REVIEW
PHYSIOLOGY 34: 169–177, 2019. Published April 10, 2019; doi:10.1152/physiol.00045.2018

Extracellular Vesicles: Exosomes and Microvesicles, Integrators of Homeostasis

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Extracellular vesicles (EVs), cell-derived membrane structures, are secreted after fusion of endosomes with the plasma membrane (exosomes) or shed from the plasma membrane (microvesicles). EVs play a key role both in physiological balance and homeostasis and in disease processes by their ability to participate in intercellular signaling and communication.

Introduction

Extracellular vesicles (EV) are secreted by all cells, and early reports indicate they play a variety of physiological functions, including commingling, communication, and defense. Indeed, the evolution of clonal multi-cellularity from single-cell precursors is thought to have occurred for reasons of security, communication, and the metabolic benefits of a "division of labor" to achieve a common goal—survival (4). What is the physiological role of EVs in multicellular organisms? Communication and commingling between and among tissues and cells is self-evident, and a role for EVs "rings true." Over the past several decades, a new field of basic research has burst onto the scene—the cell biology and physiology of EVs (and its application in diagnostics and therapeutics). One of the goals of this new frontier is to define the role of EVs in physiological and pathological states. EVs, however, comprise vesicles that originate from the endosomal network or from the plasma membrane. The former are called exosomes, whereas the latter, variously designated as microparticles, oncosomes, or ectosomes, are collectively referred to as microvesicles (52) (FIGURE 1). A community effort, virtually global in participation, has been undertaken to understand EVs: to elucidate their mode and mechanism of biogenesis, secretion, and targeting, and to improve methods for their isolation and characterization.

Our understanding of the nature and mechanisms by which EVs mediate physiologically relevant communication activities and commingling events is still at an early stage and remains incomplete. The French physiologist Claude Bernard introduced the concept of homeostasis (the word being coined later by WB Cannon) as those forces that tend to maintain the constancy of the internal environment or *milieu interieur*. EVs may represent a new level of physiological control in multicellular organisms and may open a new level of understanding of homeostasis.

Breakthroughs That Serve as Pillars of a New Field of Research

The exosome secretion pathway was discovered in 1983 (15, 40). Harding and colleagues (15) used transferrin-colloidal gold particles to track transferrin receptor trafficking. Transferrin-gold particles were internalized into the tubular-vesicular endosomal network and gathered into multivesicular bodies (MVB). Following a pulse-chase experiment, a serendipitous moment revealed that MVBs enriched with transferrin-gold-decorated intraluminal vesicles fused with the plasma membrane releasing vesicles (now called exosomes) into the extracellular environment. Although exosomes arise from within the endosomal network during MVB formation, microvesicles, which vary from 50 to 1,000 nm in diameter, appear to have multiple points of origin, ranging from the selective outward pinching of the plasma membrane to membrane shedding and/or vesicles resulting from cell death.

The nascent field of EV biology came into sharper focus with the discovery that exosomes are mediators of cell signaling. Secreted by B lymphocytes, exosomes are immunologically active and both present antigen in vitro (46) and have the ability, in vivo, to stimulate anti-tumoral immune responses (60). These reports introduced a new concept—exosomes operate as signal transducers. A decade later, a new development extended the concept of exosomes and microvesicles as signal-transducing agents. EVs were found to harbor genetic material, both miRNA and mRNA but also other RNAs (38), that can be transmitted between cells in a functional way. This discovery extended the concept that exosomes were cogs in a signaling network in multicellular organisms capable of transmitting information between and among cells (51).

Taken together, these findings suggest that all modes of communication, via proteins, lipids, nucleic acids, and potentially glycoconjugates, were

open to investigation and were part of a large communication system that operates to oversee both local and long-range homeostatic mechanisms in multicellular organisms.

EV Biogenesis and Secretion

Exosomes arise as intraluminal vesicles (ILVs) within maturing endosomes. MVBs were first observed in 1963 (11, 44) and were considered part of a one-way path to lysosomes until the exosome secretion pathway was discovered (FIGURE 1). Originally described in yeast, the biogenetic pathway of ILVs, including cargo selection, budding, and scission, requires the ESCRT (endosomal sorting complexes required for transport) family of proteins (see FIGURE 2). Later, other components involving lipid rafts enriched in ceramide (50), tetraspanin-enriched microdomains (8, 52), and other proteins such as syntenin and syndecans (2) have been shown to be involved in ILV generation and therefore in exosome biogenesis (FIGURE 2). MVBs have long been shown to fuse with the lysosomal compartment, resulting in the degradation of the contents. An important question is why some MVBs fuse with the lysosomal compartment, whereas others fuse with the plasma membrane resulting in exosome secretion. The mechanisms that control MVB fusion with the plasma membrane are just now coming into view. There are subpopulations of MVBs (6, 35) that have certainly distinct fates dictated by the association with particular effectors, allowing fusion with target membranes (lysosome and/or plasma membrane). A large body of work has uncovered parts of the molecular machinery that regulates MVB fusion with the plasma membrane, including the endosomal V0V1-ATPase (14, 29), and low molecular weight GTPases, including the RabGTPases, Arf6, and Snares (reviewed in Refs. 30, 52). Meanwhile, imaging of MVB fusion with the plasma membrane in real time using a CD63-pH fluorin construct has been reported (54). Model organisms such as the zebrafish embryo are valuable tools to follow exosome biogenesis, targeting, and fate in vivo (53).

Microvesicles, on the other hand, emerge from the plasma membrane where multiple mechanisms seem to be at play (FIGURE 1). One set of microvesicles is derived from budding events nucleated by the protein ARRDC1, which is recruited to the plasma membrane along with elements of the ESCRT pathway generating 50-nm vesicles (37). Other microvesicles have been reported to utilize Bin-1 (ampiphysin), a protein that forms molecular curvatures when recruited to the membrane. Larger vesicles sometimes referred to as oncosomes are associated with cancer cells (34). Although the mechanism of formation is unclear, these 200- to 500-nm vesicles have been shown to contain relevant content, including EGFR (1).

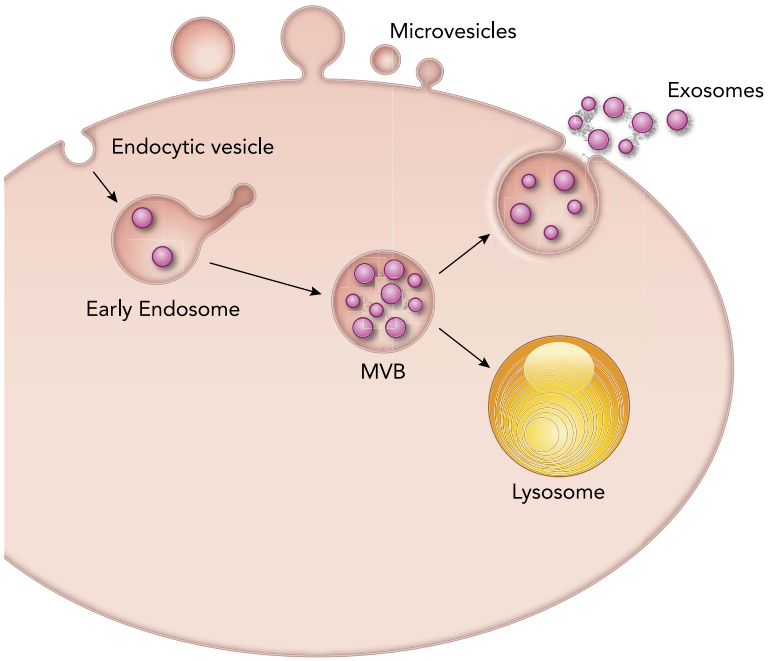

FIGURE 1. **Generation of exosomes and microvesicles**
Multivesicular bodies (MVB) are formed during endosomal maturation, a process through which intraluminal vesicles are generated by invagination of the limiting membrane, which results in the apparent selective sequestration of a small portion of cytosol. MVBs can fuse with lysosomes for degradation of their contents or fuse with the plasma membrane to release their intraluminal vesicles, then called exosomes. Microvesicles bud directly from the plasma membrane, sequestering portions of cytosol.

Selective Packaging of EV Cargo

The selective packaging of signaling proteins and nucleic acids into nascent exosomes or microvesicles is an area of intense interest. Reports that ubiquitinated proteins are commonly found in EVs (5) and the well-established role of ubiquitin to identify and recruit proteins to forming MVBs by the ESCRT system point to ubiquitination as one mechanism by which proteins are selectively targeted to the EV pathways. All options are open, since there are many forms of UB conjugation. Recent work suggests that mono-ubiquitination is important for targeting CD133 to EVs. Nevertheless, several reports also indicate that non-ubiquitinated MHC class II molecules are targeted to MVBs fated for secretion (6). Other candidates include palmitoylation and farnesylation (36) that bring proteins to lipid rafts that are implicated in EV biogenesis. The selective packaging of RNA into nascent EVs has seen significant progress with the discovery that some miRNAs contain a targeting sequence recognized by a sumoylated hnRNPA2B1 (55) and that YB-1 proteins may selectively recruit specific miRNAs into newly forming exosomes (48). Given the wide collection of RNA molecules in

EVs (mRNA, miRNA, lncRNAs, and snoRNAs) also observed in biofluids, this is a field still in its infancy (47). Last, successful cell-to-cell commingling or cell signaling requires that vesicles be tagged with recognition molecules that will direct them to the proper target. Candidates such as the integrin family of proteins are implicated, but the mechanism by which they are packaged into newly formed vesicles along with appropriate content is poorly understood.

EVs and Homeostasis

Among the factors that affect cellular and tissue homeostasis in multicellular organisms is their mutual dependence on the utilization of energy resources and metabolites. Metabolites and substrates may flow freely between cells via an extracellular pathway (by diffusion or active secretion and re-uptake) or between cells in close proximity via channels or junctions. However, recent work from a number of perspectives demonstrates that the sharing of metabolites and other materials may occur via extracellular vesicles—exosomes and microvesicles.

Many advances in understanding cell-to-cell transfer of metabolites via EVs have been uncovered in the study of cancer cells in tumors and the supporting normal cells present in the microenvironment. It is well established that a hallmark of cancer cells, particularly in tumors, is their reliance on the availability of glucose for their energy and proliferative needs. The necessary metabolites and intermediates can be provided by nearby normal cells that package amino acids, lipids, and other building blocks necessary for the recipient's well-being under hypoxic conditions into exosomes for delivery (59). The secreted EVs presumably encode the necessary targeting information to dock on the appropriate acceptor cells and deliver cargo via endocytosis or direct fusion (10).

Expanding the idea that tumor cells can receive cargo via EVs produced by normal and potentially tumor cells, Lyden and colleagues (19) showed that exosomes isolated from different tumor cell lines, whose origins were known, tended to target, when injected into live animals, their tissues of origin (e.g., lung to lung and liver to liver) (41). Moreover, integrin molecules on the surface of the aforementioned exosomes were likely responsible for the tissue-selective targeting (10, 19). Interestingly, the targeting of exosomes to certain anatomic locations (e.g., bone marrow) also has been implicated in the process called "seeding," where secondary metastasis develop almost exclusively in certain tissues. Also of interest, EVs/exosomes released by metastatic melanoma cells increase the metastatic properties of non-metastatic melanoma. Knowledge on the mechanisms underlying these effects will certainly help to design future therapeutic applications (26).

An important question is whether the selective uptake or production of exosomes exhibited by tumor cells is an exclusive feature of proliferative disorders like cancer or whether it is simply the amplification or exaggeration of a normal physiological process. There is an argument to be made that low-level commingling between and among tissues via exosomes is an ongoing process and is happening pari passu with other transfer processes, such as diffusion under normal physiological conditions in multicellular organisms. To this end, a set of observations reported by Whitham and colleagues is relevant (56). By using rodents running on treadmills and human volunteers on stationary bicycles, they showed that exosome secretion by muscle was significantly elevated during exercise. Moreover, the secreted exosomes targeted to liver in an apparent integrin-dependent manner where they delivered their cargo, among which were glycolytic enzymes.

A recent study by Schaffer and colleagues examined the distribution of exosomal small nuclear

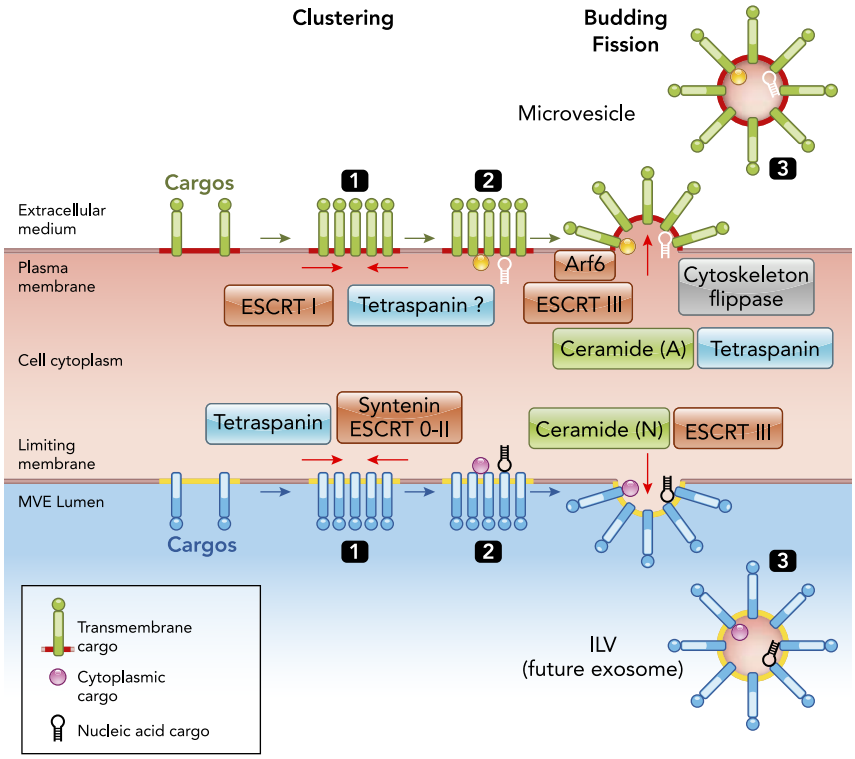

FIGURE 2. **Mechanisms involved in the biogenesis of EVs**
The formation of microvesicles and exosomes involves clustering of cargo, budding, and scission, and release into the environment. Transmembrane proteins present on both types of EVs maintain the same topology as at the plasma membrane, while cytosolic proteins and genetic material are contained within the bilayer. Some of the major mechanisms are displayed. Although they mostly appear distinct for the generation of microvesicles and exosomes, some are shared.

RNAs (snoRNAs) in a human inflammatory model and in an animal model parabiosis experiment (18). SnoRNAs are non-coding RNAs that play a role in the processing of other structural RNAs. In humans, a local exposure of LPS led to a significant increase in serum exosomes containing several snoRNAs. In the parabiosis experiment, snoRNAs from the donor animal were found both widely distributed and functional in the recipient animals.

Exosomes could be involved in generic low-level delivery of cargo to various sites in the organism, including protected sites like the brain, since exosomes are reported to cross the blood-brain barrier (7), although the binding, internalization, and transcytosis have yet to be studied. The question is whether there is a rational basis for tissue-specific exchange.

A variation of the theme of commingling can be found in exosomes called upon to alter microenvironments by enhancing the availability of substrates for energy production or signaling. In the former case (12), glucose-deprived myocardial cells secrete exosomes with glucose transporters (both Glut1 and Glut4) that are promptly delivered to nearby endothelial cells. Endothelial cells with a new allotment of Glut molecules enhance their uptake and metabolism of glucose, and release metabolic intermediates such as pyruvate. The released metabolites in turn are utilized by the cardiomyocytes for oxidative metabolism. Similarly, stem cells release exosomes enriched in a L-asparaginase-like enzyme that is specific for asparagine (20). The released exosomes are thought to operate as "independent metabolic units" enhancing the generation of aspartate (and not glutamine), which has important implications for growth.

Cell Signaling: EVs and Homeostasis

The concept of exosomes as intermediates in intercellular communication was introduced in the 90s with the findings that they activate the immune system and induce anti-tumoral immune responses in vivo making way for phase I and phase II clinical trials that are extremely promising as immunotherapy protocols (43). This has now extended to many different therapeutic applications, particularly in regenerative medicine (27). These findings introduced the concept of exosomes as a new mode of signaling in intercellular communication (FIGURE 3). The concept has now been widely confirmed in many physiological and disease models including neurodegenerative disorders such as prion disease and Alzheimer (9, 30, 45).

From the foregoing, it seems reasonable to propose that EVs and exosomes represent a broad new set of homeostatic signaling mechanisms whose actions operate hand in hand with the great physiological systems, including endocrine and paracrine, that maintain constancy in the internal environment or the milieu interieur. The elements of this new EV signaling pathway include the packaging and secretion of signals by the donor cell in the form of membrane vesicles, transport to a distant site, and, last, delivery to a recipient cell or the deposition of a product in close proximity of the target site to serve as an activator or a guidance queue. Biological fluids, such as blood plasma, saliva, CSF, intravascular fluids, etc., are replete with EVs. Upon reaching their destinations, signaling exosomes must interact with the target cell with high fidelity and either fuse directly or be subject to endocytosis. Much is known about how signaling exosomes might enter cells, but how signals are transmitted to the proper intracellular target (e.g., mitochondria or nucleus) remains an enigma.

Melanocytes, Macrophages, and Mesenchymal Stem Cells as Exemplars

EV-dependent signaling events are found aplenty in the current literature, although, for the most part, they represent snapshots of still undiscovered regulatory pathways. Certain tissues and cell types that have been exemplar in revealing EV signaling functions are melanocytes and keratinocytes in the epidermis, macrophages, and mesenchymal stem cells.

In the skin epidermis, melanocytes and keratinocytes communicate with each other by direct contacts but also indirectly by soluble factors. Keratinocytes influence melanin synthesis in lysosome-related organelles called melanosomes, which are transferred to keratinocytes. In addition to soluble compounds, keratinocytes secrete exosomes, enclosing particular miRNAs that are specifically targeted to the melanocytes to control the expression of melanosomal proteins and to, consequently, modulate pigmentation (FIGURE 3). Any deregulation in these pathways may underlay pigment disorders. Exosomes and possibly other EVs released by keratinocytes and melanocytes may control direct cell-cell contacts that influence melanosome transfer and ultimately skin homeostasis (Lo Cicero A, Raposo G, unpublished observations). Moreover, they may be involved in skin carcinoma or melanoma (31, 39). Clearly, they are important components of the overall homeostasis of the skin (23). EVs and their signaling capacities could be used in therapeutic strategies in skin-regenerative medicine by exploiting stem cells (25).

Macrophages are widely invested in short-distance EV signaling, and this is particularly true of macrophages embedded in adipose tissue (referred to as adipose tissue macrophages or ATM).

Macrophages are among the most pleiotropic of cells displaying multiple phenotypes dependent on the tissue in which they reside and the environment (e.g., cytokines) that surrounds them (13). In general, macrophages are broadly subdivided into two functional types (M1 and M2, of which there may be multiple subtypes). M1 macrophages are generally associated with an activation state induced by interferon-γ producing ROS and other metabolites. M2 macrophages are thought to be alternatively activated, expressing characteristic markers including the antigen F4-80 and the mannose receptor. Both M1 and M2 macrophages secrete exosomes that influence the insulin sensitivity of nearby adipocytes. Olefsky and colleagues (57) have shown that M1 macrophages secrete exosomes that target adipocytes by delivering miRNA-155 that suppresses expression of PPARγ that renders them insulin resistant. M2 macrophages secrete exosomes (putatively containing miR-223) that also target adipocytes, rendering them more insulin sensitive. In vivo studies following administration of the two types of exosomes and examining insulin sensitivity seem to confirm these initial findings. To add another layer of complexity, Zhao et al. (58) have studied adipose-derived stem cells (ADSC) that are present in adipose tissue. These ADSC secrete exosomes containing the activated transcription factor STAT3, among other intermediates. ADSC exosomes target the macrophage population in adipose tissue and enhance the conversion or the accumulation of M2 macrophages. Interestingly, in response to stimulation by stem-cell exosomes, M2 macrophages in adipose tissue produce factors that increase the proliferation of ADSC forming a positive feedback loop and thereby maintaining the population of adipocyte precursors. These findings present a compelling case for exosome signaling in a complex homeostatic pathway by shuttling both miRNA and protein regulatory factors between and among cells that lie at a major crossroads in energy metabolism.

As mentioned earlier, macrophage differentiation is complex, and multiple states exist at different tissue sites. In injured axons in both the peripheral and central nervous systems, a type of activated macrophage (CX3CR1-positive) secretes exosomes whose cargo includes NOX2 (ROS-generating NADPH-dependent oxidase) (17). These exosomes are internalized by damaged axons, and NOX2 is transported to the cell body where it regulates PTEN and assists in axon repair. In yet another example, a major macrophage subtype in bone is the osteoclast, which, in addition to bone resorption, also regulates osteoblasts and bone formation. Osteoclasts reportedly use exosomes to transfer a key miRNA to osteoblasts (miR-214-3p) to inhibit bone formation (28). This picture has similarities to the yin-yang of M1 and M2

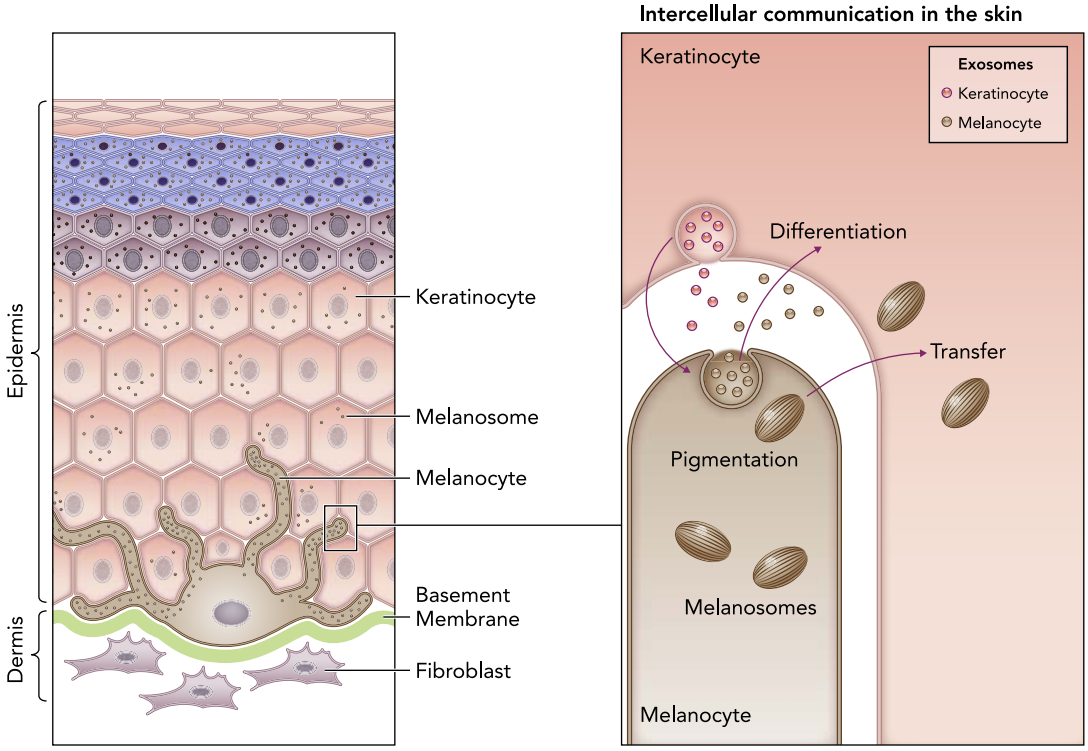

FIGURE 3. **Intercellular communication in the skin**
In the skin epidermis, melanocytes connect with keratinocytes by direct contacts and indirectly via exosomes. Exosomes, secreted by keratinocytes, modulate pigmentation. Melanocytes also secrete exosomes that are likely to be involved in keratinocyte behavior (e.g.,differentiation, melanosome transfer).

macrophages and the regulation of energy metabolism in adipose tissue.

A physiologically relevant function of EVs only recently appreciated is their role in cell guidance. Positional queues are very important for tissue organization and assembly, cell migration and motility, and the response to injury and infection (49). EVs play a central role in assisting dendritic cells in their mission to acquire and process antigens (3). Lymphatic endothelial cells secrete EVs, decorated with the ligand CX3CL1, that engage the cognate receptor CX3CR1 on nearby dendritic cells and serves as a guidance tool to enhance their surveillance, especially during inflammation. Also during the infection cycle, recent work has shown that neutrophil chemotaxis is enhanced by a novel exosome guidance mechanism. During inflammation, migrating neutrophils respond to a gradient of leukotriene B4 laid down by early arriving cells. Parent and colleagues (24, 32) have shown that neutrophils secrete exosomes from the aft position (the back of the moving cell) that harbor as cargo the enzymatic machinery to generate LTB4. The exosomal footprints so created produces a continuous generation of LTB4 that provides a chemotactic signal for newly arriving neutrophils (FIGURE 4).

Mesenchymal stem cells are key to embryogenesis and regenerative processes in tissues, and have emerged as high-visibility targets for therapy. However, they are equally interesting in the context of EV-dependent cell signaling during development and beyond. The interactions and cross talk between mesenchyme and epithelia are part of the sequence of events necessary for normal organogenesis. EVs and exosomes are emerging as significant players, although research is still at an

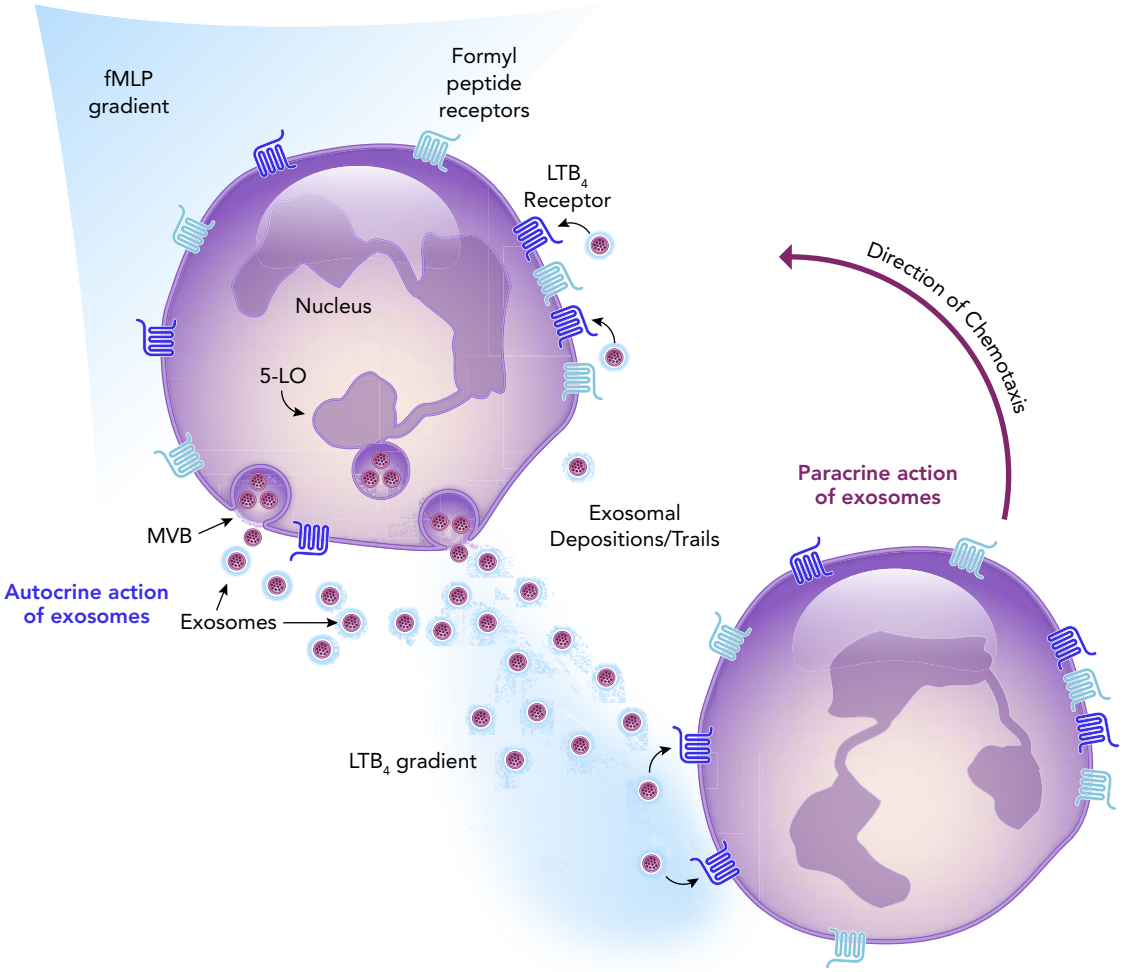

FIGURE 4. **Exosomes play a role as guidance queues in directed neutrophil movement during infection**
In this illustration, the team of Dr. C. Parent (32) shows the migration of two neutrophils moving in a fMLP gradient. The model proposes that neutrophils release exosomes containing the enzymatic machinery to synthesize and release LTB4, a chemoattractant. The released exosomes operate in an autocrine manner by sensitizing cells toward the primary chemoattractant (fMLP) and in a paracrine manner by serving as a lodestar for newly arriving cells.

early stage. Hayashi et al. (16) reported that mesenchymal cells secrete exosomes with miRNAs that regulate salivary gland development. The developing tooth organ is another experimental microcosm where EV-based interactions have been studied (22). Mesenchymal stem cell-derived exosomes interact with epithelial cells in culture and affect their developmental program and vice versa. These findings suggest that exosomes can actually replace cells in driving or enhancing parts of the developmental program and that different signals are delivered in each direction. The concept that emerges from these and other studies is that EVs are an important part of the signaling between and among cells during developmental organogenesis. For this reason, in part, mesenchymal stem cell-derived exosomes have emerged as promising treatments in many clinical applications and pathologies (42). One in particular is the use of cardiospheres or organoids for the treatment of cardiac anomalies. In one model of induced myocardial infarction, Mattapally et al. (33) showed that a patch of cardiac organoids attached to the site of infarction enhanced the recovery of cardiac function and that exosomes, secreted by the patch, were a significant part of the recovery mechanism. The latter is one of many studies, still at the early phase, but highly suggestive of a therapeutic application of EVs.

Conclusions: Cell Signaling—Two-Way Communication Integrating EVs Into Physiology

Communication in biological systems is a two-way process where the recipient or target cell responds to the signal received by generating a response—in either a negative or a positive feedback manner—to the donor. It is too early to frame EV communication in that context, but there are signs that feedback mechanisms are in play. Several examples alluded to above suggest a two-way communication pathway between adipose-derived stem cells and local macrophages in fat tissue, and between melanocytes and keratinocytes in skin may serve as examplars. Jalabert and colleagues (21) indicated that exosome-like vesicles secreted by insulin-resistant muscle regulates the proliferation of pancreatic β-cells that produce insulin. In the 19th century, Berthold, one of the founders of endocrinology, carried out the first definitive experiments that correlated the presence of testosterone with the development of secondary sex characteristics in chickens. A similar definitive experiment in EV signaling is not out of the question, and first principles are not yet formed. However, the weight of evidence that EVs will be important elements in the regulation of homeostasis and general organismal physiology and behavior is growing. ■

We are grateful to our colleagues for their many contributions in the EV field: Dr. G. van Niel (U-1266 INSERM Institute of Psychiatry and Neurosciences of Paris) for FIGURE 2, Dr. C. Delevoye (CNRS UMR144, Institut Curie) for contribution to FIGURE 3, and Dr. C. Parent and her team (University of Michigan) for the work depicted in FIGURE 4.

We thank the National Institutes of Health, Clarins, l'Oréal, Institut Curie, and Centre National de la Recherche Scientifique for support.

No conflicts of interest, financial or otherwise, are declared by the author(s).

P.S. and G.R. prepared figures; P.S. and G.R. drafted manuscript; P.S. and G.R. edited and revised manuscript; P.S. and G.R. approved final version of manuscript.

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