy and any biases specific to that method should be taken into consideration. For example, the popular RNA extraction reagent TRIzol has been shown to exhibit strong anti-GC-content bias in small RNA extraction from low quantities of total RNA, a caveat which is highly relevant for EV-RNA researchers working with similarly low RNA quantities.[82] Sources of bias in RNA extraction and sequencing discussed in this section are summarised in Table 3.

Downstream of RNA extraction, deep sequencing analysis of EV-RNA is prone to biases at several different steps in the analysis. First, sequence biases occur during library preparation, for which many kits are available. For small RNA, for example, these include NEBNext multiplex small RNA library preparation kit (New England Biolab, Ipswich, MA, USA), NEXTflex small RNA sequencing kit (Bioo Scientific, Austin, TX, USA), TruSeq small RNA sample preparation kit

Table 3. Common sources of bias in RNA isolation and sequencing methods.

Source	Example	Solution
Size selection	Underrepresentation of mid-size RNAs in RNA sequencing experiments	Tailor size selectivity of RNA purification technique to size of RNA of interest. If analysing total RNA, perform multiple extractions for differently sized populations.
Extraction reagent	TRIzol induces GC content bias in small RNAs	Use alternative RNA extraction reagents for comparison.
Library preparation kit or protocol	Adaptor ligation bias	Use newly developed strategies to control for ligation bias, i.e. 4N adapter-based kits.
Sequencing platform	Different biases in different sequencing platforms	Use of identical platforms for experiments to be directly compared. Corroborate important conclusions with a second technique.
Bioinformatics	Mapping order	Map to concatenated databases and clearly indicate order of steps if mapping to multiple databases.

(Illumina, San Diego, CA, USA), and others. [72,74,83,84] One study presented at the meeting also used the CleanTag small RNA library kit (TriLink, San Diego, CA, USA) which has been proposed to reduce background adapter-dimers that can negatively influence the analysis of low-input samples. Most small RNASeq kits require RNAs to be captured by ligation, rather than priming, and these ligations are prone to biases.[85,86] Newly developed methods, such as CATS,[87] 4N adapter-based kits and the NEXTflex and SMARTer smRNA-Seq kits, have introduced strategies to avoid adaptor-ligation biases. The type of kit or protocol used heavily influences library preparation and the observed RNA profile, as was for example shown for plasma-derived EVs.[83] One challenge of deep sequencing analysis is that some "medium" length non-coding RNAs (e.g. snoRNAs of 60–300 nt) are difficult to capture either by small RNA sequencing kits or long RNA sequencing kits, and therefore require the use of kit-free protocols.

A nearly unavoidable second source of bias is that introduced due to size selection after cDNA synthesis and adapter ligation. This is performed in most small RNA sequencing protocols and thus particularly relevant for small non-coding RNAs. Almost all nucleic acid purification strategies have some size selectivity. For example, Zymo Clean & Concentrate kits yield small RNA with one concentration of ethanol and long RNA with another; SPRI (solid phase reversible immobilisation) beads isolate DNA fragments of different lengths according to the bead:DNA ratio, and even RNA precipitation in different volumes of ethanol will yield different size distributions in the precipitate. By selecting for a subset of the isolated RNA, one does not obtain a global small RNA profile of the vesicles under study. This can be an advantage if focusing on the analysis of a specific subset of small RNA, due to a gain in sequencing depth and thus the possibility of identifying low abundance RNAs, but it makes comparisons of RNA levels across differently sized transcripts challenging.

A third source of bias is introduced by the sequence platform used (e.g. HiSeq or MiSeq systems from Illumina, Ion Torrent or SOLiD system from Life Technologies, and others) and the subsequent bioinformatics analysis of obtained data. For example, it was discussed during the workshop that the order in which reads are mapped to multiple databases can have a dramatic effect on results: this is particularly pertinent for small RNAs, i.e. piRNA and tRNA. Because small RNASeq reads are 30 bp or less in length, many reads fall into a category of "multi-mappers". The sequences cannot be confidently assigned to any one RNA biotype, but instead could be counted in many categories of small RNA. In general it is recommended to map to a concatenated database if possible, or, if mapping to multiple databases with stepwise removal of mapped sequences, to state clearly which order was used. Further considerations for bioinformatic analysis of EV-RNA sequencing data are outlined in the previous ISEV position paper on "extracellular vesicle RNA analysis and bioinformatics".[9] As described in that paper, various parameters including the set-up of the analysis pipeline and data normalisation are crucial steps in interpreting sequencing data for EVs. Thus, processing steps applied to the raw data such as trimming/clipping of adapters, cut-off values and specific databases used for sequence annotation should be clearly described in publications in order to allow comparison of studies. Sharing of pipelines and raw data, as is often performed in genetics research, would lead to even better reproducibility and standardisation in the field.

It should above all be noted that none of the available methods is completely unbiased, and therefore all libraries in a given project should be created in a consistent manner throughout, and expression of key transcripts validated using a second platform, e.g. RT-qPCR. When comparing across projects from different

laboratories using different sequencing kits, the data should be examined with consideration of the differing biases that may be present. Many problems encountered by EV-RNA researchers are familiar to practitioners of single-cell RNA sequencing, given the shared challenge of low input material, and it would thus perhaps be prudent to keep an eye out for solutions from the single-cell field to some of these issues.

2.4. Normalisation strategies and reference transcripts

A number of strategies are in use for normalising EV-RNA data, as was discussed in the previous position paper.[9] Commonly used methods include normalisation over the total number of mapped reads or the number of reads mapping to a specific class of RNAs (e.g. miRNAs). During RT-qPCR validation of EV-RNA data, it is important to include validated reference gene transcripts, preferably three or more.[88] Reference genes should preferably be in the same size range as the transcript of interest. To evaluate the best and most stable reference gene combination for expression normalisation, various mathematical algorithms are recommended, such as Genorm, Normfinder or "Pattern Recognition Analysis".[88–90] Alternatively, the geometric mean of all mRNA or miRNA analysed in the study (geomean) has been suggested to serve as an accurate normalisation factor.[88,91] Other authors recommend a combination of various algorithms to identify the perfect normalisers.[92] Reference transcripts often used to normalise expression levels between cellular RNA samples are not necessarily reliable for normalising EV-RNA data. The reason for this is that RNAs stably expressed between cells in different conditions could still be differentially sorted into EVs released by these cells. Therefore, EV-RNA reference gene candidates should be extensively evaluated for their stable presence in each of the different experimental conditions tested. Various researchers rely on non-coding RNAs such as snoRNA or U-RNA as endogenous reference genes for normalisation,[92,93] although these RNAs differ substantially in length and stability from miRNAs. Moreover, other reports have described sno- and U-RNAs as extracellular biomarkers in other contexts.[94] Justification of the choice of particular transcripts as expression normalisers must be included in manuscripts, following the MIQE guidelines.[55] During the workshop, RNA spike-ins were discussed as a normalisation strategy, since these were recommended in the previous EV-RNA position paper. Synthetic miRNA spike-ins have been used for normalisation in several studies.[46,95,96] Addition of spike-in RNA to EV samples during RNA extraction serves to normalise RNA isolation efficiencies between samples. Adding a spike-in to equal amounts of isolated RNA does not control for technical variations in RNA quantification, but may be used to compare PCR efficiencies between samples.

A final factor to consider, especially when attempting to compare relative RNA expression levels of a given species between the cellular and EV sample, is the PCR efficiency in different sample types. Depending on the RNA sample composition and the levels of possible contaminants, the PCR efficiencies of a particular primer set can differ considerably. In these cases, the simplified ΔΔCt method [97] can lead to erroneous results, especially if cellular and EV-RNA amplification curves (and corresponding Ct values) differ significantly. These issues may be addressed by determining the PCR reaction efficiencies using calibration curves or other relative quantification methods, or by conducting absolute quantitation of studied transcripts.[98,99]

New advances in both EV isolation techniques and RNA quantification, including careful optimisation and standardisation of existing techniques and protocols, will certainly foster progress towards more reliable EV-RNA characterisation and identification of specific biomarkers.

3. Diversity in EV-RNA content and mechanisms underlying RNA sorting into EVs

As mentioned in section 1, it is currently thought that the RNA content of EVs likely differs according to the parent cell type, cell status, and the subcellular location where the EVs were formed. Although there are several indications that sorting of RNAs into EV is a regulated process, discussions at the EV-RNA workshop suggested that the biological mechanisms underlying this distribution of RNAs among EVs are largely unknown and that the relative contributions of passive and active loading of RNAs into EVs remain unclear. There is also limited information on exactly how RNA loading varies by cell activation status and pathological conditions, due to the technical challenges in gathering this data. As most of the common EV isolation methods are not able to distinguish between different vesicle subpopulations, and the exact subpopulations relevant to a particular disease state are not known, differential sorting of RNA during cell activation or diseases may be difficult to detect in heterogeneous EV mixtures. Understanding the molecular mechanisms underlying specific incorporation of RNAs into EVs is not only important for understanding potential biological functions, but is also an important prerequisite to rationalising their use as disease biomarkers. In the following section, we summarise recent developments in the field of EV-RNA loading, identify the major questions

within this topic, and provide suggestions for how to address these questions in the near future.

3.1. What is known and what is suggested by recent findings?

3.1.1. Intracellular versus EV-associated RNA profiles

It is now well established that the activation and differentiation status of cells are reflected in EV-associated RNA released by those cells. In cell culture, environmental stressors such as hypoxia [100–102] and oxidative stress [103,104] alter EV-RNA profiles in a manner consistent with shifts to the RNA profile inside the cell. Modification of EV miRNA profiles have been associated with cancers,[105–110] autoimmune diseases,[111–113] asthma [114] and cardiovascular disease.[115] There is growing evidence that microbial infections lead to changes in the RNA content of host EVs, and that pathogens and commensals may modulate the host's immune system via EVs. Macrophages secrete EVs with different RNA content after infections with e.g. HIV [116] or Epstein-Barr virus.[117] Similar findings have been observed for Hepatitis C,[118] Enterovirus 71,[119] and Mycobacterium tuberculosis.[120] Of note, the isolation of virus-free EV preparation from infected cells is challenging,[121,122] and may impact the observed diversity of EV-associated RNA.

Although EV-RNA content can clearly reflect changes in the RNA profile of parent cells, the extent of this association and its applicability to all or only certain RNA species remain open to debate. Independent studies, for example, have reported profound differences between intracellular RNA profiles of cell lines and EVs recovered from these cells. Recurrent observations include an increased relative abundance of small RNAs in EVs, [22,123] and the prevalence of ncRNA fragments (from e.g. vault RNA, Y RNA, and specific tRNAs) among small RNA species.[70,72,73] Thus, observational studies support that certain RNA subtypes are preferentially detected in EVs over others. We will return to possible explanations for this in the following sections.

With regard to the specificity of miRNA inclusion in EVs, some labs have found a strong correlation ($r > 0.9$) between intracellular and extracellular miRNA profiles,[46,73] while others found weaker correlations ($r = 0.5–0.7$), observing that certain EV-enriched miRNAs were common to different cell types.[22,124] This discrepancy may be explained by differences in experimental procedures, causing differential sensitivity for detecting low abundant RNA in EVs or cells or biases in isolation of different EV subpopulations and RNA-containing contaminants (see section 2 for more discussion). For example, recent data suggest that co-isolation of contaminating RNA present in foetal bovine serum, generally used in cell culture medium, may directly affect observed abundances of miRNAs in EV-RNA preparations.[28] Depicted in Figure 2 and described below are molecular players and pathways that are currently thought to be involved in incorporation of RNAs into EVs.

3.1.2. RNA binding proteins are likely involved in EV-RNA sorting mechanisms

As emphasised by an overview speaker at the workshop, RNases are ubiquitous in the cell and in the various extracellular spaces of the body; individual RNAs are protected from degradation chiefly through association with RNA binding proteins (RBPs) and their molecular interaction partners. For example, while unprotected mature miRNA is destroyed within seconds to minutes in biological settings (e.g. in blood [125]), AGO-bound but inactive miRNAs can persist for as long as several weeks.[126] Therefore, any discussion of RNA sorting is necessarily a discussion of RNA–protein association and complexing with other binding partners. As noted at the workshop, until now the field has mostly focused on EV-associated miRNAs and their association with RBPs, including those involved in the biogenesis and function of miRNAs.

The canonical binding partners and effectors of miRNAs are AGO proteins, four versions of which are encoded in the human genome (AGO1–4). Mature miRNAs are loaded into AGO during the pre-miRNA maturation process mediated by Dicer. Once associated with AGO, the miRNA is "committed" and cannot easily be competed away by excess molecules.[127] This, combined with the fact that an unloaded miRNA is rapidly degraded,[128] suggests that miRNAs associated to EV may only be functional in a recipient cell if an entire AGO-miRNA complex is transferred. As such, it is of interest to investigate whether the abundance and availability of AGO- or miRNA-interacting proteins like human embryonic lethal abnormal visual (ELAV proteins or human antigen R (HuR),[129,130] Dicer,[131] trinucleotide repeat-containing gene 6 (TNRC6) proteins [132] or fragile X mental retardation syndrome-related protein 1 (FXR1) [133] play a role in selecting miRNAs for EV export. There is evidence that these AGO- and other miRNA-interacting proteins interact with endomembranes. MVB [134,135] and endoplasmic reticulum (ER), [136] for example, were shown to associate with components of the miRNA effector complex (AGO2, TNRC6A, Dicer) and modulate miRNA complex assembly and activity. Moreover, AGO2 and Dicer were shown to be selectively degraded by the autophagosome,[137] and AGO2 directly interacts with a transmembrane form of the

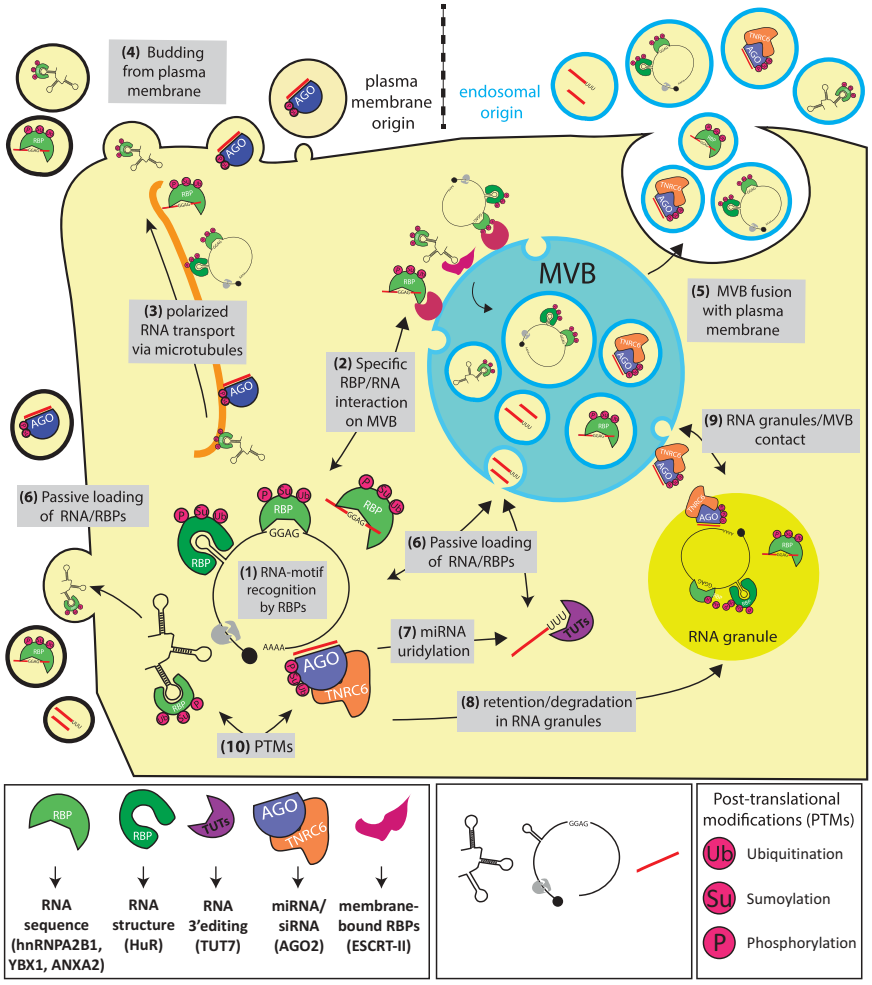

Figure 2. Suggested mechanisms for EV-RNA sorting by RNA-binding proteins (RBPs). RNA may be packaged into EVs via active or passive mechanisms. RNA-binding proteins (RBPs) could bind intracellular RNAs bearing certain motifs or signals (structure, sequence or size) (**1**). Specific interaction between RNA/RBPs and endomembranes through docking receptors (**2**) or microtubule-docking receptors (**3**) may result in the local enrichment of RNA close to membrane compartments, thereby modulating their selective incorporation into EVs (**4 and 5**). Alternatively, RBPs may also be passively incorporated into EVs and protect their cargo in the extracellular space (**6**). Non-templated RNA modifications (e.g. uridylation) known to regulate RNA-turnover in cells (**7**), are also hypothesised to impact EV-RNA sorting by a still unknown mechanism. Upon viral infection, cellular stress, or miRNA-induced silencing, RNA can be selectively stored in cytoplasmic RNA granules (e.g. P/GW bodies) (**8**). This may balance their passive/active incorporation into EVs, either negatively by decreasing their soluble pool, or positively by interactions between GW-bodies and MVBs (**9**). The depicted processes may be tightly regulated by distinct signalling pathways (e.g. RAS, AKT) that trigger specific post-translational modification (PTMs) on RBPs or RNA-editing on transcripts, thereby affecting the stability and subcellular localisation of RNA/RBP complexes (**10**).

endogenous prion protein (PRNP), which allows the assembly and stabilisation of AGO complexes on the endolysosomal membrane network.[138] Interestingly, while both TNRC6A and AGO2 interact with MVBs, Gibbings et al. [134] reported that only TNRC6A was enriched in MonoMac-derived EVs, thereby suggesting that additional mechanisms are required for their preferential incorporation into EVs. Several studies have reported a relative depletion or even absence of AGOs from EVs,[32,139] while others detect significant amount of AGOs.[76,140,141] Workshop participants discussed various reasons for the difficulties in detecting EV-associated AGO, and there was a consensus that it is still an open question whether AGO is generally associated with EVs or not. Apart from differences in the purity of EV preparations used in these studies, the discrepant findings may also be explained by context- or cell type-specific regulation of AGOs on endomembranes.[140] It is also worth noting that Squadrito et al. [142] established a correlation between the level of "free" intracellular AGO-miRNA complex (not associated with mRNA targets), and their preferential enrichment in EVs. Interestingly, while *in vitro* cultured proliferating cells have a majority of their AGO-associated miRNAs engaged into a high molecular weight complex RNA-induced silencing complex (HMC-RISC) containing mRNAs, most cells in differentiated

mammalian tissues express AGO-miRNA as a "free" low-molecular weight complex (LMC-RISC).[143] Moreover, cell stimulation (e.g. T-cell activation), could induce the mobilisation of AGOs from its LMC- toward the HMC-pool.[143] Moreover, alteration in cell signalling (e.g. oncogenic RAS) was shown to alter the pool of P-body- vs. MVB-associated AGO2, by modulating AGO phosphorylation.[140] Altogether, these observations suggest that AGO2 post-translational modifications and the modulation of the level of endogenous miRNAs targets, may directly modulate the export of AGO2-loaded miRNAs into EVs,[144,145] either by promoting the association of AGO2 to MVBs, or by modulating the pool of AGO2 that could be passively or actively engulfed into EVs.

Beyond AGOs and their direct partners, other proteins have been proposed to directly affect miRNA sorting. hnRNPA2B1 has been suggested to regulate EV sorting of miRNAs containing a specific motif [146] when modified by a small ubiquitin-like modifier, SUMO, which in mono-attachment does not target proteins for degradation, but instead regulates stability and subcellular localisation.[147,148] Interestingly, constitutive activation of mutated forms of KRAS, frequent in certain cancers, was recently shown to promote EV-associated release of specific miRNAs, while inducing intracellular retention of others.[149] Downregulation of Annexin A2 (ANXA2), a protein exhibiting Ca2+-dependent phospholipid-binding protein and RNA-binding properties,[150] was shown to decrease the level of EV-associated miRNAs, without significantly impacting the abundance of released EVs.[151] In addition, HuR protein, an ELAV RBP family protein, blocks particular miRNA binding sites on target transcripts [152] and facilitates RISC dissociation from target mRNA. [153] The first author of the latter study reported at the workshop and now in publication [129] that in stressed hepatic cells, HuR also binds miR-122 after dissociating it from AGO2, and would promote its release at the surface of the MVB to favour its incorporation into EVs. A similar mechanism involving the RNA-binding protein Y-box protein 1, a known EV-associated protein,[154–157] was recently identified by Shurtleff et al. [139] using cell-free assays for exosome biogenesis. It was shown that YBX1 binds to specific miRNAs (e.g. miR-223) and promotes their selective packaging into CD63+ EVs. Interestingly, knocking out YBX1, alone or in combination with additional knockdown of its homologue YBX2, led to a strong depletion of YBX1-bound siRNA into EVs, and their remobilisation into cellular AGO2. Future work should address whether miRNA dissociation from AGO and subsequent binding by non-AGO RNA-binding proteins (such as hnRNPA2B1, Annexin A2, HuR and YBX1) is a general phenomenon that may contribute to EV-RNA sorting. Moreover, as these RBPs are known to bind to other type of RNAs (including mRNA, tRNA and snoRNAs) it will be important to decipher whether they could also promote the packaging of these RNA biotypes into EVs.

Finally, proteins implicated in EV biogenesis can influence RNA loading into EV. Modulation of endosomal sorting complex required for transport (ESCRT) activity can impact both the number of released EV and EV-RNA abundance. For example, Vps4 was found to influence the secretion of oncogenic miRNAs by human hepatoma cells.[158] Interestingly, knocking down Alix, another ESCRT protein, did not affect the number of released EVs but induced a decrease of secreted miRNAs.[159] It should be noted that ESCRT complex is also involved in the regulation of the RNA silencing pathway,[134] thereby complicating the interpretation of these observations.

3.1.3. RNA motifs and modifications associated with EV incorporation

As intimated above, several RNA motifs have been identified that correlate positively with release in EVs. A GGAG motif in miRNAs, recognised by hnRNPA2B1, has been suggested to promote packaging into EVs.[146] Studies involving analysis of transcripts enriched in EVs over their secreting cells have also identified motifs that positively correlate with RNA targeting to EVs.[160,161]

Besides the recognition of specific RNA secretion motifs, the addition of non-templated nucleotides has been reported to determine RNA fate; 3′ adenylation of miRNAs has been associated with intracellular retention whereas uridylated isoforms are more enriched in the small vesicles fraction.[84] This could suggest that the 3′ end of the miRNA sequence contains a critical signal for sorting into EVs, or that the specific subcellular localisation of RNA-editing enzymes directly impacts this sorting. Alternatively, the 3′ uridylation may indicate the presence of apoptotic bodies in the EV isolate, since this is also a signature of RNA undergoing degradation during apoptosis.[162] The possibility of mutating the packaging motif of specific EV-associated RNA (using CRISPR-mediated homologous recombination) or mutation/overexpression of key RNA editing enzymes (e.g. TUT7) may allow this question to be addressed.

In conclusion, as typified by strong recent work on miRNA, EV-RNA sorting mechanisms are likely to involve RNA binding proteins and their partners, although other mechanisms may exist (Figure 2). The focus on miRNAs at the workshop and in this article is simply a reflection of the field; indeed, diverse other classes of cellular RNAs, or their fragments, have been

reported in EVs.[70] Below, we specify potential research directions to uncover how these diverse RNA species are packaged into EVs.

3.2. Remaining questions and how we can answer them

3.2.1. How can the varying correlations between cellular and EV-associated RNA contents be explained?

As previously mentioned for the case of miRNAs, different studies report differing degrees of correlation between intracellular and EV-RNA content.[22,46,73,124,163] At the most basic level, all EV-RNAs originate in cells, and thus any EV-RNAs must also exist at least transiently in the parent cell. We can readily envision four distinct but non-exclusive scenarios that might contribute to observed relationships between cellular and EV-associated RNA:

(1) Cellular RNAs are randomly included in the vesicle lumen during EV biogenesis, yielding an EV-RNA profile representative of the relative RNA abundance at close proximity of the EV luminal engulfment site. Note that this scenario does not necessarily mean that EV and cellular RNA would correlate perfectly. Also, random RNA packaging might be limited by the length of prospective packaged RNAs and the size of RNPs. In a pure example of this case, the EV-RNA profile would be roughly identical to that of cellular membrane-associated RNAs;[164] for example, those near MVBs, or endosome-like domains of the plasma membrane. The recent development of a high throughput method to determine subcellular localisation of RNA *in situ* [165] may facilitate the identification of such bias in the future.
(2) Cellular RNAs are included in the vesicle lumen based on their proximity to the site of engulfment, but with enrichment provided by affinity of RNA motifs and/or RNP components for protein and lipid elements preferentially found at sites of EV biogenesis.[139] Here, EV-RNAs would approximate a subset of membrane-associated RNAs, enriched in a particular sequence or protein association.
(3) RNAs harbouring specific structural or sequence signals are loaded into EVs in an energy-dependent, i.e. active, manner. Interestingly, some RNAs might "bring their own" ATP, as ATP-binding motifs have been identified.[166] Depleting ATP from parent cells would presumably be an experimental approach to identify active loading processes, although careful controls would be necessary. However, workshop participants were unaware of any reported ATP dependence in EV-RNA sorting pathways.
(4) Finally, it remains a possibility that recycling mechanisms operating in cells (e.g. RNautophagy) would lead to continuous EV incorporation of a large range of cellular RNAs at a given time.[167,168]

As discussed, it is anticipated that more than one of the above are likely to define the relationship of cellular and EV-RNA, complicating the picture substantially. Resolving the relative contributions of different scenarios in different contexts is an area of much-needed research.

3.2.2. Can further research into RBPs and their partners lead to discovery of mechanisms underlying RNA sorting into EVs?

It may be informative to explore the roles and interaction partners of RBPs that are already known to be present in EVs, as these may be involved in the stabilisation and/or sorting of EV-RNA. As an example of this approach, a list of known curated RBPs [169] could be compiled based on overrepresentation in databases that compile EV proteomics data, such as Vesiclepedia,[170] Exocarta [171] and Evpedia.[172] In a preliminary exercise using Vesiclepedia (see Table 4), we note that, apart from enrichment for general translation machinery factors, EVs contain tRNA associated factors (EEF1A1, AARS, GARS, DARS, TARS, WARS) and more specialised RBPs such as CNP, PARK7, RNH1, RUVBL1/2, HNRP-K/A2B1/C/D/R, PCBP1, DHX9, NPM1, PARP1, SND1, SYNCRIP, and MVP (major vault protein). Future investigation will be required to validate these observations, and notably to exclude possible contamination of EV preparation with co-fractionating RBPs. It should also be emphasised that there are caveats to this approach. For low abundance EV-RNAs, RBPs with one-to-one binding stoichiometry might be difficult to detect in EVs. Furthermore, shuttling proteins may exist that transfer RNAs from cellular to EV binding partners while remaining themselves excluded from EVs.

3.2.3. Which EV-associated RNAs are inside vs. outside EVs?

In addition to the question of whether specific RNAs are destined for release via EVs, it is also unclear in a given preparation whether EV-RNAs localise both in the EV lumen and on the outside of the EV membrane. Though the possibility of a functional association of RNA with the outside of the EV membrane cannot be formally ruled out, no evidence for it has been observed, and this topology would be inconsistent with the traditional model of RNA

Table 4. Top 80 RNA-binding proteins in Vesiclepedia datasets.

ribosome function			translation	tRNA function	Poly-A RNA-binding		unclear RNA-binding	
RPS8	RPS5	RPL4	EEF2	EEF1A1	CNP	DHX9	GAPDH	IPO5
RPS16	RPS24	RPL7	EIF4A1	AARS	PARK7	HNRNPA2B1	PRDX1	ACO1
RPS18	RPS6	NCL	EEF1G	GARS	RNH1	HNRNPC	UBA1	CANX
RPS27A	RPL15	RPS9	EEF1A2	DARS	RUVBL2	PA2G4	RAN	XRCC6
RPLP0	RPS3A	RPL6	EIF5A	TARS	HNRNPK	HNRNPD	PSMA6	CRYZ
RPSA	RPL18	RPL27	EEF1D	WARS	RUVBL1	NPM1	KPNB1	
RPS3	RPS13	RPS7	EIF2S1		PCBP1	SND1	PSMA1	
RPS4X	RPL30	RPS11	Vault complex			PARP1	DYNC1H1	
RPL12	RPL3	RPL19	MVP			SYNCRIP	CALR	
RPS14	RPS25	RPL13				HNRNPR		
RPLP2	RPS2	RPL23						
RPL7A	RPS15A							

Top 80 RNA-binding proteins in Vesiclepedia datasets. The occurrence of each protein within a census of 1542 manually curated RBPs [169] was queried within the Vesiclepedia database (http://www.microvesicles.org; v3.1; restricted to *Homo sapiens* samples). Proteins are clustered by functional group with colours indicating the number of occurrences in the database (30–39 blue; 40–49 orange; >50 red).

delivery by vesicle fusion with the recipient cell. In addition, observed outside associations with RNPs may be technically induced by isolation procedures (i.e. force of centrifugation) rather than biological in nature. RNase treatment of EVs has been applied to deplete non-encapsulated RNA;[27,73] however, even RNAs exterior to EVs are likely to be protected from RNase degradation by protein complexes, as there are already high levels of RNase in biological media such as blood plasma.[26] To selectively study RNA residing in the vesicle lumen, it is critical to eliminate any source of RNA outside the vesicles. We therefore recommend that investigators quantify the amount of EV-associated RNAs (for example by RT-qPCR) before and after treatment of the vesicle with a proteinase followed by an RNase digestion step. By combining this approach with a negative control (proteinase/RNase digestion in the presence of detergent, as in Royo et al. [173]), it is possible to determine the proportion of a specific RNA residing within the vesicle in a given preparation.

3.2.4. Does subcellular localisation affect sorting into EVs?

It has been hypothesised that the subcellular distribution of RNAs can promote the inclusion of particular molecules in EVs. Similarly, the location of RNA species in the different compartments – i.e. cytoplasm, nucleus or plasma membrane – could determine their in/exclusion in different types of EVs (large microvesicles, apoptotic bodies, or exosomes). Increased knowledge on sub-cellular localisation of different RNA species is needed to further address this issue. In general terms, crucial decisions on maturation of both mRNAs and ncRNAs (i.e. splicing, polyadenylation) are made during their time in the nucleus, suggesting that the nuclear history of an RNA can be fundamental for its fate in the cytoplasm. Once in the cytoplasm, RNP complexes containing mRNAs and/or other ncRNAs interact with motor proteins, which mediate their trafficking, mostly in inactive form, along the cytoskeleton tracks to their final destinations.[174] It is possible that the same mechanisms active in RNA pre-localisation also function in specific sorting of RNP to nascent EVs. Notably, in the nervous system, intracellular RNP trafficking seems to be regulated by neurotransmission (reviewed in [175]) and the same has been also reported for EV trafficking among different brain cell types (reviewed in [176]). It is likely that the mechanisms governing subcellular RNA localisation are important for their specific packaging into EVs. Future experiments characterising these processes will be key to validating this hypothesis. For example, detecting changes in subcellular RNA localisation by fluorescent *in situ* hybridisation (FISH) [177] or using indirect RNA-binding protein detection (e.g. with the MS2 system) [178] could provide insight on the relevance of localisation within the cell for the packaging of a given RNA transcript.

3.2.5. Differential stability of RNA in EVs?

It has also been proposed that differences in EV-RNA profiles versus intracellular RNA profiles are not the result of a selective process, but rather represent differences in the extracellular stability of particular RNA species.[179] Certain RNases may be present at higher concentration in EVs than in the cell, inducing the relative depletion of RNAs targeted by these RNases in EVs.[180] Moreover, the portion of RNAs bound by RBPs inside EV might be preferentially stabilised and resistant to RNases. This may be addressed by searching for known RBP binding motifs specifically enriched in RNA isolated from EV compared to the parent cells.

3.2.6. Do RNA modifications impact their sorting into EVs?

Accumulating evidence indicates that an array of RNA modifications (5′-end cap, m^6A: N^6-methyladenosine, m^5C: 5-methyl cytidine, A-to-I editing, pseudo-uridine, 5hmC: 5-hydroxylmethyl cytidine) directly control RNA metabolism (reviewed in [181]). These RNA modifications are tightly controlled by cellular enzymes (e.g. m6A "writers" METTL3, m6A "eraser" FTO), and could be recognised by RNA-modification "readers" (such as YTHDF1) that could impact the stability [182] of modified RNA. As for the addition of non-templated nucleotides to the 3′ end of the RNA (adenine, uridine, see also section 3.1.3), an interesting hypothesis would be that these modifications may favour specific packaging of these RNAs into EV. Novel techniques have recently been developed to detect specific RNA modification (e.g. MeRIP-seq [183]). Applying these approaches to EV-associated-RNAs would enable analysis of whether RNA modifications are enriched or depleted in EV-associated RNA.

3.3. Next challenge: qualitative and quantitative RNA analysis in individual EVs

Since EV populations can be highly heterogeneous, a full understanding of RNA incorporation into EVs will require analysis at the EV subpopulation or individual EV level rather than bulk analysis. However, detecting and enumerating RNA molecules in individual EVs face unmet challenges. As technologies for analysis and isolation of individual EVs are rapidly improving,[19,184–187] the opportunities to investigate the EV-RNA cargo of single EVs are expanding. Attempts have been made to apply fluorescent RNA-tracking dyes (e.g. acridine orange, Syto84 or Syto-RNASelect) for high-resolution flow cytometric analysis of the RNA content of individual EVs. However, the use of such an approach remains challenging due to limited probe specificity, limited fluorescence per EV, and poor signal-to-background ratio. For example, fluorescent small molecules that are highly specific for RNA tend to be charged and thus membrane impermeable, hampering labelling of RNA in the EV lumen. Table 5 lists several membrane-permeant fluorescent dyes that may be used to detect the presence of RNA associated with EVs, although their specificity and efficiency in labelling EV-RNA remain to be validated.

It is important to mention that none of the currently utilised single EV analysis cytometry-based methods are capable of detecting a single fluorescein molecule. When using molecular equivalents of soluble fluorescein (MESF) calibration, it has been demonstrated that hundreds or thousands of fluorescein equivalents are commonly required for detection, depending on the instrument and configuration (ISEV-ISAC-ISTH working group, ISEV 2016). Moreover, most RNA dyes are intercalating agents and it is not expected that the structure of a small miRNA would support intercalation of hundreds of dye molecules. Labels with higher quantum yield (and better signal:noise ratio), such as quantum nanocrystal-labelled oligonucleotides, may be more suitable for detection of molecular components in single EVs. In addition to flow cytometry, Raman spectroscopy was recently used to analyse single EVs in solution, allowing distinction of several distinct EVs populations differing in chemical properties.[195,196] However, it is currently unclear whether this technique may allow detection of RNA in single EVs.

Innovative methods were recently developed to indirectly detect RNA in EV by microscopy, notably by using RNA-binding fluorescent protein probes. One multiplexed approach [197] combines the labelling of vesicle membranes using a palmitoylated fluorescent protein, and the indirect labelling of RNA molecules using the bacteriophage MS2 coat protein-GFP:MS2 RNA binding sequence system.[178] Although this method may be restricted to the labelling of long RNA (e.g. lcnRNA or mRNA) and the target RNA must be modified to include the MS2 loop, it is a promising strategy to monitor the packaging of extracellular RNAs into EVs in living cells. It would be interesting to further develop this approach to investigate the percentage of RNA-loaded EVs or to

Table 5. Membrane-permeant fluorescent dyes that may be used to detect the presence of RNA associated with EVs.

Dye	Ex/Em (nm)	Known nucleic acid specificity	Publication for cell staining	Publication EV staining
Acridine orange (Thermo Fisher Scientific)	460/650 (RNA) 500/526 (DNA)	dsDNA (Green) and ssDNA/dsRNA (Red) (orange emission in acidic organelle compartments)	[188]	[189]
Pyronin Y (Sigma, Saint Louis, MO, USA)	555/580	ssRNA	[190]	No
Syto14 (Thermo Fisher Scientific)	517/549 (DNA), 521/547 (RNA)	RNA/DNA	[191]	No
SYTO-RNASelect (Thermo Fisher Scientific)	490/530	RNA selective	[192]	[193]
E36	497/548	RNA selective	[192]	No
Styryl-TO	520/531	RNA selective	[194]	No

combine with the use of fluorescent-tagged markers of sub-cellular compartments in order to infer the endomembrane origin of EVs containing RNA.

During the workshop, participants suggested applying digital droplet PCR methods to quantify the RNA content in single EVs, although it is not currently clear whether this methodology will be applicable to single vesicle analysis. Another approach would be to perform PCR on limiting dilutions of EV preparations, so that each PCR is initiated from the RNA content of a single vesicle. A microfluidic PCR platform would likely be necessary to analyse such a small amount of RNA. Finally, it would be interesting to be able to "visualise" specific RNA within the vesicular context. Protocols developed to detect RNA within virus particles by RNA FISH [198] or *in situ* hybridisation-electron microscopy (ISH-EM) [199] could likely be applied to detect EV-associated RNA given their comparable size and chemical nature. In fact, the high resolution of the ISH-EM method may allow determination of whether specific EV-RNA are located inside or at the surface of vesicles, a point remaining controversial in the field.

4. Assessing functional transfer of EV-RNA

In the past 10 years, EVs were not only shown to contain RNA, but also to be able to functionally transfer RNA into target cells. It is important that this process is studied using different experimental models and by mimicking physiological EV(-RNA) concentrations and conditions, both *in vitro* and *in vivo*. The formal demonstration that EV-mediated effects on target cell behaviour are caused by functional transfer of EV-RNA is technically challenging and requires a number of important controls in order to validate observations. Below, we describe the current approaches used to investigate EV-RNA transfer and discuss advantages and pitfalls of these methods. During the workshop, there were also fruitful discussions about the implementation of novel models/approaches in the field, of which the most promising are also reported in the sections below.

4.1. Strategies to study functional transfer of EV-associated mRNA and miRNA in vitro

The first observation that mRNA could be transferred via a pelletable fraction of cell conditioned medium and translated in target cells was made by Ratajczak et al. [200]. Soon after, Valadi et al. [201] reported that RNA was indeed associated to low-density EV, and further demonstrated RNase-resistance of this RNA. In this study, mouse mRNA transferred via EVs to recipient human mast cells could be translated into corresponding mouse proteins *in vitro*. [201] Skog et al. [202] provided a confirmatory study showing that, upon EV-mediated transfer, *Gaussia* luciferase mRNA was translated within recipient cells. *Gaussia* luciferase (Gluc) is a flash-type reporter which emits a bioluminescent signal 100-fold higher than typical luciferases such as firefly or *Renilla* luciferases,[203] thereby enabling detection of minute biological events such as EV-RNA translation. Later on, Pegtel et al. [117] showed that viral miRNAs secreted by EBV-infected cells were transferred to uninfected recipient cells via EVs and could repress the EBV target genes using a luciferase sensor assay. Shortly after, Kosaka et al. [204] and Zhang et al. [141] documented functional cell-to-cell miRNA transfers in destination cells using EVs isolated from miRNA-overexpressing cells, by using a luciferase sensor assay,[204] or by showing direct repression of endogenous miRNA targets at the protein level.[141] Another method to investigate spatial and temporal aspects of EV uptake and EV-mRNA translation is via the use of bioluminescent reporters. More recently, it was demonstrated that EV-mRNA encoding a membrane-bound Gluc reporter (GlucB) can be translated in as soon as 30 minutes following EV uptake by recipient cells.[197] While the GlucB signal began to increase substantially from 30 min, it reached a plateau at 12 h post-EV uptake, perhaps suggesting that there is a limit to the amount of mRNA which EVs can transfer.

4.1.1. Considerations for assessing functional transfer of EV-associated miRNAs

A plethora of studies have confirmed the initial observations, strengthening the idea that functional transfer of EV-associated mRNAs and miRNAs can occur *in vitro* between mammalian cells. In several studies, EVs from cells overexpressing endogenous miRNAs were shown to cause target mRNA repression in recipient cells transfected with a luciferase reporter construct using the 3′UTR of the mRNA target (examples include [22,205,206]). Besides effects on miRNA reporter systems, EV-mediated transfer of miRNA was also shown to cause changes in target gene expression in recipient cells.[205,207,208] These experiments are generally performed with donor cells expressing miRNAs for which the expression levels in the recipient cell are very low or undetectable in order to maximise the window in which miRNA transfer can be observed. In order to exclude the possibility that the EVs/co-culture treatment itself would induce the endogenous transcription of the tested miRNA in destination cells, which is a potential confounding effect in this assay, some studies tested the level of miRNAs precursors (pri-miRNA and/or pre-miRNA) in destination cells. [209,210] Although the lack of changes in miRNA precursor level would strengthen the EV-mediated transfer hypothesis, it should be noted that: (1) pre-

miRNAs can also be transferred by EVs;[76,211] and (2) because the pre-/pri-miRNA are continuously processed into mature miRNA by the RNA silencing machinery, a transient increase in pre-/pri-miRNA levels caused by EV treatment may easily be overseen at a later time point, while mature miRNA may stably accumulate for hours/days.[212,213] Therefore, detecting changes in precursor miRNAs in destination cells may not be a reliable method to control for spurious miRNA transcriptional activation caused by EV treatment or in co-cultures. In other studies, the contribution of specific miRNAs in EV-induced effects has been investigated by blocking the function of the candidate miRNA in donor cells using antimiRs [109,214,215] or by using miRNA genomic knockout donor and recipient cells.[208] Since knockdown of miRNAs may lead to changes in the phenotype and/or function of cells, it is important to address whether the observed changes in the function of EVs released by these cells are only due to the lack of this particular miRNA or whether changes in other functional molecular components contributed to these effects. Moreover, when using anti-miRs it is essential to use adequate controls such as a control anti-miR that targets an unrelated miRNA expressed at a similar level.

4.1.2. Stoichiometry of miRNAs associated with EVs

Since a population of EVs can contain many different miRNAs, they may suppress functionally related genes in parallel, leading to effective paracrine control over neighbouring cells. Nonetheless, a quantitative assessment of the miRNA content of EVs by a stoichiometric approach found the miRNA/vesicle ratio in a blood sample to be lower than 1, even for the most abundant miRNAs, meaning that there are vesicles that do not contain a copy of a given miRNA.[25] Based on these observations, the notion was put forward that individual EVs were unlikely to be functionally important vehicles for miRNA-based communication. However, the workshop attendants felt that there was a need for a more nuanced consideration of these data. First, the stoichiometry assessment relies on careful quantification of EVs, for which there are no gold standards and which heavily depends on EV purity and the experimental approach. In addition, this study assessed only small EVs (50–200 nm), while larger vesicles, such as microvesicles and oncosomes, also contain miRNAs as well as longer molecules of RNA that can contribute to EV-RNA mediated communication. With regard to EV-miRNA function, it may well be that EV uptake is an infrequent but very selective event, in which rare EVs carrying many copies of a given miRNA may lead to miRNA-mediated silencing of mRNAs in specific target cells. It is also possible that, by taking up multiple EVs carrying a low concentration of miRNA, sufficient quantities of transferred miRNA are reached to modify mRNA levels in target cells. This hypothesis is consistent with the observation that different miRNAs often operate in concert to simultaneously control different players in the same signalling pathway. Finally, as was also suggested by Chevillet et al. [25], very low quantities of miRNAs may be compatible with non-conventional functions, such as binding to Toll-like receptors as well as affecting DNA transcription and/or epigenetic states.[216–219] More complete knowledge of EV heterogeneity, target cell specificity, and RNA transfer efficiency is therefore needed to further address issues of EV-RNA stoichiometry.

4.2. Assessing functional EV-RNA transfer in vivo

It currently remains to be determined whether functional transfer of EV-RNA occurs and is relevant *in vivo* during physiological or pathological processes. EV-RNA transfer assays in the context of cancer cell xenografting or immune cell function are among the currently used experimental set-ups *in vivo*.[220,221] In these studies, effects of EVs *in vivo* were assessed by *in vitro* isolation of EVs followed by their re-introduction *in vivo*. However, this approach is unlikely to reflect the physiological properties, concentration, and distribution of EVs released in (patho)physiological conditions. Recently, other approaches have been introduced to more directly address whether EV-RNA functional transfer occurs *in vivo*. During the workshop, the most intensively discussed *in vivo* approach was the Cre-loxP-based EV tracing system to study functional transfer of mRNA. Possibilities and limitations of this approach are described in the following sections.

4.2.1. Assessing EV-associated mRNA translation using the Cre-loxP system

The Cre-LoxP system is a recombination system, originating in the bacteriophage P1, consisting of the DNA recombinase Cre, and the recognition sequence loxP. Sequences that are flanked by loxP sites will be excised by Cre and re-joined leaving a single loxP site. EV-mediated transfer and translation of the Cre mRNA in target cells can be visualised by using donor cells expressing Cre recombinase and recipient cells containing a Cre-inducible open reading frame coding for a fluorescent protein, e.g. with a stop signal terminating transcription that is flanked by LoxP sites. If functional Cre mRNA is transferred by EVs to the recipient cells, the "transcription block" impairing fluorescent protein (FP)

transcription is genomically eliminated leading to the irreversible production of the FP in the recipient cell.

Using this Cre-loxP system, functional transfer of EV-RNA between distant cells has been demonstrated between blood and brain,[220] as well as other non-haematopoietic organs as a proof of principle. Later, these findings were extended to EV signalling between tumour cells and healthy cells as well as among tumour cells.[221,222] In both studies, Cre protein levels in EV were below detection levels on Western blots, suggesting that recombination observed in target cells was caused predominantly by EV-mediated transfer of mRNA encoding the Cre protein (see also below). The major advantages of this approach are its high sensitivity (a single transferred Cre mRNA may lead to the permanent transcription of a marker protein), and its ability to detect transfer events in living cells both *in vitro* and *in vivo*. Other important advantages implicit to this system are knowledge of the specific cell type (i.e. Cre expressing cells) from which EVs originate and the ability to monitor changes in phenotype and molecular composition induced by EVs down to the level of an individual target cell. In the following paragraphs we will discuss what we currently know about the system and outline both future directions and limitations.

4.2.2. Interpretation of Cre-loxP based tracing data

Recombined cells observed after EV uptake using the Cre-LoxP system almost certainly only represent a subpopulation of cells that interact with EVs, given that EVs can exert biological effects on their target cells that are independent of full translation of transferred RNA, e.g. via binding of membrane bound molecules or the transfer of protein or lipids.[57,223–225] It was discussed during the meeting that addition of fluorescent (lipid) dye labelled EVs to target cells *in vitro* generally leads to a much higher percentage of target cells positive for the fluorescent dye compared to the number of target cells that start to express fluorescent marker proteins due to recombination upon uptake of Cre-containing EVs. It is not clear whether cells expressing a marker gene after Cre-induced recombination therefore represent only a subset of EV-targeted cells, or perhaps whether the higher labelling rates observed with *in vitro* fluorescence experiments are artefactual (e.g. due to incorporation of dye aggregates).

Additionally, researchers present at the meeting had not found a correlation between the proximity of donor cells and the frequency of loxP-recombination events. This last observation may suggest that cell-to-cell transfer of EV-RNA is likely to be systemic and involves EVs without the need for direct cell-to-cell contact. However, within the tumour context, the local release of EV by Cre-positive cells and their subsequent migration away from the recombined acceptor cells cannot be excluded.

The fact that recombined cells in multiple experimental settings display clearly separated populations with differences in their miRNA profile, phenotypic markers or cellular behaviour compared to their non-recombined counterparts of the same or similar lineage,[221,222] suggests that the number of non-recombined false positives cannot be very high. However, this does not exclude that non-recombined target cells were modulated by (other) EV subpopulations that did not confer Cre mRNA-mediated recombination. Another important question is whether uptake and processing of Cre mRNA-containing EVs merely reflects the current physiological state of the recipient, i.e. that the EV-targeted cell is already distinct from other cells, in contrast to the notion that EV uptake actively changes the status of the cell. At least for the case of the induction of an immunosuppressive phenotype of myeloid derived suppressor cells (MDSCs), experimental evidence supports the latter: EV uptake actively changes MDSCs to become more immunosuppressive [226,227] although the mechanisms responsible for inducing this change are unknown.

4.2.3. Limitations of Cre-loxP based tracing of EVs

Although elegant, the Cre recombinase based EV tracking technique has its limitations. First, it is not quantitative for the number of EVs transferred, as it uses a permanent genetic switch. In addition, it is not possible to distinguish the type of EVs mediating the transfer. As discussed previously,[221] loading of Cre mRNA in subpopulations of vesicles might be very much cell type dependent. In EVs that were isolated from peripheral blood serum and then fractionated according to sucrose density ultracentrifugation, Cre mRNA could be detected in several density fractions.[222] Sorting of Cre mRNA may occur preferentially into specific EV subpopulations, but this is difficult to assess given the lack of standardisation of vesicle numbers in the different subfractions and the fact that the method of detection of Cre (by PCR or even nested-primer PCR) is considerably more sensitive than the ability to physically separate EV subtypes by density gradients. It is also important to address whether the relatively low frequency (0.2–3%) of observed recombination events are representative of the real frequency of EV transfer *in vivo* or whether they represent a low sensitivity of this technique.

Using the Cre-loxP system, it is also imperative to consider alternative explanations to EV-mediated transfer of Cre. In a chimeric system where Cre and Cre reporter expression occurs in physically separate cell population such as between tumour cells, Cre mRNA or protein could also be transferred via cellular membrane protrusions, such as tunnelling nanotubes (for a review see [228]). Also, it should be mentioned that while in some studies using Cre-loxP for testing EV-RNA functional transfer the

Cre protein could not be detected in EVs,[220,221] others found Cre protein in EVs after overexpression in donor cells.[57] Although Cre is a nuclear protein not expected to be abundant in EVs, involvement of mRNA vs. protein must be verified. Cell fusion is another process that would give similar observations, although in chimeric systems it can be controlled for relatively easily by co-expressing Cre with a marker gene (e.g. fluorescent protein). Transgenic systems where Cre is expressed under a cell type-specific promoter in the same cells that carry the Cre reporter require careful assessment to exclude that observed recombination events are due to a leaky expression of the promoter, rather than transfer of Cre from one cell to the other. A precise characterisation of promoter activity extending to all tissues is rarely available and might not apply to certain pathologies that are used. On the other hand, some cases of apparent "misexpression" of Cre might instead be the result of a horizontal transfer of Cre. As a minimal requirement for this type of experiments we propose that evidence is provided that the cells in question release Cre mRNA and/or Cre protein containing EVs and that these EVs are sufficient to induce recombination in target cells. The use of distinct Cre models, each allowing Cre expression in specific donor cells, may help in ruling out unspecific Cre expression. In addition, the use of chimeric mice where Cre-donor cells are grafted into loxP-reporter mice (or the reciprocal experimental set-up where loxP-acceptor cells are grafted into Cre-expressing mice) can avoid the possibility of spurious expression of genomically encoded Cre in acceptor cells.

In summary, the Cre-loxP system has been and will be a valuable tool for uncovering EV-based communication between cells and tissues in a (patho)physiological setting. While it requires careful implementation to address specific questions in EV biology, it is relatively easy to use and benefits from the availability of a large number of cellular and animal models.

4.3. Expanding and refining our experimental models

During the EV-RNA workshop, there was a consensus that we should not restrict the functional analysis of EV-RNA transfer to one particular type of EV. Physical properties like size and surface chemistries of different types of EVs may impact their capacity to functionally transfer RNA. Although most studies perform EV-RNA transfer assays using EVs collected from *in vitro* cell cultures, it would also be interesting to investigate EV directly extracted from their natural environment, e.g. those isolated from body fluids and tissues. These EV may differ in surface characteristics, possibly due to binding of lipid or protein moieties present in tissues or body fluids to the EV membrane. This may influence functional RNA transfer in destination cells, e.g. due to changes in EV binding to docking proteins or in the delivery of EV cargo through endosomal escape or direct membrane fusion. Isolation of EV from tissues is technically challenging. Mild tissue dissociating reagent (e.g. liberase) without mechanical disruption could perhaps be used to isolate EVs from tissues without co-isolating intracellular vesicles released by broken cells. However, it is important to keep in mind that such protease treatments may affect the proteins displayed on the surface of EVs, potentially affecting their ability to dock on or fuse with recipient cells. Alternatively, EV may be isolated from *in vitro* maintained tissues. Another interesting approach would be to use novel technology combining both microfluidics, which facilitate concentration of the EVs in the microenvironment, and organoid cultures, which have been highly recommended to mimic *in vivo* cellular organisation.[229] Indeed, 2D-grown cells may not recapitulate certain features required for proper release or reception of functional EV-RNA (e.g. cellular polarisation [230]). Knowledge acquired in this area could ultimately be used to optimise EV-mediated functional RNA transfer in prospective therapeutics for gene delivery.

4.4. Usable knowledge for RNA delivery by EV-inspired synthetic mimics

4.4.1. Preparation of RNA-loaded EV or EV-mimics

Since EVs are increasingly regarded as natural carriers of nucleic acids, it is of interest to explore EVs as vectors for delivering both endogenous as well as foreign genetic material into recipient cells. For this approach, several challenges remain, one of the most prominent being the introduction of an RNA of interest into EVs. Several reports describe loading procedures, based on electroporation of EVs directly,[231–233] loading of EVs by virus-infected cells,[234] or targeted embedding of specific RNA-sequences into EVs via sequence-recognition domains fused to vesicular proteins.[235] Although promising in concept, these loading procedures are in practice less than ideal, either loading RNA with low efficiency or showing artificially high loading caused by formation of electroporation-induced nanoprecipitates.[61] In addition, it is challenging to remove the nanoparticle-forming chemical transfection reagents from the EV preparation after loading.

Even if the challenge of RNA loading is met, the subsequent steps in functional delivery of RNA content into the target cells present significant hurdles. Upon intravenous injection, EVs are generally rapidly taken up by cells of the mononuclear phagocyte system, particularly in the liver and spleen. This limits the distribution of EVs to other cell types. In addition, not all target cells are accessible via the vascular

lumen, as the endothelial barrier prevents extravasation. Only at sites of fenestrated or sinusoidal endothelium, or at sites of increased permeability, can extravasation be expected. Finally, specific recognition of the target cell should be followed by appropriate delivery of the RNA cargo in its cytoplasm.

In addition to natural EV-based delivery systems, EV-mimics have emerged as alternative delivery systems.[236] The design of EV-mimics is inspired both by understanding of the properties of synthetic liposomes and by knowledge of the structure and function of EVs. Since not all components of EVs may be required for proper delivery, the synthetic mimics of EVs could be developed through assembly of liposomes having crucial components of natural EVs in order to reduce structural complexity.[237] Although Kooijmans et al. [237] have provided insight into which EV components could be used to enhance the drug delivery properties of EV-mimetics, EV components that are essential for the assembly of functional EV-mimetics remain to be identified.

In theory, the EV-mimics could be designed with enhanced circulation time and increased specificity for target cells. Such EVs could be applied for the cytoplasmic delivery of wide variety of biomolecules such as oligonucleotides, DNA, proteins and peptides.[238] In this regard, modifying EVs with nanobody-PEG-lipids could potentially protect the biomolecules loaded inside from macrophages and give the EVs high affinity for the receptor of a particular target cell type. This prolonged circulation time and target cell specificity could increase EV accumulation in targeted tissues and may improve the cargo delivery. [239] Most recently, Sato et al. [240] developed hybridosomes (hybrid EVs) by fusing their membranes with liposomes, showing that it is possible to control the degree of interaction between hybridosomes and recipient cells through a membrane-engineering strategy. These developments will open up new avenues for developing improved RNA delivery systems and may advance the field of nanomedicine.

4.4.2. Delivering EV-RNA cargo: understanding mechanisms of EV fusion with recipient cells

The fusion and entry of EVs into recipient cells may follow several routes (Figure 3). The EV uptake mechanism may depend on the target cell type and recognition of proteins or receptors present on the surface of both EVs and on recipient cells, as well as environmental conditions. Notably, the protein molecules present on both the surface of EVs and on the plasma membrane of recipient cells potentially modulate vesicle binding and uptake by recipient cells. It has for example been shown that heparin sulphate proteoglycans (HSPGs) on the plasma membrane of the recipient cell efficiently regulate EV uptake.[241,242] Furthermore, it was recently demonstrated that various isoforms of integrins present on tumour-derived EVs influence the uptake of EVs in an organ-specific manner.[223] Environmental factors that

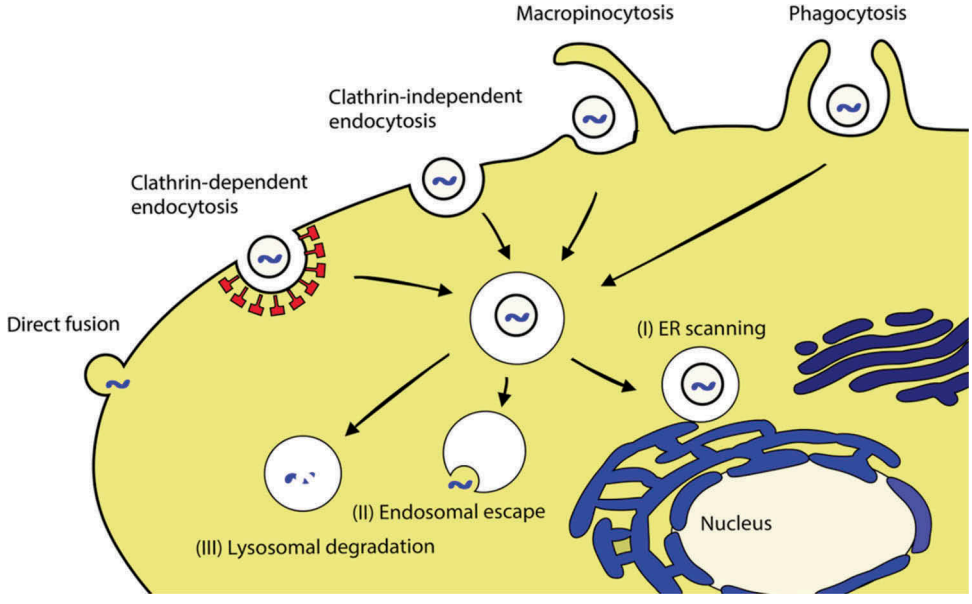

Figure 3. Extracellular vesicle uptake and cargo delivery in recipient cells. EVs may release their cargo into the cytosol through direct fusion with the plasma membrane. Alternatively, EVs may be internalised via a variety of endocytic mechanisms, including clathrin-dependent endocytosis, clathrin-independent endocytosis, macropinocytosis and phagocytosis. Subsequently, EVs are transported into the cytoplasm in endocytic vesicles. These vesicles may proceed to scan the endoplasmatic reticulum (I), which has been reported to be a site for translation and RNA interference. EVs may fuse with endosomal membranes after acidification to release their RNA content (II), or be directed to lysosomes where they are degraded (III).

influence EV uptake include temperature and radiation exposure,[243] as well as pH conditions, which affect direct fusion of EVs with the plasma membranes of recipient cells.[244,245] Intracellular availability of ATP in recipient cells has also been shown to affect EV uptake, indicating that the internalisation of EVs into recipient cells is an energy-requiring process.[246]

Different mechanisms of internalisation have been described. In a classical way, EVs could enter cells via clathrin-dependent endocytosis, micropinocytosis or phagocytosis.[247] Additionally, EV uptake may also follow the non-classical, lipid raft-dependent endocytosis involving caveolin-mediated entry.[248] Interestingly, Heusermann et al. [62] recently reported that purified EVs labelled with GFP-CD63 surf filopodia of primary human fibroblasts, before being endocytosed at the cell basis in a process reminiscent to entry of specific viruses. In the cytoplasm, internalised EVs particles establish transient contact with the endoplasmic reticulum (ER) before being eventually being trafficked to the lysosomal compartment. Converging evidence indicate that ER is a cellular nucleation site for RISC complex miRNA/siRNA loading and targeting of mRNA.[136,249] As such, it could be speculated that internalised EVs may deliver their miRNA cargo in close proximity to the ER in order to favour their loading into AGOs.

5. Conclusions and considerations

The EV-RNA research field has clearly progressed since the previous ISEV workshop on EV-RNA held in 2012. There is a better understanding of the heterogeneity of EV subpopulations and of non-EV RNA-carrying structures that potentially contaminate EV preparations and their distribution among different body fluids. In addition, several factors influencing sorting of RNA into EV have been identified and experimental systems have been designed to address functional transfer of EV-RNAs *in vitro* and even *in vivo*. Nevertheless, the field continues to experience numerous technical challenges: the large variety in EV isolation procedures, the small size of EV and the minute amounts of RNA that are recovered from these vesicles, and biases in the analysis of these trace amounts of RNA. In addition, it remains challenging to obtain direct proof for the role of EV-associated RNA

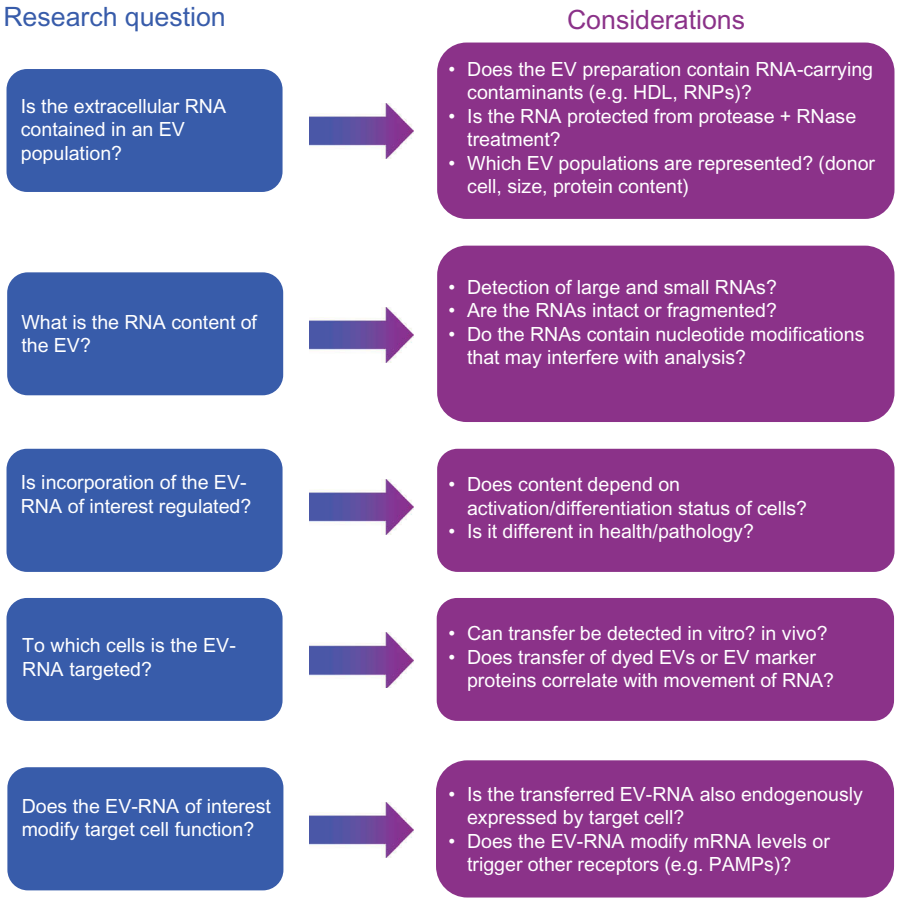

Figure 4. Considerations for analysing the nature and function of EV-associated RNA. Overview of research questions aimed at unravelling the nature and function of EV-RNA and considerations in addressing these questions, as discussed at the 2015 ISEV workshop on EV-RNA.

Table 6. Checklist experimental details to be included in publications.

Step	Parameters to be described
EV isolation	
Cell culture	Cell type
	Confluency
	Cellular activation/differentiation status
	Use of EV-depleted serum (include depletion protocol) or serum-free medium
	Cell viability
	Mycoplasma test
Body fluid	Health/disease status
	Collection method (disposables, chemicals, procedure)
	Removal of body fluid specific contaminants
	Storage
EV isolation	Differential centrifugation steps
	Filtration steps
	Size exclusion chromatography
	Density gradient
	Commercially available kits (e.g. chemical, column based)
	EV quantification method
EV-RNA characterisation	
EV-RNA sample preparation	RNase/DNase/proteinase treatment of EV
	Use of RNA spike
	RNA isolation method /kit
	RNA quantification method
	Bioanalyzer profile of EV-RNA
Library preparation	Enzymatic treatment to remove phosphates, caps, etc.
	Method /kit for ligating adapters
	Pre-amplification steps
	Size selection steps
	Bioanalyzer profile of library
Sequencing	Platform
	Maximum read length
	Direction (paired or single end)
	Number of cycles
	Number of replicates (biological or technical)
Bioinformatics	Pre-processing software (trimming/clipping, cut-off values)
	Reference genome assemblies (release numbers)
	Primary analysis software (alignment and mapping criteria)
	Browsers and annotation tools
	Normalisation methods and software
	Statistical methods and tools for differential expression analysis
Validation	Validation technique
	Normalisation procedure; validation of reference genes
Deposition in database	Name of database
EV-RNA functional analysis	
EV-RNA transfer	Target cell type and activation/differentiation status
	Methodology (EV-RNA labelling, target cell RT-qPCR)
	Quantity of EV per target cell
EV-RNA function	Methodology (transcriptome analysis, functional read-out)
	Endogenous expression levels of EV-RNA in target cell
	Proof to attribute functional effects to the RNA component of EV

in effects that EVs exert on target cells *in vitro* and *in vivo*. As an overview of what was discussed at the 2015 research seminar on EV-RNA and to prepare researchers from inside or outside the EV field for working in this area, we provide a list of important considerations that could guide future experiments (Figure 4). Furthermore, we provide a list of experimental details (an updated version of the list provided in the previous EV-RNA position paper [9]) that should be reported in publications addressing the composition and function of RNA associated to EV (Table 6). This checklist may aid in augmenting the reproducibility of studies in the EV-RNA field and may also be used by reviewers and editors to aid the review process of articles in the EV-RNA research field.

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