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REVIEW

In vivo imaging of EVs in zebrafish: New perspectives from "the waterside"

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Abstract

To harmoniously coordinate the activities of all its different cell types, a multicellular organism critically depends on intercellular communication. One recently discovered mode of intercellular cross-talk is based on the exchange of "extracellular vesicles" (EVs). EVs are nano-sized heterogeneous lipid bilayer vesicles enriched in a variety of biomolecules that mediate short- and long-distance communication between different cells, and between cells and their environment. Numerous studies have demonstrated important aspects pertaining to the dynamics of their release, their uptake, and sub-cellular fate and roles in vitro. However, to demonstrate these and other aspects of EV biology in a relevant, fully physiological context in vivo remains challenging. In this review we analyze the state of the art of EV imaging in vivo, focusing in particular on zebrafish as a promising model to visualize, study, and characterize endogenous EVs in real-time and expand our understanding of EV biology at cellular and systems level.

KEYWORDS

exosomes, extracellular vesicles, homeostasis, live-imaging, zebrafish

1 | EXTRACELLULAR VESICLES, AN EVERGREEN FIELD

For more than four decades, extracellular vesicles (EVs) have been attractive study objects in cellular and molecular biology, with a dazzling increase in the number of scientific publications since the last decade onwards. From the definition of "platelet dust" (1967)[1] to nowadays, the scientific community has been steadily unravelling their roles in various biological processes, from embryonic development[2,3] to body homeostasis[4] up to the development and progression of many diseases, including neurodegenerative diseases and cancer.[5-9]

Within the large group of EVs, we classically distinguish apoptotic bodies, microvesicles (or "ectosomes"), and exosomes.[10] Between these three, apoptotic bodies constitute the largest class with diameters ranging from 200 nm to 5 μm, and are formed directly at the plasma membrane (PM) of cells undergoing programmed cell death. Another PM-derived EV subclass is microvesicles (MVs), that originate from viable cells and have diameters ranging from 100 to 800 nm. Exosomes, by contrast, are generated as intra-luminal vesicles (ILV) within intracellular multivesicular bodies (MVB) by invagination of their limiting membrane, and have a diameter ranging from 30 to 150 nm. The ILVs generated through this process can be

released into the extracellular environment as "exosomes" when MVBs fuse with the PM.[11] However, these classifications are still prone to adaptation, as new categories of EVs of different cellular or even unknown origins and novel roles frequently emerge, such as exophers,[12] autophagy pathway-derived EVs,[13-15] mitochondrial-derived vesicles (mitovesicles),[16-18] and migrasomes[19-21] (Figure 1). On all these different EV subtypes, there is still a lot to be clarified, opening up an ever-increasing field of exploration that attracts more and more researchers. Yet, one should consider that the EV field is still immature compared to other research fields and is constantly being refined. EV-related studies can therefore be easily susceptible to misinterpretations due to the mere complexity of EVs and several substantial technological gaps that we are still facing today.

2 | "EVS, ET AL.": WHAT ARE WE REALLY LOOKING AT?

The most common approach to study the role of EVs is based on their isolation from large volumes of biological fluids or conditioned cell culture media, often followed by further characterization by different means. These methods are associated with various technical challenges. Differential (ultra)centrifugation (DUC),[22] one of the earliest EV isolation techniques, is prone to induce EV aggregation, deformation, or loss of functional integrity and cargo,[23] including loss of the integrity of the glycan crown (an ensemble of O- and N-linked glycans, GPI-anchors, glycolipids and glycoproteins covering the EV, mediating processes as EV adhesion, targeting and uptake).[24,25] Additionally, DUC can easily result in co-isolation of indistinguishable EV subpopulations (exosomes, small microvesicles, or mitovesicles) and contaminants such as viruses, protein aggregates, and nucleic acids (RNA and DNA) associated or not to the outer membrane (rather than loaded into EVs by their donor cells).[26] In recent years, many techniques have been refined to allow separation of EVs from soluble proteins and other contaminants, using immune-capture, density gradient, ultrafiltration, or size-exclusion chromatography.[27-29] These techniques and their combinations can lead to very good recovery yield of pure or subpopulations of EVs based on their size, density or on optimal expression of a targetable marker. However, these techniques still cannot fully discriminate collected EVs based on their biogenesis (MVB-derived exosomes from PM-derived microvesicles for example) and will thus benefit from more refined characterization approaches that are currently being explored.[30,31] As a consequence, uncertainties in the isolation strategies challenges, to some extent, the interpretation of data with respect to the exact EV subpopulation that is responsible for the functional effects that are observed, both in vitro and in vivo.

To further explore EV biology, complementary imaging strategies are developed to label and track EVs during intercellular communication. Broadly applied methods require a post-isolation labelling step of exogenous EVs using fluorescent dyes that bind nucleic acids (such as SYTO 13, H33342, and Thiazole Orange[32,33]) or are integrated in the lipid bilayer of EVs (e.g., PKH family, MemBright, Rhodamine B, or carbocyanin dyes like DiL and DiR[34-36]), or by incorporation of radioactive or magnetic tracers. While these techniques have the advantage

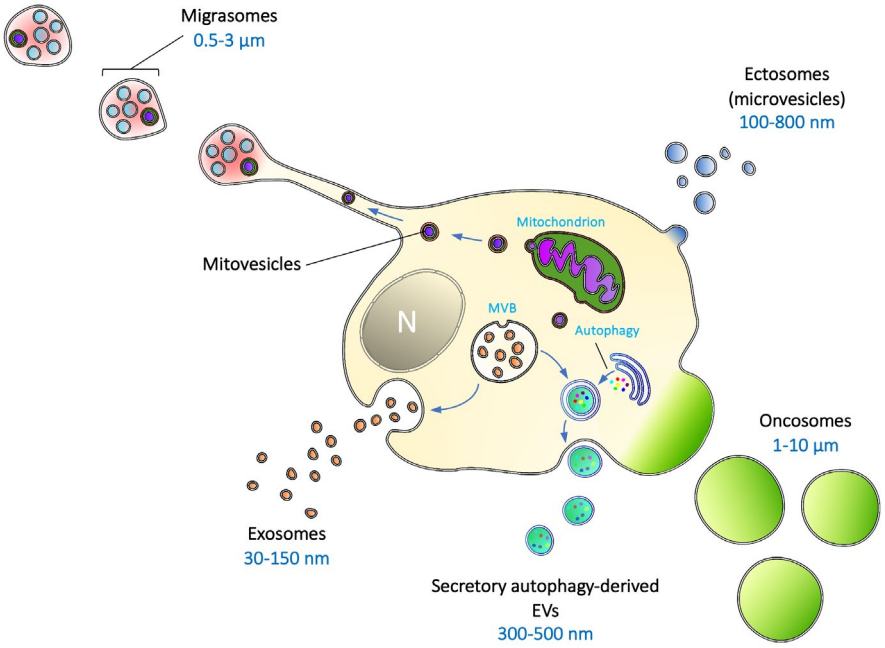

FIGURE 1 EV sub-populations released by a single cell, with their respective diameters. EV, extracellular vesicles; MVB, multivesicular body; N, nucleus

to label the whole EV population, they require robust controls as some of these dyes can induce further aggregation and formation of micelles, or can label contaminants, such as nucleic acids externally attached to EVs or membrane debris derived from cell damage, poor isolation, or poor storage. In addition, the relative stability of the dyes suggests their signal can persist over time, even after the degradation of the EVs,[37,38] which might complicate the interpretation of fluorescent signals in long-term experiments.

3 | NEW PERSPECTIVES REVEAL NEW DETAILS: THE ENDOGENOUS INSIGHT

Since the last decade, the EV community is therefore slowly expanding its approaches, moving toward genetic labelling of EVs.[39] This helps not only to improve their detection by having a fluorescent signal uniquely associated with EV-marker proteins, but also to address fundamental questions about the subcellular location(s) and dynamics of EV biogenesis, secretion, and degradation after uptake in recipient cells.

Among the most widely adopted strategies of genetic EV-labelling are fluorescent protein (FP)-tagging of EV-marker proteins, guaranteeing a more specific and reliable association of a fluorescent signal to the EV, and allowing the visualization of EV release, uptake and degradation in vitro, with the possibility to define the half-life of the EVs in recipient cells ex vivo.[40] EV-markers often fused with an FP include tetraspanins (TSPANs, a protein family often found in EVs, characterized by four transmembrane domains, two extracellular loops, and a number of highly conserved amino acid residues) such as CD63 or CD9, that form relevant targets for cellular and molecular studies. Association of TSPANs with pHluorin (a pH sensitive GFP variant) for instance, allows to observe individual MVB-PM fusion events in living cells using total internal reflection fluorescence or spinning-disk microscopy.[41,42] With these methods, fundamental questions can be addressed such as how often and at which location on the PM exosomes are secreted, as well as the characterization of natural and synthetic triggers and inhibitors that modulate this process in cancer cells.[42] This approach can likewise be applied to study exosome secretion in polarized cells. In specialized cells like neurons for example, where specific functions are associated to specific regions of the cell, mapping the location, frequency, and triggers of exosomal exocytosis events could bring novel insights to neuronal cell biology.

Looking at endogenous, genetically labelled EVs has been an important step in the field, potentially resulting in more precise imaging data compared to singular use of fluorescent dye-labelled EVs. This allowed new insights not only in uptake and clearance studies, but also more importantly enabled for the first time the study of biogenesis and secretion of endogenous EVs in real-time. Yet researchers should keep in mind that this approach may target EV subpopulations, whereas at the same time their overexpression or the addition of a genetic label may result in their exclusion from- or mis-localization to other EV-subtypes, not indigenous to the marker in question. In addition, genetic labelling strategies may be susceptible to (proteolytic) cleavage,[43] and may not always be accessible to immune-capture or -labelling approaches when the tag is embedded within the 3D structure of the protein. Finally, genetic tagging is incompatible with the study of EVs isolated from patient-derived body fluids.

4 | A NEW IMAGING SCENARIO IN 3D DEVELOPMENT

Although very informative, it is not clear whether studies performed with purified and *a posteriori*-labelled EVs actually reflect what naturally happens in living organisms. Indeed, relatively little is known on EV biology in vivo in terms of biogenesis, mode- (induced, pulsed or constitutive) and quantities of secretion, their bio-distribution, cognate target cells, clearance, and functional effects. To address these issues in vivo, significant breakthroughs are necessary to explore EV biology beyond the use of isolated EVs for studying their cellular and molecular effects. A first step is therefore to transpose this "endogenous perspective" on EVs in vitro to more complex structures, such as 3D co-cultures (e.g., transwells) and "mini organ" formations like spheroids and organoids. These models are physiologically more faithful to the in vivo situation compared to 2D cell culture systems, since they better recapitulate tissue architecture and interactions with the extracellular matrix (ECM).

As for spheroids, organoids, and assembloids,[44] the community is still mostly focussing on the isolation of EVs from these structures followed by *a posteriori* analyses, for example by proteomics or RNA-omics. Interestingly, compared to EVs derived from cells grown in 2D monolayers, acquisition of a complex 3D structure by the exact same cell type is known to modify the protein and nucleic acid cargo content of the released EVs, as well as their size distribution.[45-48] This indicates that the spatial and geometric organization, and therefore the complex cell-cell/cell-ECM interactions impact the (sub)type of EVs that is secreted. It is clear that these 3D systems are a qualitative improvement over 2D systems and therefore

rightly deserve more attention in EV research.[49] More recently, attempts have been made to visualize genetically tagged endogenous EVs released in simple 3D systems such as transwells to study the blood brain barrier,[50] and further improvement can be expected from live-imaging of organoids or assembloids.[44] These approaches will help clarify how the architecture of an organoid impacts EV size and cargo at molecular level, in terms of mechanical triggers or biological cues that might impact EV biogenesis, cargo sorting, and endosomal trafficking. Furthermore, exploiting systems such as pHluorin-associated TSPANs might help to clarify if within the 3D topographic context of an organoid some cells—or even specific parts of single cells (e.g. the apical or basolateral membrane)—are more prone to secrete EVs than others, and if and how this is linked to composition and/or cell-fate specification within the organoid. This could help to instrumentalize the further exploration of potential roles for EVs in tissues or cell populations seen as complex superstructures. Akin to *quorum-sensing* mechanisms found in the microbial world, mammalian cells could coordinate their activity according to cell number, a postulation that has been explored in various fields, including immunology, cancer biology and stem cell behavior.[51-53] Interestingly, various of these studies directly or indirectly alluded to an important role for cytokines in these processes, many of which can be associated to EVs, extending their half-life and potency compared to their soluble counterparts.[54-56] Apart from this "quantitative" mode of sensing, EVs may also be implicated in a more "qualitative" fashion to support tissue architecture, by providing positional cues. Indeed, EVs, known for their capacity to modify the ECM, to provide anchoring points and migration cues, could likewise allow a cell to perceive its own position within a complex three-dimensional environment, or constitute (polarized) environmental cues for cell specialization/differentiation.[57,58]

To study the role of EVs in the communication of a cell with its environment, 3D models thus appear a cogent choice. Within the spectrum of available 3D models, one should then consider the complexity, costs, throughput and availability of imaging methods.[59] Yet, depending on the research question, one could still consider 3D models as simplified representations of reality. While it is certainly true that these models can bring crucial insights into the relationship between cellular complexity, space-geometry, and EV biology, they are commonly not vascularized and/or do not contain stroma as found in tissues within in a living organism. Hence, they might be less suited to study EVs as endocrine messengers mediating the cross-talk between certain cell types or communication over long distances.

5 | IMAGING EVS IN VIVO: ARE MAMMALIAN MODELS ENOUGH?

To study the (patho) physiological roles of EVs in complex fields like cancer biology, often classical rodent models such as mice and rats are used. These approaches typically involve repeated injections of exogenous EVs in an orthotopic or heterotopic intravenous, intra-peritoneal, or intra-footpad fashion. Prior to injection, these EVs can be labelled with dyes or by incorporation of radioactive or magnetic tracers, by virtue of which these strategies allow to distinguish the main bulk accumulation points of EVs by PET, MRI, or SPECT/CT,[60-63] or to evaluate their accumulation in more detail in *post mortem* tissues. Despite the benefits and relevant insights they can bring us, especially in studying EV function, these studies rely on exogenous EVs often labelled *a posteriori*, associated with the various criticalities as discussed above, that is, the quality of the isolated material, labelling issues, dosage-, timing-, and injection sites that might not be faithful to physiology.[39] This may cause biases in our extrapolations toward how endogenous EVs behave in vivo, as their release by cells, dynamics and spread in the organism are likely heavily impacted by the intrinsic 3D architecture of the tissues. A breakthrough toward a more "endogenous perspective" in vivo was accomplished with the generation of genetically modified rat models featuring endogenous EV labelling by expression of CD63-GFP in a tissue-specific manner, thus bypassing the isolation and injection of exogenous EVs steps.[64] Yet, when it comes to observing nm-sized objects such as EVs, these animal models do not generally allow for robust live-tracking, or the imaging is restricted to the area immediately adjacent to the imaging window, such as in intra-vital microscopy.[65] Alternatively, one could rely on organ extraction and optical clearing of the tissue, allowing for more detailed ex vivo (post-fixation) analysis of the fate of endogenous EVs, even though this approach is incompatible with live-tracking.[66] Therefore, these models are less suited to obtain a detailed, dynamic understanding of the "in vivo EV mediated cross-talk" paradigm, for example, with respect to rare events compared to the "main" EV flow, or functional events occurring before EV uptake. Moreover, the site of (bulk) accumulation might not necessarily be identical to the site of function.

While studies carried out in rodents have been very enlightening in various aspects of EV (patho) biology, tracking the whole life cycle of single EVs in real-time in a living organism requires animal models with superior optical accessibility. Because of its transparency, various groups have used the larvae and the adults of *Caenorhabditis elegans* to investigate EV biogenesis and dynamics in vivo, visualizing endosomal membranes dynamics and fusion

of MVBs with the PM in vivo by electron microscopy, with consequent release of endogenous exosomes from stem cells into the extracellular environment.[67-69] *Drosophila melanogaster*, covered extensively elsewhere in this special collection, is likewise an attractive model system for studying EVs in tissue organization, development, and systemic crosstalk. However, these models will not recapitulate all aspects of vertebrate biology, as for example, they lack complex organs, vascular- and parenchymal systems. This could in turn limit EV dynamics and spread in vivo.

With zebrafish, the EV community recently adopted another transparent in vivo model that combines the single cell precision of *C. elegans* but in a vertebrate system that is endowed with a more complex vasculature, interstitial-, and organ architecture, as well as diverse blood cell populations.

6 | THE "ZEBRAFISH REVOLUTION"

Zebrafish (*Danio rerio*) (ZF) is a tropical freshwater fish native to India. It has been used for decades now as a model for basic and applied human related research, as it shares more than 70% orthologous genes with *Homo sapiens*, notably including genes involved in organ-specific genetic programs and cancer-biology.[70]

Beyond its ease of management, short breeding intervals, large number of offspring, and reduction of housing costs compared to mammalian models, the ZF embryo adds optical transparency that enables the live-tracking of any type of fluorescent labelled object in real-time by live-microscopy. Indeed, this model organism has allowed the observation of objects at single-particle level as small as viruses,[71] nanoparticles,[72] or even (single) proteins,[73] and hence has rightfully entered the ranks of suitable animal models to study EV biology.[74] In certain mutant strains such as *casper* and *crystal*, a degree of transparency is maintained even throughout the adult life stage.[75]

Recently, a study used EVs isolated from cancer cell conditioned supernatant, that were labelled, injected in the *duct of Cuvier* and followed over-time in the ZF embryo. Doing so, researchers were able to map individual tracks of tumor-derived EVs and describe their speed and flow in the bloodstream using high-speed confocal microscopy. This helped to clarify aspects related to the mechano-dynamic behavior of EVs in the blood circulation at the whole organism scale, and a possible role for these EVs in pre-metastatic niche formation. This study also identified endothelial cells and patrolling macrophages as major recipient of these tumor EVs. By using a correlative light and electron microscopy approach, they further demonstrated that these EVs ended up in degradative subcellular compartments.[76]

To observe endogenous EVs naturally released by cells in vivo, a back-to-back study reported the use of the EV reporter CD63-pHluorin in the ZF embryo. During the first 2–3 days of development, a significant part of endogenous EVs were released into the bloodstream by an embryonic structure called the yolk syncytial layer (YSL), as demonstrated using tissue-specific expression of CD63-pHluorin. By interfering with syntenin, a protein critically involved in EV biogenesis as first identified in vitro,[77] this work demonstrated that YSL-EVs were released in a syntenin-dependent manner in vivo. Once released into the circulation, these EVs were specifically endocytosed by macrophages and endothelial cells of the caudal vein plexus (CVP), as shown using inhibitors in vivo. Finally, using a combination of immune-capture, ex vivo proteomics and site-specific syntenin interference, these EVs were implicated in trophic support of the recipient tissue.[78] Similar approaches using tagged TSPANs in vivo revealed key features of genetically labelled (endogenous) migrasomes, an interesting new class of EVs derived from retraction fibers, highlighting a role for migrasomes in organ morphogenesis during gastrulation in the ZF embryo in real-time.[21] Combined, these studies amply demonstrate the power of the ZF model to study the dynamics of (endogenous) EV subpopulations from producing to receiving cells in a living organism, by simultaneously overcoming a number of significant issues related to the isolation, injection site, dosage, timing, and labelling of EVs (Figure 2). Future studies capitalizing on these developments could be instrumental in better understanding the involvement of EV sub-populations in embryonic development, but can also elucidate the relationship between EVs and other physiological processes or environmental factors, such as tissue repair, circadian rhythm, physical activity, and stress.[79,80]

7 | IMAGING INTER ORGAN CROSS-TALK IN ZEBRAFISH: THE EVE OF A NEW ERA

The use of tissue-specific endogenous fluorescent EVs reporters in the ZF transparent vertebrate model in vivo will thus aid in addressing long-standing fundamental questions in the EV field. For example, in a fully physiological in vivo context, we could uncover the modality of EV release by single cells or within a single tissue, and decipher whether this happens in a continuous fashion or in spikes at certain intervals, and in response to what kind of stimuli (mechanical, chemical, voltage-dependent...). This real-time insight could help in unravelling the involvement

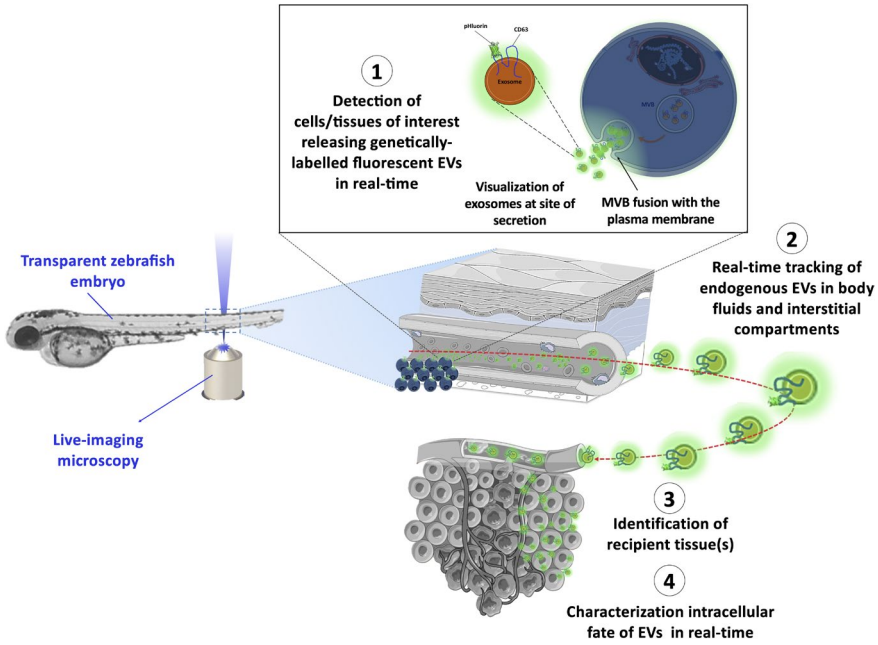

FIGURE 2 Zebrafish (ZF) embryos as comprehensive model to investigate EV-biology in vivo. Live-imaging of genetically labelled endogenous EVs in the ZF embryo allows to (1) study their release by producing cells, (2) follow their journey in the bloodstream and interstitial compartments, (3) follow their uptake by their natural targets and (4) characterize their intracellular fate. EV, extracellular vesicles

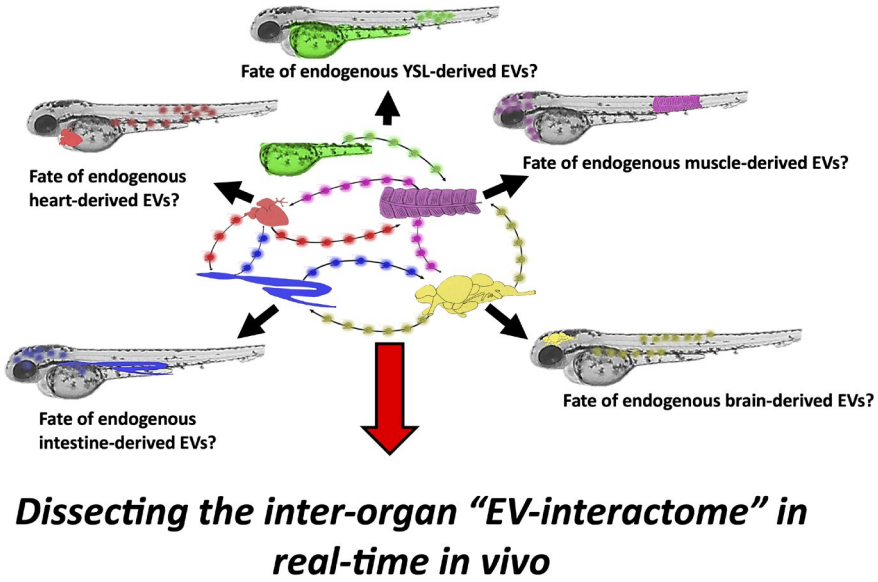

FIGURE 3 Visualization and mapping of the endogenous "inter organ EV-interactome" in the ZF embryo. Tissue-specific expression of EV (-subpopulation) reporter-proteins as well as cargo-transfer reporter systems could help unravel EV-mediated communication pathways existing between different tissues and organs. EV, extracellular vesicles; ZF, zebrafish

of EV-mediated cross-talk in inter-organ homeostasis at single-vesicle resolution, and facilitate the characterization of inter-tissue and inter-organ EV-mediated interaction pathways in ZF. This mapping of the "endogenous inter-organ EV-interactome" will be highly insightful and a significant advance from the current *status quo*, with a high potential to uncover novel physiological functions of EVs (Figure 3).

One of the most exciting parts of these new developments is their potential to further characterize the fate of EV population(s) of interest in real-time in vivo. For example, we previously exploited the pH sensitivity of CD63-pHluorin to observe whether the uptake of endogenous YSL-derived EVs by the endothelial cells in the ZF cardinal vein resulted in late-endosomal targeting in vivo. The disappearance of the pHluorin signal suggested that YSL-derived endogenous EVs end their journey in acidic compartments of endothelial cells, possibly to be degraded. Indeed, using Bafilomycin A1 (a drug that neutralizes V-ATPase, a proton pump responsible for endosomal acidification to allow degradation of endo-lysosomal content) we observed the accumulation of pHluorin-EVs in this endothelial cell population.[78]

With some adaptations, this approach could also be exploited to determine EV half-life in near physiological-conditions. Subsequent studies could also be used to understand what fraction of endocytosed EVs is degraded or for instance may cross the cell by transcytosis,[81,82] and if certain EV subpopulations differ in this respect. At cellular and tissue level it will allow us to distinguish cell types

constitutively taking up EVs from cells that only do so upon a specific (physiological or pathological) trigger. Likewise, we could distinguish recipient cells that take up EVs exclusively from a specific donor cell type versus cells taking up EVs in a nondiscriminatory manner. Furthermore, we could study which cells use EVs for metabolic and trophic support,[83,84] or are carrying out a (signalling) function by functional transfer of their content(s). In recent years, Cre-Lox and CRISPR recombination technologies established the molecular basis to map which cells functionally receive the cargo of the EV population of interest in their cytoplasm, requiring currently ill-understood processes such as back-fusion/endosomal-escape, where internalized EVs fuse with the limiting membrane of late endosomes before their cargo can be degraded.[65,85-87] It could thus prove very informative to apply these molecular approaches to the ZF embryo endogenous EV tracking-model system, and interrogate EV-intraluminal content release at the level of the whole embryo.

7.1 | Manipulating endogenous EV secretion in vivo

A detailed characterization of endogenous EV release and fate is a highly useful yet descriptive approach. Therefore, the possibility to control EV secretion and fate will be determinant to critically demonstrate their roles in inter organ cross-talk, homeostatic maintenance and other processes. An exciting challenge for the EV field is therefore to generate new tools able to manipulate EVs in space and time. The application of conditional gene control and synthetic biology tools in vivo could prove very fruitful. The ZF embryo model is an ideal basis for such developments, and could expediate subsequent transposition of these tools to other model systems.

A first much needed development concerns the manipulation of EV-secretion in a tissue-specific manner in vivo. So far, several methods to manipulate EV secretion have been published, such as the use of chemical compounds affecting EV-biogenesis and/or -release, including Bafilomycin A1 and GW4869, or the interference with proteins crucial for EV biogenesis, such as syntenin.[88-90] In a previous study in ZF, our group blocked EV secretion from the YSL in a tissue-specific manner by using a syntenin-a morpholino injected locally in the YSL. This resulted in an impairment of CVP angiogenesis during ZF embryo development, suggesting a role for these EVs in vascular development and growth.[78] Whereas this strategy may not suit other tissues due to lower accessibility or the involvement of different biogenesis pathways, alternative developments targeting various biogenesis pathway by tissue-specific RNA interference in vivo could be a powerful approach to study EV function.[91,92] Conditional expression could provide an even "cleaner" system and further reduce any potential detrimental or embryonically lethal effect of interference with the targeted genes.

Tissue-specific control over EV secretion can be accomplished by deploying genetically encoded "switches" expressed in a conditional manner or controlled in an optogenetic fashion to modulate EV release specifically in the tissue of interest in the embryo (Figure 4). Ideally, such an

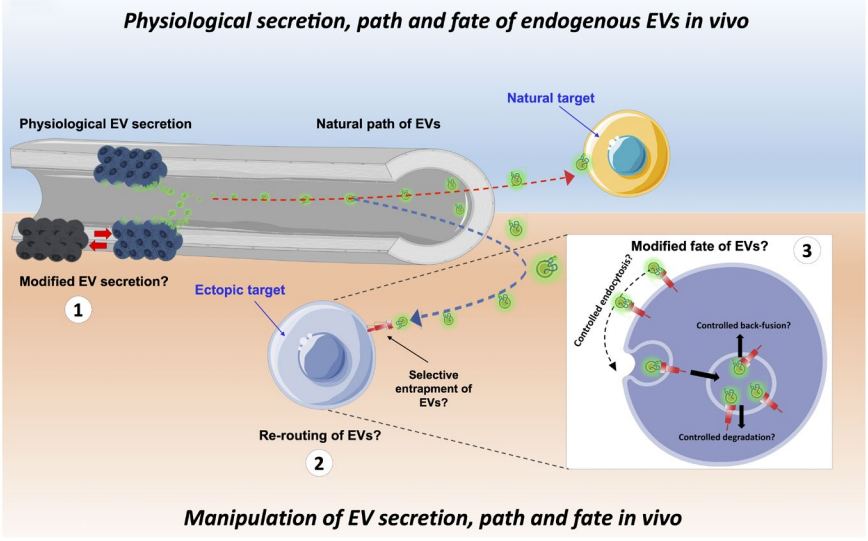

FIGURE 4 Options to interfere with endogenous EV biology in vivo. To better understand and pinpoint the (patho) physiological roles of endogenous EVs in vivo, various developments are necessary. (Upper half) Hypothetical model of the various steps during the normal life-span of endogenous EVs in vivo. (Lower half) Opportunities to interfere. (1) Spatial- and/or temporal modulation of EV secretion. (2) Modulation of the natural "default" EVs trajectory toward a different, ectopic target. (3) Genetic control of endocytosis and of the intracellular fate in recipient cells. EV, extracellular vesicles

"EV-switch ON/OFF" system should be able to selectively modulate the secretion of a specific EV subpopulation in space and time, for instance by acting on proteins involved in a particular EV biogenesis process. The problem, however, is that often these biogenesis-involved proteins are shared between EVs of different sub-cellular origin (e.g., endosome-derived exosomes and PM budding microvesicles), meaning the separation will still be inaccurate. Interestingly, some refined molecular tools are currently emerging that aim to identify specific markers for EV subpopulations,[31,93] and could thus prove very helpful here. Alternative strategies to modulate EV secretion could be based on the control of their compartments or location of biogenesis. Acting on MVB positioning or turnover using optogenetic tools[94] for example, could potentially give us the ability to more precisely modulate exosome secretion in ZF embryos, while discriminating them from other EV subpopulations (e.g., microvesicles) in vivo. This level of control over the secretion of endogenous EV sub-populations would constitute a significant step in the field, and would allow to refine our understanding on the roles of different EV subpopulations in inter organ cross-talk and homeostasis maintenance in vivo, which in turn could have potential implications for translational research.

7.2 | Manipulating the path and fate of endogenous EVs in vivo: futuristic utopia or possible reality?

A second challenge to better understand the natural functions of tissue-specific EV subpopulations would be to interfere with the normal (physiological) spread and targeting of endogenous EVs in vivo. This "hijacking" of specific endogenous EVs would constitute a valuable tool if we want to attain an intricate understanding of the involvement of EV cross-talk in processes such as maintenance of homeostasis or their role(s) in various pathologies.

So far, literature reported the in vitro use of optical tweezers for selective displacement and manipulation of single EVs, compatible with live-imaging.[95] Approaches like this can be very useful to study molecular uptake mechanisms in detail. However, this changes the fate of just one EV at a time in vitro, and therefore it not suitable to study the effect of tissue-specific endogenous derived EVs released in high numbers in vivo.

One possibility to modify the fate of endogenous EVs is to play with the receptors involved in the EV uptake in the target region. In a previous study, various groups including our own have implicated scavenger receptors in EV clearance from the circulation.[78,96,97] Blocking class A-scavenger receptors using DexSO4-500K strongly diminished EV uptake and caused an impairment of the caudal vasculature development of the ZF embryo, mainly by affecting cellular proliferation.[78] Further analysis showed that a general block of dynamin-dependent endocytosis was likewise able to starkly reduce EV uptake in the CVP. While informative, the main problem associated with general uptake blockers in vivo is that we affect all tissues that take up EVs or express a particular receptor simultaneously. In addition, receptors like scavenger receptors are a diverse family known for their broad target range, such that their interference may simultaneously lead to a block in uptake of other molecules and nutrients. Likewise, the use of general blockers of EV secretion means we also interfere with the secretion of EVs other than the EV population of interest.

To investigate the effect of a specific subtype of EVs on a specific target tissue, it thus seems clear that we need an approach where EV uptake can be controlled specifically in the region of interest. Would it be possible to devise a molecular system to "hijack" tissue-specific endogenous EVs of interest, allowing their capture in a selected region in vivo? Once again it seems that genetic approaches will be most straight-forward. One approach would be to generate a forced ligand-receptor system that can be expressed in specific tissues to capture endogenous EVs, and can be controlled in space and time. This would then allow the "hijacking" of an EV population of interest, redirecting it toward an ectopic location. Additional tools could then be developed to ascertain a degree of control over the downstream fate of EVs, for example, by controlling the internalization pathway or promoting endosomal escape. We are still far from this level of precision, but making efforts in this direction would guarantee a more detailed exploration of the concept of EV-mediated cross-talk in a dynamic way in vivo by controlling each step of the fate of EV in recipient cells (Figure 4). In a more distant future, and on a more speculative note, one could imagine applications in the field of tissue-repair or regeneration, for example, by diverting EVs from stem cell pools to specific sites in vivo, such as necrotic or inflamed sites, or in case of pathological EVs, trying to reduce or intercept their spread through the organism. While appealing, these developments would necessitate the identification and use of (highly) selective EV subpopulation markers, for which further developments are required.

8 | FROM TANK TO BEDSIDE: USING ZEBRAFISH TO STUDY THE ROLE OF EVS IN HUMAN PATHOLOGIES

All these aspects, though primarily focusing on fundamental aspects of EV biology in a zebrafish organism, can potentially have interesting translational applications, especially

toward human pathologies. In fact, we know that EVs are involved in various patho-physiological aspects in humans, such as in embryonic development,[2,3] tissue regeneration and repair,[98,99] inter-organ homeostasis and communication[4] and microbiota-host interactions.[100] Moreover, accumulating evidence in literature shows the involvement of EVs in human pathologies too. Indeed, EVs are now emerging as novel therapeutic targets in cancer,[101] as a source of disease biomarkers[102] (for example, neurotoxic spreading of Aβ in Alzheimer's and α-synuclein in Parkinson's disease[5-8]), metabolic diseases,[103] inflammatory diseases,[104] cancer,[9] Down syndrome etiologies,[18,105] and so on.

So far, no one has ever recorded the EV release dynamics of cell types of interest in these patho-physiological contexts from an "endogenous" in situ perspective. Now more than ever, the tracking of their spread and fate in real-time in vivo appears feasible using ZF embryos. To fully exploit these possibilities, we advocate the use of ZF models of human pathologies, such as transgenic or drug- induced models, or by xenografts of human cells. Indeed, the application of ZF models is gaining *momentum* in the study of developmental disorders, mental disorders, metabolic diseases,[106] hematopoietic disorders, cardiovascular diseases[107] neurological disorders,[108,109] cancer biology, and precision cancer therapy[110] as well as for screening platforms in drug discovery.[111] The latter might also prove useful in medium/high throughput screens to identify critical modulators of EV secretion, targeting, uptake, and fate in vivo.

However, there are still some limitations in the application of ZF models with respect to (human) transgene expression and xenografts that must be considered here. First of all, when studying EVs, we are mostly limited to the use of a model that is still at an embryonic state (as adults lose transparency), with many tissues still undergoing further development and maturation. The blood– brain barrier for instance has a relatively high permeability at early stages compared to adulthood, being therefore potentially more permissive to the passage of EVs. These and other issues could impact the biodistribution of EVs through the organism, resulting in distribution patterns that might not reflect normal homeostasis. Moreover, certain brain areas such as the cortex are not developed as far as in rodents and humans, and some central nervous system structures in ZF are still difficult to map to their human counterparts.[108,109] Furthermore, ZF have gills instead of lungs, which could be a *caveat* in the study of EVs derived from tumors that metastasize toward the lungs, or derived from lung cancer xenografts. ZF also lack important glandular organs such as a localized endocrine compartment of the pancreas (that in ZF appears dispersed in endocrine islets scattered throughout the exocrine pancreas) and a prostate. On the other hand, ZF show several useful homologies in heart,[112] liver,[113] and even skin[114] development and organization compared to rodents and humans. As such, ZF is a highly versatile pre-mouse and even preclinical model that can help reinforce and fast-track developments in both basic and applied research in the EV field.[115]

9 | CONCLUSIONS

Methodologies of isolation, purification, labelling, and injection of exogenous EVs are still in need of further refinement, as they are associated with various limitations that preclude us from better understanding the physiology of EV-mediated inter-organ cross-talk in a living organism. To do so, we need new methods and foremost a change of perspective in how we study EVs. Looking at genetically labelled endogenous EVs has brought us the possibility to study the regulation of their biogenesis, secretion, uptake, and fate in vitro. Applying this approach in a transparent vertebrate model like zebrafish, enabled us for the first time to visualize EV release events, but also to assess the dynamics of distribution, uptake and fate of endogenous EVs in vivo in real-time, allowing us to address cell biological questions in an in vivo setting. This opens the door to a myriad of possibilities in terms of mapping and manipulation of the "inter organ EV-interactome", as well as applications in basic and applied research focussed on human pathologies. In parallel to other in vivo models, the introduction of zebrafish thus constitutes a significant revolution in EV research, and we expect the near future will bring exciting developments and findings to the field.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

VV wrote the first draft and drew the pictures, AB provided insightful questions, GvN and FJV edited the manuscript.

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