Feature Review

Extracellular Vesicles: Novel Mediators of Cell Communication In Metabolic Disease

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Metabolic homeostasis emerges from the complex, multidirectional crosstalk between key metabolic tissues including adipose tissue, liver, and skeletal muscle. This crosstalk, traditionally mediated by hormones and metabolites, becomes dysregulated in human diseases such as obesity and diabetes. Extracellular vesicles (EVs; including exosomes) are circulating, cell-derived nanoparticles containing proteins and nucleic acids that interact with and modify local and distant cellular targets. Accumulating evidence, reviewed herein, supports a role for extracellular vesicles in obesity-associated metabolic disturbance, particularly the local and systemic inflammation characteristic of adipose and hepatic stress. As the practical and conceptual challenges facing the field are tackled, this emerging and versatile mode of intercellular communication may afford valuable insights and therapeutic opportunities in combatting these major threats to modern human health.

EVs: an Alternative Mode of Cellular Communication

The past decade has witnessed growing interest in the role of EVs, particularly exosomes (see Glossary), in physiology and disease. First described in the 1980s, exosomes are circulating, membrane-bound nanovesicles secreted from the endosomal pathway of cells [1–3]. Originally considered a discarding mechanism for membrane proteins, exosomes are gaining recognition as essential conveyers of cellular information, which, by virtue of their bioactive cargo, modify the activity or properties of specific target cells [4–7]. A wide range of stimulatory or inhibitory functional outcomes has been induced by exosomes, including cell proliferation, apoptosis, cytokine production, immune modulation, and metastasis [8]. They thus add an alternative mode of paracrine and endocrine communication to the conventional strategies of direct cell–cell contact and soluble, receptor-targeted hormones and cytokines.

Exosomes, and to a growing extent other EVs, are proposed to play an important albeit variably described role not only in human physiology and homeostasis, but also in the pathogenesis of major human diseases. In addition, as discussed below, they offer a promising source of disease-associated biomarkers and may eventually be used as cell-free delivery vectors for targeted biological therapies [9,10]. Research examining the role of EVs in human metabolism and metabolic disease is in its infancy. Emerging work, however, indicates that their continued interrogation may afford important insights and potential therapeutic strategies. Here, after providing an introduction to EVs and reviewing existing research in this field, we identify potential

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future research themes and highlight the challenges and outstanding questions that may be faced moving forward.

Assembly and Release of EVs
The umbrella term ‘extracellular vesicle’ includes exosomes, microvesicles (also known as shedding vesicles, ectosomes, and nanoparticles) and apoptotic bodies, traditionally distinguished by their size and biogenesis. Exosomes are complex 20–100 nm vesicles formed by the inward budding of endosomal membranes to form large multivesicular bodies (MVBs; Figure 1). These vesicles are released extracellularly when MVBs fuse with the plasma membrane. By contrast, larger microvesicles (100 nm–1 μm) and apoptotic bodies (1–5 μm) result from the direct outward budding and fission of the plasma membrane. EVs carry an assorted bioactive cargo of soluble and membrane-bound protein, lipids, metabolites, DNA, and RNA (miRNA, miRNAs, and other small regulatory RNA), contained within a protective lipid bilayer. These cargos represent a nonidentical subset of the contents of the parent cell of origin [4]. In recent years, EVs originating from multiple cell types have been subject to transcriptomic, proteomic, and lipidomic analyses; many of which are freely available on public repositories, leading to an increasingly comprehensive view of their nucleic acid, protein, and lipid contents [11–13]. EVs contain a heterogeneous array of cytosolic proteins derived from the parent cell. They are particularly enriched in proteins such as integrins, MHC molecules, and cytoskeletal proteins, and also express a selection of relatively vesicle-specific proteins often used as EV markers (such as the tetraspanins TSG10 or CD63 found in exosomes) [14,15]. Such markers are used in immunoaffinity-based EV isolation techniques or for assessing the purity of EV isolates after other separation techniques, most of which (including ultracentrifugation, density gradient separation, and polymer-based precipitation methods) rely on vesicle size or buoyancy [16] (Box 1). The lack of unambiguous physical properties or unique EV markers, however, and the use of variable isolation protocols and parameters, limits the generalizability and comparability of many research studies in the field.

The molecular machinery regulating the assembly and loading of EVs has been described in detail [17]. The traditional model for exosome secretion requires the sequential assembly of four ESCRT (endosomal sorting complex required for transport) complexes on the endosomal membrane. These complexes, which are conserved across the eukaryotic lineage, are an ancient system for membrane remodelling, budding and fission to form free vesicles [18,19]. Exosome formation is triggered by the accumulation of tetraspanins (CD9 and CD63) in the endosomal membrane [20]. Next, sequential recruitment of ESCRT 0, I, and II drives membrane curvature and budding, as well as recruitment of ubiquitinated proteins for sorting into the vesicle. Protein programmed cell death 6 interacting protein (PDCD6IP or ALIX), which interacts with the tetraspanin TSG101 in ESCRT I, in turn recruits ESCRT III, which is ultimately responsible for membrane constriction and fission [19,20].

ESCR- and protein-independent pathways of exosome formation, which may operate in parallel with ESCRT-dependent mechanisms, have also been described [21,22]. According to one model, exosome formation is triggered by the budding of ceramide-rich intraluminal vesicles from sphingolipid rafts in endosomal membranes, in a process dependent on sphingomyelinase-2 [23,24].

Following their assembly, release of exosomes from the cell occurs via endosomal fusion with the plasma membrane, under the regulation of several Rab-GTPases (including RAB11, RAB35, RAB27A, and RAB27B) [25]. The biogenesis and release of microvesicles and apoptotic bodies follows a distinct pathway; key events include redistribution of phospholipids, repositioning of phosphatidyserine to the outer leaflet of the plasma membrane, and contraction of the actin–myosin machinery by the activation of myosin light chain kinase [26,27].
EVs and Target Cell Interactions

A large body of evidence indicates that, once released, an exosome can interact with a recipient cell, deliver its cargo to the cytosol of the recipient cell, and modulate its phenotype. Not only do exosomes transfer functional protein and translatable mRNA [4,5,28], but their miRNA cargo can silence recipient target genes [7], as can exosome-mediated delivery of exogenous siRNAs [29,30]. The induction of luciferase activity in luciferase-transfected dendritic cells treated with luciferin-loaded exosomes provides further support for the cytosolic delivery of EV contents [7]. In addition, use of fluorescent EV membrane dyes (e.g., PHK67) or GFP-tagged EV proteins (e.g., GFP-CD63) with confocal microscopy has allowed direct EV visualisation, confirming their functional delivery.

**Glossary**

**Adipose tissue macrophages (ATMs):** Differentiated macrophages resident in adipose tissue that express a characteristic profile of pro- or anti-inflammatory genes, referred to as M1 or M2 polarisation, respectively.

**Apoptotic bodies:** 1–5 μm membrane-bound fragments formed by the outward budding of the plasma membrane of apoptotic cells. They are phagocytosed by neighbouring cells and degraded by phagolysosomes.

**Caveolae:** Specialised plasma membrane pits, located within lipid rafts, enriched in cholesteryl, sphingolipids, and caveolin proteins. They have signalling roles in addition to their key role in clathrin-independent endocytosis.

**Clathrin-mediated endocytosis:** A dominant form of endocytosis mediated by small (100 nm) membrane pits coated with the cytosolic protein clathrin. Vesicle formation is triggered by the engagement of extracellular ligands with specific membrane receptors.

**Endocytosis:** An active process by which cells internalise material in the extracellular fluid by invagination and pinching of the plasma membrane from internal vesicles (endosomes).

**Exosomes:** 20–100 nm membrane-bound vesicles containing protein, RNA, and lipids, secreted from cells via the endosomal pathway.

**Integrins:** Superfamily of heterodimeric transmembrane cell adhesion receptors that link the actin cytoskeleton of cells to their surrounding extracellular matrix, and also bind cell surface and soluble ligands.

**Lipid rafts:** Specialised, cholesterol-enriched membrane microdomains consisting of glycosphingolipids and protein receptors.

**Macropinocytosis:** Nonspecific endocytosis of extracellular fluid, solutes, and small particles.

**Major histocompatibility complex (MHC):** Cell surface proteins that bind peptide fragments derived from pathogens and display them on the cell surface for recognition by T cells. miRNA: Small (19–22 nucleotides), noncoding RNAs that form short hairpin structures and act post-transcriptionally to modulate gene expression.
Box 1. Tools and Techniques in EV Research

Nanosized EVs can be isolated from cell supernatants and other biological fluids including blood, urine, and saliva [16]. Isolation methods, summarised below, rely on size, buoyancy or surface marker expression [115,116]. They are typically followed by a confirmation step involving direct visualisation by electron microscopy, fluorescence staining followed by confocal microscopy, size analysis by nanotrackng particle analysis, or assessment of surface marker expression using western blotting or flow cytometry.

Differential ultracentrifugation is the most common approach to EV isolation, and often described as the gold standard. After an initial centrifugal force spin (1000–3000 g) to remove cells and cell debris, large microvesicles and apoptotic bodies are pelleted by centrifugation at 10 000 g. Exosomes and smaller microvesicles are subsequently separated by ultracentrifugation at 100 000 g for ~1 h. This technique does not separate small vesicles from large protein oligomers, RNA–protein complexes or viruses, and inconsistencies in protocols may lead to isolation of different EV subsets. The technique also suffers from low yield (5–25% efficiency).

Sucrose gradient centrifugation separates EVs from similarly sized membrane-free protein and macromolecular aggregates based on their low buoyant density, using a noncontinuous sucrose density gradient (or equivalent solution). This approach affords superior exclusion of nonvesicular contaminants but discriminates less well between exosomes and microvesicles. Sucrose density centrifugation may be used as an extra step after ultracentrifugation to provide a cleaner exosome population.

Immunoaffinity techniques make use of differential protein marker expression on vesicle subclasses, such as CD63 or TSG101 on exosomes, phosphatidyl serine on microvesicles, and annexin-V on apoptotic bodies. Vesicles are usually separated by affinity chromatography with antibodies fused to magnetic beads. With prudent marker selection this technique offers promising selectivity, however currently suffers from lack of scalability.

Polymer-based precipitation methods in commercially available kits (e.g., ExoQuick) are advertised for their speed and ease of use. They are growing in popularity but specificity is low and cell of origin cannot be discriminated. This technique is most useful for rapid isolation or concentration of EVs in conjunction with other separation techniques.

Microfiltration technologies use nanomembrane filters along with low protein-binding membranes to enrich exosomes and microvesicles from small sample volumes. They are sometimes used within ultrafiltration spin columns using low centrifugal force. This emerging technique mainly suffers from EV trapping within the nano- or micropores of the membrane.

Microfluidic devices operate by means of specific binding of EVs to antibody-coated surfaces. The biofluid of interest is loaded on a pump that slowly pushes fluid through the chip, allowing one-step targeted isolation of EVs.

rapid incorporation into by target cells, and allowing their dynamic localisation and spatiotemporal characteristics to be characterised [7,31–34]. However, given the limit of resolution of light microscopy, the ability to detect small EVs and EV clusters is challenging, and there is concern that dyes and tags may interfere with normal EV function and cycling.

Although exosomes have attracted the most attention for their functional effects on target cells, it is recognised that microvesicles and even apoptotic bodies may have largely analogous physiological and pathological roles [35]. Apoptotic bodies have been investigated particularly in the context of communication between cancer cells, with evidence that they too can carry RNA to neighbouring cells [36]. It has been reported, for example, that fibroblasts transfected with and expressing oncogenes released apoptotic bodies containing the same oncogenes. The apoptotic bodies were taken up by target cells, which then exhibited tumourigenic properties, including loss of contact inhibition [37].

The mechanism by which EVs and their bioactive cargo are delivered into, and influence a recipient cell has been subject to debate. One possibility is that they become incorporated as part of the background, continuous recycling of the plasma membrane. Various observations argue against this, including the rapidity of EV uptake, the disruptive effect of cooling, and the requirement for an intact cytoskeleton [8]. Indeed, the long list of proteins that appear to participate in EV uptake (including tetraspanins, immunoglobulins, and integrins), argue instead...
for an active uptake process involving specialised intracellular transport machinery [38]. Various endocytic pathways are now implicated in EV uptake, including traditional clathrin-mediated endocytosis (CME), clathrin-independent endocytosis (e.g., caveolin-dependent endocytosis associated with caveolae within lipid rafts), phagocytosis, and macropinocytosis (Figure 1) [7,38]. The dominant pathway operating at any given time likely depends on expression of surface proteins on the recipient cells, its internal signalling milieu, and the protein complement on the EV. Furthermore, the extent to which some of these pathways represent EV clearance mechanisms, rather than drivers of a cellular response, remains unclear. Indeed, although responses elicited by RNA rely on EV internalisation, the phenotype of a target cell can be modulated by direct protein–protein interactions between ligands and cell receptors without the need for internalisation [39,40]. The specificity of the protein–protein interactions is presumably what targets EVs to certain cell types, although the evidence for specificity of EV uptake is variable, and the mechanisms regulating it largely unknown [6,29,32].

The carriage of RNA within EVs allows them to circulate within a protected microenvironment and, upon delivery into a recipient cell, act in a combinatorial manner to modulate the expression of specific target genes; this involves both de novo translation of EV mRNAs and post-transcriptional regulation of target mRNAs by EV miRNAs [41]. As such, a large body of research has focused on understanding the mechanisms by which specific RNAs are sorted together into developing exosomes (Box 2). After endocytic uptake by a target cell, the mechanisms by which RNA and other EV cargoes escape the intrinsically degradative endosomal pathway and access the cytosol of the recipient cells is uncertain. There is evidence, however, that exosomal peptides and lipids do ultimately end up on the recipient cell surface, indicating that such an exit mechanism exists [34,42,43]. This may involve EV fusion with the endosomal membrane, however, this issue awaits further investigation with direct imaging of exosome fusion and nucleic acid labelling [33].

**EVs in Human Metabolic Disease**

EVs are implicated in a growing range of human diseases, including the spectrum of conditions associated with obesity and the metabolic syndrome. Owing to their ubiquitous presence and stability in various human biofluids, and because their contents reflect the characteristics of the parent cell, circulating EVs (particularly exosomes) and their constituent miRNAs have been explored as a readily accessible source of novel diagnostic and prognostic biomarkers in metabolic disease. Flow cytometric analyses have revealed a quantitative increase in circulating EVs in obesity and associated disease states including insulin resistance, diabetes, and nonalcoholic fatty liver disease (NAFLD) [44–50]. Qualitative differences in exosome protein (including adipokines) and RNA content have been described in obese rodents and humans [51], with the lipid droplet protein perilipin A recently highlighted as a potential biomarker of adipocyte EVs and adipose tissue stress [52]. In a study of the EV miRNA profiles of 219 patients, distinct EV miRNA profiles were observed for the metabolic syndrome, type 2 diabetes mellitus (T2DM), hypercholesterolaemia, and hypertension [53]. Circulating platelet-derived EVs from patients with T2DM had higher levels of CD42 and CD41a positivity compared to healthy controls, and higher CD14 positivity in monocyte-derived EVs [9]. In particular, the latter were particularly increased in patients with diabetic nephropathy. A large body of work has focussed on the identification of urinary exosomal biomarkers for diabetic nephropathy, with dipeptidyl peptidase IV (DPPIV), Wilm’s Tumour-1 (WT-1) and alpha-1-microglobulin/bikunin precursor (AMBP) among the putative exosomal proteins [54–56].

The need for biomarkers of NAFLD is particularly pressing, since diagnosis and monitoring of this condition relies on either invasive biopsy or nonspecific surrogate markers of liver inflammation and synthetic function. Numerous putative EV-associated miRNA biomarkers have been reported, including liver-derived mir-122 and mir-192, endothelium-derived CD144 and
adipose-derived adiponectin, adipose-derived adiponectin, interleukin (IL)-6, monocyte chemotactic protein 1 (MCP1) and macrophage migration inhibitory factor (MIF); their correlation with histological NAFLD grade has not been verified [50]. However, a detailed transcriptomic study of liver disease-associated EVs identified a panel of 12 miRNAs whose expression profile, assessed by principle component analysis, allowed NAFLD to be distinguished from viral hepatitis and normal liver [57]. Furthermore, in a study of circulating EVs in patients with NAFLD, an EV profile was identified characterised by elevated CD14 (a monocyte/macrophage marker) and Vx24/Vx11 (an invariant natural killer cell marker), both of which correlated with alanine aminotransferase (ALT) and, importantly, histological grade of NAFLD, and both of which are implicated as major drivers of hepatic inflammation and fibrosis [58]. Although promising, the clinical utility of these and other EV biomarkers has yet to be verified.

**Exosomes and Obesity-Associated Adipose Dysfunction**

Dysfunction of white adipose tissue is considered a central driver of obesity-associated metabolic disease [59]. Exosomes and microvesicles are secreted by primary adipocyte cultures, adipocyte cell lines (e.g., 3T3-L1) and adipose tissue explants in vitro [60–63]. Analysis of the RNA and protein contents of adipose EVs reveals expression of adipocyte-specific and adipocyte-dominant proteins, such as fatty acid binding protein 4 (FABP4), also adipocyte Protein 2 or aP2) and adiponectin [64–66]. Expression of some adipocyte markers, such as microvesicle-associated adiponectin, reportedly change over the course of adipogenic differentiation [65]. Others, such as
exosomal fatty acid synthase (FAS) or FABP4 are regulated by physiological stimuli (including fatty acids and hypoxia [67,68]) or altered in obesity [66]. Adipose-specific proteins (such as adiponectin) have also been used to select specifically for, or confirm the presence of, adipose-derived EVs from a pool of EVs in whole blood or a mixed cell culture in vitro [51].

A small collection of studies have emerged in recent years that investigate the potential role of adipose-derived EVs (or exosomes specifically) in the metabolic consequences of obesity and adipose tissue dysfunction [51,69,70] (Figure 2). Several studies have reported that EVs collected from obese adipose tissue, when applied to a target cell population in vitro, induce changes consistent with the obese phenotype. In the most comprehensive study to date, exosomes collected from ex vivo adipose tissue explants of obese ob/ob mice were fluorescently labelled then injected intravenously into wildtype mice on a high-fat diet (HFD). Subsequent fluorescence-activated cell sorting (FACS) analysis of single cell suspensions of several tissues revealed that ~80% of fluorescent exosomes were taken up by peripheral blood monocytes [69] (Figure 2 and Box 2). These monocytes showed a greater degree of activation, and secreted more pro-inflammatory IL-6 and TNFα, compared to mice injected with exosomes from wildtype adipose tissue. Adipose exosomes from ob/ob mice also caused a greater degree of macrophage activation when applied to bone marrow-derived macrophages (BMDMs) in vitro. When injected in vivo, these macrophages showed increased homing to adipose tissue and liver [69]. Furthermore, conditioned medium from BMDMs preincubated with ob/ob adipose exosomes from obese mice impaired insulin-stimulated AKT phosphorylation and glucose transport in myocytes in vitro [69]. This was not observed when BMDMs were incubated with thymus exosomes. Most importantly, adipose-derived exosomes from ob/ob mice were associated with systemic insulin resistance and cytokine activation when delivered intravenously into lean, wildtype mice.

In a comparable study in humans, adipose EVs isolated from explants of human white adipose cultured ex vivo, or mature primary adipocytes differentiated in vitro, caused primary monocytes to adopt properties characteristic of adipose tissue macrophages (ADMs), as defined by their profile of pro- and anti-inflammatory gene expression and cytokine release [49]. The supernatant from these ADM-like macrophages impaired insulin signalling when applied to cultured adipocytes [49]. In a subsequent study, it was demonstrated that activated macrophages themselves secrete EVs which, when applied to human primary mature adipocytes, impaired insulin signalling [71] (Figure 2B).

Collectively, these data support a hypothetical model in which stressed adipocytes (in the context of obesity) alter their EV secretion in order to communicate this cell stress to other tissues. These EVs, some of which are taken up by immune cells, result in activation of proinflammatory macrophages that drive local and systemic inflammation and insulin resistance (Figure 2). While appealing, this model required further testing in vivo, and the molecular cargo within adipose EVs responsible for this effect is unidentified. Furthermore, while the association between systemic inflammation and IR is well established, a causal role for inflammation in humans is unproven. A subsequent study in which human adipose EVs were directly applied to hepatocytes or myocytes in vitro failed to demonstrate a convincing effect on either insulin-stimulated AKT phosphorylation in hepatocytes or myocytes, or gluconeogenic gene expression in hepatocytes. This was possibly because of donor heterogeneity, although it could reflect omission of EV-activated immune cells [70]. Caveats notwithstanding, these studies indicate that ongoing research into the systemic effects of adipose-derived exosomes may yield important insights into specific channels of systemic communication between obese adipocytes, immune cells and other metabolic tissues (Figure 2).

The role of adipocyte EVs has also been investigated at the level of local cell–cell communication within adipose tissue. In an elegant series of studies, the application of lipogenic stimuli
Induced the release of CD73-containing EVs from primary rat adipocytes in culture [60]. Upon incubation with EVs, recipient adipocytes took up CD73, which was translocated to lipid droplets, with subsequent upregulation of lipid esterification [72]. Notably, large adipocytes were more efficient at releasing CD73+ EVs, but less efficient at translocating CD73 to...
lipid droplets. Depletion of CD73-containing EVs from the culture medium of adipocytes abrogated the proestrogenic effects of lipogenic stimuli [73]. These EVs contained, in a dose-dependent manner, transcripts and miRNAs involved in upregulation of lipogenesis (e.g., diacylglycerol acyltransferase-2) and lipid droplet assembly (e.g., caveolin-1 and perilipin-A) and, when applied to adipocytes in culture, had a greater effect on small than on large adipocytes [73]. Collectively, these studies raise the possibility that the nutritional and lipid-filled status of adipocytes might be communicated to their neighbours by the regulated release of EVs (Figure 2A and Box 1). Importantly, the regulation (and dysregulation) of adipocyte size and lipid droplet assembly is a likely key determinant of adipose and metabolic health [59]. A detailed examination of EV exchange between neighbouring adipocytes may help contribute to our understanding of how hyperplasia and hypertrophy is regulated within calorically challenged adipose tissue [74].

Exosomes and NAFLD
The liver is a producer of and target for systemic EVs, and EV-mediated paracrine communication within the liver has been implicated in a range of local processes including tumour growth, cell migration, viral infection, and hepatocyte regeneration [75–78]. Quantification by flow cytometry or nanotrack particle analysis has demonstrated a time-dependent increase in circulating EVs in experimental models of NASH [48,79]. The difficulty in identifying liver-derived EVs within the circulating pool, however, has complicated efforts to understand their systemic physiological and pathological role, and has demanded a more ‘tissue-centric’ approach to research. Similar to adipose EVs, hepatocyte EV release is responsive to metabolic signals: treatment of primary mouse/human hepatocytes or cultured hepatoma cells (Huh7 or HepG2) with palmitate, which acts as lipotoxic signal, increased EV release into culture medium [48,79]. Furthermore, when EVs (exosomes and microvesicles) were harvested from primary hepatocytes in a diet-induced mouse model of NASH, then applied to BMDMs in vitro, the macrophages showed a greater degree of activation, as assessed by secretion of proinflammatory IL-1β and IL-6 [79] (Figure 2D). Treatment of these mice with the Rho-associated coiled-coil containing protein kinase 1 (ROCK1) inhibitor fasudil, which purportedly blocks plasma membrane blebbing and microvesicle release [80], decreased lipotoxicity-stimulated EV release in vitro, decreased both total and liver-specific circulating EV levels, decreased diet-induced elevation of ALT, reduced the degree of macrophage-associated hepatic inflammation and reduced fibrosis [79]. Collectively, these data suggest that hepatic EVs, and microvesicles in particular, may play a key role in local macrophage activation, and subsequent inflammation and fibrosis, in response to lipotoxic signals reaching the liver. This is comparable to the proposed role of adipose EVs in local macrophage activation [69], suggesting that EVs may play a general role in immunological activation in the context of metabolic stress; more research is required to test this hypothesis.

The extent and relevance of EV-mediated communication between liver and other metabolically active tissues remains unclear. A small study in which adipose-derived EVs harvested from visceral adipose tissue of lean and obese human individuals were fluorescently labelled and applied to hepatocytes in culture failed to produce a convincing, consistent effect on hepatocyte gene expression [81]. Furthermore, little is currently known about the potential role in obesity and metabolic disease of EVs secreted by other key metabolic tissues.

EV Activity in Skeletal Muscle and Pancreas
EVs have been isolated from a range of pancreatic cell preparations, including insulinoma cell lines and isolated human islets [82,83,125]. A range of stimuli effect quantitative and qualitative changes in EV secretion from islets, including glucose, calcium and inflammatory cytokines [84,125]. Exosomes released from cytokine-treated pancreatic β cells induced apoptosis when applied to naïve islet cells; this was prevented by disrupting Argonaute ( Ago)-2 in the RNA-induced silencing complex (RISC), required for miRNA-mediated gene targeting, thereby
suggesting a miRNA-mediated effect [83]. Given the direct apoptotic effect of high-dose cytokines on β cells, this implies pancreatic EVs including exosomes might carry distress signals to neighbouring cells. These observations raise the possibility that pathological EV secretion may contribute to the development or progression of β cell dysfunction in inflammatory states such as obesity and diabetes (Figure 2E).

Skeletal muscle is the major site of postprandial insulin-stimulated glucose disposal, and obesity-associated resistance to the actions of insulin by myocytes is a major contributor to systemic insulin resistance. Similar to adipocytes, hepatocytes, and pancreatic islets, skeletal myocytes release and take up EVs [85–87]. Skeletal muscle of mice fed a high fat diet secreted more exosomes than those fed a standard chow diet [88]. When applied to myoblasts in vitro, however, EVs from skeletal muscle of HFD mice did not convincingly affect insulin-stimulated AKT phosphorylation (Figure 2G). However, when EVs from palmitate-treated C2C12 myotubes (suggested to represent an in vitro model of HFD skeletal muscle) were applied to myoblasts, they increased myoblast proliferation, increased the expression of genes involved in the cell cycle, reduced those involved in muscle differentiation, and interfered with insulin-stimulated AKT phosphorylation [88]. Recently, the same group has demonstrated that fluorescently tagged EVs from skeletal muscle of HFD mice were detectable within the pancreatic tissue of naïve mice within 24 h of intravenous administration [89]. When applied to islets in vitro, these exosomes stimulated islet cell proliferation and modulated gene expression. One identified mechanism was the EV-mediated transfer of miR-16, which upregulated the islet protein patched homolog 1 (Ptc1), known to play a role in pancreatic development [89]. These data illustrate how exosomes may mediate a link between peripheral insulin resistance and pancreatic dysregulation.

New Opportunities for EVs in Metabolic Research
Future research into the metabolic roles of EVs is likely to focus on the fundamental biology and regulation of EV assembly, release, and uptake, as well as the extent to which they carry information between metabolically relevant tissues in the obese state. In addition, we suggest that emerging concepts from other branches of EV research may be relevant to the study of metabolic disease. We review four of these concepts below.

Novel Paradigms in Cell Signalling
Exosomes offer an alternative mode of communication between neighbouring and distant cells, differing from conventional mechanisms both in its (largely unexplored) temporal and spatial properties, and in its unique potential to group together multiple signals. The extent to which exosomes, microvesicles, and even apoptotic bodies interact with and modulate intracellular signalling pathways within target cells is mysterious [90]. On one level, EVs carrying proteins and lipids within their cargo, including cytokine and growth factors, can activate downstream canonical signalling pathways in target cells. Examples include activation of signal transducer and activator of transduction (STAT) signalling by exosomal interferon-γ, or Notch signalling by exosomal lipids [91,92]. In addition, more complex mechanisms are emerging. An analysis of exosomes from retinal pigment epithelial cells subject to oxidative stress has revealed >40 active phosphoproteins, including phosphoinositide-dependent kinase 1 (PDK1), mammalian target of rapamycin (mTOR) and AKT [93]. More generally, the varied protein and RNA (including miRNA) contents of EVs raises the possibility that they may act in nonlinear ways at multiple stages in a single signalling pathway or, indeed, on more than one pathway [94,95]. They can also exert indirect actions by stimulating release from target cells of signalling peptides or receptor ligands, or by mediating the intracellular transfer of lipid-insoluble signalling species [96–98]. The impact of each of these options depends critically on the precise composition of the individual EVs in question. Future efforts to investigate the role of exosomes in insulin resistance and impaired insulin signalling need to be mindful of these possibilities.
EV Mediators of Tissue Homeostasis
A second function for which EVs are gaining recognition, which may be applicable to metabolic disease, is in maintenance of tissue homeostasis. Rat insulinoma (INS-1) cells rendered apoptotic by an inducible inactivating mutation in the transcription factor hepatocyte nuclear factor 1x released EVs that stimulated the proliferation of naïve INS-1 cells; an effect that was blocked when cell supernatant was filtered to remove EVs [99]. Given the continual cell death and renewal taking place within the pancreas, this study raises the possibility that EVs play an important paracrine role in regulating cell number and homeostasis. An equivalent paradigm has been posited for muscle development, based on the exchange of exosomes between myoblasts and myotubes [85]. This is particularly relevant to the study of obesity given the importance attributed to the regulation of adipocyte numbers as a determinant of adipose tissue health [100,101].

Microenvironmental Modulators – Local and Distant
Research into the role of EVs (particularly exosomes) in cancer has provided potentially transferrable examples of local and distant EV-mediated communication between different cell types [102]. First, EVs are implicated in stromal activation; the process by which cancer cells communicate in a bidirectional manner with, and activate, stromal fibroblasts. For example, exosomal transforming growth factor β secreted by prostate cancer cells triggers fibroblasts to differentiate into a myofibroblast phenotype resembling those present in the stroma of cancerous prostate tissue [103]. This was abolished by eliminating exosomes from the cancer secretome by transfection with an anti-Rab27a ribozyme transgene; stroma-associated tumour growth in vivo was similarly diminished. Conversely, exosomes released by fibroblasts reportedly promote proliferative activity, migration, and invasion of breast cancer cells [104]. Obesity involves a comparable remodelling of adipose tissue; it is therefore plausible that the crosstalk between adipocytes and supporting cells is EV mediated. Indeed, adipose stromal cell exosome have already been studied in the context of angiogenesis, immunomodulation, and tumour development [105].

In addition to modulating the local environment, a series of reports has demonstrated the extent to which tumour cells exert tissue-specific effects on distant sites via secreted exosomes [106,107]. Recently, a central role for tumour exosomes in determining cancer metastasis organotropism has been demonstrated [6]. In this study, exosomes collected from different cancer cell lines, containing different integrin signatures, fused preferentially with resident cells at their target destination and initiated the process of metastatic niche formation. Critically, treatment with exosomes from lung-tropic cancer cells redirected the metastasis of traditionally bone-tropic cancers towards the lung. Furthermore, modification of specific integrin signatures, which predicted organotropism, allowed this tropism to be disrupted [6]. These findings provide compelling evidence that some exosomes may be specifically tagged towards destination cell types. These insights support the possibility that key metabolic tissues, such as adipose tissue, liver, and skeletal muscle, influence each other in a targeted manner via EV exchange (Figure 2).

Therapeutic Horizons
EVs, and particularly exosomes, have attracted considerable interest for their potential use as efficient, targeted and nonimmunogenic delivery systems for biological molecules or pharmacotherapies. Various studies have explored the use of stem-cell-derived exosomes in tissue regeneration and therapy in disease models, including myocardial infarction, inflammatory CNS disorders, and stroke [108]. Moreover, recent years have witnessed the use of engineered or synthetic exosomes as delivery tools for proteins and miRNAs. In one of the earliest examples, exosomes containing siRNAs were synthesised using self-derived dendritic cells, with neuronal targeting achieved by engineering the dendritic cells to express the exosomal membrane protein Lamp2b fused to a neuron-specific RGV peptide. These exosomes were then loaded with exogenous siRNA (targeting GAPDH) by simple electroporation. Intravenous injection of these
exosomes resulted in brain-specific GAPDH knockdown [29]. A similar technique used deliver to the brain siRNAs again the µ opioid receptor prevented relapse in a mouse model of morphine addiction [109]. Although these represent early studies, the potential for using EVs as customisable delivery vehicles has appealing potential.

Challenges in Exosome Research

Although research into exosomes and metabolism is gaining ground, the past decade has highlighted three underlying challenges facing scientists in this field. First, the selective isolation of distinct EV subtypes poses ongoing difficulties, in part because of widespread inconsistencies in nomenclature and isolation protocols, and the risk of co-isolation of non-exosomal EV and protein complexes. Efforts to standardise nomenclature and protocols are ongoing [110].

Second, exploration of the role of EVs in vivo are complicated by difficulties in labelling endogenous EVs, tracing their movement, and identifying target cells without interfering with their function. Recent progress has been made in imaging single exosomes in vivo. One promising technique uses the Cre-loxP system to fluorescently mark Cre-reporter cells that take up EVs released by Cre recombinase-expressing cells in vitro and in vivo [111,112]. This and other techniques will allow the movement of individual and populations of exosomes to be studied more precisely in a physiologically relevant context. Assessment of their function in vivo, however, remains challenging and will require development of techniques that allow specific EV subpopulations (such as those from adipose tissue) to be targeted and disrupted, or specific proteins, RNAs, or miRNAs to be deleted from EVs, for example, by interfering with their incorporation into developing exosomes (Box 3).

Third, partly because of a lingering reputation as inactive and physiologically irrelevant shedding vesicles, the functional significance of EVs remains contested. Although their modulatory action on target cells has been demonstrated repeatedly, their physiological relevance is difficult to

Box 3. Dissecting Mechanisms of EV Uptake

Multiple mechanisms are proposed to contribute to EV uptake, including clathrin-mediated endocytosis (CME), caveolin-dependent endocytosis, phagocytosis, and macropinocytosis [30]. A range of experimental techniques, including use of antibodies to block ligand/receptor interactions, chemical inhibitors, and RNAi technologies, have sought to dissect the underlying molecular events. Inhibition of CME by the drug chlorpromazine, for example, which prevents the formation of clathrin-coated pits at the plasma membrane, decreases EV uptake by ovarian cancer cells [31]. Inhibition of dynamin-2, a key regulator of membrane curvature and fission in CME, has a similar effect, as does expression of a dominant negative mutant of epidermal growth factor receptor pathway substrate clone 17; an integral component of the clathrin pit [31,123]. A more recent study, however, failed to detect exosome colocalisation with either transferrin or acetylated low-density lipoprotein; both classical ligands of conventional CME. Furthermore, 80% knockdown of clathrin itself had no apparent impact on exosome uptake, nor did blockade of Na/H exchange by amiloride, which disrupts macropinocytosis [124]. Twenty percent of exosomes colocalised, however, with the lipid raft marker cholera toxin B, and membrane cholesterol depletion by MβCD or simvastatin disrupted exosome uptake [32].

Collectively, these observations point towards a nonclassical, clathrin-independent endocytic process; an established example of which is dependent on caveolin-1 (CAV1). Knockout of CAV1 enhances exosome uptake in mouse embryonic fibroblasts (without altering clathrin-mediated endocytosis), suggesting that caveolin plays a negative regulatory role in this process. In line with this hypothesis, rescue with Cav1–YFP in knockdown cells, and stable overexpression of CAV1 in HeLa cells both significantly reduce exosome uptake. Further studies have identified a signalling role for Cav1 in which it negatively regulates exosome-activated extracellular signal-regulated kinase (ERK1/2) signalling (and downstream heat shock protein (HSP)27), while ERK1/2 inhibition (by U0126) reverses the enhanced exosome uptake observed in Cav1 knockout fibroblasts [32]. This has led to an alternative model in which endocytosis of exosomes occurs at lipid rafts, associated with and dependent on ERK1/2 and HSP27 phosphorylation, with Cav1 acting as a negative regulator at the plasma membrane (although a direct interaction between Cav1 and Erk1/2 has not been identified). This work highlights the existence of a multiplicity of parallel mechanisms that permit EV uptake and access to the cytosol; the dominant mechanism is likely to be situation-specific.
prove. This is complicated further by intrinsic challenges in studying miRNAs; potentially the most important of EV constituents yet contained at potentially low copy numbers [113,114]. As our understanding of exosome assembly and targeting becomes more advanced, the armory of tools available to manipulate exosomes and interrogate such controversies will expand.

Concluding Remarks and Future Perspectives
Exosomes, microvesicles, and even apoptotic bodies offer an alternative means of communication between cells, based on the combination of proteins, lipids, and nucleic acids they deliver. Recent years have witnessed advances in our understanding of the molecular mechanisms governing their assembly, release, and uptake. Nevertheless, the central question as to whether EVs play an active role in physiology and disease, or whether they are merely passive bystanders, is unresolved; this is in part caused by practical challenges in their isolation and characterisation. As reviewed herein, evidence for their role in human metabolism remains preliminary. However, qualitative and quantitative changes in EVs observed in various metabolic diseases, and accumulating evidence for their role in other pathological states such as cancer suggests that further research in this field is warranted. Ongoing investigation of EV regulation and function in vivo will better equip us to understand their role in metabolism (see Outstanding Questions), and may one day provide new therapeutic opportunities in combatting the heavy burden of metabolic disease.

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Supplemental Information
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Outstanding Questions
To what extent are the functional roles of exosomes, microvesicles, and apoptotic bodies similar, and to what extent do they differ? Does their relative importance depend on the tissue type in question and physiological context?

What processes regulate the specific recruitment of protein and RNA into EVs in response to different physiological and pathological stressors? What is the molecular machinery that packages specific combinations of proteins and nucleic acids into EVs? What pathways regulate these mechanisms?

How similar, and how different, are EVs released from (i) different tissues (e.g., adipose tissue and liver); (ii) different cells (e.g., individual adipocytes); and (iii) the same cell over time?

How does the ‘EV map’ (the collection of target tissues modulated via EVs) of one tissue differ from that of another, and over time? What, for example, is the EV map of different adipose tissue depots?

What defines the EV map of an individual vesicle? How generalisable is the recently described mechanism of exosomal integrin-directed metastatic niche formation?

Which components of adipose EV cargo are altered in the obese state?

Are adipose-derived EVs taken up by other metabolic tissues in vivo? If so, what effects do they have? Does inhibition of adipose exosome in vivo ameliorate the consequences of obesity?

Is there scope for using exosome-mediated delivery of specific miRNAs or siRNAs to reverse adipose tissue inflammation or NAFLD?
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