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Suresh Mathivanan
Pamali Fonseka
Christina Nedeva
Ishara Atukorala *Editors*

New Frontiers: Extracellular Vesicles

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Suresh Mathivanan • Pamali Fonseka •
Christina Nedeva • Ishara Atukorala
Editors

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Editors
Suresh Mathivanan
Department of Biochemistry and Genetics
La Trobe Institute for Molecular Science
La Trobe University
Melbourne, Victoria, Australia

Pamali Fonseka
Department of Biochemistry and Genetics
La Trobe Institute for Molecular Science
La Trobe University
Melbourne, Victoria, Australia

Christina Nedeva
Department of Biochemistry and Genetics
La Trobe Institute for Molecular Science
La Trobe University
Melbourne, Victoria, Australia

Ishara Atukorala
Department of Biochemistry and Genetics
La Trobe Institute for Molecular Science
La Trobe University
Melbourne, Victoria, Australia

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Preface

In multicellular organisms, intercellular communication is critical for the maintenance of homeostasis. The discovery of the pivotal role of extracellular vesicles (EVs) in intercellular communication has ignited significant interest in the exploration of EV-mediated signalling in physiological and pathological conditions. EVs are heterogeneous in nature as they differ in their biogenesis and secretion, cargo content and the cell type of origin. Despite the heterogeneity, their biophysical properties overlap to an extent. The rich cargo content of EVs often reflects the cell of origin and their physiological status. They are stable, membranous nanovesicles that have been shown to participate in many disease conditions. However, the role of EVs in normal physiological conditions is poorly understood. In this book, the fundamental molecular mechanisms involved in EV biogenesis, cargo sorting and secretion will be discussed first. In the second half of the book, the functional role of EVs in various disease settings is highlighted.

EVs: A Diverse Community

EVs can be classified into several subtypes including exosomes, apoptotic bodies, ectosomes or shedding microvesicles, large oncosomes, migrasomes and exomeres. An introduction to various subtypes of EVs is provided in Chaps. 1 and 2. Amongst the EV subtypes, exosomes initially received significant attention due to their role in intercellular communication. The other EV subtypes including ectosomes and apoptotic bodies are also gaining more interest amongst biomedical researchers. Due to the heterogeneity of EVs, prior to exploring the functional properties of EVs, it is important to understand the EV subtypes and the various methods employed for isolation. Depending on the specific EV subtype and the biological source (bodily fluid or conditioned media), the EV isolation method varies. Chapter 1 discusses the different EV subtypes and the current techniques involved in isolation and characterisation of EVs. It should be noted that, in order to differentiate between the EV

subtypes, it is necessary to adhere to guidelines outlined by the International Society of Extracellular Vesicles (ISEV).

Chapter 2 discusses the molecular mechanisms involved in the biogenesis and secretion of EV subtypes. Even though the EV field is rapidly evolving, the mechanism by which cargo content is sorted into EVs, before secretion, is poorly understood. Chapter 3 discusses the role of post-translational modifications in the sorting of cargo into EVs. Gaining deeper insight into the pathways involved in biogenesis, cargo sorting and secretion of vesicles can aid in enhanced understanding of EV biology, thereby aiding in EV manipulation for clinical applications.

EVs are secreted from a plethora of cell types and at different stages of the cell cycle. With the recently gained importance of apoptotic bodies in cellular communication and apoptotic cell clearance, apoptotic bodies have attracted significant interest amongst biomedical researchers. Chapter 4 discusses the regulators of apoptotic cell disassembly, specific techniques involved in isolation of apoptotic bodies and functional role of these vesicles. Another new EV subtype that was recently discovered is exomeres. These vesicles are nanoparticles that lack a lipid bilayer, and current knowledge about the isolation methods and protein cargo associated with exomeres will be discussed in Chapter 5.

EVs released from non-mammalian cell types have often been shown to alter pathology in disease settings. Bacterial vesicles released from both gram-positive and gram-negative bacteria are known to participate in bacterial communication and can be potentially employed for therapeutic purposes. The physical and functional aspects of bacterial EVs are discussed in Chapter 6. With the emergence of the connection between fungal EVs and fungal virulence, fungal EVs have attracted significant attention in recent years. Chapter 7 gives a brief overview of the impact caused by fungal EVs on the host and elucidates their functional role.

Functional Role and Clinical Implications of EVs

EVs are considered key players in cell-to-cell communication. These vesicles carry a rich cargo content that often mirrors the cell of origin. EVs modulate phenotypic changes in the recipient cells by the delivery of cargo in a non-selective manner. Hence, it is important to understand the various EV-based cargo delivery mechanisms. Chapter 8 discusses various means of EV-mediated cargo delivery. Amongst the proposed functions of EVs, their ability to transfer chemoresistance to the recipient cells is discussed in Chapter 9.

During the process of cancer progression, vascular changes occur in tumours to aid in metastasis. Chapter 10 discusses the importance of EVs in vascular remodelling, and Chaps. 11 and 12 discuss the involvement of EVs in metastasis and organotropism. EVs released from the tumour microenvironment have shown to communicate with neighbouring vascular systems and educate metastatic sites. In-depth understanding of the role of EVs in disease settings will provide insights into prognostic evaluation of diseases. The overlap between the contents of cells of

origin and EVs will allow EVs to be explored as reservoirs of potential biomarkers. The unique biomolecules encapsulated in EVs are often specific to diseased tissues and, hence, can give an indication of the disease status of that tissue. This aspect of EVs is highlighted in Chapter 13. In recent years, EVs have been exploited as drug delivery vehicles. EVs have shown to protect their content from harsh environmental conditions, making them attractive nanocarriers for therapeutic drugs. Chapter 14 discusses the progress that has been made in utilising EVs for the treatment of cancer whilst addressing the hurdles associated with the adaption of EVs as feasible drug delivery systems.

EVs have also been implied in other complex diseases such as metabolic disease (Chap. 15), neurodegeneration (Chap. 16), cardiovascular disease (Chap. 17), pre-eclampsia (Chap. 18) and cerebral malaria (Chap. 20). With most of these diseases, it is highlighted that not only the cargo content of EVs changes depending on the disease stage, but also the number of EVs released. It is important to understand distinctive properties of EVs secreted in each disease condition when investigating methods of targeting either the EV cargo or EV secretion. EVs have also been shown to play a pivotal role in physiological processes such as gamete production and fertilisation. Chap. 19 gives an overview of the current knowledge pertaining to the involvement of EVs in sperm maturation and male fertility.

The final chapter (Chap. 21) of the book discusses the role of EVs in cross-species and inter-individual communication. The discovery of dietary EVs as mediators of cross-species communication has paved the way for research in employing dietary EVs as drug delivery vehicles. In this chapter, the benefits and arising concerns associated with the utilisation of EVs from different species or kingdoms are discussed. Overall, *New Frontiers: Extracellular Vesicles* comprises various topics pertaining to EVs. The book will help readers to better understand the biological significance of EVs, which will contribute to the progression of EV research.

Melbourne, Australia
October, 2020

Pamali Fonseka
Ishara Atukorala
Christina Nedeva
Suresh Mathivanan

Contents

About the Editors

Suresh Mathivanan Suresh Mathivanan completed his PhD in proteomics and bioinformatics at the Institute of Bioinformatics, India, and Johns Hopkins University, USA. Suresh joined Ludwig Institute for Cancer Research, Melbourne, Australia, for his first postdoctoral position. In 2011, he received a NH&MRC Peter Doherty fellowship to study the role of exosomes in cancer cells. In 2015, he was awarded an ARC DECRA to study the role of exosomes in intercellular communication. Suresh established his own research group in the Department of Biochemistry, La Trobe Institute for Molecular Science (LIMS), at La Trobe University after receiving a LIMS fellowship in 2011. Mathivanan's laboratory is focused in exploring exosomes and their role in cancer and intercellular communication. Currently, he is funded by an ARC Future Fellowship (FT2: 2018–2022) to investigate exosome biogenesis. He has authored over 94 papers that are cited more than 20000 times (Google Scholar; July 2020) and has been recognised as a highly cited researcher in 2018 and 2019. Currently, he serves as the Director of the Research Centre for Extracellular Vesicles (RCEV) in La Trobe University.

Pamali Fonseka Pamali Fonseka is an early career scientist at the La Trobe Institute for Molecular Science. Dr Fonseka was awarded PhD in Dec 2018 from La Trobe University. Her PhD thesis was on the role of exosomes in intra-tumour heterogeneity and cross-species communication. She has been working in the field of extracellular vesicles for the past 7 years. Dr Fonseka has made numerous contributions to the field of extracellular vesicles and has published 11 manuscripts in well-reputed journals. Dr Fonseka was awarded CASS foundation science/medical grant and ECR travel grant by the Research Centre for Extracellular Vesicles in 2020. She is the current secretary of the La Trobe Institute for Molecular Science postdoctoral society and ECR representative of the Research Centre for Extracellular Vesicles. Currently, her research focuses on exosome biogenesis and the role of extracellular vesicles in the transfer of chemoresistance to recipient cells.

Christina Nedev Christina's research career stems from that of an infection and immunity background. Her PhD and first postdoctoral studies at La Trobe University (LIMS) focused on identifying apoptotic factors involved in immune cell death during sepsis. She identified a crucial receptor responsible for signalling cell death of innate immune cells during the early phase of sepsis leading to host immune suppression and ultimately death in severe cases. This research has been published in *Nature Immunology*, 2020. In more recent times, she has shifted focus of research which currently entails using exosomes for cancer therapy.

Ishara Atukorala Ishara Atukorala is an early career researcher at the La Trobe Institute for Molecular Science. Ishara completed her PhD in 2019 at La Trobe University where she explored the role of cadherins in colorectal cancer progression and metastasis and developed an antibody to block the active binding sites of them. She has been working on extracellular vesicles with specific focus on biogenesis and secretion. Dr Atukorala is exploring novel protein candidates and biological pathways involved in the biogenesis of extracellular vesicles. Her research further extends to understand the role of cadherins in extracellular vesicle-mediated metastasis of colorectal cancer.

Part I
Subtypes, Biogenesis, and Secretion of Extracellular Vesicles

Chapter 1
Introduction to the Community of Extracellular Vesicles

Pamali Fonseka, Akbar L. Marzan, and Suresh Mathivanan

Abstract Since the discovery that extracellular vesicles (EVs) mediate intercellular communication, there is an exponential increase in the interest on EVs, especially in pathological settings. EVs are membranous vesicles that are secreted by various cell types and the release of EVs is conserved in every prokaryotic and eukaryotic organism tested to date. These vesicles were initially thought to be garbage disposal vehicles and subsequent studies over the past 4 decades have attributed several functional roles to EVs, some of which are critical for homeostasis. The molecular cargo of nucleic acids, proteins, lipids and metabolites packaged in EVs often mirror the host cells phenotypic status. EVs can be taken up by recipient cells and upon uptake, EVs through its molecular cargo, can induce a cascade of signal transduction events in recipient cells. EVs are categorised into several subtypes depending on their biogenesis and secretion. Due to several subtypes, differing sizes within a subtype and varying cargo, EVs are heterogenous in nature and the biophysical and biochemical properties of EVs often overlap between EV subtypes. Hence, it is important to be cautious when selecting the method of EV isolation and characterisation. This chapter provides a brief introduction to EVs and their subtypes.

Keywords Extracellular vesicles · Exosomes · Ectosomes · Apoptotic bodies · Microvesicles

Introduction

Direct cell-cell contact or secreted molecules are key regulators of intercellular communication in multicellular organisms (Mathivanan et al. 2010; Gangoda et al. 2015). Over the past two decades, the role of extracellular vesicles (EVs) in mediating intercellular communication is beginning to emerge (Zhao et al. 2019;

P. Fonseka (✉) · A. L. Marzan · S. Mathivanan
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia
e-mail: P.Fonseka@latrobe.edu.au

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Peinado et al. 2012). EVs consist of a lipid bilayer membrane and are secreted from cells under various pathophysiological conditions (Yuana et al. 2013). EVs encapsulate a compendium of molecules including proteins, lipids, nucleic acids and metabolites (Lunavat et al. 2015; Zaborowski et al. 2015; Palviainen et al. 2019; Vagner et al. 2018; Pathan et al. 2019). The cargo content of EVs often reflect the molecular signature of the host cells (Gangoda et al. 2017; Maas et al. 2017; Katsuda and Ochiya 2015; Anand et al. 2019; Samuel et al. 2017). Depending on the mode of biogenesis and release, EVs can be classified into several subtypes (Kalra et al. 2016; Zijlstra and Di Vizio 2018).

Subtypes of EVs

EVs can be classified as exomeres (<50 nm), exosomes (30-150 nm), ectosomes or shedding microvesicles (100-1000 nm), apoptotic bodies (1000-5000 nm), migrasomes (500–3000 nm) and large oncosomes (1000–10,000 nm) (Fig. 1.1). Among the subtypes of EVs, exosomes are the relatively well studied nanovesicles. Exosomes are formed by inward budding of the endosomal membrane which results in the accumulation of intraluminal vesicles (ILVs) within the multivesicular bodies (MVBs) (Théry et al. 1999; Piper and Katzmann 2007; Kowal et al. 2014). MVBs play a pivotal role in protein sorting, storage, recycle and transport of molecules (Babst et al. 2002; Nikko et al. 2003). These MVBs can either be directed to degradation by fusing with lysosomes or directed to exocytosis by fusing with the plasma membrane (Fig. 1.2). Upon fusion with the plasma membrane, the ILVs are

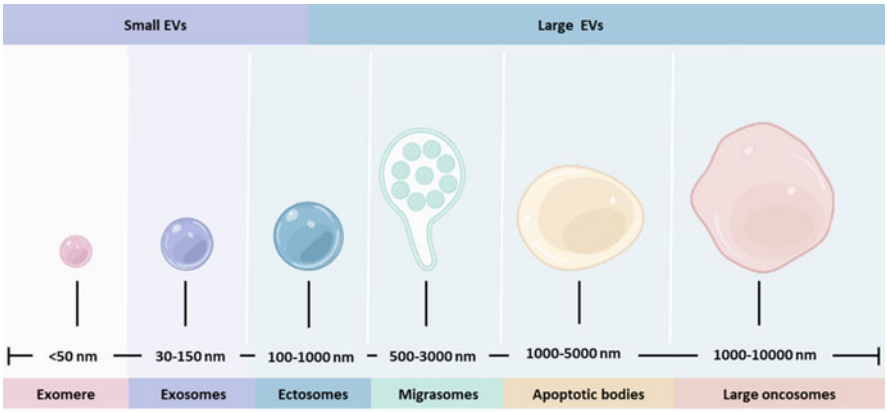

Fig. 1.1 Size ranges of EVs. EVs can be categorised as small and large EVs. Relatively well studied exosomes range from 30 to 150 nm in size while ectosomes (shedding microvesicles) are 100–1000 nm. Apoptotic bodies are extremely heterogeneous in nature and are 1–5 μm in size. Exomeres are nanoparticles that are less than 50 nm while migrasomes and large oncosomes are much larger vesicles. Created using BioRender.com

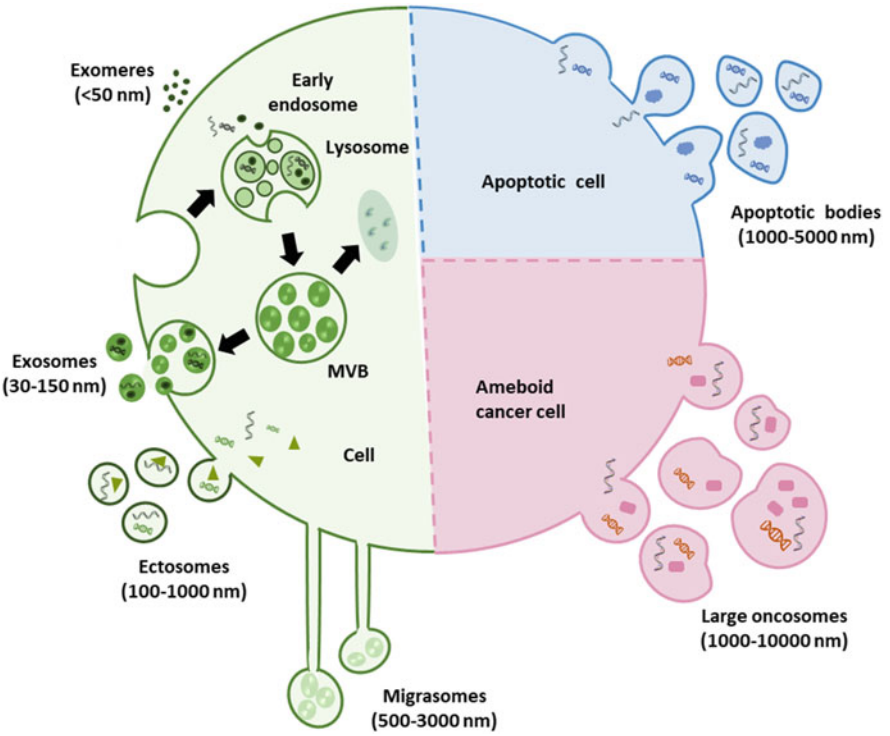

Fig. 1.2 Schematic representation of EV biogenesis and secretion. Exosomes are formed by inward budding of the endosomal membrane which results in the accumulation of intraluminal vesicles (ILVs) within the multivesicular bodies (MVBs). These MVBs can either be directed to degradation by fusing with lysosomes or fuse with the plasma membrane and release ILVs to the extracellular space as exosomes. Ectosomes are released by budding of plasma membrane while apoptotic bodies are released from dying cells. Migrasomes are released by migrating cells due to cell migration and actin polarization. Large oncosomes are carriers of oncogenic cargo and are released from amoeboid cancer cells. Exomeres are non-membranous vesicles and the exact pathway involved in their biogenesis is yet to be understood. Created using BioRender.com

released into the extracellular space and referred to as exosomes (Kalra et al. 2016). Mechanism that govern the exact fate of MVBs and sorting of specific cargo into ILVs are poorly understood (Woodman and Futter 2008; Hu et al. 2015; Anand et al. 2019). However, it has been suggested that the endosomal sorting complexes required for transport (ESCRT) machinery may regulate some parts of these mechanisms (Colombo et al. 2013; Gan and Gould 2011; Baietti et al. 2012). Another pathway by which ILVs are formed is through the involvement of ceramide. The conversation of sphingomyelin to ceramide followed by clustering of lipid rafts triggers the formation of ILVs (Guo et al. 2015; Verderio et al. 2018; Fukushima et al. 2018). Nevertheless, exosomes have a buoyant density of 1.10–1.14 g/mL and range in size from 30 to 150 nm (Mathivanan et al. 2010; Keerthikumar et al. 2015).

In contrast to exosomes, ectosomes or shedding microvesicles are larger vesicles (100–1000 nm) that are being released from the plasma membrane via budding (Fig. 1.2) (Choi et al. 2013; Keerthikumar et al. 2015). The term "ectosytosis" was coined by Stain and Luzio to describe the shedding of vesicles from neutrophils in early 90s (Stein and Luzio 1991). However, the exact mechanism by which ectosomes are formed and released is poorly understood. Recurring evidences have shown that the alteration of local membrane curvature, due to rearrangement of the actin cytoskeleton and plasma membrane content both vertically and laterally cause the release of ectosomes (Tricarico et al. 2017; Pollet et al. 2018). Prior to outward budding of plasma membrane, cells undergo a series of changes. Increase in accumulation of Ca^{2+}, activation of cytoskeletal components degrading enzymes (e.g. calpain) and externalization of phosphatidylserine (PS) are the most common steps in this phenomenon (Fox et al. 1991; Meldolesi 2018; Pang et al. 2018). Similar to exosomes, ectosomes also carry the host cell specific signature (Keerthikumar et al. 2015; Kalra et al. 2016).

Apoptotic bodies are large EVs (1000–5000 nm) and are released from apoptotic cells (Mathivanan et al. 2010; Kalra et al. 2016). It is known that this group of EVs act as corpse clearers, removing damaged cell debris (Caruso and Poon 2018). Apoptotic bodies are eventually eliminated via phagocytosis (Poon et al. 2014). Prior to blebbing of apoptotic bodies, cells undergo a series of distinct events causing the dying cells content to be packed inside apoptotic bodies (Jiang et al. 2017b; Caruso and Poon 2018). Among the key changes are fragmentation of DNA, plasma membrane externalization, shrinking of cells, detaching from the extracellular matrix and plasma membrane blebbing (Fig. 1.2) (Atkin-Smith et al. 2015). Formation of apoptotic bodies is important for the survival of organisms as this mechanism prevents toxins and degrading enzymes from leaking to healthy cells (Liu et al. 2018; Gavrilescu and Denkers 2003). Followed by the activation of caspase mediated apoptotic pathway, formation of apoptotic bodies is activated by Rho-associated coiled-coil-forming kinase I (ROCK1) (Shi and Wei 2007; Tixeira et al. 2020). When the cellular content is packed in apoptotic bodies, concatenation of events occur such as enucleation, expansion and retraction (Orlando et al. 2006). Discovery of apoptopodia (beads-on-a-string-like membrane protrusions) has opened a new avenue in forming apoptotic bodies. This was first observed in monocytes where the beads often detach from the strings to form apoptotic bodies (Atkin-Smith et al. 2015). It was noted that the formation of apoptopodia are negatively affected by caspase-activated pannexin 1 (PANX1) (Atkin-Smith et al. 2019).

Other than these subtypes of EVs, exomeres, migrasomes and large oncosomes are also described in the literature (Fig. 1.2). Exomeres (<50 nm) were discovered only recently and, unlike other EVs, they are non-membranous nanoparticles (Zhang et al. 2018). These particles however have shown to be enriched in amyloid precursor protein, argonaute 1–3 and metabolic enzymes. Additionally, other proteins, lipids and nucleic acids were also detected in exomeres (Zhang and Lyden 2019). It is possible that exomeres could potentially be aggregation of molecules and hence additional research is needed to uncover the biogenesis and functional properties of these nanoparticles (Zijlstra and Di Vizio 2018). Migrasomes (<3000 nm)

were discovered by Ma et al. (2015) and is a subtype of EVs that are generated by migrating cells (Fig. 1.2) (Ma et al. 2015). The process of formation of migrasomes is coined as "migracytosis" and is dependent on cell migration and actin polymerization (da Rocha-Azevedo and Schmid 2015; Tavano and Heisenberg 2019).

Molecular Composition of EVs

EVs serve as intercellular transport vehicles and due to the presence of lipid bilayer membrane, the content of EVs are often protected from the harsh degrading environment (Huang et al. 2013; Ridder et al. 2014; Sanwlani et al. 2020). However, the composition of this lipid bilayer is distinct from the plasma membrane of the donor cell (Balaj et al. 2011; Lydic et al. 2015). Exosomes are enriched with cytosolic proteins from the cell of origin (Mathivanan et al. 2010). As the endosomes and ESCRT complex contribute to the formation of exosomes, proteins such as Alix, TSG101, HSP70 and integrins are always identified in exosomes regardless of the cell type of origin (Mathivanan et al. 2010; Thery et al. 2002, 2009; Keerthikumar et al. 2016). Similarly, members of tetraspanin family, CD63, CD9 and CD81 are enriched in exosomes compared to host cells (Kowal et al. 2016; Andreu and Yáñez-Mó 2014; Liem et al. 2017). Exosomes are also enriched with lipids such as cholesterol, ceramide and sphingolipids (Trajkovic et al. 2008; Subra et al. 2007). Exosomes also contain, polysaccharides and glycan on the outer surfaces (Batista et al. 2011). In addition, exosomes are reported to carry mRNA and miRNA that can regulate gene expression in recipient cells (Valadi et al. 2007; Michael et al. 2010; Batagov and Kurochkin 2013; Samuel et al. 2015). Furthermore, exosomes from cancer cells also contain double-stranded DNA with mutations specific for the cancer patient (Thakur et al. 2014; Qu et al. 2019; Wang et al. 2018). Hence, exosomes with disease state specific cargo including mutant proteins, RNA and DNA are considered as reservoirs of biomarkers (Mathivanan et al. 2012; Boukouris and Mathivanan 2015; Al-Nedawi et al. 2008).

Even though a subset of the cargo content of ectosomes are similar to exosomes, their composition is relatively poorly studied. Ectosomes are reported to contain heterogenous cargo which are distinctive from the donor plasma membrane. Several studies have highlighted the presence of matrix metalloproteinases (MMP2), integrins and cytoskeletal proteins such as α-actin and β-actin in ectosomes (Bernimoulin et al. 2009; Li et al. 2013; Gasser et al. 2003). Unlike exosomes, ectosomes are enriched with ribosomal, centrosomal and mitochondrial proteins (Keerthikumar et al. 2015; Anand et al. 2018). Ectosomes also contain several RNA and lipids some of which are also detected in exosomes (Ratajczak et al. 2006; Weerheim et al. 2002; Lunavat et al. 2015).

Though apoptotic bodies were considered as cell debris, recent studies have implicated them in intercellular communication and hence their cargo has been characterised in some conditions. The composition of the apoptotic bodies can vary according to the size of the vesicle, donor cell, cause of apoptosis and the

selection of cargo. It has been reported that apoptotic bodies derived from lymphocytes and monocytes are enriched with annexin and coagulation factor III (Mallat et al. 1999; Zarà et al. 2019). Similarly, a proteomic evaluation of apoptotic bodies derived from thymocytes have identified cytosolic and heat shock proteins (Turiak et al. 2011). Among the other components reported, Annexin A6, low density lipoprotein receptor-related protein 1, proteins involved in immune response and RAB11 proteins were also detected in apoptotic bodies (Lleo et al. 2014; Jiang et al. 2017a). In 2015, a total of 1028 proteins were found to be differentially abundant in apoptotic bodies marking the depletion of nuclear components in these vesicles (Atkin-Smith et al. 2015). Apoptotic bodies have also shown to carry microRNA, DNA and lipids (Jiang et al. 2017b; Bergsmedh et al. 2001; Berda-Haddad et al. 2011).

Isolation and Characterisation of EVs

One of the major challenges in the EV field is optimizing and standardizing the isolation protocols to purify EV subtypes to homogeneity. Moreover, these protocols vary depending on the type and quantity of samples used for isolating EVs (Momen-Heravi et al. 2013). Hence, prior to selecting a method of EV isolation, several factors including EV subtype, size, buoyant density, physical properties, surface molecules, sample source and sample amount should be taken into consideration (Konoshenko et al. 2018). Most commonly used EV isolation protocols include differential centrifugation, ultracentrifugation, ultrafiltration, density gradient centrifugation, precipitation, immunoaffinity based pull-down and size exclusion chromatography (Konoshenko et al. 2018; Furi et al. 2017; Momen-Heravi et al. 2013). Differential centrifugation coupled with ultracentrifugation has been used in a vast majority of the studies (Thery et al. 2006; Furi et al. 2017; Kang et al. 2017; Simpson et al. 2012). However, it is accepted that none of the currently employed protocols can isolate the EV subtypes to homogeneity (Kalra et al. 2016; Simpson and Mathivanan 2012). This is due to the complexity of the tissue samples, biochemical and biophysical overlap between the EV subtypes and the heterogeneity within a EV subtype (Kowal et al. 2016; Willms et al. 2018).

Currently, most of the EV isolation techniques couple different protocols in order to enrich a specific subtype, though the presence of other EV types cannot be completely ruled out (Furi et al. 2017). For instance, when isolating exosomes from conditioned media, sample is subjected to $2000\times g$ (20 min) and $10{,}000\times g$ (30 min) centrifugation and an addition filtration step is added to the protocol in order to remove apoptotic bodies and ectosomes. EV sample is further subjected to 100,000–120,000 g ultracentrifugation step for 1 h. However, co-sedimentation of other vesicles is often seen in final exosomes preparation (Witwer et al. 2013). Multiple studies report pure population of exosomes when the final ultracentrifugation step is followed by a density gradient centrifugation (sucrose or OptiPrep™) (Tauro et al. 2012; Konoshenko et al. 2018). However, major draw backs of this

method are sample loss, fragmentation of EVs due to filtration and centrifugal force, time consumption and tediousness (Konoshenko et al. 2018; Ayala-Mar et al. 2019).

In addition, size-based isolation methods has also been utilized to isolate exosomes with less contamination from other EV subtypes (Gámez-Valero et al. 2016; Li et al. 2017). Some of these methods are size exclusion chromatography, sequential filtration, ultrafiltration, hydrostatic filtration dialysis and flow field-flow fractionation (Benedikter et al. 2017; Stranska et al. 2018; Heinemann et al. 2014; Musante et al. 2014; Zhang and Lyden 2019). Commercial kits such as qEV exosomes isolation kit (iZON) and Invitrogen™ total exosomes isolation kit isolate EVs from less sample volume in a time efficient manner (Moon et al. 2019; Helwa et al. 2017; Stranska et al. 2018; Andreu et al. 2016). However, the finalized EV preparation often suffer due to co-isolation of other EV subtypes, protein complexes such as lipoproteins and reduction in stability (Patel et al. 2019; Ayala-Mar et al. 2019; Li et al. 2017). The antibody-based precipitations of EVs from samples are widely used and immunobeads and flowcytometry is employed in the technique (Nakai et al. 2016; Pugholm et al. 2015; Shao et al. 2018). When isolating EVs, surface markers such as CD9, CD63 and EPCAM (Tauro et al. 2012; Contreras-Naranjo et al. 2017) are used but caution is needed to not neglect EVs that are negative to some of these surface molecules (Simpson and Mathivanan 2012; Tauro et al. 2012).

There are relatively less contaminants when isolating apoptotic bodies as they are released from dying cells. Due to size of apoptotic bodies, low speed centrifugation can be utilised to isolate them (Atkin-Smith et al. 2015, 2019). To obtain pure population of apoptotic bodies, samples are often subjected to flowcytometry based on immune-affinity (Atkin-Smith et al. 2015; Serrano-Heras et al. 2020). Due to the lack of specific markers that can assist in confidently distinguishing between the EV subtypes, it is important to adhere to the guidelines published by the International Society of Extracellular Vesicles (ISEV) (Lötvall et al. 2014; Witwer et al. 2017; Thery et al. 2018). Hence, EV-based studies need to characterise the EVs using both biophysical and biochemical techniques (Lötvall et al. 2014; Witwer et al. 2013, 2017). For instance, whilst characterising the biochemical properties of EVs, Western blotting or flow cytometry can be employed to detect transmembrane/lipid bound proteins, cytosolic proteins, intracellular proteins and contaminants such as lipoproteins (negative control) (Witwer et al. 2017; Lötvall et al. 2014). The biophysical properties (morphology and size) of EVs can be assessed using transmission electron microscopy, atomic force microscopy, nanoparticle tracking analysis and high resolution flowcytometry (Kalra et al. 2019; Fonseka et al. 2019).

Concluding Remarks

With the recent advances in technology, significant progress has been made in understanding the biogenesis and function of EVs. A significant issue in the field of EVs relates to the lack of isolation procedures that can isolate EV subtypes to

homogeneity and markers that can identify specific EV subtypes. As EVs are implicated in intercellular communication and disease progression, further understanding on the biogenesis of EV subtypes are needed. Perhaps, it can be speculated that manipulation of biogenesis and secretion of EVs could result in the suppression of tumour progression and metastasis.

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Chapter 2
Biogenesis of Extracellular Vesicles

Taeyoung Kang, Ishara Atukorala, and Suresh Mathivanan

Abstract Extracellular vesicles (EVs) refer to vesicles that are released by cells into the extracellular space. EVs mediate cell-to-cell communication via delivery of functional biomolecules between host and recipient cells. EVs can be categorised based on their mode of biogenesis and secretion and include apoptotic bodies, ectosomes or shedding microvesicles and exosomes among others. EVs have gained immense interest in recent years owing to their implications in pathophysiological conditions. Indeed, EVs have been proven useful in clinical applications as potential drug delivery vehicles and as source of diagnostic biomarkers. Despite the growing body of evidence supporting the clinical benefits, the processes involved in the biogenesis of EVs are poorly understood. Hence, it is critical to gain a deeper understanding of the underlying molecular machineries that ultimately govern the biogenesis and secretion of EVs. This chapter discusses the current knowledge on molecular mechanisms involved in the biogenesis of various subtypes of EVs.

Keywords Extracellular vesicles · Apoptotic bodies · Shedding microvesicles · Exosomes · Oncosomes · Migrasomes · Nanoparticles · Exomeres · Biogenesis and secretion · Tetraspanins

Introduction: Extracellular Vesicles

Extracellular vesicles (EVs) are nanoparticles that are secreted by cells and their presence has been confirmed in a range of biological fluids such as blood, urine, saliva and breast milk (Admyre et al. 2007; Michael et al. 2010; Cheng et al. 2014; Villarroya-Beltri et al. 2014; Boukouris and Mathivanan 2015; Gangoda et al. 2015). Broadly classified as large and small EVs, several subtypes of EVs including exosomes, large oncosomes, apoptotic bodies, migrasomes, ectosomes/shedding

T. Kang · I. Atukorala · S. Mathivanan (✉)
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia
e-mail: S.Mathivanan@latrobe.edu.au

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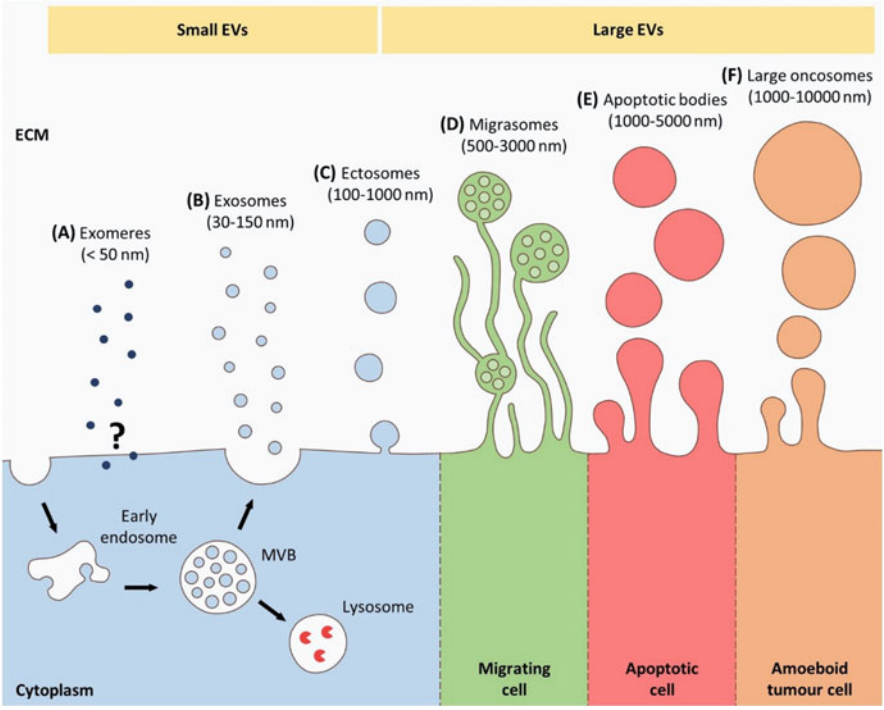

Fig. 2.1 The biogenesis of various extracellular vesicles and extracellular vesicle-like nanoparticles. (**a**) Exomeres are extracellular vesicle-like non-membranous nanoparticles and the specific processes involved in the biogenesis of exomeres are currently unknown. (**b**) Exosomes biogenesis involves endocytosis and subsequent fusion of endocytic vesicles with early endosomes. During maturation of an early endosome into a multivesicular body, intraluminal vesicles form via inward budding of the endosomal membrane. Intraluminal vesicles can either be degraded upon fusion of multivesicular bodies with lysosomes or be secreted into the extracellular matrix through fusion of multivesicular bodies with the plasma membrane. Once released, intraluminal vesicles are referred to as exosomes. (**c**) Ectosomes or shedding microvesicles are directly shed into the extracellular matrix via outward budding of the plasma membrane. (**d**) Migrasomes are generated at the tips or intersections of retraction fibres during cell migration and are secreted into the extracellular matrix upon detachment from retraction fibres. (**e**) Apoptotic bodies are directly secreted into the extracellular matrix from membrane protrusions/blebs that occur in apoptotic cells. (**f**) Large oncosomes are typically released from membrane blebs of amoeboid tumour cells and have been identified to encapsulate oncogenic cargo. *EVs* extracellular vesicles; *ECM* extracellular matrix; *MVB* multivesicular body

microvesicles and exomeres have been described so far (Fig. 2.1). Large EVs are considered to be greater than 200 nm in diameter, which includes large oncosomes, apoptotic bodies, migrasomes and ectosomes/shedding microvesicles. On the other hand, small EVs consists of vesicles that are less than 200 nm in diameter, such as exosomes, exomeres and a subpopulation of ectosomes/shedding microvesicles (Kalra et al. 2016; Chuo et al. 2018; Théry et al. 2018; Margolis and Sadovsky 2019). However, as the size of these secretory vesicles may overlap with one

another, there are inherent challenges when attempting to characterise a distinct subtype of EVs by solely relying on their size (Anand et al. 2019). Thus, the classification of EVs can further be assisted by the presence of particular markers that highly represent specific organelles that EVs originate from (Kowal et al. 2016; Théry et al. 2018). For example, exosomes originate from the endocytic pathway and are enriched in tetraspanins such as cluster of differentiation CD9, CD63 and CD81 as well as constituents of endosomal sorting complex required for transport (ESCRT) such as tumour susceptibility gene 101 (TSG101) (Andreu and Yáñez-Mó 2014; Pathan et al. 2019). However, markers or enriched proteins for other EV subtypes are poorly characterised (Anand et al. 2019).

Most of the EVs, baring exomeres, contain lipid bilayer membrane that enables EVs to encase functional biomolecules such as proteins, nucleic acids and lipids to safely transport them from host to recipient cells that reside nearby or in distant locations (Kalra et al. 2016; Mondal et al. 2019). Hence, EVs provide an efficient mode of intercellular communication and are considered as attractive tools for novel therapeutic strategies in many pathological conditions (Boere et al. 2018; Borrelli et al. 2018; Kalra et al. 2019; Murphy et al. 2019; Upadhya and Shetty 2019; Sanwlani et al. 2020). However, despite the growing body of evidence elucidating the clinical utility of EVs, the underlying molecular mechanisms that govern the process of EV biogenesis is poorly understood. This chapter will discuss current findings in regard to the formation of the above-mentioned subtypes of EVs, with particular focus on exosomes.

Large Oncosomes

The word “oncosomes” was first introduced by Janusz Rak and colleagues in 2008 in a study describing vesicle-mediated circulation of epidermal growth factor receptor variant III (EGFRvIII), an oncogenic form of the EGFR among glioma cells (Al-Nedawi et al. 2008). Oncosomes are any EV subtype that are secreted by cancerous cells and contain oncogenic cargo (Di Vizio et al. 2009; Minciacchi et al. 2017). They carry the unique signature of the tumour cell it is originating from in the form of proteins, lipids and nucleic acids. They can contribute to a horizontal propagation of oncogenes and aid in transforming phenotype among subsets of cancer cells (Al-Nedawi et al. 2008; Lee et al. 2011; Minciacchi et al. 2017). The precise role of these circulating messengers in the tumour microenvironment, metastasis and for diagnostic uses, are areas currently under active investigation.

Large oncosomes were reported in 2009 by Di Vizio et al., and were described as uncommonly large EVs secreted by prostate cancer cells upon stimulation by EGFR activation and overexpression of membrane-targeted Akt1 (Di Vizio et al. 2009). They are 1000–10,000 nm in diameter and are derived from membrane blebs of amoeboid tumour cells (Di Vizio et al. 2012; Kim et al. 2014). It has to be noted that large oncosomes are a specific subtype of EVs secreted only by amoeboid tumor

cells and differ from the term oncosomes which refer to any EVs that has oncogenic cargo. Large oncosomes are reported to be detected in tumour tissues and plasma of advanced cancer patients and their presence in blood has been speculated to serve as an indication of tumour metastasis (Di Vizio et al. 2012). As such, these large oncosomes carry a signature that promotes tumour metastasis made of certain RNAs, miRNAs (miR-1227 and miR-125a), caveolin-1, metalloproteinases and ADP ribosylation factor 6 (ARF6) (Di Vizio et al. 2012; Morello et al. 2013; Kim et al. 2014). This cargo is capable of inducing changes in the tumour microenvironment, bringing in immunoevasive effects and upregulation of pro-oncogenic phenotype in recipient cells (Morello et al. 2013; Kim et al. 2014; Minciacchi et al. 2017). Moreover, enzymes implicated in metabolic processes related to cancer such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase B (LDHB), heat shock 70-kDa protein 5 (HSPA5), malate dehydrogenase (MDH) and glucose-6-phosphate isomerase (GPI) have been identified in abundance by proteomics studies of large oncosomes (Coller 2014; Li et al. 2014; Minciacchi et al. 2015b).

The phenotypic transition of cells from epithelial-to-mesenchymal to amoeboid nature increases the plasticity of cells and has been described as a method of cancer cell migration leading to metastasis. Moreover, amoeboid cancer cells demonstrate a blebbing morphology as a result of GTPase RhoA or Rho-associated coiled-coil containing protein kinase (ROCK) activation (Oppel et al. 2011). The biogenesis of large oncosomes has been characterised as the shedding of the non-apoptotic blebs, from these amoeboid cells, suggesting that amoeboid cells may employ more than one mechanism of migration and metastasis (Di Vizio et al. 2009; Minciacchi et al. 2015a). Furthermore, the enrichment of ARF6 in membrane-derived microvesicles, which is implicated in the abscission of microvesicles from the cell membrane, may suggest further alternate pathways important in large oncosome biogenesis (Muralidharan-Chari et al. 2009, 2010; Minciacchi et al. 2015a). Internalisation of large oncosomes has been observed in stromal cells via phagocytosis which results in reprogramming events in the fibroblasts, stimulating tube formation in endothelial cells and promoting tumour growth in vivo (Minciacchi et al. 2017).

Apoptotic Bodies

Apoptotic bodies range from 1000 to 5000 nm in diameter and are typically released from membrane blebs of apoptotic cells (Caruso and Poon 2018). Apoptosis is a crucial cellular process, whereby regulated execution of genetically programmed cell death aids in the maintenance of physiological homeostasis (Gavrilescu and Denkers 2003). During apoptosis, a number of intrinsic changes occur in a cell that ultimately result in the formation of apoptotic bodies. These include nuclear chromatin condensation, DNA fragmentation, caspase-mediated cleavage of intracellular components, such as the Golgi apparatus and cytoskeleton, as well as the formation of membrane blebs (Kerr et al. 1972; Chiu et al. 2002; Lu et al. 2005; Seo and Rhee

2018). Hence, unlike other subtypes of EVs, apoptotic bodies encapsulate a spectrum of cargo, ranging from fragments of DNA to entire organelles such as mitochondria (Xu et al. 2019).

Mechanistically, the blebbing of the plasma membrane in apoptotic cells is thought to be driven by the cleavage of ROCK1 by caspase-3 (Table 2.1) (Leverrier and Ridley 2001). Once cleaved, ROCK1 phosphorylates myosin light chain (MLC) to induce contraction of cortical actin-myosin, which, in turn, culminates in the build-up of hydrostatic pressure to allow the plasma membrane to detach from the cytoskeleton and swell up upon uptake of cytosol (Charras et al. 2005, 2008; Sharanek et al. 2016). A key alteration in the membrane of an apoptotic cell is the exoplasmic exposure of phosphatidylserine (PS), which is mediated by scramblases such as calcium-dependent transmembrane protein 16F (TMEM16F) and Xk-related protein 8 (Xkr8) (Suzuki et al. 2010, 2013). Once externalised, PS interacts with Annexin V, milk-fat-globule-EGF factor 8 (MFG-E8) as well as growth arrest-specific 6 (Gas6) (Erwig and Henson 2008). Moreover, oxidation of phospholipids, such as PS, assists in the binding of thrombospondin (TSP) and complement proteins C1q and C3b (Erwig and Henson 2008). These alterations, in addition to serving as signals for phagocytic clearance, are also reflected in the membrane composition of apoptotic bodies, hence providing a useful means of segregating apoptotic bodies from other subtypes of EVs (Carnino et al. 2019; Masvekar et al. 2019).

Migrasomes

In 2015, Ma et al. first described the presence of migrasomes in the extracellular matrix (ECM), where they observed secretion of oval shaped membrane-bound structures from migrating cells (Ma et al. 2015). This requires the initial formation of retraction fibres, which are long tubular projections rich in actin filaments that eventually detach from the rear end of cells during migration (Porter et al. 1945; Taylor and Robbins 1963; Svitkina 2018). Subsequently, the biogenesis of migrasomes occurs at the tips or at the branching points of retraction fibres through a newly defined cellular process known as migracytosis. During migracytosis, cytosol and its contents are transferred to the lumen of migrasomes and upon detachment of retraction fibres, migrasomes are released into the ECM (da Rocha-Azevedo and Schmid 2015; Ma et al. 2015). Secreted migrasomes range from 500 to 3000 nm in diameter and, interestingly, migrasomes harbour multiple smaller vesicles within their lumen, hence projecting as open pomegranate-like structures (Ma et al. 2015). Due to the enclosure of luminal vesicles, migrasomes show a close morphological resemblance to multivesicular bodies (MVBs), which is a late endosomal structure that gives rise to exosomes (Piper and Katzmann 2007; Ma et al. 2015). However, the expression of MVB marker, lysosomal-associated membrane protein 1 (LAMP1), is typically absent in migrasomes (Ma et al. 2015).

Since discovery, mammalian cells derived from various sources, such as normal rat kidney, mouse embryonic fibroblasts, human adenocarcinoma, human breast

Table 2.1 Summary of molecules that regulate the biogenesis of various EV subtypes

Protein/lipid	EV subtype	Function	References
Actin-myosin	Large oncosomes	Non-apoptotic bleb formation (membrane deformation)	Paluch et al. (2006)
MRCK	Large oncosomes	Maintenance of blebbing morphology	Wilkinson et al. (2005)
ROCK	Large oncosomes	Myosin-II light chain (MLC2) phosphorylation	Wilkinson et al. (2005)
ROCK1	Apoptotic body	Myosin light chain phosphorylation	Leverrier and Ridley (2001)
TMEM16F	Apoptotic body	Scramblase; PS externalisation	Suzuki et al. (2010)
Xkr8	Apoptotic body	Scramblase; PS externalisation	Suzuki et al. (2013)
Caspase-3	Apoptotic body	ROCK1, Xkr8 cleavage	Leverrier and Ridley (2001, Suzuki et al. (2013)
Actin-myosin	Apoptotic body Ectosomes	Membrane neck scission	Muralidharan-Chari et al. (2009), Wickman et al. (2013)
TSPAN4	Migrasome	TEM formation	Ma et al. (2015)
ABCA1	Ectosomes	Floppase: PS externalisation	Albrecht et al. (2005)
ARF6	Ectosomes	PLD activation	Muralidharan-Chari et al. (2009)
PLD	Ectosomes	ERK activation	Muralidharan-Chari et al. (2009)
ERK	Ectosomes	MLCK activation	Muralidharan-Chari et al. (2009)
MLCK	Ectosomes	Myosin light chain phosphorylation	Muralidharan-Chari et al. (2009)
ARRDC1	Ectosomes	TSG101/ALIX/NEDD4 interaction	Rauch and Martin-Serrano (2011), Nabhan et al. (2012)
CD81	Ectosomes	Induction of microvilli formation and extension	Bari et al. (2011)
Rab5/VPS21	Exosomes	Early endosomal fusion	Gorvel et al. (1991), Lachmann et al. (2012), Nickerson et al. (2012)
VPS-HOPS	Exosomes	Rab5 to Rab7 conversion	Rink et al. (2005)
Rab7	Exosomes	Early endosome maturation	Rink et al. (2005), Poteryaev et al. (2010)
HRS	Exosomes	ESCRT-0 subunit; ubiquitin binding; TSG101 interaction	Bache et al. (2003a, b), Pornillos et al. (2003), Takahashi et al. (2015)
STAM	Exosomes	ESCRT-0 subunit; mono-ubiquitin binding	Bache et al. (2003b), Takahashi et al. (2015)
TSG101	Exosomes Ectosomes	ESCRT-I subunit; HRS/ALIX interaction; ubiquitin binding	Katzmann et al. (2001), Bache et al. (2003a), Pornillos et al. (2003), Strack et al. (2003),

(continued)

Table 2.1 (continued)

Protein/lipid	EV subtype	Function	References
			Rauch and Martin-Serrano (2011), Nabhan et al. (2012)
VPS28	Exosomes	ESCRT-I subunit; VPS36 interaction; ubiquitin binding	Katzmann et al. (2001), Teo et al. (2006)
VPS37	Exosomes	ESCRT-I subunit; UBAP1 interaction	Curtiss et al. (2007), Colombo et al. (2013)
UBAP1	Exosomes	ESCRT-I subunit; VPS37 interaction; ubiquitin binding	Curtiss et al. (2007), Agromayor et al. (2012)
VPS22	Exosomes	ESCRT-II subunit; VPS25 interaction; ILV budding	Teo et al. (2004), Malerød et al. (2007), Wollert and Hurley (2010)
VPS25	Exosomes	ESCRT-II subunit; VPS22/VPS36/CHMP6 interaction; ILV budding	Teo et al. (2004), Wollert and Hurley (2010), Colombo et al. (2013)
VPS36	Exosomes	ESCRT-II subunit; VPS25/VPS28/CHMP6 interaction; ILV budding	Babst et al. (2002a, b), Teo et al. (2004, 2006), Wollert and Hurley (2010)
CHMP1	Exosomes Ectosomes	ESCRT-III subunit; IST1 interaction; membrane neck scission	Rauch and Martin-Serrano (2011), Colombo et al. (2013), Schöneberg et al. (2017)
CHMP2	Exosomes Ectosomes	ESCRT-III subunit; VPS4 interaction; membrane neck scission	Babst et al. (2002a, b), Fabrikant et al. (2009), Wollert et al. (2009), Rauch and Martin-Serrano (2011)
CHMP3	Exosomes Ectosomes	ESCRT-III subunit; VPS4 interaction; membrane neck scission	Babst et al. (2002a, b), Wollert et al. (2009), Rauch and Martin-Serrano (2011)
CHMP4	Exosomes Ectosomes	ESCRT-III subunit: ALIX interaction; ESCRT-III membrane association; membrane neck scission	Babst et al. (2002a, b), Katoh et al. (2003), McCullough et al. (2008), Wollert et al. (2009), Rauch and Martin-Serrano (2011)
CHMP5	Exosomes Ectosomes	ESCRT-III subunit; membrane neck scission	Rauch and Martin-Serrano (2011), Colombo et al. (2013), Son et al. (2019)
CHMP6	Exosomes Ectosomes	ESCRT-III subunit; VPS25/VPS36 interaction; ESCRT-III membrane association; membrane neck scission	Babst et al. (2002a, b), Teo et al. (2004), Wollert et al. (2009), Rauch and Martin-Serrano (2011)
IST1	Exosomes Ectosomes	ESCRT-III subunit; CHMP1 interaction; ESCRT-III assembly	Rauch and Martin-Serrano (2011), Frankel et al. (2017), Schöneberg et al. (2017)
ALIX	Exosomes Ectosomes	ESCRT accessory component; TSG101/CHMP4/Syntenin interaction	Katoh et al. (2003), Strack et al. (2003), McCullough et al. (2008), Rauch and Martin-Serrano (2011), Baietti

(continued)

Table 2.1 (continued)

Protein/lipid	EV subtype	Function	References
			et al. (2012), Nabhan et al. (2012)
VPS4	Exosomes	ESCRT accessory component; CHMP2/CHMP3/VTA1 interaction; ESCRT-III disassembly	Babst et al. (2002a, b), Wollert et al. (2009), Yang and Hurley (2010)
VTA1	Exosomes	ESCRT accessory component; VPS4 interaction; ESCRT-III disassembly	Yang and Hurley (2010)
Syntenin	Exosomes	ALIX interaction; ILV budding	Baietti et al. (2012)
Syndecan	Exosomes	Syntenin-ALIX recruitment, ILV budding	Baietti et al. (2012)
Sphingomyelin	Exosomes	Lipid raft formation	Ando et al. (2015)
nSMase	Exosomes	Sphingomyelin cleavage	Trajkovic et al. (2008)
Ceramide	Exosomes	ILV budding	Trajkovic et al. (2008)

cancer and human gastric carcinoma among others, revealed secretion of migrasomes, where the process of biogenesis is dependent on the polymerisation of actin filaments, enrichment of integrins as well as the formation of microdomains containing clusters of cholesterol and tetraspanins (TSPANs), in particular TSPAN4 (Table 2.1) (Ma et al. 2015; Wu et al. 2017; Huang et al. 2019; Zhao et al. 2019b). Migrasomes are highly abundant in N-deacetylase and N-sulfotransferase 1 (NDST1), phosphatidylinositol glycan anchor biosynthesis class K (PIGK), carboxypeptidase Q (CPQ) and EGF domain-specific O-linked N-acetylglucosamine transferase (EOGT) (Zhao et al. 2019b). Agreeing with this notion, previous reports show that NDST1 and CPQ are implicated in cell migration and tumour invasiveness, whereas PIGK and EOGT are involved in cell adhesion and cell-ECM interaction, respectively (Zacks and Garg 2006; Sakaidani et al. 2011; Lee et al. 2016; Qazi et al. 2016). Although these proteins, along with TSPAN4, may serve as potential markers for migrasomes, the overall physiological functions as well as the precise processes involved in the biogenesis of migrasomes remain to be explored (Ma et al. 2015; Zhao et al. 2019b).

Ectosomes/Shedding Microvesicles

Ectosomes or shedding microvesicles are a subtype of EVs that range from 100 to 1000 nm in diameter and are secreted into the ECM upon scission of plasma membrane evagination (Kalra et al. 2016). Ectosomes have shown to encapsulate a myriad of cargo components, for example, EGFR, matrix metalloproteinases (MMPs) and vascular epithelium growth factor (VEGF), suggesting their

implications in cell growth, ECM remodelling and angiogenesis among others (Surman et al. 2017).

The biogenesis of ectosomes is primarily achieved through coordinated alterations in the phospholipid distribution within the plasma membrane as well as the contractility of cytoskeletal components (Table 2.1) (Kalra et al. 2016). During the formation of ectosomes, floppase such as ATP binding cassette transporter 1 (ABCA1) mediates the exoplasmic translocation of PS, thereby inducing asymmetric distribution of phospholipids, which, in turn, results in structural imbalance within the plasma membrane (Oram and Vaughan 2000; Albrecht et al. 2005; Hankins et al. 2015; Bevers and Williamson 2016). Furthermore, the binding of GTP to a member of the small GTPase family, ARF6, initiates a downstream signalling cascade that results in contraction of actin-myosin, allowing scission of ectosomes from the plasma membrane. In detail, the binding of GTP to ARF6 results in activation of phospholipase D (PLD) to aid in recruitment and activation of extracellular signal-regulated kinase (ERK) at the plasma membrane, which subsequently phosphorylates myosin light chain kinase (MLCK). Upon activation, MLCK phosphorylates MLC, thereby inducing contraction of actin-myosin (Muralidharan-Chari et al. 2009). Interestingly, the ESCRT machinery that gives rise to exosomes also partakes in ectosome biogenesis. During arrestin domain-containing protein 1 (ARRDC1)-mediated formation of ectosomes, TSG101 of ESCRT-I and apoptosis-linked gene 2 interacting protein (ALIX) associates with ARRDC1 via its PSAP and PPXY motifs, respectively. ARRDC1 also interacts with neural precursor cell expressed, developmentally downregulated 4 (NEDD4) via PPXY motif during ectosome biogenesis, suggesting a ubiquitin-based cargo sorting mechanism (Rauch and Martin-Serrano 2011; Nabhan et al. 2012; Anand et al. 2018). ALIX then interacts with ESCRT-III complex to mediate membrane scission, resulting in secretion of ectosomes into the ECM (Rauch and Martin-Serrano 2011).

Exosomes

Whilst the current understanding supports that exosomes perform various cellular functions via intercellular communication, they were initially believed as mere waste secreted by cells (Johnstone et al. 1987). Indeed, as part of the eukaryotic endomembrane system, biogenesis of exosomes and autophagic processes occur in conjunction to assist in the maintenance of cellular homeostasis (Hessvik et al. 2016). The biogenesis of exosomes involves endocytosis and subsequent MVB formation. MVBs harbour intraluminal vesicles (ILVs) that are either degraded by lysosomes or exocytosed into the ECM as exosomes (Kalra et al. 2016). Exosomes were first identified in the early 1980s, where two independent studies indicated that differentiating reticulocytes utilise vesicles of endosomal origin to shed transferrin receptors (Harding et al. 1983; Pan and Johnstone 1983). A growing body of evidence has since suggested that various cell types secrete exosomes that enclose functional biomolecules (Liem et al. 2017). For example, B-lymphocytes and

dendritic cells secrete exosomes that are composed of T-cell stimulatory molecules to initiate immune response (Raposo et al. 1996; Zitvogel et al. 1998). Glial and neuronal cells of the central nervous system also release EVs that express specific markers of endosomal origin to augment signal transduction (Potolicchio et al. 2005; Lachenal et al. 2011; Chivet et al. 2014; Zappulli et al. 2016). Furthermore, mesenchymal stem cells derived from the bone marrow utilise exosomes to transfer functional mRNA that code for cell cycle regulators and growth factor receptors to promote tissue regeneration (Tomasoni et al. 2013; Grange et al. 2019).

A number of distinct cellular processes and coordination of various molecular machineries are required to regulate the biogenesis and secretion to exosomes (Table 2.1). Exosomes biogenesis begins with the internalisation of extracellular macromolecules and cell surface proteins following endocytosis (Colombo et al. 2014; Kalra et al. 2016). Upon scission of the membrane, biomolecules are enclosed within endocytic vesicles, which then fuse with early endosomes. This fusion process is largely driven by small GTPases Rab5/vacuolar protein sorting-associated protein 21 (VPS21) (Gorvel et al. 1991; Lachmann et al. 2012; Nickerson et al. 2012). Subsequent maturation from an early to late endosomal compartment, such as the MVB, occurs via class C VPS-vacuolar/lysosomal homotypic fusion and vacuole protein sorting (HOPS) complex-mediated replacement of Rab5 to Rab7 at the endosomal membrane (Rink et al. 2005; Poteryaev et al. 2010). Moreover, maturing endosomes reside nearby to other intracellular organelles such as trans Golgi network and the endoplasmic reticulum (ER) to allow for exchange, recycling as well as modification of biomolecular cargo (Tu et al. 2020). Agreeing with this notion, recent findings suggest that, throughout the maturation and trafficking, an early endosome remains in physical contact with the ER, to assist in enzymatic modification of the endosomal cargo (Friedman et al. 2013). The process of endosome maturation is accompanied by invagination of the endosomal membrane that gives rise to ILVs, which occurs via ESCRT-dependent or -independent pathway (Piper and Katzmann 2007; Trajkovic et al. 2008; Colombo et al. 2013). Furthermore, it has been illustrated that tetraspanins also play a crucial role in ILV formation, particularly during the ESCRT-independent pathway (van Niel et al. 2011; Edgar et al. 2014).

ESCRT-Dependent Pathway of ILV Biogenesis

ESCRTs are highly conserved multi-protein complexes, comprising of ESCRT-0, I, II and III, that associate with each other as well as accessory proteins to mediate ILV formation (Schmidt and Teis 2012). The ESCRT-dependent biogenesis of exosomes has been extensively studied by Colombo et al. through RNA interference of various constituents of ESCRT complexes (Colombo et al. 2013). In HeLa cells, knockdown of hepatocyte growth factor regulated tyrosine kinase substrate (HRS), signal transducing adaptor molecule 1 (STAM1) of ESCRT-0 and TSG101 of ESCRT-I resulted in attenuated secretion of exosomes. Interestingly, knockdown of ESCRT accessory

protein, ALIX, resulted in changes in exosomal cargo contents (Colombo et al. 2013). In breast cancer cells, ALIX has been previously alluded to partake in packaging of syndecan into exosomes through interactions with syntenin via LYPX(n)L motif and, in doing so, regulates the formation of exosomes (Baietti et al. 2012).

The early endosomal membrane is highly abundant in phosphatidylinositol 3-phosphate (PI(3)P), whose presence allows for the recruitment and binding of HRS of ESCRT-0 through FYVE domain (Kutateladze 2006). The subunits of ESCRT-0, HRS and STAMs, directly interact with each other and bind to mono-ubiquitinated cargo for sequestration (Bache et al. 2003b; Takahashi et al. 2015). HRS also harbours PSAP motif, which facilitates the recruitment and association of ESCRT-0 with ESCRT-I via TSG101 (Bache et al. 2003a; Pornillos et al. 2003). Subsequently, solenoid of overlapping ubiquitin-associated domain present on ubiquitin-associated protein 1 (UBAP1) of ESCRT-I allows the sorting of mono- and di-ubiquitinated cargo into ILVs (Agromayor et al. 2012). VPS36, a subunit of ESCRT-II, contains GLUE domain, via which it interacts with VPS28 of ESCRT-I, thereby mediating the recruitment of ESCRT-II and concomitant invagination of the endosomal membrane (Teo et al. 2006; Wollert and Hurley 2010). VPS36 then interacts with charged multivesicular body protein 6 (CHMP6) of ESCRT-III to facilitate the scission of the endosomal membrane neck (Babst et al. 2002a, b, Wollert and Hurley 2010). Additionally, the recruitment of ESCRT-III to the endosomal membrane may also be conducted by Alix through its association with CHMP4, in particular CHMP4B, of ESCRT-III via Bro1 domain and simultaneously with TSG101 of ESCRT-I (Katoh et al. 2003; Strack et al. 2003; McCullough et al. 2008). VPS4, upon recruitment by CHMP2-CHMP3 subcomplex of ESCRT-III, mediates the dissociation and recycling of ESCRT-III components for further ILV biogenesis (Babst et al. 2002a, b).

ESCRT-Independent Pathway of ILV Biogenesis

In mammalian cells, MVBs continue to develop without the presence of major constituents of ESCRT complexes, suggesting the occurrence of ILV biogenesis in an ESCRT-independent manner. However, MVBs that form in the absence of ESCRT machinery appear larger in size and encapsulate less number of ILVs that are also irregularly shaped and sized (Stuffers et al. 2009). Moreover, it has been previously reported that oligodendroglial precursor cells, that lack the expression of functional ESCRT components, secrete exosomes enriched in cholesterol, ceramide and sphingolipids such as sphingomyelin (Trajkovic et al. 2008). Indeed, the ESCRT-independent biogenesis of ILVs requires the initial clustering of sphingomyelin and cholesterol into lipid rafts. Upon formation of lipid rafts, sphingomyelin undergoes neutral sphingomyelinase (nSMase)-mediated hydrolytic cleavage to give rise to ceramides (Trajkovic et al. 2008; Ando et al. 2015). In line with this notion, studies have demonstrated that administration of nSMase inhibitor,

GW4869, results in a decreased number of secreted exosomes (Chairoungdua et al. 2010; Essandoh et al. 2015; Nojima et al. 2016; Menck et al. 2017). Structurally, ceramides are inverted cone-shaped, thus the accumulation of ceramides into microdomains at the endosomal membrane culminates in spontaneous inward budding to drive the formation of ILVs (Janmey and Kinnunen 2006; Trajkovic et al. 2008; Castro et al. 2014).

Role of Tetraspanins in Biogenesis of Exosomes

It is well established that EVs are enriched with tetraspanins (Yáñez-Mó et al. 2009; Andreu and Yáñez-Mó 2014). Tetraspanins are a family of relatively small integral membrane proteins that contain four transmembrane domains (Charrin et al. 2009). They are present in many metazoan cells and 33 different proteins have been identified in humans (Stipp et al. 2003; Charrin et al. 2009). They are capable of forming tetraspanin-enriched microdomains (TEMs) by homo or hetero associations among themselves or with a plethora of other transmembrane receptors and membrane-associated proteins such as integrin, immunoglobulin superfamily receptors and MMPs (Rubinstein et al. 1996; Berditchevski 2001; Boucheix and Rubinstein 2001; Stipp et al. 2001; Charrin et al. 2003; Barreiro et al. 2005; Yáñez-Mó et al. 2009, 2011; Mazurov et al. 2013). The abundance of tetraspanins found in EVs suggests a potential functional role for them in EV biogenesis (Andreu and Yáñez-Mó 2014).

As discussed earlier, biogenesis of exosomes takes place via ESCRT-dependent or independent pathways. Tetraspanins evidently play a vital role in the ESCRT-independent mechanism of exosomes biogenesis (Fig. 2.2). CD63, a tetraspanin most often used as a marker of exosomes, reportedly participates in ESCRT-independent sorting of the luminal domain of premelanosome protein (PMEL), in melanosomes, into ILVs but not in the canonical ESCRT-dependent mechanism (van Niel et al. 2011). Moreover, exosomes production was significantly increased upon knockdown of CD63 in B lymphoblastoid cells, allowing these cells to be consistently recognised by $CD4^+$ T cells (Petersen et al. 2011). In brain tissues of mice with Alzheimer's disease, elevated TSPAN6 levels enhanced the size of endosomes and increased number of ILVs, which resulted in accelerated secretion of exosomes. Moreover, increased TSPAN6 levels were responsible for the impaired degradation of proteins due to diminished fusion of autophagosomes and lysosomes (Guix et al. 2017).

Further to exosomes, tetraspanins are also known to regulate plasma membrane-derived EV formation. CD81 and CD82 have been shown to play a critical role in the development of membrane protrusions, where CD81 induces microvilli formation and extension while CD82 abrogates them (Bari et al. 2011). The discovery of CD82, CO-029 and TSPAN1 in luminal vesicles secreted from intestinal microvilli, suggested the enrichment of these tetraspanins in the microvilli (McConnell et al. 2009). Furthermore, CD9 and CD63 have been discovered on the microprotrusions

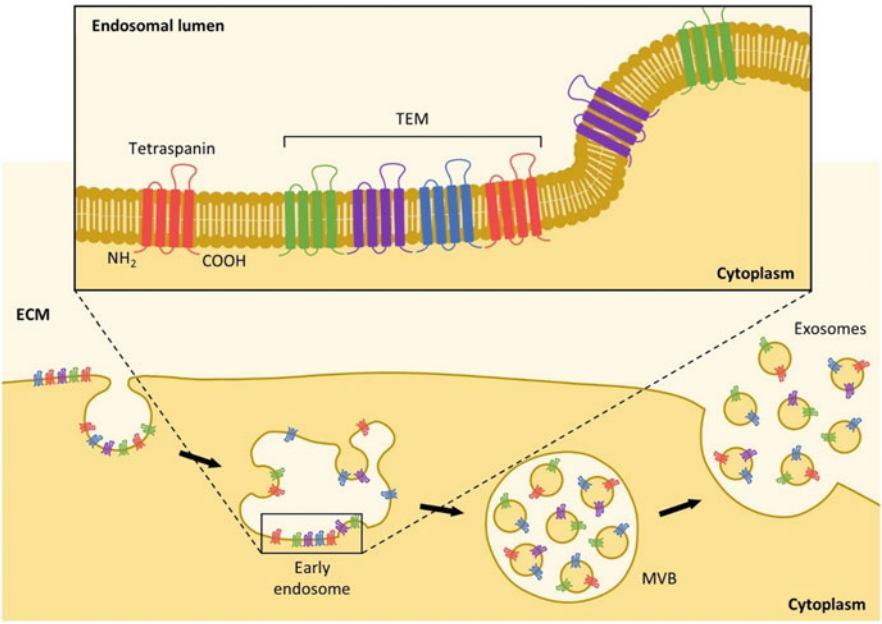

Fig. 2.2 Tetraspanins in the biogenesis of exosomes. Tetraspanins contain four transmembrane domains with both N and C termini located intracellularly. Tetraspanins assemble into tetraspanin-enriched microdomains in association with other membrane-associated proteins. Upon endocytosis, the internalised tetraspanin-enriched microdomains localise to the endosomal membrane and subsequently packaged into intraluminal vesicles that are present within multivesicular bodies. Hence, the secreted exosomes demonstrate an enrichment in certain tetraspanins. *ECM* extracellular matrix; *TEM* tetraspanin-enriched microdomain; *MVB* multivesicular body; NH_2 N-terminus; *COOH* C-terminus

of activated platelets (Brisson et al. 1997; Israels and McMillan-Ward 2007). These evidences suggest that altered membrane morphogenesis may be brought about by the TEMs on the plasma membrane, by potentially modifying the actin reorganisation via interactions with Rho GTPases (Hemler 2005; Bari et al. 2011; Richardson et al. 2011; Zhang et al. 2011).

Tetraspanins may also regulate cargo changes in EVs. For instance, CD9 knock-down and CD151 overexpressing prostate cancer cells demonstrated an altered proteome in their EVs, which caused enhanced cell migratory and invasive capabilities upon incubation with non-tumorigenic prostate cells (Brzozowski et al. 2018). Furthermore, the protein-protein interaction network of TEM has a significant influence on the exosomal proteome, where 45% of the protein cargo in exosomes was part of the interactome of the TEM components. Moreover, certain tetraspanin-associated molecules, including Rac, were found to be excluded from exosomes cargo in cells of CD81-deficient animals (Perez-Hernandez et al. 2013). Similarly, CD81- and CD9- dependent sorting of cytosolic proteins such as β-catenin into exosomes has been known to modulate Wnt signalling activity in dendritic cells

(Chairoungdua et al. 2010). It has further been demonstrated that CD81, in cancer-associated fibroblasts, is responsible for the sorting of Wnt 11 into exosomes. Tumour stroma demonstrated an induction in Wnt-PCP pathway activity upon internalisation of these vesicles, leading to increased cell migration and metastasis (Luga et al. 2012). Trafficking of metalloproteinases are also influenced by tetraspanins. The association of CD9 with metalloproteinase CD10 augments the packaging of CD10 into exosomes, where the relocalisation of CD10 from the plasma membrane to exosomes may play a pivotal role in the maturation of B lymphocytes in the tumour microenvironment (Mazurov et al. 2013). While the role of tetraspanins in EV biogenesis and cargo sorting are beginning to emerge, several biological functions of this family of proteins are poorly understood.

Secretion of Exosomes

Secretion of exosomes is a tightly controlled non-random process where vesicles of endocytic origin are released into the extracellular environment (Hannafon and Ding 2013). Upon formation of MVBs, they may be predestined for degradation intracellularly by fusion with lysosomes or be secreted out from the cell by fusion with plasma membrane leading to the release of exosomes (Kowal et al. 2014). The regulation of this latter exocytosis process is largely governed by Rab GTPases, microtubule and actin cytoskeleton and Soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE) proteins (Rodriguez-Boulan et al. 2005; Kowal et al. 2014).

The Rab family of small GTPases play a major role in multiple aspects of vesicular trafficking, including vesicle formation, movement of MVBs intracellularly and disembarking of the vesicles to their respective target compartments for degradation or membrane fusion leading to exocytosis (Stenmark 2009). A protein silencing study has unveiled that knockdown of RAB5A, RAB9A and RAB2B resulted in decreased secretion of exosomes (Ostrowski et al. 2010). Furthermore, RAB11 and RAB35 have been portrayed in association with early sorting of endosomal compartments for exocytosis (Stenmark 2009). Among the other Rab proteins, the roles of RAB27A and RAB27B in MVB docking at the plasma membrane have been well-characterised. While silencing of RAB27A increased the size of MVBs, silencing of RAB27B induced redistribution of MVBs towards the perinuclear region of the cell, demonstrating that the two isoforms play unique but complementary roles in the regulation of exosomes secretion (Ostrowski et al. 2010).

The final step of exosomes release involves MVB fusion with the plasma membrane, which is thought to be regulated by SNARE proteins (Jahn and Scheller 2006). In order to form the stable SNARE complex essential for exocytic release of vesicles, the membrane of the MVB needs to be comprised of v-SNAREs, while the cell membrane should have t-SNAREs (Yang et al. 2019). Vesicle-associated

membrane protein (VAMP) 2, VAMP3, VAMP7, and VAMP8 have been characterised as v-SNAREs that regulate exocytosis in multiple tumour cells, while synaptosome-associated protein (SNAP) 23 has been identified as a crucial t-SNARE that localises to the plasma membrane (Zhu et al. 2015; Wei et al. 2017). Furthermore, SNAP23 activation is mediated by phosphorylation, which promotes the assembly of the SNARE complex, resulting in facilitation of exosomes secretion (Puri and Roche 2006). The subject of biogenesis and secretion of exosomes is still under active investigation and future research may assist in extending our current knowledge.

Exomeres

Exomeres are secretory nanoparticles that are less than 50 nm in diameter (Chuo et al. 2018; Théry et al. 2018). The extracellular presence of exomeres was first described in 2018 by Zhang et al. and the exact processes involved in the biogenesis of exomeres is currently unknown (Zhang et al. 2018). Interestingly, exomeres are highly enriched in calreticulin (CALR), α-mannosidase 2 (MAN2A1), hexosaminidase B (HEXB), neutral α-glucosidase AB (GANAB) and mammalian target of rapamycin complex 1 (mTORC1), hinting that the uptake of exomeres potentially alters the glycosylation and metabolic activity in recipient cells (Zhang et al. 2018). Moreover, amyloid precursor protein (APP) as well as β-galactoside α2,6-sialytransferase 1 (ST6GAL1) and amphiregulin (AREG) are abundantly present in exomeres, suggesting that exomeres may have a potential role in pathological events such as Alzheimer's and cancer metastasis, respectively (Zhang et al. 2019). Uniquely, the encapsulating lipid bilayer structure, as well as the expression of ESCRT components, are proposed to be absent in exomeres, suggesting that exomeres may not originate directly from the plasma membrane or through the endocytic pathway unlike other subtypes of EVs. In agreement with this notion, the subcellular localisation of proteins enriched in exomeres revealed that they are closely associated with the ER, mitochondria as well as cytoskeletal microtubules (Zhang et al. 2018). Despite these findings, the participation of these subcellular components in the biogenesis of exomeres as well as the exact biological functions of exomeres remain currently unknown.

Concluding Remarks

EVs are vesicles produced by cells and subsequently secreted out into the ECM (Zhao et al. 2019a). The primary function of EVs is proposed to be the transfer of biomolecular cargo from host to recipient cells in order to mediate intercellular communication (Kalra et al. 2016; Fonseka et al. 2019). Since discovery, it has been highlighted that EVs play essential roles, not only in the maintenance of normal

physiology, but also in mediating pathological conditions, such as inflammation, cardiovascular and renal disease as well as cancer among many others. Due to their significant contributions during pathophysiology, EVs have attracted significant interest in clinical applications. Owing to this, many clinical benefits of EVs have been unveiled, allowing them to be exploited as potential modes of therapeutic delivery, target of disease inhibition as well as biomarkers to determine disease progression (Yuana et al. 2013; Koniusz et al. 2016; Chitti et al. 2018; Ståhl et al. 2019). However, further understanding on the exact molecular pathways that give rise to different subtypes of EVs are needed. To successfully use EVs in clinical settings, it is imperative to gain a more in-depth insight into the underlying molecular machineries that govern the biogenesis of EVs.

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Chapter 3
The Role of Post-Translational Modifications in Targeting Protein Cargo to Extracellular Vesicles

Ishara Atukorala and Suresh Mathivanan

Abstract Extracellular vesicles (EVs) are naturally occurring nanoparticles that contain proteins and nucleic acids. It is speculated that cells release EVs loaded with a selective cargo of proteins through highly regulated processes. Several proteomic and biochemical studies have highlighted phosphorylated, glycosylated, ubiquitinated, SUMOylated, oxidated and palmitoylated proteins within the EVs. Emerging evidences suggest that post-translational modifications (PTMs) can regulate the sorting of specific proteins into EVs and such proteins with specific PTMs have also been identified in clinical samples. Hence, it has been proposed that EV proteins with PTMs could be used as potential biomarkers of disease conditions. Among the other cellular mechanisms, the endosomal sorting complex required for transport (ESCRT) is also implicated in cargo sorting into EVs. In this chapter, various PTMs that are shown to regulate protein cargo sorting into EVs will be discussed.

Keywords Extracellular vesicles · Exosomes · Post-translational modifications · Cargo sorting · Phosphorylation · Glycosylation · Ubiquitination · SUMOylation · Oxidation · ISGylation · Myristoylation · Palmitoylation

Introduction

Extracellular vesicles (EVs) are nanoparticles that are released by various cells (Kalra et al. 2016; Théry et al. 2018). Several subtypes of EVs including exosomes, ectosomes or shedding microvesicles, apoptotic bodies and large oncosomes are known to be secreted by cells (Kalra et al. 2016). In this chapter, for clarity, the term EVs will be used to collectively refer to all EV subtypes from here on. Based on the host cells, EVs carry a variety of cargo including nucleic acids, proteins, lipids and

I. Atukorala (✉) · S. Mathivanan
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia
e-mail: I.Atukorala@latrobe.edu.au

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metabolites (Mathivanan et al. 2012; Pathan et al. 2019). Owing to the presence of a lipid bilayer membrane, EVs can survive the degrading conditions during transport from the site of origin to the secondary sites and hence can protect the cargo (Corrado et al. 2013; Batrakova and Kim 2015; Sanwlani et al. 2020). Upon reaching secondary sites, EVs can be internalized by the recipient cells resulting in the non-selective transfer of the cargo which subsequently exerts downstream signaling events (Bang and Thum 2012; De Toro et al. 2015; Kalra et al. 2019; Pathan et al. 2019). Hence, EVs have been implicated in various pathological processes among which cancer progression has been the most well studied (Peinado et al. 2012; Hoshino et al. 2015; Zhang et al. 2017; Chitti et al. 2018). The cargo contained within EVs is of utmost importance to orchestrate various signaling pathways in the recipient cells and thereby play a significant role in pathological conditions (Thery et al. 2002; Kalra et al. 2016; Samuel et al. 2017; Anand et al. 2019).

Cargo sorting into EVs is a tightly controlled and non-random process where proteins and RNA are thought to be selectively packaged via various mechanisms (Anand et al. 2019). Among these cargo sorting mechanisms, post-translational modifications (PTMs) that are known to activate/inactivate various proteins in a context dependent manner, can also target proteins into EVs (Uy and Wold 1977; Moreno-Gonzalo et al. 2014; Anand et al. 2019). Several widely studied PTMs in EV cargo sorting include phosphorylation, ubiquitination and SUMOylation. It is also becoming evident that there can be more than one PTM that could regulate cargo sorting into EVs. Among the other molecular machineries, ESCRT machinery and its accessory proteins are known to play a major role in identifying and sorting proteins into multivesicular bodies (MVBs), which will be either subjected to proteosomal degradation following fusion with lysosomes or secretion into extracellular space via exocytosis or membrane fusion (Babst 2011; Henne et al. 2011; Colombo et al. 2013).

Several studies have reported the presence of proteins with PTMs in EVs. However, the importance of PTMs in the process of cargo sorting into EVs is poorly understood. This may be largely due to the technological limitations as many of the PTMs are known to be unstable and hence targeted biochemical analysis may be required. Consequently, additional research is warranted to identify how PTMs regulate cargo sorting into EVs and to understand the utility of EV contained proteins with unique PTMs as potential biomarkers for disease conditions. Nevertheless, this chapter discusses the currently available knowledge on PTMs implicated in EV cargo sorting (Fig. 3.1).

Phosphorylation

Phosphorylation is the most common PTM in proteins, which may activate or deactivate their function, affecting almost all fundamental cellular processes (Hunter 2000; Jeffery et al. 2015). This dynamic event is mediated by protein kinases, which catalyses the transfer of the γ-phosphate of ATP molecules to specific residues of

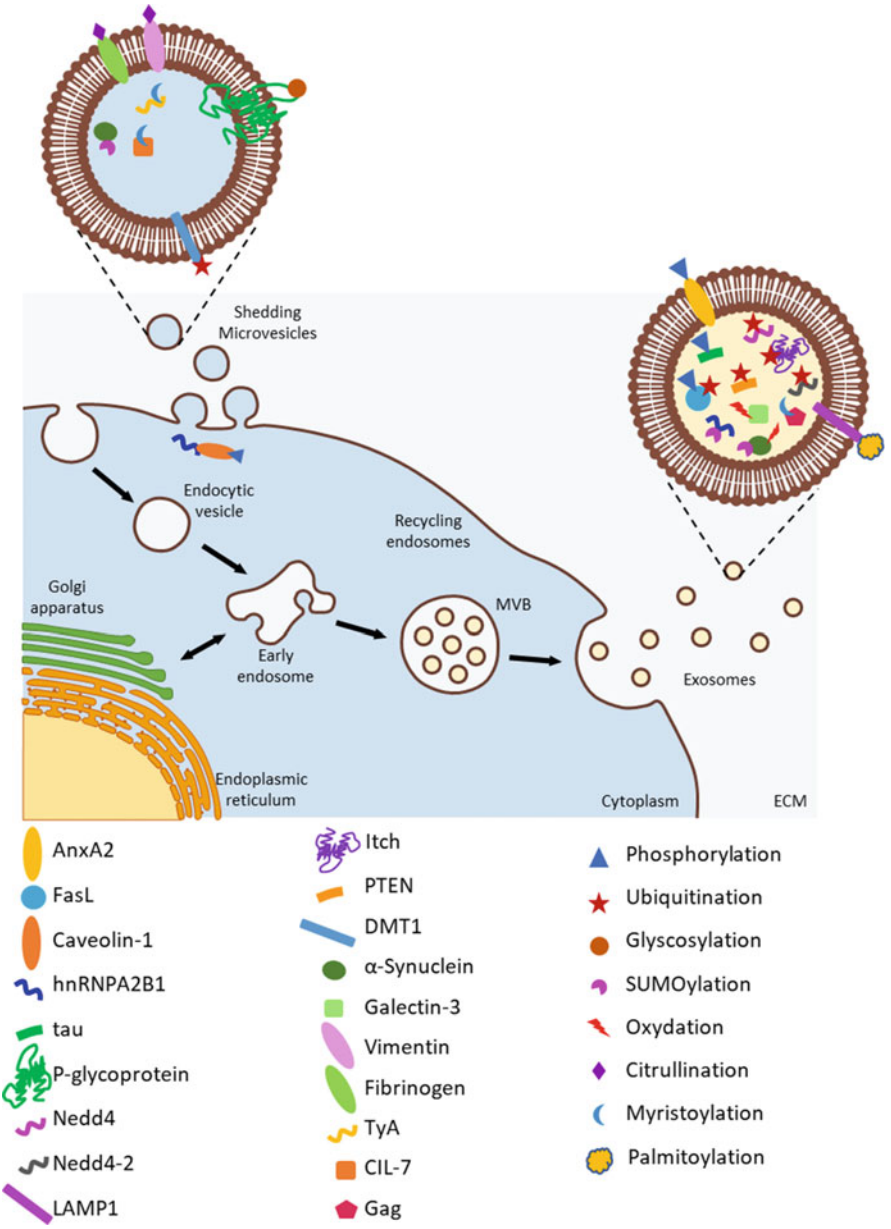

Fig. 3.1 Post-translational modifications in cargo sorting into EVs. PTMs play a significant role in the packaging of cargo, mainly proteins, to be carried within EVs. This figure summarizes the proteins that are discussed in this chapter in relation to PTMs implicated in cargo sorting

target proteins. In eukaryotes, proteins usually get phosphorylated at Serine, Threonine and Tyrosine residues (Manning et al. 2002). It has been estimated that at least 30% of the cellular proteome is phosphorylated at a given time on at least one amino acid (Pinna and Ruzzene 1996; Cohen 2000). Apart from the broad range of signal transduction processes mediated by phosphorylation, tagging of proteins with phosphate groups may also target them into EVs. For instance, phosphorylation of AnxA2 at Tyr-23 is necessary for its recruitment to plasma membrane rafts in a Ca^{2+}-dependent manner, which will be further targeted to the intralumenal vesicles (ILVs) of the multivesicular bodies (MVB) and get incorporated into EV membrane (Valapala and Vishwanatha 2011). Many proteomic studies performed on EVs derived from different cell types have shown an abundance of phosphoproteins in EV cargo (Chen et al. 2017; Rontogianni et al. 2019; Iliuk et al. 2020). A recent study has identified more than 10,000 phosphopeptides in plasma derived EVs (Chen et al. 2017).

Moreover, phosphorylation of proline-rich domain of FasL by Src family tyrosine kinases has been found to be important for internalisation of FasL into MVBs (Zuccato et al. 2007). Similarly, there is evidence for selective packaging of pAkt in EVs secreted by LIM1215 colorectal cancer cells upon activation of PI3K/Akt pathway with insulin (Liem et al. 2017). Another proteomic study performed on human urinary EVs has reported the identification of 19 phosphorylation sites corresponding to 14 proteins (Gonzales et al. 2009), however the dependency of phosphorylation on sorting these proteins into EVs is not clear. A protein implicated in Alzheimer disease (AD), tau (Vanderstichele et al. 2006), upon being phosphorylated at Thr-1801, is sorted into EVs and is capable of the horizontal propagation of the disease. Hence, cerebrospinal fluid (CSF)-derived EV phosphotau as opposed to whole CSF-derived phosphotau has been proposed as a biomarker for mild stages of AD (Saman et al. 2012). Apart from proteins, a recent study suggests the importance of caveolin-1 phosphorylation at Tyr-14 in order for the sorting of selected cargo of miRNAs into microvesicles by interacting with RNA binding protein hnRNPA2B1 (Lee et al. 2019).

Glycosylation

Protein glycosylation is a critical PTM in physiology as well as pathology. It involves the covalent binding of glycans or glycan chains to an oxygen (O-glycosylation) or nitrogen (N-glycosylation) atom of an amino acid residue of a protein, catalysed by glycotransferases (Reily et al. 2019). Several techniques such as hydrophilic interaction chromatography (HILIC), immobilized metal ion (usually TiO_2) chromatography and lectin affinity chromatography have been devised in the past to comprehensively identify glycopeptides (Chen et al. 2014). Recently, the development of a new analytical strategy which utilizes NP-HPLC with exoglycosidase digestion in combination with MALDI-TOF mass spectrometry and lectin blotting allowed for detailed profiling of glycans and elucidating their

structures in high resolution (Costa et al. 2018). Using this approach, it was revealed that EVs from HEK293 and human glioma cells exhibited an enrichment in complex N-glycans, but minor abundance of high mannose glycans in EVs compared to the cell membrane. Similarly, lectin microarray-based profiling reported the enrichment of mannose, α-2,6 sialic acid, polylactosamine, and complex N-linked glycans in EVs compared to that of the parent cell membrane. The study further suggested a major role for glycans in sorting of glycoproteins to EVs (Batista et al. 2011). Another glycopeptide analysis performed on urinary EVs identified 126 N-glycopeptides corresponding to 37 glycoproteins. Furthermore, 66 unique nonmodified N-glycan compositions and 13 sulphated or phosphorylated glycans were identified in the glycome profiles of EVs (Saraswat et al. 2015).

The importance of glycosylation in EVs was further substantiated by Saunderson et al. where they reported the enrichment of α2,3-linked sialic acid in B cell-derived EVs that allowed the identification of these EVs by CD169+ macrophages in lymph nodes and spleen (Saunderson et al. 2014). Moreover, uptake of EVs was reportedly increased by ovarian cancer cells proportional to the quantity of glycosylated proteins in EVs (Escrevente et al. 2011). Functionally, transfer of P-glycoprotein from chemoresistant tumour cells to chemosensitive cells have been observed via EVs. Intercellular transfer of EVs with P-glycoprotein between the cells induced resistance to chemotherapy in previously sensitive cells (Bebawy et al. 2009). Similarly, another study discovered the presence of glycosylated P-glycoprotein in EVs originating from chemoresistant ovarian cancer cells and upon horizontal transfer, EVs were able to induce chemoresistance in sensitive cells (Zhang et al. 2014). However, the importance of glycosylation for the protein sorting into EVs has not been clarified.

A comparative analysis of urinary EVs derived from classical galactosemia patients and healthy individuals demonstrated a shift from usually predominant high-mannose-type glycans in healthy individuals to complex-type N-linked glycosylation in galactosemia patients. This observation highlighted the potential utility of N-glycome studies in disease biomarker discovery (Staubach et al. 2012). Further to that, a recent study on breast cancer patients suggested that N-glycoproteome derived from plasma EVs as a promising source of cancer biomarker discovery compared to the whole plasma or serum derived glycoproteins. The study identified 1453 unique glycopeptides in total corresponding to 556 glycoproteins giving rise to a resourceful dataset (Chen et al. 2018).

Ubiquitination

Ubiquitination is a tightly regulated, highly versatile process which targets proteins for trafficking or proteasomal degradation (Bonifacino and Weissman 1998; Hicke 2001). It involves the covalent conjugation of a small protein unit of 76 amino acids (Wilkinson 1995). Ubiquitination is achieved through a cascade of enzyme-catalysed biological reactions that involves 3 classes of enzymes known as E1

(ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin-protein ligases) (Hochstrasser 1996; Weissman 1997). Polyubiquitination often tags proteins for proteasomal degradation (Hochstrasser 1996) or sometimes for trafficking (Martin et al. 2003). Monoubiquitination is known to regulate various cellular events including protein sorting for endocytosis, histone regulation and budding of retroviruses from plasma membrane (Hicke 2001).

Ubiquitination has been identified as a necessary PTM for sorting proteins into ILVs in MVBs. This process is mediated by the ESCRT complexes (Hirano et al. 2006; Henne et al. 2011). Among the definitive ubiquitin-binding motifs present throughout the ESCRT complex, ESCRT-0 highlights itself as the cargo binding motif due to the presence of several (5 domains identified in yeast ortholog) ubiquitinated cargo recognition modules (Hirano et al. 2006; Ren and Hurley 2010). Moreover, ESCRT-I and ESCRT-II complexes also possess ubiquitin binding domains, whereas no ubiquitin binding domain have been identified in ESCRT-III complex (Henne et al. 2011). However, the role of ESCRT machinery in cargo sorting into ILVs seems to be more complex and controversial (Villarroya-Beltri et al. 2014).

A proteomic study has reported that approximately 10% of the protein cargo in Myeloid-derived suppressor cell EVs is composed of ubiquitinated proteins in which, proinflammatory histones and other proinflammatory mediators were abundantly identified (Burke et al. 2014). There are multiple studies that reported the sorting of epidermal growth factor receptor (EGFR) into MVBs. Monoubiquitination of EGFR is identified by the ESCRT mechanism which facilitates the internalisation of EGFR into ILVs of the MVBs and transported to the lysosomes eventually (Katzmann et al. 2002; Jiang and Sorkin 2003). However, it has been demonstrated earlier that the monoubiquitination is dependent on another PTM, autophosphorylation of EGFR, which facilitates binding of ubiquitin ligases (Sorkin et al. 1992; Waterman et al. 2002).

A Nedd4 family-interacting protein 1 (Ndfip-1) overexpression study demonstrated the sorting of Nedd4, Nedd4-2, and Itch proteins into EVs in the presence of Ndfip-1, which are typically not secreted via EVs (Putz et al. 2008). Ndfip-1 is an adapter protein that leads to the ubiquitination of substrates even in the absence of PPxY motifs on them, to facilitate the direct binding of E3 ligases to achieve ubiquitination (Harvey et al. 2002; Shearwin-Whyatt et al. 2004; Mund and Pelham 2009). Furthermore, the tumour suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a generally cytoplasmic or nuclear localized protein, undergoes ubiquitination in the presence of Ndfip-1 and is sorted into EVs for secretion into extracellular environment (Putz et al. 2012).

Apart from endocytic pathways and EV secretion, ubiquitination is reportedly important in the secretion of a novel group of EVs, named arrestin-domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs). These membrane derived vesicles are known to originate due to the interaction of tumour susceptibility gene 101 protein (TSG101) with a PSAP motif in ARRDC1 (Nabhan et al. 2012; Anand et al. 2018). Though changes in protein cargo of EVs upon depletion of Arrdc1 has been reported, the importance of ubiquitination in the cargo

of these EVs has not been characterized yet (Anand et al. 2018). Moreover, divalent metal transporter 1 (DMT1) regulation in mouse gut explants was found to be achieved via ubiquitination assisted by Arrdc1 and Arrdc4 which act as Nedd4 E3 ligase adaptors. The subsequent ubiquitination of DMT1 results in its sorting from plasma membrane into EVs, which may be implicated as a mechanism of ion homeostasis (Mackenzie et al. 2016).

SUMOylation

Small ubiquitin-related modifier (SUMO) is a ubiquitin-like modifier, common to all eukaryotic organisms (Hanania et al. 1999). Human SUMO-1, SUMO-2 and SUMO-3 have been identified as the members of the SUMO family and they share a 50% sequence similarity (Müller et al. 2001). SUMOylation is the reversible process of covalently binding a SUMO group to a lysine residue of a target protein via a series of enzymatic reactions (Maejima and Sadoshima 2014). However, unlike ubiquitin, SUMOylation does not target proteins for degradation, but regulates their subcellular localisation or stabilizes the proteins (Melchior 2000; Müller et al. 2001). Even though there is a scarcity of articles about SUMOylation mediated cargo sorting into EVs, there are two key studies on the effect of SUMOylation in EV cargo sorting. Heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) is an RNA binding protein that takes part in EV sorting of miRNAs. It is reported that the sumoylated form of hnRNPA2B1 is present in EVs and the SUMOylation event is important in mediating the binding of hnRNPA2B1 to miRNAs (Villarroya-Beltri et al. 2013). Furthermore, sorting of α-Synuclein into EVs is known to be controlled by SUMOylation and this PTM sorts both cytosolic and transmembrane α-Synuclein to EVs. This SUMO-dependent sorting of α-Synuclein exploits the ESCRT machinery by interacting with phosphoinositols (Kunadt et al. 2015).

Moreover, there are evidences of interplay between SUMOylation and ubiquitination in relation to viral particle release from cells. It is established that HIV uses ESCRT machinery to release viral particles from cells in a fashion similar to secretion of EVs (Patters and Kumar 2018). Monoubiquitination of p6 domain of Gag polyprotein efficiently promotes its association with Alix and TSG101, ESCRT associated proteins, in order to promote the viral progeny release (Patnaik et al. 2000). However, competitive SUMOylation at the same Lys residue (Lys_{27}) of p6 domain can inhibit its ubiquitination, preventing its interaction with TSG101 and thereby triggering significant defects in the viral particle release (Gurer et al. 2005).

Oxidation

Protein oxidation can be a result of proteins reacting with a range of reactive oxygen, nitrogen and chlorine species (Harman et al. 1984). Moreover, thiol (–SH) groups are known to readily oxidise Cys residues on proteins (Ryan et al. 2014). Methionine oxidation to form methionine sulfoxide which may advance to methionine sulfone (Stadtman and Levine 2000) and tryptophan oxidation to hydroxytryptophan and further to formation of other species have been implicated in several age related pathologies (Kong et al. 1996). It has been demonstrated that induction of oxidative stress in human $CD14^+$ monocytes using an NADPH activity inducer resulted in the secretion of Galectin-3 via EVs, a protein otherwise contains no signalling sequences for secretion (Madrigal-Matute et al. 2014). Furthermore, γ-synuclein, an easily oxidized protein at Met38 and Tyr39, is secreted via EVs. The oxidized form is capable of further influencing α-synuclein aggregation, which is associated with Parkinson's disease and several other synucleinopathies within the cell as well as upon transportation via EVs to other glial cells (Surgucheva et al. 2012).

Citrullination

Citrullination, also known as deamination, is the process of irreversible conversion of a positively charged amino acid Arg to citrulline, reducing the net charge of proteins. The process can result in protein misfolding and increased hydrophobicity causing gain or more likely, loss of function of proteins (Tarcsa et al. 1996; Fert-Bober et al. 2015). The interest on citrullinated proteins was increased since its established role in rheumatoid arthritis (RA) and these citrullinated proteins have been identified as EV cargo in RA patients (Li et al. 2018). Skriner et al. reported the presence of an array of citrullinated proteins in synovial EVs derived from patients with RA, reactive arthritis, and osteoarthritis, establishing strong evidence of the role of protein citrullination in joint diseases. These proteins elicit autoantigen properties in RA (Skriner et al. 2006; Simpson et al. 2009). Furthermore, citrullinated vimentin and fibrinogen have been identified on the surface of EVs associated with immune complexes in RA synovial fluid, which act as autoantigens (Cloutier et al. 2013). However, further studies may facilitate the understanding of the importance citrullination in the process of sorting proteins as cargo into EVs.

ISGylation

Interferon (IFN)-stimulated gene 15 (ISG15) is a ubiquitin-like protein (Farrell et al. 1979) that contains two UBL domains. ISG15-mediated PTM of proteins is known as ISGylation where ISG15 is covalently conjugated to Lys residues of a protein in

reactions catalysed by enzymes such as UBE1L (UBA7), UbcH8, EFP and HERC5 (Villarroya-Beltri et al. 2016; Ageta and Tsuchida 2019). There are evidences of ISGylation mediated EV release and viral particle release, but their role in EV cargo sorting is poorly understood. The ability of ISGylation in the regulation of the ESCRT machinery has been revealed as ISG15 mediates blocking of viral budding from cells via ESCRT machinery (Okumura et al. 2006). Moreover, ISGylation of ESCRT protein TSG101 promoted the aggregation and colocalization of MVBs with lysosomes resulting in the consequent degradation of MVBs, thereby reducing the EV secretion (Villarroya-Beltri et al. 2016). However, there is no evidence to state that ISGylated TSG101 is secreted out via EVs as it has been observed that ISGylated TSG101 is more prone to lysosomal degradation (Villarroya-Beltri et al. 2016). Other proteins such as CHMP2A, CHMP4B and CHMP6 which are popular targets of ISGylation are known to be secreted out in EVs (Pincetic et al. 2010; Kuang et al. 2011; Sanyal et al. 2013), however, their ISGylated state in EVs is unclear.

Myristoylation

A lipid modification that involves the addition of the myristic acid via a covalent bond catalysed by N-myristoyltransferase is known as myristoylation. This lipid modification takes place exclusively at the N-terminal Gly in a protein (Boutin 1997). Introduction of a myristoylation tag to the yeast protein TyA facilitated higher order clustering of TyA, targeting it to the site of vesicle budding. The combination of the myristoylation tag along with a prenylation tag further caused the extracellular secretion of TyA via EVs from Jurkat T-cells (Shen et al. 2011). Furthermore, it is established that incorporation of retroviral Gag proteins into EVs is achieved via N-terminal myristoyl moiety in Gag which facilitates its anchoring in the inner leaflet of the plasma membrane (Briggs et al. 2004), allowing its subsequent incorporation into EVs (Fang et al. 2007). Moreover, ciliary protein CIL-7 was targeted as cargo to ciliary EVs in a myristoylation dependent manner *in vivo*, in a study that used *C. elegans* as the model organism. The study has further demonstrated the importance of an intact N-myristoylation motif for the function and sorting of CIL-7 (Maguire et al. 2015).

Palmitoylation

Palmitoylation is a PTM where protein lipidation takes place in a reaction catalysed by palmitoyl transferases and are known to target proteins into membrane microdomains (Fukata and Fukata 2010). In this process, generally, a fatty acid chain will be attached to the thiol side chain of a cysteine residue via a thioester linkage, known as the S-palmitoylation. The reversible nature of S-palmitoylation

makes it more versatile allowing the proteins to translocate among intracellular compartments (Charollais and Van Der Goot 2009; Fukata and Fukata 2010). On the other hand, N-palmitoylation modifies proteins irreversibly, but less abundantly, by the lipidation of cysteine or glycine residues of proteins via an amide linkage (Guan and Fierke 2011). Verweij et al. reported the importance of palmitoylation in sorting LAMP1 into EVs, at the known palmitoylation site of C78. Moreover, palmitoylation of LAMP1 initiated its endosomal membrane localisation and oncogenic signalling. Both these activities were unavailable for LAMP1 upon mutating the palmitoylation site (Verweij et al. 2015).

Concluding Remarks

EVs have been implicated in cell-to-cell communication and the functional aspects of EVs are largely regulated by the cargo (proteins, nucleic acids, lipids and metabolites). Therefore, characterising the EV cargo and understanding the precise mechanisms by which they are sorted into EVs are critical. Among the protein sorting mechanisms, PTMs have been shown to tag proteins for sorting into EVs. Even though several studies have highlighted the presence of proteins with various PTMs in EVs, whether a precise modification is critical for their packaging into EVs is poorly understood. Furthermore, studies have demonstrated the enrichment of proteins with certain PTMs in EVs isolated from patient plasma compared to plasma of healthy individuals. These observations led to speculations that EVs containing proteins with particular PTMs in systemic circulation may aid in disease propagation. Therefore, these EV proteins with unique modifications have been proposed as potential biomarkers for certain pathologies, which may serve as a reliable and viable source for diagnosis and prognosis.

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Chapter 4
Apoptotic Bodies: Mechanism of Formation, Isolation and Functional Relevance

Jascinta P. Santavanond, Stephanie F. Rutter, Georgia K. Atkin-Smith, and Ivan K. H. Poon

Abstract In the final stages of apoptosis, apoptotic cells can generate a variety of membrane-bound vesicles known as apoptotic extracellular vesicles (ApoEVs). Apoptotic bodies (ApoBDs), a major subset of ApoEVs, are formed through a process termed apoptotic cell disassembly characterised by a series of tightly regulated morphological steps including plasma membrane blebbing, apoptotic membrane protrusion formation and fragmentation into ApoBDs. To better characterise the properties of ApoBDs and elucidate their function, a number of methods including differential centrifugation, filtration and fluorescence-activated cell sorting were developed to isolate ApoBDs. Furthermore, it has become increasingly clear that ApoBD formation can contribute to various biological processes such as apoptotic cell clearance and intercellular communication. Together, recent literature demonstrates that apoptotic cell disassembly and thus, ApoBD formation, is an important process downstream of apoptotic cell death. In this chapter, we discuss the current understandings of the molecular mechanisms involved in regulating apoptotic cell disassembly, techniques for ApoBD isolation, and the functional roles of ApoBDs in physiological and pathological settings.

Keywords Apoptotic bodies · Apoptosis · Apoptotic body isolation · Apoptotic cell clearance · Extracellular vesicles · Intercellular communication

J. P. Santavanond · S. F. Rutter · I. K. H. Poon (✉)
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, VIC, Australia
e-mail: i.poon@latrobe.edu.au

G. K. Atkin-Smith (✉)
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, VIC, Australia

Inflammation Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia
e-mail: atkinsmith.g@wehi.edu.au

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Introduction

Similar to the ability of healthy cells to form extracellular vesicles (EVs), dying cells like apoptotic cells can also generate a variety of membrane-bound vesicles called apoptotic EVs (ApoEVs). Apoptosis is a well characterised form of programmed cell death which involves a cascade of tightly regulated processes ultimately leading to the activation of executioner caspases including caspases 3 and 7, which can then activate various downstream events (Cohen 1997; Nagata 2010). Although this process is vital to maintain tissue homeostasis, these apoptotic cells are rarely detected in physiological conditions due to rapid efferocytotic mechanisms (Henson and Hume 2006; Hochreiter-Hufford and Ravichandran 2013; Nagata 2018). In the final stages of apoptosis, apoptotic cells can dismantle and release apoptotic bodies (ApoBDs) through a process known as apoptotic cell disassembly. ApoBDs are a major subset of ApoEVs and are generally considered to range between 1 and 5 μm in diameter (Kerr et al. 1972; Poon et al. 2019). In addition to ApoBDs, smaller ApoEVs including apoptotic microvesicles (ApoMVs, described as 0.1–1 μm) (Buzas et al. 2014; Karpman et al. 2017) and exosome-like apoptotic vesicles (<150 nm) (Pavlyukov et al. 2018) have also been observed, however these two subsets of ApoEVs will not be discussed in detail in this review.

Apoptotic cell disassembly has been observed across a large number of cell types including T cells, monocytes, epithelial cells, fibroblasts, thymocytes and endothelial cells (Sebbagh et al. 2001; Coleman et al. 2001; Berda-Haddad et al. 2011; Poon et al. 2014a; Atkin-Smith et al. 2015). By using a combination of flow cytometry and time-lapse confocal microscopy, the complete disassembly process including blebbing, protrusion formation and ApoBD release can be monitored and accurately quantified (Poon et al. 2014a; Jiang et al. 2016). Although most studies have investigated the disassembly process under in vitro or ex vivo conditions, there is now increasing evidence of ApoBD formation in physiological conditions in vivo. For example, through live confocal imaging of various transgenic zebrafish embryos, apoptotic epithelial, endothelial and neuronal cells were shown to bleb and undergo rapid cell fragmentation into ApoBDs (Ham et al. 2010; Morsch et al. 2015; Zhang et al. 2018; Zhu et al. 2019; Brock et al. 2019). Furthermore, intravital imaging of murine lymph nodes revealed that apoptotic germinal centre B cells also undergo blebbing and fragmentation into smaller ApoBDs (Mayer et al. 2017). Together, in vivo imaging techniques have provided a fundamental insight into the occurrence of cell disassembly in vivo.

Although recent advances in the field have led to the direct visualisation of ApoBD formation under in vivo settings as mentioned above, the functional role of ApoBDs have predominantly been demonstrated in vitro. Specifically, these studies have shown that ApoBDs are important for two key processes including apoptotic cell clearance and intercellular communication. The ability of apoptotic cells to dismantle into smaller ‘bite-sized’ ApoBDs has been shown to enhance engulfment rates by phagocytes and may therefore aid the prevention of diseases associated with inadequate clearance of apoptotic debris such as systemic lupus

erythematosus (Gaipl et al. 2004; Tixeira et al. 2019). Furthermore, similar to other EV subtypes, ApoBDs have been shown to harbour a range of biomolecules including DNA, RNA and proteins, suggesting that ApoBDs may also have the ability to facilitate intercellular communication (Berda-Haddad et al. 2011; Wickman et al. 2013; Jiang et al. 2017). Although much progress has been made in this field, further studies are required to fully understand the functional significance of ApoBD formation and its potential implications in homeostatic and disease settings. This chapter will provide a comprehensive description of the known molecular mechanisms driving ApoBD formation, the methods used to isolate them and their functional roles.

Mechanisms of Apoptotic Cell Disassembly

It has become increasingly clear that ApoBD formation relies on several tightly regulated morphological steps orchestrated by a range of molecular factors and the rearrangement of cytoskeleton. This process, termed apoptotic cell disassembly, involves three main morphological steps including plasma membrane blebbing (step 1), the formation of thin apoptotic membrane protrusions (step 2) and finally, the fragmentation into individual ApoBDs (step 3) (Poon et al. 2014a; Atkin-Smith et al. 2015).

Step 1: Plasma Membrane Blebbing

The first step of apoptotic cell disassembly involves the formation of large circular bulges (known as blebs) on the surface of the apoptotic cell (Fig. 4.1). This step, termed plasma membrane blebbing, is a well-known hallmark of apoptosis (Wyllie et al. 1980) and is the most well-characterised step of the disassembly process. Previously, it has been proposed that hydrostatic pressure within apoptotic cells may work in concert with cytoskeletal rearrangement to cause inflation of membrane blebs (Charras et al. 2005). More specifically, the compressive stresses generated during actomyosin contraction was shown to increase intracellular hydrostatic pressure, causing cytosolic fluid to push out in regions where the membrane is weakly attached, resulting in bleb formation (Charras et al. 2005). At the molecular level, apoptotic plasma membrane blebbing is thought to be regulated by various kinases including the serine/threonine caspase-activated kinases p21 activated kinase 2 (PAK2), Lim domain kinase 1 (LIMK1) and Rho-associated kinase 1 (ROCK1) (Lee et al. 1997; Rudel and Bokoch 1997; Sebbagh et al. 2001; Coleman et al. 2001; Tomiyoshi et al. 2004). Previously, caspase 3 was shown to activate PAK2 in T cell, leukemia cell and epithelial cell lines by cleaving and separating its regulatory and catalytic domain (Lee et al. 1997; Rudel and Bokoch 1997). This leads to the activation of Rac1 and Cdc42, both of which are involved in regulating cytoskeletal

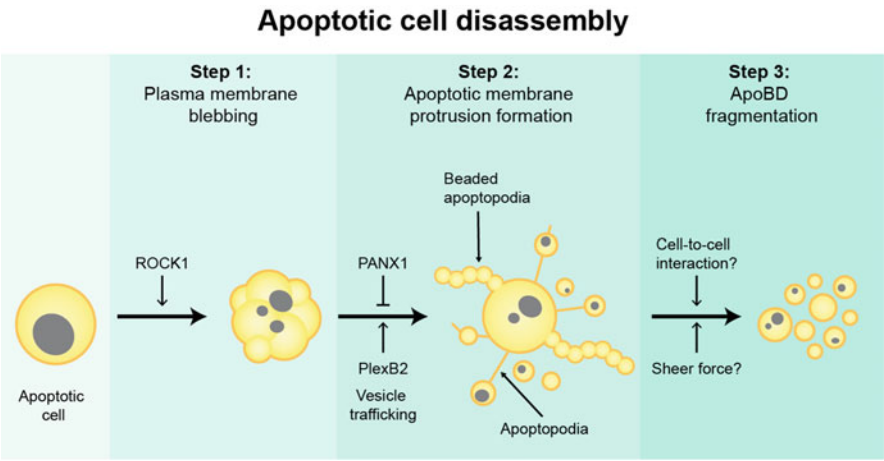

Fig. 4.1 Morphological steps and molecular mechanisms of apoptotic cell disassembly. Apoptotic membrane blebbing (step 1) is positively regulated by ROCK1. The formation of apoptotic membrane protrusions including apoptopodia and beaded apoptopodia (step 2) is negatively regulated by PANX1 channels and positively regulated by PlexB2 receptors and vesicle trafficking. Finally, step 3 includes the fragmentation of the cell and protrusions into an abundance of individual ApoBDs

reorganisation (Lee et al. 1997; Rudel and Bokoch 1997). Similarly, caspase 3 can also activate LIMK1 and is thought to regulate plasma membrane blebbing in T cells by deactivating cofilin and inhibiting depolymerisation and severance of actin filaments (Tomiyoshi et al. 2004). Finally, the third kinase that has been widely described as a key regulator of plasma membrane blebbing is ROCK1 (Sebbagh et al. 2001; Coleman et al. 2001). ROCK1 is activated by caspase 3 cleavage of its auto-inhibitory domain which in turn, leads to the phosphorylation of myosin light chain and triggers the actomyosin contraction that drives plasma membrane blebbing (Sebbagh et al. 2001; Coleman et al. 2001). Recently, a study has found that pharmacological inhibition of PAK2 or LIMK1 with FRAX-1036 and BMS-5, respectively, in apoptotic T cell, monocytic cell and epithelial cell lines did not alter membrane blebbing during apoptosis (Tixeira et al. 2019). However, pharmacological inhibition of ROCK1 activity (through Y-27632 and GSK-269962) in mouse fibroblast, human T cell, monocytic cell and epithelial cell lines led to a substantial decrease in apoptotic membrane blebbing (Sebbagh et al. 2001; Tixeira et al. 2019). Additionally, disrupting ROCK1 expression in Jurkat T cells using CRISPR/Cas9 technology also led to a significant decrease in cells undergoing blebbing during apoptosis (Tixeira et al. 2019). In comparison, disruption of PAK2 or LIMK1 expression had no effect on the ability of cells to undergo plasma membrane blebbing (Tixeira et al. 2019). Together, this demonstrates that ROCK1 is the key positive regulator of plasma membrane blebbing during apoptotic cell disassembly.

Step 2: Apoptotic Membrane Protrusion Formation

Following dynamic membrane blebbing, some cells can continue to generate long apoptotic membrane protrusions known as apoptopodia and beaded apoptopodia (Fig. 4.1). The formation of these apoptotic membrane protrusions has been observed in a wide variety of cell types including T cells and thymocytes, epithelial cells, monocytes, and fibroblasts (Poon et al. 2014a; Atkin-Smith et al. 2015; Caruso et al. 2019). It is also important to note that apoptopodia and beaded apoptopodia are generated exclusively during apoptosis. Apoptopodia, first identified in 2014, extrude from the plasma membrane following membrane blebbing and have a long string-like appearance (Poon et al. 2014a). Interestingly, ApoBD-like structures were also observed along the length or the ends of these protrusions demonstrating that apoptopodia can contribute to ApoBD formation by separating membrane blebs (Poon et al. 2014a). In comparison, beaded apoptopodia consists of several ApoBDs connected in a row with each measuring between 1 and 3 μm in diameter and can collectively stretch multiple times longer than the apoptotic cell (Atkin-Smith et al. 2015). Remarkably, the fragmentation of a single beaded apoptopodia strand can lead to the release of 10–20 individual ApoBDs that are generally uniformed in size (Atkin-Smith et al. 2015).

With the advancements in in vivo imaging techniques, a number of recent studies have captured various stages of apoptotic cell disassembly in vivo, namely plasma membrane blebbing and ApoBD formation (see Table 4.1). However, there is still a lack of studies that focus on examining in vivo protrusion formation and thus, further studies are required to investigate this phenomenon and to determine its functional significance in vivo. Although apoptotic membrane protrusions are predominately characterised under in vitro culture conditions with cells dying in suspension, a recent study has shown the ability of both apoptopodia and beaded apoptopodia to form in a 3D matrix model that are largely consisted of laminin and collagen IV (Caruso et al. 2019), suggesting that these protrusions have the potential to form in an in vivo environment. To date, a number of key molecular factors and processes have been implicated in regulating apoptotic membrane protrusion formation including Pannexin 1 (PANX1) channels, Plexin B2 (PlexB2) receptors, the cytoskeletal network and vesicular trafficking, and will be discussed further below.

Pannexin 1

Among the three members of the pannexin family (PANX1, 2 and 3), PANX1 is the most widely expressed and exists as a heptameric transmembrane protein which forms a pore-like structure in the plasma membrane (Deng et al. 2020). This channel has been implicated in a number of functions including pain sensing, inflammation and apoptotic cell clearance (Thompson et al. 2008; Elliott et al. 2009; Chekeni et al. 2010; Seminario-Vidal et al. 2011; Gulbransen et al. 2012; Billaud et al. 2015; Weaver et al. 2017; Medina et al. 2020). During apoptosis, PANX1 channels are

Table 4.1 Morphological steps of apoptotic cell disassembly observed in various settings

Cell and/or tissue type	Species	Experimental setting	Apoptotic stimuli	Apoptotic cell disassembly step observed	Apoptotic cell clearance observed	Apoptotic experimental validation	Detection method	References
T cell line (Jurkat)	*H. sapiens*	In vitro	Anti-Fas antibody and trovafloxacin	1–3	–	Annexin V staining and TO-PRO-3 uptake assay	Confocal microscopy and flow cytometry	Poon et al. (2014a), Tixeira et al. (2017), Caruso et al. (2019)
Thymus; thymocyte	*M. musculus*	Ex vivo	Dexamethasone treatment	1–3	–	Annexin V staining and TO-PRO-3 uptake assay	DIC microscopy and flow cytometry	Poon et al. (2014a)
Lymph node; T-cell	*M. musculus*	In vivo	Homeostatic cell death	3	–	Annexin V staining and activated caspase-3 staining	Intravital two-photon imaging	Garrod et al. (2012)
Thymus; thymocyte	*R. Rattus*	In vivo	Irradiation	1 and 3	–	–	Scanning electron microscopy	Ohyama et al. (1985)
Thymus; thymocyte	*M. musculus*	Ex vivo	UV irradiation and trovafloxacin	2 and 3	–	Annexin V staining	Confocal microscopy and flow cytometry	Caruso et al. (2019)
Monocytic cell line (THP-1)	*H. sapiens*	In vitro	UV irradiation and serum starvation	1–3	–	Annexin V staining and TO-PRO-3 uptake assay	DIC microscopy, confocal microscopy and flow cytometry	Atkin-Smith et al. (2015), Tixeira et al. (2017), Baxter

								et al. (2019), Caruso et al. (2019)
Monocytic cell line (U937)	*H. sapiens*	In vitro	Hydrogen peroxide and puromycin	1	–	Annexin V staining	Flow cytometry, SEM, TEM, and confocal microscopy	Bonanno et al. (2002)
Primary mouse monocyte	*M. musculus*	Ex vivo	UV irradiation	2 and 3	–	Annexin V staining	Confocal microscopy and flow cytometry	Caruso et al. (2019)
Spinal neuron	*D. rerio*	In vivo	Single cell UV ablation	1 and 3	–	Annexin V fluorescent reporter line	Confocal microscopy	Morsch et al. (2015)
Neural crest cells	*D. rerio*	In vivo	Single cell laser ablation, and homeostatic cell death	1 and 3	Yes	Annexin V fluorescent reporter line	Confocal microscopy	Zhu et al. (2019)
Spinal cord interneuron, optic vesicle, lens, retina, and Rohon beard neurons	*D. rerio*	In vivo	UV irradiation, camptothecin treatment and homeostatic cell death	1 and 3	–	Annexin V fluorescent reporter line and TUNEL staining	Confocal microscopy	Ham et al. (2010)
Skeletal muscle; myoblast	*M. musculus*	Ex vivo	Homeostatic cell death and serum starvation	1–3	Yes	Annexin V staining, cleaved caspase 3 assay, and TO-PRO-3 uptake assay	Immunohistochemistry	Hochreiter-Hufford et al. (2013)

(continued)

Table 4.1 (continued)

Cell and/or tissue type	Species	Experimental setting	Apoptotic stimuli	Apoptotic cell disassembly step observed	Apoptotic cell clearance observed	Apoptotic experimental validation	Detection method	References
Cardiac tissue	*M. musculus*	Ex vivo	Homeostatic cell death	1 and 3	–	TUNEL staining	Immunohistochemistry	Tran et al. (2002)
Hair follicle epithelium; basal epithelial cell	*M. musculus*	In vivo	Laser ablation	3	Yes	–	Intravital multiphoton microscopy and FACS	Mesa et al. (2015)
Epithelial cell line (A431)	*H. sapiens*	In vitro	UV irradiation	1–3	–	Annexin V staining	Confocal microscopy and flow cytometry	Caruso et al. (2019)
Endothelial cell line (HUVEC)	*H. sapiens*	In vitro	Serum starvation and TNFα	3	–	Annexin V staining and cleaved caspase 3 staining	Confocal microscopy and flow cytometry	Berda-Haddad et al. (2011)
Endothelial cells	*D. rerio*	In vivo	Homeostatic cell death	1 and 2	Yes	Caspase 3 staining	Confocal microscopy and immunofluorescence	Zhang et al. (2018)
Embryonic cells (unspecified)	*D. rerio*	In vivo	Camptothecin	1	–	–	Epifluorescence and phase-contrast microscopy	Ikegami et al. (1999)
	D. melanogaster	In vivo		1 and 3	Yes	–	Electron microscopy	Abrams et al. (1993)

Embryonic cells (epidermal layer)			Homeostatic cell death and X-irradiation					
Embryonic cells (leg)	*D. melanogaster*	In vivo	Homeostatic cell death	1 and 3	–	Caspase 3 staining	Confocal time-lapse microscopy	Monier et al. (2015)
Squamous cells (imaginal disks)	*D. melanogaster*	Ex vivo	Homeostatic cell death	3	–	Annexin V staining	Confocal time-lapse microscopy	Aldaz et al. (2010)
Nurse cells (ovaries)	*D. melanogaster*	Ex vivo	Homeostatic cell death	3	–	TUNEL staining	Fluorescence microscopy	Foley and Cooley 1998)

activated by caspase 3 and 7 mediated cleavage of the carboxyl domain, leading to the release of nucleotides including ATP and UTP (Elliott et al. 2009; Chekeni et al. 2010; Sandilos et al. 2012). These nucleotides act as chemotactic 'find-me' signals which recruit macrophages to the site of cell death to initiate cell clearance (Elliott et al. 2009; Chekeni et al. 2010). Furthermore, a recent study has also demonstrated that apoptotic murine macrophages, thymocytes and human T cell lines can also release 'good-bye' signals through PANX1 channels in the form of spermidine, AMP, GMP, creatine and G3P, which can subsequently upregulate genes involved in metabolism, anti-apoptotic pathways, anti-inflammatory processes, and actin reorganisation/cell motility (Medina et al. 2020). In addition to these roles in apoptosis and cell clearance, PANX1 was also identified as the first negative regulator of apoptotic cell disassembly (Poon et al. 2014a). PANX1$^{-/-}$ murine thymocytes and human T cell lines expressing a PANX1 dominant negative mutant produced apoptopodia (as described above) following the initiation of apoptosis (Poon et al. 2014a). Furthermore, pharmacological inhibition of PANX1 during apoptosis also led to the formation of apoptopodia which promoted the efficient disassembly of dying thymocytes both ex vivo and in vivo (Poon et al. 2014a). In addition to apoptopodia, PANX1 was also found to be a negative regulator of beaded apoptopodia formation by apoptotic monocytic cells (Atkin-Smith et al. 2015). However, how PANX1 regulates apoptopodia formation remains to be defined.

Plexin B2

In contrast to PANX1, the transmembrane receptor PlexB2 was recently identified as the first positive regulator of beaded apoptopodia formation in apoptotic monocytes (Atkin-Smith et al. 2019). PlexB2 belongs to a large family of plexin proteins (Plexin A-D) which bind to semaphorins to predominately regulate cell motility and the maintenance of cellular positioning (Zielonka et al. 2010; Hota and Buck 2012). Specifically, PlexB2 receptors interact with semaphorin 4C to regulate axonal guidance cues and thus, is crucial for neurodevelopment (Hota and Buck 2012; Deng et al. 2007; Zielonka et al. 2010). PlexB2 possesses an intracellular PDZ binding domain which can interact with PDZ-domains of Rho guanine nucleotide exchange factors (RhoGEFs) and LARG to activate Rho A and regulate cytoskeletal rearrangement (Deng et al. 2007; Zielonka et al. 2010; Atkin-Smith et al. 2019). Similar to ROCK1 and PANX1 as mentioned above, PlexB2 is also cleaved by caspase 3 and 7 upon apoptosis induction and was found to be enriched in monocyte-derived ApoBDs (Atkin-Smith et al. 2019). Lack of PlexB2 significantly impaired the formation of beaded apoptopodia in human monocytic cell lines in vitro (Atkin-Smith et al. 2019). Specifically, these cells formed non-beaded protrusions which quickly retracted, demonstrating the importance of PlexB2 in regulating the beaded appearance of beaded apoptopodia (Atkin-Smith et al. 2019). Furthermore, although PlexB2 is involved in cytoskeletal rearrangement, loss of PlexB2 expression had no effect on the activation of ROCK1 therefore, suggests that PlexB2 positively

regulates beaded apoptopodia formation independently of ROCK1 (Atkin-Smith et al. 2019).

Cytoskeleton

It is well-known that healthy cells rely on cytoskeletal components to form protrusions such as lamellipodia and filopodia which are rich in F-actin and microtubules to aid in cell motility (Ballestrem et al. 2000; Pollard and Cooper 2009). Similar to this, apoptotic membrane protrusions were also found to be rich in F-actin (Caruso et al. 2019). However, despite more than 55% of epithelial, monocytic and T cell lines generating F-actin-rich apoptopodia, inhibiting actin polymerisation pharmacologically with cytochalasin-D had no significant effect on the number of cells forming F-actin-rich apoptopodia or overall protrusion formation (Caruso et al. 2019). Such observations demonstrate that despite containing F-actin, the formation of apoptopodia is not reliant on actin polymerisation and perhaps, the presence of F-actin is more important for maintaining the architecture of these apoptotic protrusions (Caruso et al. 2019).

In addition to F-actin-rich protrusions, apoptotic epithelial cell lines were also found to develop rigid microtubule-rich protrusions known as microtubule spikes (Moss 2006). Interestingly, a recent study has demonstrated that apoptopodia formed by epithelial, monocytic and T cell lines can also contain microtubules (Caruso et al. 2019). Destabilising microtubules in apoptotic cells pharmacologically with nocodazole treatment reduced the percentage of cells forming microtubule rich protrusions, and also significantly reduced the percentage of epithelial cells forming apoptopodia (Caruso et al. 2019). However, this inhibition did not have a substantial effect on apoptopodia formation in monocytic or T cell lines and only had a minimal effect on ApoBD formation in epithelial, monocytic and T cell lines (Caruso et al. 2019). These findings suggest that unlike microtubule spike formation, the assembly of microtubules may only play a minor role in apoptopodia formation however, the importance of this particular cytoskeletal component may also vary depending on cell type.

Vesicle Trafficking

One of the most striking features of apoptopodia and beaded apoptopodia is the extent to which these protrusions can rapidly elongate. Although the driving force underpinning the formation and extension of apoptotic membrane protrusions is not clear, one possible explanation is that additional membrane from internal organelles which can be tethered to the plasma membrane such as the endoplasmic reticulum (ER) (Manford et al. 2012; Prinz 2014) or Golgi apparatus may be acquired to develop these protrusions. In line with this hypothesis, vesicle trafficking, a process which involves the shuttling of cargo (including proteins) in membrane-bound vesicles to facilitate communication between various cellular compartments

(Segev et al. 2009), was found to be involved in regulating the formation of apoptopodia and beaded apoptopodia (Atkin-Smith et al. 2015). Specifically, inhibiting vesicle trafficking by disrupting cellular ion balances with the compound monensin in both human monocytic and T cell lines prevented the formation of apoptopodia and beaded apoptopodia without affecting the levels of apoptosis, membrane blebbing and PANX1 activity (Atkin-Smith et al. 2015). Additionally, treatment of apoptotic cells with another drug known as sertraline, also prevented the formation of apoptopodia and beaded apoptopodia (Atkin-Smith et al. 2015; Caruso et al. 2019). Although the primary role of sertraline is to promote serotonergic signalling by blocking selective serotonin reuptake, previous studies have shown that this drug could also interfere with vesicle trafficking and protein synthesis in yeast (Rainey et al. 2010), providing further evidence that vesicle trafficking is an important process not only for maintaining functional organisation and communication within cells, but also for the formation of apoptotic membrane protrusions.

Step 3: ApoBD Fragmentation

The final step of the disassembly process where apoptotic cells fragment into discrete ApoBDs, is not well studied. It has been suggested that both cell extrinsic and intrinsic factors within the in vitro environment such as shear force generated by the movement of culture medium and abscission like processes may be responsible, respectively (Atkin-Smith and Poon 2017). Additionally, physical interaction between apoptotic and neighbouring cells may also generate enough physical force to disrupt the apoptotic cells and membrane protrusions to release ApoBDs (Atkin-Smith and Poon 2017).

Although further investigations are required to fully understand the exact mechanisms involved in ApoBD fragmentation, several studies have shown that many factors regulating the upstream disassembly steps can influence the amount of ApoBDs generated. For example, pharmacological and/or genetic inhibition of ROCK1, actin polymerisation, vesicle trafficking or PlexB2 not only inhibits plasma membrane blebbing or apoptopodia formation but, also leads to an overall decrease in the generation of ApoBDs (Atkin-Smith et al. 2015, 2019; Caruso et al. 2019; Tixeira et al. 2019). In contrast, inhibition of PANX1 activity enhances apoptopodia formation and subsequently leads to increased ApoBD formation (Poon et al. 2014a). Surprisingly, cells lacking LIMK1 generated significantly more ApoBDs following apoptosis induction however, the mechanism driving this phenomenon still remains unclear (Tixeira et al. 2019). The ease at which ApoBD formation can be manipulated is intriguing and is an area worth exploring for the development of future disease therapeutics.

Maintenance of Plasma Membrane Integrity to Prevent Premature Lysis

During apoptosis, maintaining plasma membrane integrity is vital to prevent premature cell lysis and thus, the progression to secondary necrosis. Not only is this important to prevent tissue damage caused by the release of proinflammatory molecules (Sachet et al. 2017), but an intact membrane is also necessary to ensure that the disassembly process goes to completion. Like other known regulators of the disassembly process (as described in Section "Step 1: Plasma Membrane Blebbing"), Gasdermin E (GSDME, also known as DFNA5) is activated during apoptosis by caspase 3 cleavage and is thought to be involved in driving the progression of apoptosis to secondary necrosis (Wang et al. 2017) and limiting ApoBD formation by apoptotic murine macrophages (Rogers et al. 2017). However, more recently studies have demonstrated that neither membrane permeabilisation nor ApoBD formation was regulated by GSDME in murine macrophages (Lee et al. 2018), human monocytic, epithelial and T cell lines (Tixeira et al. 2018).

As plasma membrane integrity is reliant on the architecture of the cell, it is not surprising that certain cytoskeletal components may also be involved in maintaining cellular integrity. During the early stages of apoptosis, interphase microtubules rapidly dismantle and are subsequently replaced with densely packed dynamic microtubules (Sánchez-Alcázar et al. 2007). These microtubules closely associate with the plasma membrane and are collectively known as the apoptotic microtubule network (AMN) (Sánchez-Alcázar et al. 2007). It is thought that the assembly of the AMN occurs following actin and intermediate filament disorganisation and could therefore be the sole cytoskeletal component remaining to support the plasma membrane at certain stages of apoptosis. Thus, the AMN may also have a role in regulating the morphological changes associated with the disassembly process by maintaining cellular integrity (Moss 2006; Sánchez-Alcázar et al. 2007).

In addition to positively regulating step 1 of disassembly, ROCK1 was also shown to play a role in regulating the onset of secondary necrosis (Tixeira et al. 2019). Although cells lacking ROCK1 remained intact during the normal period of apoptotic cell disassembly (i.e. within 2–4 h post induction of apoptosis), these cells underwent membrane permeabilization more readily and released cytosolic components 7–10 hours post apoptosis induction (Tixeira et al. 2019). This suggests that ROCK1 may be involved in both regulating plasma membrane integrity and plasma membrane blebbing during apoptosis, and reveals an important relationship between maintaining plasma membrane integrity and blebbing (Tixeira et al. 2019).

Methods of ApoBD Isolation

In order to gain further insight into the functional importance of ApoBDs, it is vital that these ApoBDs are isolated appropriately and to a high purity to increase the accuracy and validity of downstream analysis. Without this, it is difficult to draw appropriate conclusions on the role of ApoBDs in certain settings. Although a wide variety of methods may be used to isolate ApoBDs, the most commonly used techniques include differential centrifugation (with or without filtration) and fluorescence-activated cell sorting (Fig. 4.2).

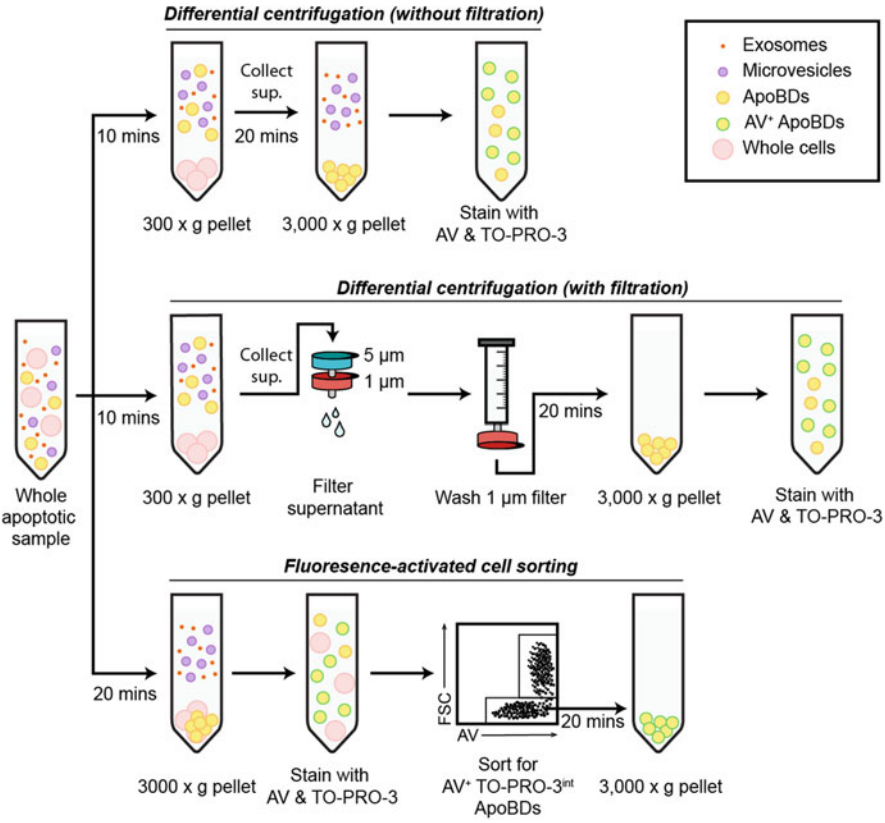

Fig. 4.2 Approaches for ApoBD isolation. ApoBDs can be isolated from whole cells and smaller EVs based on density (differential centrifugation) and size (filtration). Furthermore, a range of markers and parameters including annexin V (AV) positivity, TO-PRO-3 uptake, cell granularity and size can also be used in conjunction to isolate ApoBDs by fluorescence-activated cell sorting

Differential Centrifugation

Differential centrifugation is the most widely used approach and is generally considered as a gold standard technique for EV isolation. This method aims to separate particles based on density through several rounds of centrifugation with each increasing in force and duration (Livshts et al. 2015). For most studies, ApoBD isolation generally begins with an initial centrifugation at 300–500×*g* to remove whole cells and debris, followed by centrifugation of the supernatant at 1000–3000×*g* to pellet ApoBDs (Berda-Haddad et al. 2011; Crescitelli et al. 2013; Atkin-Smith et al. 2017; Phan et al. 2018) (Fig. 4.2). Using this technique an ApoBD purity of ~84–98% can be achieved, as determined by flow cytometry analysis (Atkin-Smith et al. 2017; Phan et al. 2018). The ability to isolate ApoBDs by differential centrifugation is favourable as it is an efficient approach (total isolation time of ~30 mins) and therefore, may limit ApoBD lysis during the isolation process (Poon et al. 2019). However, despite being a gold standard technique, it is evident that many discrepancies exist in the field. For example, following the removal of whole cells and debris at 800×*g*, some studies attempted to pellet ApoBDs at 16,000×*g* (Hristov et al. 2004). However, due to these high centrifugation forces both ApoBDs and smaller EVs such as microvesicles could be isolated together and thus, the resulting data should be interpreted with caution. Furthermore, as this method separates vesicles purely on density, a heterogenous population of vesicles is generally obtained and thus, cell-type specific ApoBDs cannot be isolated from complex samples such as tissues or biofluids (Atkin-Smith et al. 2017). Interestingly, studies have also shown that different centrifuge rotor types can also have an influence on the yield and type of vesicles isolated (Cvjetkovic et al. 2014; Livshts et al. 2015). Specifically, centrifugation using a swing bucket rotor results in a significantly higher yield of vesicles which are also generally larger in size when compared to samples isolated in centrifuges with fixed angle rotors (Livshts et al. 2015). Therefore, it is clear that these rotors require different centrifugation settings in order to pellet samples equally (Cvjetkovic et al. 2014). However, it is important to note that these studies focused on the isolation of exosomes and the influence of rotor type on ApoBD isolation is yet to be determined.

Filtration

In addition to the differential centrifugation method discussed above, an additional filtration step may also be included to further purify ApoBDs based on size. As ApoBDs have been generally described as 1–5 μm in diameter (Poon et al. 2014a; Atkin-Smith et al. 2015), passing the ApoBD-containing supernatant obtained from the first centrifugation step (300×*g*) through filters of corresponding sizes will allow for the collection of particles that falls within the 1–5 μm size range (Liu et al. 2018) (Fig. 4.2). However, the choice of filter size and subsequent centrifugation force is

extremely important, as the use of filters with smaller pore size (e.g. 1.2 μm) followed by centrifugation at 100,000×*g* will most likely lead to the co-isolation of small ApoBDs, microvesicles and exosomes (Schiller et al. 2008; Fehr et al. 2013). Furthermore, forceful filtration may also lead to artificial fragmentation or lysis of the cell or vesicle and thus, this method should be performed with caution. Although size is a useful criteria in EV characterisation, isolating ApoBDs solely using this parameter may not always be appropriate as a recent study has demonstrated that some human cell line-derived ApoBDs can be as large as 8–10 μm thus, falling outside the typical ApoBD size range of 1–5 μm and within the larger vesicle range that includes EVs such as large oncosomes (Meehan et al. 2016; Poon et al. 2019). Additionally, human T cell lines undergoing primary necrosis and murine macrophages undergoing pyroptosis induced by plant defensin NaD1 or LPS and nigericin respectively, can also generate vesicles similar in size to ApoBDs (Jiang et al. 2016; Chen et al. 2016; Baxter et al. 2019). Therefore, it is evident that in addition to physical characteristics such as size, additional biological characteristics should also be considered to accurately identify and isolate ApoBDs.

Fluorescence-Activated Cell Sorting (FACS)

Recently, a new method has been developed to detect and isolate ApoBDs by fluorescence-activated cell sorting (FACS). This method is based on the principle that apoptotic cells and ApoBDs contain exposed phosphatidylserine (PS) and activated PANX1 channels. As a result, these events allow for the binding of fluorochrome-conjugated annexin V to externalised PS, and the selective uptake of the nucleic acid dye TO-PRO-3 through caspase-activated PANX1 channels (Koopman et al. 1994; Poon et al. 2014a). In addition to these markers, the size and granularity of particles are also considered and therefore allows the separation of ApoBDs from viable, apoptotic, and necrotic cells in a qualitative and quantitative manner (Jiang et al. 2016; Atkin-Smith et al. 2017; Phan et al. 2018). It is also important to note that prior to the sorting process, the whole apoptotic sample is collected by centrifugation at 3000×*g*, and this by default would also help reduce the amount of small EVs from the sample (Phan et al. 2018). Although relatively high purity can be achieved by differential centrifugation, FACS-based isolation methods allows for even higher ApoBD purity (~99%) and also enables for the isolation of cell-type specific ApoBDs from tissue samples, bodily fluids or blood-derived samples (Atkin-Smith et al. 2017; Phan et al. 2018). Although the methods discussed here allow for the isolation of ApoBDs to high purity, it is also important to note that other methods including size exclusion chromatography, microfluidics and laser capture approaches is also worth exploring in future studies.

Validation of ApoBDs

Whilst differential centrifugation, filtration and FACS are routinely used to isolate ApoBDs, it is also extremely important to validate the induction of apoptosis and the formation of ApoBDs (to ensure that the vesicles of interest are indeed generated from apoptotic cells), as well as the purity of the ApoBD sample (Phan et al. 2018; Poon et al. 2019). Without adopting these additional steps, it is quite difficult to draw any meaningful conclusions on the fundamental role of ApoBDs in physiological or pathological settings.

A major goal in the EV field is to identify EV-specific markers to aid in distinguishing different EV subtypes. To this end, PS exposure, DNA, nuclear proteins, calreticulin, calnexin and Bip/GRP78 are all considered as ApoBD markers (Rosen et al. 1994; Koopman et al. 1994; Buzas et al. 2014; Jeppesen et al. 2014; Lunavat et al. 2015; Thompson et al. 2016; Shin et al. 2017; Karpman et al. 2017). However, it is important to note that many of these markers are not specifically expressed by ApoBDs. For instance, studies have shown that PS can also be detected on other vesicles including microvesicles and necroptotic bodies (vesicles derived from necroptotic cells) (Hugel et al. 2005; Lima et al. 2009; Muralidharan-Chari et al. 2009; Zargarian et al. 2017; Poon et al. 2019). In addition, ApoBDs derived from different cell types can also expose varying levels of PS (Poon et al. 2019). Furthermore, the distribution of DNA and nuclear proteins into ApoBDs is dependent on cell type and the mechanism by which the ApoBDs are formed (e.g. beaded apoptopodia-dependent ApoBD formation leads to ApoBDs void of nuclear material (Atkin-Smith et al. 2015)) and thus, fragmented DNA is not an appropriate marker for all ApoBDs (Atkin-Smith et al. 2015; Jiang et al. 2017). However, as ApoBDs are only generated by apoptotic cells, it is reasonable to assume that caspase 3 and 7 cleaved proteins such as ROCK1 and PANX1 have the potential to be useful markers for ApoBDs (Poon et al. 2019). Nevertheless, this highlights that multiple markers should be used in conjunction to confirm the presence of ApoBDs in a particular sample, and further research is required to identify ApoBD specific markers.

Functional Significance of ApoBD Formation

As mentioned above, the disassembly of apoptotic cells into ApoBDs is suggested to have two primary functions including aiding the efficient clearance of apoptotic debris and intercellular communication through the trafficking of biomolecules. These particular functional roles of ApoBDs in various physiological and pathological settings will be discussed further below.

ApoBDs in Cell Clearance

After the induction of apoptosis, a series of 'find-me' and 'eat-me' signals are released and exposed to coordinate the rapid recruitment of phagocytes and the removal of dying cells (Fig. 4.3) (Poon et al. 2014b). Uncleared apoptotic cells can proceed to a pro-inflammatory cell death state known as secondary necrosis where damage-associated molecular patterns (DAMPs) are released and can trigger an inflammatory response (Bell et al. 2006; Poon et al. 2014b). Notably, impaired cell clearance has been linked to a variety of inflammatory diseases including autoimmunity (such as systemic lupus erythematosus (Cohen et al. 2002)) and cardiovascular disease (such as atherosclerosis (Kojima et al. 2016)). Thus, the efficient clearance of apoptotic cells and their debris is paramount.

The disassembly of apoptotic cells into ApoBDs has long been suggested to provide small, 'bite-sized' fragments that can be easily engulfed by surrounding phagocytes (Atkin-Smith and Poon 2017). Studies have shown that reducing apoptotic membrane blebbing and ApoBD formation through pharmacologically

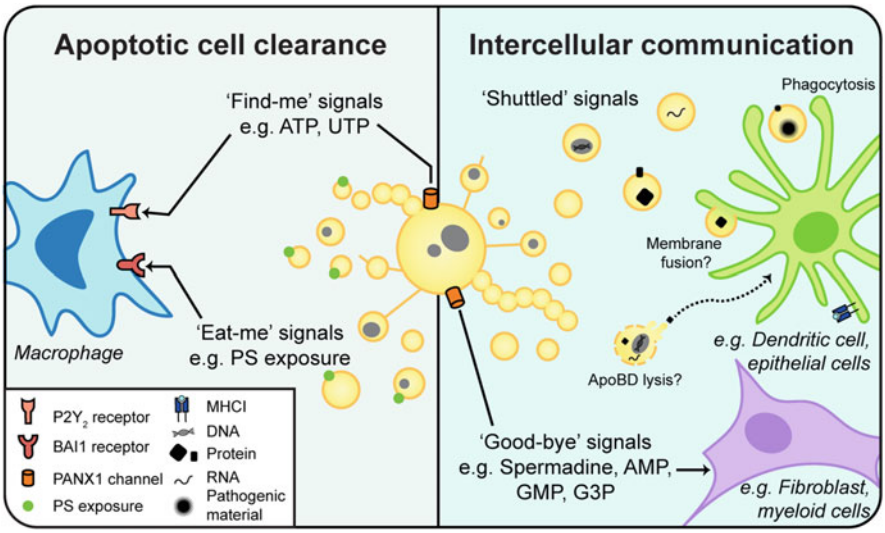

Fig. 4.3 Functional roles of ApoBDs. To aid cell clearance, apoptotic cells can release a range of 'find-me' signals including ATP and UTP through caspase-activated PANX1 channels which recruit phagocytes to the site of cell death. Apoptotic cells and ApoBDs are also known to expose 'eat-me' signals such as PS for recognition and uptake by phagocytes. In addition, ApoBDs can mediate the transfer of biomolecules such as DNA, RNA, proteins and pathogenic material to recipient cells to facilitate intercellular communication. ApoBDs can harbour an array of molecular signals, collectively packaged in/on ApoBDs as 'shuttled' signals. It is currently unclear whether the biomolecules are transferred via engulfment, membrane fusion or ApoBD lysis. Finally, apoptotic cells can also release a series of 'good-bye' signals through caspase-activated PANX1 channels to upregulate genes involved in anti-inflammatory processes, anti-apoptotic pathways, metabolism and cell motility in recipient cells

inhibiting ROCK1 (for example with the small molecule Y-27632) or cytoskeletal rearrangement could impair efficient engulfment by surrounding phagocytes (Orlando et al. 2006; Orlando and Pittman 2006; Witasp et al. 2007). More recently, a CRISPR/Cas9-based approach was also used to target the key regulators of ApoBD formation such as ROCK1, PANX1 and PlexB2 (see Sect. "Mechanisms of Apoptotic Cell Disassembly"), and subsequent effects on efferocytosis was examined. ROCK1 deficiency in apoptotic human T cell lines (thus impaired apoptotic cell disassembly) significantly compromised the engulfment efficiency by surrounding phagocytes including dendritic cells and fibroblasts (Tixeira et al. 2019). In contrast, the engulfment efficiency of monocyte-derived macrophages was significantly enhanced when co-incubated with PANX1-deficient apoptotic human T cell lines which readily form apoptopodia and ApoBDs (Tixeira et al. 2019). Similarly, apoptotic human monocytic cell lines deficient in PlexB2 (a positive regulator of beaded apoptopodia formation) also significantly impaired their engulfment by a range of phagocytes in vitro, as well as in an in vivo model investigating alveolar macrophage engulfment (Atkin-Smith et al. 2019). Altogether, these studies clearly demonstrate a fundamental role of ApoBD formation in aiding cell clearance during apoptosis. However, whether disruption to the apoptotic cell disassembly process contributes to the initiation or severity of inflammatory diseases such as those linked to impaired cell clearance is yet to be determined.

ApoBDs in Intercellular Communication

As ApoBDs are generally a large fragment of the apoptotic cells, they can contain a wide variety of cellular components including the plasma membrane, fragmented organelles as well as various biomolecules (including membrane-bound or intracellular proteins, RNA and DNA) and partake in intercellular communication with surrounding cells (Bergsmedh et al. 2001; Coleman et al. 2001; Schaible et al. 2003; Croft et al. 2005; Lane et al. 2005; Wang et al. 2008; Zernecke et al. 2009; Berda-Haddad et al. 2011; Singh et al. 2012; Thomas et al. 2015; Jiang et al. 2017; Zhu et al. 2017; Brock et al. 2019; Atkin-Smith et al. 2020). Thus, the range of molecular signals present on/in ApoBDs can collectively be termed as 'shuttled' signals (Fig. 4.3). It is also important to note that the way in which cells disassemble (e.g. through apoptopodia or beaded apoptopodia dependent ApoBD formation) can also consequentially dictate the contents of ApoBDs (Atkin-Smith et al. 2015).

Intercellular Communication Through DNA as Cargos

The presence of DNA in ApoBDs has been noted since the first description of apoptosis and ApoBD formation by Kerr et al. 1972. The trafficking of DNA into apoptotic membrane blebs (Croft et al. 2005; Lane et al. 2005) and subsequently ApoBDs has been proposed to be driven by actomyosin contraction during the first

stage of apoptotic cell disassembly and has been demonstrated in several in vitro experimental models. First, ApoBDs generated from apoptotic Burkitt's lymphoma cells with integrated copies of the Epstein-Barr virus (EBV) was suggested to traffic EBV-encoded genes to recipient human fetal fibroblasts (Holmgren et al. 1999). Remarkably, through this co-culture study, the expression of EBV protein was detected in recipient fibroblasts (Holmgren et al. 1999). In subsequent studies from the same group, it was also proposed that HIV genes as well as oncogenes could be transferred to a variety of recipient cells via ApoBDs (Spetz et al. 1999; Bergsmedh et al. 2001). However, it should be noted that the conclusion derived from these studies (Holmgren et al. 1999; Spetz et al. 1999; Bergsmedh et al. 2001) are based on the direct co-culture of cells undergoing apoptosis with recipient cells, rather than the co-culture of purified ApoBDs with recipient cells. Although the formation of discrete ApoBDs by apoptotic cells were also not addressed directly (Holmgren et al. 1999; Spetz et al. 1999; Bergsmedh et al. 2001), these studies do suggest the possibility of ApoBDs mediating the transfer of DNA in disease settings. Furthermore, nuclear DNA is cleaved by nucleases such as caspase-activated deoxyribonuclease and endonuclease G during apoptosis (McIlroy et al. 1999; Li et al. 2001) and thus, whether functional RNAs or proteins can subsequently be expressed from DNA which was packaged into ApoBDs should be investigated further in future studies.

Intercellular Communication Through RNA as Cargos

Distribution of mRNA inside healthy cells is heterogenous, with ample evidence demonstrating that certain mRNA species can be distributed at a specific subcellular localisation (Shav-Tal and Singer 2005). Although global degradation of mRNA occurs during the progression of apoptosis, some noncoding RNAs can remain stable during this process (Thomas et al. 2015). More specifically, certain noncoding RNAs like microRNAs (miRNAs) can be detected in ApoBDs, and could therefore have a potential role in intercellular communication (Zernecke et al. 2009; Zhu et al. 2017). ApoBDs generated from LPS-stimulated macrophages were shown to contain miRNA-221/222 and the transfer of these miRNAs-containing ApoBDs to recipient lung epithelial cells promoted cell proliferation by targeting CDKN1B-cyclin D3/CdK4 pathways (Zhu et al. 2017). Furthermore, miRNA-126, an endothelial cell-restricted miRNA (Wang et al. 2008), was found in the ApoBD-enriched sample derived from serum-starved human umbilical vein endothelial cells (Zernecke et al. 2009). This study demonstrated that the EVs present in the endothelial ApoBD-enriched samples facilitated the transfer of miRNA-126 to recipient endothelial cells to promote the expression of CXCL12, which subsequently mediated the recruitment of progenitor cells to aid endothelial repair (Zernecke et al. 2009). Interestingly, a recent study reported that the RNA cargo in different subsets of ApoEVs can vary greatly, with endothelial ApoBD-enriched samples containing predominately ribosomal RNAs, whereas exosome-like apoptotic vesicles harbour mainly non-ribosomal non-coding RNAs (Hardy et al. 2019). Although there is no doubt that

ApoBDs can traffic RNA molecules to recipient cells, the mechanism underpinning how these RNA molecules could be released from ApoBDs requires further investigation.

Intercellular Communication Through Proteins as Cargos

The ability for ApoBDs to traffic proteins in a range of settings has been more widely described, with plasma membrane-associated molecules (including cell-type specific CD markers (Jiang et al. 2017)), intracellular signalling factors, as well as cytokines being found on or in ApoBDs. For example, the presence of RANK (receptor activator of nuclear factor kappa B ligand) on the surface of osteoclast-derived ApoBDs was found to promote the differentiation of pre-osteoblastic cells in an in vitro model (Ma et al. 2019). Additionally, utilising a zebrafish-based model, ApoBDs generated from apoptotic basal stem cells in the epidermis was described to harbour Wnt8a, which could activate Wnt signalling in neighbouring stem cells to maintain tissue homeostasis by driving stem cell proliferation (Brock et al. 2019). Furthermore, ApoBDs generated from serum-starved and TNFα-treated endothelial cells was shown to contain the proinflammatory cytokine IL-1α, which could promote inflammation when ApoBDs were administered into mice intraperitoneally (Berda-Haddad et al. 2011). Collectively, this demonstrates that ApoBDs can carry a range of proteins from the parental cell and aid intercellular communication in a number of physiological and pathological settings.

Intercellular Communication Through Pathogen-Derived Material

Given the strong link between cell death and infectious disease (Zychlinsky 1993), ApoBDs may also have key roles in trafficking pathogenic material and contributing to disease pathogenesis or resolution. For example, efferocytosis of HIV-infected apoptotic T cells and infected cell-derived ApoBDs could mediate the transfer of HIV proteins and genomic material to the engulfing cell (Singh et al. 2012). Consequently, this promoted the expression of HIV proteins and viral replication within the engulfing epithelial cells and highlighted a role of efferocytosis in viral transmission (Singh et al. 2012). Similarly, ApoBDs derived from influenza A virus (IAV)-infected monocytes were also shown to aid viral propagation both in vitro and in vivo (Atkin-Smith et al. 2020). Such ApoBDs were shown to harbour a series of IAV RNA, proteins and lethal virions which as a consequence, could elicit viral infection and induce cell death in neighbouring cells (Atkin-Smith et al. 2020). Importantly, IAV propagation via ApoBDs could be impaired by pharmacologically inhibiting ApoBD formation from apoptotic monocytes, providing an exciting therapeutic direction (Atkin-Smith et al. 2020).

The engulfment of apoptotic cells (for example of IAV- or salmonella-infected apoptotic cells) is a well-known mechanism required to aid antigen presentation through the cross-presentation pathway (Albert et al. 1998; Yrlid and Wick 2000;

Blachère et al. 2005). However, whether the engulfment of ApoBDs specifically can also aid cross-presentation is not well defined. Nevertheless, ApoBDs were shown to have a functional role in antigen presentation during *Mycobacterium tuberculosis* (TB) infection whereby TB-infected macrophages could generate ApoBDs containing TB-antigen. Such ApoBDs could then be taken up by surrounding dendritic cells to mediate cross presentation (Schaible et al. 2003). Altogether, given that a single apoptotic cell can fragment into abundance of ApoBDs, these studies highlight the importance of further investigating the functional consequence of ApoBDs during infectious disease that induce apoptosis and ApoBD formation.

Concluding Remarks

Apoptotic cell disassembly is a complex process involving a series of tightly regulated morphological steps. Much progress has been made to better understand the regulatory factors involved in apoptotic cell disassembly, as well as on the development of new methodologies to study ApoBD formation and determining the functional role of ApoBDs. However, the apoptotic cell disassembly process must be further explored in order to truly understand the significance of ApoBD formation in vivo, in particular in different physiological and pathological settings.

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Chapter 5
Exomeres: A New Member of Extracellular Vesicles Family

Sushma Anand, Monisha Samuel, and Suresh Mathivanan

Abstract Extracellular vesicles (EVs) are described as membranous vesicles that are secreted by various cell types. EVs can be categorised as exosomes, ectosomes, apoptotic bodies, large oncosomes and migrasomes. EVs are heterogeneous in nature according to their origin, mode of release, size, and biochemical contents. Herein, we discuss a recently discovered subpopulation of EVs called 'exomeres'. Unlike the other subtypes of EVs, exomeres are defined as non-membranous nanovesicles with a size $\leq$50 nm. They can be isolated using asymmetric-flow field-flow fractionation as well as ultracentrifugation. The cargo of exomeres are beginning to be unravelled and are highlighted to be enriched with proteins implicated in regulating metabolic pathways. Consistent with other types of EVs, exomeres also contain nucleic acids and lipids which can be delivered to recipient cells. These discoveries highlight the complex heterogeneity of EVs and thereby necessitates further attention to understand the nature of each subpopulation more exclusively. Overall, this chapter describes the current knowledge on exomeres.

Keywords Extracellular vesicles (EVs) · Exosomes · Exomeres · Asymmetric flow field-flow fractionation

Introduction

Cells release extracellular vesicles (EVs) under pathophysiological conditions to facilitate various biological functions (Mathivanan et al. 2010; Witwer et al. 2017; Sanwlani et al. 2020). EVs are implicated in intercellular communication and are

S. Anand · S. Mathivanan (✉)
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia
e-mail: S.Mathivanan@latrobe.edu.au

M. Samuel
Division of Immunology and Allergy, Department of Medicine, Karolinska Institute, Solna, Sweden

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known to regulate various diseases including cancer (Peinado et al. 2012). Even though our understanding on EV biology is rapidly growing, it has become apparent that a significant level of heterogeneity exists in the context of EV subtypes and subpopulations (Thery et al. 2018). Currently, it has been acknowledged that cells release various subtypes of EVs, however separation of a specific EV subtype to homogeneity is challenging owing to lack of methods to isolate specific EV subtypes alone and the unavailability of bona fide markers (Kalra et al. 2016; Thery et al. 2018). Based on the mode of biogenesis, EVs have been broadly classified into exosomes (50–150 nm), ectosomes or shedding microvesicles (100–1000 nm), migrasomes (500–3000 nm), large oncosomes (1000–10,000 nm) and apoptotic bodies (1000–5000 nm). Among these EV subtypes, exosomes are most well studied and they are of endocytic origin formed within multivesicular bodies (Kalra et al. 2016).

EVs carry scrupulously sorted cargo of proteins, nucleic acids (mRNAs, miRNAs, lnRNAs, DNA), metabolites and lipids (Keerthikumar et al. 2015, 2016; Kalra et al. 2016, 2019; Anand et al. 2019). Several seminal studies have shown that EVs essentially participate in horizontal transfer of biologically functional cargo to the recipient cells (Al-Nedawi et al. 2009; Peinado et al. 2012; Hoshino et al. 2015; Kowal et al. 2016; Fonseka et al. 2019). EVs also can stimulate various signalling pathways in the recipient cells under normal and diseased state (Kalra et al. 2019; Zhang et al. 2019; Hoshino et al. 2020). In addition to intercellular communication, they are investigated extensively as reservoirs of disease biomarkers and drug delivery vehicles for therapeutic applications (Boukouris and Mathivanan 2015; Urbanelli et al. 2015; Tai et al. 2018; Anand et al. 2019; Sanwlani et al. 2020).

One of the major challenges in the field of EVs relate to the heterogeneity of EVs, both with different subtypes of EVs and within a specific subtype (Thery et al. 2018). In this context, signature proteins that could efficiently distinguish EV subtypes are not yet identified and the overlapping EV size are the major limiting factors (Willms et al. 2018). Hence, various studies use novel methods to characterise EV subtypes to identify better methods of isolation and markers of EV subtypes. Recently, while using novel methods to isolate EVs, David Lyden and colleagues described a new subtype of EVs called exomeres which are described as non-membranous particles (Zhang et al. 2018).

Exomeres

Exomeres are tiny vesicles (<50 nm) that are secreted by cells (Zhang et al. 2018). Due to the recent discovery, the precise molecular machinery implicated in the biogenesis and secretion of exomeres is unclear. Unlike all the other subtypes of EVs, exomeres are not enclosed by lipid bilayer membrane and are significantly smaller (Zhang et al. 2018, 2019; Zhang and Lyden 2019). To gain clarity on exomeres, unique features of the most well studied EV subtype exosomes and exomeres are listed in Table 5.1. To date, only two studies have isolated and

Table 5.1 Distinguishing features of exosomes vs exomeres

Features	Exosomes	Exomeres	References
Biogenesis	MVBs	Unknown	Kalra et al. (2016), Zhang et al. (2018, 2019)
Size	30–150 nm	≤50 nm	Chuo et al. (2018), Zhang et al. (2018, 2019), Zhang and Lyden (2019)
Shape	Heterogeneous	Heterogeneous	Zhang et al. (2018, 2019), Anand et al. (2019)
External membrane	Lipid bilayer	Absent	Zhang et al. (2018, 2019)
ESCRT proteins	Present	Absent	Zhang et al. (2018, 2019)
Enriched proteins	PDCD6IP, TSG101, FLOT1, FLOT2, CD9, CD63, CD81	Unclear – Need more studies	Keerthikumar et al. (2015), Kowal et al. (2016), Zhang et al. (2018, 2019)
Lipid content	Unesterified cholesterol, phospholipids, Sphingomyelin, Phosphatidylcholine	Esterified cholesterol triglycerides, Ceramide, Phosphatidylcholine	Kalra et al. (2016), Zhang et al. (2018, 2019), Anand et al. (2019)
Nucleic acid content	Non- coding RNA, miRNA and DNA	DNA, RNA, miRNAs	Zhang et al. (2018, 2019), Anand et al. (2019)

characterised exomeres from mammalian cell lines and human plasma. However, the respective studies have used different protocols such as asymmetric-flow field-flow fractionation (AF4) (Zhang and Lyden 2019) and ultracentrifugation (Zhang et al. 2019) to isolate exomeres. Hence, the results obtained from the cargo-based characterisation of exomeres may be significantly influenced by the method used. Nevertheless, exomere isolation methods will be more explicitly discussed in the following sections.

Proteomic Cargo of Exomeres

It has been well established that EVs contain a rich cargo of proteins (Pathan et al. 2019). Consistent with other EV subtypes, exomeres also contain proteins (Zhang et al. 2018, 2019). However, extensive proteomic analysis of exomeres highlighted the lack of membrane-associated proteins, consistent with biophysical studies. Remarkably, exomeres were highly enriched with proteins involved in hypoxia, coagulation and metabolic pathways such as glycolytic and mTOR pathways. Hence, the authors hypothesised a potential metabolic role for exomeres, though additional studies are needed to validate this speculation. Interestingly, exomeres were associated with proteins localised to endoplasmic reticulum, mitochondria and

microtubules, which potentiates their probable role in biogenesis and secretion of these nanovesicles (Zhang et al. 2018). Exomeres were also packed with proteins involved in post-translational modifications such as sialoglycoprotein galectin-3-binding protein (LGALS3BP), hexoaminidase A, glycogen phosphorylase L, glucuronidase beta, fructose-bisphosphatase and galactosamine-6-sulfatase. Hence, it has been speculated that exomeres facilitate target protein glycosylation in recipient cells. Similarly, colorectal (DiFi), glioblastoma (Gli36), and canine kidney (MDCK) cell line-derived exomeres were enriched in Argonaute 1–3, proteins implicated in Alzheimer's disease such as amyloid precursor protein (APP) and the rate determining enzyme in proteolytic cleavage, BACE-1 (Zhang et al. 2019).

Isolation of Exomeres

Various EV isolation and separation techniques such as differential ultracentrifugation coupled with density gradient centrifugation, immuno-affinity capture, size-exclusion chromatography, precipitation and ultrafiltration have been constantly used either alone or in combination to isolate EVs (Théry et al. 2006; Merchant et al. 2010; Lässer et al. 2012; Tauro et al. 2012; Zhao et al. 2019; Huang et al. 2020). Currently, exomeres are isolated by two different techniques and will be described below.

(a) **Asymmetric-flow field-flow fractionation (AF4)**

AF4 is an advanced sample fractionating technique that separates particles based on hydrodynamic radius and size. Its principle is based on analytical fractionating techniques. Low field-flow fraction has proven to have exceptional abilities to separate samples with great resolution (nm to 100 μm) (Giddings et al. 1976). AF4 technology is widely used in biopharmaceutics industry sector to characterise polymeric nanoparticles but has not been used for EV isolation until 2018. AF4 is known to display exceptionally robust capability to separate and purify nanovesicles. The chromatographic separation is attained inside a very thin flow against which a perpendicular force field is applied. This force is applied by a parabolic channel flow and cross flow with a permeable ultrafiltration membrane at the bottom plate that holds the analyte that is bigger than membrane cut-off (Granger et al. 1986). This technique is efficient, smooth, fast, label free and uses no columns. Hence, no stress on sample integrity has been observed as it does not interact with the stationary phase. Above all, AF4 technique produce high-resolution separation of EV subpopulations, especially 'exomeres' (Zhang and Lyden 2019). Recently, to isolate exomeres and other small molecules, AF4 was further coupled with an on-line immunoaffinity chromatography (Multia et al. 2020).

(b) **Ultracentrifugation**

Although, AF4 method is efficient and isolates homogeneous exomeres population, it depends on sophisticated instruments and user handling skills, thereby a

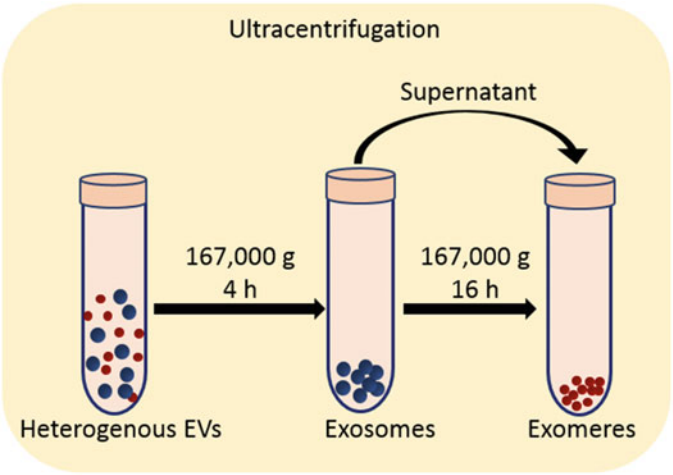

Fig. 5.1 Schematic illustration of ultracentrifugation to isolate exomeres. Conditioned media containing heterogenous EV population are subjected to ultracentrifugation at 167,000×*g* for 4 h to isolate exosomes (blue pellets). Next, supernatant is subjected to a longer ultracentrifugation (16 h). The resulting pellet is proposed to contain exomeres (red pellet)

much simpler method is more desirable. Using ultracentrifugation, exosomes are first removed from conditioned media, followed by a second round of ultracentrifugation for 16 h to separate exomeres (Fig. 5.1) (Zhang et al. 2019). Consistent with previous reports from AF4 method, exomeres isolated using ultracentrifugation also revealed to be non-membranous functional vesicles and were < 50 nm in size.

Concluding Remarks

In summary, better understanding of EV heterogeneity is essential to uncover the role of various EV subpopulations in disease progression. This could be further translated clinically for therapeutic benefits. In addition, deeper understanding on biogenesis and secretion of various EV subtypes is needed. Improvements in existing protocols or novel methods are essential for isolation of pure subpopulation of EVs. Exomeres are a recently identified subtype of EVs that lack the lipid bilayer but contain proteins and nucleic acids. Additional studies are needed to characterise the exomeres from various cells and by different protocols. In addition, studies focused on uncovering the biogenesis and the biological functions of exomeres are needed.

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Part II
Functional Role and Clinical Implications of Extracellular Vesicles

Chapter 6
Pathogenesis Mediated by Bacterial Membrane Vesicles

William J. Gilmore, Natalie J. Bitto, and Maria Kaparakis-Liaskos

Abstract The release of extracellular vesicles (EVs) is a process conserved across the three domains of life. Amongst prokaryotes, EVs produced by Gram-negative bacteria, termed outer membrane vesicles (OMVs), were identified more than 50 years ago and a wealth of literature exists regarding their biogenesis, composition and functions. OMVs have been implicated in benefiting numerous metabolic functions of their parent bacterium. Additionally, OMVs produced by pathogenic bacteria have been reported to contribute to pathology within the disease setting. By contrast, the release of EVs from Gram-positive bacteria, known as membrane vesicles (MVs), has only been widely accepted within the last decade. As such, there is a significant disproportion in knowledge regarding MVs compared to OMVs. Here we provide an overview of the literature regarding bacterial membrane vesicles (BMVs) produced by pathogenic and commensal bacteria. We highlight the mechanisms of BMV biogenesis and their roles in assisting bacterial survival, in addition to discussing their functions in promoting disease pathologies and their potential use as novel therapeutic strategies.

Keywords Bacterial membrane vesicles (BMVs) · Outer membrane vesicles (OMVs) · Membrane vesicles (MVs) · Bacterial pathogenesis · Therapeutic applications of BMVs

W. J. Gilmore · N. J. Bitto · M. Kaparakis-Liaskos (✉)
Department of Physiology, Anatomy and Microbiology, La Trobe University, Melbourne, VIC, Australia

Research Centre for Extracellular Vesicles, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, VIC, Australia
e-mail: m.liaskos@latrobe.edu.au

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Introduction

What are Bacterial Membrane Vesicles?

The release of extracellular vesicles (EVs) is a process conserved across the three domains of life (reviewed in (Deatherage and Cookson 2012)). Microbiologists first identified the ability of Gram-negative bacteria to release EVs from their cell membrane more than 50 years ago (Knox et al. 1966), and since then their production by both Gram-negative and Gram-positive bacteria has become widely accepted. Due to bacterial EVs originating from the cell membrane of bacteria, EVs produced by both Gram-negative and Gram-positive bacteria are collectively referred to as bacterial membrane vesicles (BMVs). BMVs can be further classified on the basis of the cellular architecture of the bacterium from which they are derived, whereby Gram-negative and Gram-positive bacteria produce vesicles known as outer membrane vesicles (OMVs) and membrane vesicles (MVs), respectively. Specifically, the release of OMVs by Gram-negative bacteria occurs at the cells outermost membrane, whilst MVs released by Gram-positive bacteria are derived from the single cytoplasmic cell membrane, which is surrounded by a peptidoglycan-rich cell wall (reviewed in (Kaparakis-Liaskos and Ferrero 2015; Bitto and Kaparakis-Liaskos 2017)).

OMVs are nanoparticles released by Gram-negative bacteria ranging between 30–300 nm in size, therefore having a far narrower size distribution compared to eukaryotic EVs (Fig. 6.1a) (reviewed in (Zavan et al. 2020; Raposo and Stoorvogel 2013)). OMVs are capable of packaging cargo such as proteins, nucleic acids and toxins within a lipid membrane (Fig. 6.1b) (reviewed in (Kaparakis-Liaskos and Ferrero 2015)). First discovered in 1966, the production of OMVs by the Gram-negative bacterium *Escherichia coli* was initially considered an artefact of bacterial growth, without substantive physiological relevance (Knox et al. 1966). In 1975, interest in OMVs was sparked by the identification of OMVs within the cerebrospinal fluid of patients with meningococcal disease (DeVoe and Gilchrist 1975). Subsequent studies of patients infected with Gram-negative pathogens, including *Helicobacter pylori, Haemophilus influenzae* and *Neisseria meningitidis* identified the presence of OMVs within patient tissue or biological fluids (Fiocca et al. 1999; Ren et al. 2012; Stephens et al. 1982; Namork and Brandtzaeg 2002). However, the functions of OMVs from these pathogens in a disease setting remained unclear.

More recent studies have confirmed that OMVs significantly contribute to fundamental biological processes of their parent bacterium. For example, it has been shown that OMVs can function to facilitate horizontal gene transfer (HGT), the formation of bacterial biofilms and the sequestration of antibiotics (Renelli et al. 2004; Schooling and Beveridge 2006; Manning and Kuehn 2011; Kulkarni et al. 2015). Furthermore, OMVs may promote bacterial pathogenesis by acting as vehicles for the delivery of toxins and immunomodulatory products (reviewed in (Ellis and Kuehn 2010)), or by packaging conserved microbe-associated molecular patterns (MAMPs) from their parent bacterium, that can be detected by host pathogen

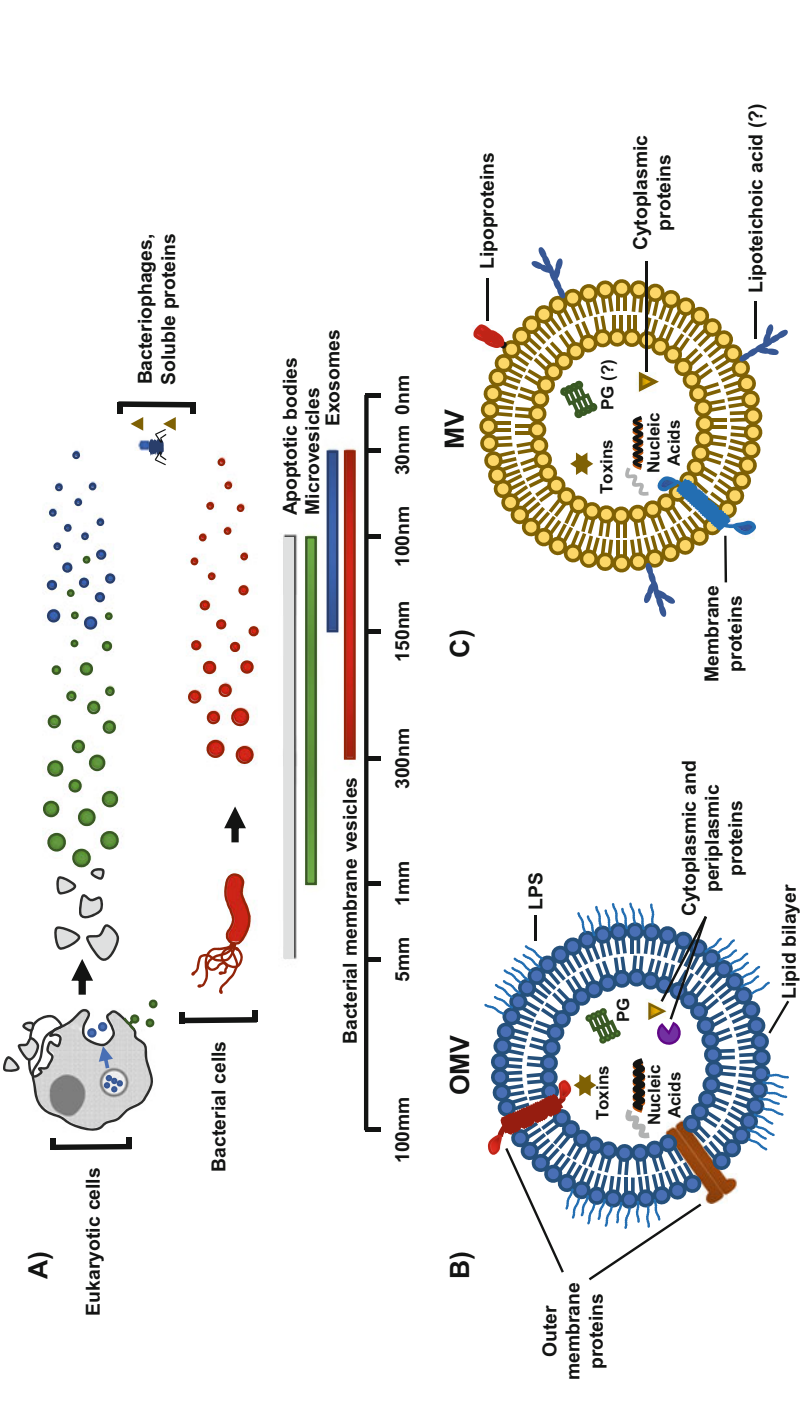

Fig. 6.1 Size and composition of Bacterial membrane vesicles. (**a**) Schematic overview of the size and characteristics of EVs secreted by eukaryotic and prokaryotic cells. Eukaryotic EV release is defined by populations of vesicles emanating from intracellular compartments, termed exosomes (30–150 nm), or from the cell membrane, termed microvesicles (100–1000 nm) or apoptotic bodies (<1–5μm) which are produced during apoptotic cell death. By contrast,

recognition receptors (PRRs) and mediate an innate immune response (reviewed in (Kaparakis-Liaskos and Ferrero 2015; Johnston et al. 2020)). Non-pathogenic bacteria, such as those constituting the human microbiota, also produce OMVs containing MAMPs and research in this field has increased significantly within the last 5 years. It was first identified that OMVs from the commensal bacterium *Bacteroides fragilis* could modulate host immunity (Shen et al. 2012) and numerous studies have since investigated the effects of OMVs produced by the human microbiota in shaping immunological processes within the gut and in other organs (reviewed in (Stentz et al. 2018)). Due to their intrinsic immunogenic properties, OMV-based vaccines have been developed and licenced for human use (Oster et al. 2005), with considerable interest in furthering their development to protect against antibiotic-resistant bacterial infections.

MVs are nanoparticles produced by Gram-positive bacteria that range between 30–300 nm in size (Fig. 6.1c) (reviewed in (Brown et al. 2015)). The release of MVs from Gram-positive bacteria was first reported as early as 1990 (Dorward and Garon 1990). However, in an era where prokaryotic extracellular vesicles were viewed as cellular artefacts, the suggestion of vesicle release from the thick and rigid cell wall of Gram-positive bacteria was unaccepted by the scientific community. While interest in OMVs gained momentum, MV research stalled, with limited studies reporting the characterisation and functions of MVs (Klieve et al. 2005; Mayer and Gottschalk 2003). It was not until 2009, nearly 20 years after their initial identification, that this dogma was challenged by electron microscopy images demonstrating the release of MVs by *Staphylococcus aureus* (Lee et al. 2009). Following this finding, subsequent studies identified MV production by a range of Gram-positive bacteria, including the pathogens *Bacillus anthracis* (Rivera et al. 2010), *Streptococcus pyogenes* (Resch et al. 2016), *Listeria monocytogenes* (Vdovikova et al. 2017) and *Clostridium perfringens* (Jiang et al. 2014) and even the commensal strains *Lactobacillus reuteri* (Grande et al. 2017) and *Enterococcus faecium* (Wagner et al. 2018). Akin to OMVs, MVs have been implicated in a range of biological functions including inter-bacterial communication and host-pathogen interactions (Liao et al. 2014; Obana et al. 2017; Wang et al. 2020). Yet there still remains a significant gap in knowledge surrounding the biogenesis, composition and biological functions of MVs, with significant efforts focused on expanding knowledge in this area.

Fig. 6.1 (continued) prokaryotic bacteria produce bacterial membrane vesicles (BMVs) via their cell membrane (30–300 nm). For comparison, the size of bacteriophages and soluble proteins are depicted at right (<30 nm). (**b**) Gram-negative bacteria produce OMVs which package cargo within a lipid bilayer. OMVs package contents derived from their parent bacterium which include membrane-bound proteins, cytoplasmic or periplasmic proteins, toxins, nucleic acids and peptidoglycan. (**c**) Gram-positive bacteria produce MVs which package cargo including nucleic acids, toxins, cytoplasmic or membrane-bound proteins and lipoproteins. MVs may also contain cell wall components such as peptidoglycan and lipoteichoic acids

The Study of BMVs

Despite more than 50 years of BMV research, difficulties remain within the field in terms of uniformity and standardisation of techniques. By comparison to the field of eukaryotic EVs, the field of bacterial membrane vesicles has not seen the development of guidelines regarding appropriate methods and minimal standards that need be applied for their isolation and characterisation. This is compounded by the isolation and examination of BMVs from a vast number of Gram-negative and Gram-positive bacterial species of interest with varying growth and culturing conditions. Therefore, the techniques used to isolate and characterise vesicles produced by one bacterial species may not be inherently identical to those produced by another. As a result, variables in bacterial strains, culture conditions and vesicle purification methods prevent effective comparisons to be made between BMV studies. With the rapid expansion of studies examining the characteristics and functions of OMVs and MVs, it is becoming increasingly necessary to ensure standardisation and uniformity across the field. Below we outline general techniques used to isolate, purify and analyse OMVs and MVs.

Isolation and Purification

The majority of the first reported OMV preparations were achieved by ultracentrifugation of bacterial supernatants from broth cultures. Whilst succeeding in harvesting OMVs from these species, these seminal studies were unaware of the contaminating debris, including soluble proteins and bacterial structures such as flagella and pili within their vesicle preparations (reviewed in (Kulp and Kuehn 2010)). Gradually, researchers have improved the purity and yield of their vesicle preparations by adopting techniques including ultrafiltration and low-speed differential centrifugation prior to ultracentrifugation (reviewed in (Klimentová and Stulík 2015)). Density-gradient separation of crude BMV preparations, using iodixanol or sucrose mediums, has allowed for greater purification of vesicles for proteomic and immunological analyses (reviewed in (Klimentová and Stulík 2015)), in addition to the separation of different sized OMVs (Kaparakis et al. 2010; Turner et al. 2018). Other groups have also implemented size exclusion chromatography (SEC) techniques to isolate vesicles (Post et al. 2005; Hong et al. 2019; Schulz et al. 2018). A recent study comparing BMV purification methods reported that vesicle purification by density-gradient separation or SEC were both equally suitable for the isolation of highly purified BMV preparations (Dauros Singorenko et al. 2017). However, irrespective of the technique used to isolate and purify BMVs, scientific rigor and careful reporting of the techniques used to isolate and purify these vesicles are necessary to publish meaningful and reproducible findings that can enable comparisons to be made between independent studies.

Characterisation & Analysis

As researchers begin to investigate the functions of BMVs more broadly, it has become increasingly important to thoroughly detail their methods of production and characterisation before reporting BMV functions. For example, the protein content of OMVs and MVs can vary between bacterial species, strains and even by the same strain cultured using different growth media and to varying growth stages (Yara et al. 2019; Taheri et al. 2019; Klimentova et al. 2019; Zavan et al. 2019; Jeon et al. 2016; Jiang et al. 2014). Furthermore, genetic engineering of bacteria enables the generation of hypervesiculating bacterial mutants, which has greatly increased the yield of OMV preparations (Bernadac et al. 1998; Turner et al. 2015; Moon et al. 2012; Ojima et al. 2020; Mitra et al. 2016). These differences in BMV production highlight that it is imperative that researchers clearly define the conditions used to generate, isolate and purify their BMVs, as this may impair the reproducibility of results and limit comparisons between studies if they are not clearly reported.

There are numerous techniques employed to characterise OMVs and MVs which include electron microscopy (EM) to visually assesses vesicle morphology, and nanoparticle tracking analysis (NTA) instruments such as NanoSight™ and ZetaView™ that assess the yield and size of BMVs. Additionally, techniques to characterise vesicular cargo include Western-immunoblot or mass-spectrometry (proteins) (Kaparakis et al. 2010; Zavan et al. 2019; Lee et al. 2009; Choi et al. 2011), lipidomics (lipids) (Jasim et al. 2018; Roier et al. 2015; Jeon et al. 2018) and next-generation DNA and RNA sequencing methods (Koeppen et al. 2016; Bitto et al. 2017). Collectively, the tools and techniques used to isolate and characterise BMVs vary, and this highlights the need for detailed methodology and uniformity in the purification, characterisation and functional analyses of BMVs to enable better comparisons between studies.

BMV Biogenesis

Mechanisms of OMV Biogenesis

The production of OMVs is common to all Gram-negative bacteria, and although many key determinants of OMV biogenesis have been suggested, the molecular pathways underlying vesiculation are only recently being uncovered. Unlike eukaryotic EVs, BMVs do not originate from an intracellular organelle. Two mechanisms of OMV biogenesis have been identified to date. First, OMVs secreted by Gram-negative bacteria may be released via the outer membrane (OM) of their parent bacterium by OM bulging, eventuating in the formation and budding of a new vesicle. OMV budding from the bacterial OM has been described for numerous species and is thought to be a conserved mechanism of OMV biogenesis amongst Gram-negative bacteria (reviewed in (Schwechheimer and Kuehn 2015)).

Blebbing of the bacterial OM can be regulated via several different pathways and OM molecules such as peptidoglycan (PG), proteins and lipids. The bacterial OM is anchored to the periplasmic peptidoglycan layer via strong covalent bonding. Thus, disruptions to the linkages between the OM and PG layer can affect OMV biogenesis, as demonstrated by investigations of *Acinetobacter baumannii, E. coli* and *Porphyromonas gingivalis*, amongst other bacterial species (Moon et al. 2012; Schwechheimer et al. 2014; Iwami et al. 2007; Suzuki et al. 1978). Furthermore, degradation of the PG layer itself, or accumulation of PG fragments within the periplasm, were shown to augment the production of *E. coli* and *P. gingivalis* OMVs (Schwechheimer et al. 2014; Hayashi et al. 2002). OMV biogenesis may also be upregulated in response to other envelope stresses, such as the accumulation of misfolded proteins or OM curvature-inducing molecules (McBroom and Kuehn 2007; Mashburn-Warren et al. 2008; Mashburn and Whiteley 2005). Additionally, the transmembrane Tol-Pal protein system, an important regulator of bacterial membrane integrity, has been identified to play a role in the regulation of OMV biogenesis of both non-pathogenic *E. coli* and pathogenic bacteria including *H. pylori* and *Shigella boydii* (Bernadac et al. 1998; Turner et al. 2015; Mitra et al. 2016).

Another regulator of OMV production is the composition of LPS at the site of vesicle budding, as demonstrated by lipid remodelling in the OM of the pathogen *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) being necessary for OMV release (Elhenawy et al. 2016). Interestingly, another recent study identified a universal regulator of OMV blebbing common to most Gram-negative bacteria via a retrograde lipid transporter system (Roier et al. 2016). Deletion or repression of the VacJ/Yrb ABC phospholipid transporter system in *H. influenzae* and *Vibrio cholerae* increased OMV production due to phospholipid accumulation in the bacterial OM of both species (Roier et al. 2016). The authors suggested that this system may serve as a universal regulator of OMV production by Gram-negative bacteria.

Finally, OMV production can also be modulated by environmental stresses including exposure to antibiotics or unfavourable bacterial growth conditions such as aberrant temperature or pH (McBroom and Kuehn 2007; Kadurugamuwa and Beveridge 1995; Klimentova et al. 2019). Overall, these studies highlight the role of alterations to the cell membrane in regulating OMV biogenesis via blebbing from the OM.

An alternative mechanism of OMV biogenesis was recently identified via stress-induced explosive cell lysis of bacterial cells (Turnbull et al. 2016). The production of *Pseudomonas aeruginosa* OMVs via explosive cell lysis is mediated by a prophage-encoded endolysin whereby membrane fragments of exploded bacteria re-fused to form vesicles, thus capturing cytoplasmic contents (Turnbull et al. 2016). However, it remains to be determined if this mechanism of OMV biogenesis is common to all Gram-negative bacteria or specific to *P. aeruginosa.*

Mechanisms of MV Biogenesis

In contrast to the vast knowledge surrounding OMV biogenesis, few studies defining the mechanisms of MV biogenesis exist to date. The structural differences between the Gram-positive and Gram-negative cell wall imply that there are mechanistic differences between MV and OMV biogenesis (reviewed in (Brown et al. 2015)). While OMVs can be released from the outer membrane of the cell surface, MVs are derived from the cytoplasmic membrane and therefore must traverse the thick layer of peptidoglycan to be released (Brown et al. 2015). However, recent evidence has shown that accumulation of phenol-soluble modulin (PSMs) in the cytoplasmic membrane leads to MV formation by increasing membrane fluidity (Wang et al. 2018; Schlatterer et al. 2018). The first electron microscopy images depicting the release of MVs by *S. aureus* suggest that disruption of the peptidoglycan layer allows for the protrusion of the cytoplasmic membrane through the cell wall, and therefore MV release (Lee et al. 2009). This was followed by similar findings in other species of Gram-positive bacteria and is thought to occur via protrusions through the cell wall mediated by autolysins that hydrolyse peptidoglycan crosslinks (Wang et al. 2018; Toyofuku et al. 2017; Resch et al. 2016; Lee et al. 2009).

An alternative model of MV biogenesis has been observed in *Bacillus subtilis* that involves the disruption of the peptidoglycan cell wall via a prophage-encoded endolysin (Toyofuku et al. 2017). Similar to the formation of OMVs via prophage-mediated explosive cell lysis reported in *P. aeruginosa* (Turnbull et al. 2016), *B. subtilis* MV production by this mechanism is dependent on activation of a defective prophage triggered by genotoxic stress. However, unlike the process in *P. aeruginosa*, explosive cell lysis was not observed in *B. subtilis*, but rather cell death eventuated from the loss of membrane integrity (Toyofuku et al. 2017). This alternative mechanism of MV biogenesis is believed to occur in addition to other mechanisms that do not result in cell death (Toyofuku et al. 2017; Andreoni et al. 2019). Furthermore, a putative phage-encoded endolysin is enriched in *S. pyogenes* MVs (Resch et al. 2016), suggesting a similar mechanism may occur in this species. More studies are required to determine whether the prophage-mediated liberation of MVs via cell death is common to other Gram-positive species.

Bacterial extracellular vesicles are becoming increasingly recognised as a bacterial secretion system for the long-distance delivery of effector molecules. While there have been significant breakthroughs in our understanding of MV biogenesis over the last 10 years, there is still much to uncover. Elucidating the mechanisms by which MV biogenesis occurs can provide a better understanding of the role of MVs in bacterial survival and pathogenesis.

Cargo Packaging into BMVs

BMVs are often enriched in cargo that facilitate specific biological functions. Certain molecules may act as chaperones to facilitate packaging of necessary structural components, while others may directly enact functions such as toxins packaged by pathogens.

Since their discovery, research in the field of BMVs has predominantly focused on defining their contribution to pathogenesis. Consequently, numerous studies have identified virulence effectors associated with OMVs and MVs from various pathogens. Indeed, proteomic profiling of BMVs produced by pathogenic bacteria including *E. coli*, *S. aureus, Streptococcus sp., Neisseria gonorrhoeae* and even members of the non-pathogenic *Bacteroidetes* genus has revealed selective enrichment of proteins in BMVs compared to the outer membrane of their parent bacteria (Lee et al. 2007, 2009; Thay et al. 2013; Olaya-Abril et al. 2014; Biagini et al. 2015; Zielke et al. 2014; Elhenawy et al. 2014). However, the mechanisms underpinning the packaging of these virulence effector proteins into OMVs and MVs remain largely unclear.

One proposed mechanism for cargo selection into OMVs is via outer membrane lipid chaperones which guide molecules to sites of OMV biogenesis. For example, *Klebsiella pneumoniae* is a nosocomial pathogen whose OMVs differentially package protein cargo in response to LPS modifications (Cahill et al. 2015). Specifically, with the use of a *K. pneumoniae* mutant strain deficient in the O antigen of LPS (Δ*wbbO* strain), it was shown that the LPS composition of the bacterial outer membrane impacted the protein composition of OMVs produced (Cahill et al. 2015). Similar results have been reported for OMV and MV production by *P. gingivalis* and Group A streptococcus, whereby the lipid composition of the bacterial membrane was responsible for the selective enrichment of specific proteins or RNA species (Haurat et al. 2011; Resch et al. 2016).

It has long been reported that BMVs can associate with bacterial genetic material including DNA, and more recently RNA (Dorward and Garon 1990; Sjöström et al. 2015; Liao et al. 2014; Choi et al. 2018; Dorward and Garon 1989; Kolling and Matthews 1999). In particular, bacterial DNA can be packaged into OMVs, delivered to eukaryotic host cells and thereafter trafficked to the host cell nucleus (Bitto et al. 2017). Similarly, the delivery of bacterial regulatory RNAs to eukaryotic host cells via OMVs was recently demonstrated, resulting in the modulation of the host immune response (Koeppen et al. 2016; Choi et al. 2017; Zhang et al. 2020). Packaging of nucleic acids into MVs has also been reported to occur by a limited number of Gram-positive species, including *C. perfringens*, *Streptococcus mutans* and *L. reuteri* (Jiang et al. 2014; Liao et al. 2014; Grande et al. 2017). Although there is limited understanding of its biological functions, evidence suggests that MV-bound DNA may serve in biofilm formation (Liao et al. 2014). In addition, RNA was recently detected in MVs from *Streptococcus sp.*, however its role is yet to be elucidated (Choi et al. 2018; Resch et al. 2016). Overall, it remains unclear how

nucleic acids are packaged into BMVs, however their functions in bacterial communications are becoming increasingly clear.

Collectively, it is clear that cargo packaging into BMVs serves to facilitate bacterial pathogenesis and contribute to disease pathology. In the following sections, we will further discuss the varied mechanisms by which the contents associated with OMVs and MVs drive pathology in the human host.

Physiological Roles of BMVs

Physiological Roles of OMVs Produced by Gram-Negative Bacteria

One of the earliest reviews about OMVs speculated that they possess bacteriolytic activity, binding capability, proteolytic activity and a possible role in genetic exchange (Mayrand and Grenier 1989). Thirty years later, researchers have unravelled many of their multi-faceted roles that facilitate pathogenesis, including inter-bacterial communication, biofilm formation, nutrient acquisition and horizontal gene transfer.

Biofilm Formation

Biofilm formation enables bacteria to thrive in a protected microenvironment and is therefore strongly linked to bacterial pathogenesis, resistance and survival (Whitchurch 2002). The role of OMVs in biofilm formation was first observed in 1987, whereby OMVs from the periodontal pathogen *P. gingivalis* were shown to facilitate adherence between bacterial cells (Grenier and Mayrand 1987). Almost 10 years later, this observation was confirmed by transmission electron microscopy imaging that revealed OMVs were common constituents of the extracellular matrix of *P. aeruginosa* biofilms (Schooling and Beveridge 2006). A clear link between biofilm formation and OMV production has since been demonstrated in a number of bacterial species (Yonezawa et al. 2017; Nakao et al. 2018; Esoda and Kuehn 2019). Furthermore, the contribution of OMVs to biofilm formation *in vivo* was demonstrated in a study that showed *P. aeruginosa* OMVs could fuse with mouse corneas to prime the ocular surface for bacterial adhesion and biofilm formation (Metruccio et al. 2016).

Recent studies have identified components of OMVs that influence biofilm formation, such as the major biofilm matrix proteins identified in *V. cholerae* OMVs (Altindis et al. 2014), leucine aminopeptidase PaAP in *P. aeruginosa* OMVs and outer membrane proteins of *H. pylori* OMVs (Altindis et al. 2014; Esoda and Kuehn 2019; Yonezawa et al. 2017). Furthermore, OMV-associated DNA forms part of the biofilm matrix and its negative charge confers adhesive

properties to OMVs (Turnbull et al. 2016; Grande et al. 2015; Schooling et al. 2009). Advances in super-resolution microscopy have led to insights into the release of OMV-bound DNA within biofilms by explosive cell lysis (Turnbull et al. 2016). While these studies shed light on novel roles that OMVs serve in host colonization, more research is needed to understand how OMVs may regulate biofilm establishment.

Nutrient Acquisition

Bacteria are often faced with nutrient-limited conditions in their host, a hurdle which must be overcome to effectively establish colonisation. It is therefore not surprising that one of the earliest putative roles for OMVs was in nutrient acquisition (Forsberg et al. 1981; Thompson et al. 1985). Upregulation of OMV production is observed in nutrient-limited conditions, whilst bacterial enzymes required for nutrient acquisition including proteases, glycosidases and ion-chelators are common components of OMVs, suggesting that nutrient acquisition is a key function (Gerritzen et al. 2019; Kadurugamuwa and Beveridge 1995; Mashburn et al. 2005; Schaar et al. 2011; Elhenawy et al. 2014). However, there have been few studies investigating this aspect of OMV function. Studies have identified OMVs that possess enzymatic activity to liberate energy sources from the environment, such as lignin-degrading ability to liberate carbon and redox-reactivity to sequester metal ions (Salvachúa et al. 2020; Gorby et al. 2008; Subramanian et al. 2018). A recent study showed that the human opportunistic pathogen *P. aeruginosa* uses OMVs to sequester iron and supplement bacterial growth in iron-deficient media, suggesting that OMVs may play a role in iron acquisition in the host environment (Lin et al. 2017). In this study, the delivery of iron to bacteria occurred via receptor-mediated entry, a finding that sheds light on the poorly understood process of OMV entry into bacterial cells and suggests selectivity in nutrient delivery (Lin et al. 2017). Whilst nutrient acquisition by OMVs is a poorly understood process, it presents a novel therapeutic target to limit the growth of bacteria in host systems and therefore warrants further investigation.

Competition and Predation

In their natural environment, bacteria must compete with other species for nutrients and space. This is particularly important in the mixed microbial communities within the host, where bacteria can use BMVs to establish a niche in the host. OMVs can be trafficked out of biofilms (Schooling and Beveridge 2006) and therefore have the potential to serve as long distance antimicrobials against competing species. In addition to their role in inter-species competition, OMVs are also utilised by predatory bacteria to lyse surrounding bacterial species (Berleman et al. 2014; Evans et al. 2012; Vasilyeva et al. 2008; Schulz et al. 2018). The lytic function of OMVs is conferred by peptidoglycan hydolases that are delivered to target cells by

membrane fusion and are capable of destroying the cell wall of Gram-positive and Gram-negative species (Li et al. 1998; Kadurugamuwa and Beveridge 1996; Kadurugamuwa et al. 1998). However, rather than indiscriminately lysing all bacteria, peptidoglycan hydrolases contained within OMVs possess specificity in their mode of action by targeting the peptidoglycan of particular bacterial species (Li et al. 1998). The antimicrobial specificity of OMVs towards certain species is of relevance for the development of novel, targeted OMV-based antibiotics; a potential application of OMVs that remains largely unexplored (Schulz et al. 2018). Overall, there is much to uncover about the antimicrobial properties of OMVs in maintaining homeostasis within mixed microbial communities, particularly in the context of the microbiome, and their possible application as novel antibiotics.

Bacterial Defence

Bacteria have developed sophisticated ways to use OMVs to evade recognition and attack by the host immune system. OMV production increases in response to the host environment (Martinez et al. 2019) and early studies revealed that they confer protection against bacteriolytic factors in human serum (Grenier and Bélanger 1991; Pettit and Judd 1992b). Proteolytic enzymes, LPS and outer membrane proteins carried by OMVs are involved in their ability to confer serum resistance (Grenier and Bélanger 1991; Pettit and Judd 1992a; Lekmeechai et al. 2018). OMV-mediated serum resistance has been implicated in facilitating co-infections of *Moraxella catarrhalis* and *H. influenzae* (Tan et al. 2007), as well as facilitating the spread of the etiological agent of infective endocarditis, *Aggregatibacter actinomycetemcomitans*, through the bloodstream (Lindholm et al. 2020).

OMVs can also protect bacteria against antimicrobial treatment, by harbouring enzymes that neutralise antibiotics or by serving as decoys to sequester antimicrobials away from bacteria (Roszkowiak et al. 2019; Stentz et al. 2015). There are many reports identifying the presence of antibiotic-degrading β-lactamase enzymes within OMVs, a feature which is also linked to bacterial virulence (Yun et al. 2018; Stentz et al. 2015; Ciofu et al. 2000; Giwercman et al. 1992). Antibiotic treatment has also been demonstrated to increase OMV production (Devos et al. 2017; Manning and Kuehn 2011), while blocking OMV release can increase bacterial susceptibility to antibiotics (Kosgodage et al. 2019), indicating that OMVs contribute to antibiotic resistance. *P. aeruginosa* OMVs harbouring enzymatically active β-lactamase have been found to form a layer around *P. aeruginosa* biofilms in the lungs of cystic fibrosis patients, thereby protecting the underlying bacteria from antibiotics (Giwercman et al. 1992). OMVs have also been shown to confer interspecies protection against antibiotics in microbial communities, such as in the gastrointestinal tract (Stentz et al. 2015; Roszkowiak et al. 2019). These findings highlight the clinical importance of characterising the antimicrobial neutralising properties of OMVs and determining the bacterial strains that contain antimicrobial enzymes in order to understand their role in antibiotic resistance.

Additionally, OMVs can protect bacteria against infection by bacteriophages, which are ubiquitous in all natural and host environments (Barr et al. 2013). Specifically, OMVs can sequester phages to defend bacteria from infection, although there are limited studies demonstrating this interaction. Proteomic profiling has revealed OMVs contain phage-targeting receptors (Lee et al. 2007), and the rapid and irreversible binding of phages to OMVs have been observed by electron microscopy (Manning and Kuehn 2011; Biller et al. 2014). *V. cholerae* OMVs contain phage receptors that bind and inactivate phages naturally found in human stools, giving evidence of phage-OMV interaction in host environments (Reyes-Robles et al. 2018). Collectively, these studies suggest that OMVs play a protective role against bacteriophages, particularly in phage-rich environments such as the gastrointestinal tract. However, the interplay between OMVs and phages in host environments remains poorly understood and requires further research.

Horizontal Gene Transfer

OMVs are known to carry DNA that is protected from nuclease digestion and trafficked out of biofilms, suggesting the prospect of their far-reaching dissemination of virulence-related genes and their role in HGT (Schooling et al. 2009; Dorward and Garon 1989). OMV-mediated transfer of virulence genes was demonstrated in studies showing *E. coli* OMVs could transfer plasmid DNA to *S. enterica* serovar Enteritidis, thereby conferring cytotoxic activity and antibiotic resistance to recipient bacteria (Yaron et al. 2000). A similar study demonstrated OMV-mediated transfer of plasmid DNA containing antibiotic resistance genes from *Acinetobacter baylyi* to *E. coli* (Fulsundar et al. 2014). The potential for OMVs to facilitate wide-spread HGT is highlighted in a study that demonstrated *A. baumannii* OMVs transferred plasmids carrying virulence and antibiotic resistance genes to stably transform recipient strains. These transformed recipient strains were subsequently able to act as donors, producing OMVs laden with the acquired plasmid and transforming other bacteria (Rumbo et al. 2011). The rate and efficiency of OMV-mediated transformation has been suggested to be similar to that of bacterial transduction, adding further credence to its biological significance (Tran and Boedicker 2017). However, there appears to be differences in the genetic transformation potential of OMVs from different bacteria (Renelli et al. 2004; Klieve et al. 2005; Tran and Boedicker 2017), which may be due to factors such as restriction modifications conferred by the donor strain, OMV charge and natural competency of the recipient strain (Klieve et al. 2005; Tran and Boedicker 2017; Fulsundar et al. 2014; Tashiro et al. 2017). Collectively, these studies highlight the potential biological relevance of OMVs in HGT, however further studies are required to define these processes *in vivo*.

Physiological Roles of MVs Produced by Gram-Positive Bacteria

There is little known about the non-pathogenic functions of MVs produced by Gram-positive bacteria. Limited reports suggest they serve similar roles to OMVs in biofilm formation, nutrient acquisition, inter-bacterial communication and bacteria defence. However, more studies are required to build an understanding of the functions of MVs in microbial populations.

Membrane vesicles produced by *S. aureus* and *S. mutans* have been identified in biofilms (He et al. 2017; Liao et al. 2014). *S. aureus* MVs have also been shown to enhance bacterial adhesion and mediate cell aggregation to promote biofilm formation (He et al. 2017). Moreover, MV release by *S. aureus* increased upon treatment with vancomycin, which corresponded with an increase in biofilm formation (He et al. 2017). Biofilm formation by *S. mutants* can also be enhanced by the release of extracellular DNA associated with MVs, which aids in facilitating adhesion to surfaces (Liao et al. 2014). Similarly, *E. faecium* MVs contain biofilm-promoting proteins and extracellular matrix-binding proteins that point toward their role in supporting biofilm formation (Wagner et al. 2018).

The proteomic profiles of MVs from a variety of Gram-positive species suggest they function in nutrient acquisition. *S. aureus* MVs contain iron-binding factors that indicate a role in iron sequestration (Lee et al. 2009) and *Streptomyces coelicolor* MVs are enriched in proteins involved in the acquisition of phosphates, iron and carbon sources (Schrempf et al. 2011). Similarly, *Mycobacterium tuberculosis* MVs package the iron-binding compound mycobactin and have been shown to directly participate in the delivery of iron to recipient cells to support their growth in iron-limited conditions (Prados-Rosales et al. 2014).

Although MVs have not been directly implicated in inter-species competition, their contents points to a possible role in lysis of competing species. *B. subtilis* MVs contain endolysins that are known to degrade the cell wall of bacteria (Toyofuku et al. 2017), and *E. faecium* MVs contain glycopeptides that hydrolyse bacterial peptidoglycan (Wagner et al. 2018). Similarly, proteomic analysis of *S. aureus*, *Streptococcus pneumoniae* and *Streptococcus suis* MVs show they contain autolysins that also degrade bacterial peptidoglycan (Haas and Grenier 2015; Lee et al. 2009; Olaya-Abril et al. 2014; Wang et al. 2018). Clearly further studies are needed to further explore the antimicrobial role of MVs.

MVs can also contain enzymes that hydrolyse antibiotics and there is evidence that MVs play a role in bacterial defence against antibiotic treatment and host immune attack (Andreoni et al. 2019; Kosgodage et al. 2019; Lee et al. 2013; Wagner et al. 2018). *S. aureus* MVs reduce bacterial lysis in whole-blood, suggesting that they can protect bacteria against host innate immune molecules (Andreoni et al. 2019). *E. faecium* MVs contain eleven antimicrobial resistance-related proteins, including proteins that confer vancomycin resistance (Wagner et al. 2018), while β-lactamase carried by *S. aureus* MVs enables protection of bacteria, such as *E. coli*, *S. enterica* serovar Enteritidis and other *S. aureus* strains against

ampicillin (Lee et al. 2013). *S. aureus* MVs can also confer resistance against the last-resort antibiotic daptomycin, highlighting the clinical relevance of MVs in promoting antibiotic resistance (Andreoni et al. 2019). Inhibition of bacterial vesiculation may be a potential target to increase the efficacy of antibiotics, as a recent study demonstrated that inhibition of *S. aureus* MV production corresponded with an increased susceptibility to a wide range of antibiotics (Kosgodage et al. 2019).

Gram-positive MVs are reported to contain DNA, however little is known about the forms of DNA they carry and their role in HGT (Klieve et al. 2005; Liao et al. 2014; Sisquella et al. 2017). A study examining MVs from the bovine rumen-derived bacteria *Ruminococcus albus* was the first to demonstrate HGT by MVs (Klieve et al. 2005). DNA associated with *R. albus* MVs was genomic in origin and contained genes encoding for cellulose-digesting enzymes (Klieve et al. 2005). MV-mediated HGT conferred cellulase activity to strains that previously lacked cellulase activity, and this trait was stable and inheritable in subsequent bacterial generations (Klieve et al. 2005). However, *R. albus* MVs were not able to transform the rumen-derived *Butyrivibrio fibrisolvens*, suggesting a possible mechanism for selective transformation of species (Klieve et al. 2005). This study is the first to indicate a role for MV-mediated HGT in microbial communities (Klieve et al. 2005). However, further studies are required to substantiate these findings in other Gram-positive species.

We have merely touched the surface of understanding the multi-faceted functions of MVs in interbacterial communication, biofilm formation and bacterial defence that facilitate pathogenesis. Future research is needed to provide a greater understanding of the ways in which Gram-positive bacteria utilise MVs to their advantage, and to shed light on their possible antimicrobial targets and therapeutic applications.

Pathogen-Derived BMVs Can Modulate the Host Immune Response

Since the discovery of BMVs more than 50 years ago, research investigating their functions has largely centred upon their role in promoting pathogenesis and their detrimental effects on human health. Several clinically relevant Gram-negative and Gram-positive pathogens have been shown to utilise OMVs and MVs as vehicles to deliver virulence effectors to host cells (Lindmark et al. 2009; Wai et al. 2003; Elluri et al. 2014; Thay et al. 2013; Jin et al. 2011; Fiocca et al. 1999; Vdovikova et al. 2017; Rivera et al. 2010). Packaging of bacterial toxins into OMVs and MVs allows pathogenic bacteria to access distal sites within the body without directly translocating away from their environmental niche.

Pathogenic Functions of BMVs at the Mucosal Surface

Pathogenic BMVs Can Disrupt the Epithelial Barrier

The human body is protected from the external environment by skin and the mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts. These mucosal surfaces are important interfaces supporting host-microbe interactions; however, in many instances they are also the site of first contact with pathogens. Whilst some pathogenic bacteria are invasive intracellular pathogens, many bacteria mediate their virulence extracellularly whilst remaining within the mucosa (reviewed in (Acheson and Luccioli 2004)). Such bacteria have developed means to elicit pathogenic effects, via the secretion of soluble proteins or using complex secretion systems that act as a molecular syringe to 'inject' virulence factors inside host epithelial cells, or via the secretion of OMVs or MVs to deliver cargo to the host epithelium without the need for direct cell to cell contact (reviewed in (Thanassi and Hultgren 2000; Kulp and Kuehn 2010)).

Pathogen-derived OMVs and MVs may contain cargo including toxins and immunostimulatory molecules that induce cellular reprogramming and disrupt the integrity of epithelial barriers. For example, *H. pylori* is a Gram-negative pathogen that infects approximately 50% of the world's population, causing chronic inflammation and peptic ulceration (reviewed in (Correa and Piazuelo 2008)). OMVs from *H. pylori* have been studied extensively as they perform numerous functions in facilitating the pathogenesis of their parent bacterium. *H. pylori* OMVs can damage the gastric epithelium via the disruption of tight junction (TJ) proteins, such as zonula occludens (ZO)-1, that act to maintain paracellular junctions (Turkina et al. 2015). Furthermore, *H. pylori* OMVs containing the oncogenic virulence factor 'cytotoxin-associated gene A' (cagA) induce the redistribution of ZO-1 from paracellular junctions to the cytoplasm, resulting in increased epithelial permeability *in vitro* (Turkina et al. 2015).

Campylobacter jejuni is another Gram-negative intestinal pathogen whose OMVs were demonstrated to disrupt epithelial integrity. For example, treating the intestinal epithelial cell lines T84 and Caco-2 with *C. jejuni* OMVs was sufficient to induce cleavage of adherens protein E-cadherin and TJ protein occludin (Elmi et al. 2016). Notably, the disruption of intestinal epithelial cells by *C. jejuni* OMVs allowed for increased invasiveness of their parent bacteria, indicating that the production of OMVs was directly facilitating bacterial pathogenesis. Periodontal pathogens *Treponema denticola* and *P. gingivalis* also produce OMVs that have been shown to disrupt and dislodge epithelial cell layers, allowing their parent bacteria to invade into the underlying tissue and cause further damage to host tissues (Chi et al. 2003; Nakao et al. 2014).

Another mechanism used by pathogenic bacteria to disrupt the epithelial cell barrier is to cause morphological changes to the cytoskeleton of host epithelial cells. Many pathogens are known to elicit this effect via Type I-VI secretion systems or by the secretion of soluble toxins, however little is known about the roles of OMVs in

cytoskeletal rearrangements at the epithelial cell barrier. One prominent pathogen whose OMVs have been investigated is *Bordetella pertussis*, the causative agent of whooping cough. Interestingly, *B. pertussis* OMVs have been isolated post-mortem from a patient with lethal *B. pertussis* infection (Donato et al. 2012). *B. pertussis* OMVs were found to contain adenylate cyclase toxin-hemolysin (CyaA), which is responsible for decreasing epithelial barrier integrity, disrupting TJ proteins and upregulating host cell cyclic AMP (cAMP) signalling, resulting in rearrangement of the actin cytoskeleton (Donato et al. 2012; Hasan et al. 2018). The effects mediated by OMV-associated CyaA toxin facilitate bacterial translocation across the airway epithelium, demonstrating a role for *B. pertussis* OMVs in contributing to the pulmonary inflammation observed during whooping cough disease.

BMVs Can Enter Host Epithelial Cells

As well as mediating pathogenic effects by disrupting epithelial layers, BMVs can also enter epithelial cells, thereby releasing their cargo intracellularly and modulating host cell metabolism (reviewed in (O'Donoghue and Krachler 2016; Kaparakis-Liaskos and Ferrero 2015)). Several routes of cell entry have been suggested for BMVs from different bacterial species. OMV entry into host cells can occur via cholesterol-rich lipid rafts in the host cell membrane. This was first demonstrated using *P. aeruginosa* OMVs, whereby OMVs entered lung epithelial cells in a cholesterol-dependent manner (Bauman and Kuehn 2009). Lipid raft-independent mechanisms of BMV endocytosis have also been described, including macropinocytosis whereby the host cell cytoskeleton engulfs extracellular BMVs, or by receptor-mediated formation of clathrin or caveolin pits (Bomberger et al. 2009; Furuta et al. 2009; Pollak et al. 2012; Bielaszewska et al. 2013; Kunsmann et al. 2015; Vanaja et al. 2016; Kesty et al. 2004; Chatterjee and Chaudhuri 2011; Schaar et al. 2011; Wang et al. 2020; Turner et al. 2018). Direct fusion of the OMV membrane with the host cell membrane has also been observed for membrane vesicles from multiple species (Bomberger et al. 2009; Rompikuntal et al. 2012; Jäger et al. 2015). The specific endocytic mechanisms utilised by BMVs to enter host cells have been discussed extensively elsewhere and therefore will not be explored in great detail here (reviewed in (O'Donoghue and Krachler 2016)).

Interestingly, OMVs or MVs produced by the same species can enter host cells using various endocytic pathways. A study examining the mechanisms used by *H. pylori* OMVs to enter host cells revealed that small (20–100 nm) and large (90–450 nm) OMVs enter gastric epithelial cells via distinct pathways (Turner et al. 2018). Furthermore, it has been reported that the composition of OMVs can also alter their mechanism of uptake by host cells. Notably, *H. pylori* OMVs harbouring the vacuolating cytotoxin (VacA) virulence factor entered gastric epithelial cells via a different mechanism of endocytosis compared to OMVs that did not package VacA toxin (Parker et al. 2010). Furthermore, the lipid composition of OMVs produced by different *E. coli* serotypes has been reported to affect the mechanism and kinetics of OMV-mediated entry into HeLa cells (O'Donoghue

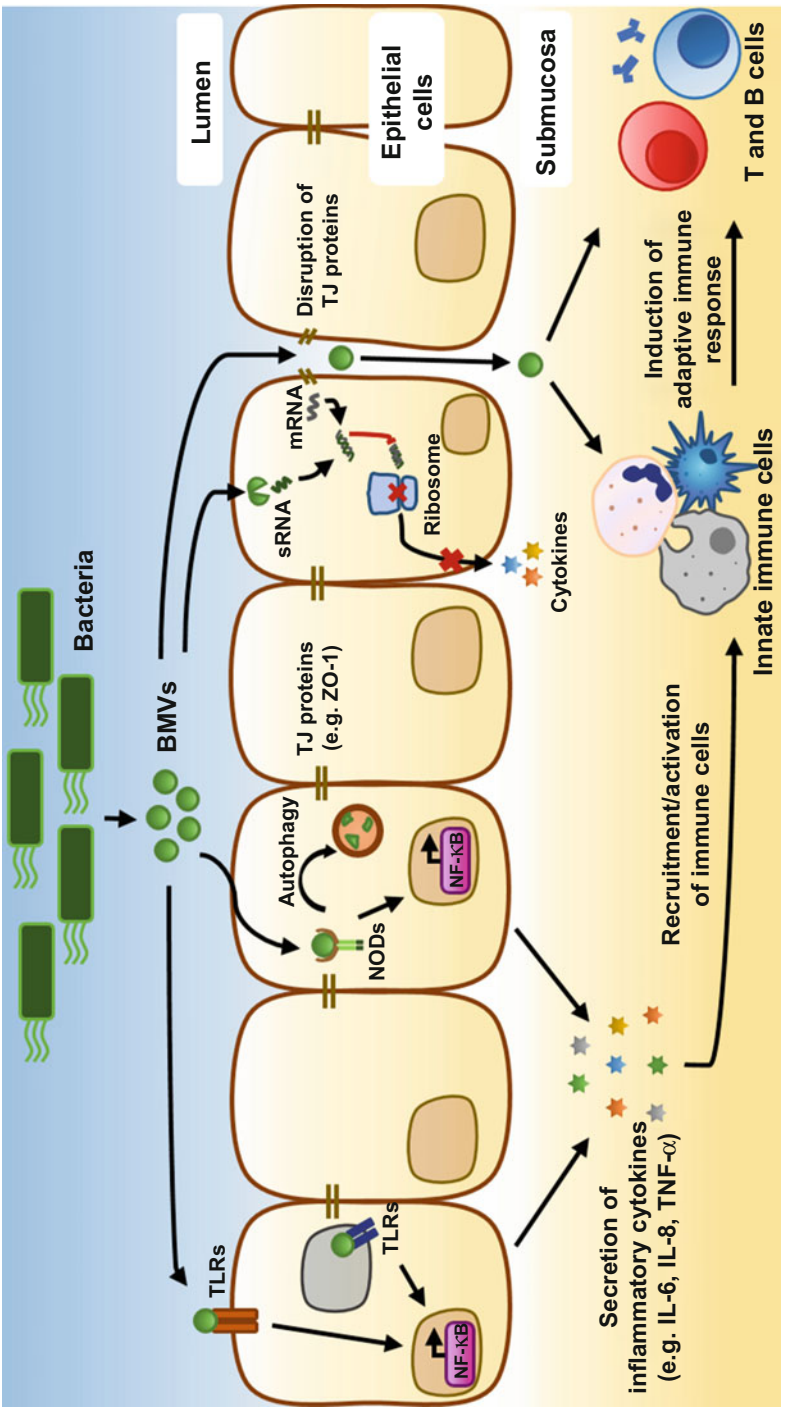

Fig. 6.2 Bacterial membrane vesicles (BMVs) facilitate bacterial pathogenesis via many mechanisms. BMVs can interact with TLRs at the apical membrane of host epithelial cells or enter host cells and interact with TLRs in the endosomal lumen. Intracellular BMVs can also release their cargo resulting in the activation of cytoplasmic NOD receptors. These processes lead to the phosphorylation and nuclear translocation of the transcription factor NF-κB, resulting

et al. 2017). In contrast to knowledge identifying that OMV entry into host cells can be regulated by their cargo or size, very few studies have investigated whether size and cargo composition of MVs produced by Gram positive bacteria can also determine their mechanisms of cellular entry. However, it is known that MVs produced by the Gram-positive pathogens *B. anthracis* and *S. aureus* are endocytosed by host cells via different pathways, entering via receptor-mediated and dynamin-dependent pathways, respectively (Rivera et al. 2010; Wang et al. 2020). These studies suggest that MVs also have the potential to enter host cells via a range of endocytic mechanisms.

BMVs Mediate Innate Immune Signalling in Epithelial Cells

OMVs and MVs produced by pathogens are loaded with immunostimulatory cargo from their parent bacteria, known as MAMPs, including molecules such as LPS, PG and nucleic acids. BMV-associated MAMPs are recognised by host innate immune pathogen recognition receptors (PRRs), which detect foreign molecules and elicit a cytokine-mediated immune response, recruiting immune cells to the site of infection. Some of the PRRs at the epithelial barrier include the transmembrane Toll-like receptors (TLRs) and cytoplasmic nucleotide-binding oligomerisation domain (NOD) receptors. Innate immune receptors of both the TLR and NOD-like receptor (NLR) families have been identified as key responders to BMVs and subsequently have important roles in OMV and MV-mediated pathologies which we discuss below.

TLRs at the Apical and Endosomal Membranes Detect BMVs

Epithelial cells expressing Toll-like receptors are the innate immune system's first responders to pathogenic microbes. The TLR family constitutes 10 transmembrane receptors, which can be located on the apical membrane of epithelial cells, detecting MAMPs such as LPS, flagella and lipoproteins associated with bacterial cells or BMVs (reviewed in (Janeway Jr and Medzhitov 2002)). Ligand-mediated TLR activation leads to the recruitment of adaptor proteins such as Myeloid Differentiation primary response protein (MyD)-88, inducing a signal cascade that eventuates in the phosphorylation and nuclear translocation of pro-inflammatory transcription factors including nuclear factor kappa B (NF-κB) (reviewed in (Akira et al. 2006)).

Fig. 6.2 (continued) in the production and release of pro-inflammatory cytokines including IL-6, IL-8 and TNF-α. Additionally, BMVs can disrupt TJ proteins, resulting in a loss of epithelial barrier integrity and ultimately allowing BMVs and bacteria to cross the epithelial barrier. In this way, BMVs can indirectly and directly modulate innate and adaptive immune cell functions. However, BMVs may also deliver bacterial sRNA to host epithelial cells, which upon binding to host mRNA, may dampen host inflammatory responses

These transcription factors subsequently mediate the secretion of inflammatory cytokines from epithelial cells, which is critical for the recruitment of immune cells to the site of infection.

As BMVs produced by pathogenic bacteria are loaded with MAMPs from their parent bacteria, they are capable of stimulating a pro-inflammatory immune response in the host (Fig. 6.2). This was first reported by Ismail et al. who identified a fundamental role for *H. pylori* OMVs in stimulating an interleukin (IL)-8-mediated inflammatory response by gastric epithelial cells *in vitro* (Ismail et al. 2003). Many other pathogen derived BMVs have since been discovered to elicit inflammatory immune responses by epithelial cells. One clinically relevant example is the opportunistic pathogen *P. aeruginosa*, whose OMVs have a well characterised role in promoting inflammatory responses in the host. OMVs produced by *P. aeruginosa* isolates obtained from patients with cystic fibrosis induced IL-8 secretion by primary human bronchial epithelial cells (Bauman and Kuehn 2006). *P. aeruginosa* OMVs were also shown to engage human epithelial cells in a TLR4-dependent manner, resulting in the increased expression of TLR4 in a positive-feedback mechanism to drive further inflammation, while also increasing host expression of MyD88, NF-κB and IL-1β (Zhao et al. 2013). Thus, it is proposed that the aberrant inflammation driven by OMVs during *P. aeruginosa* infection may contribute to the pathology mediated by this opportunistic pathogen.

TLRs are also located at the endosomal membrane and are capable of detecting microbial products that have entered intracellularly. Once endocytosed by epithelial or immune cells, microbial products are trafficked to endosomes where they are processed for degradation (Nakamura et al. 2014). Similarly, upon entry into host epithelial cells, OMVs produced by Gram-negative bacteria can be degraded via the host cellular degradation pathway of autophagy (Fig. 6.2) (Irving et al. 2014). OMV-mediated autophagy was found to be essential for the initiation of an inflammatory immune response to OMVs (Irving et al. 2014). In addition, OMVs may also be detected by intracellular TLRs -7, −8 and − 9, which respond to microbial nucleic acids (Fig. 6.2) (Cecil et al. 2016; Akira et al. 2006). Recent studies investigating the PRRs activated by OMVs produced by the three prominent periodontal pathogens *P. gingivalis*, *T. denticola* and *Tannerella forsythia* revealed that they could all induce inflammatory immune responses by host immune cells, characterised by the release of IL-1β, as well as tumour necrosis factor (TNF)-α and IL-8 (Cecil et al. 2017). Moreover, the activation of TLR7, TLR8 and TLR9 by *P. gingivalis* and *T. forsythia* OMVs were found to play a direct role in pathogen detection and the subsequent generation of an inflammatory immune response by the host (Cecil et al. 2016).

Bacterial sRNA Delivery by OMVs and MVs

The delivery of bacterial RNA to host cells via the release of membrane vesicles is an emerging and exciting field. RNA was first identified within OMVs only 5 years ago, though the importance of RNA delivery by BMVs has quickly become apparent

(Sjöström et al. 2015). Bacterial small RNA (sRNA), which is similar in size to eukaryotic micro (mi)RNA, can regulate gene expression by binding to host cell messenger RNA (mRNA) (reviewed in (Repoila and Darfeuille 2009)). Furthermore, recent evidence suggests bacterial pathogens differentially package sRNAs into OMVs and MVs to modulate the host immune response. Notably, *P. aeruginosa* OMVs differentially package regulatory sRNAs that can be stably transferred into human airway epithelial cells (Koeppen et al. 2016). It was reported that *P. aeruginosa* OMVs containing sRNA52320 were able to downregulate both LPS-mediated and OMV-mediated inflammatory cytokine production by airway epithelial cells. Furthermore, OMVs carrying sRNA52320 attenuated OMV-induced cytokine secretion and neutrophil infiltration *in vivo* (Koeppen et al. 2016). The immunomodulatory roles of OMV-associated sRNAs may also extend to adaptive immune responses, whereby sRNAs associated with OMVs produced by several periodontal pathogens were isolated, characterised and their immunological effects on T cells were investigated (Choi et al. 2017). Isolation of these sRNAs and their transfection into Jurkat T cells resulted in reduced basal expression of IL-5, IL-13 and IL-15 compared to cells that were transfected with a non-coding sRNA as a control (Choi et al. 2017). The authors proposed that OMV-mediated delivery of sRNAs facilitate the pathogenic functions of their parent bacterium (Choi et al. 2017).

More recently, a role for small non-coding RNAs (sncRNA) in *H. pylori* pathogenesis and evasion of the host immune system has been identified (Zhang et al. 2020). The authors demonstrated that two sncRNA species, sR-2509025 and sR-989262 were enriched in *H. pylori* OMVs compared to their parent bacterium (Zhang et al. 2020). With the use of deletion mutant strains, it was shown that OMVs produced by *H. pylori* strains lacking these sncRNA species induced a heightened immune response when introduced to gastric epithelial cells, compared to OMVs produced by the wild-type strain that were enriched with these immunomodulatory sncRNA species (Zhang et al. 2020). Therefore, it was suggested that differential packaging of particular sncRNA into OMVs is a strategy employed by *H. pylori* to dampen host immunity and promote long-term persistence in the host.

Finally, the delivery of sRNA to host cells by BMVs is not unique to Gram-negative bacteria. The Gram-positive species group A streptococcus differentially regulates RNA packaging into MVs (Resch et al. 2016). However, very few studies have investigated the biological significance of RNA packaging into MVs produced by Gram-positive bacteria and this remains an interesting avenue for further research.

Intracellular Activation of NLRs

The cytoplasmic innate immune receptors NOD1 and NOD2 are responsible for the detection of bacterial PG, a complex molecule constituting the cell walls of both Gram-negative and Gram-positive bacteria (Girardin et al. 2003a, b). Examination of OMVs produced by the mucosal pathogens *H. pylori*, *P. aeruginosa* and

N. gonorrhoeae revealed that OMVs could package PG that could be detected by the host innate immune receptor NOD1 (Kaparakis et al. 2010). Specifically, it was identified that once OMVs entered human epithelial cells, PG contained within OMVs was detected by NOD1, resulting in the induction of a NOD1-dependent pro-inflammatory immune response (Fig. 6.2) (Kaparakis et al. 2010). Furthermore, NOD1 was also found to be essential for the generation of OMV-specific adaptive immune responses *in vivo*, as oral administration of *H. pylori* OMVs to mice lacking the NOD1 receptor did not result in the induction of innate and OMV-specific adaptive immune responses (Kaparakis et al. 2010). Collectively, these findings revealed that OMVs are a conserved mechanism used by Gram-negative pathogens to mediate NOD1-driven inflammation and pathology *in vivo* (Kaparakis et al. 2010). Subsequently, studies also identified the ability of NOD1 and NOD2 to mediate immune responses to both OMVs produced by *A. actinomycetemcomitans, V. cholerae* and MVs from Gram-positive *S. aureus,* amongst other bacteria (Thay et al. 2014; Bielig et al. 2011; Jun et al. 2017).

BMVs can also interact with another member of the NLR family known as NLR-pyrin domain containing 3 (NLRP3) (Wang et al. 2020; Cecil et al. 2017; Fleetwood et al. 2017; Bitto et al. 2018). Activation of NLRP3 by OMVs or MVs leads to the formation of an intracellular complex termed the inflammasome, a protein complex responsible for the maturation of caspase-1 and the subsequent secretion of mature IL-1 family cytokines including IL-1β and IL-18 (reviewed in (Schroder et al. 2010)). For example, *S. aureus* MVs were internalised by human macrophages which resulted in activation of the NLRP3 inflammasome via K^+ efflux (Wang et al. 2020). Additionally, *P. gingivalis* OMVs were found to induce inflammasome activation both *in vitro* and *in vivo*, leading to recruitment of inflammasome adaptor proteins and the secretion of mature IL-1β from murine peritoneal macrophages (Cecil et al. 2017). Collectively, these studies highlight that there are a broad range of PRRs and multiple mechanisms by which BMVs mediate the induction of pro-inflammatory responses in the host.

BMVs can cross the epithelial cell barrier

At the mucosal interface, BMVs produced by pathogenic bacteria may face various fates. As described above, OMVs and MVs can interact with epithelial cell surface receptors, or they may be internalised by host cells and release their cargo into the cell cytoplasm. However, BMVs can also directly cross the epithelial layer and access immune cells within the submucosa (Fig. 6.2) (Kaparakis-Liaskos and Ferrero 2015). It has been hypothesised that BMVs can cross epithelial barriers by different methods including transcytosis or by diffusion through paracellular junctions (reviewed in (Stentz et al. 2018)). Once the epithelial barrier has been crossed, bacterial OMVs and MVs can modulate innate immune cells, which can subsequently affect T and B cell functions and ultimately manipulate host immunity.

BMVs and Macrophages

Macrophages are innate immune cells which survey host tissues and rapidly detect and respond to invading pathogens and their secreted products. Macrophages can phagocytose extracellular bacterial products including BMVs, resulting in the production of inflammatory cytokines to ultimately facilitate the induction of a pathogen-specific adaptive immune response (reviewed in (Arango Duque and Descoteaux 2014)). However, some pathogens can specifically target macrophages to facilitate the onset of pathogenesis in the host. One such bacterium that mediates pathogenesis by macrophages is *P. gingivalis* which produces OMVs that can interact with macrophages in the subgingival cavity (Imayoshi et al. 2011). It was found that *P. gingivalis* OMVs induced the production of inflammatory mediators such as nitric oxide (NO) and inducible nitric oxide synthase (iNOS) by murine macrophages *in vitro* (Imayoshi et al. 2011). Furthermore, *S.* Typhimurium OMVs were also found to interact with murine macrophages via their ability to induce the production of TNF and NO production, whilst OMVs from a range of periodontal pathogens induced the secretion of TNF-α, IL-8 and IL-1β from THP-1 monocytes (Alaniz et al. 2007; Cecil et al. 2017). Additionally, BMVs may deliver their cytotoxic cargo to macrophages, as is the case with OMVs produced by the pathogen *A. baumannii* (Jin et al. 2011). Specifically, OMVs produced by wild-type *A. baumannii* were found to package the virulence effector protein AbOmpA, resulting in a significant increase in cytotoxic cell death of U937 macrophage-like cells compared to OMVs isolated from Δ*OmpA* mutant *A. baumannii* strains lacking AbOmpA (Jin et al. 2011).

Whilst BMVs facilitate pathogenesis by driving inflammation in the host to promote tissue damage, they can also downregulate the immune response generated by macrophages to prolong bacterial survival within the host. For example, OMVs produced by *H. pylori* induce anti-inflammatory IL-10 secretion by human peripheral blood mononuclear cells, whilst *P. gingivalis* OMVs can degrade the CD14 receptor, rendering macrophages incapable of responding to bacterial LPS and thus avoiding hyperinflammatory immune responses (Winter et al. 2014; Duncan et al. 2004).

In contrast to BMVs produced by gastrointestinal or periodontal pathogens, BMVs produced by respiratory pathogens can interact with alveolar macrophages. For example, *Legionella pneumophila*, a causative agent of severe pneumonia, produces OMVs that induce TLR2- and NF-κB-dependent increases in inflammatory cytokine production by alveolar macrophages (Jung et al. 2016). However, by contrast, prolonged exposure to *L. pneumophila* OMVs may facilitate bacterial replication within alveolar macrophages by downregulating host immune responses (Jung et al. 2016). Similarly, *M. tuberculosis* is a chronic intracellular pathogen that infects alveolar macrophages (reviewed in (Pieters 2008)). EVs secreted by *M. tuberculosis*-infected macrophages are immunostimulatory to the human host, however it was unclear until recently whether this immunogenicity was attributed to host-derived exosomes containing bacterial cargo or directly attributed to BMVs

emanating from the intracellular bacterium (Athman et al. 2015). Using density-gradient separation to resolve prokaryotic BMVs from eukaryotic EVs, it was elucidated that *M. tuberculosis* BMVs are responsible for this immunogenicity (Athman et al. 2015). Collectively, these studies highlight the roles of BMVs in promoting bacterial pathogenesis via their interactions with macrophages (Fig. 6.2).

BMVs and Neutrophils

The interactions between BMVs and neutrophils can be categorised into two groups: immunomodulatory effects and Neutrophil Extracellular Trap (NET)-related. Foremost, neutrophils are involved in inflammatory immune responses as they migrate to the site of infection in response to chemoattractant cytokines and chemokines release by epithelial cells. However, BMVs can also directly mediate inflammatory cytokine production by neutrophils, as demonstrated using *N. meningitidis* OMVs and *S. aureus* MVs (Lapinet et al. 2000; Askarian et al. 2018). Second, NETs are released by neutrophils upon cell death, whereby fibrillar networks composed of bacterial DNA and antimicrobial peptides (AMPs) extend from the cell membrane to trap and kill surrounding pathogens, preventing their spread throughout the body (reviewed in (Papayannopoulos 2018)). The pathogens *S. pneumoniae* and *P. aeruginosa* are captured by NETs to prevent their dissemination through the host, however they have evolved mechanisms to evade NET entrapment via the secretion of BMVs (Jhelum et al. 2018; Shan et al. 2014). Specifically, *S. pneumoniae* packages the extracellular DNase TatD into its MVs to degrade the structure of neutrophil NETs, whereas OMVs produced by highly cytotoxic strains of *P. aeruginosa* bind NETs to reduce the available amount of NET that can bind to the parent bacteria (Jhelum et al. 2018; Shan et al. 2014). Overall, BMVs can interact with neutrophils to potentiate disease pathologies by eliciting inflammatory immune signalling, or they may subvert normal immune responses by disrupting NET formation and thereby modulating the host's response to infection.

BMVs and DCs

Dendritic cells (DCs) are professional antigen presenting cells and are considered to bridge the innate and adaptive immune systems. One of the earliest reports of DC activation by BMVs was demonstrated using the pathogen *S.* Typhimurium (Alaniz et al. 2007). The authors showed that *S.* Typhimurium OMVs induced the maturation of murine bone marrow-derived DCs (BMDCs) and stimulated pro-inflammatory cytokine signalling (Alaniz et al. 2007). Interestingly, it has also been reported that the commensal bacterium *B. fragilis* produces OMVs that can interact with DCs, inducing anti-inflammatory immune signalling which may represent an important regulator of host immunity by the microbiota (Shen et al. 2012; Chu et al. 2016). These studies suggest that the outcome of the interactions between OMVs and DCs may be dependent on the pathogen from which the OMVs are derived. By contrast,

there is still very limited knowledge regarding the interactions between DCs and MVs released by Gram-positive bacteria and how these may modulate immunity in the host.

BMVs and Adaptive Immune Cells

The innate immune system functions as an early response to mitigate infection, whereas adaptive immunity functions to provide the host with long term protection from pathogens. The following section will briefly describe the ability of BMVs to modulate T cell and B cells responses of the humoral immune system.

While direct interactions between humoral B cells and BMVs are not yet well elucidated, BMVs from multiple pathogens have been implicated in modulating B cell responses. OMVs from pathogens including *N. meningitidis* and *M. catarrhalis* have been shown to promote B cell activation via OMV-associated DNA delivery to intracellular TLR9, and to induce the production of long-term memory B cells, respectively (Vidakovics et al. 2010; Romeu et al. 2014). More recent studies examining the efficacy of BMV-based vaccines have demonstrated significant antibody production by B cells in response to OMV or MV administration (Wang et al. 2017; Stevenson et al. 2018).

By contrast, the roles of T cells in BMV-mediated immune responses are better understood. T cells are critical in the response to pathogenic infection and several studies have indicated that BMVs from various pathogens can induce T cell activation (Alaniz et al. 2007; Kim et al. 2013; Schetters et al. 2019). However, in contrast, chronic pathogens including *H. pylori* and *M. tuberculosis* release BMVs which can inhibit T cell proliferation and function. For example, *H. pylori* OMVs can induce apoptosis of Jurkat T cells or supress T cell function in an IL-10 and cyclo-oxygenase-2 (COX-2)-dependent manner (Winter et al. 2014; Hock et al. 2017). Similarly, *M. tuberculosis* vesicles inhibit $CD4^+$ T cell responses to facilitate bacterial survival *in vivo* (Athman et al. 2017). Collectively, these studies and others have demonstrated that BMVs modulate cellular immune responses. The therapeutic potential of BMVs for use in stimulating protective immunity against bacterial infections will be covered in greater detail later in this chapter.

Microbiota-Derived BMVs Modulate Immune Responses

The human microbiota is a community of organisms that inhabit various sites throughout the body. This complex community is comprised of bacteria, viruses, fungi and bacteriophages whose metabolic functions are closely linked to the environment they inhabit. In particular, both Gram-negative and Gram-positive bacteria occupy niches within the gastrointestinal tract, lungs, skin, urinary tract and other such mucosal surfaces. The mutualistic relationships between host and microbiota are broad, whereby the microbiota facilitates the human host in the

acquisition of nutrients and the maintenance of immunological homeostasis. Interestingly, recent evidence suggests that microbial secreted factors, including OMVs and MVs, are key determinants in many of the health benefits conferred by the microbiota.

BMVs in Host-Microbiota Interactions at Mucosal Surfaces

Membrane vesicles produced by the microbiota are able to gain direct access to the adjacent epithelium, whereas their parent bacteria may be physically inhibited by mucus barriers. Akin to pathogen-derived OMVs, epithelial cells can endocytose commensal OMVs resulting in the release of their content into the host cell cytoplasm (Cañas et al. 2016; Cañas et al. 2018; Jones et al. 2020). The cargo associated with commensal OMVs and MVs is constituted of molecules derived from their parent bacterium, including membrane components such as LPS and PG which can be immunogenic in the host. However, despite ongoing production of BMVs by commensal bacteria, they are not regarded to elicit uncontrolled hyper-inflammatory immune responses within the host during normal conditions. Rather, commensal-derived BMVs can induce both pro- and anti-inflammatory immune responses by the host, contributing to immunological homeostasis.

OMVs produced by commensal *E. coli* strain 'C25' were identified to elicit moderate IL-8 secretion from multiple intestinal epithelial cell lines *in vitro* (Patten et al. 2017). However, pre-treatment of the intestinal cell line Caco-2 with *E. coli* C25 OMVs inhibited subsequent internalisation of live bacteria by Caco-2 cells, indicating that OMV-mediated priming of the intestinal epithelium may confer protection against challenge by invasive bacteria (Patten et al. 2017). The authors suggested that the mild inflammatory response induced by these commensal OMVs mimicked the homeostatic conditions of the healthy intestine, implicating a role for OMVs in immunological maintenance in the gut.

Membrane vesicles from other commensal and probiotic *E. coli* strains have also been studied extensively to elucidate their functions in promoting intestinal homeostasis. For example, OMVs produced by the probiotic *E. coli* strain Nissle 1917 and commensal *E. coli* strain ECOR12 are internalised by intestinal epithelial cells via clathrin-mediated endocytosis (Cañas et al. 2016). Subsequent studies by the same group identified that OMVs from both *E. coli* Nissle 1917 and ECOR12 modulated innate immune responses in multiple epithelial cell models toward an anti-inflammatory profile by regulating the expression of cytokines such as IL-10 and antimicrobial peptides including human beta-defensin-2 (Fábrega et al. 2016). Furthermore, OMVs produced by the probiotic *E. coli* Nissle 1917 improved intestinal barrier integrity by upregulating the expression of TJ proteins ZO-1 and claudin-14 and downregulating the expression of pro-inflammatory transcription factors iNOS and COX-2, as well as various pro-inflammatory cytokines (Alvarez et al. 2016; Fábrega et al. 2017). Moreover, probiotic *E. coli* Nissle 1917 OMVs conferred protection against chemically induced colitis in mice (Fábrega et al. 2017).

Similar to pathogen-derived OMVs, it was reported that OMVs produced by both *E. coli* strains Nissle 1917 and ECOR12 induced NOD1-dependent innate immune responses characterised by the activation of NF-κB and the secretion of IL-6 and IL-8 (Cañas et al. 2018). These findings indicate that the interkingdom communications mediated by BMVs are complex, whereby both pro-inflammatory and anti-inflammatory signalling pathways may be induced by the commensal microbiota to maintain immunological homeostasis. Further studies are required to better elucidate the signalling pathways induced by BMVs produced by the microbiota to broaden our understanding of their fundamental roles in maintaining homeostasis in the dynamic environment of the gut.

Members of the Gram-positive commensal *Lactobacillus* genus have long been considered to be probiotic due to the health benefits they confer to the host. Recently, MVs produced by several *Lactobacillus* species have been shown to elicit similar probiotic effects. *Lactobacillus plantarum* MVs were first reported to upregulate antimicrobial defence genes in Caco-2 cells *in vitro* (Li et al. 2017). Interestingly, in a *Caenorhabditis elegans* model of infection, *L. plantarum* MVs upregulated antimicrobial defence genes, conferring moderate protection against vancomycin-resistant *E. faecium* infection (Li et al. 2017). This study demonstrates the potential for the incorporation of *L. plantarum* MVs into novel therapeutics against Enterococcal infection. Amongst other *Lactobacillus* species, *Lactobacillus sakei*-derived MVs promote the production of IgA antibodies from Peyer's Patch cells of the murine intestine, which function to neutralise extracellular toxins and protect epithelial cells from bacterial invasion (Yamasaki-Yashiki et al. 2019). Furthermore, in an *ex vivo* model of HIV-1 infection of human cervico-vaginal and tonsillar tissues, incubation with *Lactobacillus crispatus* or *Lactobacillus gasseri* MVs for 1 hour was sufficient to protect against viral replication (Ñahui Palomino et al. 2019). These studies indicate the protective properties of MVs produced by the *Lactobacillus* genus and provide insights into the mechanisms by which these probiotic bacteria confer health benefits to the host.

The Gram-negative anaerobe *B. fragilis* is a member of the intestinal microbiota. *B. fragilis* bacteria are encapsulated by a polysaccharide A (PSA) capsule which mediates anti-inflammatory effects via a TLR2-dependent interaction with dendritic cells, leading to the activation of regulatory T cells and subsequent secretion of the immunosuppressive cytokine IL-10 (Mazmanian et al. 2008; Ochoa-Repáraz et al. 2010; Round and Mazmanian 2010). However, *B. fragilis* is a non-motile bacterium and early studies could not identify the method of PSA delivery to the host immune system (Shen et al. 2012; Cerdeño-Tárraga et al. 2005). Ultimately, OMVs produced by *B. fragilis* were found to associate with PSA and elicit immunosuppressive effects via PSA-TLR2 dependent interactions (Shen et al. 2012). Oral administration of *B. fragilis* OMVs to mice conferred protection against colitis via the activation of regulatory T cells and the production of IL-10 (Shen et al. 2012). This study was the first to identify a specific bacterial species and mechanism by which microbiota-derived OMVs conferred health benefits to the host (Shen et al. 2012).

With the advent of more powerful DNA sequencing technologies, alleles for Crohn's disease susceptibility have been implicated in the immunomodulatory

pathway subverted by *B. fragilis* and their OMVs (Chu et al. 2016). Specifically, the genes *Atg16l1* and *Nod2* involved in the non-canonical autophagy pathway are essential to suppress mucosal inflammation via dendritic cell-dependent induction of regulatory T cells (Chu et al. 2016). Immune cells from human subjects with a major Crohn's disease risk variant in the *Atg16l1* allele were defective in regulatory T cell responses to *B. fragilis* OMVs in an *ex vivo* model, highlighting that gene-microbiota interactions mediated by BMVs are critical for the induction of mucosal tolerance and may be relevant to the aetiology of inflammatory bowel diseases (Chu et al. 2016).

Finally, *B. fragilis* has been implicated in mediating protection from multiple sclerosis, a demyelinating disease of the central nervous system (CNS). Using a murine model of multiple sclerosis, administration of *B. fragilis* bacteria was sufficient to confer protection against neuronal demyelination, however this protection was abrogated in IL-10 deficient mice or upon the administration of PSA-deficient *B. fragilis* strains (Ochoa-Repáraz et al. 2010). The author's suggested that the protection conferred by *B. fragilis* was dependent upon PSA. Notably, the authors did not investigate the potential for OMV-associated PSA in protection against neuronal demyelination. The potential for *B. fragilis* OMV-based therapies against demyelinating conditions such as multiple sclerosis remains an avenue for further research.

Commensal BMVs Mediate Long-Distance Anti-Inflammatory Effects

There is mounting interest in the potential for extracellular factors secreted by the gut microbiota to travel to distal sites within the body to mediate their effects (reviewed in (Ahmadi Badi et al. 2017)). BMVs may represent one mechanism responsible for long-distance transport of such factors, protecting them from degradation by the host. However, whilst many studies have speculated on the effects of microbiota-derived BMVs outside of the intestinal lumen, very few studies have investigated these interactions experimentally. Nonetheless, microbiota-derived BMVs have been collected from both urine and blood samples, highlighting that they can cross the epithelial boundary and enter the bloodstream to be distributed throughout the body (Yoo et al. 2016; Lee et al. 2017; Park et al. 2017). Using DNA extraction and sequencing methods, these studies provide insights into the composition of the microbiota during states of both health and disease. A more recent study provided a protocol to isolate BMVs from biological materials and eukaryotic EVs found within bodily fluids (Tulkens et al. 2020). The authors reported that orthogonal implementation of ultrafiltration, size-exclusion chromatography and density-gradient ultracentrifugation resulted in the isolation of BMVs from bodily fluids within 72 hours, commenting that this method may prove valuable in the

characterisation of BMVs within bodily fluids and drive the development of novel clinical diagnostic kits (Tulkens et al. 2020).

Depressive and neurological disorders have been linked to dysregulated metabolic functions of the microbiota, termed 'dysbiosis', the symptoms of which may improve with restoration of a more normal microbiota community. Thus, many commensal bacteria and their secreted BMVs are under investigation as novel strategies for the treatment of extra-intestinal diseases. For example, administration of *L. plantarum* MVs to mice was found to reduce stress-induced depressive behaviours and restored the normal expression of brain-derived neurotrophic factor (BDNF), a molecule depleted during depression (Choi et al. 2019). Additionally, *Akkermansia muciniphila* is a Gram-negative commensal bacterium with anti-inflammatory properties that are becoming increasingly well recognised. As well as eliciting immunomodulatory effects directly in the gut (Kang et al. 2013), *A. muciniphila* OMVs administered to C57BL/6 mice were shown to alleviate symptoms of cardiovascular disease such as weight gain, adipose tissue inflammation and increased blood glucose and cholesterol (Ashrafian et al. 2019). Interestingly, the beneficial effects mediated by *A. muciniphila* OMVs were stronger than those mediated by their parent bacteria, however it should be noted that it remains difficult to normalise between administration of OMVs and live bacteria.

Recent technological advances have revealed that areas of the body that were previously perceived to be sterile, such as the brain, have been found to harbour microbial products (Branton et al. 2013; Emery et al. 2017). Notably, studies investigating Alzheimer's disease have discovered that brains of both healthy and diseased patients showed evidence of microbial products including *E. coli* LPS and K99 pili protein (Zhan et al. 2016). However, it remains contentious whether the brain may harbour its own resident microbiota or if the microbial molecules discovered in the brain were secreted from bacteria at distal sites, potentially via BMVs.

Evidently, the potential health benefits conferred by microbiota derived OMVs and MVs are broad and our understanding of their implications is still in its infancy. With the advent of more powerful resolving technologies being employed within the EV research field, we may soon be able to precisely identify the specific origins of BMVs within the bloodstream and characterise their functions after crossing the epithelial boundary. Limited studies have thus far demonstrated tissue tropism for microbiota derived BMVs in the bloodstream and this remains an avenue of important future research. Furthermore, few studies have investigated whether OMVs or MVs within the blood can cross the endothelial blood-brain barrier (BBB). Answering these emerging questions raises exciting new research avenues aiming to better understand the contribution of BMVs to systemic and neurological disorders.

Applications of BMVs

BMVs as Vaccine Candidates

A global rise in the incidence of antibiotic resistance necessitates that new therapies be developed to combat drug-resistant microbes. Such therapies may involve the use of BMVs either as novel vaccine candidates or as therapeutics to facilitate drug delivery.

OMVs and MVs have become interesting vaccine candidates in the last 20 years due to many of their innate immunostimulatory properties discussed throughout this chapter. First, they are relatively simple and cost-effective to produce, and more recent studies have demonstrated that upscaling their production is within the means of modern technologies (Baart et al. 2007; van de Waterbeemd et al. 2013). Second, BMVs package antigenic contents from their parent bacterium in a non-replicative form and are able to protect their cargo from degradative enzymes and potentially deliver them to specific sites within the body. Accordingly, BMV-associated proteins can be delivered to host cells in their native form which avoids the need for attenuation or inactivation of live bacterial cells. Finally, OMVs in vaccine formulations have proven to be stable for long periods of time and more importantly, safe for administration to humans (Alves et al. 2016; Arigita et al. 2004; Oster et al. 2005).

As discussed previously in this chapter, OMVs and MVs induce innate and adaptive immune responses in the host as they contain MAMPs which can interact with host innate immune PRRs to mediate an immune response. Indeed, vaccination of mice with *E. coli* OMVs was shown to not only mediate the production of cytokines that are indicative of Th1 and Th17 responses, but could also protect mice from lethal challenge via the generation of T cell-dependent immunity (Kim et al. 2013). Similar observations have also been observed using murine models whereby OMVs and MVs produced by *S.* Typhimurium, *V. cholerae* and *S. aureus* have been used to protect against subsequent bacterial infection (Alaniz et al. 2007; Schild et al. 2008; Sinha et al. 2015; Pritsch et al. 2016; Choi et al. 2015). A more recent study identified a role for *A. baumannii* OMVs as a potential therapeutic to combat multi-drug resistant bacterial infection (Huang et al. 2019). Mice were immunised with *A. baumannii* OMVs, resulting in the production of high titre IgG antibodies which, when purified from vaccinated animals and re-introduced in combination with quinolone antibiotics, significantly improved bacterial susceptibility to antibiotics in multiple murine models of infection (Huang et al. 2019). The authors noted that anti-*A. baumannii* OMV antibodies were likely exhibiting their function by blockade of outer membrane porins, thus preventing antibiotic efflux and improving antibiotic susceptibility (Huang et al. 2019). This study provides an interesting example of the use of BMVs as a novel therapeutic agent to combat antibiotic resistant pathogens (Huang et al. 2019).

The ability of OMVs and MVs to deliver antigenic cargo to host cells makes them dually useful in vaccine development as both an antigen and an adjuvant. To date,

the only OMV-based vaccine licensed for human use confers protection against meningococcal serotype B disease, caused by infection with the Gram-negative pathogen *N. meningitidis*. The MeNZB OMV-based vaccine was first implemented in New Zealand whereby reports indicated that the vaccine was protective against infection in both adults and children (Oster et al. 2005; Oster et al. 2007). OMV-based vaccines against meningococcal serogroup B disease are now available globally (reviewed in (Holst et al. 2013)). Interestingly, a retrospective study investigating the efficacy of MeNZB in New Zealand identified that protection conferred by vaccine administration was also 31% effective against the closely related *N. gonorrhoeae* (Petousis-Harris et al. 2017). This was the first report of a vaccine showing effectiveness against gonorrhoea, albeit limited.

BMVs as Novel Therapeutics

Another advantage of OMV and MV-based vaccines is the ease with which bacteria can be genetically manipulated to express certain antigenic epitopes. These antigenic epitopes can be anchored to native OM proteins, including the ClyA protein of *E. coli* or fHbp protein of *N. meningitidis*, resulting in their association with OMVs at a high concentration (Kim et al. 2008; Chen et al. 2010; Huang et al. 2016; Rappazzo et al. 2016; Salverda et al. 2016). Using this method, it is hoped that a more successful influenza vaccine may be developed using *E. coli* OMVs as a platform (Rappazzo et al. 2016). The current influenza vaccine is designed against a strain specific glycoprotein haemagglutinin, however these molecules are highly variable and thus the vaccine must be re-designed annually (reviewed in (Treanor 2015)). The conserved ectodomain matrix 2 (M2e) ion channel motif is a highly conserved membrane protein of the influenza A strain (Mezhenskaya et al. 2019). Engineering of the commensal *E. coli* Nissle 1917 to express a multimeric construct of the M2e receptor via fusion with ClyA, a transmembrane protein enriched in *E. coli* OMVs, conferred 100% protection against lethal challenge of a mouse-adapted H1N1 influenza strain PR8 (Rappazzo et al. 2016). Additionally, an *E. coli* OM autotransporter (Hbp) was also engineered to perform as a platform to display multiple antigenic epitopes. The Hbp protein associated with *E. coli* OMV surfaces at a high density, indicating that this model may benefit researchers wanting to display heterologous antigens in OMV-based vaccines in the future (Daleke-Schermerhorn et al. 2014).

An alternative model of engineering BMVs for their use as vaccines is via the generation of hypervesiculating mutants by disruption of bacterial outer membrane integrity (Turner et al. 2015; Watkins et al. 2017). However, as many outer membrane proteins and lipids are essential for cargo trafficking into vesicles, conflicting reports raise doubts as to whether the contents, structure and composition of BMVs produced by hypervesiculation, via the disruption of the bacterial outer membrane, remain identical to their counterpart wild-type BMVs (Schager et al. 2018; Pérez-Cruz et al. 2016). Collectively, these studies highlight the potential for the genetic

engineering of antigenic epitopes into BMVs to provide benefit in future vaccine research. Moreover, consideration must to be given to how these vesicles are produced as this may ultimately define their composition and functions.

Drug Delivery

Whilst the biodistribution of BMVs in the human body has not been fully elucidated, multiple recent studies have identified tissue-specific tropisms (Jang et al. 2015; Jones et al. 2020). As such, the potential for OMVs and MVs to serve as drug delivery vehicles has attracted significant attention. Compared to more well-established methods of drug delivery using nanoparticles including liposomes or metal-based nanoparticles, OMVs and MVs have the advantage that they can target specific tissue types, and even cell types, before entering host cells and releasing their cargo (reviewed in (Yoo et al. 2011)). This concept was first described using *E. coli* engineered to express the enzyme phosphotriesterase (PTE), demonstrating that the activity of PTE was better preserved when associated with OMVs, compared to its soluble form (Alves et al. 2016). These results show that OMVs could be utilised in drug delivery to protect enzymatic function with applications spanning to environmental remediation and potential industrial applications.

Of particular interest to the field is the potential use of engineered OMV- and MV-based therapies as novel cancer treatments. The ability to selectively hone OMVs or MVs to specific tumour cells via the targeting of cancer cell receptors may allow heightened specificity compared to the indiscriminate chemotherapeutics currently in use. Recent studies have materialised this potential in the targeting of HER2 receptors expressed at high density on tumour cell surfaces, and EGF receptors overexpressed on epithelial tumours, by *E. coli* OMVs loaded with anti-tumour siRNA and anti-tumour drugs, respectively (Gujrati et al. 2014; Kim et al. 2017). In both cases, tumour-specific effects and a significant reduction in tumour size were observed, with no apparent side effects. Furthermore, MVs produced by commensal bacteria *Lactobacillus rhamnosus* GG were shown to display cytotoxic effects on hepatic cancer cell line HepG2 *in vitro* and may prove beneficial as drug delivery vehicles (Behzadi et al. 2017).

Novel Technologies Utilising BMVs

Researchers are beginning to realise the potential applications of BMVs using novel technologies. For example, a recent study highlighted a role for BMVs in optoacoustic imaging, whereby *E. coli* OMVs were bioengineered to encapsulate high levels of melanin suitable for imaging applications *in vivo* (Gujrati et al. 2019). The authors identified that BMVs containing melanin were sufficient to facilitate imaging of tumours *in vivo* (Gujrati et al. 2019). Another study identified that

bioengineering of both the internal lumen and external surface of *E. coli* OMVs allowed for biosensing applications by functional capturing of antigen and simultaneous reporting via luminescence (Chen et al. 2017). Finally, a recent study revealed the potential use of BMVs in antibiotic delivery, as antibiotic-treated bacteria efflux antibiotics via OMVs, resulting in their potential use as bactericidal therapies (Huang et al. 2020). Indeed, oral administration of antibiotic-loaded vesicles provided protection against intestinal bacterial infections *in vivo*, demonstrating their efficiency in delivering antibiotics to distal sites (Huang et al. 2020).

These studies highlight the current and potential future applications of BMVs in designing novel therapeutics to presently untreatable infectious and non-communicable diseases. Whilst these seminal studies have provided the premise for future work, further research is needed to realise the broad therapeutic applications of BMVs.

Conclusions and Perspectives

In recent years, BMVs have emerged as key mediators of bacterial communication. OMVs and MVs have been demonstrated to contribute to several biological functions ranging from supporting nutrient acquisition from the external environment to the delivery of virulence effectors to host cells. In particular, a wealth of literature now supports the notion of pathogen-derived OMVs in driving disease pathologies either by the delivery of toxins or by the packaging of MAMPs to promote inflammation within the host. Furthermore, due to their native immunostimulatory properties, OMVs have been successfully utilised in vaccine development and remain promising candidates for drug delivery in cancer settings. In the last 15 years, MVs released by Gram-positive bacteria have become recognised as *bona fide* secretion systems. Since their discovery, studies have demonstrated roles for MVs in promoting antibiotic resistance, the formation of bacterial biofilms and the packaging of toxins to promote pathogenesis. However, whilst these functions of MVs are increasingly being appreciated, a significant knowledge gap still remains regarding their physiological roles in disease, as well as their mechanisms of biogenesis. Furthermore, OMVs and MVs produced by commensal bacteria have recently emerged as important messengers that function to promote host-microbiota communications, thereby preventing the onset and severity of pro-inflammatory diseases of bacterial or autoimmune aetiology. However, we have only begun to uncover the broad scope of host-microbiota interactions, including the roles for microbiota-derived BMVs in inter-kingdom communications. Whilst the field looks ahead to utilise BMVs in novel technologies such as vaccine development, drug delivery and rapid screening techniques for infectious and non-communicable diseases, it is important to continue to improve our fundamental understanding of the biogenesis and functions of both OMVs and MVs to ultimately limit the pathologies they mediate and to harness their therapeutic potential.

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Chapter 7
Fungal Extracellular Vesicles in Pathophysiology

Donovan Garcia-Ceron, Mark R. Bleackley, and Marilyn A. Anderson

Abstract Fungal pathogens are a concern in medicine and agriculture that has been exacerbated by the emergence of antifungal-resistant varieties that severely threaten human and animal health, as well as food security. This had led to the search for new and sustainable treatments for fungal diseases. Innovative solutions require a deeper understanding of the interactions between fungal pathogens and their hosts, and the key determinants of fungal virulence. Recently, a link has emerged between the release of extracellular vesicles (EVs) and fungal virulence that may contribute to finding new methods for fungal control. Fungal EVs carry pigments, carbohydrates, protein, nucleic acids and other macromolecules with similar functions as those found in EVs from other organisms, however certain fungal features, such as the fungal cell wall, impact EV release and cargo. Fungal EVs modulate immune responses in the host, have a role in cell-cell communication and transport molecules that function in virulence. Understanding the function of fungal EVs will expand our knowledge of host-pathogen interactions and may provide new and specific targets for antifungal drugs and agrichemicals.

Keywords Fungi · Extracellular vesicles · Yeast · Plant pathogens · Filamentous fungi

D. Garcia-Ceron · M. A. Anderson (✉)
La Trobe University, Melbourne, Australia
e-mail: 18538197@students.latrobe.edu.au; m.anderson@latrobe.edu.au

M. R. Bleackley
Incannex, Melbourne, Australia
e-mail: m.bleackley@latrobe.edu.au

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The Impact of Fungi on Human Health

Of the estimated 1.5 million total fungal species (Hawksworth 2001), there are 300 severe human pathogens, most of which are members of the genera *Candida, Aspergillus*, and *Cryptococcus* (LIFE 2017). Almost a billion people have fungal infections of skin, nails and hair that are non-life-threatening. However, 150 million people suffer severe fungal diseases, and 1.5 million die each year as a consequence of fungal infections (Bongomin et al. 2017; Brown et al. 2012).

Invasive fungal diseases also impose a huge burden on healthcare systems since they have mortalities of 30 to 90% (Fisher et al. 2020). The estimated cost of hospital care for patients with fungal infections was USD 7.2 billion in 2017 in the US alone (Benedict et al. 2019).

Candida spp. cause infections of mouth, vagina and skin, as well as life-threatening systemic blood infections. Drug-resistant *Candida* strains have a reported mortality of around 25% (CDC 2019) and the cost of hospitalizations due to *Candida spp.* is estimated to be USD 1.4 billion (Benedict et al. 2019). The ability of fungi to infect different tissues and the similarities in the biochemistry of fungal and host cells have created challenges for the generation of new antifungal treatments (Rodrigues and Nosanchuk 2020; Fisher et al. 2020). Right now, a potential crisis is developing due to the spread of drug-resistant *Candida auris.* This species is often resistant to all three main classes of available antifungal treatments (CDC 2019). With an increase of 318% in infection rates in the US from 2015 to 2018, ease of transmission, and challenges in elimination by disinfection, *C. auris* is rapidly becoming a global threat (CDC 2019).

Aspergillus spp. typically cause respiratory diseases such as allergic bronchopulmonary aspergillosis (ABPA) and severe asthma with fungal sensitization (SAFS). The main pathogenic species are *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus* (Lockhart et al. 2020). There are 4.8 million estimated cases of ABPA around the world, and 1.2 million patients develop chronic pulmonary aspergillosis after suffering from tuberculosis (Denning et al. 2011, 2013). The healthcare cost of patients affected by *Aspergillus spp.* is estimated around USD 1.2 billion in 2017, placing a huge load on health infrastructure (Benedict et al. 2019), and although current antifungal therapy is usually effective against these pathogens, there are emerging reports of antifungal resistance (Lockhart et al. 2020). Approximately one third of drug-resistant isolates have no identifiable resistance mechanism, although it is hypothesized that efflux pumps aid in this process (Snelders et al. 2008; da Silva Ferreira et al. 2004).

Cryptococcus spp. cause lung infections in immunocompromised patients, and the disease can evolve into meningoencephalitis particularly in HIV-infected individuals (Rodrigues et al. 1999). It is estimated that almost a quarter of a million of new cases of cryptococcal meningitis occur each year causing 180,000 deaths (Rajasingham et al. 2017).

The Impact of Fungi on Agriculture

There are 8000 estimated species of fungal plant pathogens in the world (Hawksworth 2001). From these, the top 5 most devastating pathogens are *Magnaporthe oryzae, Botrytis cinerea, Puccinia spp., Fusarium graminearum* and *Fusarium oxysporum* (Dean et al. 2012).

M. oryzae causes rice blast disease. The fungus is responsible for global losses of 30% per year, which would have fed 60 million people (Nalley et al. 2016). The disease is present in 85 countries and there is no comprehensive access to resistant varieties of rice (Ryan 2016; Nalley et al. 2016). Reports indicate that if rice blast is not managed, demand for this crop will not be met in future years (Ray et al. 2013).

B. cinerea, also known as gray mold, can infect more than 200 plant species. This broad host spectrum leads to crop losses of about USD10 billion each year (Williamson et al. 2007; Dean et al. 2012). Pesticides are employed extensively to control *B. cinerea* and represent 10% of the world's pesticide market (Dean et al. 2012).

Puccinia spp. cause rust on a variety of crops. The most important members of this genus are those that infect wheat, such as *Puccinia graminis* f. sp. *tritici* (stem rust), *P. graminis* f. sp. *striiformis* (stripe rust), and *P. triticina* (leaf rust) (Dracatos et al. 2018). In 1998 a highly virulent race of *P. graminis* f. sp. *tritici*, named Ug99, emerged in Uganda and migrated into east and southern Africa and the middle east (Saunders et al. 2019). Since then, stem rust epidemics have occurred in Germany, Sweden, the UK, Italy, and western Siberia with losses of 30–40% (Saunders et al. 2019; Bhattacharya 2017; Shamanin et al. 2016). Ug99 can affect 80% of the world's wheat varieties, making this fungus a serious threat to global food security (Singh et al. 2011).

Fusarium graminearum is a pathogen of cereals, such as wheat, barley and corn. It causes a disease named head blight that is characterized by loss of grain quality and a potential accumulation of mycotoxins (Goswami and Kistler 2004). Mycotoxins, such as zearalenone (ZEA) and deoxynivalenol (DON) are secondary metabolites that are particularly toxic for animals and humans (Sobrova et al. 2010). In the US, epidemics of *Fusarium* head blight of wheat caused losses of almost USD 2.4 billion between 1993 and 2001 (Nganje et al. 2004).

The *Fusarium oxysporum* species complex includes more than 100 plant pathogens that each show strong affinity to specific host plants, as well as human and animal hosts. The term *forma specialis* (f. sp.) is used to indicate this host specificity (DeIulio et al. 2018). *F. oxysporum* f. sp. *cubense* (Foc) causes Panama disease of banana. Race 1 of Foc infections almost eradicated *Gros Michel* bananas in the mid-1950s causing losses of USD 2.3 billion (Altendorf 2019). Now, race 4 of Foc is one of the biggest threats to the USD 400 billion banana industry worldwide, with presence in Australia, China, south east Asia, the middle east and Africa (O'Neill et al. 2016; Zheng et al. 2018; Ordoñez et al. 2015).

Characteristics Unique to Fungal EVs

EVs have been isolated from yeast and filamentous fungi (Table 7.1). They are similar in morphology and cargo to EVs from other species (Bleackley et al. 2019a; Rizzo et al. 2020a). Studies on fungal extracellular vesicles lagged remarkably behind studies on human EVs, until a role was proposed in pathogenesis for fungal EVs and in facilitating the colonization of a host (Fig. 7.1) (Freitas et al. 2019; Rodrigues et al. 2007). This hypothesis is based on observations that fungal EVs are loaded with virulence-associated proteins, pigments, cell wall degrading enzymes, immunogenic carbohydrates, and nucleic acids and different types of lipids (Rodrigues et al. 2007, 2008; Alves et al. 2019; Vallejo et al. 2011, 2012b; Albuquerque et al. 2008; Rizzo et al. 2020a). EVs from filamentous fungi are understudied compared to yeast, and there is a gap in the knowledge surrounding the role of EVs in host-pathogen interactions.

Similar to mammalian EVs, the cargo of fungal EVs consists of proteins, nucleic acids and lipids involved in metabolism or basic cytoplasmic processes (Fig. 7.2). Most research on fungal EV cargo has focused on proteins (Bleackley et al. 2019a). Like EVs from other kingdoms, many of the proteins that are packaged into fungal EVs work in basic physiological processes. However, there are proteins in fungal EVs that are specific to fungi, such as those involved with the synthesis and maintenance of the fungal cell wall (Bleackley et al. 2019a; Dawson et al. 2020).

The Fungal Cell Wall Impacts EV Content

The fungal cell wall distinguishes fungi from other eukaryotic cells as it contains polysaccharides that are unique to fungi and hence ideal targets for specific antifungal drugs. Fungal EVs carry proteins involved in cell wall architecture, synthesis and integrity (Bleackley et al. 2019a; Zhao et al. 2019; Brown et al. 2015; Rodrigues et al. 2008). Several synthases of cell wall polysaccharides such as glucan and chitin have been detected in EVs from *C. neoformans*, *H. capsulatum*, *C. albicans*, *P. brasiliensis*, *S. cerevisiae* and *F. oxysporum* f. sp. *vasinfectum*, among others (Rodrigues et al. 2008*;* Gil-Bona et al. 2015*;* de Paula et al. 2019*;* Zhao et al. 2019*;* Bleackley et al. 2019b). EVs from yeast-form *C. albicans* transport 1,3 beta-glucanosyltransferase and interestingly, EVs from biofilm-form *C. albicans* also carry cell wall-degrading enzymes with exo-1,3-beta-glucosidase activity. This supports the observation that the spectrum of EV proteins that affect cell wall architecture changes depending on fungal morphology (Dawson et al. 2020). Other cell wall degrading enzymes found in EVs include chitinases and glucanases (Bleackley et al. 2019b; Oliveira et al. 2010b). These enzymes may be involved in providing cell wall elasticity to allow EV release (Brown et al. 2015) and in stress-triggered cell wall remodeling (Hopke et al. 2018). This cell wall remodeling allows

Table 7.1 All fungal EV studies reported to date with a focus on pathophysiology.

Organism	Studied Morphology	Host	EV Isolation Method	Characterization	Pathophysiological Role of EVs	Main Sources
Candida albicans	Yeast, biofilm	Humans	UC, SEC	Proteomics, RNA analysis	Macrophages and dendritic cells: Increased production of cytokines. *G. mellonella*: Reduction of fungal burden after EV exposure. Biofilm EVs: Role in drug resistance.	Vargas et al. (2015, 2020) Zarnowski et al. (2018), Dawson et al. (2020)
Pichia fermentans	Yeast, biofilm	–	UC	RNA analysis	Not yet reported	Leone et al. (2018)
Cryptococcus gattii	Yeast	Humans	UC	–	Macrophages: Phagocytosis of *C. gattii* EVs	Bielska et al. (2018)
Cryptococcus neoformans	Yeast	Humans	UC	Proteomics, lipidomics, RNA analysis	Macrophages: Increased production of cytokines. Brain endothelial cells: Membrane fuses with EVs from *C. neoformans*.	Huang et al. (2012), Oliveira et al. (2010a), Peres da Silva et al. (2015a), Rodrigues et al. (2007, 2008)
Exophalia dermatitidis	Yeast	Humans	UC, sucrose gradient	–	Neuroblastoma cells: Increased cytotoxicity of melanized EVs	Lavrin et al. (2020)
Histoplasma capsulatum	Yeast	Humans	UC	Proteomics, lipidomics, metabolomics	Macrophages: Reduced phagocytosis	Albuquerque et al. (2008), Baltazar et al. (2016, 2018); Cleare et al. (2020)
Malassezia sympodialis	Yeast	Humans	UC	Proteomics, RNA analysis	PBM cells: Increased cytokine production. Keratinocytes and monocytes: Internalization of *M. sympodialis* EVs	Gehrmann et al. (2011), Johansson et al. (2018)
Paracoccidioides brasiliensis	Yeast	Humans	UC	Proteomics, lipidomics, glycomics, RNA analysis	Macrophages: M1 polarization	da Silva et al. (2016), Peres da Silva et al. (2015a, b), Vallejo et al. (2011)

(continued)

Table 7.1 (continued)

Organism	Studied Morphology	Host	EV Isolation Method	Characterization	Pathophysiological Role of EVs	Main Sources
Paracoccidioides lutzii	Yeast	Humans	UC	Proteomics, glycomics	Not yet reported	Peres da Silva et al. (2015b)
Saccharomyces cerevisiae	Yeast	–	UC	Proteomics, RNA analysis	Prion Sup35: Transported via EVs.	Kabani and Melki (2015), Oliveira et al. (2010b), Zhao et al. (2019)
Sporothrix brasiliensis	Yeast	Humans	UC	Proteomics	Dendritic cells: Increased phagocytic index; higher CFU of EV-exposed cells; increased cytokine production. Mice: Increased skin lesion; higher fungal load in organs	Ikeda et al. (2018)
Sporothrix schenckii	Yeast	Humans	UC	–	Not yet reported	Albuquerque et al. (2008)
Alternaria infectoria	Filamentous	Plants	UC	Proteomics	Not yet reported	Silva et al. (2014)
Aspergillus flavus	Filamentous	Humans	UC	–	Macrophages: Increased production of cytokines; M1 polarization. *G. mellonella*: Reduction of fungal burden after EV exposure.	Brauer et al. (2020)
Aspergillus fumigatus	Filamentous	Humans	UC	Proteomics	Macrophages: Increased EV phagocytosis; increased cytokine production. Neutrophils: Increased EV phagocytosis.	Souza et al. (2019)
Fusarium oxysporum f. sp. vasinfectum	Filamentous	Plants	UC, sucrose gradient	Proteomics	Not yet reported	Bleackley et al. (2019b)
Rhizopus delemar	Filamentous	Humans	Total exosome isolation reagent	RNA analysis	Not yet reported	Liu et al. (2018)

Trichoderma reesei	Filamentous	Humans	Modified UC	Proteomics	Not yet reported	de Paula et al. (2019)
Trichophyton interdigitale	Filamentous	Humans	UC	-	Macrophages: Increased production of cytokines; M1 polarization. Keratinocytes: Increased production of cytokines	Bitencourt et al. (2018)

Production of fungal EVs has been reported for 20 species, and 12 studies have shown that EVs have the ability to modulate immune responses in the host. For recent reviews refer to (Freitas et al. 2019; Rizzo et al. 2020a)

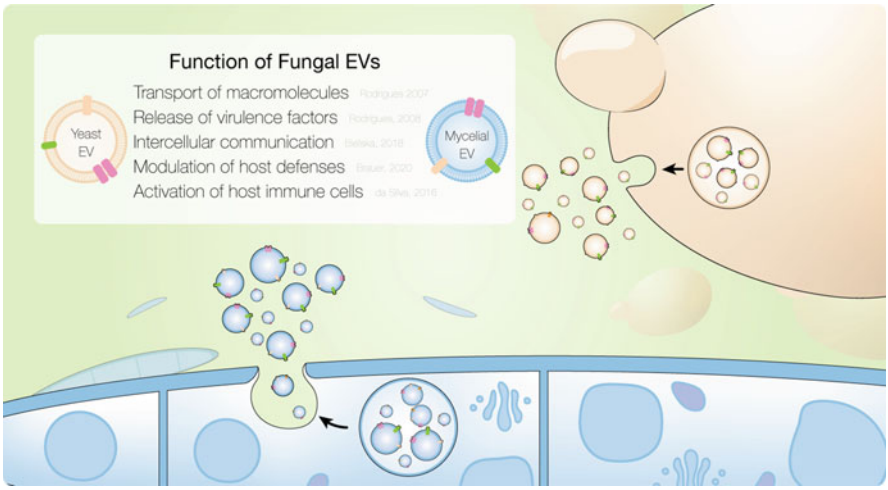

Fig. 7.1 Functions of fungal EVs. Yeast and filamentous fungi secrete EVs that work by transporting macromolecules (Rodrigues et al. 2007), releasing virulence factors (Rodrigues et al. 2008), in intercellular communication (Bielska et al. 2018), by modulating the host defenses and activating immune cells (da Silva et al. 2016; Brauer et al. 2020)

fungi to escape the host immune surveillance and resist biotic and abiotic stresses (Hopke et al. 2018).

Fungal EVs Must Traverse the Cell Wall to Reach the Host

The fungal cell wall represents a significant barrier for EV release. It is often considered a rigid, protective barrier that shields the fungal cell from biotic and abiotic stresses. However, the fungal cell wall is a dynamic organelle that interacts with the extracellular environment and the cell.

There are three main mechanisms thought to be involved in EV release through the cell wall (Wolf et al. 2014). The first is that EVs are discharged by simple turgor pressure after they are released from the plasma membrane. Fungal cell walls have pores that allow molecules of up to 5.8 nm, or 400,000 Da, to diffuse across freely (De Nobel et al. 1989, 1990). This process would not allow free diffusion of EVs. However, Walker and colleagues described fungal cell walls as viscoelastic after observing the transit of liposomes of 60–80 nm, carrying the antifungal amphotericin B across the cell walls of *C. albicans* and *C. neoformans* (Walker et al. 2018). EVs may cross the cell wall in a similar manner.

The second mechanism for fungal EV release uses cell wall-degrading enzymes contained in EVs, such as glucanases, chitinases and mannosidases, to loosen the cell wall and enable passage of the EVs (Albuquerque et al. 2008; Gil-Bona et al. 2015; Zhao et al. 2019; Bleackley et al. 2019b). These proteins have been detected in

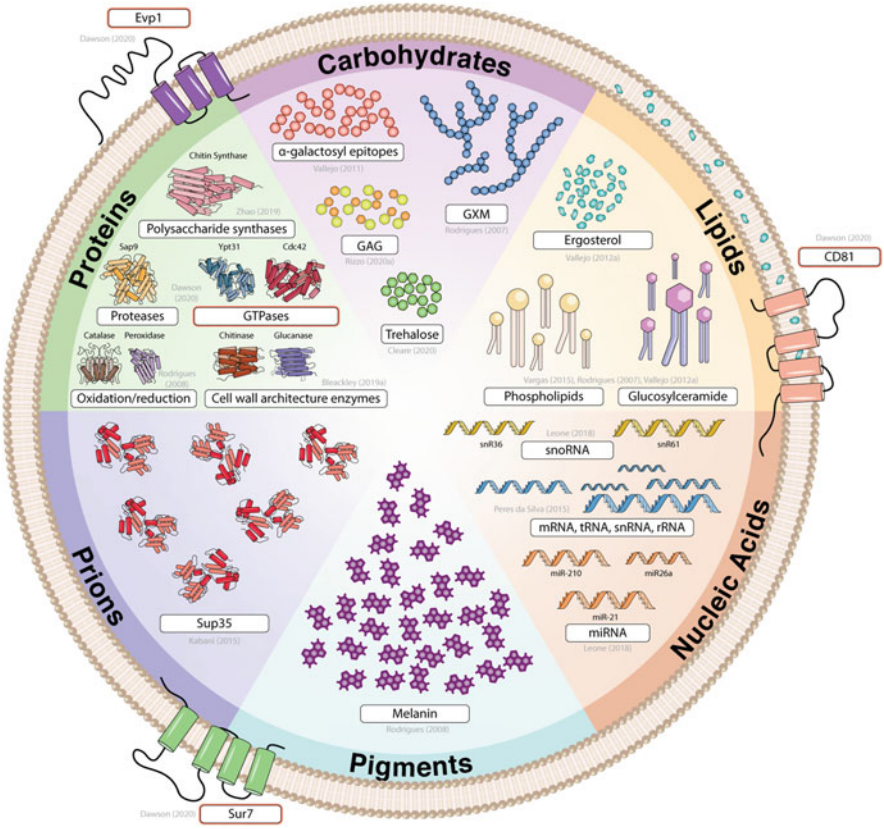

Fig. 7.2 Typical contents of fungal EVs. Carbohydrates include immunogenic molecules such as glucuronoxylomannan (GXM) (Rodrigues et al. 2007), galactosaminogalactan (GAG) (Rizzo et al. 2020b), and α-galactosyl epitopes (Vallejo et al. 2011); trehalose was associated to fungal adaptation (Cleare et al. 2020). Several proteins associated with virulence, such as catalase/peroxidase (Rodrigues et al. 2008), and with cell wall remodeling (Bleackley et al. 2019b), like glucanases and chitin synthase (Zhao et al. 2019), have been reported for different fungi. Potential protein markers from *C. albicans* (highlighted with red) include GTPases and eisosomal membrane proteins like Sur7, CD81 and Evp1 (Dawson et al. 2020). Lipid content includes glucosylceramide (Vargas et al. 2015; Rodrigues et al. 2007) ergosterol and general phospholipids (Vallejo et al. 2012b). Small RNAs have been described in EVs from several fungi. snR36 and snR61 have roles in rRNA synthesis and O-methylation of RNA, respectively (Peres da Silva et al. 2015b; Leone et al. 2018). miRNAs like miR-210, miR26a and miR21, function in cell differentiation and proliferation (Leone et al. 2018; Asangani et al. 2008). Melanin, a pigment used by *C. neoformans*, has been detected in EVs from this pathogen (Rodrigues et al. 2008). Transport of the prion Sup35 was characterized in EVs from *S. cerevisiae* (Kabani and Melki 2015)

most fungal species studied to date, supporting an important role in EV release depending on their location in the vesicle. Interestingly, since *C. neoformans* forms complexes of chitin and glucuronoxylomannan (GXM) that induce the production of IL-10, IL-17 an TNF-α in macrophages (Ramos et al. 2012), some authors have

suggested that cell wall-degrading enzymes transported by EVs generate smaller polysaccharides from the cell wall that elicit immune responses (Nimrichter et al. 2016).

The third proposed mechanism for EV release is through cell wall channels, suggested after observations of tubulin and actin were detected in EV preparations (Wolf et al. 2014; Rodrigues et al. 2008). Microscopy evidence does not support the presence of channels for EV release in *C. neoformans*, but rather a free transit through the cell wall (Wolf et al. 2014).

The importance of the cell wall in EV function is gaining traction. EV trafficking is an opportunity for the exchange of macromolecules within the cell wall and with the extracellular compartment (Zhao et al. 2019; Champer et al. 2016; Vallejo et al. 2012b), suggesting a facilitating role of EVs for adaptation of fungal cells to the environment. *S. cerevisiae* strains lacking genes associated with cell wall biosynthesis produced more EVs than wildtype, showing that the cell wall also works as a partial barrier to EV release. EVs may also function in cell wall strengthening under conditions that affect cell wall integrity (Zhao et al. 2019).

The reports discussed above have built a better understanding of the role of the fungal cell wall during EV production. Still, other points remain unanswered such as the effect of cell walls with different polysaccharide composition and thickness on EV release and content, and the role of the cell wall on EV release from filamentous and plant pathogenic fungi.

Emergence of Protein Markers for Fungal EVs

Mammalian and fungal EVs display different EV-specific protein markers. Typical mammalian EV markers such as tetraspanins and ESCRT proteins are not present in fungal EVs (Fig. 7.3) (Bleackley et al. 2019a) which has hampered the identification and isolation of fungal EVs. A recent study on EVs from *C. albicans* has provided a list of 47 proteins that could be employed as markers (Dawson et al. 2020). Some of these are eisosome-related proteins Sur7, Nce101 and Evp1. The fungal eisosome is a site of endocytosis located at the plasma membrane (Walther et al. 2006), which supports the hypothesis of EV formation via multivesicular bodies (Raposo and Stoorvogel 2013; Wolf et al. 2014). Other potential fungal EV markers include Rab GTPases Sec4 and Ypt31, and Rho GTPases Cdc42, Rac1, Rho1 and Rho3. It remains to be determined whether these markers are present in EVs from other fungal species.

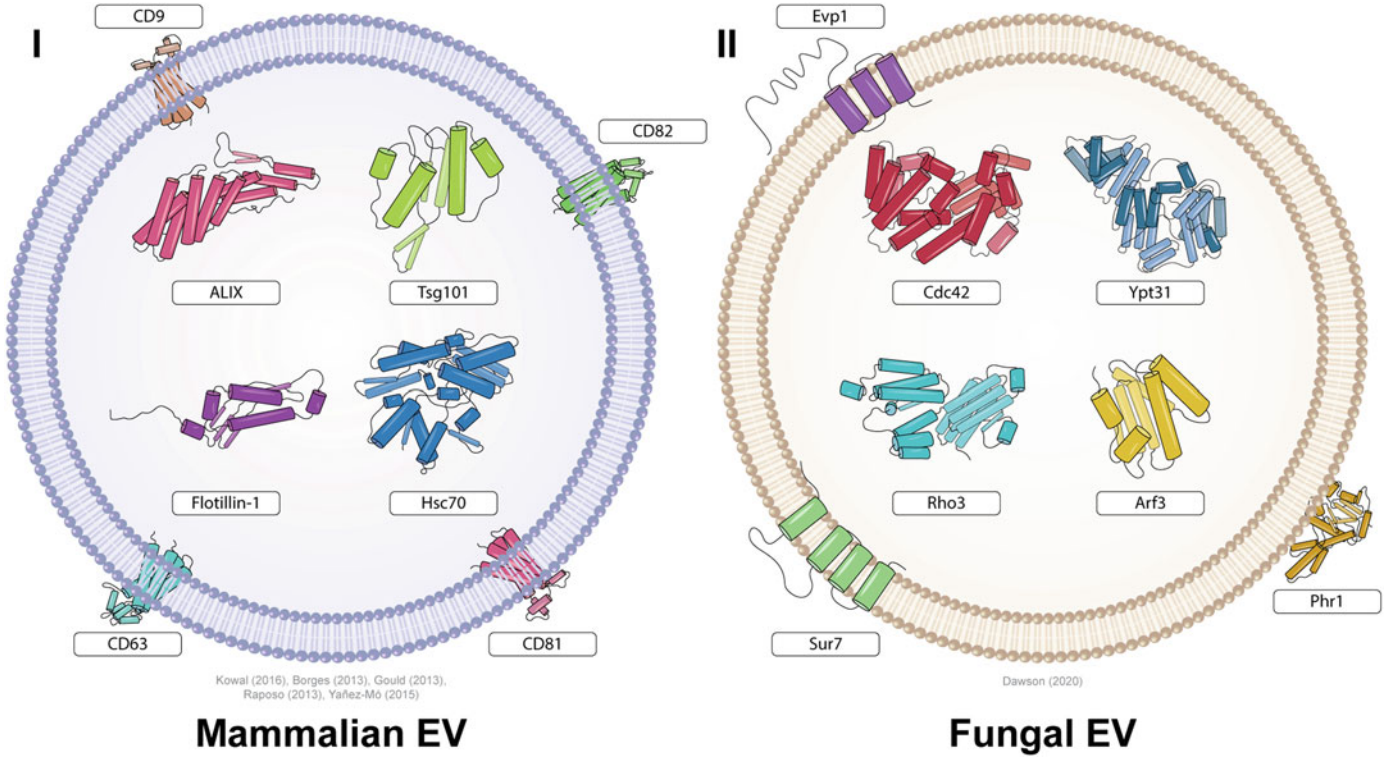

Fig. 7.3 Protein markers for mammalian and fungal EVs. I. Some of the most employed markers for mammalian EVs in isolation and characterization include ALIX, and tetraspanins such as CD63 and CD82 (Kowal et al. 2016; Borges et al. 2013; Gould and Raposo 2013; Raposo and Stoorvogel 2013; Yáñez-Mó et al. 2015). Such proteins have not been detected as markers in fungal EVs. II. Protein markers for fungal EVs have been proposed for *C. albicans*. These include eisosomal proteins EVp1, Sur7 and Nce101; GTPases such as Rho3, Arf3 and Ypt31 (Dawson et al. 2020)

Role of Fungal EVs in Pathophysiology

The role of fungal EVs in host-pathogen interactions has been reviewed widely (Joffe et al. 2016; Rodrigues and Casadevall 2018; Samuel et al. 2015). There are several reports showing direct links between EV production by human pathogens and changes in immune responses from the host (Fig. 7.4). These reports are discussed below.

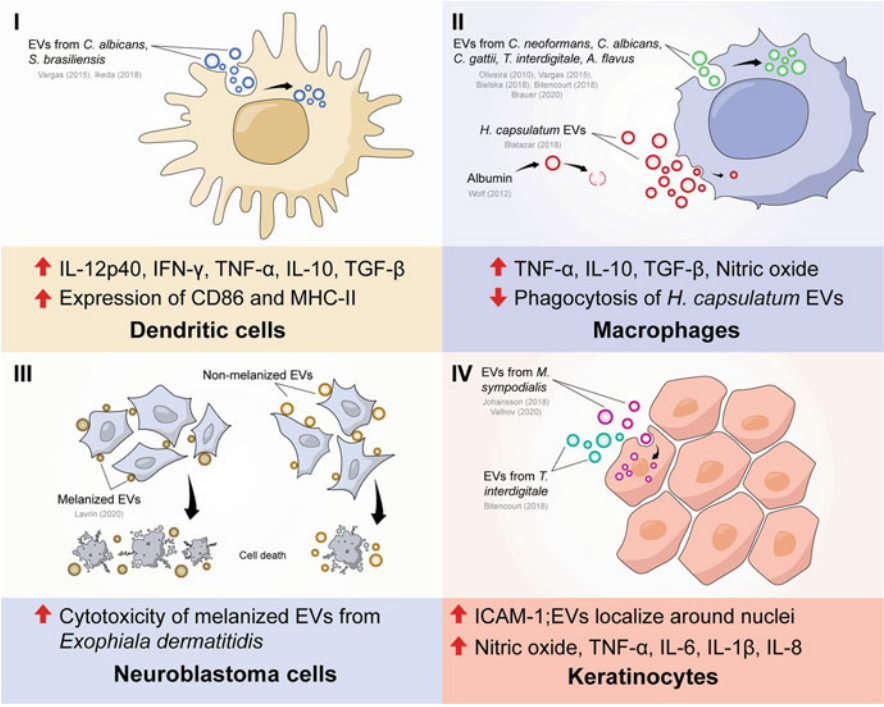

Fig. 7.4 Fungal EVs modulate the immune response of host cells. I. EVs from *C. albicans* and *S. brasiliensis* activate dendritic cells and increase the production of cytokines such as IL-12p40, IFN-γ, TNF-α, IL-10 and TGF-β. Other proteins upregulated in response to the presence of EVs were CD86 and MHC-II (Vargas et al. 2015; Ikeda et al. 2018). II. EVs from pathogenic fungi increase the production of TNF-α, TGF-β and nitric oxide by macrophages (Bielska et al. 2018; Bitencourt et al. 2018; Brauer et al. 2020; Oliveira et al. 2010a; Vargas et al. 2015). Incubation of macrophages with EVs from *H. capsulatum* reduced the ability of macrophages to phagocytize *H. capsulatum* (Baltazar et al. 2018). These studies revealed that albumin disrupts EVs (Wolf et al. 2012). III. EVs from the yeast *E. dermatitidis* were more cytotoxic than non-melanized EVs and had a greater ability to cause cell death (Lavrin et al. 2020). IV. Expression of the intracellular adhesion molecule 1 (ICAM-1), involved in immune defense of skin cells, was increased in keratinocytes after incubation with *M. sympodialis* EVs, which localized around the nuclei (Johansson et al. 2018; Vallhov et al. 2020). Similarly, keratinocytes increased production of cytokines after exposure to *T. interdigitale* EVs (Bitencourt et al. 2018)

Interactions of Yeast EVs with the Host Immune System

Fungal EVs have the ability to modify the immune response from the host (Zamith-Miranda et al. 2018). EVs from *C. neoformans* were internalized by murine macrophages, resulting in the production of TNF-α, a fungicidal cytokine, IL-10 and TGF-β (Fig. 7.4, II) (Oliveira et al. 2010a). Importantly, *C. neoformans* EVs appear to cross the blood-brain barrier. This observation is relevant since the infection mechanism that leads to cryptococcal meningoencephalitis is largely unknown and it may be mediated by *C. neoformans* EVs (Huang et al. 2012). Finally, EVs from *C. neoformans* (and from the bacterium *Bacillus anthracis*) are destabilized by human albumin and may have a short life *in vivo*, suggesting that content might be discharged shortly after EV release (Fig. 7.4, II) (Wolf et al. 2012).

Fungal EVs also protect *Histoplasma capsulatum* from the host defense arsenal by inhibiting phagocytosis by human bone marrow macrophages (Fig. 7.4, II) (Baltazar et al. 2018). The interaction between *H. capsulatum* and host cells also caused changes to EV content, suggesting that the composition of EVs is actively regulated (Baltazar et al. 2016, 2018).

C. albicans EVs modulate the immune response of murine macrophages and dendritic cells (Fig. 7.4, I and II), where they cause production of nitric oxide and several cytokines. EVs from *C. albicans* also conferred a protective effect in the infection model *Galleria mellonella.* Inoculating *G. mellonella* larvae with EVs from *C. albicans* prior to a yeast challenge reduced the survival of viable yeast compared to larvae that had not been pre-exposed to EVs, confirming the ability of EVs to modify immune responses (Vargas et al. 2015). A similar protective effect from *C. albicans* EVs was reported for mice, suggesting that fungal EVs may be used as a vaccination (Vargas et al. 2020).

Host cells also produce EVs in the presence of fungi. For instance, macrophages produce EVs with a different proteome after interaction with *C. albicans*. They have more proteins involved in immune responses, signaling and cytoskeleton reorganization, showing a role for EVs in cell communication (Reales-Calderon et al. 2017).

An important example of the involvement of EVs in cell-cell communications and in pathophysiology has come from a study with *Cryptococcus gattii*, the causative agent of respiratory infections (Bielska et al. 2018). This fungus uses a "division of labor" mechanism whereby cells with higher virulence due to mitochondrial changes facilitate the proliferation of cells without the mitochondrial modification, creating a pool of pathogenic fungi inside macrophages (Voelz et al. 2014). This process is mediated by EVs from *C. gattii* (Bielska et al. 2018). Similarly, *C. neoformans* can transition to the more virulent lineage VNIa-5, through a process that involves secreted proteins from the more virulent strain that are enriched in EVs (Hai et al. 2020).

Further evidence for the role of EVs in communication between fungi and host are the observations that dendritic cells increase phagocytosis rates of the yeast *Sporothrix brasiliensis* after they have been exposed to EVs from this pathogen (Fig. 7.4, I) (Ikeda et al. 2018). The same study reported that subcutaneous lesions in mice

were more severe, and the fungal load was higher at the time of euthanization, for mice that were administered EVs from *S. brasiliensis* prior to infection with the fungus (Ikeda et al. 2018). These findings are contrary to reports of a protective role of fungal EVs in *G. mellonella* (Vargas et al. 2015).

Fungal pigments carried by EVs can also enhance the virulence of fungal pathogens. For example, melanized EVs from the black yeast *E. dermatitidis* reduced the viability of neuroblastoma cells significantly more than non-melanized EVs (Fig. 7.4, III) (Lavrin et al. 2020). The authors suggest that these type of fungal infections might be involved in triggering neurodegenerative diseases such as Parkinson's disease (Lavrin et al. 2020).

EVs from fungi associated with non-life-threatening diseases have also been studied. *Malassezia sympodialis* is a yeast that causes atopic eczema (Bieber and Novak 2009). EVs from this fungus carry allergens and antigens with the capacity to activate human keratinocytes and monocytes (Fig. 7.4, IV) (Gehrmann et al. 2011; Johansson et al. 2018; Vallhov et al. 2020).

Interactions of Mycelial EVs with the Host Immune System

EVs from filamentous fungal pathogens are understudied compared to EVs from yeast. As mentioned above, *Aspergillus spp.* are a severe threat to human health and the role of EVs during infection of these pathogens is mostly unknown. However, EVs from *A. fumigatus* accelerate phagocytosis by neutrophils and macrophages, and macrophages increase production of cytokines TNF-α and CCL2, showing that EVs from filamentous fungi also modulate immune responses in the host (Souza et al. 2019).

Human granulocytes also produce EVs in response to *A. fumigatus* (Shopova et al. 2020). These mammalian EVs are enriched with antifungal molecules and were able to limit fungal growth, showing that the exchange of EVs modulates immune responses from the host and the pathogen (Shopova et al. 2020).

Similar to *C. albicans*, EVs from *Aspergillus flavus* increased the production of cytokines in bone marrow macrophages, which also increased phagocytic rates and developed the M1 phenotype (Brauer et al. 2020). Prior exposure to *C. albicans* EVs also protects *G. mellonella* larvae from a lethal fungal infection (Brauer et al. 2020), showing that both mycelial and yeast EVs can enhance the antifungal response from the host.

Human keratinocytes increase production of nitric oxide and cytokines after incubation with EVs from the dermatophyte *Trichophyton interdigitale*. Bone marrow macrophages responded in the same way as the keratinocytes after exposure to EVs from this fungus, and an M1 phenotype was also observed (Bitencourt et al. 2018). Preconditioning of bone marrow macrophages with EVs from *T. interdigitale* conidia also increased the fungicidal activity of the macrophages when they were incubated with the fungus (Bitencourt et al. 2018). Similar findings have been reported for *Paracoccidioides brasiliensis* (da Silva et al. 2016).

EV Content from Human Fungal Pathogens Suggests a Role in Virulence

Protein

Fungal EVs from human pathogens contain proteins that modulate the immune responses of host cells and that facilitate infection (Rodrigues et al. 2008; Peres da Silva et al. 2015a; da Silva et al. 2016; Rizzo et al. 2020a). For instance, proteins involved in protection against oxidative stress have been reported in EVs from plant and human pathogens (Vallejo et al. 2012a; Rodrigues et al. 2008; Bleackley et al. 2019b; Dawson et al. 2020). It is known that redox proteins, such as catalase and peroxidase, are used by fungi during colonization of the host (Warris and Ballou 2019). Another example of transport of virulence-associated proteins found in fungal EVs is phospholipase b, which disrupts phospholipids of the host and is essential for *C. albicans* virulence (Ghannoum 2000).

Fungal EVs carry a variety of proteases that function by enhancing fungal virulence (Yike 2011). For instance, the *C. albicans* protease Sap9 facilitates biofilm formation (Dutton et al. 2016; Dawson et al. 2020) and the peptidase YscII from *S. cerevisiae* is used to obtain leucine from environmental sources (Hirsch et al. 1988; Oliveira et al. 2010b).

Fungal EVs also transport enzymes involved in the production of pigments such as melanin, which can act as virulence factors. Melanin reduces phagocytosis by immune cells, alters cytokine production and reduces the toxicity of antifungal compounds (Taborda et al. 2008; Liu and Nizet 2009; Nosanchuk and Casadevall 2003; Panepinto et al. 2009; Nosanchuk et al. 2015). Laccase, involved in melanin production, was detected in EVs from *C. neoformans* (Rodrigues et al. 2008) and Eisenman and colleagues proposed melanization of *C. neoformans* occurs via synthesis inside EVs (Eisenman et al. 2009). Melanized EVs isolated from the black yeast *Exophiala dermatitidis* had a more potent cytotoxic effect on neuroblastoma cells than non-melanized EVs, showing that melanin is an important component for cytotoxicity (Lavrin et al. 2020).

RNA

EVs from *C. neoformans*, *P. brasiliensis*, *C. albicans* and *S. cerevisiae* carry RNA sequences of less than 250 nt. composed of mRNA, tRNA, rRNA, snRNA, snoRNA and miRNA (Peres da Silva et al. 2015a). Some of these sequences are involved in splicing control and RNA degradation (Peres da Silva et al. 2015a; Leone et al. 2018). EVs from *P. fermentans*, *C. neoformans*, *C. albicans*, *S. cerevisiae* and *P. brasiliensis* contain snR36 and snR61, snoRNAs involved in rRNA synthesis and O-methylation of RNA, respectively (Peres da Silva et al. 2015a; Leone et al. 2018). Other RNAs detected in EVs from *Pichia fermentans* with human orthologues, miR-210 and miR-26a, are induced during cell differentiation and hypoxia, and have a

potential role in pseudohyphal cell formation (Leone et al. 2018; Ramteke et al. 2015; Lee et al. 2015; Leeper et al. 2011). miR-21, also found in *P. fermentans* EVs, has a role in cell proliferation and apoptosis in human cells (Asangani et al. 2008).

The RNA content of EVs from the pathogen *Rhizopus delemar* included around 400 sequences of 18–30 bases, with roles in regulation of carbohydrate metabolism and biosynthesis of secondary metabolites, which in some fungal species is tightly related to virulence (Liu et al. 2018; Pusztahelyi et al. 2015). Analysis of RNA from *Paracoccidioides spp.* EVs revealed sRNA sequences that map to genes encoding fungal proteins such as α-amylase and β-glucanase, with a proposed role of regulating virulence genes (Peres da Silva et al. 2019).

The Golgi reassembly and stacking protein (GRASP) has a role in RNA export via EVs in *C. neoformans*, which may involve a tightly regulated mechanism (Peres da Silva et al. 2018). GRASP also functions in polysaccharide secretion in *C. neoformans* (Kmetzsch et al. 2011) suggesting an important role in vesicle loading.

Carbohydrates

There has been limited work on the carbohydrate content of EVs, partly due to the relatively large amount of material needed for glycomic analysis.

C. neoformans has an extracellular capsule made of glucuronoxylomannan (GXM) which decreases the phagocytic ability of immune cells and is associated with virulence (Vecchiarelli 2000). EVs have a fundamental role in the transport of GXM from the cytosol to the cell surface (Rodrigues et al. 2007, 2008).

A comparative analysis of the cargo of *H. capsulatum* EVs produced in different growth media revealed macromolecules associated with virulence, such as the glycolipid ergosterylglucoside. Trehalose, also detected in *H. capsulatum* EVs, is a carbohydrate associated to thermotolerance and growth under limited carbon sources (Cleare et al. 2020; François and Parrou 2001; Thammahong et al. 2017; Hare et al. 1998).

EVs from *A. fumigatus* also transport polysaccharides. Carbohydrate analysis revealed the presence of glucans, galactomannan and galactosaminogalactan (GAG) in EVs (Rizzo et al. 2020b). GAG is a cell wall polysaccharide that has potent immunomodulatory effects in mice (Fontaine et al. 2011; Gresnigt et al. 2014), supporting the role of fungal EVs in altering immune responses in the host.

Further glycomic studies on EVs from *Paracoccidioides* revealed α-galactosyl epitopes, which have the ability of activating immune cells (Vallejo et al. 2011). Also found in EVs were different types of cell wall oligosaccharides like 4,6-α-glucan and galactofuranosylmannan (da Silva et al. 2015; Peres da Silva et al. 2015b).

Lipids

Similar to the carbohydrate analysis, high amounts of EV material are needed to perform lipid analyses and hence there are few reports on the lipid composition of fungal EVs, since lipids may have a relatively low abundance based on EV size (Bleackley et al. 2019a).

EVs from *C. neoformans* contain sterol derivatives that, in other fungi, are involved in resistance to different types of stress and hence are targets of antifungal molecules (Lv et al. 2016; Rodrigues et al. 2007). Glucosylceramide, with a well-described role in fungal virulence, has also been detected in EVs from *C. neoformans* and from *C. albicans* (Rodrigues et al. 2007; Vargas et al. 2015; Rittershaus et al. 2006).

A lipidomic analysis of EVs from *P. brasiliensis*, revealed the presence of a plethora of phospholipids, fatty acids and sterols, with the most abundant being brassicasterol (Vallejo et al. 2012b). Brassicasterol has been linked to resistance to azole antifugal drugs (Camacho and Niño-Vega 2017), suggesting a potential link between EV production and drug resistance.

Prions

The transmission of prion Sup35 via EVs from *S. cerevisiae* has been reported recently (Liu et al. 2016; Kabani and Melki 2015). Although prions are best spread by direct contact between cells, EVs transport Sup35 into the extracellular space, creating a new mechanism of prion transmission in fungi. The transport of prions via EVs and their role in disease is well characterized in mammalian systems (Quek and Hill 2017).

Fungal EVs in Drug Resistance

Recent research has shed light on the role of EVs in drug resistance and in the morphological changes between yeast and biofilms. A *C. albicans* study revealed that EVs from planktonic cells are morphologically different and have a different proteome compared to biofilm-derived EVs. Also, WT EVs are able to confer resistance to antifungal-susceptible *C. albicans* strains. These data show that EVs have a role in biofilm matrix biogenesis and that there is a direct link between EV production and drug resistance (Zarnowski et al. 2018; Dawson et al. 2020). A role for morphological shifts was also reported for the industrial fungus *Pichia fermentans*, in a process that my mediated by EVs (Leone et al. 2018). Similarly, EVs were able protect *S. cerevisiae* from the antifungal caspofungin and the plant defensin NaD1 (Zhao et al. 2019). The authors proposed two mechanisms for EV-mediated drug resistance: the first is that EVs act as decoys and interact with these lipid-binding antifungals, effectively sequestering them. The second mechanism relies on EV internalization by host cells that derives in reinforcement of the fungal cell wall and in protection against the antifungal agents (Zhao et al. 2019).

EVs from Fungal Plant Pathogens Contain Molecules Related to Virulence

Little is known about the content and function of EVs from fungal plant pathogens compared to human fungal pathogens. Proteomic data is available only for EVs from *Alternaria infectoria* (Silva et al. 2014), and *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) (Silva et al. 2014; Bleackley et al. 2019b). However, some proteins indicate a potential role for fungal EVs during infection. For instance, Fov EVs contain a Hsp70-like protein. Hsp70 is involved in conidiation and plant infection in *Fusarium pseudograminearum*, an emerging plant pathogen (Chen et al. 2019).

Fov EVs are enriched with two types of polyketide synthases that might be involved in the production of the pigment bikaverin. EVs and/or this pigment are thought to be responsible for causing a hypersensitive response on cotton cotyledons (Bleackley et al. 2019b). Polyketide synthases are used extensively in toxin production from plant pathogens and have a clear role in virulence (Choquer et al. 2005; Yang et al. 1996; Noar et al. 2019; Ruocco et al. 2018; Woo et al. 2012).

Proteases from plant pathogenic fungi can also increase the severity of plant diseases (Podder et al. 2019). Fov EVs were enriched with various proteases such as carboxypeptidases D and F, glutamate carboxypeptidase II, Pep1, vacuolar protease a, and aspartic-type signal peptidase a (Bleackley et al. 2019b). The role of these proteases in the context of a plant infection still needs to be addressed.

A. infectoria EVs contain proteins involved in polysaccharide metabolism, pigment synthesis, and trafficking of vesicles (Silva et al. 2014).

Conclusion

The production of fungal extracellular vesicles has been reported for pathogenic and non-pathogenic fungal species. Fungal EVs are similar in morphology and content to EVs from other organisms, and multi omic analyses have revealed that EVs supports a role in cell wall remodeling, infection, and interactions between yeast and filamentous fungi with their animal or plant host.

The field of fungal EVs has consistently advanced towards a better understanding of EV function. The absence of universal fungal markers to assist in isolation and tracking in biological systems, the lack of knowledge on EV heterogeneity from a single fungal species, as well as little information on specific pathways represent exciting challenges. The diversity of fungi provides the opportunity to investigate new pathogens and interactions with their hosts, which in time will lead to elicit a more complete role of fungal EVs in the context of infection.

A better understanding of EV cargo and how they enhance virulence of fungal pathogens or prime the immune response of the host for protection against pathogens is highly likely to lead to the development of new drugs for treatment of fungal diseases.

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Chapter 8
Socially Distanced Intercellular Communication: Mechanisms for Extracellular Vesicle Cargo Delivery

Stephanie J. Popa and Sarah E. Stewart

Abstract Extracellular vesicles (EVs) are increasingly being recognised as players in intercellular communication within the human body. EVs are nano-sized vesicles that are secreted by virtually all cells, primarily arising from either the plasma membrane or the endocytic system. They contain a wide range of proteins and nucleic acids in their lumen, as well as cell surface proteins on their exterior. The proteins and nucleic acids within are the 'cargo' that EVs deliver into the cytosol of recipient cells to elicit a response or phenotypic change. For delivery to occur, the cargo needs to cross two lipid bilayers; one that makes up the vesicle itself, and the other of the recipient cell. Exactly how this process works is a topic that is poorly understood, despite being pivotal for their function. Furthermore, extracellular vesicles have therapeutic potential as drug delivery vehicles. Therefore, understanding their delivery mechanism and harnessing its action for drug delivery is of great importance. This chapter will focus on the proposed mechanisms for cargo delivery and discuss existing evidence for cargo delivery from EVs into the cytosol of recipient cells.

Keywords Extracellular vesicles · Exosomes · Microvesicles · Intercellular communication · Fusion · Cargo delivery

Intercellular Communication

Intercellular communication is critical for coordinating many cellular processes. Cells in the human body can communicate at a distance using protein or nucleic acid messengers, which are released from cells via three major pathways. The first, and most well characterised, is conventional protein secretion via the endoplasmic reticulum and Golgi apparatus. Conventional protein secretion involves directed

S. J. Popa · S. E. Stewart (✉)
University of Cambridge, Metabolic Research Laboratories, Wellcome Trust-Medical Research Council Institute of Metabolic Science, University of Cambridge, Cambridge, UK
e-mail: ss2340@cam.ac.uk

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synthesis of proteins into the secretory pathway for expression at the cell surface or release into the extracellular space. Generally, proteins secreted via this route are soluble, and once free in the extracellular space will elicit their effects on other cells via receptor mediated interactions. Major examples include the secretion of cytokines, growth factors and hormones. By contrast, unconventional protein secretion (UPS) is poorly understood, with interleukin-1β (IL-1β), fibroblast growth factor-2 (FGF-2) and annexin A2 being examples of proteins secreted in this way, as reviewed (Popa et al. 2018; Sitia and Rubartelli 2018; Rabouille 2017). UPS includes any protein that is secreted into the extracellular space that cannot be secreted through the conventional secretory pathway described above. UPS can be further categorised into several general secretion routes: direct translocation across the plasma membrane, secretion via a vesicular intermediate, and Golgi bypass. The specific pathway used for secretion of proteins by UPS is often controversial as there is overlap in the pathways by which each protein can be secreted. For example, it is widely accepted that FGF-2 is secreted by directly crossing the plasma membrane, whereas annexin A2 and IL-1β have both been reported to be able to directly cross the plasma membrane and be secreted encapsulated within extracellular vesicles (Stewart et al. 2018; Stewart et al. 2016; Sitia and Rubartelli 2018; Steringer and Nickel 2018). Extracellular vesicles (EVs) are interesting mediators of UPS. While EVs could be classified as vesicular intermediates of UPS, EVs ultimately represent a distinct third pathway for intercellular communication in their own right due to their complex nature.

EVs are small vesicles composed of a lipid membrane that encapsulates proteins and nucleic acids from the secreting cell. EVs act as a vehicle for the unconventional secretion of many proteins simultaneously; additionally, they also carry nucleic acids (primarily RNA) and lipids, making them a very interesting mediator of both UPS and non-protein based intercellular communication. EV mediated cell-cell communication will be the focus of this chapter.

Extracellular Vesicles

EVs were originally thought to be a waste management system for cells to get rid of unwanted material (in particular, plasma membrane proteins), and therefore of little physiological consequence in the extracellular space (Johnstone et al. 1987). However, over the last few decades it has become apparent that EVs can mediate intercellular communication. Furthermore, the process of packaging proteins and nucleic acids onto or into EVs is more specific than was previously thought (Colombo et al. 2014). The term ‘EVs’ refers to a heterogeneous pool of membrane bound nanovesicles secreted by virtually all mammalian cell types (Gyorgy et al. 2011). EVs have been isolated from almost all known biological fluids and cell lines. Therefore, the potential of EVs as intercellular communicators is vast, and many studies are now investigating the consequence of EVs in the human body. EVs have been reported to mediate many different functions, including roles in pregnancy,

immunity, neurodegeneration and cancer (Buca et al. 2020; Boomgarden et al. 2020; Hill 2019; Veerman et al. 2019). These functional effects are generally attributed to specific proteins or RNA. Proteins may be on the surface or within EVs, while RNA components, including mRNA, miRNA, tRNA and long non-coding RNA, are within EVs (O'Brien et al. 2020). The cargo content of EVs depends on the cell type and biogenesis of the EVs. EVs can be classified according to their sub-cellular origin (as described below).

EV Biogenesis and Cargo Packaging

EVs are thought to be secreted via three major pathways and can be categorised according to their biogenesis (Fig. 8.1). Here, these biogenesis pathways are briefly

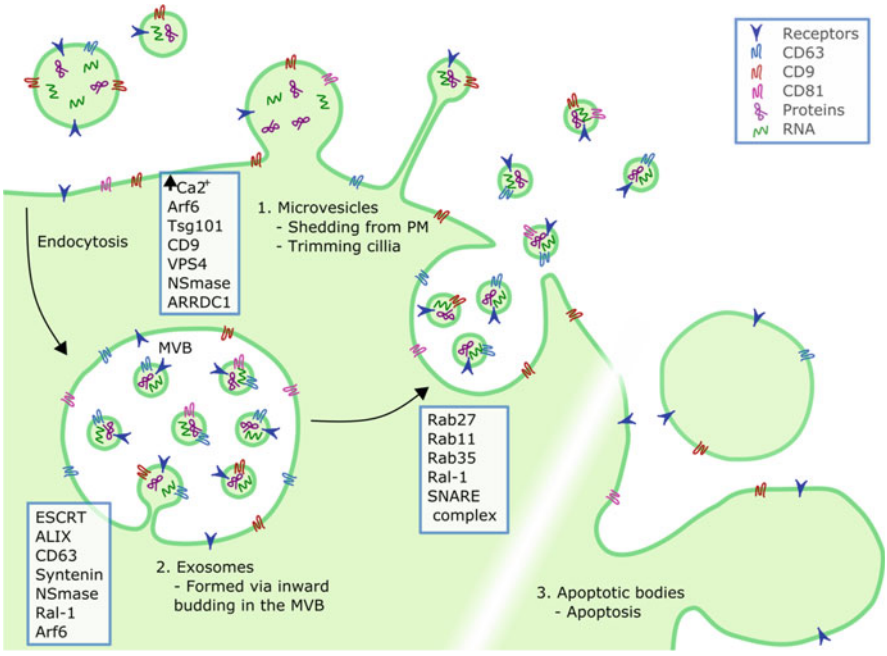

Fig. 8.1 Extracellular vesicle biogenesis pathways. EVs are secreted from cells via three major pathways. (1) Microvesicles are secreted directly from the plasma membrane through plasma membrane blebbing and release. (2) Exosomes are formed within the endocytic pathway through the inward budding to form intraluminal vesicles within the multivesicular body. Exosomes are secreted when the multivesicular body fuses with the plasma membrane. (3) Apoptotic bodies are formed during the orchestrated process of apoptotic cell death. Apoptotic bodies contain proteins, RNA, DNA and organelles which are not depicted here. For all three pathways, the architecture and content of EVs should reflect that of the secreting cell, with the exception of lipid asymmetry. Once released into the extracellular space, EVs can mediate intercellular communication

described to highlight differences and similarities in these pathways; however, there are several excellent reviews available that describe these processes in detail (van Niel et al. 2018; Mathieu et al. 2019; Pollet et al. 2018; Hessvik and Llorente 2018; Jadli et al. 2020).

Microvesicles

Microvesicles (MVs; also called ectosomes, oncosomes or shedding vesicles) are secreted by direct shedding of vesicles from the plasma membrane (Fig. 8.1). These vesicles range in size from 50–1000 nm in diameter, and the membrane protein and lipid content should in theory reflect that of the plasma membrane, although the plasma membrane asymmetry is not maintained in MVs (Kastelowitz and Yin 2014). MVs are formed through the outward budding of the plasma membrane or in some instances by trimming cilia, and a number of proteins have been identified to facilitate their formation and released into the extracellular space. The biogenesis, lipid profile and cargo of MVs has been recently reviewed in more detail (Pollet et al. 2018).

Shedding of MVs can be driven by different stimuli, and several mechanisms for membrane scission have been described. A well-known stimulus for MV shedding is increased intracellular calcium (Taylor et al. 2017; Pollet et al. 2018). In this scenario, the rise in intracellular calcium stimulates several pathways that disrupt plasma membrane anchorage to the cytoskeleton. These pathways include activation of the protease calpain and/or phospholipid asymmetry disruption, leading to the formation of outward blebs (Pollet et al. 2018; Fujii et al. 2015; Taylor et al. 2017). The plasma membrane blebs are then excised and released into the extracellular space. Scission can be mediated by several proteins in different situations. These include members of the endosomal sorting complex required for transport (ESCRT) complex, such as CHMP4/VPS4; neutral sphingomyelinase activity, or ADP-ribosylation factor 6 (ARF6) (Jackson et al. 2017; Muralidharan-Chari et al. 2009; Xu et al. 2017; Menck et al. 2017; Nabhan et al. 2012; Matusek et al. 2014). However, the exact details of these processes, and which of these pathways is dominant, remain unclear.

Additionally, arrestin domain containing protein-1 (ARRDC1) can drive the formation and shedding of MVs by causing membrane deformation and constriction at the base of the forming vesicle (Nabhan et al. 2012). ARRDC1-mediated MVs (ARMMs) are approximately 50 nm in diameter, which is considered small for MVs, and their formation is facilitated by ubiquitination of ARRDC1. Membrane scission is driven by the recruitment of tumour susceptibility gene 101 (TSG101) and VPS4 ATPase prior to release into the extracellular space (Nabhan et al. 2012). Furthermore, evidence suggests that ARRCD1 is also responsible for specific protein recruitment and loading of cargo into MVs (Wang and Lu 2017; Anand et al. 2018). Finally, the transmembrane tetraspanin CD9 is thought to be enriched in MVs and has been reported as a driver of microparticle shedding (Dale et al. 2009).

Exosomes

Another prominent source of EVs arise from the endocytic pathway (Fig. 8.1), where vesicles bud inward to form intraluminal vesicles (ILVs) within the multivesicular body (MVB). Once loaded with ILVs, the limiting membrane of the MVB then fuses with the plasma membrane, releasing the vesicles into the extracellular space. These EVs of endocytic origin are known as exosomes and are thought to range in size from 50–150 nm in diameter (Gyorgy et al. 2011). The exosome biogenesis pathways and cargo sorting are reviewed in detail (Hessvik and Llorente 2018; Jadli et al. 2020), and are briefly outlined below.

Exosome Biogenesis

The formation of exosomes within the MVB can be ESCRT dependent or independent. ESCRT proteins have a diverse range of functions in the cell, including in cell surface receptor signalling, signal transduction and cell proliferation (Tu et al. 2011), in addition to their role in membrane scission. There are 30 proteins that make up the ESCRT complex which can be further broken down into four subcomplexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III (Vietri et al. 2020). ESCRT-0 includes TSG101 which is also involved in MV release. These components, along with other associated proteins including the AAA ATPase VPS4, programmed cell death 6-interacting protein (ALIX) and syntenin, are recruited to the MVB to drive the biogenesis of ILVs (Vietri et al. 2020; Baietti et al. 2012; Jackson et al. 2017). The ESCRT independent mechanism, in contrast, has been reported to be mediated by various proteins including neutral sphingomylinase, which catalyses the hydrolysis of sphingomyelin into phosphocholine and ceramide; the tetraspanin CD63; the small GTPases Ral-1, and ARF6 and/or phospholipase D2 (Hyenne et al. 2018; Trajkovic et al. 2008; Gauthier et al. 2017; Ghossoub et al. 2014). While exosome biogenesis may be described as ESCRT dependent or independent, there are overlaps between these pathways (Ghossoub et al. 2014; Baietti et al. 2012; Larios et al. 2020) and it is unclear whether the use of either pathway gives rise to any differences in the vesicles formed. Presumably, the mechanism by which the ILVs are formed will have ramifications for the lipid, protein and RNA content of the exosomes.

Exosome Secretion

Regardless of how they are formed, once within the MVB, exosomes are only released when the limiting membrane of the MVB fuses with the plasma membrane. This process is not a certainty, as the MVB can alternatively deliver its cargo for degradation within the lysosome. What governs whether a particular MVB is directed to the plasma membrane or to the lysosome remains unclear, although

there is evidence that post-translational modifications, such as ubiquitination and acidification, direct MVBs towards one or the other (Moreno-Gonzalo et al. 2018; Guo et al. 2017). Those that are destined for the plasma membrane again require various proteins to mediate trafficking and fusion of the limiting MVB membrane with the plasma membrane. These proteins include cytoskeletal proteins; small GTPases, specifically Ral-1, Rab27 and Rab11; soluble N-ethylmaleimide-sensitive fusion attachment protein receptors (SNARES), such as synaptosomal-associated protein 23 (SNAP-23); and syntaxin-1 (van Niel et al. 2018). Once the MVB fuses with the plasma membrane, the ILVs are released into the extracellular space.

Cargo Loading

During formation, cargo is recruited into exosomes by various mechanisms. Post translational modifications on proteins and RNA all increase the likely hood of recruitment into exosomes, including, ubiquitination, acetylation, methylation, phosphorylation and sumoylation, as reviewed in detail (Anand et al. 2018; Moreno-Gonzalo et al. 2018). A specific sequence of miRNA has also been identified that enables loading into exosomes (Villarroya-Beltri et al. 2013). Additionally, it has been shown that there is an enrichment of specific lipids—cholesterol, sphingomyelin, ceremide and glycosphingolipids—in exosomal membranes (Trajkovic et al. 2008; Skotland et al. 2017). Therefore, proteins that bind to these lipids may also be recruited. This may be part of the reason proteins specific proteins are enriched in exosomes, such as tetraspanins and flotillin, which have affinity for cholesterol or cholesterol and sphingolipids in lipid rafts, respectively (Silvie et al. 2006; Dermine et al. 2001). ALIX is reported to influence the sorting of cargo into exosomes, independent of post translational modifications, through binding the specific YPX_3L motif on proteins such as protease-activated receptor 1 (PAR1) (Dores et al. 2012). While some of the mechanisms and regulation of cargo recruitment have been described, the sorting process is multifaceted, and many aspects remain poorly understood.

Apoptotic Bodies

The third and less well studied source of EVs are apoptotic bodies (Fig. 8.1). Apoptotic bodies are 500–2000 nm in diameter, larger than the other EV types secreted by healthy cells. As the name suggests, apoptotic bodies are formed during apoptosis and contain a wide range of proteins, nucleic acids and organelles. While apoptotic bodies are often rapidly cleared by phagocytes such as macrophages, apoptotic bodies have also been reported to have roles in cell-cell communication. Examples of this include inducing stem cell proliferation for epithelial tissue maintenance, mediating tumour cell migration, and regulating autophagy in myocardial infarction (Zweemer et al. 2017; Liu et al. 2020; Brock et al. 2019). This chapter will mainly focus on microvesicles and exosomes because most of the work has been

done with these EV subtypes; however, it is important to note that for apoptotic bodies to have a role in intercellular communication the same challenges apply with regards to cargo delivery.

EV Isolation and Origins

Once secreted into the extracellular space, EVs act as mediators of intercellular communication. To investigate the underlying cellular mechanisms by which EVs function, most studies isolate EVs from cell culture medium and biological fluids to study their effects. However, there are some important technical challenges to consider when studying EVs. Given there are three major pathways for EV biogenesis, it is reasonable to assume that they should be identifiable as discreet entities, but current isolation techniques to study microvesicles, exosomes and apoptotic body preparations are generally based on size, density and surface markers, all of which are overlapping features of the different types of EVs. Similarly, thus far no protein has been found to be specific to any sub-population, likely due to the fact that the plasma membrane is dynamic and contributes to the endosomal system.

The field still has some way to go in understanding the biogenesis and cargo loading of EV populations. Regardless, if EVs function as intercellular communicators, EVs must deliver their cargo into recipient cells. The delivery of functional cargo will be the focus of the rest of the chapter. Given that once they have been released from cells, there is as yet no specific protein marker or distinct size range that allows their categorisation as microvesicles, exosomes or apoptotic bodies, it has been proposed by the International Society of Extracellular vesicles in a position paper that, unless the origin is unequivocally demonstrated, all EVs should be referred to as the heterogenous term 'EV' with the option of further description based on their size (small EVs, for example), composition or cell type origin (Thery et al. 2018).

Evidence for Cargo Delivery by EVs

There are many reports of EV-mediated effects in both physiological and pathophysiological settings; however, many of these reported EV cargo functions are derived by inference. While these functions may be mediated through the delivery of EV cargo into the cytoplasm of the recipient cells, this is almost never specifically demonstrated; directly demonstrating cargo delivery into the cytoplasm has proven to be technically difficult. Furthermore, directly demonstrating the site of delivery in the cell remains extremely difficult (discussed below). While direct evidence is somewhat elusive, there are convincing examples both *in vivo* and *in vitro* that demonstrate delivery of functional RNA and proteins. Of particular interest are the models that have been developed to specifically for this purpose, most of which

utilise genetic modification for EV delivery of reporter molecules. A selection of these are further discussed here and are summarised in Table 8.1.

Evidence for Cargo Transfer by EVs

EVs were shown to contain RNA in 2007, and it was suggested that this RNA cargo, including messenger RNA (mRNA) and microRNA (miRNA), was able to be transferred from EVs into recipient cells (Valadi et al. 2007). In this original report, EVs from murine cells were incubated with human cells; following this incubation, mouse proteins were detected in recipient human cell samples by mass spectrometry (Valadi et al. 2007). Therefore, it was concluded that the mRNA from mouse EVs must have accessed the cytoplasm of the human cell where the RNA was translated into protein. This was the first report of its kind, and sparked major interest in EVs as intercellular communicators. However, it has since been suggested that the murine proteins detected could have been be synthesised in murine cells and packaged into the EVs, and it would be hard to distinguish between these proteins and newly synthesised proteins. Therefore, it is possible that the murine EVs simply remained associated with the cell surface of human cells and thereby the proteins were detected, rather than delivering murine mRNA to the recipient cells (Somiya 2020).

More recently, an example of protein cargo transfer by EVs arose when Crewe and co-workers were attempting to generate an adipocyte-specific knockout of caveolin-1 *in vivo*. Despite generating an adipocyte specific knockout for caveolin-1 genetically, caveolin-1 protein remained readily detectable at the plasma membrane of adipocytes. With further analysis, Crewe et al. determined that the source of caveolin-1 in adipocytes was neighbouring endothelial cells and that if trafficked via EVs. Isolation of EVs from endothelial cells reveal that caveolin-1 protein is transferred to adipocytes in small EVs (Crewe et al. 2018). The authors describe the plasma membrane sharing of adipocytes and endothelial cells under fed and fasted conditions. Interestingly, they found that endothelial small EVs containing caveloin-1 were predominantly targeted to adipocytes and very little was found in preadipocytes or macrophages (Crewe et al. 2018). However, the authors note that although caveolin-1 was detected in the plasma membrane of adipocytes, it did not seem to form characteristic flask-shaped caveolae, suggesting that while it localised to the plasma membrane, the transferred protein was not fully functional. Therefore, while this is a clear example of protein transfer *in vivo*, it does not demonstrate functional cargo delivery, despite the cargo localising to the correct subcellular location.

Furthermore, as described in detail below, Joshi and co-workers demonstrated that the luminal of the transmembrane protein GFP-CD63 was detectable on the cytosolic face of the late endosome in the recipient cell cytoplasm using fluobodies (Joshi et al. 2020). While this does not directly show that other cargo was delivered into the cytoplasm, it indicates that the EV had fused with the endosomal membrane

Table 8.1 Examples of functional cargo delivery by engineered EVs

Functional EV cargo	Reporter system	EV donor cell line	Recipient cell line/mouse model	EV terminology	Comments on system design/ molecules delivered	Reference
mRNA						
Cre recombinase	β-Galactosidase/GFP	Hematopoietic cells	Multiple tissues in murine model; focussing on Purkinje neurons	EVs	Low recombination events in mouse brain (1–7 per hemisphere of cerebella)	(Ridder et al. 2014)
Cre recombinase	β-Galactosidase	Murine tumour cell lines: TU22449 (from a spontaneous tumour in a murine glioma model) and Lewis lung carcinoma cell line LLC2	Tumour implantation or EV injection into mouse brain	EVs	Total recombined events tumour in infiltrating leukocytes range from 0.1–2.2%	(Ridder et al. 2015)
Gaussia luciferase (GlucB) reporter	Luciferase activity	Human embryonic kidney 293 T	Human glioma cell line Gil-36	EVs	EV membranes fluorescently labelled using a palmitoylation signal (PalmGFP/ PalmtdTomato)	(Lai et al. 2015)
Cas-9	Cas-9 activity (INDELs) targeting miR-125a sequence and GFP sequence	Red blood cells	Human acute myeloid leukemia MOLM13 and NOMO1	EVs	Cas9 mRNA/gRNA targeting miR-125 or GFP loaded EVs lead to a > 90% reduction of miR-125a/b expression and ~ 32% reduction in GFP expressing cells, respectively.	(Usman et al. 2018)

(continued)

Table 8.1 (continued)

Functional EV cargo	Reporter system	EV donor cell line	Recipient cell line/mouse model	EV terminology	Comments on system design/ molecules delivered	Reference
Luciferin	Luciferase activity	Bone marrow derived murine dendritic cells	Bone marrow derived murine dendritic cells	Exosomes	Detection of fusion at the plasma membrane with R18	(Montecalvo et al. 2012)
p53	Cell death Gene transcription	Human embryonic kidney 293 T	p53-null-H1299 cells and *p53* knockout mice (I.V. injection and post irradiation analysis of sensitive tissues)	ARMM	Estimates of delivery based on protein delivery below	(Wang et al. 2018)
GFP	Fluorescence	Human embryonic kidney 293 T	Human lung carcinoma A549 cells	ARMM	Estimates of delivery based on protein delivery below	(Wang et al. 2018)
Small RNA						
CRISPR/Cas-9 single guide RNA	Fluorescence: CROSSFIRE system	Human breast adenocarcinoma MDA-MB-231 cells	Human embryonic kidney 293 T cells		~0.2% reporter activation in co-culture, likely due to low abundance of 1 sgRNA per ~$4.5x10^5$ EVs	(de Jong et al. 2020)
siRNA/antisense oligonucleotides	miRNA125a	Red blood cells	Human acute myeloid leukemia MOLM13 and NOMO1	EVs	Reported 80–95% suppression of miR-125b levels due to EV delivery of 125b-ASOs in cells	(Usman et al. 2018)
DNA						
Cas-9 + sgRNA-GFP	Loss of fluorescence	Red blood cells	Human embryonic kidney 293 T cells expressing GFP	EVs	Reported lower efficiency of targeting using plasmid transfer from EVs, although different cell types used in each experiment	(Usman et al. 2018)

Protein						
Tumour suppressor p53 and GFP control	Cell death Gene transcription	Human embryonic kidney 293 T cells	p53-null- human non-small cell lung carcinoma H1299 cells and *p53* knockout mice (I.V. injection and post irradiation analysis of sensitive tissues)	ARMM	Estimated that each ARMM vesicle contains ~540 cargo protein molecules and that in some assays each recipient cell received ~3.1×10^6 cargo protein molecules from 5.8×10^3 ARMMs based on GFP	(Wang et al. 2018)
CRISPR-Cas9/guide RNA complex	Loss of fluorescence	Human embryonic kidney 293 T cells	Human embryonic kidney 293 T cells and human bone osteosarcoma U2OS-GFP+ cells	ARMM	GFP expression decreased in ~13% of recipient cells	(Wang et al. 2018)
Cre recombinase	Fluorescence	Human glioblastoma cell line LN18	mT/mG mouse embryonic fibroblasts, Ai14 mice (intranasal delivery followed by assessment of brain sections)	Exosomes	Cre recombinase activity detected in ~1% recipient cells	(Sterzenbach et al. 2017)
CD63-GFP	Fluobody fluorescence	Human embryonic kidney 293 T	Human embryonic kidney 293 T	EVs	~10–24.5% EVs exposed cargo to cytoplasm	(Joshi et al. 2020)

and therefore the contents of the EV should have been delivered into the recipient cell cytoplasm.

Evidence for Functional Cargo Delivery

The use of reporter systems has helped to provide evidence of both functional RNA and protein delivery into recipient cells. The advantage of using a specifically engineered cargo to measure functional delivery is that it eliminates some of the ambiguity when using endogenous proteins. One example of this, from Ridder and colleagues, used a Cre recombinase system to demonstrated the transfer of Cre recombinase mRNA from hematopoietic cell EVs into neural cells by detecting recombination of LoxP sites (Ridder et al. 2014). This system was validated both *in vivo* and using *ex vivo* derived EVs injected into the mouse brain. Recombination events were detected in Purkinje neurons, and took place at a low frequency in healthy animals. Recombinase activity was thought to be due to the transfer of mRNA and not the Cre recombinase protein itself, as the levels of Cre protein detected in EVs was negligible. In an additional study, Ridder et al. also demonstrated that this Cre/Lox system also allows for the tracking of mRNA transfer between tumour cell explants and the immune system *in vivo* (Ridder et al. 2015). Similar to the Valadi et al. study described above, these studies are convincing in showing delivery of cargo to recipient cells, because in order for a recombination event to happen the mRNA in the EVs must be transferred from the EV lumen into the cytoplasm of the recipient cell for translation into an active protein *in vivo*. A further example of mRNA delivery by EVs was demonstrated *in vitro* using the luciferase activity of the GlucB reporter. In this study from Lai and co-workers, the translation of EV-delivered mRNA was monitored in parallel with fluorescently labelled EV uptake. Data showed that EVs were efficiently taken up by recipient cells, and luminescence was detected as early as 1 hour after EV exposure. The luminescent signal was inhibited by the translation inhibitor cycloheximide, whereas uptake of EVs was not affected (Lai et al. 2015). This again provides strong evidence for the delivery of functional mRNA by EVs.

As mentioned above, EVs loaded with mRNA generally also carry the translated protein and therefore delivery of the active protein rather than mRNA cannot be excluded. One study that specifically rules out this confounding factor was from Usman and co-workers, who demonstrated the ability of red blood cell-derived EVs to deliver Cas9 mRNA that was electroporated into EVs post isolation (Usman et al. 2018). Cas9 activity was detected in leukaemia recipient cells, targeting miR-125 or GFP, and this must be due to translation of Cas9 protein from the externally-loaded mRNA (Usman et al. 2018). Additionally, this study also described the functional delivery of plasmid DNA, although at significantly lower efficiency (Usman et al. 2018).

The delivery of Cre recombinase protein has also been demonstrated, although its delivery is variable in different settings. Similar to the reports described above using

mRNA, if delivered into cells, the functional Cre recombinase will initiate recombination events that are reported by fluorescent protein expression (Sterzenbach et al. 2017). However, another study using HEK293T cells reported that the delivery of Cre recombinase was not detectable unless an endosomal disrupting agent was present, suggesting that the EVs were taken up by the cells but the cargo was unable to access the cytoplasm (Heath et al. 2019). This raises questions about whether all EVs and cell types can effectively deliver and receive EV cargo.

Other convincing data for functional cargo deliver include the arrestin domain containing protein 1 [ARRDC1]-mediated microvesicle (ARMM) transfer of functional proteins and RNAs, described by Wang et al. (Wang et al. 2018). These engineered microvesicles recruit specific cargo including the tumour suppressor p53 protein, p53 and GFP mRNAs, and Cas9/sgRNA complex, which were shown to be released as biologically active cargo into the cytoplasm of the target cells (Wang et al. 2018). *In vitro,* they showed that p53 protein was able to induce the transcription of multiple target genes; furthermore, p53 packaged into ARMMs was sufficient to recapitulate its function, as *ex vivo* p53-containing ARMMs were able to induce apoptosis following DNA damage in multiple tissues in p53 knockout mice. Similarly, the mRNA of p53 and GFP was translated into functional protein when delivered by ARMMs into target cells lacking these proteins. Additionally, an assay that has gained significant interest for potential use of EVs for gene editing is the delivery of Cas9/sgRNA complexes. Here the authors targeted GFP expression in a haploid cell line engineered to contain a single copy of GFP. When incubated with ARMMs containing the Cas9/sgRNA complex targeting GFP, the percentage of GFP positive cells decreased in around 13% of the recipient population. This suggests that the complete ribonucleoprotein complex was delivered from the EV lumen into the cytosol of the recipient cell and, followed by translocation into the nucleus, the Cas9/sgRNA complex then created INDELs in the genome to disrupt GFP.

Questions Regarding Biological Relevance

As described above, delivery of mRNA by EVs has been convincingly demonstrated using *in vitro*-cultured cells and reporter systems, but whether this occurs *in vivo* remains controversial. Several reports suggest that the endogenous mRNA content in EVs is relatively low compared to small RNA species (>200 nt) (Jenjaroenpun et al. 2013; Willms et al. 2016). While this may be true, a single mRNA can be translated into a functional protein many times over; therefore, the original delivered cargo is amplified during this process so the low mRNA content may still be functional.

Indeed, there are many reports of miRNA-mediated effects as a consequence of treating cells with isolated EVs, and there is significant interest in the role of miRNA transfer by EVs particularly during cancer progression, as reviewed (Bayraktar et al. 2017). However, often studies do not specifically show that the miRNA responsible is exclusively present in EVs and not associated with copurified contaminants, such

as lipoproteins, which have also been shown to carry miRNA (Vickers et al. 2011). Furthermore, the amount of miRNA encapsulated in EVs has also been reported to be relatively low in some instances, and there is some argument as to whether there are enough copies of a single miRNA species in each EV to deliver a high enough concentration to have a biologically relevant effect in the recipient cell (Chevillet et al. 2014; He et al. 2019; Somiya 2020). Other small RNA species have also been identified in EVs, such as transfer RNA and Y-RNA, and it is becoming apparent that the major RNA species in EVs differ depending on the source of EVs (Nolte-'t Hoen et al. 2012; Godoy et al. 2018).

Montecalvo and colleagues isolated EVs from dendritic cells and demonstrated miRNA transfer into recipient cells using a luciferase activity assay (Montecalvo et al. 2012). In addition, compelling evidence that small RNA molecules are delivered into recipient cells by EVs comes from the use of a CRIPSR-Cas9 reporter system. de Jong and colleagues demonstrated delivery of single guide RNA (sgRNA) cargo into recipient cells using a fluorescent CRISPR-Cas9 reporter system (de Jong et al. 2020). In this study, EVs from MDA-MB-231 cells were used to transfer sgRNA molecules between cells in culture and using isolated EV preparations. The recipient cells were engineered to contain an mCherry sequence and the target sequence; if frame shift mutations are introduced upon sgRNA targeted Cas9 cleavage, recipient cells will then express GFP. Using this 'CROSS-FIRE' system, the delivery of small RNA molecules (~100 nt) was effectively demonstrated (de Jong et al. 2020). The EVs in this study were isolated using filtration followed by size exclusion chromatography. This method should in theory help to decrease the contamination of lipoproteins that may carry small RNA; however, lipoprotein removal was not directly demonstrated. The authors did demonstrate that the sgRNA was not degraded during RNase treatment, indicating the sgRNA were protected, presumably in EVs (de Jong et al. 2020). Interestingly, despite the high sensitivity due to low background, the reported proportion of cells that became GFP positive after incubation with EVs was less than 0.2% of the total recipient cell population. This may be due to a combination of inefficient loading of sgRNA into EVs (1 sgRNA molecule per 4.5×10^5 EVs) and the frequency of insertion or deletions mutations (INDELs). However, inefficient delivery of cargo from EVs cannot be ruled out as a factor, potentially calling into question the importance of this pathway i*n vivo*.

Taken together, the above examples of EV-mediated RNA and protein transfer demonstrate that functional molecules can be delivered from the lumen of EVs into recipient cells. However, questions remain surrounding the number of molecules needed to be delivered for biological relevance.

Underlying Cellular Mechanisms Proposed for EV Communication and Cargo Delivery

As mentioned above, the mechanisms that underpin how EVs deliver molecules to mediate intercellular communication are poorly understood, despite being critical for EV function. While demonstrating the delivery of functional cargo has been challenging, there are now several reports, described above, providing good evidence of this phenomenon. However, directly observing the sub-cellular location of delivery and describing the mechanisms that govern delivery from EVs into the cytoplasm remains elusive.

Presently, it is accepted that there are three sites of action for EVs; peripherally, at the cell surface and inside recipient cells (Fig. 8.2). Peripherally, EVs act indirectly by activating pathways outside cells in the circulation. In this scenario, the EVs do not act directly on recipient cells; rather, they activate pathways in the extracellular space, which in turn leads to effects on cells. To kick off blood clotting, for example, EVs can activate the coagulation cascade via phosphatidylserine and tissue factor on the exofacial leaflet of their membranes (Owens and Mackman 2011). In addition to this example, EVs can also carry proteins and receptors that may have functions in the extracellular space, such a matrix metalloproteinases (Shimoda 2019). These peripheral or indirect functions are important, and could be the simplest way that EVs can act extracellularly, but are less numerous than their functions involving direct interactions with target cells.

Binding to Cell Surface Receptors

EVs contain surface bound proteins and transmembrane receptors on their surface, similar in profile to those found on the cell surface. Depending on their biogenesis and the cell type, there may be also an enrichment in specific transmembrane proteins due to targeting and recruitment by lipid rafts and tetraspanin-enriched microdomains. When EVs bind to cells, the proteins on their surface can act as a ligand or binding partner for a surface receptor on the recipient cell (Fig. 8.2). In this case, it is not the intraluminal cargo that is important for intercellular communication, but the surface proteins and receptors. Early examples of this were identified in the immune system, where dendritic cell EVs bind to effector cells, such as T cells, and elicit an antigen-specific immune response (Thery et al. 2002; Segura et al. 2005). This is dependent on MHC class II and ICAM-1, two important transmembrane proteins present in dendritic cell EVs that bind to receptors on the surface of effector cells to activate them (Segura et al. 2005). Another example of EVs activating cell surface receptors is through the transport and loading of interferon-γ. Here, interferon-γ loaded on EV interferon-γ receptors can be transferred from EVs onto interferon-γ receptors on recipient cells, leading the activation of STAT1 signalling in the recipient cell (Cossetti et al. 2014). Whether all EV subtypes have

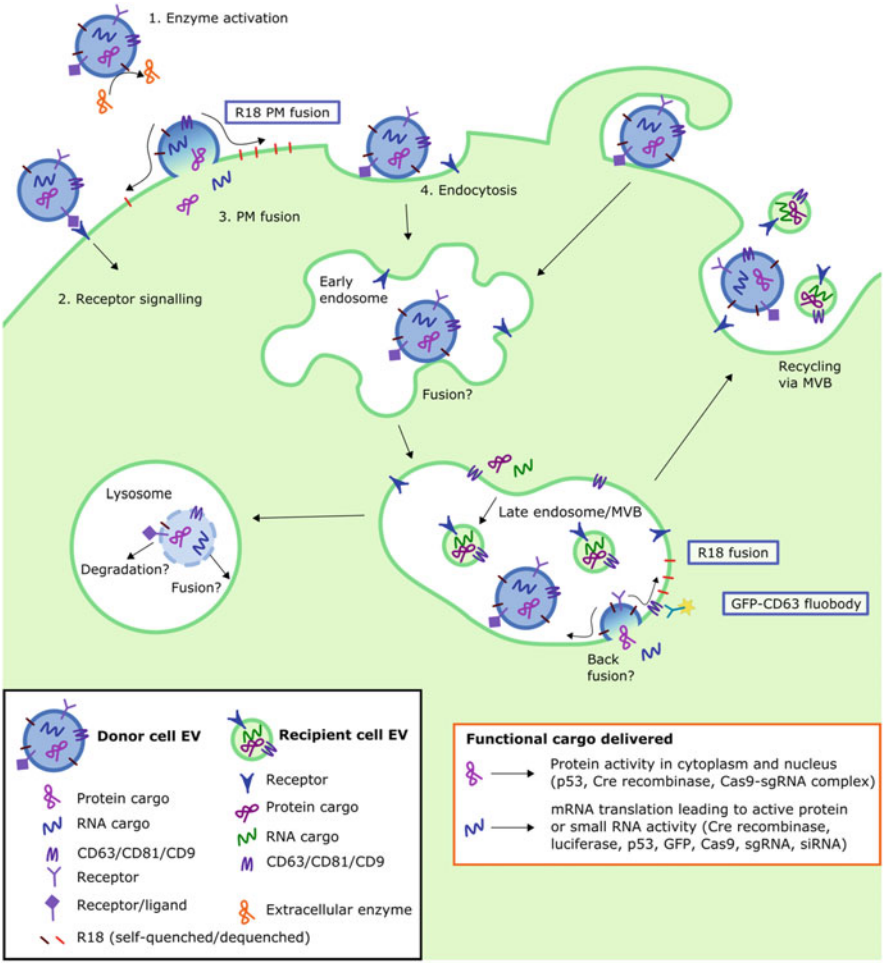

Fig. 8.2 Extracellular vesicle functions and intercellular communication routes. Once secreted into the extracellular space, EVs can act at three sites: (1) peripherally, activating proteins in the blood; (2) at the cell surface, binding to receptors and activating signalling cascades; and within cells, delivering their contents into the cytoplasm, with (3) plasma membrane fusion and (4) endocytosis as proposed mechanisms. Here, EVs originating from donor cells are shown in blue, while EVs originating from recipient cells are shown in green. How EVs from donor cells deliver their contents is poorly understood. EVs have been proposed to fuse with the plasma membrane, and studies using the R18 reporter support this notion, as indicated. EVs have also been proposed to fuse in the endo-lysosomal system at various points. These include the early endosome, late endosome or MVB and lysosome. Evidence for delivery in the late endosome or MVB includes R18 fluorescence and the detection of the luminal portion of GFP-CD63 at this site, as depicted. Once in the cytoplasm, protein and RNA can mediate their effects, which has been demonstrated using engineered EVs that deliver specific functional cargo

the capability to act in this way remains unclear; presumably it would depend on the type of secreting cell and its plasma membrane proteome. This type of signalling allows for specificity in the types of cells targeted, as not all cells would possess the correct receptors to respond.

Cargo Delivery within the Recipient Cell

EVs contain a range of proteins and nucleic acids from the cytoplasm of the secreting cell. Some of these proteins and RNA are differentially expressed by specific cell types and can therefore have effects on a recipient cell of a different tissue. Or, in the case of pathological states, EVs may contain proteins and RNA responsible for the aberrant phenotype. In most cases, for these cargos to be functional, they need to be delivered into the cytoplasm of the recipient cell. This involves a complex process of crossing several membranes; first, the membrane encapsulating the EV, and second, the membrane of the recipient cell. There have been several mechanisms proposed for how this may occur, but data is generally limited. This section will describe the possible sites where EVs might fuse with the recipient cell to deliver their cargo into the cytoplasm, such as at the plasma membrane or within the endo-lysosomal system.

Plasma Membrane Fusion

Arguably the most direct, and potentially efficient way of delivering their cargo, is for EVs to fuse directly with the plasma membrane (Fig. 8.2). In theory, this process would involve (i) the EV binding to the surface of the recipient cell (ii) the membrane of the EV and the recipient cell being in very close proximity to each other and (iii) the membranes fusing, incorporating the lipids from the EV into the plasma membrane while delivering the EV cargo into the cytoplasm. This proposed mechanism seems straightforward, but there are many open questions regarding how this might work mechanistically, and little evidence to suggest this membrane fusion occurs either *in vitro* or *in vivo*.

EVs are able to bind to cells in many ways. As discussed above, specific ligands on EVs can bind to their corresponding receptors at the cell surface and in certain settings proteins are important for EV binding to the cell surface (French et al. 2017). Similarly, carbohydrates on the recipient cell surface have been identified as EV binding partners (Christianson et al. 2013; Purushothaman et al. 2016) and it is thought that the lipid composition may also play a role (Zakharova et al. 2007; Buzas et al. 2018). Therefore, it appears that EV binding to the cell surface can be mediated by several EV surface constituents, but it remains unclear if they are all required for efficient binding and subsequent cargo delivery. It is also possible that EV association with the cell surface may be mediated by general electrostatic interactions rather than specific binding partners. Again, this could be through a combination of

the charged motifs on proteins, carbohydrates and lipids. However, given that EVs are should reflect the parental cell membrane, both membranes' surface charge should also be negative overall, which would not necessarily promote interactions between EVs and the plasma membrane.

The biggest question regarding this pathway is: how would membrane fusion occur? Intracellularly, membrane fusion events are mediated by many proteins, as described briefly for biogenesis of EVs. On the cell surface, however, these proteins are not available as they should reside inside the cell, and if present, also be retained in the lumen of EVs. Without these membrane fusion mediators, it is unclear exactly how fusion would occur. Fusion may be driven by lipids and their affinity for each other, as is the case *in vitro* for liposomes when forming a lipid bilayer. But in this scenario, there may be an issue of membrane curvature. EVs are very small with prominent membrane curvature (Kastelowitz and Yin 2014); therefore, is it energetically favourable for them to fuse and incorporate with the plasma membrane unfacilitated? These questions all need to be considered and addressed when considering fusion with the plasma membrane as a mechanism for EV cargo delivery.

Supporting Evidence for Plasma Membrane Fusion

Supporting evidence for this route of delivery is sparse. Several groups have used the lipophilic dye R18 to monitor the incorporation EV lipids into the plasma membrane upon fusion (Montecalvo et al. 2012; Parolini et al. 2009; Del Conde et al. 2005). R18 is a self-quenching fluorescent probe that can be used to label the lipids of EVs. If EVs fuse with a membrane, the lipids from the EV should incorporate into the bilayer and dissipate within the membrane due to its fluid nature; this then promotes dequenching of the fluorescently labelled lipids. In recipient dendritic cells, confocal microscopy showed that the R18 specific fluorescent signal appeared to localised to the plasma membrane (Montecalvo et al. 2012). Furthermore, cargo from these EVs was released into the recipient cell measured using a luciferase activity assay (Montecalvo et al. 2012). Interestingly, in this study the authors attempted to visualise incorporation of EV membranes with the plasma membrane by electron microscopy, but they were unable to detect these events (Montecalvo et al. 2012). Another study showed that R18 fluorescence was increased in the acidic extracellular conditions as seen in tumour micro-environments (Parolini et al. 2009). Furthermore, incorporation of R18, but not binding of EVs (specifically calcium induced microvesicles) to the recipient cell surface, decreased in the presence of annexin A5, implicating phosphatidylserine in the membrane fusion step specifically (Montecalvo et al. 2012; Parolini et al. 2009; Del Conde et al. 2005).

These data provide some evidence that EV fusion with the cell membrane is possible; however, the mechanisms remain elusive and there are no other studies that do not use R18 that confirm these findings. Furthermore, other studies found that R18 did not incorporate into the plasma membrane, discussed in more detail below (Yao et al. 2018; Tian et al. 2013).

Fusion within the Endo-Lysosomal System

Another avenue for cargo delivery into the recipient cell is through the endocytic pathway (Fig. 8.2). Endocytosis is the process responsible for the internalisation of receptors, cell surface proteins and macromolecules bound to the plasma membrane. The endocytic system is a complex network of vesicular and tubular structures that are required for sorting, recycling and degrading material. As EVs bind to the plasma membrane, they can enter the endocytic pathway and could deliver their contents into the cytoplasm during this process.

Endocytosis

Several types of EV uptake have been reported, including clathrin-dependent endocytosis, caveolin-mediated endocytosis, pinocytosis, lipid raft-mediated endocytosis, receptor mediated endocytosis and phagocytosis (Tian et al. 2014; Feng et al. 2010; Costa Verdera et al. 2017), and reviewed (Mulcahy et al. 2014). EVs have also been reported to "surf" the plasma membrane, being grabbed and pulled in on filopodia prior to endocytosis (Heusermann et al. 2016). The type of endocytosis responsible for EV uptake depends on the cell type, how the EVs bind to the cell surface (discussed above, 4.1), and their size and composition. For example, the type of endocytosis will be different if EVs bind in a receptor mediated fashion compared to a non-protein-receptor mediated interaction through lipids or carbohydrates. Additionally, the size of the EVs may impact how the cell engulfs them; for example, phagocytosis may be used for larger EVs while micropinocytosis may be used for smaller EVs. After binding to the cell surface, EVs are endocytosed through inward invagination of the plasma membrane and the EVs remain bound to what was the extracellular face of the membrane—now the luminal leaflet of the endosome.

Once inside the cell, the endocytic compartment will presumably follow its usual trafficking route; initially forming an early endosome, which will then mature and either be recycled back to the plasma membrane or targeted to the lysosome for degradation, as reviewed (Scott et al. 2014). This is a simplified view of trafficking along the endocytic pathway, which in reality involves a complex network of structures. However, EVs may be too big to enter narrow tubular extensions within the endocytic compartment, and therefore are more likely to localise to the larger punctate regions of endosomes that are typically trafficked toward the lysosome.

Early/Sorting Endosome

The first stage is localisation of EVs within the early endosome. The early endosome is also known as the sorting endosome, where the fate of the cargo is decided (Jovic et al. 2010; Naslavsky and Caplan 2018). The early endosome is mildly acidic at approximately pH 6.2 (Murphy et al. 1984). Several proteins are recruited to the

cytosolic leaflet of early endosomes, including Rab5 and early endosomal antigen-1 (Christoforidis et al. 1999; Jovic et al. 2010). These proteins are important for the formation, trafficking and maturation of the endosome within the cytosol (Jovic et al. 2010). At this stage the EVs could, in theory, fuse with the limiting membrane of the early endosome to deliver their cargo into the cytosol, although there is little evidence for this in the literature.

Late Endosome and MVB

The early endosome then continues maturing towards becoming a late endosome. Exactly how this process works is still controversial; the early endosome itself could acidify and mature into a late endosome, or specific parts of the early endosome could bud off and fuse with a late endosome (Naslavsky and Caplan 2018). Either way, the late endosome is a more acidic compartment, approximately pH 5.5 (Griffiths 1989; Scott et al. 2014). The proteins associated with the late endosomal compartment include and Rab7 and LAMP-1, which are required for trafficking and stability (Vanlandingham and Ceresa 2009; Scott et al. 2014). It is worth mentioning that it is hard to discriminate between a late endocytic compartment and the lysosome due to overlapping markers, and the only way of definitively identifying these compartments is by sub-cellular fractionation (Scott et al. 2014). However, the late endosome still represents a distinct compartment where EVs may fuse within the endosomal system before reaching the lysosome. Some late endosomes also contain intraluminal vesicles (as described above in EV biogenesis) and become MVBs, whose membranes are enriched with CD63 and LAMP-1 (Kobayashi et al. 2000; Piper and Katzmann 2007). Again, this is a dynamic process that may also begin in the early endosome (Scott et al. 2014). The MVB may contain ILVs with different properties, such as different lipid signatures, arising from different microheterogeneity within the late endosomal membrane (Kobayashi et al. 2002). This may also reflect the fact that there are several pathways described for ILV biogenesis.

If EVs originate in the MVB and then fuse in the recipient cell at the same point, it is termed ‘back-fusion’ as the EV is fusing back with the original compartment it was formed in, though in another cell. This may represent the relevant stage of endocytosis where EVs fuse to release their contents. This is an interesting notion, as endogenous ILVs that are formed with in the MVB also undergo back-fusion which has been proposed to by mediated by lyso-bisphosphatidic acid (LBPA) and Alix (van der Goot and Gruenberg 2006). LBPA is thought to be fusogenic at an acidic pH, and may be found in secreted EV membranes where it accounts for up to 15% of the late endosomal membrane, although this is somewhat controversial and requires further investigation (Kobayashi et al. 2002; Rabia et al. 2020; Skotland et al. 2020). Some enveloped viruses also enter the cytoplasm at this point, due to specific proteins contained on their surface that undergo conformational changes at the lower pH allowing them to penetrate the membrane (Lozach et al. 2011).

Therefore, EVs may use a similar mechanism, but it is unclear which proteins would be involved in this process.

Lysosome

The final fate of endocytosis is often fusion of the late endosome or MVB with the lysosome. The lysosome is a highly acidic and degradative organelle that functions to break down unwanted material. As highlighted above, the MVB will either fuse with the lysosome or traffic to the plasma membrane to release exosomes into the extracellular space. If destined for the lysosome, the contents of the MVB will be degraded by the acidic pH and the many enzymes that reside in this organelle. It has been shown that EVs can be detected in the lysosome by several groups, but it remains unclear at which stage in the endocytic process the EVs delivered their cargo; this may happen in the early or late endosome or in the lysosome (Tian et al. 2013; Heusermann et al. 2016; Roberts-Dalton et al. 2017).

Supporting Evidence for Fusion within the Endo-Lysosomal System

In direct contrast to plasma membrane fusion studies mentioned above, one study found that EVs were trafficked to the lysosome, and incorporation of R18 at the plasma membrane was not observed (Tian et al. 2013). In agreeance with this observation, another group using R18 to monitor fusion detected dequenching of R18 in the late endosome, MVB and lysosome but not at the plasma membrane (Yao et al. 2018). It is unclear why there are discrepancies in the literature regarding EV labelled with R18; it may be due to differences in the cell types used to both generate the EVs and as recipient cells. Further evidence for fusion of EVs in the late endosome comes from a recent paper by Joshi and co-workers, which used GFP-CD63 expressed in EVs and an anti-GFP nanobody fused to mCherry expressed in the cytosol of recipient cells to detect EV fusion events. Using correlative light and electron microscopy (CLEM), they found that luminal GFP (fused to CD63) was only detected in the cytosol of recipient cells after fusion in the endosomal system, rather than at the plasma membrane. Cytosolic nanobodies detected punctate GFP, and CLEM showed that these puncta were in endosome-like structures. Finally, the exposure of GFP in the cytosol was dependent on endosomal acidification, indicating fusion occurred in the late endosome or lysosome (Joshi et al. 2020).

Other Delivery Sites

Evidence for other delivery sites is very limited. It has been suggested that the ER is a site of cargo delivery, and that this would enhance the targeting of mRNA and miRNA cargo in EVs. Evidence for this comes from a study which showed that when HEK293T EVs are taken up in primary human fibroblasts, they surf on

filopodia and can be tracked through the endosomal system to close contacts with the ER, before finally localising at the lysosome (Heusermann et al. 2016). While interesting, it remains unclear whether this is cell type specific; furthermore, it was not demonstrated whether or not EV cargo was actually delivered to the ER (Heusermann et al. 2016). Further work is required to establish whether there are additional delivery sites for EVs other than the plasma membrane and the endosome.

Summary, Open Questions and Challenges

Intercellular communication is an important process, and EVs represent a complex, controversial and tantalising means for both UPS and cellular communication at a distance. There are numerous reports of EVs being involved in a variety of biological processes, where they function at a distance from the secreting cell. EVs contain a wide range of cargo and their mode of action varies depending on the circumstances. They are able to operate both extracellularly and intracellularly, and as such their functions are copious. Despite all this, their mechanisms of action at a cellular level are elusive and many major questions remain.

Given the ability of all cells to secrete EVs, one question that needs to be addressed is whether EVs from different cell types are equally able to deliver their cargo, or is it only a subset? This is a relatively hard question to address but it is an important consideration, particularly when studying the function of EVs from cell lines *in vitro*. Not all EVs from all cell types express the same proteins, carbohydrates and lipids in the same ratios, and therefore those that may be involved in delivery or fusion may not be available on all EVs. Therefore, unless the delivery mechanism is conserved across all cell types, which indeed it may be, some EVs from certain cell types may not be particularly effective at intercellular delivery of cargo molecules. Furthermore, there are several subtypes of EVs, including exosomes, small exosomes, microvesicles and apoptotic bodies. It is unclear if they are all equally effective as agents of intercellular communication. Assuming delivery of cargo is required for their function, is the delivery mechanism the same for each subpopulation of EVs? Or do microvesicles and exosomes contain different surface lipids and proteins and therefore have differing delivery mechanisms? On the flipside, it is unclear whether all cells are equally able to receive EV cargo. Some cell types are more endocytic, and will therefore take up more EVs, but it is unclear if this increased uptake translates into more delivery of functional cargo. The use of cell lines in studying the cell biology of EVs is important, and it is becoming apparent that a biologically relevant pairing of donor and recipient cell lines should be considered to measure uptake and cargo delivery.

Following this theme, another question that needs to be addressed is how many EVs are required to have a biologically or physiologically relevant effect? *In vitro* assays often concentrate EVs from tissue culture medium and use this heavily-concentrated pool of EVs to elicit effects on recipient cells. Whether these assays reflect the number of EVs that would be available *in vivo* remains unclear. One

review used existing data to estimate how many protein and RNA effector molecules EVs contain (Somiya 2020). The results of these calculations suggest that EVs contain very low concentrations of individual miRNA and proteins. The concentration of a transfected miRNA mimic (where a clear effect was observed) was estimated at $1x10^6$ molecules per cell, while the concentration available for delivery by EVs was estimated to be 1000 times lower at $1x10^3$ per cell (Somiya 2020). When comparing the amount of miRNA available for delivery in EVs to a miRNA mimic by transfection (where a clear effect is observed) there is a large discrepancy in the estimated number of molecules delivered per cell, $1x10^3$ compared to $1x10^6$ molecules per cell respectively (Somiya 2020). This suggests that despite numerous reports of miRNA being responsible for phenotypic changes when delivered by EVs, the amount of a specific miRNA delivered would be less than one molecule per EV. It is unlikely that the large number of EVs that would be required to deliver a functionally relevant concentration would occur *in vivo*.

Furthermore, the potential of the limited cargo that is actually delivered is not equal when comparing proteins and RNA. To elicit an effect, mRNA needs to be translated into a protein, and a single mRNA can be translated into a functional protein many times over. Therefore, there is an element of amplification for mRNA that is not achievable for proteins. Conversely, enzymes such as proteases and nucleases are able to continue to be active longer and target their substrates many times over, therefore for some proteins only a small amount may be required to have an effect on the cell. The question of how many EVs are required; and in turn, whether enough effector molecules are delivered, remains very much open and specific to the action of the potential cargo.

Despite our lack of understanding regarding cargo delivery, it is clear that EVs represent an exciting mode of multifaceted intercellular communication. Some standardisation across the field and clear reporting will help toward answering many of the questions posed in this chapter. Additionally, the development of novel technologies, particularly sensitive imaging techniques coupled with functional studies, to investigate these questions will also shed light on the process of cargo delivery by EVs.

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Chapter 9
Extracellular Vesicles in Chemoresistance

Gabriele De Rubis and Mary Bebawy

Abstract Chemotherapy represents the current mainstay therapeutic approach for most types of cancer. Despite the development of targeted chemotherapeutic strategies, the efficacy of anti-cancer drugs is severely limited by the development of drug resistance. Multidrug resistance (MDR) consists of the simultaneous resistance to various unrelated cytotoxic drugs and is one of the main causes of anticancer treatment failure. One of the principal mechanisms by which cancer cells become MDR involves the overexpression of ATP Binding Cassette (ABC) transporters, such as P-glycoprotein (P-gp), mediating the active efflux of cytotoxic molecules from the cytoplasm. Extracellular vesicles (EVs) are submicron lipid-enclosed vesicles that are released by all cells and which play a fundamental role in intercellular communication in physiological and pathological contexts. EVs have fundamental function at each step of cancer development and progression. They mediate the transmission of MDR through the transfer of vesicle cargo including functional ABC transporters as well as nucleic acids, proteins and lipids. Furthermore, EVs mediate MDR by sequestering anticancer drugs and stimulate cancer cell migration and invasion. EVs also mediate the communication with the tumour microenvironment and the immune system, resulting in increased angiogenesis, metastasis and immune evasion. All these actions contribute directly and indirectly to the development of chemoresistance and treatment failure. In this chapter, we describe the many roles EVs play in the acquisition and spread of chemoresistance in cancer. We also discuss possible uses of EVs as pharmacological targets to overcome EV-mediated drug resistance and the potential that the analysis of tumour-derived EVs offers as chemoresistance biomarkers.

Keywords Biomarkers · Cancer · Chemotherapy · Extracellular vesicles · Immune evasion · Intercellular communication · Metastasis · Microparticles · miRNA · Multidrug resistance · P-glycoprotein

G. De Rubis · M. Bebawy (✉)
Discipline of Pharmacy, Graduate School of Health, University of Technology Sydney, Sydney, Australia
e-mail: mary.bebawy@uts.edu.au

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Introduction

Among the many different modalities available for the treatment of cancer, chemotherapy remains the mainstay treatment of most cancers (Nikolaou et al. 2018). Since the first recorded use of chemotherapy in 1942, when nitrogen mustard was administered intravenously in a lymphosarcoma patient (Fenn and Udelsman 2011), anticancer drugs have undergone extensive development towards molecular targeted approaches with improved specificity towards cancer targets. This approach has significantly improved patient survival rates in cancer (Chabner and Roberts Jr. 2005; Chung et al. 2010). In the United States, there has been a 20% increase in the overall 5-year relative survival rate for people diagnosed with cancer since the seventies (Howlader et al. 2020). Despite this, in many cancers the efficacy of anticancer drugs is still limited by the development of drug resistance, or chemoresistance, which represents a major hindrance to the success of cancer therapy even when the initial response to treatment is promising (Gottesman 2002; Holohan et al. 2013; Nikolaou et al. 2018).

Chemoresistance involves a reduced effectiveness of a given chemotherapeutic regimen which results in disease reoccurrence, poor prognosis and poor overall survival (Nikolaou et al. 2018). Drug resistance can develop after an initial sensitivity of the tumours to treatment, however further drug treatment does not ensure tumour suppression or the prevention of cancer metastasis (Gillet and Gottesman 2010; Nikolaou et al. 2018; Madden et al. 2020). Chemoresistance can be intrinsic or acquired. Intrinsic resistance is attributed to the existence of resistance factors in the cancer cells prior to exposure to chemotherapy. In this case, the initial response to treatment is generally poor. Intrinsic resistance is often observed in malignancies such as renal cancer, malignant melanoma and hepatocellular carcinoma (Gottesman 2002). Conversely, in the case of acquired resistance, mechanisms are developed or 'acquired' in cancer cells following exposure to chemotherapeutic drugs. In this case, the drug resistance mechanisms are considered to be drug-induced (Gottesman 2002). The development of drug resistance is multifactorial and complex in nature. Interestingly, in addition to alterations in the cellular machinery resulting in drug resistance following drug exposure, resistance can also be acquired through cell-to-cell communication, from resistant cancerous cells to drug-sensitive cells through the transfer of membranous vesicles known as extracellular vesicles (EVs) (Bebawy et al. 2009; Lu et al. 2016; Zhang et al. 2014).

EVs are submicron vesicles enclosed by a phospholipid membrane bilayer. EV-mediated cell communication is evolutionarily conserved, having been identified in virtually all living species, including animals, plants and microorganisms (Lawson et al. 2017). They are secreted into the extracellular space by all living cells, innately and following cell exposure to different types of stimuli (Taylor and Bebawy 2018). EVs can be found in all body fluids including blood and urine and now comprise important biomarkers of disease (Samir et al. 2013; De Rubis et al. 2019; Torrano et al. 2016). EVs were first discovered by Peter Wolf in the last century during plasma ultracentrifugation and were described as "platelet dust"

(Wolf 1967). However, technological advancements enabled researchers to uncover their role and EVs are now considered critical mediators of intercellular communication. EVs are enriched with coding and non-coding RNA, DNA, proteins and lipids and they allow cells to communicate with both neighbouring and distant cells through the transfer of cargo. This type of cell-to-cell communication plays important roles in cell homeostasis as well as in many physiological and pathological cellular processes (van Niel et al. 2018; Yáñez-Mó et al. 2015; Samir et al. 2013; Raposo and Stoorvogel 2013).

EVs can be classified based on their size and biogenic origin and include among others; exosomes, microvesicles or microparticles (MPs), apoptotic bodies and oncosomes (van der Pol et al. 2012). Importantly, EVs reflect the parental cells' molecular characteristics (Margolis and Sadovsky 2019), and this assists in understanding their role in disease development. Exosomes are endosomal in origin and are of 50–150 nm in size. They are released in the extracellular space through the fusion of multivesicular endosomes with the plasma membrane (Théry et al. 2002; van der Pol et al. 2012). EVs are typically characterised by the presence of tetraspanin proteins, such as CD9, CD63, CD81 and CD82 (Théry et al. 2002; Kowal et al. 2016). MPs are plasma membrane-derived vesicles which are formed following loss of phospholipid asymmetry and calpain-mediated cleavage of the underlying cytoskeleton. MPs are generally larger than exosomes, ranging between 100 and 1000 nm in diameter (van der Pol et al. 2012). Apoptotic bodies comprise the largest EVs and range in size from 1 to 5 μm in diameter (van der Pol et al. 2012). As their name suggests, they are released by cells undergoing programmed cell death and contain DNA fragments and histones (Théry et al. 2002; van der Pol et al. 2012).

Several studies have reported an increase in peripheral EV numbers in many types of cancer (Melo et al. 2015; Krishnan et al. 2016; Wang et al. 2017). In the cancer context, EVs have been shown to facilitate cancer development and progression through the transfer of oncogenic material, through priming of the premetastatic niche, through support of angiogenesis and through effects on tumour immune evasion (Xavier et al. 2020). These, together with their role in conferring resistance to chemo-sensitive cells (Bebawy et al. 2009; van Niel et al. 2018; Yáñez-Mó et al. 2015; Samir et al. 2013), place EVs in a central role in cancer pathology. In this chapter, we focus on the multifaceted role EVs play in the acquisition and spread of drug resistance in cancers. We also describe possible strategies to circumvent EV-mediated chemoresistance and the opportunities arising from the analysis of EVs as chemoresistance biomarkers.

Multidrug Resistance

In cancer, multidrug resistance (MDR) is a unique type of resistance arising from an innate or acquired cross-resistance to a wide spectrum of functionally and structurally unrelated chemotherapeutic drugs (Holohan et al. 2013; Szakacs et al. 2006). The development of MDR is a significant obstacle in cancer treatment, being

associated with poor prognosis and survival in most cancers (Saraswathy and Gong 2013). Furthermore, 90% of patients with metastatic cancer become unresponsive to treatment due to MDR (Longley and Johnston 2005; Dillekas et al. 2019). Classic MDR is attributed to the overexpression of transmembrane efflux proteins belonging to the ATP Binding Cassette (ABC) superfamily of membrane transporters (Szakacs et al. 2006), with typical members including P-glycoprotein (P-gp) (Juliano and Ling 1976), Multidrug resistance Protein 1 (MRP1) (Cole et al. 1992) and Breast Cancer Resistance associated Protein 1 (BCRP) (Doyle et al. 1998).

ABC Transporters

The ABC transporters are a superfamily of transmembrane proteins that actively translocate solutes across cell membranes, generally against a concentration gradient, in an ATP-dependent manner (Dean et al. 2001). They are evolutionary conserved and are represented from prokaryotes to humans. In humans, 48 distinct genes encode for ABC transporter proteins, which are categorised into 7 subfamilies, from ABCA to ABCG (Dean et al. 2001). ABC transporters are physiologically distributed throughout the human body, particularly at important pharmacological interfaces and sanctuary sites such as the intestinal epithelium, the blood-brain barrier and the blood-testis barrier (He and Liu 2002). At these sites, the physiological role of these proteins is protective in nature, as they prevent the uptake of xenobiotics and endogenous toxic metabolites from entering these barriers (Bart et al. 2000). A vital feature of these proteins is their ability to transport a wide range of structurally and chemically unrelated compounds (Szakacs et al. 2006). This mechanism is exploited by cancerous cells to prevent the intracellular accumulation of chemotherapeutic agents at sufficiently cytotoxic levels (Longley and Johnston 2005). Furthermore, a significant degree of substrate and functional redundancy among different members of the ABC family of transporters ensures a fail-safe survival mechanism by cancer cells (Lu et al. 2017; Jaiswal et al. 2012).

The typical "core" functional unit of ABC transporters comprises a four-domain structure which includes two highly conserved intracellular ATP-binding domains, called nucleotide-binding domains (NBD), and two variable transmembrane domains (TMD), each composed by 6 alpha helices, which are the sites of substrate recognition, binding and translocation (Szollosi et al. 2018) (Fig. 9.1). An exception to this is represented by the ABCC family of transporters, whose members have one extra TMD composed by 5 alpha helices which doesn't participate to substrate translocation (Liu 2019) (Fig. 9.1b). There are two general typologies of ABC transporters: the "full" transporters, in which these four domains are present on a single polypeptide chain (Fig. 9.1a, b), and the "half" transporters, which have one NBD and one TMD on each polypeptide chain and function upon homo- or heterodimerization (Szollosi et al. 2018) (Fig. 9.1c). The mechanism of action of ABC transporters is composed of alternating cycles of ATP hydrolysis and substrate translocation. The transport cycle is initiated by the binding of the substrate to the

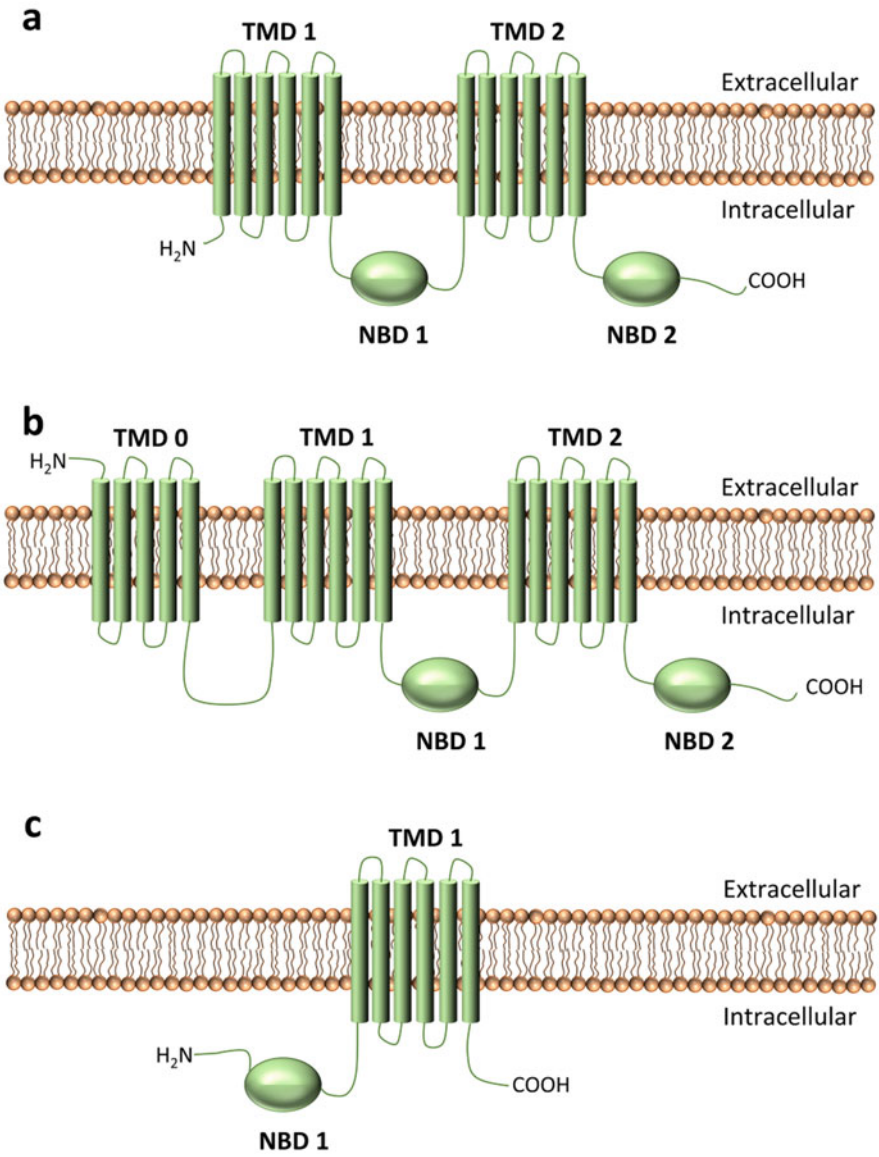

Fig. 9.1 Domain composition and topology of the three most studied families of ABC transporters**.** (**a**) ABCB Family (comprising P-gp). ABC transporters belonging to the ABCB family are composed by two TMDs, each one with 6 putative transmembrane alpha helices, and two intracellular NBDs. (**b**) ABCC Family (comprising MRP1). ABC transporters belonging to the ABCC family are composed by the "core" structure with two TMDs and two NBDs (similar to the ABCB family) with an additional N-terminal TMD (TMD 0), with 5 putative transmembrane alpha helices, which do not participate in substrate translocation. (**c**) ABCG Family (comprising BCRP). ABC transporters belonging to the ABCG family are composed of one putative TMD and one N-terminal NBD. They function upon homo- or heterodimerization of two monomers. *P-gp* P-glycoprotein; *ABC* ATP-Binding Cassette; *TMD* Transmembrane Domain; *NBD* Nucleotide Binding Domain; *MRP1* Multidrug Resistance-associated Protein 1; *BCRP* Breast Cancer Resistance Protein

TMDs, which are in a high-affinity inward-facing orientation. This stimulates changes in the NBDs, which initiate binding and hydrolysis of two ATP molecules, in turn exciting the TMDs to switch conformation into a low-affinity outward-facing orientation and therefore translocating the substrates to the other side of the membrane against the concentration gradient. The release of ADP and Pi allows the TMDs to assume the initial inward-facing conformation and start a new cycle (Liu 2019; Szakacs et al. 2006; Szollosi et al. 2018).

Among the numerous ABC transporter proteins, the first identified and most investigated in the context of chemoresistance is P-glycoprotein (P-gp) (Juliano and Ling 1976; Shapiro and Ling 1994), also known as MDR1, and which is encoded by the ABCB1 gene (Liu 2019). Two other important efflux transporters of this class are multidrug resistance-associated protein 1 (MRP1) and the breast cancer resistance protein (BCRP), which are encoded by the ABCC1 and ABCG2 genes respectively (Szollosi et al. 2018; Liu 2019). Overexpression of these efflux transporters confers MDR in most cancers and, for this reason, these proteins are also known collectively as MDR proteins (Cole 2014; Holohan et al. 2013).

P-Glycoprotein

The role of P-glycoprotein (P-gp) in conferring the MDR phenotype in cancer was first identified in the early ‘70s (Gottesman and Ling 2006). It is considered to be the most important ABC transporter in the context of MDR, as it confers resistance to the widest range of substrate compounds (Szakacs et al. 2006; Callaghan et al. 2014). P-gp is a 170 kDa protein comprised of 1280 amino acids (Schinkel 1999). It is an ABC transporter with two NBD and two TMD domains (Kunjachan et al. 2013) (Fig. 9.1a). P-gp is involved in excretory functions in many organs including brain, lungs, liver, kidney and intestine (Schinkel 1999). Localised on the apical membranes of epithelial cells, it acts to regulate the absorption, distribution, metabolism and elimination of xenobiotics across pharmacological barriers (Pokharel et al. 2017; Kumar and Jaitak 2019). Numerous segmental regions of the TMDs provide P-gp with a broad spectrum of substrate binding capacity (Schinkel 1999). There is no consensus structural requirement for molecules to be classified as its substrates other than they are large, hydrophobic, amphipathic and bear a positive charge at physiological pH (Schinkel 1999). Among its substrate repertoire there are numerous commonly used chemotherapeutic drugs including taxanes (paclitaxel), anthracyclines (doxorubicin, daunarubicin), vinca alkaloids (vinblastine), immunomodulatory agents and proteasome inhibitors (Krishnan et al. 2016; Kumar and Jaitak 2019; Kathawala et al. 2015; Tamaki et al. 2011).

Multidrug Resistance Associated-Protein 1

MRP1 is a 190 kDa, 1580 amino acid protein (Cole et al. 1992) comprised of the core two NBD and two TMD structure with an additional N-terminal TMD domain

(Kunjachan et al. 2013) (Fig. 9.1b). MRP1 is encoded by the *ABCC1* gene and, similar to P-gp, MRP1 is also found physiologically expressed in various cell types and organs (Flens et al. 1996). MRP1 is often colocalised with P-gp and this functional redundancy has been shown to be regulated by extracellular vesicles (Lu et al. 2017). Unlike P-gp, MRP1transports organic anion hydrophobic conjugates such as glutathione conjugates, thereby limiting oxidative stress and mediating cellular detoxification (Flens et al. 1996). MRP1-mediated resistance in cancer is also conferred through the intercellular transfer of the protein in recipient drug sensitive cells (Lu et al. 2013). MRP1 also has a wide range of substrate drugs, including anthracyclines (doxorubicin), vinca alkaloids (vincristine), topoisomerase inhibitors (topotecan) and methotrexate (Cole 2014; Yin and Zhang 2011). It is shown to mediate drug resistance in many cancers including haematological cancers, prostate cancer and neuroblastoma (Cole 2014; Yin and Zhang 2011).

Breast Cancer Resistance Protein

BCRP is a 72 kDa "half" ABC transporter (Fig. 9.1c), which functions upon the dimerization of two monomers (Doyle and Ross 2003). It is localised in many organs including the brain, lungs, intestines, kidney, and placenta (Doyle and Ross 2003). It is expressed at pharmacological barriers also transporting xenobiotics and toxic metabolites across cell membranes preventing their intracellular uptake (Gutmann et al. 2005). BCRP transports both cationic and anionic compounds and among its drug substrates are the anthracyclines (mitoxantrone), topoisomerase inhibitors (topotecan and irinotecan) and antimetabolites (methotrexate) (Chen et al. 2003; Doyle and Ross 2003). In addition to its expression in breast cancer, BCRP is also overexpressed and causes MDR in lung, colon and gastric cancers (Kawabata et al. 2001; Ross et al. 1999).

Pharmacological Inhibition of ABC Transporters as a Strategy to Overcome MDR

Considering the role of ABC transporters in MDR, a valid strategy to overcome MDR in cancer is the co-administration of pharmacological inhibitors (or competing substrates as modulators) with anticancer drugs (Callaghan et al. 2014). As P-gp was the most thoroughly characterized member of the ABC transporter superfamily, as well as due to its prominent role in conferring MDR, most efforts focussed on inhibiting the function of P-gp (Szakacs et al. 2006; Callaghan et al. 2014). The first molecule identified to modulate the function of P-gp was verapamil, an L-type calcium channel blocker (Tsuruo et al. 1983). Although this drug was shown to successfully circumvent MDR *in vitro*, clinical trials revealed serious cardiac side effects (Callaghan et al. 2014). In the following three decades, three generations of

P-gp modulators were developed and tested in clinical trials, however success was limited due to various reasons, including poor specificity for P-gp, low potency and high toxicity (Callaghan et al. 2014). The third generation of P-gp inhibitors, including tariquidar, also caused adverse drug reactions when tested in combination with anticancer drugs, often due to an overlapping specificity of substrate between P-gp and the cytochrome P450. This resulted in unpredictable pharmacokinetic issues requiring anticancer dose reductions (Callaghan et al. 2014; Jaramillo et al. 2018). Although novel promising P-gp inhibitors are currently under evaluation, such as compounds targeting the protein's NBDs (Nanayakkara et al. 2018), the presence of "physiological" P-gp in tissues other than in cancer limits their specificity. For this reason, alternative approaches to overcome these MDR proteins are required (Callaghan et al. 2014; Szakacs et al. 2006).

Extracellular Vesicles in the Dissemination of Cancer Drug Resistance

EVs comprise discrete packages of cellular information which, after being released by a donor cell, are taken up by recipient cells, influencing the recipient cell's proteome, metabolism and gene expression (Raposo and Stoorvogel 2013). By the same principle, EVs play a role as central effectors of intercellular communication between cancer cells and between cancer and the tumour microenvironment (TME) (Rak 2013). Several studies have reported EV-mediated influences on every step of the tumorigenic process, including cell proliferation, epithelial-mesenchymal transition (EMT), angiogenesis, metastasis and immune evasion (Gopal et al. 2017; Gong et al. 2015). Substantial evidence also shows that EVs are widely recognised as chemoresistance-conferring effectors in a wide range of cancers including breast (Xu et al. 2016; Jaiswal et al. 2012; Jaiswal et al. 2013), colorectal (Hu et al. 2019), pancreatic (Muralidharan-Chari et al. 2016), ovarian (Samuel et al. 2018), lung cancer (Cohen et al. 2018), melanoma (Vella et al. 2017), glioblastoma (Shao et al. 2015), osteosarcoma (Torreggiani et al. 2016) and haematological malignancies such as multiple myeloma (Rajeev Krishnan et al. 2020), acute lymphoblastic leukaemia (Bebawy et al. 2009; Lu et al. 2013) and acute myeloid leukaemia (Bouvy et al. 2017). The EV-mediated transfer of MDR between cancer cells is multimodal, and the main mechanism by which it occurs involves the transfer of bioactive cargo (including proteins, nucleic acids and lipids) from drug resistant "donor" cells to drug sensitive "recipient" cancer cells (Fig. 9.2) (Bebawy et al. 2009; Jaiswal et al. 2012; Lu et al. 2016). It often involves activation of signalling pathways or alteration of gene expression (Gong et al. 2014; Jaiswal et al. 2012). In this section, we detail the mechanisms by which EVs mediate the dissemination of the cancer drug resistance trait.

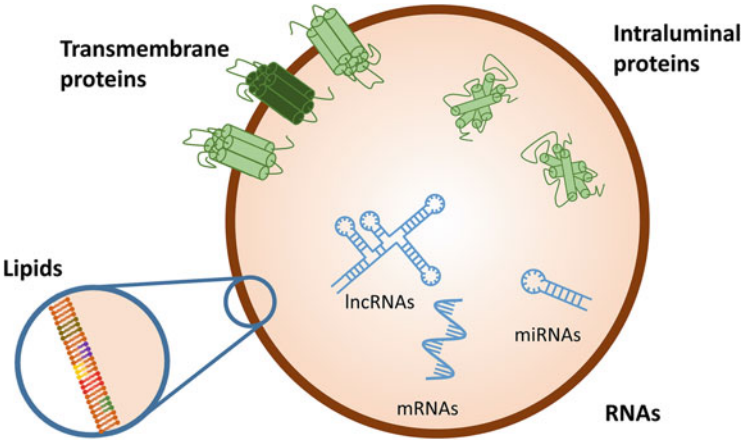

Fig. 9.2 Chemoresistance-conferring extracellular vesicle cargo. Different components of the EV cargo mediate the transfer of chemoresistance from drug resistant donor cells to drug sensitive recipient cells. Transmembrane proteins include functional ABC transporters, such as P-gp, which are expressed on the surface of recipients cells upon uptake. These transporters also contribute to chemoresistance by accumulating chemotherapeutic drugs inside the EVs lumen. Other transporters, such as the calcium channel TrpC5, do not affect drug accumulation but are involved in MDR through the calcium-dependent activation of signalling pathways leading to the expression of P-gp in recipient cells. Examples of intraluminal proteins include anti-apoptotic proteins, such as survivin, and other proteins such as pGSN and phosphorylated STAT-3. These proteins generally induce chemoresistance in recipient cells by activating survival signalling pathways. Numerous classes of nucleic acids, including mRNAs, miRNAs and lncRNAs, induce chemoresistance through different mechanisms, including the direct or indirect upregulation of the expression of resistance proteins as well as the modulation of signalling pathways involved in cell survival, replication, apoptosis and DNA repair. These RNAs are responsible for the "re-templating" of the transcriptional landscape of the recipient cells so as to reflect the donor cell resistance trait. Finally, EVs derived from drug resistant tumour cells have a distinctive lipid content which can be involved in the induction of resistance upon transfer to sensitive cells. *EVs* Extracellular Vesicles; *ABC* ATP-Binding Cassette; *P-gp* P-glycoprotein; *TrpC5* Transient receptor potential Channel 5; *pGSN* Plasma Gelsolin; *STAT-3* Signal Transducer and Activator of Transcription 3; *mRNA* messenger RNA; *miRNA* microRNA; *lncRNA* long non coding RNA

Transfer of Functional ABC Transporter Proteins

In a seminal study, Levchenko and colleagues showed nongenetic acquisition of MDR through the intercellular transfer of P-gp from P-gp$^+$ donor cells to P-gp$^-$ drug sensitive cells in an *in vitro* co-culture model. The authors proposed the transfer of P-gp to be a result of direct cell-cell contact (Levchenko et al. 2005). Bebawy and coworkers showed for the first time that MDR can be acquired through the intercellular transfer of P-gp and MRP1 by extracellular vesicles (Bebawy et al. 2009; Lu et al. 2013). This was followed by several *in vitro* studies which demonstrated that different classes of EVs, including small EVs (exosomes), mediated horizontal transfer of MDR proteins into recipient cells, conferring MDR (Corcoran et al. 2012; Zhang et al. 2014; Lv et al. 2014; Jaiswal et al. 2013; Kharaziha et al.

2015; Pasquier et al. 2012; Wang et al. 2016). Despite the complex tumour microenvironment conditions, some studies could recapitulate the *in vitro* horizontal transfer of drug resistance to be sustainable *in vivo*. In a breast xenograft model, a single subcutaneous injection of microparticles (MPs) was shown to confer P-gp transfer within the tumour core, with expression being stable for at least 2 weeks thereafter in the absence of any selective pressure (Jaiswal et al. 2013). There are many different mechanisms by which EV cargo is internalised by the recipient cells. These mechanisms include different endocytic pathways as well as direct membrane fusion, and it is likely that internalisation occurs via different pathways simultaneously (Mulcahy et al. 2014). An alternative parallel pathway by which EV-associated ABC transporters mediate MDR involving active and passive vesicular drug sequestration was also identified (Gong et al. 2013). The vesicles shed from MDR cells were found to contain P-gp in both an inside out and a rightside out orientation. The inside out orientation with exposed catalytic domains meant that, in the presence of ATP, active drug sequestration into the vesicles could occur resulting in drug sequestration and a reduction of the available free drug concentration available to cancer cells (Gong et al. 2013). The mechanisms governing protein orientation on EVs remain unknown (Mulcahy et al. 2014).

EVs also display a certain tissue selectivity in transferring resistance proteins to recipient cells. In particular, even though EVs released from drug resistant breast cancer cells were found to bind to both malignant and non-malignant recipient cells, they were selective in the transfer of P-gp to malignant cells only (Jaiswal et al. 2013). Contrary to this, leukemic cell-derived EVs transferred MDR proteins to both non-malignant and malignant recipient cells (Jaiswal et al. 2013). The basis of this differential selectivity was proposed to occur through CD44 (Jaiswal et al. 2013). Moreover, the transfer of P-gp via EVs is a regulated process and involves the FERM domain binding proteins Ezrin, Radixin, Moesin (ERM proteins) and CD44, which are colocalised with P-gp within the vesicular cargo and are proposed to stabilise P-gp *en route* to the recipient cell (Pokharel et al. 2016; Pokharel et al. 2014). In another report, again MPs derived from drug-resistant leukemic cells displayed no cell type selectivity and transferred P-gp to sensitive breast and lung cancer cells, together with specific miRNAs and apoptosis inhibitors, supporting earlier findings (de Souza et al. 2015). Others showed the selective uptake of exosomes released by leukemic cells by endothelial and pancreatic cells was dependent on the expression of specific exosomal tetraspanin complexes (Rana et al. 2012). These findings collectively indicate that EV binding and cargo uptake is a complex process which involves receptor-ligand signalling. Besides P-gp, also the EV-mediated transfer of functional MRP1 has been shown to be involved in conferring MDR to drug sensitive recipient leukaemia cells (Lu et al. 2013; Jaiswal et al. 2013; Bouvy et al. 2017).

The transfer of functional MDR proteins is only one of many mechanisms by which EVs mediate MDR and, more broadly, influence cancer cell survival and spread. Our group characterized the role of MPs in these processes. We demonstrated that MPs play a fundamental role in regulating the functional redundancy that exists amongst MDR transporters (Lu et al. 2017), and that MPs shed by MDR cells alter the biomechanical properties of recipient cells (Pokharel et al. 2016) and

enhance their migration and invasion capacity (Gong et al. 2014). Finally, we showed that these MPs contribute to cancer immune evasion by inducing the functional incapacity of macrophages and stimulating their engulfment by MDR cancer cells (Jaiswal et al. 2017). All these examples will be discussed in greater detail in the relative subsections of this chapter.

Transfer of Other Proteins

Despite the predominant role of ABC transporters, particularly P-gp and MRP1, in the intercellular transfer of chemoresistance, other proteins have been implicated in drug resistance through EV-mediated pathways. The calcium channel transient receptor potential channel 5 (TrpC5) was shown to be actively transferred from adriamycin-resistant breast cancer cells to endothelial (Dong et al. 2014) and drug sensitive breast cancer cells (Ma et al. 2014), and inducing P-gp expression in recipient cells through calcium-dependent activation of the transcription factor NFATc3 (nuclear factor of activated T cells isoform c3) (Dong et al. 2014). In this context, Ma and colleagues also showed the presence of TrpC5-positive EVs in mouse and human breast cancer samples, as well as in plasma samples of breast cancer patients with prior exposure to chemotherapeutics (Ma et al. 2014). Both these findings highlight the potential role of TrpC5-positive EVs in the transfer of drug resistance (Ma et al. 2014). Further to this, in the same report, Ma and colleagues showed that TrpC5 also induced vesiculation. This may contribute to the dissemination of the chemoresistant phenotype by virtue of an increased overall number of secreted EVs as well as through the accumulation of anticancer drugs inside the EVs, thus reducing the intracellular concentration of drugs to sublethal levels (Ma et al. 2014). This finding is consistent with earlier reports by Gong et al., where EVs released from MDR breast cancer cells were shown to accumulate cytotoxic drugs through both passive and active mechanisms, the latter depending on the presence of P-gp in an inside out orientation on the surface of EVs (Gong et al. 2013).

Survivin is an anti-apoptotic protein which was found to be involved in the EV-mediated transfer of drug resistance. In an *in vitro* study, triple negative breast cancer cells resistant to the drug paclitaxel were found to secrete EVs enriched with survivin when treated with paclitaxel (Kreger et al. 2016). The survivin-bearing EVs conferred paclitaxel resistance to fibroblasts and drug sensitive breast cancer cells upon transfer (Kreger et al. 2016). In another study survivin, together with other anti-apoptotic proteins and P-gp, were transferred via MPs from drug resistant chronic myeloid leukaemia cells to drug sensitive cells, conferring a multifactorial resistance phenotype (de Souza et al. 2015). Furthermore, survivin was shown to be associated with P-gp in late phase chronic myeloid leukaemia (Reis et al. 2011), and its overexpression in many cancers correlates with tumour progression and drug resistance (Cheung et al. 2013).

In a recent study, plasma gelsolin (pGSN), an actin scavenger protein, was shown to be transferred through exosomes from drug resistant to drug senstive ovarian cancer cells, inducing resistance to cisplatin (Asare-Werehene et al. 2020). Through gain- and loss-of-function studies, the authors showed that pGSN regulates the α5β1 integrin-FAK-Akt-HIF1α signalling pathway, upregulating pGSN expression in an autocrine manner and conferring cisplatin resistance to sensitive cells in a paracrine manner (Asare-Werehene et al. 2020). Moreover, upregulation of pGSN in ovarian cancer patients correlated with poor overall survival and reduced relapse-free survival (Asare-Werehene et al. 2020).

Other proteins have been reported to confer EV-mediated chemoresistance. For example, in a recent study, the signal transducer and activator of transcription (STAT) protein, phosphorylated STAT-3, was shown to be enriched in exosomes derived from fluorouracil resistant colorectal cancer cells and to confer resistance to fluorouracil to sensitive cells (Zhang et al. 2019). Furthermore, the exosome-mediated transfer of the chloride intracellular channel 1 (CLIC1) from resistant cells was shown to contribute to vincristine resistance in drug sensitive gastric cancer cells following EV transfer (Zhao et al. 2019). Considering the mechanistic role of these vesicular proteins in the transfer of drug resistance, these studies demonstrate the potential EVs have as biomarkers for monitoring response to chemotherapy.

Transfer of RNA

The EV cargo contains a variety of nucleic acids, such as functional mRNA, miRNAs and lncRNAs, which can be transferred to sensitive recipient cells and confer drug resistance traits (Jaiswal et al. 2012; Jaiswal et al. 2012; Lu et al. 2016). These effectors induce drug resistance through different mechanisms, including the upregulation of the expression of the proteins responsible for the resistance phenotype (Xavier et al. 2020) and the regulation of the functional redundancy amongst members of the ABC superfamily of resistance proteins (Lu et al. 2017). The role of nucleic acids in sustaining expression of resistance proteins (Lu et al. 2017) explains observations whereby the expression of ABC transporters was sustained for long periods of time after EV-mediated transfer, well beyond the transporters' protein half-life (Jaiswal et al. 2013). In this subsection, we review the current knowledge on the role of EV-mediated RNA transfer in the intercellular transfer of drug resistance. Although we will describe three RNA species separately, the reader must keep in mind that, often, the resistance phenotype induced by EVs is complex and multifactorial, consisting of the combined effect of different classes of mediators, including other RNA species together with proteins (de Souza et al. 2015; Lu et al. 2013; Torreggiani et al. 2016; Lu et al. 2016; Yang et al. 2017). As an example of the complexity of this process, Bebawy and coworkers showed that the EV cargo was capable of "re-templating" the transcriptional landscape of recipient cells so as to reflect the donor cell resistance trait and that the dominance of such in the recipient

cells was dependent on mRNAs and miRNAs transferred by the MPs (Jaiswal et al. 2012; Jaiswal et al. 2012; Lu et al. 2013; Lu et al. 2017).

mRNA

Together with the direct transfer of functional resistance proteins, another obviously viable pathway is the transfer of functional messengers encoding the aforementioned proteins. The functionality, in terms of successful translation, of mRNAs transferred between cells through EVs has been demonstrated in different studies. Valadi and colleagues were the first to show that mouse exosomal mRNA was transferred to human mast cells resulting in the recipient cells expressing novel mouse proteins (Valadi et al. 2007). Although the authors proposed that the presence of mouse proteins was due to the translation of the transferred mRNAs, this study did not distinguish between the translation of the conferred transcripts and the transfer of endogenous mouse proteins (Valadi et al. 2007). In a successive study, Skog and colleagues successfully demonstrated the translation of a luciferase reporter mRNA contained within the EV cargo (Skog et al. 2008). In a more recent study, our lab first reported the translation of a human native mRNA, *ABCB1* (encoding for P-gp), in recipient human acute lymphoblastic leukaemia cells following intercellular transfer by MPs (Lu et al. 2016). In this study, surface shaving of MPs using proteinase K was used to distinguish between newly synthesized P-gp in the recipient cells and the P-gp protein transferred from donor cells (Lu et al. 2016). The EV-mediated transfer of both functional P-gp and its corresponding mRNA has also been shown in human osteosarcoma cells (Torreggiani et al. 2016) and in breast cancer cells (Jaiswal et al. 2012).

The mRNA encoding for ΔNp73, an isoform of the tumour suppressor protein p73 lacking the N-terminal transactivation domain, was shown to be selectively packaged in colon cancer cell-derived EVs, and its function as drug resistance promoter was confirmed in both *in vitro* and *in vivo* models (Soldevilla et al. 2014). In another study, the exosomal transfer of the DNA Methyl Transferase 1 (DNMT1) mRNA was shown to confer cisplatin resistance to ovarian cancer cells, both *in vitro* and *in vivo* (Cao et al. 2017). Finally, both the transcript encoding for Glutathione S-transferase P1 (GSTP1) and the functional GSTP1 protein were found to be transferred through EVs from adriamycin resistant to drug sensitive breast cancer cells, inducing GSTP1 expression and drug resistance in an EV dose-dependent manner (Yang et al. 2017).

miRNA

MicroRNAs (miRNAs) are small non-coding RNAs with important roles in the post-transcriptional regulation of gene expression in physiologic and pathologic contexts (Mills et al. 2019). The discovery that EVs, particularly those released from cancer

cells, contain miRNAs (Valadi et al. 2007), represented a paradigm shift in the understanding of miRNA biology (Mills et al. 2019). Among the different classes of RNA packaged in EVs, miRNAs are the most represented (Turchinovich et al. 2019). In the context of cancer, EV-associated miRNAs function as autocrine, paracrine and endocrine modulators of intercellular communication (Mills et al. 2019). Due to the fact that EV-associated miRNAs can be found in virtually all biological fluids, particularly blood (Garcia-Romero et al. 2018), and due to the fact that the miRNAs enclosed in vesicles are protected from RNAse degradation (Koga et al. 2011), this class of molecule is a promising source of cancer biomarkers (Mills et al. 2019). Similarly to other classes of EV cargo, miRNAs packaged into EVs can be transferred to sensitive recipient cancer cells and confer drug resistance through different mechanisms. These include the modulation of the expression of MDR-related proteins (Lu et al. 2017), the interference with the apoptotic homeostasis (Steinbichler et al. 2019) and the induction of dormancy in cancer cells (Steinbichler et al. 2019). Several studies have analysed the role of miRNAs in EV-induced drug resistance. Exosomal miRNA-221 and -222 shed by tamoxifen resistant breast cancer cells were reported to induce tamoxifen resistance in their sensitive counterparts through downregulation of p27 and the Estrogen Receptor alpha (ERα) (Wei et al. 2014). Similarly, exosomal miR-222 was found to induce adriamycin resistance in sensitive breast cancer cells (Yu et al. 2016). Together with miR-223, exosomal miR-222 was also found to be an important mediator of the communication between mesenchymal stem cells (MSCs) and breast cancer cells in the bone marrow (Bliss et al. 2016). In particular, breast cancer cells were found to prime MSCs to release exosomes loaded with a specific cargo of miRNAs, including miR-222/223, which in turn promoted a state of dormancy in the breast cancer cells, conferring resistance to chemotherapeutic treatments (Bliss et al. 2016). In another study, adriamycin and docetaxel-resistant breast cancer cells transferred miR-100, miR-222 and miR-30a to recipient cells inducing resistance to chemotherapeutic treatments (Chen et al. 2014). Exosomal miR-425-3p derived from cisplatin-resistant non-small cell lung cancer cells was shown to induce drug resistance in recipient cells by downregulating the expression of AKT1 and activating autophagy. In this study, the authors also demonstrated that the expression of miR-425-3p was induced by cisplatin through the activation of the transcription factor c-Myc (Ma et al. 2019). In another study, exosomal miR-96 has been shown to induce cisplatin resistance in lung cancer cells through downregulation of LIM-domain only protein 7 (LMO7) (Wu et al. 2017).

In the context of EV-associated miRNAs, our group demonstrated that drug resistant cells selectively package specific miRNAs in their MPs (Jaiswal et al. 2012). Upon transfer of the MPs to recipient cells, these miRNAs play important roles in "re-templating" the transcriptional landscape of recipient cells to regulate the acquisition of specific MDR traits as present in the donor cell (Jaiswal et al. 2012; Jaiswal et al. 2012; Lu et al. 2017; Lu et al. 2013). Specifically, we showed that MPs released from cells with a P-gp-dominant resistant profile were capable of re-templating a pre-existing MRP1-dominant profile in recipient cells, thereby downregulating the expression of MRP1 in favour of the overexpression of P-gp

(Lu et al. 2013; Jaiswal et al. 2012). A few candidate MP-transferred miRNAs were proposed to be involved in this pathway (Jaiswal et al. 2012; Jaiswal et al. 2012), including miR-27a and miR-451 in the upregulation of P-gp expression and a possible role for miR-326 in the downregulation of MRP1 expression (Jaiswal et al. 2012). In a successive study, the role of miR-326 in the cellular suppression of MRP1 expression was established, showing that the EV-mediated transfer of the ABCB1 transcript (encoding for P-gp), together with miR-326, facilitates the suppression of ABCC1 (encoding for MRP1) by miR-326 in recipient cells (Lu et al. 2017). This showed for the first time an additional mechanism for the regulation of miRNA suppressive function through the presence of non-target transcripts. (Lu et al. 2017). Furthermore, MPs released by both drug sensitive and drug resistant leukaemia cells carry the transcript encoding for the miRNA biogenesis enzymes Drosha, Dicer and Argonaute, implicating MPs as potential key intercellular regulators of miRNA biogenesis in recipient cancer cells (Jaiswal et al. 2012).

Besides the mechanism by which a drug resistant cell regulates resistance via EV-packaged miRNAs, another way through which EV-associated miRNAs can contribute to drug resistance is the selective packaging of miRNAs into EVs released from cancer cells. This mechanism was shown by Akao and colleagues, who showed that colorectal cancer cells released small EVs containing the tumour suppressive miR-34a and miR-145. This resulted in lower intracellular levels of these miRNAs and reduced cells' sensitivity to treatment with 5-fluorouracil (Akao et al. 2014).

Finally, some studies have comprehensively evaluated EV miRNA expression profiles in the context of chemoresistance (Chen et al. 2018; Zhong et al. 2016; Jaiswal et al. 2012). For example, our group used microarray profiling to explore the miRNA expression profiles of MPs and their donor and recipient cells from both leukaemia and breast cancer cells. In this study we demonstrated that several miRNA, including miR-1246, miR-1308, miR-1228*, miR-149*, miR-638 and miR-923, were selectively packaged into MPs from MDR cells and, upon transfer to drug sensitive cells, induced a miRNA expression profile which was similar to that of MDR donor cells (Jaiswal et al. 2012). This is consistent with studies showing the "re-templating" of recipient cells to reflect donor cell traits following MP-mediated transfer (Jaiswal et al. 2012). Chen and colleagues analysed differentially expressed exosomal miRNAs between adriamycin-resistant breast cancer cells and their sensitive counterparts, finding 309 miRNAs to be upregulated and 66 miRNAs to be downregulated in the adriamycin-resistant cell derived exosomes. Functional enrichment analysis showed that the miRNAs were associated with transcriptional misregulation pathways, MAPK, and Wnt signalling pathways in cancer (Chen et al. 2018).

lncRNA

Long noncoding RNAs (lncRNAs) represent an important class of regulatory RNA molecules which influence almost every stage of gene expression (Mercer and

Mattick 2013). Deregulation of lncRNAs is implicated in the pathogenesis of many cancers (Spizzo et al. 2012). Similarly to other classes of RNA, lncRNAs are selectively packaged in EVs and can be transferred from donor to recipient cells (Hinger et al. 2018). Recent studies demonstrated their role in the EV-mediated acquisition of chemoresistance. One study reported increased expression of the lncRNA linc-VLDLR in hepatocellular carcinoma cells exposed to doxorubicin, camptothecin and sorafenib. EVs derived from these cells transferred linc-VLDLR to sensitive cells and conferred resistance through increased expression of ABCG2 (encoding BCRP) (Takahashi et al. 2014). More recently, a very similar finding involving linc-VLDLR was described in the EV-mediated induction of resistance to adriamycin in oesophageal cancer cells (Chen et al. 2019). Similarly, the transfer of EV-associated linc-ROR to sensitive hepatocellular carcinoma cells was shown to induce resistant traits through the enhancement of TGFβ-induced stimulation of tumour-initiating cells (Takahashi et al. 2014). In renal cell carcinoma, the transfer of exosomal lncARSR to drug sensitive recipient cells induced sunitinib resistance by acting as a competing endogenous RNA (ceRNA) and binding miR-34/miR-449, thus facilitating the expression of AXL and c-MET (Qu et al. 2016). Furthermore, the lncRNA-small nucleolar RNA host gene 14 (SNHG14) was found to be enriched in exosomes released by trastuzumab-resistant breast cancer cells and, upon exosome uptake, induced resistance to trastuzumab in sensitive cells by targeting the Bcl-2-associated X protein (BAX) signalling pathway (Dong et al. 2018). A similar role in conferring exosome-mediated resistance to trastuzumab in HER2-positive breast cancer was also described for the lncRNA AGAP2 Antisense RNA 1 (AGAP2-AS1) (Zheng et al. 2019). In glioblastoma, the exosomal transfer of the lncRNA SBF2 antisense RNA 1 (SBF2-AS1) was shown to induce temozolomide resistance to sensitive cells by acting as a ceRNA for miR-151a-3p, resulting in enhanced repair of double strand breaks in temozolomide-treated cells (Zhang et al. 2019). In a similar fashion, two other lncRNAs transferred by exosomes, HOXA transcript at the distal tip (HOTTIP) and HNF1A antisense RNA 1 (HNF1A-AS1), were reported to induce resistance to cisplatin in gastric (Wang et al. 2019) and cervical cancer (Luo et al. 2019), respectively. These studies collectively demonstrate the great potential of lncRNAs as targets for the inhibition of chemoresistance in various cancers, as well as their potential role as biomarkers.

Transfer of Lipids

EVs are encapsulated by a phospholipid bilayer which is mainly composed of lipids such as ceramide, cholesterol, sphingomyelin, phosphoglycerides, glycosphingolipids, phosphatidylserine (PS), phosphatidylethanolamine, and sialic acid (Trajkovic et al. 2008). The study of the lipidic content of EVs is a relatively new and emerging field (Skotland et al. 2020). In the context of EV-mediated drug resistance, Jung and colleagues reported that a specific signature of EV-associated lipids was capable of differentiating gefitinib-resistant non-small cell lung cancer

cells from sensitive cells (Jung et al. 2015). The lipid ceramide plays an essential role in exosome biogenesis and cargo loading (Trajkovic et al. 2008; Janas et al. 2015), and its glycosylation has been shown to mediate MDR in breast cancer cells (Liu et al. 2001; Liu et al. 2000). Kong and colleagues showed that treatment of breast cancer cells with the farnesoid X receptor antagonist guggulsterone (gug) and the retinoid X receptor agonist bexarotene (bex) resulted in increased levels of ceramide, which in turn stimulated the secretion of exosomes containing the ABC transporter BCRP (Kong et al. 2015). Although the authors reported that the exosome-mediated secretion of BCRP resulted in a decreased drug resistance due to lower cellular levels of BCRP, the BCRP-loaded exosomes might function as shuttles mediating the transfer of BCRP to recipient cells, similarly to what has been observed for other EV-loaded ABC transporters. A more direct link between ceramide and EV-mediated MDR has been recently proposed by Faict and colleagues, who demonstrated that the treatment of multiple myeloma cells with melphalan or bortezomib induced the upregulation of acid sphingomyelinase (ASM) in both the cells and their exosomes. ASM catalyses the conversion of sphingomyelins to ceramides, resulting in increased ceramide levels (Faict et al. 2019). Furthermore, the authors showed that the ASM-loaded exosomes could transfer MDR to chemosensitive cells, suggesting a chemoprotective role for vesicle-associated ASM (Faict et al. 2019).

Beyond the Transfer of Drug Resistance: EVs Mediate a Complex Survival Phenotype

In the previous section we discussed the fundamental role of EVs as mediators of the drug resistant trait and the many different modalities by which EVs accomplish this function. The studies discussed so far collectively describe EVs as discrete "packages", transporting mediators which confer resistance in drug sensitive recipient cell populations. As mentioned above, the role of EVs in cancer is not limited to this task. In fact, it is well known that cancer cells can exploit the physiological EV signalling network in order to support tumour progression and spread. EVs play important roles across every cancer hallmark, including the promotion of cell proliferation, angiogenesis, metastasis, the escape from the immune system and the modulation of the tumour microenvironment (Xavier et al. 2020; Gong et al. 2015) (Fig. 9.3). All these "cancer-supportive" functions of EVs may directly or indirectly influence the occurrence of chemotherapeutic treatment failure. In this section, we will discuss further roles of EVs in supporting cancer survival beyond simply the transfer of drug resistance.

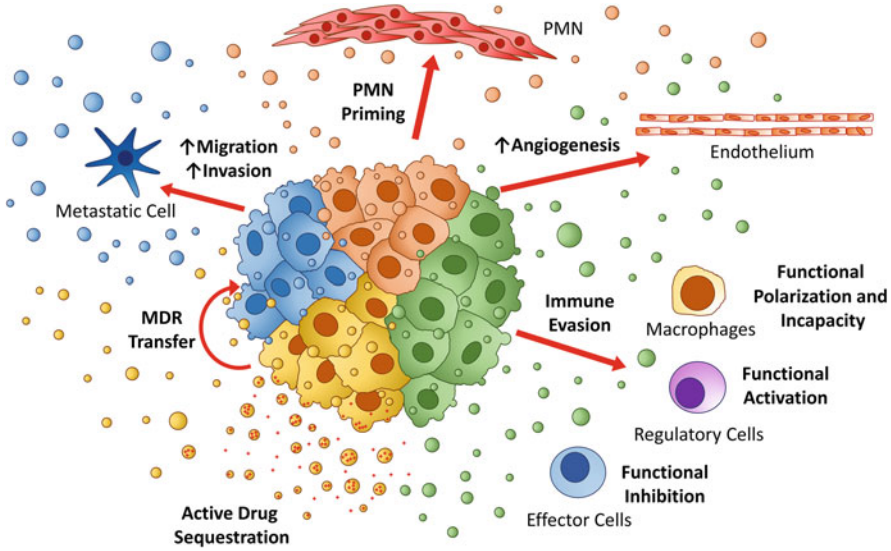

Fig. 9.3 EVs mediate a complex survival phenotype. Tumour cells release EVs that are heterogeneous in terms of size and components of their cargo. Besides their role in transferring MDR to drug sensitive cells, tumour-derived EVs play important roles across every cancer hallmark with many cancer-supportive effects exerted through their modulation of the tumour microenvironment. EVs can contribute to the detoxification of anticancer drugs through the passive and active sequestration of cytotoxic molecules from the extracellular space into the vesicle lumen. In this context, cancer cells are also capable of using vesiculation as a means of reducing intracellular drug levels. Furthermore, EVs stimulate the metastatic potential of cancer cells by enhancing their migratory and invasive capacity. Part of this effect is also achieved through the modulation of the recipient cells' biomechanical properties. In the context of metastasis, EVs are also involved in the preparation and priming of the premetastatic niche (PMN). Finally, EVs are known to induce angiogenesis and to be fundamental mediators of tumour evasion through regulation of the immune response. This "immune-suppressive" effect is primarily exerted through three mechanisms: the activation and stimulation of cells with immunosuppressive activity, such as regulatory T and B cells and myeloid-derived suppressor cells (functional activation); the inhibition of antitumour effector cells such as NK lymphocytes and cytotoxic T lymphocytes (functional inhibition); and the polarization of macrophages towards an anti-inflammatory, M2 phenotype (functional polarization). MDR tumour-derived EVs also induce functional incapacity of macrophages and make them susceptible to engulfment by cancer cells. *EVs* Extracellular Vesicles; *MDR* Multidrug Resistance; *PMN* Pre-Metastatic Niche; *NK* Natural Killer

Sequestration of Cytotoxic Drugs and EV-Mediated Drug Efflux

Besides their action as intercellular messengers, EVs released by cancer cells in the extracellular space have been shown to contribute to drug resistance by accumulating anticancer drugs in their lumen, therefore reducing the free drug concentration. We showed that MPs released from MDR breast cancer cells could actively and passively sequester cytotoxic drugs providing a parallel survival pathway. Passive

sequestration occurred through binding to phospholipid and nucleic acid constituents within the vesicle cargo. Active drug sequestration occurred through the presence of vesicles with an inside out topographical form of P-gp with exposed catalytic domains. In the presence of ATP, this form thus can actively pump drug within the intravesicular compartment reducing the amount of free drug available to interact with cells (Gong et al. 2013).

With regards to therapeutic antibodies, EVs can reduce their extracellular concentration, therefore their availability to cancer cells, by exposing bait targets on their surface. For example, exosomes expressing the CD20 receptor have been shown to bind to therapeutic anti-CD20 antibodies, protecting the target lymphoma cells from the antibody-mediated cytotoxicity (Aung et al. 2011). Similarly, exosomes released by HER2-overexpressing breast cancer cells were found to contain HER2 and bind to trastuzumab, an anti-HER2 therapeutic antibody, thereby reducing efficacy (Ciravolo et al. 2012). Finally, tumour exosomes expressing the epithelial cell adhesion molecule (EpCAM) were shown to sequester the EpCAM-specific antibody C215 limiting its availability (Battke et al. 2011).

Another mechanism by which EVs accumulate cytotoxic drugs, contributing to drug resistance, is by sequestering drug from within the cytoplasm of cancer cells, therefore reducing free drug intracellular concentration. For example, breast cancer cells were shown to release BCRP-rich EVs which had sequestered mitoxanthrone, topotecan, imidazoacridinones and methotrexate, (Ifergan et al. 2005; Goler-Baron and Assaraf 2012; Goler-Baron and Assaraf 2011) within the cell. Similarly, pancreatic cancer cell-derived MPs were found to accumulate different cytotoxic drugs following treatment (Muralidharan-Chari et al. 2016). The sequestration of drugs into EVs appears to be an important mechanism of drug detoxification. This interestingly is consistent with the fact that cancer cells which secrete more EVs appear to achieve greater levels of drug resistance (Xavier et al. 2020).

EV-Mediated Stimulation of Cancer Cell Invasion and Metastatic Capacity

Numerous studies have shown that EVs are involved in cancer metastasis by stimulating cancer cell migration and invasion. For example, EVs released by mesenchymal-like prostate cancer cells were shown to promote mesenchymal features in recipient epithelial-like prostate cancer cells, enhancing their migratory and invasive capacity (El-Sayed et al. 2017). In breast cancer, EVs containing Caveolin-1 were found to increase the migratory and invasive capacity of recipient cells (Campos et al. 2018). Further, exosomes released by highly invasive hepatocellular carcinoma cells were found to induce EMT through the TGF-β/Smad pathway and to promote invasion and migration of recipient cells, also promoting lung metastasis in a hepatocellular carcinoma xenograft model (Qu et al. 2019). In another study, the exosome-mediated transfer of CXC chemokine recepter-4 (CXCR4) from highly

metastatic mouse hepatocellular carcinoma cells was shown to enhance migratory and invasive capacities of cells with low metastatic potential (Li et al. 2018). Our group proposed that MPs could represent a conduit between MDR and metastasis, as we showed that the MP-mediated acquisition of MDR in breast cancer cells was associated with an enhanced migration and invasion capacity of recipient cells due to the MP-induced downregulation of miR-503 expression and upregulation of Proline rich tyrosine kinase-2 (PYK-2) expression (Gong et al. 2014). Tissue rigidity or stiffness is a biomechanical property that plays a key role in cell function such as adherence, motility and invasion, therefore contributing to the metastatic capacity of cancer cells (Cross et al. 2008; Swaminathan et al. 2011). Our group investigated the biomechanical properties of tissues with MP-mediated acquired MDR and showed that the transfer of MPs from MDR cells to drug sensitive breast cancer cells caused an increase in biomechanical stiffness of recipient cells, likely due to the transfer of P-gp and CD44 (Pokharel et al. 2016). In doing so, we described a role for EVs in the regulation of the biomechanical properties of malignant tissues, with potential implication on their metastatic potential (Pokharel et al. 2016).

Besides the activation of signalling pathways pertaining to EMT and cells' migrating/invasive potential, EVs have also been described as being direct mediators of tumour cell invasion due to the presence of the metalloproteinases MMP-2 and MMP-9, which are involved in the degradation of the extracellular matrix, in the cargo of EVs shed by ovarian and cancer cells (Dolo et al. 1999; Ginestra et al. 1998; Graves et al. 2004). More recently, nasopharyngeal carcinoma cells cultured in hypoxic conditions were shown to release exosomes containing MMP-13, which induced EMT in recipient normoxic cells (Shan et al. 2018). Another way through which EVs can promote metastasis is by inducing the remodelling and preparation of pre-metastatic niches. For example, in a recent study, Deep and colleagues showed that the injection of exosomes released by hypoxic prostate cancer cells in mice stimulated the expression and activity of MMPs in selective putative pre-metastatic niche organs (Deep et al. 2020).

EV-Induced Evasion from the Immune System

Physiologically, EVs are important mediators of the antigen-dependent and independent cross-talk and interaction between immune cells occurring during the immune response (Théry et al. 2009). In order to support their growth and development, tumour cells are known to adopt numerous strategies to manipulate their surrounding microenvironment. One of these strategies involves the modulation and evasion from the antitumor immune response (Vinay et al. 2015), and this can be one of the causes of cancer immune treatment failure (Sambi et al. 2019). It has been widely demonstrated that tumour-derived EVs play a key role in promoting tumour-mediated immune suppression by interacting with the immune system at both the innate and the adaptive level (Raimondo et al. 2020). There are three general ways by which tumour-derived EVs act on the components of the immune system:

"functional activation", which includes the stimulation of the expansion of cells with an immunosuppressive activity such as myeloid-derived suppressor cells (MDSCs) and regulatory T and B cells; "functional inhibition", which includes the inhibition of Natural Killers T cells and the induction of apoptosis in antitumour effector T cells; and "functional polarization", consisting in the induction of the polarization of macrophages toward an anti-inflammatory (M2) phenotype (Raimondo et al. 2020). An in-depth discussion of the numerous reports describing the mechanisms by which tumour-derived EVs aid cancer cells in escaping immune surveillance is beyond the scope of this chapter, and the topic has been recently reviewed (Raimondo et al. 2020; Benito-Martin et al. 2015). In the context of drug resistance, a recent study demonstrated that tumour-derived exosomes carry functional Programmed death-ligand 1 (PD-L1) and are able to present PD-L1 to the surface of T cells, suppressing their activation *in vitro* and *in vivo* and promoting tumour progression across different tumour types (Poggio et al. 2019). In this study, the authors showed that exosomal PD-L1 contributes to resistance to anti-PD-1/PD-L1 blockade therapy, proposing that PD-L1 exposure on the surface of exosomes makes it less targetable by therapeutic antibodies or that the high levels of exosomal PD-L1 make the therapeutic dose of anti-PD-L1 antibody not sufficient to achieve an effect (Poggio et al. 2019). Similarly, more recently Timaner and colleagues showed that breast cancer cells exposed to radiation release MPs loaded with various immunomodulators, including PD-L1, which inhibited cytotoxic T lymphocyte activity in vitro and in vivo, thus promoting tumour escape from the immune system, in a way that was partially dependent on PD-L1 (Timaner et al. 2020). These studies suggest that the presence of EV-associated PD-L1 could be a promising biomarker of immune therapy unresponsiveness.

Our group discovered a novel pathway by which MDR tumour-derived EVs contribute to cancer immune evasion through the functional incapacity and engulfment of macrophages (Jaiswal et al. 2017). In this study, we demonstrated that MPs shed by MDR breast cancer cells bind to THP-1 macrophages, modulating the release of pro-inflammatory cytokines and impairing macrophage functional characteristics such as phagocytosis and chemotaxis (Jaiswal et al. 2017). Further, we showed that hyaluronic acid is selectively packaged in MPs shed from MDR cells and that it induces macrophage aggregation and loss of surface adhesion through the activation of CD44 (Jaiswal et al. 2017). Finally, we showed that MDR cells, unlike drug sensitive cells, were able to engulf macrophages *in vitro* and that this ability was acquired by drug sensitive cells upon uptake of MPs shed from MDR cells (Jaiswal et al. 2017). This study showed for the first time that, besides their role as mediators of the transfer of the MDR trait, MPs serve as conduits in a parallel pathway supporting the cellular survival of MDR cells through immune evasion.

EVs as a Therapeutic Target in the Treatment of Multidrug Resistance

Considering their central role as effectors and mediators of the acquisition and spread of deleterious cancer traits, particularly chemoresistance, EVs represent a promising pharmacological target to be exploited in circumventing MDR. Two general strategies are mainly considered in circumventing EV-mediated MDR. The first one consists in the modulation of EV biogenesis with the aim of blocking EV production. The second strategy is to specifically target tumour-derived EVs by virtue of the specific cargo molecules they contain (Sharma 2017; Maacha et al. 2019). Both strategies have been pursued in various studies.

In order to pursue the first strategy, knowledge of the cellular processes and machinery involved in the biogenesis of EVs is necessary. With regards to MPs, cellular activation and consequent increase in intracellular calcium seem to be the most common initiating stimuli to their biogenesis and release (Taylor and Bebawy 2019). Our group showed that, in breast cancer cells, MP biogenesis at rest is predominantly dependent on the calcium-mediated activation of calpain whilst, in non-malignant cells, basal levels were mediated by another pathway (Taylor et al. 2017). This evidences the potential for a tumour-selective strategy. Furthermore, we demonstrated the efficacy of modulating calcium channels in inhibiting MP secretion using a range of molecules such as calpain inhibitors, inhibitors of the enzyme Rho-associated, coiled-coil containing protein kinase (ROCK) and the vitamin B5 derivative pantethine on MP shedding (Roseblade et al. 2015). Other groups also obtained reduced MP secretion by targeting signalling molecules such as extracellular signal-regulated kinase (ERK) (Muralidharan-Chari et al. 2016) and the enzyme peptidyl arginine deiminase, which is highly expressed in cancer (Kholia et al. 2015). With regards to exosomes, they are generated through the inward budding of late endosomes which results in the formation of intraluminal vesicles within multivesicular bodies. Numerous mediators have been described as being involved in this process, including components of the endosomal sorting complex required for transport (ESCRT) and sphingomyelinases, which convert sphingomyelins into ceramides (McAndrews and Kalluri 2019; Faict et al. 2019). Blocking exosome biogenesis has been proposed in many studies (Sharma 2017). In a recent study, a drug repurposing strategy was used to identify novel putative inhibitors of exosome biogenesis (Datta et al. 2018). Federici and colleagues described the effect of proton pump inhibitors (PPI) on both cisplatin uptake and exosomes release *in vitro* and *in vivo*, showing that treatment with a PPI decreases the release of exosomes loaded with cisplatin and increases tumour cell sensitivity to cisplatin (Federici et al. 2014). In other studies, blocking exosome biogenesis with indomethacin was shown to restore the therapeutic effects of the CD20-targeted antibody rituximab (Aung et al. 2011) and the intracellular accumulation of doxorubicin (Koch et al. 2016) in B cell lymphoma, and the inhibition of acid sphingomyelinase with amitriptyline was found to restore the sensitivity of multiple myeloma cells to melphalan and bortezomib (Faict et al. 2019). Furthermore,

cannabidiol has been recently found to inhibit the production of both exosomes and MPs in prostate, hepatocellular and breast cancer cells (Kosgodage et al. 2018).

The second main strategy, consisting in exploiting the presence of specific cargo components to selectively target tumour-derived EVs, seems to also be a viable approach. For example, the extracorporeal removal of HER2-positive exosomes through plasmapheresis has been proposed as an adjuvant therapy in breast cancer (Marleau et al. 2012), while the selective photodestruction of tumour-derived EVs accumulating photosensitive chemotherapeutic drugs was shown to overcome MDR in breast cancer cells through the generation of reactive oxygen species, causing severe damage to EVs and the cells from which they are released (Goler-Baron and Assaraf 2012).

A possible third, and indirect, strategy to overcome EV-mediated MDR consists in targeting specific cellular signalling pathways activated in recipient cells upon EV transfer. For example, knowing that EV-transferred lncARSR induces resistance to sunitinib through the activation of AXL and c-MET, the use of c-MET and AXL inhibitors in association with sunitinib has been proposed in renal cell carcinoma (Qu et al. 2016).

Although the strategies discussed are all very promising, further work is currently needed to identify compounds capable of fulfilling the promise of selectively targeting EV-mediated MDR.

EVs as Biomarkers of Multidrug Resistance

In the studies discussed previously we have seen how particular components of the cargo of tumour-derived EVs drive their numerous effector functions in cancer. Generally, these components also allow for the detection and analysis of tumour-derived EVs. EV cargo can be considered as "molecular fingerprints" of the cells from which they are released (Torrano et al. 2016), and due to their presence in large quantities in bodily fluids (Garcia-Romero et al. 2018), EVs hold tremendous potential as a source of biomarkers in the expanding field of liquid biopsies (De Rubis et al. 2019). In the context of MDR and more widely of resistance to anticancer treatment, generally the same EV-associated molecules responsible for the resistance trait are exploitable as biomarkers of MDR in the monitoring of treatment response and cancer prognosis. For example, in breast cancer patients, high levels of circulating EVs containing TrpC5 were found to correlate with stable or progressive disease following chemotherapy (Ma et al. 2014), and the levels of exosomal pGSN have been suggested as a potential prognostic biomarker of drug resistance in ovarian cancer (Asare-Werehene et al. 2020). In lung cancer, the circulating levels of exosomes containing the miRNA miR-425-3p were found to correlate with resistance to platinum-based therapy (Ma et al. 2019), while the levels of exosomal miR-96 correlated with tumour grade and lymph node involvement (Wu et al. 2017). Furthermore, exosomal lncRNA-SNHG14 has been shown to be a potential biomarker of trastuzumab resistance in HER2-positive breast cancer

patients (Dong et al. 2018), while the circulating levels of exosomal lncRNA-HOXA transcript at the distal tip (HOTTIP) correlated with poor response to cisplatin treatment in gastric cancer patients (Wang et al. 2019). Finally, a particular signature of up- and downregulated EV-associated lipids was shown to function as predictive biomarker of gefitinib resistance in nonsmall cell lung cancer, and the circulating levels of MPs expressing PD-L1 have been suggested as potential biomarker of therapeutic success for the combination of radiotherapy and immune checkpoint inhibition in breast cancer (Timaner et al. 2020).

We recently described a liquid biopsy for the detection and analysis of circulating EVs for the longitudinal, minimally-invasive and personalized detection of multidrug resistance and disease progression in multiple myeloma. In an earlier study, we detected the presence of elevated levels of circulating $CD138^+$ MPs in multiple myeloma patients and demonstrated that the levels of these correlated with disease burden and treatment response to therapy (Krishnan et al. 2016). In a more recent study, we identified a specific molecular signature on circulating MPs which correlated with the presence of multidrug resistance, disease progression and relapse (Rajeev Krishnan et al. 2020).

Numerous other studies have demonstrated the incredible potential of EVs as analytes in liquid biopsies for cancer diagnosis and real-time monitoring of tumour burden, tumour progression, treatment response and the prediction of drug resistance (Torrano et al. 2016; Vasconcelos et al. 2019). Despite this, the development of EV-based biomarker assays is still in its early days. Advances in standardized and reproducible protocols for EVs isolation and sensitive analytical techniques and protocols will undoubtedly further serve to support the utility of the approach (Vasconcelos et al. 2019).

Summary

EVs are membranous vesicles released by all cell types, both constitutively and following an inducing stimuli, which play a fundamental role in mediating intercellular communication in both physiological and pathological conditions. In cancer, EVs have been shown to support cancer cell growth and disease progression by mediating pathways associated with the cancer hallmarks, from cancer initiation to metastasis, invasion, treatment response and escape from the immune system. In the context of drug resistance, tumour-derived EVs act as fundamental mediators of drug resistance through their intercellular cargo, which comprises functional drug transporters and other resistance proteins as well as nucleic acids and lipids. Furthermore, EVs contribute beyond simply resistance to support a complex survival pathway in recipient cancer cells through active and passive drug sequestration, by supporting metastasis and invasive capacity, by facilitating biomechanical alterations in recipient cells and through immune evasion. Given the multifaceted role EVs have in mediating these deleterious cancer traits, EVs are important therapeutic targets and predictive biomarkers in the management of cancer.

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Chapter 10
Extracellular Vesicle Mediated Vascular Pathology in Glioblastoma

Cristiana Spinelli, Nadim Tawil, Lata Adnani, Janusz Rak, and Dongsic Choi

Abstract Glioblastoma (GBM) is an incurable, infiltrative high-grade brain tumour associated with dramatic vascular responses observed both locally (angiogenesis, vascular cooption, angiocrine effects, microthrombosis) and systemically (venous thromboembolism). GBM-associated vascular pathology is diagnostically relevant and constitutes a source of morbidity, mortality and progressive changes in tumour biology. Extracellular vesicles (EVs) have emerged as unique mediators of vascular effects in brain tumours acting as vehicles for intercellular transfer of oncoproteins (e.g. EGFRvIII), RNA, DNA and molecular effectors of angiogenesis and thrombosis. Vascular effects of GBM EVs are regulated by cancer cell genome, epigenome and microenvironment and differ between subtypes of cancer cells and stem cells. Understanding and targeting EV-driven vascular processes in GBM may offer new approaches to diagnose and treat these intractable tumours.

Keywords Brain tumours · Glioblastoma · Extracellular vesicles · Exosomes · Oncogenes · EGFR · Angiogenesis · Thrombosis · Cell communication · Endothelial cells · Coagulation system · Blood vessels

Introduction: Vascular Consequences of Malignant Transformation

In cancer, normal cellular functions are compromised by three types of transforming events, often acting sequentially or concurrently. First, over time cells with replicative potential may sustain mutational alterations to their genomes, which may predispose them to (but not necessarily trigger) overtly aberrant growth (Martincorena et al. 2015). Those changes often occur in molecular signalling

C. Spinelli · N. Tawil · L. Adnani · J. Rak (✉) · D. Choi (✉)
McGill University and the Research Institute of the McGill University Health Centre, QC, Canada
e-mail: janusz.rak@mcgill.ca; choi.dongsic@mail.mcgill.ca

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centres critical for a given cell type, and the affected genes act as either dominant oncogenes (e.g. RAS, BRAF, EGFR) or loose their regulatory function as tumour suppressors (e.g. TP53, PTEN, CDKN2A), or regulators of genomic stability (ATM, BRCA1, MSH2). Second, cells may undergo reprogramming of their epigenome due to either mutational or regulatory queues, whereby lineage-specific cellular programs are stably altered due to chromatin modifications, and a new phenotype (e.g. stemness) may emerge. Third, cell may receive regulatory queues from the microenvironment, including hypoxia, chronic inflammation, or tissue repair, which may trigger proliferative, migratory or more permanent phenotypic changes, as well as unmask or enhance transformed potential of already genetically altered cellular clones.

These cellular changes were traditionally viewed as cell-autonomous in nature with the remaining hallmarks of cancer resulting from 'unspecific' responses of the affected tissues. However, over the past three decades mounting evidence suggested a co-evolution of cancer cells and their stromal microenvironments, whereby the aforementioned oncogenic alterations would encompass the signalling apparatus involved in non-autonomous intercellular interactions, including elements of the cancer cell secretome. Historically some of the earliest observations in this regard included the notion that oncogenic RAS mutations directly deregulate the expression of vascular endothelial growth factor (VEGF), a potent inducer of vascular growth (Rak et al. 1995), while loss of TP53 expression precipitate down-regulation of the angiogenic inhibitor, thrombospondin 1 (TSP1)(Dameron et al. 1994).

Cellular transformation was also found to impact the ability of cancer cells to interact with the coagulation system (Yu et al. 2005) or to receive paracrine (angiocrine) signals from endothelial cells (Rak et al. 1994). Similarly, epigenetic changes responsible for stemness were linked to the expression of VEGF (Bao et al. 2006) and affected coagulant properties of cancer cells (Milsom et al. 2007), while angiogenic effects of hypoxia are often exacerbated in cells harbouring mutant oncogenes (Mazure et al. 1996). Although the nexus between cellular transformation and vascular responses in cancer is not yet entirely mapped, it likely encompasses a wide spectrum of vascular processes. These include, but are not restricted to, different forms of angiogenesis (sprouting, intussusception), local vaso-occlusive microthrombosis, systemic coagulopathy, alterations in platelet function and bone marrow-dependent regulation of blood vessel formation, the role of blood vessels in immunoregulation and multiple other events (De Palma et al. 2017; Rak 2009).

Diversity of vascular responses to progression of different cancers reflects, at least in part, the impact of underlying oncogenic alterations. For example, the risk of cancer-related thrombosis varies between cancer types (Hisada and Mackman 2017), as do patters of blood vessels and responsiveness to blood vessel targeting therapies (e.g. VEGF inhibitors) (Khan and Kerbel 2018). These differences and their mediators are of interest as blood vessel access (by definition) defines the transition between localized and metastatic (systemic) cancer (Folkman 2007). Blood vessels also play a central role in metabolic processes occurring in the tumour mass, formation of cancer stem cell niches, immune cell infiltration, inflammation,

morbidity and mortality due to cancer-associated thrombosis, drug delivery and radiation response, among many other aspects of cancer patho-biology and progression. Again, the specific contributions, mediators, mechanisms and therapeutic implications of these processes differ between cancer types. In the remainder of this article, these questions will be discussed mainly in the context of high-grade brain tumours, which combine the unique vascular properties of the central nervous system with those imposed by distinct molecular mechanisms of cellular transformation.

Brain Tumour Vasculature

Brain receives a disproportional fraction of the cardiac blood output and exhibits unique vascular properties reflecting metabolic demands and functional properties of the central nervous system (CNS) (D'Asti et al. 2014). Cerebral blood vessel networks emerge during embryogenesis, mainly though the process of sprouting angiogenesis, defined as an outgrowth of new blood capillaries from the pre-existing vessels (Carmeliet and Jain 2011). Fully developed brain vasculature contains a dense network of capillaries, the distinct property of which is the maintenance of the blood-brain barrier (BBB) function that protects CNS from blood-borne toxins. The BBB includes a combined effect of high endothelial expression of ABC transporters (molecular pumps), well-developed inter-endothelial junctions, and unique structure of the capillary wall composed of a continuous basement membrane, embedded pericytes and astrocytic foot processes separating blood from neurons. While BBB is often disrupted during neoplastic processes, the formation of dysfunctional poorly perfused tumour microcirculation creates a blood-tumour barrier (BTB), which along with the remaining BBB protects cancer cells throughout the brain from blood borne therapeutics (Arvanitis et al. 2020).

Cancers occurring in the brain profoundly impact the structure and function of the vasculature. The nature and degree of these changes differ depending on the tumour type and molecular subtype, as defined by oncogenic alterations and other factors. For example, in the subtype of pediatric medulloblastoma driven by oncogenic activation of the WNT signalling pathway blood vessel density is especially high and associated with hemorrhagic appearances, whereas these features are absent in other molecular subtypes of this tumor (SHH, Group 3 and Group 4), which inhibits drug delivery and worsens prognosis (Phoenix et al. 2016). Indeed, different subtypes of medulloblastoma express different profiles of angiogenic and coagulant factors depending on the nature of the underlying malignant transformation (D'Asti et al. 2014). In rare embryonal brain tumour with multilayered rosettes (ETMR), disease driven by C19MC microRNA cluster, vascular permeability and intratumoural hemorrhage dominate the histological appearances, while in other embryonal brain tumours capillary networks are more structured and less leaky (D'Asti et al. 2016). Adult patients with glioblastoma (GBM) are at high risk of peripheral venous thromboembolism (VTE) and exhibit prevalent vaso-occlusive thrombosis in

the tumour microcirculation, but these features are rare in low grade gliomas in pediatric and adult patients (Brat and Van Meir 2004; Tehrani et al. 2008; Unruh et al. 2016). Thus, given the exuberance and complexity of vascular changes occurring in GBM, we will focus our discussion on this class of brain tumours where a better understanding of vascular mechanisms and their mediators is badly needed, including the emerging role of extracellular vesicle (EV)-mediated intercellular communication pathways (Rak 2013).

Glioblastoma

GBM is the most common type of primary astrocytic brain tumor in adults, associated with severe morbidity, average 5-year survival of only 6.8% and median overall survival of only approximately 15 months (Wen et al. 2020). This dismal outlook persists in spite of an aggressive standard of care involving surgical resection, radiation and courses of chemotherapy involving mostly temozolomide (TMZ) (Stupp et al. 2005). Multiple attempts at alternative, targeted, anti-angiogenic and immunotherapy treatments remained futile and invariably failed to prevent the disease relapse and lethality (Wen et al. 2020). GBM affects mostly older adults, but GBM-like tumours (high grade gliomas) occur also in children and, while molecularly different, are also associated with poor prognosis, (Reifenberger et al. 2017). GBM-like tumours carrying oncogenic mutations of IDH1/2 genes have been recently excluded from GBM classification due to distinct biology, including low incidence of thrombosis and somewhat better prognosis (Unruh et al. 2016; Wen et al. 2020). In over 90% of cases, GBMs emerge without preceding low grade lesions leading to full blown infiltrative malignancy, likely in a matter of months (Chittiboina et al. 2012). While GBMs form distinctive lesions readily identified by MRI imaging, cancer cells are found throughout the brain, including the contralateral hemisphere due to invasion along neural track and vascular channels (Wen et al. 2020).

Despite morphological similarities among GBM lesions, these tumours exhibit stark molecular differences and are broadly classified as: proneural, classical and mesenchymal GBM subtypes (Wen et al. 2020). At the cellular level, each GBM consists of cellular subsets with features of all molecular subtypes (or intermediate), the varying proportions of which define tumour assignment to a particular subtype (Patel et al. 2014). GBM cells also assume gene expression profiles (states) dictated by underlying oncogenic alterations. For example, the preponderance of PDGFRA or CDK4 amplifications favour generation of either oligodendrocyte progenitor-like or neural progenitor-like cells, respectively, both enriched in proneural GBM. EGFR amplification/mutation favours formation of astrocytic-like cells, while NF1 loss and abundant stroma associate with mesenchymal-like cells, prevalent in classical and mesenchymal GBM subtypes, respectively (Neftel et al. 2019). Furthermore, GBMs contain distinct stem cell subpopulations (Singh et al. 2004) capable of generating

cell subsets committed to specific differentiation programs (roadmaps) (Couturier et al. 2020).

These features of GBM cell populations may define their diverse interactions with the vascular system. For example, molecular GBM subtypes at both tissue (Magnus et al. 2013) and single cell levels (Tawil et al. 2019) are associated with distinct repertoires of genes involved in vascular regulation. This may imply corresponding differences in mechanisms of vascular responses to GBM progression underneath the acknowledged generic roles of blood vessels in tumour growth, invasion, delivery of oxygen and nutrients and systemic comorbidities (Arvanitis et al. 2020; Perry 2012; Waziri 2010).

GBM progression is associated with a wide spectrum of vascular responses. Among those, the most extensively studied are: angiogenesis and endothelial hyper-proliferation, perivascular invasion and blood vessel cooption by cancer cells, formation of glioma stem cell (GSC) niches in proximity of tumour blood vessels, changes in the vascular structure (distension, tufting, regression, remodelling), trans-differentiation of GSCs to form endothelial-like cells and pericyte-like cells within the blood vessel wall as well as several others (Arvanitis et al. 2020; Cheng et al. 2013; Ricci-Vitiani et al. 2010; Zadeh et al. 2010; Zagzag et al. 2000).

The diversity of tumour-vascular interactions in GBM poses significant challenges in defining relevant actionable mechanisms. Since regions of hypoxia and pseudopalisading necrosis surrounding occluded tumour microvessels are hallmarks of GBM, the expression of hypoxia-inducible pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF) has long been at the centre of attention (Plate et al. 2012). The potent angiogenic activity of VEGF and experimental evidence for its role in GBM neovascularization, (Bao et al. 2006; Benjamin et al. 1999; Lu et al. 2012) along with promising results of early clinical trials, (Vredenburgh et al. 2007) supported the role of this factor GBM progression. However, several phase III clinical trials with drugs targeting the VEGF pathway failed to improve overall survival of GBM patients (Wen et al. 2020) suggesting that alternative regulators of vascular processes in GBM should be considered. In this regard EVs represent a unique and attractive category (Rak 2013).

Brain Tumour EVs: Heterogeneity and Function

GBM represents a paradigm for the role of EVs in forming a complex web of intercellular interactions (Abels and Breakefield 2016; Broekman et al. 2018; Rak 2013). The involvement of EVs in the biology of GBM and tumour microenvironment have been reviewed in detail elsewhere (Broekman et al. 2018; Quail and Joyce 2017), but some key points of interest deserve to be mentioned.

First, EVs have a unique potential to mediate the release and intercellular transfer of both naturally secretable and non-secretable molecular entities, including transmembrane receptors, transcription factors, nucleic acids, bioactive lipids and their

combinations (Broekman et al. 2018). Second, GBM cells and their stroma exhibit elevated vesiculation activity, which leads to simultaneous release of multiple EV subtypes and is under the influence of transforming events (Choi et al. 2018). Third, EVs contain GBM driver oncoproteins (Al-Nedawi et al. 2008; Graner et al. 2009), transcripts (Skog et al. 2008) and DNA sequences (Figueroa et al. 2017), as well as other cancer-specific cargo. These molecules could be transferred to recipient normal cells, or to indolent cancer cell populations changing their phenotypes, including features relevant to vascular responses in GBM, such as angiogenesis (Al-Nedawi et al. 2008; Skog et al. 2008). Fourth, while cancer-specific EV content exhibits detectable biological activity in GBM, it also offers unique diagnostic opportunities (liquid biopsy) upon recovery of GBM EVs from CSF or circulating blood (Zachariah et al. 2018).

Harnessing the various functional and diagnostic properties of GBM EVs is challenged by their diversity. This is reflected by traditional EV classification based on subcellular sites and mechanisms of their biogenesis. Thus, one broad EV subset referred to as exosomes is derived from endocytic multivesicular bodies (MVBs). MVB membrane undergoes intraluminal invagination forming intraluminal vesicles (ILVs) or exosome precursors ranging from 50 nm to 150 nm in size (van Niel et al. 2018). Some MVBs are subsequently shuttled to the plasma membrane through mechanisms recently reviewed elsewhere (Mathieu et al. 2019; van Niel et al. 2018) and ILVs are released into perivascular space as proper exosomes enriched for discernable (though not unequivocal) molecular markers such as tetraspanins: CD9, CD63 and CD81 and other cargo (Kowal et al. 2016). In contrast, ectosomes (or microvesicles) are shed directly through the outward budding from the cellular plasma membrane, mostly as larger vesicles (150–1000 nm in diameter) often positive for Annexin A1 (Jeppesen et al. 2019). However, the actual magnitude of EV heterogeneity based on size, source and molecular composition is far greater than this model predicts (Choi et al. 2019; Zijlstra and Di Vizio 2018). For example, the proteome analysis of standard EV preparations from conditioned media of relatively uniform cultured cells may reach between 1000 and 5000 protein signals, which predicts up to 25 non-overlapping protein cargo combinations and likely much larger number of molecularly distinct EV subsets along with the spectrum of their associated biological activities (Choi et al. 2017; Choi et al. 2018).

In view of this complexity several approaches to EV fractionation and single EV analysis have been developed in recent years to better map and understand the landscape of cellular ‘EV-ome’. Advanced technologies of single EV detection include nano-flow cytometry, imaging flow cytometry, super-resolution microscopy, and chip-based methods, many already applied in the context of brain cancer EVs (Table 10.1). In many cases the results of such studies have been quite revealing, as could be illustrated by a handful of experimental observations. For example, contrary to earlier predictions nano-flow cytometry of EVs derived from glioma cells, U373, showed only a partial positivity for canonical EV maker proteins including CD9 (~35%) and CD81 (~14%) (Choi et al. 2018). Also, ectosomal marker BSG (known as EMPIRIN or CD147) (Sidhu et al. 2004) was presented on ~28% glioma

Table 10.1 Heterogeneity and function of brain cancer EVs

Model	Methods	Description	Ref.
U373 and U373vIII (EGFRvIII overexpressing isogenic U373)	Nano-flow cytometry	Increased CD44 and BSG positive population in U373vIII EVs	(Choi et al. 2018)
Human glioblastoma stem-like cells (GS-5, GS-8, GS-57, GS-60, GS-74, BT112, NCH644, GSC168, GSC233, GSC407)	Imaging flow cytometry, correlative light- and electron microscopy	Differential CD9, CD63, CD81 positive single EV derived from different glioblastoma cell-lines and affected from various cell stimuli (hypoxia and serum)	(Ricklefs et al. 2019)
Serum of malignant glioma patients	Imaging flow cytometry	CD9/GFAP/SVN triple positive EVs in serum of malignant glioma patients with anti-survivin vaccine treatment	(Galbo Jr. et al. 2017)
Gli36-WT, Gli36-EGFRvIII, and Gli36-IDH1R132H	Single EV capture and detection on the chip	Increased CD9 in Gli36-EGFRvIII and CD81 in Gli36-IDH1R132H	(Lee et al. 2018)
GL261, GBM8 cells, and plasma of glioma patients	Single EV capture and detection on the chip	Different composition of single EV depends on EV marker (CD81, TSG101, CD63, Alix, integrin beta 1, CD9, CD40, Arf6, VAMP-3) and glioblastoma marker (EGFR, EGFRvIII, EpCAM, IDH1-R132H) in glioma EVs	(Fraser et al. 2019)
Mouse glioma model	Single EV capture and detection on the chip; EV subpopulation analyses by different size filter (0.8, 0.22, and 0.02 μm)	Differential Gja1, CD9, CD81 positive single EV in large extracellular vesicles (>0.8 μm), small EVs (>0.02 μm)	(Gyuris et al. 2019)
U251, U87MG cells and serum of glioma patients	Flow cytometry by EVs on the beads	EGFR positive EVs in glioma cells and patients	(Wang et al. 2019)

EVs (Choi et al. 2018). Moreover, these molecular markers only partially overlapped in single EVs, which is to say that ~65% of U373 EVs were CD9/CD81-negative, 7% were CD81 positive, 21% were BSG positive, and 7% double positive (Choi et al. 2018), reflecting a high degree of combinatorial EV heterogeneity even with the use of limited number of markers.

This EV landscape is further altered by oncogenic transformation. In the case of U373 glioma cells the expression of mutant epidermal growth factor receptor variant III (EGFRvIII), a frequent event associated with classical glioblastoma (Wen et al. 2020), leads to the onset of a highly aggressive phenotype (Magnus et al. 2014) in

the resulting cells (U373vIII). These cells produce ample EVs with increased content of mesenchymal markers, including CD44 and CD151 tetraspanin, while the expression of another tetraspanin, CD82, involved in EV biogenesis is profoundly downregulated (Choi et al. 2018). Interestingly, while clonal U373vIII cells were uniformly positive for the EGFRvIII oncogenic driver, this oncoprotein was detected in only 10–30% the related EVs and split between CD9-positive and CD9-negaitve EV populations. These observations made possible by the advent of nano-flow cytometry and other single EV techniques suggest a hitherto unexpected complexity of GBM EV landscapes (Choi et al. 2019).

GSCs represent a preferred model of the intrinsic complexity of GBM cell populations, and reflect their molecular subtypes. While GSC lines likely exhibit a continuum of cellular phenotypes, the extremes of this spectrum are exemplified by the recently described proneural (PN) and mesenchymal (MES) GSCs (Mao et al. 2013). As expected, EV repertoires of PN-type *versus* MES-type cells are vastly different and further change is imposed by the ability of GSC to undergo serum-induced differentiation (Spinelli et al. 2018). For example, EVs from PN GSCs grown in stemness-preserving neurosphere cultures generally lack the canonical EV markers, including key tetraspanins: CD9, CD63, and CD82. However, these markers are abundant on the surface of EVs from MES GSCs (Spinelli et al. 2018). Notably, induction of GSC differentiation resulted in changes in the expression of EV markers including the emergence of CD63 expression in EVs from PN GSCs and a decline in CD82 levels in EVs from MES GSCs. Once again, the analysis of these populations using nano-flow cytometry and electron microscopy revealed subsets of EVs with different status of tetraspanins and other cargo (Spinelli et al. 2018).

Imaging flow cytometry enables direct imaging of single EVs labelled with fluorophore-conjugated antibodies. This technology offers a high throughput capacity to analyse both EV morphology and molecular diversity *in vitro* (Ricklefs et al. 2019) and *in vivo* (Galbo Jr. et al. 2017). This approach can also be used to identify distinctive CD9/GFAP/SVN triple positive EVs in plasma of glioma patients (Galbo Jr. et al. 2017), or for similar applications.

Single EV capture on the chip or their detection by high resolution microscopy are also among the emerging technologies with high sensitivity and of considerable diagnostic promise in GBM (Fraser et al. 2019; Gyuris et al. 2019; Lee et al. 2018). Recently Lee et al., applied the Single EV analysis (SEA) chip technology for molecular profiling of glioma-derived EV populations. In this case biotinylated EVs were captured on avidin coated chips and detected by fluorescent antibodies (Lee et al. 2018). This single EV detection method confirmed the very complex nature of individual glioma EVs present in plasma of patients (Fraser et al. 2019). Also, Gyuris et al., reported on the molecular heterogeneity of EVs derived from mouse glioblastoma cells. In this case, EVs were found to contain different proteome and RNA compositions, depending on the size of EVs. Also in this study higher NonO (non-POU domain-containing octamer-binding protein) expression by larger single EVs (>0.8 μm) contrasted with higher Gja1 (GAP junction alpha 1) expression by smaller EVs (>0.02 μm), with closer co-localization with CD9 compared to

CD81 (Gyuris et al. 2019). Once again, this finding suggests a divergent tetraspanin distribution at the single EV level challenging the notion of tetraspanin clusters as unequivocally linked to specific EV subtypes (Choi et al. 2019).

EV heterogeneity can also be captured, at least to some extent using functional assays, such as selective vesicle uptake by recipient cells. For example, the aforementioned U373vIII glioma cells preferentially (though not exclusively) internalize their own EVs rather than EVs from their isogenic but non-aggressive U373 counterparts (Choi et al. 2018). Exosome-like EVs enriched for ALIX and syntenin-1, showed a more efficient uptake by recipient cells than ectosome-like EVs from the same cellular source (Choi et al. 2019). EVs derived from GSCs induced to partially differentiate in the presence of serum were taken up less avidly by endothelial cells than their counterparts from undifferentiated neurosphere cultures (Spinelli et al. 2018). Moreover, numerous studies documented distinct biological activities of glioma EV subpopulations, including differential stimulation of endothelial cells (Skog et al. 2008; Spinelli et al. 2018).

In cancer, including glioma, the magnitude of EV emission and their heterogeneity are subjects to regulatory influences by oncogenic mutations (Al-Nedawi et al. 2008; Choi et al. 2018), epigenetic processes of cellular differentiation, lineage commitment and mesenchymal transition (Garnier et al. 2012; Spinelli et al. 2018). Also cellular evolution in the presence of cytotoxic stress (Garnier et al. 2018) and microenvironmental influences such as hypoxia may contribute to diversity of the EV output by a given cellular population (Kucharzewska et al. 2013; Wang et al. 2014). It follows that the glioma cells exposed to these influences may produce EV 'clouds' with a complex and dynamic information content that acts on recipient cells in a combinatorial manner. While this is an attractive possibility some of the biological effects of GBM EVs exerted on the multiple facets of the vascular system are increasingly well defined in specific molecular terms.

Evidence for the Role of EVs in Tumour Angiogenesis

Angiogenesis is defined as formation of new capillaries from pre-existing blood vessels. This mechanisms assumes several forms, the best defined of which involves the process of capillary sprouting. In this setting postcapillary vessels exposed to a gradient of angiogenic factors (such as VEGF) deploy structures (sprouts) composed of specialized invasive endothelial tip cells that direct a cohort of stalk endothelial cells to migrate, proliferate and form a new capillary loop (Carmeliet and Jain 2011). Once such immature endothelial tube attracts supporting cells such as pericytes it acquires functional stability and independence of angiogenic growth factors (Betsholtz 2018). This multicellular program is essential for normal development, organogenesis and tissue repair (Carmeliet and Jain 2011). However, angiogenesis is also a key correlate and pathogenetic factor involved in inflammatory diseases, tumor progression and metastasis (Carmeliet and Jain 2011). This does not imply an absolute dependence of tumour growth on angiogenesis. In fact, cancer

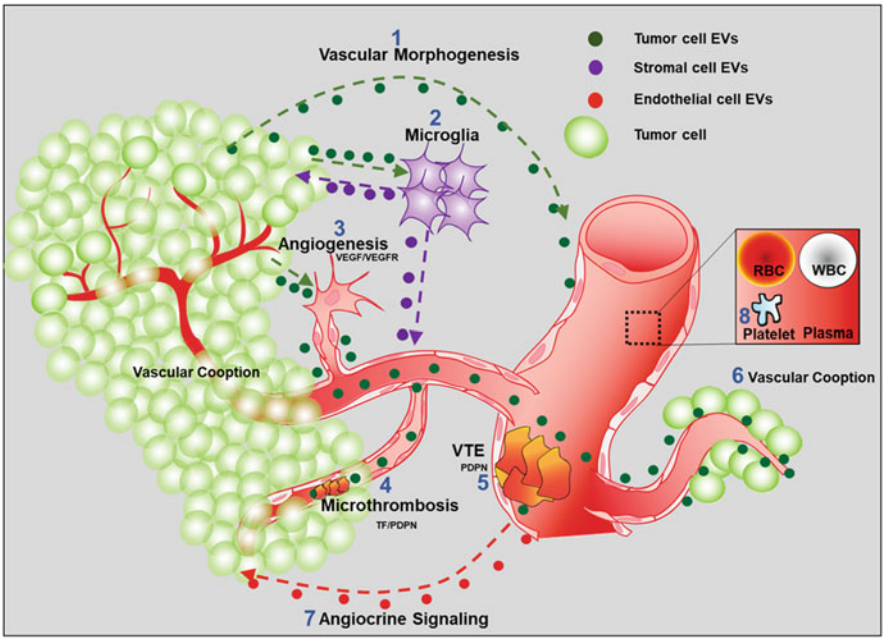

Fig. 10.1 EV-mediated tumor-vascular interaction network in GBM. GBM cells are able to interact with vascular endothelial cells via EVs in several ways that are poorly defined and either experimentally documented, or hypothetical in nature (see text): (1) GBM EVs alter the surrounding endothelial cells, which potentially remodels the blood vessels into becoming enlarged. (2) Inflammatory cells such as macrophages or microglia may interact with the tumor cells and vascular endothelial cells by secreting EVs. In turn tumor cells have been also thought to secrete EVs, which are taken up by inflammatory stromal cells and modulate their function. (3) GBM EVs have been implicated in inducing angiogenesis, which is defined by endothelial cell proliferation, tip cell formation and sprouting of *de novo* capillaries due to the VEGF and other factors carried by GBM EVs. (4 and 5) GBM EVs carrying tissue factor (TF) and/or podoplanin (PDPN) may have a role in microthrombosis in the tumour microcirculation and could also be implicated in peripheral vascular thromboembolism (VTE). (6) Vascular cooption (engulfment of pre-existing blood vessels) and perivascular invasion by GBM cells may be modulated by EVs in a manner independent of VEGF/VEGFR signaling. (7) Endothelial cells also secrete EVs that may modulate GBM cells (angiocrine effect). (8) GBM cells may also take up EVs from platelets and other cellular populations

progression depends on the access to blood vessels rather their formation, and it may or may not depend on the extent of angiogenesis *per se*, due to a wider range of processes that may allow cancer cells to position themselves near the vasculature (Kuczynski et al. 2019) (Fig. 10.1). Nonetheless, mechanisms and pathways triggering angiogenesis in cancer may serve as a paradigm for regulatory influences operative between tumour and vascular cells, including those dependent on GBM EVs (Rak 2013).

Cancer-derived EVs may impact angiogenic machinery either indirectly or directly. In the first instance, oncogenic proteins, transcripts, regulatory RNA and

other cargo may be shuttled by EVs from angiogenic to non-angiogenic cancer cell populations and stroma altering salient features of their phenotype, including VEGF production (Al-Nedawi et al. 2008). Cancer EVs may also act directly on endothelial cells (Kim et al. 2002; Skog et al. 2008) in concert with soluble angiogenic growth factors (Lucero et al. 2020). Oncogenic mutations, hypoxia and other influences impact the expression and release of VEGF and other soluble mediators of vascular growth (Rak 2009; Rak et al. 1995), along with EVs (Al-Nedawi et al. 2008; Lee et al. 2014; Yu et al. 2005), whose molecular composition is profoundly altered by cellular transformation (Choi et al. 2018). These changes are not merely an exacerbation of physiological blood vessel growth processes, but rather a qualitatively distinct process reflected in the abnormal morphology, cellular state and function of the cancer vasculature (Arvanitis et al. 2020).

Multiple studies implicated EVs as direct mediators of tumour angiogenesis (Aslan et al. 2019; Bussolati et al. 2011; Kikuchi et al. 2019). This influence is considered either to be linked to the content of canonical angiogenic factors on the surface, or in the lumen of tumour-derived (or stromal) EVs (Feng et al. 2017; Taraboletti et al. 2006; Treps et al. 2017), or reflect non-canonical effects and mediators leading to reprogramming of endothelial cells in ways that do not occur during physiological blood vessel growth (Al-Nedawi et al. 2009; Bussolati et al. 2011; Skog et al. 2008). In relation to the latter, EVs, could transfer coding and noncoding RNA, DNA and proteins, to endothelial cells thereby imposing lasting changes in their functional profile. Both of these scenarios are supported by experimental data (Al-Nedawi et al. 2008; Chennakrishnaiah et al. 2020; Lang et al. 2017; Skog et al. 2008; Spinelli et al. 2018).

Thus, several lines of evidence suggest that EVs may contain key pro-angiogenic factors directly linked to endothelial cell proliferation, migration and new blood vessel formation. Canonical isoforms of VEGF were found to be delivered in this manner to endothelial cells resulting in their stimulation (Han et al. 2019; Taraboletti et al. 2006; Treps et al. 2017). In addition, larger and poorly secretable VEGF isoforms (VEGF-90kD or VEGF189) were also found to exert their angiogenic activity following their incorporation into tumour EVs (Feng et al. 2017; Ko et al. 2019). Similarly, a transmembrane neural guidance receptor molecule with blood vessel forming activity, EPHB2, was found to be released as cargo of EVs from head and neck cancer cells. Transfer of these EVs to endothelial cells induced STAT3 phosphorylation and stimulation of angiogenic responses (Sato et al. 2019).

Intriguingly, packaging into EVs may alter the mode of angiogenic signalling of vascular mediators. For example, Angiopoietin-2 (ANG2) contained in EVs and released from hepatocellular carcinoma cells may interact with endothelial cells in a manner leading to an increase in angiogenic responses, which are ostensibly unrelated to the activation of the ANG2 receptor (TIE2)(Xie et al. 2020). Tumour EVs have also been found to carry Delta-like ligand 4 (Dll4), a cell-associated agonist for the NOTCH receptor, and a potent regulator of endothelial tip cells (Carmeliet and Jain 2011). Interestingly, exposure of recipient cells to EV-associated Dll4 triggered paradoxical inhibition rather than stimulation of NOTCH signalling (Sheldon et al. 2010).

In some cases, EVs and growth factors may cooperate in inducing proangiogenic responses, at least in part, through interdependent autocrine effects in endothelial cells. For example, interleukin 3 exerts some of its pro-angiogenic activity through triggering EV release and uptake by tumour associated endothelial cells resulting in activation of the WNT pathway (Lombardo et al. 2018). Another example is the EV-mediated transfer of EGFR from cancer cells to endothelium stimulating endogenous production of VEGF and the phosphorylation of VEGFR2 in these cells (Al-Nedawi et al. 2009).

Recent studies have demonstrated that exosome-like EVs may contribute to tumour angiogenesis by transferring genomic DNA sequences to endothelial cells. In this regard, Balaj et al. demonstrated for the first time that EVs contain small fragments of single-stranded DNA that may contain oncogenic amplicons and transposons and deliver them to cultured endothelial cells (Balaj et al. 2011). More recently, evidence was obtained that also double-stranded DNA-enriched EVs are capable of interacting with endothelial cells resulting in proangiogenic responses (Lee et al. 2014; Lee et al. 2016). Moreover, EVs containing genomic DNA and derived from genetically unstable cancer cells stimulated endothelial cell migratory activity, while also inducing abnormal formation of micronuclei and triggering activation of P53 pathway (Chennakrishnaiah et al. 2020). This finding is consistent with the observation that in a subset of cancers tumour-associated endothelial cells may exhibit aneuploidy unrelated to genomic alterations found in cancer cells themselves (Hida et al. 2004). These examples illustrate how unique traits of cancer EVs may produce cancer-specific forms of vascular responses and pathology.

Another cancer-specific mode of EV-mediated vascular reprogramming may stem from the transfer of oncogenic signalling/regulatory modules from cancer cells to their vascular counterparts. For example, the uptake of cancer EVs harbouring EGFR oncoprotein by endothelial cells results in activation of downstream MAPK and AKT signalling and triggers endogenous activation of the VEGF signalling pathway (Al-Nedawi et al. 2009). Endothelial cells exposed to tumour EVs also acquire procoagulant activities due to the uptake of tissue factor (TF), a transmembrane receptor for clotting factor VII, mediator of cellular signalling and a potent regulator of angiogenesis related genes (Albrektsen et al. 2007) TF and VII are frequently upregulated in cancer cells including GBM (Magnus et al. 2010). This conversion of normally anticoagulant phenotype of endothelial cells is further exacerbated following epithelial-to-mesenchymal transition occurring in transformed EV donor cells (Garnier et al. 2012). Despite major changes induced in endothelial cells exposed to cancer EVs harbouring oncogenic macromolecules no overt malignant transformation of these cells has been reported to date (Lee et al. 2016).

Angiogenic responses have also been linked with EV-mediated release of cellular RNA (exRNA) (Ratajczak et al. 2006; Skog et al. 2008; Valadi et al. 2007). For example, EVs from human colorectal cancer cells have the ability to release mRNA for genes involved in cell cycle regulation and the uptake of this material was reported to promote proliferation of cultured endothelial cells (HUVEC) (Hong et al. 2009). EV transfer of miR-21 from cancer cells to normal epithelium has been

shown to increase VEGF production leading to enhanced potential to trigger angiogenesis (Liu et al. 2016). Similarly, cancer EVs enriched for miR-23a and miR-494 enhanced angiogenesis through suppression of PTEN and activation of the Akt/eNOS pathway in recipient endothelial cells (Du et al. 2020; Liu et al. 2016). In leukemic cells induction of cellular differentiation using all-trans retinoic acid (ATRA) led to enrichment in exosome-like EVs and shift in their content of angiogenic and procoagulant transcripts, along with reduced endothelial stimulating activity (Fang et al. 2016).

In various biological settings cancer EVs may either stimulate or inhibit angiogenesis. For example, exosomal miR-9 released from nasopharyngeal carcinoma cells suppressed angiogenesis by triggering MDK gene inhibition in endothelial cells (Lu et al. 2018). Also miR-135b was found to exert a similar inhibitory effect on angiogenic mechanisms (Bai et al. 2019). The effect of exosomal miR-100 released from mesenchymal stem cells (MSC) attenuated hypoxia signalling and VEGF expression by recipient breast cancer cells thereby suppressing their proangiogenic activity (Pakravan et al. 2017). In another study, similar VEGF suppressing effect in breast cancer was attributed to miR-16 contained in MSC-derived EVs (Lee et al. 2013). Thus, EVs are capable of delivering signals that modulate tumour-vascular interface in a wide range of cancers and through several mechanisms.

EV-Related Processes of Blood Vessel Formation in Glioblastoma

EV-mediated vascular effects observed across the spectrum of cancers are also applicable to the unique milieu of brain tumours, including GBM. In this microenvironment the angiogenic behavior of endothelial cells is strictly dependent on dynamic interactions between stromal and tumor cells and their and soluble factors (Fig. 10.1) (Arvanitis et al. 2020; Bissell and Radisky 2001; Eilken and Adams 2010). As mentioned earlier, neural stem cells (Stiles and Rowitch 2008) and a subset of glioma stem cells (GSC) naturally occupy perivascular niches in the close proximity to endothelial cells on which they depend for maintenance and paracrine stimulation (Calabrese et al. 2007). GSCs are also believed to represent one of the richest sources of pro-angiogenic growth factors as exemplified by their high expression of VEGF (Bao et al. 2006).

While the tumour-vascular interface entails multiple processes, angiogenesis remains one of the most studied and therefore represents a convenient paradigm to consider the role of EVs. To this effect studies show that GSCs release EVs carrying bioactive VEGF, ostensibly capable of activating endothelial cell responses (Treps et al. 2017). It remains to be established how EV-VEGF comes into contact with surface VEGF receptors on endothelial cells, and whether it represents a meaningful addition to the massive production of soluble VEGF by hypoxic GBM cells and stroma (Arvanitis et al. 2020). It should also be born in mind that VEGF antagonists

did not curtail GBM progression casting a shadow on the role of VEGF in disease pathogenesis.

Unlike in the case of soluble angiogenic growth factors, such as soluble VEGF, which is readily secreted from cancer cells, EVs represent a unique pathway for the release of membrane-associated or intracellular regulators of vascular function. As mentioned earlier certain GBM cell lines produce Dll4-enriched EVs, which can present this ligand to NOTCH receptors on the surface of endothelial cells. This cell-dissociated Dll4 ligand causes inhibition of NOTCH signaling and deregulates angiogenic responses (Sheldon et al. 2010). Whether other juxtacrine ligands associated with EVs exert similar effects on GBM vasculature has not been extensively studied.

The vascular role of GBM EVs may depend on microenvironmental, epigenetic and genetic factors. For example, hypoxic glioma cells release EVs containing transcripts for several angiogenesis and inflammation regulating genes, and are able to stimulate angiogenic responses of endothelial cells *in vitro* (Kucharzewska et al. 2013). Activation of epigenetic mechanisms driving GSCs toward astrocytic differentiation results in profound reprogramming of their EV repertoire, proteome, uptake by endothelial cells, and enhanced ability to stimulate endothelial cell growth. These effects are also modulated by GSC molecular subtype and differ between proneural and mesenchymal GSCs (Spinelli et al. 2018). Interestingly, this greater endothelial stimulating activity of EVs derived from differentiated GSCs contrasted with reportedly weaker production of VEGF by these cells relative to their undifferentiated counterparts (Bao et al. 2006). This may suggest that differentiated and undifferentiated glioma cells use different mechanisms of angiogenic stimulation, such as EVs and VEGF, respectively (Spinelli et al. 2018). Indeed, in a recent study the effects of canonical angiogenic growth factors and microRNA-containing EVs produced by GSCs were found to be different, an observation signifying the distinct contribution of EV-communication to GBM-vascular interactions (Lucero et al. 2020).

Notably, a non-coding RNA known as HOTAIR was also found to control VEGF expression in GBM cells and, at the same time impacted endothelial cells following EV-mediated transfer (Ma et al. 2017). Other long non-coding RNAs expressed in glioma cells, such as linc-POU3F3 and linc-CCAT2, were also found to be delivered into endothelial cells where they stimulated angiogenesis-like responses by upregulating the expression of multiple pro-angiogenic growth factors, such as TGF-β, bFGF, VEGF -A and angiogenin (Lang et al. 2017; Lang et al. 2017).

At the level of cancer cell genome, oncogenic mutations also impact the vascular effects of GBM EVs. For example, mutant EGFRvIII has been detected in glioma cell-derived EVs at the protein (Al-Nedawi et al. 2008), mRNA (Skog et al. 2008), and DNA level (Figueroa et al. 2017), as well as in exosome fractions of plasma and CSF in GBM patients (Figueroa et al. 2017; Graner et al. 2009; Montermini et al. 2015; Skog et al. 2008).

Apart from the possible utility of the EV-mediated EGFRvIII release for biomarker purposes (Al-Nedawi et al. 2008; Nilsson et al. 2011; Skog et al. 2008), the biological activity of the oncogenic cargo of such oncogene-carrying EVs

(oncosomes) (Meehan et al. 2016) may have significant implications for angiogenesis and other responses of the vascular compartment associated with GBM (Rak 2009). First, it was documented that the transfer of mRNA from GBM cells harbouring EGFRvIII mutations to cultured endothelial cells may lead to proliferative and morphogenetic responses reminiscent of angiogenesis (Skog et al. 2008). These effects were similar to the aforementioned endothelial reprogramming processes following the EV-mediated delivery of cancer-derived EGFR oncoprotein to endothelial cells (Al-Nedawi et al. 2009). Second, since the expression of EGFRvIII in GBM cells is always heterogenous and restricted to specific cellular subpopulations (Inda et al. 2010), it was of interest whether the passage of this oncoprotein may occur between EGFRvIII-positive and -negative cell subsets and to what effect. In fact, such EV-mediated EGFRvIII-transfer between glioma cell populations has recently been described and found to activate oncogenic signalling pathways and EGFRvIII target genes in recipient cells, including expression of VEGFR (Al-Nedawi et al. 2008). This observation may suggest that cancer cell populations may share their proangiogenic activities by exchanging oncogenes and their effectors (Al-Nedawi et al. 2008). While EV-mediated cooperation between glioma cells has, indeed, been described (Al-Nedawi et al. 2008; Ricklefs et al. 2016), the significance of these processes in clinical settings remains to be elucidated.

Mounting evidence suggests that GBM EVs play multiple roles in modulating tumour-associated vasculature. Which of these diverse effects are consequential, targetable and relevant *in vivo* remains to be studied further. Similarly, not all effects on endothelial cells are tantamount to *bona fide* angiogenesis, and not all angiogenic effects are crucial for GBM progression, as amply demonstrated by the inability of agents targeting VEGF to prolong survival of GBM patients (Wen et al. 2020). Therefore, the role of EVs, perhaps, needs to be seen also in the context of non-angiogenic processes relevant to the tumour-vascular interface in GBM, such as vascular cooption (Griveau et al. 2018; Kuczynski et al. 2019), perivascular invasion (Cheng et al. 2013), angiocrine stimulation (Calabrese et al. 2007) and systemic deregulation of vascular homeostasis, as exemplified by GBM-associated thrombosis (Tawil et al. 2019).

Brain Tumour Associated Coagulome and Coagulopathy

GBM epitomizes the nexus between cancer progression, blood vessel alterations and pathological changes in the circulating blood. In comparison to the general population cancer patients are at five to seven fold higher risk of occlusive blood clots (thrombi) often in blood vessels distant from the site of tumour growth. This condition known as cancer-associated thrombosis (CAT) may entail the systemic activation of coagulation factors (hypercoagulability) often compounded by the effects of vascular stasis (bed rest), and vascular damage, including the iatrogenic use of central venous catheters and anticancer therapy (Falanga et al. 2017; Perry 2012). CAT is clinically manifested by the elevated incidence of venous

thromboembolism (VTE), which consists of deep vein thrombosis (DVT), often in extremities, and life threatening pulmonary embolism (PE), when the venous clots lodge in large pulmonary vessels (Elice et al. 2009; Falanga et al. 2017; Hisada and Mackman 2017; Kuderer and Lyman 2014). Cancer patients are also at higher risk of arterial thromboembolism (ATE)(Navi et al. 2017). Approximately 20% of sporadic cases of VTE are associated with cancer, and VTE is found in 50% of cancer cases at autopsy (Timp et al. 2013). While CAT presents considerable clinical concerns it may also alter the biology of cancer, through the impact of activated platelets and clotting proteins on cellular signalling (Ruf et al. 2011), angiogenesis (Coughlin 2005; van den Berg et al. 2009), formation of hypoxic regions (Brat and Van Meir 2004), cancer cell invasion (Labelle et al. 2011), immunomodulation (Servais et al. 2018), and reprogramming of the tumor cell gene expression profile (Albrektsen et al. 2007), stemness (Gur-Cohen et al. 2015) and the epigenome (Magnus et al. 2014).

Although VTE risk assessment is challenging, CAT is not a random or 'unspecific' occurrence, as indicated by different frequencies of thrombosis in different tumours types and subtypes (Hisada and Mackman 2017; Timp et al. 2013). While brain microenvironment is inherently procoagulant, central nervous system tumours differ widely in their ability to trigger CAT. Prothrombotic properties are especially pronounced in GBM, which is associated with 20–30% incidence of VTE (second only to pancreatic cancer) and microthrombosis throughout the tumour microcirculation in over 90% of cases (Brat and Van Meir 2004; Perry 2012; Tehrani et al. 2008). However, in histologically similar high grade gliomas harbouring IDH1 mutation, VTE risk is low and microthombosis far less prevalent (Unruh et al. 2016), suggesting that events driving cancer cells transformation at the level of genome and epigenome impact coagulation-related effector genes (coagulome) and control the occurrence, severity and possibly the nature of CAT in GBM. Indeed, the analysis of GBM tissues at the bulk (Magnus et al. 2013) and single cell level (Tawil et al. 2019) reveal different profiles of coagulation-related genes in association with proneural, classical or mesenchymal cellular classifiers.

CAT involves unscheduled triggering of core hemostatic mechanisms including the coagulation cascade, platelets and damage to the vascular wall (Adams and Bird 2009). GBM cells may engage this network indirectly, through stimulation of angiogenesis, endothelial activation and inflammation, resulting in procoagulant changes in blood vessels and tumour microenvironment. However, GBM cells may also directly influence thrombosis by expression of various elements of the coagulome (Magnus et al. 2010). For example, the aberrant expression of tissue factor (TF) on the tumour cell surface may lead to contact with plasma containing coagulation zymogens that can thereby be activated leading to clotting. TF is a transmembrane receptor that forms complexes with plasma-derived, activated coagulation factors VIIa and Xa, capable of catalysing activation of plasma prothrombin to thrombin (IIa) (Adams and Bird 2009). This critical event leads to cleavage of plasma fibrinogen to form the insoluble fibrin clot. At the same time thrombin activates platelets and triggers cellular signals through thrombin receptors (protease-activated receptor 1 – PAR-1) resulting in multiple consequential changes in the

tumour microenvironment. For example, PAR-1 is involved in tumour initiation by GSCs (Auvergne et al. 2016), while platelets may not only contribute to clot formation, but also release a plethora of growth factors, including VEGF and TGFb impacting cell growth, angiogenesis and immunoregulation (Haemmerle et al. 2018). GBM cells also activate platelets through another mechanism involving the expression of surface podoplanin (PDPN) a ligand for platelet CLEC2 receptor (Riedl et al. 2017). This is but a fragment of a more complex interface as other hemostatic proteins are also expressed by GBM cells in a manner regulated by the molecular subtype, epigenetic state (e.g. differentiation) and the expression of mutant oncogenes such as EGFRvIII and IDH1 (Tawil et al. 2019; Unruh et al. 2016).

While the variable repertoire of these effectors may suggest corresponding differences in the nature of CAT in different GBM subtypes, few were found to predict the VTE risk. Indeed, despite the appealing biochemical potential of TF, its central role in the coagulation cascade, and down regulation in non-thrombotic tumours with IDH1 mutation, TF levels were not found to be a reliable predictor of VTE in GBM patients (Thaler et al. 2013). In contrast, recent studies suggested that high levels of GBM cell associated PDPN correlate with formation of extensive intravascular platelet thrombi in the tumour microcirculation and significantly elevated incidence of VTE (Costa et al. 2019; Riedl et al. 2017). Interestingly, PDPN is downregulated in proneural GBMs, which are traditionally included among IDH1 mutant tumours (Tawil et al. 2018). Whether PDPN is mechanistically essential, sufficient and targetable in the context of CAT in GBM remains to be further investigated.

Procoagulant EVs in Brain Tumours

One major outstanding question in relation to vascular pathology associated with GBM is how this strictly intracranial (albeit highly infiltrative) tumour triggers peripheral thrombosis in extremities and large extracranial vessels with which tumour mass has no physical contact (Perry 2012)? In this regard, potential mechanisms involve either a ‘spill-over’ effect of the intratumoural activation of clotting mechanisms, or the export of key coagulant effectors to the periphery, where they may trigger formation of thrombi. The latter possibility is especially attractive in view of the fact that GBM cells are known to release EVs carrying pro-thrombotic proteins, such as TF or PDPN (Tawil et al. 2020; Thaler et al. 2012).

The role of EVs in hemostasis and thrombosis is backed by a substantial body of literature delineating their procoagulant characteristics and mechanisms by which they influence the thrombotic process (Geddings and Mackman 2013; Hisada and Mackman 2017; Zarà et al. 2019). Indeed, EVs may have a role in physiological hemostasis as indicated by hemorrhagic disorders linked to genetic defects in platelet vesiculation (e.g. Scott syndrome) (Burnier et al. 2009) and bleeding phenotype of

mice deficient for Rab27a/b genes (Tolmachova et al. 2007), crucial effectors in exosome biogenesis (van Niel et al. 2018).

In cancer various types of EVs have been implicated in thrombosis through their content of bioactive TF, mucins, phosphatidylserine (PS) and other components involved in clotting events (Geddings and Mackman 2013). While in certain cancers TF-carrying EVs have been shown to possess procoagulant activity (Yu and Rak 2004) and were implicated in triggering thrombosis (Wang et al. 2012), the analysis of blood samples from GBM patients failed to demonstrate a predictive value of TF-EVs (also referred to as microparticles) in VTE (Thaler et al. 2012). It is possible that while TF is readily released from cancer cells as cargo of EVs (Garnier et al. 2012), in some instances this process might involve TF encryption (steric modification) (Bach 2006) resulting in the absence of the related procoagulant activity in the circulation (Thaler et al. 2012).

To date, PDPN remains the strongest correlate of VTE in the context of GBM (Riedl and Ay 2019; Riedl et al. 2017; Watanabe et al. 2019) and the current body of literature points to PDPN carrying EVs as potential effectors of peripheral thrombosis. Recent studies indicate that PDPN is released by GBM cells *in vitro* and *in vivo* and retains platelet stimulating activity in experimental settings (Tawil et al. 2020). However, further research is required to determine whether PDPN-EVs also contain other accessory molecules involved in thrombosis and are true predictors of VTE. Moreover, such EVs may possess other biological activities which are presently largely unstudied.

Conclusions

The vascular system represents a regulatory continuum encompassing macro- and microvasculature, blood cells and their bone marrow derived predecessors, inflammatory cells and immune effectors, platelets and multimolecular cascades of the coagulation system. This delicate network connecting the parenchymal cells in different organs with each other and with the outside world, while protecting their integrity, is profoundly disrupted, distorted and highjacked in cancer, leading to several pathological vascular alterations. Angiogenesis and thrombosis are but the most studied examples of vascular changes occurring in cancer and prominent in GBM. However, it should be kept in mind that other relevant effects of EVs, presently poorly studied, may include important processes such as vascular invasion and cooption (Li et al. 2019) (perhaps the most important mode of GBM neovascularization (Kuczynski et al. 2019)), remodelling of larger supply vessels, vascular permeability, formation of primary and secondary (post-therapeutic) stem cell niches (Calabrese et al. 2007; Garnier et al. 2018) driven by angiocrine communication mechanisms, vascular hyperproliferation, permeability (Treps et al. 2016) and other effects. While the role of EVs released from various cellular sources and subpopulations in evolving tumours is intriguing and actionable (as targets and biomarkers), their effects are likely complementary to those of other modes of

intercellular communication, including membrane bridges (Osswald et al. 2015), synaptic connections (Venkatesh et al. 2019) and soluble factors, all of which may influence the responses of the vasculature. Understanding these complexities beyond angiogenesis and thrombosis is crucial for development of rational therapies targeting vascular system to curtail progression of brain tumours.

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Chapter 11
Extracellular Vesicles Regulate Cancer Metastasis

Sanjay Shahi, Cassandra Cianciarulo, Christina Nedeva, and Suresh Mathivanan

Abstract Metastatic cancer is a complex disease associated with poor prognosis and accounts for the majority of cancer related deaths. To date, many of the molecular mechanisms driving metastatic disease remain elusive and require further investigation for the development of effective treatment strategies. Recent studies have shown that extracellular vesicles (EVs) can be exploited by tumors to assist in cancer cell growth, proliferation, migration, invasion and metastasis. Cancer cells have proven efficient in educating fibroblasts, within their microenvironment, to secrete EVs as communicative vessels for mediating phenotypic changes in recipient cells. Using this vesicular delivery system, cancer cells can establish a new metastatic niche within distant sites, away from the primary tumor, thus favoring cancer progression. These findings demonstrate the availability of a new route for therapeutic intervention in the inhibition of cancer dissemination. Although, several approaches to target cancer cell secretion of EVs are detailed in the literature, there is still no defined way to currently apply them in clinical settings. Hence, further studies are required to unravel the molecular mechanisms of metastasis - governed by the establishment and release of cancer associated EVs.

Keywords Cancer · Metastasis · Extracellular vesicles · Exosomes · Pre-metastatic niche · Angiogenesis

Introduction

Cancer is a disease characterized by uncontrolled growth of abnormal cells and is responsible for millions of deaths every year and it is the second leading cause of death worldwide. Most cancer related deaths are due to metastasis of the primary tumor to distant sites (Seyfried and Huysentruyt 2013). The various mechanisms

S. Shahi · C. Cianciarulo · C. Nedeva (✉) · S. Mathivanan (✉)
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia
e-mail: C.Nedeva@latrobe.edu.au; S.Mathivanan@latrobe.edu.au

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driving cancer metastasis are yet to be fully understood, however, studies have shown that secreted extracellular vesicles (EVs) contribute to the pathogenesis of the disease (Becker et al. 2016; Wortzel et al. 2019; Gangoda et al. 2015; Boukouris and Mathivanan 2015). The following chapter will summarise current understanding of the various roles of EVs in aiding cancer progression and metastasis. The precise mechanisms that have been documented pertaining to EV regulated proliferation, growth, migration and invasion of cancer cells is discussed. Additionally, this chapter will also focus on how EVs protect cancer cells from immune clearance and aid in angiogenesis to connect the primary tumor to distant organs, being the hallmark of the metastatic cancer (Wortzel et al. 2019). As growing evidence suggests that EVs regulate various aspects of metastasis, they can therefore potentially serve as a target for the management or suppression of cancer progression and metastasis (Becker et al. 2016; Vader et al. 2014; Kalra et al. 2019). Hence, it is pivotal to achieve in-depth knowledge about approaches to inhibit EVs for therapeutic purposes by targeting key proteins responsible for synthesis and secretion of EVs using drugs or gene editing.

What Are Extracellular Vesicles?

EVs are secreted membranous vesicles which vary in size (30–10,000 nm in diameter). Based on various characteristics and following MISEV2018 guidelines, EVs have been categorized into various subtypes namely exosomes, ectosomes or shedding microvesicles, apoptotic bodies, large oncosomes, migrasomes and exomeres (Fig. 11.1) based on mode of secretion (Théry et al. 2018). Exosomes range from 30–150 nm in diameter and are secreted by various cell types eliciting an efficient intercellular communication (Kalra et al. 2016; Sanwlani et al. 2020). They are formed via the endocytic pathway and are released by the fusion of multivesicular bodies with the plasma membrane as shown in Fig. 11.1 (Sinha et al. 2016). Ectosomes or shedding microvesicles are released by budding of the plasma membrane while the apoptotic bodies are released by dying cells. However, ectosomes and apoptotic bodies may also appear as smaller EVs with overlapping size as that of exosomes. EVs also consist of a smaller population of vesicles called exomeres which are less than 50 nm in size (Zhang et al. 2018). In addition, larger vesicles such as migrasomes and large oncosomes, that range from 500 to 10,000 nm in diameter are also reported to be secreted by migrating and ameboid cancer cells, respectively (Zijlstra and Di Vizio 2018; Ciardiello et al. 2016).

Many of the important pathophysiological roles central to cancer and metastasis development are proposed to be regulated by exosomes or small EVs. This chapter discusses the role of exosomes and small EVs in cancer progression and from here onwards will be collectively referred to as EVs. Following early discovery of EVs, these nanovesicles were considered to function as a waste disposal system for cells undergoing maturation (Johnstone et al. 1984). However, with additional research, EVs are now known to play a pivotal role in several biological processes by

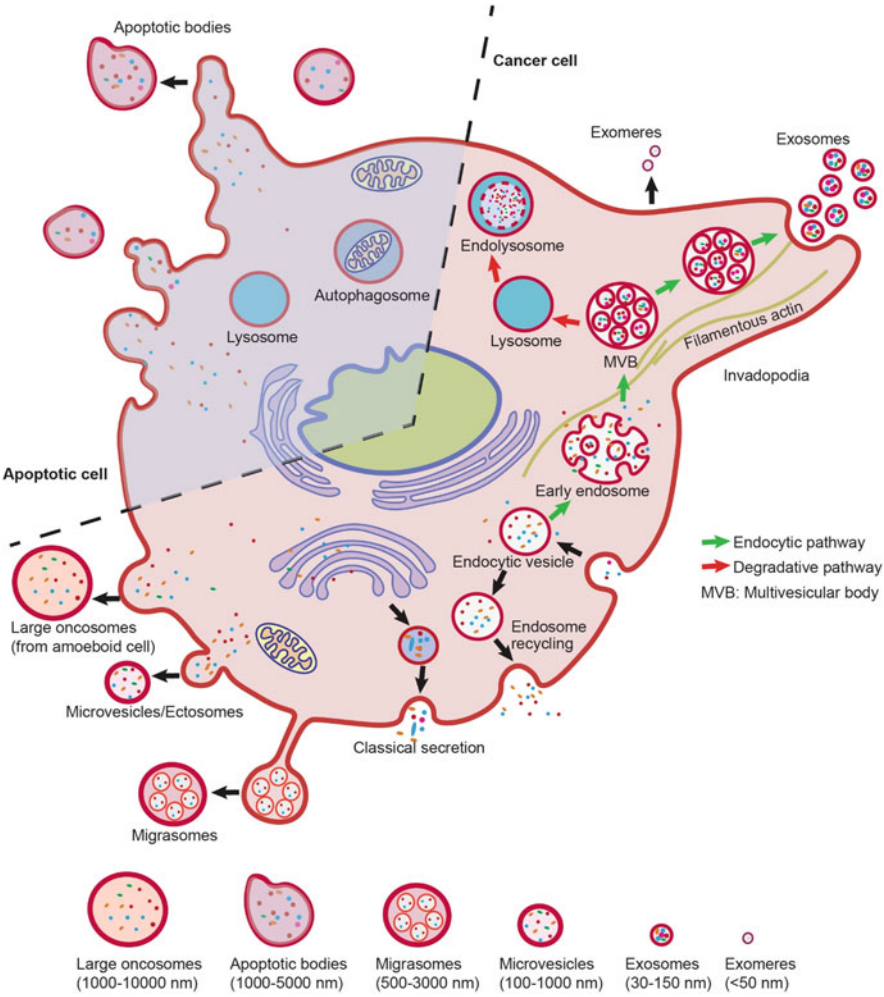

Fig. 11.1 Biogenesis of various types of EVs. (**a**) EVs are secreted by a plethora of cells via various pathways. Depending on the process of biogenesis and size, they can be classified into different subtypes such as exosomes, exomeres, large oncosomes, migrasomes, ectosomes/shedding microvesicles and apoptotic bodies. Biogenesis of exosomes occurs via the endocytic pathway within multivesicular bodies (MVB). During maturation, endosomes give rise to intraluminal vesicles (ILVs) by the inward budding of the endosomal membrane. Inside the ILVs, cargo content including proteins, lipids, metabolites and nucleic acids are packaged. Within a single MVB, multiple ILVs are formed. MVBs can either fuse with lysosomes and undergo degradation or they can be transported via the actin tubular network to undergo cell membrane docking. The MVB and plasma membrane later fuse releasing the ILVs, as exosomes, into the extracellular environment. Apoptotic bodies are released by dying cells, and shedding microvesicles or ectosomes are formed by budding from the plasma membrane. Large oncosomes are secreted by plasma membrane blebbing from ameboid cancer cells. Migrasomes originate from retraction fibers of migrating cells. The biogenesis of the newest EV subtype exomeres remains unclear. (**b**) Various subtypes of EVs with their size range

transferring functional cargo contents from one cell to another, thus aiding in cellular communication (McAndrews and Kalluri 2019; Fonseka et al. 2019; Chitti et al. 2018; Anand et al. 2019). EV cargo includes proteins, lipids and metabolites in addition to genetic material in the form of RNA and DNA (Mittelbrunn and Sánchez-Madrid 2012; Yokoi et al. 2019; Valadi et al. 2007; Anand et al. 2019; Pathan et al. 2019). Hence, the cargo content of the EVs are thought to play a key role in cancer progression and metastasis.

Metastatic Cancer

Metastasis of cancerous cells from the primary site to other organ/s is a notable characteristic of aggressive cancer progression. This also applies to instances where metastatic tumors are observed in various parts of the body without an identified primary site. Such a condition occurs in only 5% of total documented metastatic cancers and this form of metastasis is called carcinoma of unknown primary origin (Seyfried and Huysentruyt 2013). Tumors that are limited to a single site, or a 'benign form' are not cancerous and are manageable. Whilst, tumor cells that have invaded surrounding tissues and spread to secondary sites, are classified as cancerous with the ability to metastasise. Even with advanced treatment strategies such as chemotherapy, radiation, surgery and other therapeutic options, metastatic cancers are generally not curable and are mostly fatal (Weidle et al. 2017). Hence, most cancer related deaths are attributed to metastasis, and it accounts for almost 90% of cancer deaths worldwide (Seyfried and Huysentruyt 2013). Hence, there is an unmet need to unravel the unknown mechanism of metastasis and find a way to manage this hallmark of cancer progression (Fares et al. 2020).

Though decades of research have identified several ways by which cancer cells in the primary site can invade neighboring tissues, various mechanisms at the molecular level underlying cancer metastasis are currently unclear (Leong Hon et al. 2014). Multiple factors are required to operate and proceed concordantly to favor disease progression such as; growth in primary site, invasion from the primary tissue into the basement membrane, then to blood and lymphatic circulation (intravasation), immune evasion, extravasation from the circulation to secondary organs and growth to form a whole new tumor at a secondary site (Seyfried and Huysentruyt 2013). Emerging evidences suggest that EVs are implicated, at least in part, in governing various aspects of this metastasis cascade.

EVs as Communicative Vessels of Cancer Progression

The precise function of EVs is context dependent and thought to vary based on the cargo content and the recipient cells (Gangoda et al. 2015). Cargo of EVs vary depending on the type of cell they are released from (Mathivanan et al. 2010; Anand

et al. 2019), for instance, protein cargo of breast cancer cell-derived EVs can appear as signature of the breast cancer subtypes (Rontogianni et al. 2019). Nucleic acids such as DNA, present in EVs, is being studied as a potential biomarker for diseases and micro RNAs (miRNAs) have also been found to be useful biomarkers in the diagnosis of cholangiocarcinoma and gall bladder carcinoma (Xue et al. 2020; Wang et al. 2018). Furthermore, these EVs have been proposed as potential targets for therapeutic management of cancer (Kalluri and LeBleu 2020; Zitvogel et al. 1998). This is because EVs govern several functions as communicative vessels driving cancer progression and metastasis as shown in Fig. 11.2.

Cellular Communication Influencing Recipient Cell Phenotype

EV-mediated intercellular communication occurs among cancer cells, stem/stromal and normal cells either present within vicinity (via paracrine signaling) or distant sites (via endocrine signaling). EVs transfer pro-metastatic components such as proteins and nucleic acids from cancer cells to recipient cells (Wortzel et al. 2019; Kalra et al. 2019). Hence, EV cargo components can induce phenotypic changes in the recipient cells, which can favor disease progression (Liem et al. 2017). For instance, cells that are normally immotile and fully differentiated can become hyperproliferative and motile following the uptake of EVs secreted by cancer cells and cancer associated stem cells. Melanoma EVs, for instance, have been shown to transfer tumor metastatic marker Met-72 to poorly metastatic F1 cells. Injection of F1 cells, pre-treated with B16-derived EVs into mice resulted in the occurrence of lung metastasis suggesting that EVs indeed transfer pro-metastatic factors (Hao et al. 2006). Similarly, EVs from colon cancer cells with mutated K-Ras gene (Kristen rat sarcoma gene) (KRAS) have been implicated in the transfer of mutant KRAS as well as other proteins such as integrins and epidermal growth factor receptor (EGFR) to normal or wild-type KRAS cells. Uptake of such EVs was shown to induce oncogenic properties in these recipient cells, thereby promoting tumor growth (Mannavola et al. 2019).

Cancer cell-derived EVs are also known to contain proteins such as transforming growth factor β (TGFβ)-1, wingless-related integration site (WNT), epidermal growth factor (EGF), hematopoietic growth factor (HGF) and matrix metalloproteinases (MMPs) that are involved in the induction of epithelial-to-mesenchymal transition (EMT), thereby promoting motility and invasiveness (Suchorska and Lach 2016). As mentioned earlier, protein cargo of cancer cell-derived EVs are known to be enriched with pro-metastatic factors (Gowda et al. 2020). It has been demonstrated that cargo from EVs released by highly metastatic breast cancer cells are significantly diverse compared to non-metastatic breast cancer cells. The difference is due to the presence of abundant proteins implicated in proliferation, migration, invasion, as well as receptor recognition for specific metastatic sites in EVs derived from metastatic cancer cells (Gangoda et al. 2017; Lo Cicero et al. 2015). In contrast, the non-metastatic cancer cell-derived EVs were

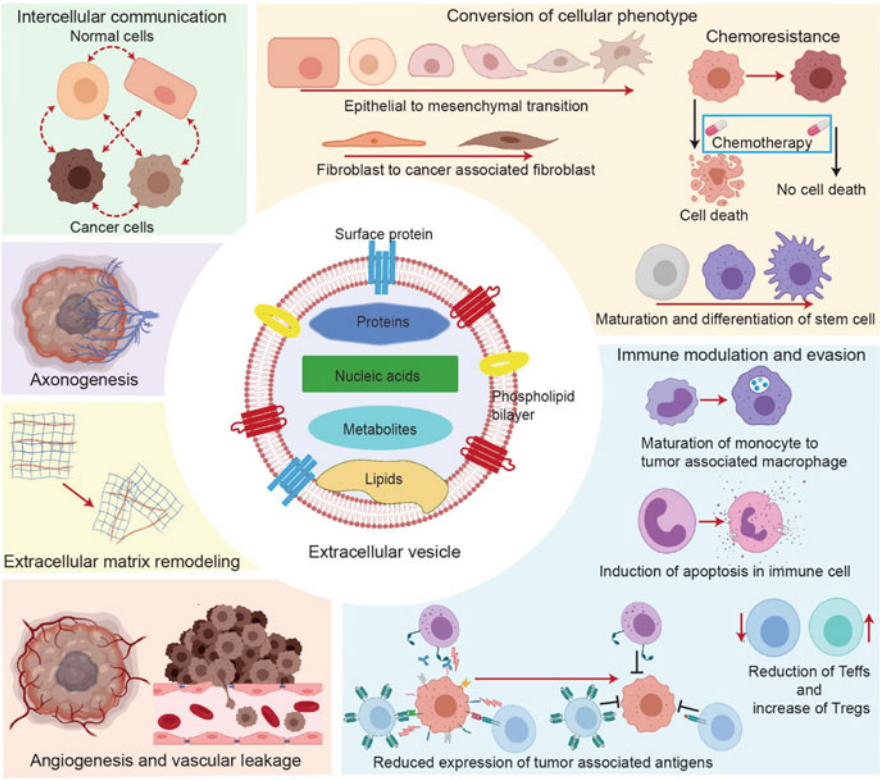

Fig. 11.2 Roles of EVs in cancer progression and metastatic niche formation. EVs play a significant role in cancer progression and metastasis. Functional effects of EVs are exerted by the cargo contained within the EVs. EVs can initiate epithelial-to-mesenchymal transition (EMT) to promote cell migration and can induce conversion of fibroblasts into cancer associated fibroblasts. Similarly, EVs aid in immune evasion by suppressing immune response against cancer cells. This is achieved by conversion of monocytes into immune suppressive tumor associated macrophages, influencing the conversion of effector T-cells (Teffs) into regulatory T-cells (Tregs) and reduced expression of surface antigens in cancer cells. Furthermore, it has been established that cancer cell-derived EVs induce apoptosis in anti-tumor immune cells. EVs also aid circulating cancer cells to extravasate into the secondary organ by inducing vascular leakage. The growth of cancer cells that extravasate into secondary site requires extracellular matrix (ECM) modulation, as well as formation of nurturing blood vessels (angiogenesis), driven by small EVs. EVs induce tumor innervation, a phenomenon that may favor metastasis. EVs are also known to play a role in the development of chemoresistance. The role of EVs in each individual step of cancer development suggests that they drive metastasis by allowing formation of a metastatic niche in the secondary site. Figure is created using BioRender.com

enriched with proteins implicated in cell-to-cell adhesion and polarity (Gangoda et al. 2017). Furthermore, TGFβ mRNA or protein induced the conversion of fibroblasts to myofibroblasts that act as cancer associated fibroblasts within the tumor microenvironment (Webber et al. 2010b). These cancer associated fibroblasts also release EVs that have been implicated in the activation of WNT-planar cell

polarity (PCP) signaling in recipient cells, clarifying that EVs released from cancer associated fibroblasts (CAFs) also play role in the pathophysiology (Suchorska and Lach 2016). Besides proteins, several miRNAs are carried within EVs, that are also implicated in the promotion of EMT, as well as drug resistance, in both normal or cancerous cells (Wang et al. 2017; Kim et al. 2020). The transfer of various miRNAs has been shown to activate the phosphoinositide-3 kinase (PI3K)/Akt, Notch, extracellular signal-regulated kinase (ERK) pathway resulting in the induction of EMT in recipient cells (Kim et al. 2020). One such miRNA is miRNA-21 which is enriched in EVs that significantly aid in the proliferation and metastasis of breast cancer by persuading EMT (Wang et al. 2019). Overall, these findings demonstrate that EVs transfer functional cargo to recipient cells and influence their behavior in favor of cancer progression and metastasis.

Angiogenesis and Vascular Leakiness

Angiogenesis is an essential process to sustain nutrient demand for tumor growth, as well as for tumor cell dissemination to distant site for metastasis. This complex process involves several molecular mediators and activation of several pro-angiogenic pathways in endothelial cells (Ucuzian et al. 2010). Here, the tumor microenvironment remains under hypoxic conditions, during the growth of cancer cells, which in-turn induces the secretion of vascular endothelial growth factor (VEGF) from the tumor cells initiating angiogenesis. This process of tumor adaptation in an hypoxic environment is known as the "angiogenic switch" (Krock et al. 2011). EVs secreted by cancer cells are enriched with VEGF, interleukin (IL)-8, insulin-like growth factor (IGF) β1, IGFβ3, Caveolin1, MMP9 and platelet-derived growth factor (PDGF) which are all implicated in angiogenesis. Furthermore, it has been shown that hypoxia and multiple stress conditions induce the release of excessive amounts of EVs from tumor cells. For instance, hypoxic glioblastoma-derived EVs were found to be enriched with proangiogenic proteins IGFβ1/3 that aid in the migration of endothelial cells (Suchorska and Lach 2016). Furthermore, the contents within EVs which are released from stressed tumor cells are enriched with cell-cycle related mRNAs and WNT related proteins. These cargo components are implicated in the activation of β-catenin intercellular signaling within endothelial cells, enhancing their proliferation (Gowda et al. 2020). Similarly, melanoma cells with an elevated levels of WNT5A release large numbers of EVs enriched with proangiogenic factors (Ekström et al. 2014). These pro-angiogenic factors are involved in migration, proliferation and network formation of the endothelial cells, aiding in blood vessel formation. Furthermore, melanoma EVs can carry miRNA-9 resulting in increased angiogenesis via the activation of the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway. Similarly, hepatocellular carcinoma and breast cancer cell-derived EVs are known to contain several miRNAs including miRNA-210 that can promote angiogenesis (Lin et al. 2018; Jung et al. 2017). It seems apparent that these angiogenic EVs are key

regulators for the formation of blood vessels not only in primary tumors but also in metastatic sites thus favoring the development of secondary tumors.

In addition to angiogenesis, vascular permeability is an essential factor inherited by cancer cells to promote invasion. In support of this, EVs released by melanoma cells have shown to increase vascular permeability of mouse lungs, following intravenous injection, suggesting that EVs aid in extravasation of circulating cancer cells to organs such as the lungs (Peinado et al. 2012). Additionally, EVs can transfer miRNA-105 and miRNA-23a to endothelial cells, which targets surface expression of Zona occludens-1 (ZO-1), causing disruption of endothelial tight junctions. This leads to vascular leakage in the lungs and liver thereby facilitating metastasis at these two sites (Wortzel et al. 2019; Hsu et al. 2017).

Extracellular Matrix Remodeling

During cancer pathogenesis, degradation and remodeling of the extracellular matrix (ECM) assists in the growth of the primary tumor (Mathivanan 2017; Mathivanan et al. 2012). Tumor cell-derived EVs with their corresponding cargo such as metalloproteinases, a disintegrin and metalloproteinases (ADAM) and ADAMs with thrombospondin motifs (ADAMTS) contribute to this remodeling. Additionally, invadopodia are known to act as key mediators for matrix remodeling and invasion of tissue by cancer cells during metastasis (Williams et al. 2019; Gowda et al. 2020). These actin-rich protrusions of the membrane are one of the prime sites in which EV release occur (Fig. 11.1). Colocalization studies suggest that EVs enriched with MMPs cause matrix degradation at the site where invadopodia are located (Beghein et al. 2018; Sinha et al. 2016). These findings suggest that invadopodia-mediated matrix degradation and invasion may be, at least in part, mediated by EVs.

Invasion of cancer cells is facilitated by activation of MMP2 via the secretion of heat shock protein (HSP)90α containing EVs, as demonstrated with models of breast cancer. Breast cancer cell-derived EVs containing HSP90α are shown to induce motility and alter the morphology of cells to facilitate migration *in vitro*. Moreover, these EVs demonstrated invasive properties by digesting collagen blocks, *in vitro*, clarifying their role in ECM remodeling (Suchorska and Lach 2016; McCready et al. 2010). Similarly, melanoma cell-derived EVs were found to be enriched with WNT protein, WNT5A, which is associated with metastatic disease and poor prognosis (Ekström et al. 2014). The quantity of WNT5A protein was also proportional to IL-6, IL-8, VEGF and MMP2 levels within melanoma EVs. All of these proteins are implicated in matrix remodeling, angiogenesis and progression of disease, and involved in facilitating metastasis suggesting positive correlation between EV mediated WNT signaling and metastasis (Suchorska and Lach 2016).

As evidenced by Libring and colleagues, breast cancer cells release EVs that educate fibroblasts inducing robust stiffness of the developing tumor by fibronectin matrix accumulation. This form of tumor-integrated rigidity is considered as

hallmark of cancer progression (Libring et al. 2020). Furthermore, this phenomenon of matrix remodeling is not exclusive to the primary tumor as it also occurs within secondary metastatic tumor disseminated sites via the activity of ECM-remodeling proteins such as MMP1, tetraspanin (Tspan) 8, podocalyxin, and cluster of differentiation (CD)151 and others packaged within EVs (Wortzel et al. 2019).

Immune Modulation and Evasion

The immune system normally clears cells that have the potential to become malignant. Immune cells perform this task by recognizing surface markers expressed by cells undergoing malignant or oncogenic transformation, then proceed to eradicate damaged cells usually via phagocytosis. It is therefore essential for tumors to evade these defense mechanisms, imparted by the host immune system in order to persist (Wortzel et al. 2019). This is achieved by reducing the expression of tumor associated antigens on the cell surface to avoid being recognized by immune cells and by secreting soluble proteins that are immunosuppressive. Immunosuppressive CD39 and CD37 expressed in EVs, and on cancer cell surface, convert ATP into immune suppressive adenosines that attenuate the T-cell response thereby protecting cancer cells (Allard et al. 2017; Yang et al. 2020). Along with this, TGFβ carried by cancer cell-derived EVs, is able to attenuate the function of natural killer (NK) cells by reducing the expression of the receptor-NK group 2 member D (NKG2D receptor) (Espinoza et al. 2012; Lazarova and Steinle 2019). Similarly, tumor-derived EVs increase the presence of negative immune system regulators such as myeloid-derived suppressor cells (MDSCs) and monocytes in tumor microenvironment to assist in immune evasion. During tumor development, monocyte internalization of EVs containing miRNA-203 mediates the expression of M2 markers, in-turn switching the differentiation status of these monocytes to that of immunosuppressive tumor associated macrophages (TAMs) (Mannavola et al. 2019). This evidence suggests that TAMs are essential components of the tumor microenvironment crucial for regulating cancer metastasis and progression (Biswas et al. 2019; Mannavola et al. 2019). Cancer cell-derived EVs have shown to additionally induce conversion of effector T-cells (Teff) to regulatory T-cells (Treg) which is essential for immune evasion as well as growth of cancer cells (Yamada et al. 2016; Yin et al. 2014). As mentioned earlier, EVs containing TGFβ are shown to reduce the expression of anti-apoptotic B-cell leukemia (BCL)-2 protein in immune cells leading to programmed cell death (Kim et al. 2005). Similarly, cancer cells can induce apoptosis of cytotoxic T-cells via the Fas ligand and Tumor necrosis factor-related apoptosis-inducing *ligand* (TRAIL) (Ichinose et al. 2001; Martınez-Lorenzo et al. 2004; Weidle et al. 2017). The vesicles released from cancer cells expressing FasL on their surface bind to the Fas receptor on the activated immune cells and direct them to undergo apoptosis preventing the cancer cells from anti-cancer immune response (Suchorska and Lach 2016). This clearly highlights that EVs are responsible for reduced immune

response against the cancer cells by either killing the immune cells, masking the cancer specific antigens and modulating the immune cell phenotypes.

Role of Extracellular Vesicles in Axonogenesis

Tumor nerve innervation, known as axonogenesis, has been found to be associated with the progression of tumors. Tumors that are extensively innervated have shown to have significantly higher metastatic potential (Madeo et al. 2018). Studies have demonstrated that EVs released from head and neck squamous cancer cells contain EphrinB1, that can stimulate the robust formation of neurites by PC12 cells (rat pheochromocytoma cell). *In vivo* investigation performed in a murine model showed that innervation of head and neck squamous cell tumor is significantly reduced when EV release is blocked using a neutral sphingomyelinase 2 (nSMase2) inhibitor GW4869 (Madeo et al. 2018; Cheng et al. 2018). It is possible that EVs might be playing a role in inducing metastasis by influencing the innervation of developing tumor. However, extensive research is necessary to validate these preliminary findings.

Recruitment and Education of Stem/Progenitor Cells

One key function of EVs is to educate normal or stromal cells, eliciting changes in their phenotype favoring the growth and development of cancer. It has been reported that EVs aid in the expansion and maturation of stem cells rendering them motile and therefore enabling them to be recruited to metastatic sites for various tumorigenic functions (Wortzel et al. 2019). Similarly, melanoma cell-derived EVs, expressing cMet, aid in the recruitment of the bone marrow-derived progenitor cells (BMDCs) to the lungs supporting lung metastasis of melanoma (Peinado et al. 2012). Additionally, EVs released from educated mesenchymal stem cells have been implicated in the differentiation of immature monocytic myeloid precursors into immunosuppressive TAMs and aiding in immune evasion (Biswas et al. 2019).

Several studies have suggested that induction of EMT can be regulated by proteins such as forkhead box protein (FOX) C2, Twist2 and zinc-finger E homeobox-binding (ZEB) 1 (Aigner et al. 2007; Hollier et al. 2013; Li et al. 2012). Activation of EMT leads to the conversion of cancer cells into cancer-associated stem cells (Xu et al. 2018). More importantly, proteins implicated in EMT, are evidently enriched in EVs from several malignant cancers. Uptake of these EVs by epithelial cancer or normal stem cells results in the differentiation of these cells into mesenchymal cancer or cancer stem cells favoring cancer metastasis (Conigliaro and Cicchini 2018; Vella 2014). In contrast, breast and prostate cancer cell-derived EVs play an important role in differentiation of fibroblasts to myofibroblasts via induction of mesenchymal to epithelial transition (MET). This phenomenon

suggests phenotype conversion for recruitment of cancer associated cells to tumor microenvironment (Vella 2014; Cho et al. 2012; Webber et al. 2010a). Furthermore, studies have shown that mesenchymal stem cells (MSCs)-derived from adipose tissue and umbilical cord from ovarian cancer and gastric cancer models, respectively, form cancer associated fibroblasts upon activation by EVs (Vella 2014). EVs released by melanoma cells have been shown to deliver Gm26809 to fibroblasts leading to conversion into CAFs (Gowda et al. 2020). These CAFs in turn release EVs enriched with miRNA-21 that increases aggressiveness of recipient cancer cells (Donnarumma et al. 2017). These findings show that education of stem/stromal cells by cancer cell-derived EVs occurs via several mechanisms bringing about phenotypic changes in the recipient cells that favor tumor progression and metastasis.

Organ Specificity in Cancer Metastasis, 'Seed, Soil and Fertilizer Concept'

The tumor microenvironment is made up of heterogenous cancer-associated cells, including stromal, immune and organ specific cells. The process of heterogenous cell recruitment to secondary sites allows for the formation of a secondary tumor microenvironment, which is not a random event. This concept is substantiated by the fact that all cancers metastasise to predetermined sites or organs and exhibit specific tropism. For instance, breast cancer primarily metastasises to bone, the lungs, the liver and the brain while colon cancer metastasizes to the liver, peritoneum and the lungs (Meleth et al. 2013). Cancers spreading to specific organs was first described by a surgeon Stephen Paget, in 1989, who coined this phenomenon as the "seed and soil" hypothesis. It was proposed that specific cancer cells that intravasate into the circulation (the seeds) can extravasate and adhere to specific organ sites of which harbor a favorable environment for dissemination (the soil) therefore allowing for tumor growth (Fig. 11.3) (Fidler 2003; Paget 1989). Numerous studies have shown that key regulators of this phenomenon are EVs secreted by tumor and cancer associated cells. They aid in the formation of pre-metastatic niche at the secondary site before the arrival of cancer cells as well as aid in the growth of secondary tumor and hence act as fertilizers in the seed and soil hypothesis (Guo et al. 2019; Peinado et al. 2012).

Mechanisms of Metastasis Via EV Mediated Pre-Metastatic Niche Formation

It has been proposed that cancer cells from primary tumor can initiate a cross talk with cells in secondary organ including local stem cells, immune cells and progenitors in bone marrow (Hoshino et al. 2015; Peinado et al. 2012). This crosstalk

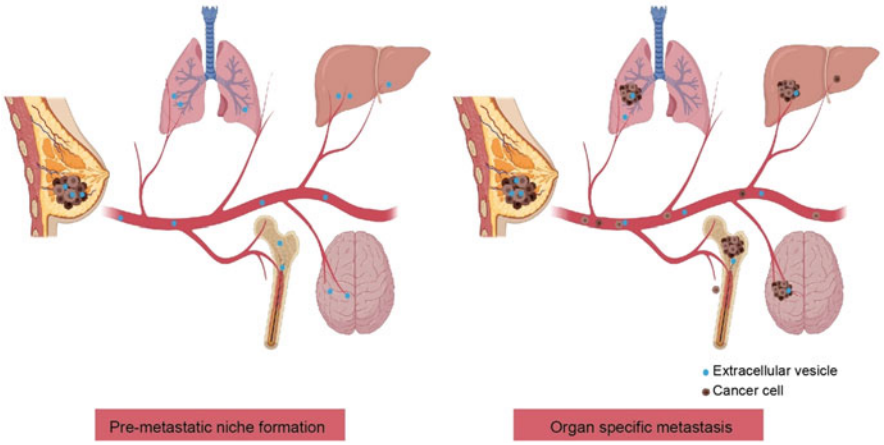

Fig. 11.3 EVs form pre-metastatic niche in specific organs and drive organ specific metastasis. (**a**) EVs released from breast cancer cells into the circulation travel throughout the body forming a pre-metastatic niche in specific organs. (**b**) Circulating tumor cells, intravasate secondary sites where a pre-metastatic niche is formed allowing for growth of secondary tumors. Figure is created using BioRender.com

results in the conversion of phenotypes, recruitment of cancer supportive cells in secondary sites and their expansion forming pre-metastatic niche to support metastatic cancer cells. As discussed earlier, for metastasis, EVs can induce migration and invasion of cancer cells. For instance, treatment of weakly metastatic MCF7 breast cancer cells with the EVs-derived from highly metastatic breast cancer cell lines, MDA-MB-231, increases the migration capacity of MCF7 cells. Furthermore, treatment of MDA-MB-231 cells with the EVs-derived from the MDA-MB-231 itself induced further robust cell migration showing the potential of EVs in inducing the cell motility, the key initial step of metastasis (Harris et al. 2015). This and several studies also suggested that EVs from highly metastatic cancer cells have distinct cargo content than that of non-metastatic cells suggesting that the ability to induce metastasis is limited to EVs released from malignant cells (Harris et al. 2015; Gangoda et al. 2017; Wortzel et al. 2019; Plebanek et al. 2017).

EVs enriched in miRNA-21-p induced mesothelial to mesenchymal transition (MMT) of the peritoneal cells as preliminary step to form pre-metastatic niche to facilitate peritoneal metastasis of gastric cancer (Li et al. 2018). Furthermore, progenitor cells such as BMDCs educated with EVs are recruited to the secondary site to aid in pre-metastatic niche formation (Peinado et al. 2012). These cells are known to regulate the expression of receptors in the pre-metastatic niche stromal cells so that the incoming tumor cells can be recognized by stromal cells (Wortzel et al. 2019). Furthermore, formation of a premetastatic niche involves the recruitment of immune suppressive cells to the secondary site. Hematopoietic stem cells are recruited to the metastatic sites where they can engage in immunosuppressive mechanisms against anti-cancer immune cells (Kaplan et al. 2005). EVs from

melanoma cells have been known to contain programmed death-ligand1 (PD-L1) which aid in immune suppression at the metastatic site via suppression of cytotoxic $CD8^{+}$T cells response (Chen et al. 2018). Endorsement of cancer specific secondary metastasis originates from studies performed by the Lyden group. EVs contain surface integrins which are recognized by receptors on the surface of specific tissues, thereby, driving organ specific metastasis (Hoshino et al. 2015). This finding highlighted the importance of EVs as metastatic drivers playing a vital role in formation of the pre-metastatic niche in specific organs. EVs with surface markers integrin (ITG)α6β1 and ITGα6β4 are lung trophic, EVs with ITGβ3 are brain trophic and ITGαVβ6 are liver trophic. The study also demonstrated that upon treatment with EVs-derived from lung metastatic tumor, cancer cells that were prone to metastasize to the bone would instead form secondary tumors in lungs which unraveled the role of EVs in driving organotropism and metastasis (Hoshino et al. 2015). Similarly, EV cargo protein cell migration-inducing and hyaluronan-binding protein (CEMIP) was able to regulate invasion, migration, and modulate blood vasculature in favor of cancer development. EVs containing CEMIP were also able to induce inflammation by upregulating pro-inflammatory cytokines in the perivascular niche. However, EVs lacking CEMIP protein inhibited brain tropism of the EVs. This study further validates that EVs are organ specific and create pre-metastatic niches in a selective manner (Rodrigues et al. 2019). Taken together, several studies support the role of EVs in the formation of pre-metastatic niche and in the regulation of metastatic organotropism.

Targeting EVs for the Inhibition of Cancer Metastasis

As EVs have been implicated in several pro-metastatic mechanisms, several groups have considered targeting the release of EVs secreted by the tumor and cancer associated cells as means to reduce metastasis. Hence, inhibition of EVs has been proposed as a potential mechanism to impede disease progression and open up novel avenues for cancer therapy (Tang et al. 2019; Yuana et al. 2013). There are various methods to inhibit the secretion of EVs from cells. These methods can either target the genes that regulate the biogenesis/secretion of EVs by genetic approach or pharmacological using the drugs. For instance, silencing of the Rab27a, which is involved in the fusion of the MVBs to the plasma membrane for release of ILVs as exosomes, has been shown to reduce the secretion of EVs (Iguchi et al. 2016; Ostrowski et al. 2010; Peinado et al. 2012). Inhibition of EV release by targeting Rab27a prevented both primary tumor growth and lung metastasis in a murine mammary carcinoma models (Bobrie et al. 2012).

In HeLa cells, knockdown of ESCRT-0/1 proteins; HRS, STAM1 and TSG101, revealed to be beneficial in the reduction of EV secretion by inhibiting their biogenesis. On the other hand, using a pharmaceutical approach, utilizing drugs such as GW4869 could potentially inhibit EV release. The ceramide inhibitor GW4869 has been widely studied as component in the reduction of EV release in

both *in vitro* and *in vivo* studies (Faict et al. 2018; Iguchi et al. 2016; McAndrews and Kalluri 2019; Panigrahi et al. 2018). However, controversies remain for use of GW4869 in the *in vivo* settings due to its solubility issue. GW4869 is soluble in high concentration of DMSO, which is toxic to rodents. Furthermore, GW4869 has not been tested in any clinical trials for safe administration in humans and hence has no clinical utility at the moment. Several drugs are currently being identified by high-throughput studies to target EV biogenesis or release. Some of the drugs that have been shown to reduce EVs are Tipifarnib, Imipramine, Calpeptin and Manumycin A. These drugs can target various molecules involved in the EV pathway ultimately reducing the amount of EVs released from cells (Catalano and O'Driscoll 2020; Datta et al. 2018). Recently, FDA approved antibiotic SFX was proposed to decrease the release of EVs from breast cancer cells and thereby inhibit metastasis (Im et al. 2019). However, the drug SFX was proposed to not inhibit EV release and hence the efficacy of the drug in attenuating EV release remains controversial (Mathivanan, unpublished observations). Even though targeting of EV release to treat cancer metastasis is warranted, it cannot be ignored that EVs may play several important roles in the normal physiology and homeostasis. Thus, targeting EV release to prevent cancer metastasis might bring about other undesired side effects. Hence, pre-clinical and clinical studies are needed to test the side effects of blocking the EV biogenesis and secretion pathways.

Conclusion

EVs play a vital role in intercellular communication. They orchestrate several functions not only in maintaining normal physiology but are also involved in several disease pathologies. EVs are known to aid in multiple steps of cancer development, progression and metastasis. Additionally, EVs play a pivotal role in pre-metastatic niche formation which is essential for the establishment and growth of metastatic tumor. Therefore, blocking EV release could be the key to control metastasis and cancer progression. However, further studies are needed to identify therapeutic targets and drugs that can halt EV driven metastasis in a clinical setting.

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Chapter 12
Extracellular Vesicle-Mediated Bone Remodeling and Bone Metastasis: Implications in Prostate Cancer

Kalyani C. Patil and Carolina Soekmadji

Abstract Bone metastasis is the tendency of certain primary tumors to spawn and dictate secondary neoplasia in the bone. The process of bone metastasis is regulated by the dynamic crosstalk between metastatic cancer cells, cellular components of the bone marrow microenvironment (osteoblasts, osteoclasts, and osteocytes), and the bone matrix. The feed-forward loop mechanisms governs the co-option of homeostatic bone remodeling by cancer cells in bone. Recent developments have highlighted the discovery of extracellular vesicles (EVs) and their diverse roles in distant outgrowths. Several studies have implicated EV-mediated interactions between cancer cells and the bone microenvironment in synergistically promoting pathological skeletal metabolism in the metastatic site. Nevertheless, the potential role that EVs serve in arbitrating intricate sequences of coordinated events within the bone microenvironment remains an emerging field. In this chapter, we review the role of cellular participants and molecular mechanisms in regulating normal bone physiology and explore the progress of current research into bone-derived EVs in directly triggering and coordinating the processes of physiological bone remodeling. In view of the emerging role of EVs in interorgan crosstalk, this review also highlights the multiple systemic pathophysiological processes orchestrated by the EVs to direct organotropism in bone in prostate cancer. Given the deleterious consequences of bone metastasis and its clinical importance, in-depth knowledge of the multifarious role of EVs in distant organ metastasis is expected to open new possibilities for prognostic evaluation and therapeutic intervention for advanced bone metastatic prostate cancer.

K. C. Patil
Translational Research Institute, Academic Health System, Hamad Medical Corporation, Doha, Qatar

C. Soekmadji (✉)
Department of Cell and Molecular Biology, QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia

School of Biomedical Sciences, Faculty of Medicine, University of Queensland, Brisbane, QLD, Australia
e-mail: Carolina.Soekmadji@qimrberghofer.edu.au

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Introduction

Bone is a very well-adapted, metabolically active tissue that is constantly in a dynamic equilibrium of synthesis and resorption of cellular matrices. It hosts finely-tuned metabolic processes, that are mandatory for maintaining homeostasis, through coordinated activities of several cell types including osteoclasts, osteoblasts, osteocytes, vascular endothelial cells, and their precursors (Tao and Guo 2019). Beyond these connections, it is well-established that a cross-communication exists between bone and different organs that together form an organic unit linked to human health and pathological diseases.

Prostate cancer (PCa) is the most common non-cutaneous malignancy in men and the second most common cause of male cancer-related deaths in the developed world (Ottewell et al. 2014). Although recent decades have seen improved clinical outcomes and survival in men with localized PCa, yet, in comparison, gains in overall survival has been more modest in men with bone metastatic PCa (Croucher et al. 2015). Relative survival statistics from 2009-2015 has shown an increase in a five-year relative survival rate for localized and regional PCa to 100% (Saraswati et al. 2011). On the contrary, distant metastatic cancer has shown a poor prognosis with a five-year relative survival rate of 30.5% (Saraswati et al. 2011). Out of the 3857 patients with metastatic PCa studied between 1991 and 2009, 2.8% of patients were diagnosed with lymph node, 80.2% with bone, 6.1% with visceral, and 10.9% with bone plus visceral metastasis (Gandaglia et al. 2015). Moreover, the median survival of men from diagnosis of bone disease was found to be 6.1 months in comparison to 18.2 months in men with no bone metastasis and 7.1 months in men with the visceral disease (Pezaro et al. 2014). These numbers distinctly indicate the dominance of bone metastasis in the clinical picture of advanced PCa and its significant impact on overall survival. Despite ongoing research efforts, the molecular and cellular mechanisms of bone metastasis and the underlying avidity of PCa cells for the bone microenvironment (osteotropism) remains largely unknown.

In the early twentieth century, Stephen Paget introduced the classical ‘seed-and-soil’ hypothesis where metastasis depends on the crosstalk between selected cancer cells (the ‘seeds’) and specific organ microenvironment (the ‘soil’) (Paget 1989). Research on the identification of factors promoting osteotropism in PCa has focused on the tumor-stroma interface in addition to identifying distinct Bone Metastatic Gene Signature (BoMS) of up- or down-regulated genes associated with metastasis (Chen et al. 2018). Empirical evidence has identified signaling programs that regulate PCa cell homing to and colonization of the bone such as Stromal-Derived Factor-1 (SDF-1)/C-X-C motif chemokine 12 (CXCL12)-C-X-C Chemokine Receptor type 4 (CXCR4) chemokine axis (Sun et al. 2005). Diverse studies have led to the identification of an array of bone-derived growth factors that support the

establishment and orchestrate expansion of PCa in the bone microenvironment (Cook et al. 2014). The pro-tumorigenic role of Hematopoietic stem cells (HSCs)/ Bone marrow stromal cells (BMSCs, also known as mesenchymal stem cells or MSCs) and that of Myeloid-Derived Suppressor Cells (MDSCs), macrophages, dendritic cells, T cells, and nerve cells in immune-regulation of the prostate to bone metastasis have been defined (Cook et al. 2014). Intriguingly, research has also highlighted the active roles of megakaryocytes and platelets, previously thought to be the bystanders in the bone marrow microenvironment, in promoting extravasation and colonization of the bone compartment during PCa bone metastasis (Cook et al. 2014). However, in recent years, these findings have been enlivened with the discovery of extracellular vesicles (EVs) as critical components of the tumor secretome that are involved in crucial steps of metastatic spread of a primary tumor, ranging from oncogenic reprogramming of malignant cells to formation of bone metastasis (Syn et al. 2016).

In aggressive and therapy-resistant malignancies such as advanced metastatic PCa, the regulatory transport circuit involving EVs (vesicular transport) is adopted to support cancer cell survival and outgrowth (Syn et al. 2016). Small EVs (vesicles with diameter < 150 nm), including exosomes, are secreted into the extracellular milieu by most, if not all cell types (Syn et al. 2016; Soekmadji et al. 2017). These vesicles behave as paracrine effectors and guide the export of major types of proteins and transcription factors to the outer-cellular milieu (Gonzalez-Begne et al. 2009) to induce distant signal transduction or mediate the horizontal transfer of information in specific immune cells, such as recipient T cells (Syn et al. 2016). There is a consensus that tumor-derived small EVs carry a functional molecular cargo consisting of oncogenic virus-derived molecules, DNA fragments, and proteins such as Heat Shock Proteins (Hsps), Dicer, Phosphatase and Tensin Homolog (PTEN), p53, and Adenomatous Polyposis Coli (APC) (Syn et al. 2016; Azmi et al. 2013), which mediate adaptive cancer responses (Syn et al. 2016). Studies have shown that small EVs also shuttle mRNAs and microRNAs (miRNA/miR) and mediate epigenetic reprogramming to promote tumor growth, invasiveness, and progression (Camussi et al. 2011). In addition, small EVs have been demonstrated to participate in the bi-directional crosstalk between tumor cells and cells of the host microenvironment to facilitate the adaptation of tumor cells to the new environment (Syn et al. 2016; Beach et al. 2014).

In the current chapter, we will discuss the cellular and molecular mechanisms that regulate physiological bone remodeling, outlining the traditional remodeling dogma in light of emerging EV-mediated regulation. Specifically discussed in detail are the pathophysiological processes orchestrated by EVs to direct tumor cell homing to the bone, colonization of the metastatic site, and development of overt bone metastasis in PCa.

The Bone Remodeling Cycle: Cellular and Molecular Mechanisms

Bone remodeling is an intricate, highly coordinated event that relies on the synchronized activities of multiple cellular participants for repairing microscopic skeletal damage, replacing aged bone and maintaining the integrity of adult skeletal, and mineral homeostasis (Deng et al. 2015). This process is driven by coordinated actions of clusters of bone-resorbing osteoclasts, bone-forming osteoblasts, and bone-sensing osteocytes to achieve the correct balance between bone resorption and bone deposition at the same anatomical location to maintain a constant bone mass (Cappariello et al. 2018; Raggatt and Partridge 2010). The cellular activities of osteoblasts and osteoclasts are tightly coupled—quantitatively and in time and space to maintain the specific architecture of bone throughout adult life (Rucci 2008). In contrast, in bone modeling, the activities of osteoblasts and osteoclasts are not necessarily coupled anatomically or temporally as bone formation occurs independently of osteoclastic resorption resulting in the transformation of the size, shape, and/or micro-architecture of bone (Langdahl et al. 2016; Zhu et al. 2018). This modeling-based bone formation contributes to the periosteal expansion, whereas in bone remodeling, osteoclastic resorption promotes medullary expansion seen at the long bones with aging (Langdahl et al. 2016). While both modeling and remodeling affect the overall bone structure, bone remodeling affects material properties such as microdamage, collagen cross-linking, and mineralization (Allen and Burr 2014).

Anatomically, bone remodeling occurs within a Bone Remodeling Unit (BRU) or Basic Multicellular Unit (BMU) that is characterized as focal and discrete packets of bone throughout the skeleton (Fig. 12.1) (Cashman and Ginty 2003). These temporary anatomical structures are composed of osteoclasts, osteoblasts, osteocytes, and capillary blood supply within a bone-remodeling cavity (Raggatt and Partridge 2010; Kenkre and Bassett 2018). The structure and composition of the BMU vary depending on whether it is located on the trabecular bone surface or in Haversian systems (Kenkre and Bassett 2018). In cancellous bone, the BMU appears as discrete osteonal units or packets that move across the trabecular surface, such that a 'trench' of bone, without perforating the trabeculae. By contrast, in cortical bone, BMU appears as osteonal units wherein osteoclasts dig a 'tunnel' into the cortex which is immediately filled in by the osteoblasts to remove damaged bone (Manolagas 2016; Zhou and Lu 2010). In both instances, the BMU is traversed and encased by a canopy of bone-lining cells that creates a specialized vascular structure—the Bone Remodeling Compartment (BRC) (Eriksen 2010; Hauge et al. 2001). Functionally, the canopy structure and subsequent BRC provides a defined area of remodeling to facilitate 'coupled' osteoclast resorption and osteoblast formation. This close anatomical coupling of osteoclasts and osteoblasts ensures the minimal net change in bone volume during the physiological bone remodeling (Andersen et al. 2004).

Bone remodeling is regulated by numerous local biomechanical factors, systemic/endocrine hormones (Table 12.1), and an array of signaling pathways (Fig. 12.2) that exerts its effect on multiple cell types, and their precursors, in the bone

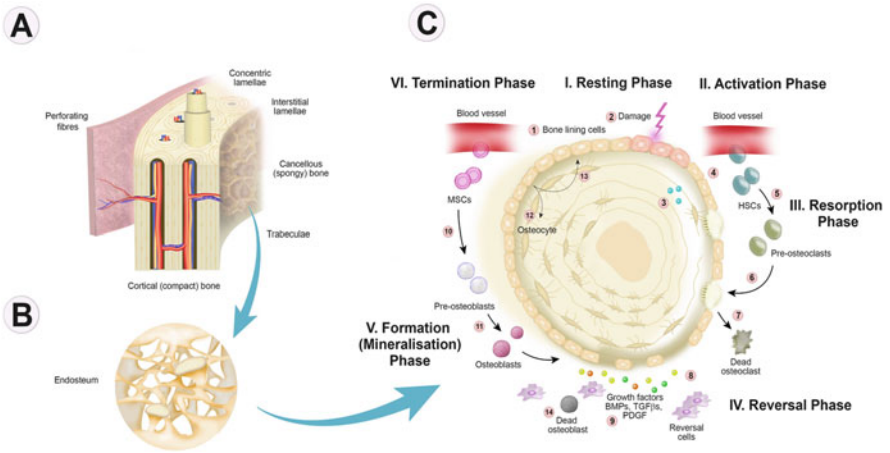

Fig. 12.1 Bone remodeling cycle at a glance. (**a**) Histology of cancellous and cortical bone. (**b**) Enlarged section of cancellous bone trabeculae. (**c**) Cross-sectional diagram of BRU in cancellous bone. The process of bone remodeling that occurs within the discrete osteonal units in cancellous bone consists of sequential and distinct phases of cellular events: Resting Phase (I), Activation phase (II), Resorption phase (III), Reversal Phase (IV), Formation Phase (V), and Termination Phase (VI). Refer text for more details

microenvironment and facilitates: (1) the replication of undifferentiated cell; (2) the recruitment of cells; and (3) the differentiated function of cells (Cashman and Ginty 2003). However, bone remodeling is also controlled by the spatial and temporal arrangement of multiple bone cells within the BMU that govern the coordination of the distinct and sequential phases of bone remodeling cycle—resting, activation, resorption, reversal, formation, and termination (Fig. 12.1c).

In the resting phase, no remodeling activity occurs, instead, the bone surface is lined by a layer of attenuated cells. During the activation phase, different inputs, such as osteocyte apoptosis induced by bone matrix microdamage, changes in mechanical strain sensed by the osteocytes, or release of paracrine factors in the bone microenvironment, such as Insulin-like Growth Factor-1 (IGF-1), Tumor Necrosis Factor-alpha (TNF-α), Parathyroid Hormone (PTH), and Interleukin (IL)-6, cause selective activation of new BMUs on the bone surface (Rucci 2008). In response to these pro-resorption stimuli generated by osteocytes or direct endocrine activation, osteoblasts promote differentiation of mononuclear osteoclast precursors into multinucleated functional osteoclasts via secretion of Receptor Activator of Nuclear Factor-Kappa B ligand (RANKL) and Macrophage Colony-Stimulating Factor (M-CSF) (Zhou and Lu 2010; Kurata et al. 2006). The osteoclasts (both mononucleated and multinucleated) then adhere to the mineralized bone matrix through integrins (ITGs) to form annular sealing zones, called hemivaculae, around bone-resorbing compartments that are isolated from the surrounding bone (Kenkre and Bassett 2018; Zhou and Lu 2010). The formation of hemivaculae and ruffled border, attained through osteoclast plasmalemmal and cytoskeleton rearrangement,

Table 12.1 Summary of endocrine control of bone remodeling

Hormone	Target cell	Mechanism/mode of action	Effect	Net effect on bone	Refs.
Parathyroid hormone	T Lymphocytes	• Stimulates T-cell production of TNF-α • Increases IL-17 expression which stimulates RANKL expression	Increased osteoclast number and activity	Increased bone resorption	Kotake et al. (1999); Wein and Kronenberg (2018); Weitzmann (2013)
		• Stimulates Wnt10B gene expression • Increases CD40L expression that induces expression of the anti-resorptive factor OPG by B lymphocytes	• Increased osteoblast activity • Anabolic osteoblast differentiation	Increased bone formation	Weitzmann (2013); Li et al. (2007)
	Osteoblasts	• Reduces osteoblast apoptosis • Stimulates canonical Wnt/β-catenin signaling • Upregulates osteoblastic MMP-13	Increased osteoblast survival and activity	Increased bone formation	Wein and Kronenberg (2018)
		• Induces osteoblastic expression of MCP-1 • Induces RANKL-mediated recruitment, differentiation, and fusion of monocytes and pre-osteoclastic cells	• Transient increase in osteoclastic activity • Subsequent increase in bone formation	Increased bone mass	Tamasi et al. (2013)
	Osteocytes	• Downregulation of anti-osteoblastogenic Wnt inhibitor sclerostin	Increased osteoblast differentiation and activity	Increased bone formation	O'Brien et al. (2008)
		• Induces osteocytic production and secretion of IL-6 which in turn stimulates RANKL expression in other cells, such as stromal-osteoblastic cells	Increased osteoclast formation	Increased bone resorption	Bellido et al. (2013)

		• Directly stimulates osteocyte RANKL production			
	Bone-lining cells	• Promotes direct conversion into osteoblasts		Increased bone formation	Wein and Kronenberg (2018)
	Skeletal stem cells	• Reduces apoptosis of osteoblast precursors • Increases Runx2 expression in BM-MSPCs	Induction of osteoblastogenesis		Wein and Kronenberg (2018); Yang et a. (2017); Balani et al. (2017)
Estrogen	Osteoclasts	• Inhibits RANKL-induced osteoclast differentiation by blocking RANKL/M-CSF-induced AP-1-dependent transcription and reducing c-Jun activity • Decreases production of pro-resorptive cytokines, including IL-1, IL-6, TNF-α, M-CSF, and PGE2 • Increases production of anti-resorptive cytokines, including IL-1 receptor antagonist and TGF-β • Suppresses RANKL production by osteoblasts, T- and B-cells • Increases OPG secretion by osteoblasts • Suppresses production of TNF-α from T cells • Induces osteoclast apoptosis	Attenuated osteoclastogenesis	Decreased bone resorption	Ammann et al. (1997); Cenci et al. (2000); Kimble et al. (1996); Manolagas and Jilka (1995); Pacifici et al. (1991); Shevde et al. (2000); Hofbauer et al. (1999); Khosla et al. (2012)
	Osteoblasts	• Inhibits osteoblast apoptosis • Increases osteoblast lifespan via activation of the Src/Shc/ERK signaling pathway • Decreases oxidative stress • Decrease NF-κB activity		Maintenance of bone formation	Kousteni et al. (2001, 2003); Khosla et al. (2012)
Vitamin D (active	Osteoblasts/ Osteoclasts	• Increases the rate and expression of osteoblast-mediated osteoclastogenesis by increasing the	Increased osteoclastogenesis	Increased bone resorption	Okada et al. (2002); Medhora et al. (1993); Horwood et al. (1998)

(continued)

Table 12.1 (continued)

Hormone	Target cell	Mechanism/mode of action	Effect	Net effect on bone	Refs.
metabolite 1,25 $(OH)_2D$)		expression of RANKL and decreasing the expression of OPG • Facilitates adhesion of osteoclast precursors to stromal osteoblasts via increased expression of intercellular adhesion molecule-1 • Induces osteoclast precursor differentiation through modulation of osteoclast adhesion molecule, αvβ3 integrin			
	Osteoblasts	• Increases Runx2 expression • Stimulates production of collagen, osteopontin, osteocalcin, and matrix Gla protein • Stimulates activity of ALP • Induces expression of the Wnt signaling regulator LRP-5	Enhanced differentiation and mineralization	• Increased bone formation • Maintenance of bone homeostasis	van Driel and van Leeuwen (2014); Fretz et al. (2007); Haussler et al. (2013)
	Osteocytes	• Induces expression of FGF-23 • Regulates SOST expression	• Response to mechanical loading • Regulation of Vitamin D and phosphate homeostasis • Increased mineralization		Kato et al. (2015); Lanske et al. (2014)

Calcitonin	Osteoclasts	• Causes loss of ruffled border • Induces cell retraction • Reduces osteoclast numbers over time • Modulates secretion of Wnt10b and S1P in osteoclasts	• Decreased osteoclast activity • Paracrine modulation of osteoblast mineralization	• Decreased basal and stimulated bone resorption • Bone formation	Chambers et al. (1986); Chambers and Magnus (1982); Hsiao et al. (2020)

Calcitonin, parathyroid hormone, vitamin D, and estrogen are the major hormones that play significant roles in systemic regulation of bone remodeling. Besides, these hormones act as endocrine regulators of calcium and phosphate homeostasis in the bone. Other hormones such as glucocorticoids, thyroid hormone, and gonadal steroids also regulate physiological bone remodeling (For more information refer (Siddiqui and Partridge 2016). Abbreviations: *BM-MSPCs* Bone marrow-Mesenchymal Stem/Progenitor Cells

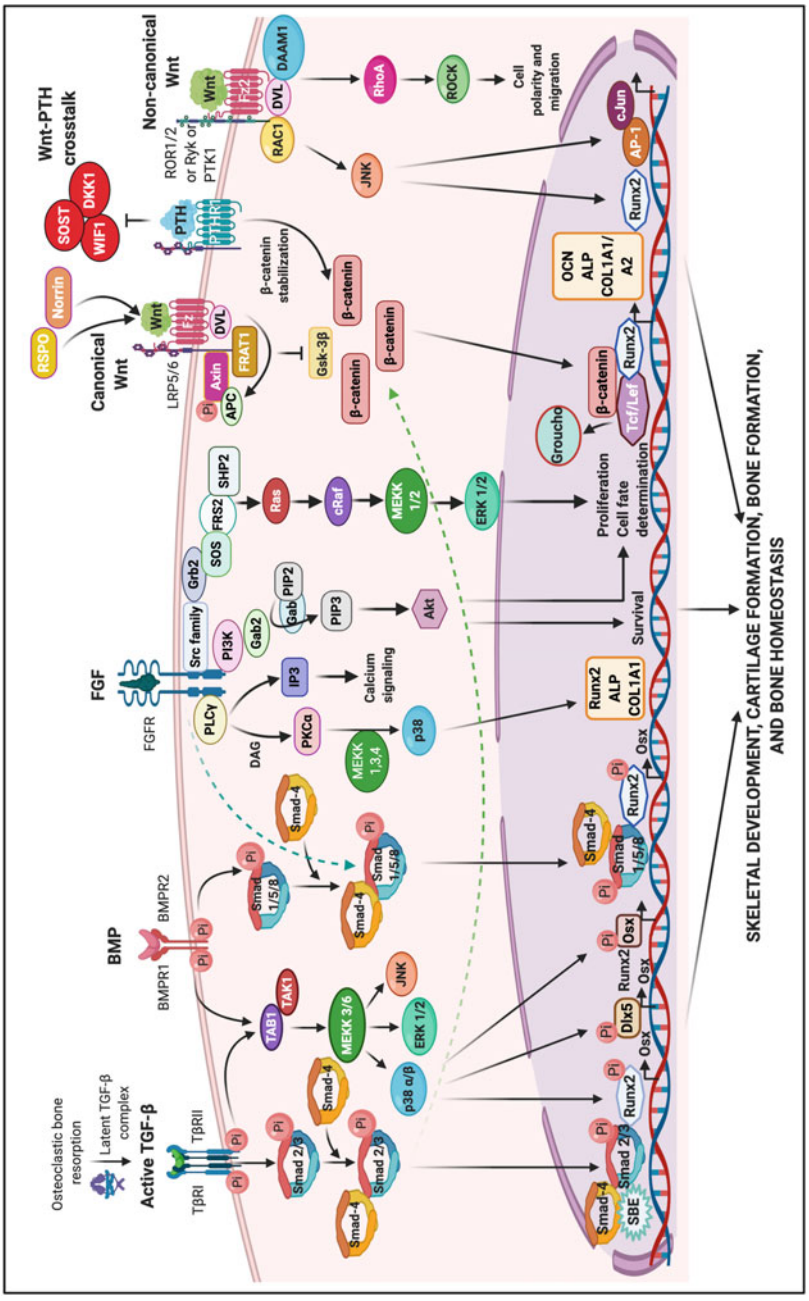

Fig. 12.2 Schematic representation of some of the crucial signaling pathways involved in bone homeostasis. **TGF-β signaling and BMP signaling**: TGF-β and BMP are powerful bone inductive factors enriching the bone matrix and have fundamental roles in both embryonic skeletal development and postnatal bone homeostasis. TGF-βs and BMPs regulate MSC differentiation during skeletal development, bone formation, and bone homeostasis by acting on a tetrameric receptor complex and transducing signals to both the canonical Smad-dependent signaling pathway (that is, TGF-β/BMP ligands, respective receptors, and

R-Smads such as Smad-1, −2, −3, −5, and − 8) and the non-canonical-Smad-independent signaling pathway (involving PI3K/Akt, ERK1/2, JNK, and p38-MAPK cascades) that converge at transcription factors such as Runx2, Dlx5, and Osx (Wu et al. 2016)**. FGF signaling**: The secreted FGFs and four receptor tyrosine kinase receptors (FGFRs) essentially regulate vertebrate skeletal development which occurs through the processes of endochondral and intramembranous bone formation (Ornitz and Itoh 2015) (For more information on the skeletal system and endochondral and intramembranous bone formation, refer (Provot et al. 2013)). During intramembranous bone formation, FGFR-mediated recruitment of intracellular signal transduction proteins, including PLCγ, FRS2, and Src family members initiate several intracellular signaling pathways, including the Ras/ERK pathway, PLCγ/PKCα pathway, and PI3K/Akt pathway. These pathways modulate the intracellular calcium concentrations as well as the expression of transcription factors such as Runx2 that control proliferation, differentiation, migration, adhesion, and survival in cells of the osteoblast lineage (Dailey et al. 2005; Marie et al. 2005). **Wnt signaling**: The Wnt proteins and the different Wnt-signaling pathways such as the canonical (cWnt) or Wnt/β-catenin dependent pathway and the non-canonical or β-catenin-independent pathway have well-defined and fundamental roles in regulating embryological bone development, bone homeostasis, as well as bone repair and regeneration following injury. (For a detailed understanding on Wnt signaling, refer to more extensive reviews (Baron and Kneissel 2013; MacDonald et al. 2009; Clevers and Nusse 2012; Lerner and Ohlsson 2015)). The trans-pathway crosstalk is depicted by dotted arrows

provides a greatly enhanced secretory surface area for bone resorption (Kenkre and Bassett 2018). Several transport systems are localized in the ruffled border including the Vacuolar-type H^+-ATPase (V-ATPase) pump and chloride channel that transport protons and acidify the osteoclastic resorption lacunae. The production of protons for the V-ATPase and the necessary levels of H^+ and Cl^- is ensured by the catalytic activity of Carbonic Anhydrase II (CAII) that mediates the hydration of CO_2 to H_2CO_3, which ionizes into H^+ and HCO_3^-. Meanwhile, basolateral exchange of HCO_3^- ions for Cl^- via an electroneutral chloride/bicarbonate exchanger provides Cl^- ions needed for the intense acidification and degradation of alkaline salts of bone mineral hydroxyapatite in the resorptive microenvironment. Dissolution of mineral crystals allows proteolysis of the type I collagen matrix in bones by acidic proteases such as cathepsin K (catK), gelatinase, Tartrate-Resistant Acid Phosphatase (TRAcP), MMP-9, and MMP-13. The resorbed material is removed from the resorption pit by osteoclast-mediated uptake and transcytosis, after which, osteoclasts either undergo apoptosis or get ready for another round of resorption (Rucci 2008; Henriksen et al. 2011). Following bone resorption, an osteogenic environment is generated at remodeling sites, which is achieved through the release of osteogenic signals by the osteoclasts, ordered cell-cell and cell-matrix rearrangements, and the consequent activation of osteoprogenitors, particularly reversal cells, located on the reversal bone surface (Kristensen et al. 2014). The reversal cells, characterized as osteoblast lineage cells (albeit, controversial), act as the natural intermediates of interactions between the osteoclasts and osteoblasts, as well as specialized candidates that switch the molecular properties of the bone surface from resorbing to reconstructing bone matrix (Andersen et al. 2013; Delaisse 2014). A recent surge in research has indicated that reversal cells may represent the missing link necessary to understand the resorption-formation balance (Andersen et al. 2013). These cells modify the surface of the eroded matrix, left behind by the osteoclast, into a new matrix environment that is critical for the osteoblast maturation and subsequent bone formation (Andersen et al. 2004). The early tasks of reversal cells are to clean the Howship's lacunae covered by unmineralized collagen matrix and generate non-collagenous mineralized matrix 'cement-lines', thereby enhancing osteoblastic adherence and rendering the bone surface favorable to bone formation. The bone formation phase is a two-step process that involves synthesis and secretion of type 1 collagen-rich osteoid matrix by the osteoblasts followed by osteoid mineralization. Mineralization involves the deposition of hydroxyapatite crystals amongst collagen fibrils into the newly deposited osteoid (Kenkre and Bassett 2018) and is regulated by the ratio of inorganic pyrophosphate to phosphate concentrations and relative activities of tissue-nonspecific alkaline phosphatase and ectonucleotide pyrophosphatase (Kenkre and Bassett 2018; Harmey et al. 2004). Systematic evaluation of nature of coupling mechanisms that coordinates the transition of bone resorption to formation has identified several 'coupling stimulators' as well as candidate-coupling mechanisms, including the soluble osteoclastic factor Sphingosine 1-Phosphate (S1P) and the cell anchored Erythropoietin-producing human hepatocellular receptor 4 (EphB4)/ephrinB2 bi-directional signaling complex (Raggatt and Partridge 2010; Matsuo and Otaki 2012). The multimerization of the

Eph-ephrin complex and their bi-directional signaling along with the S1P-S1P receptor interaction are involved in osteoclast-osteoblast communication which initiates the formative phase of bone remodeling. Following mineralization, osteoblasts that have reached the end of their synthetic activity suffer one of the three fates: (1) incorporate in the mineralized matrix and differentiate into osteocytes; (2) convert to a bone-lining phenotype; or (3) undergo apoptosis (Raggatt and Partridge 2010). During the termination phase, osteocytes transmit the 'termination' signal via secretion of antagonists, specifically SOST (Sclerostin) that antagonizes the Wingless-int (Wnt) signaling pathway (Bonewald 2011). Studies have also shown that osteocytes can sense changes in mechanical strain in bone and respond by transmitting signals to the bone-lining cells to initiate targeted remodeling (Zhou and Lu 2010). Osteocytes eventually die by apoptosis, which has been suggested to play an important role in targeted bone remodeling. At the end of the bone remodeling cycle, the resting bone surface environment is re-established and maintained until the next cycle is initiated (Raggatt and Partridge 2010).

Regulation of Bone Remodeling Via CrossTalk Amongst Bone Cells

Osteoclast-Osteoblast Communication

The osteoblast, long recognized as a bone-building cell, is a multifunctional cell that actively regulates osteoclast formation and function as well as HSC homeostasis. Osteoblasts arise from MSCs which, under the influence of a defined suite of regulatory transcription factors, are committed to differentiate to osteoprogenitors in the periosteum (a process termed osteoblastogenesis) (Fig. 12.3) (Rucci 2008; Ottewell 2016). In vitro experiments have shown the importance of direct cell-to-cell interaction of bone marrow stromal cells or osteoblasts and osteoclast progenitor cells in osteoclastogenesis (Udagawa et al. 1989). Osteoclastogenesis is a complex multi-step process involving the commitment, differentiation, multinucleation, and activation of immature osteoclasts (Fig. 12.4).

The osteoblastic stromal cell expression of members of the Tumor Necrosis Factor (TNF) superfamily: Receptor activator of nuclear factor kappa-B ligand (RANKL), its decoy receptor osteoprotegerin (OPG), and its receptor RANK mediate osteoclast differentiation and maturation (Boyce and Xing 2008). RANKL is a type II membrane protein that is typically membrane-bound on osteoblastic and activated T cells (Boyce and Xing 2008). Its receptor, RANK is a type I membrane protein and also a member of the Tumor Necrosis Factor Receptor (TNFR) superfamily. RANK lacks intrinsic protein kinase activating capacity necessary for activating downstream signaling molecules (Cheng et al. 2003); therefore, the signal initiated by RANKL binding to RANK in the early stage is transduced by recruiting adapter molecules belonging to the TNFR-associated factors (TRAFs) family and kinases such as Mitogen-Activated Protein Kinases (MAPKs) (Fig. 12.5). These

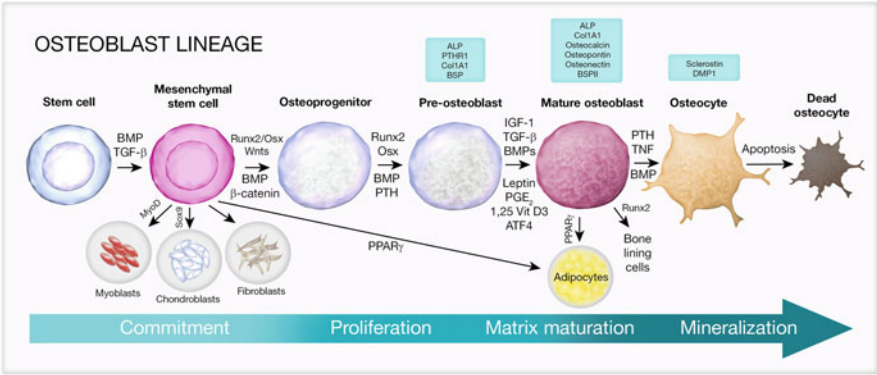

Fig. 12.3 Ontogeny of osteoblast. Schematic representation of osteoblast lineage commitment of MSCs. Like other cells of the connective tissues (fibroblasts, myoblasts, chondrocytes, and adipocytes), osteoblasts arise from common MSCs which, under the influence of Runx2 and Osx are committed to osteoprogenitors in the periosteum. Runx2 is a master gene of osteoblast differentiation and maturation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression (Levanon et al. 1994). Osx is an osteoblast-specific transcription factor and a downstream target of Runx2, that is involved in the genetic program of osteoblast differentiation, bone formation, and bone homeostasis (Sinha and Zhou 2013). Upon stimulation by local growth factors, regulatory transcription factors, and pro-osteogenic pathways (as indicated), the osteoprogenitors differentiate into pre-osteoblast cells expressing progressively mature markers such as ALP and Col1A1 and then finally into active, mature osteoblasts expressing bone matrix proteins such as type I collagen, osteocalcin, osteopontin, osteonectin, and BSPII. Matrix synthesizing mature osteoblasts may have three potential fates: undergo apoptosis, convert to quiescent bone-lining cells, or get buried within lacunae of the mineralized matrix as osteocytes expressing DMP1. At each stage in the lineage, apoptotic cell death is probably an alternative fate (Karsenty et al. 2009). Abbreviations: BSP, Bone Sialoprotein; BSPII, Bone Sialoprotein II; and Col1A1, Collagen type I Alpha 1 chain

signaling complexes control the spatiotemporal regulation of downstream signaling, which results in the RANKL-mediated osteoclastogenesis (Park et al. 2017).

In response to the stimuli generated by the osteotropic hormones and factors such as 1,25(OH)2D, PTH, Prostaglandin E2 (PGE_2), and IL-11, expression of RANKL is upregulated on the membrane surface of osteoblasts. Fuller et al. have shown that the expression of Tumor Necrosis Factor-Related Activation-Induced Cytokine (TRANCE), secreted by osteoblasts and stromal cells, is necessary and sufficient for PTH-mediated activation of mature osteoclasts (Fuller et al. 1998). TRANCE has been identified as an osteoblast-expressed ligand that stimulates osteoclastic differentiation from immature hematopoietic precursors (Lacey et al. 1998). In populations of osteoclasts isolated from neonatal rat bone that were unresponsive to PTH (resembling the cultures with an inability to generate endogenous osteoclast-stimulating activity), TRANCE induced dose-responsive stimulation of bone resorption. This TRANCE-mediated stimulation was found to be equivalent to that induced by the maximally active concentration of PTH in the presence of osteoblastic cells. Moreover, this stimulation can be neutralized by OPG, an osteoblast secreted

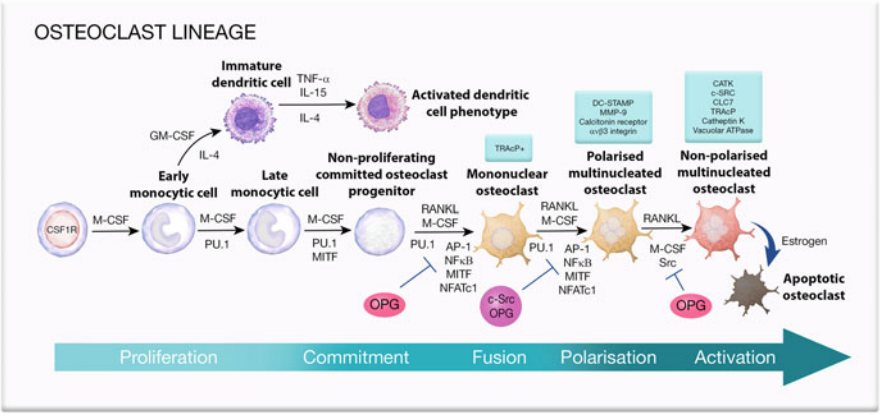

Fig. 12.4 Ontogeny of osteoclast. Schematic representation of osteoclast lineage commitment of HSCs. Osteoclasts arise from HSCs that give rise to common myeloid progenitors as a result of the activation of early factors, such as PU.1 and MITF. With the expression and stimulation of CSF-1R and the activation of intracellular proteins including c-Fos, these myeloid precursors further differentiate into cells of the monocyte/macrophage lineage, which are considered as osteoclast precursors. The commitment, proliferation, and survival of both early-stage and late-stage precursor cells is regulated by the sequential expression of RANK and c-Fms tyrosine kinase, the cognate receptors of RANKL and M-CSF respectively (Raggatt and Partridge 2010; Arai et al. 1999). Co-operative actions of M-CSF and RANKL, expressed in osteoblasts and stromal cells in response to PTH and stimulation by the active dihydroxy form of vitamin D (1,25(OH)2 D), are critical for osteoclast formation. Together with the activation of the principal transcriptional mediators NFκB and NFATc1, expressions of essential osteoclast genes, such as DC-STAMP, cat, TRAcP, MMP-9, and β3 integrin, drive the final differentiation and fusion of the precursors into a mature osteoclast phenotype. Inhibitors of osteoclastogenesis, that is c-Src and OPG, are shown. Abbreviations: PU.1, Spleen focus-forming virus (SFFV) Proviral Integration 1; MITF, Microphthalmia-associated transcription factor; PPARγ, Peroxisome Proliferator-Activated Receptor Gamma; DC-STAMP Dendritic Cell-Specific Transmembrane Protein; and CSF-1R, Colony Stimulating Factor Receptor 1

molecule with an osteoprotective role (Rucci 2008; Lacey et al. 1998). Cellular and molecular studies by Glass and colleagues have shown that the T-cell factor (Tcf) proteins, Tcf 1 and Tcf 4, β-catenin, and presumably Wnt signaling regulate expression of OPG in differentiated osteoblasts, thereby imposing control on osteoclast differentiation (Glass et al. 2005). Considering the role of OPG in inhibiting RANKL/RANK interaction and also TRANCE-mediated bone resorption, it can be deduced that OPG inhibits both osteoclast formation as well as osteoblast-stimulated bone resorption by mature osteoclasts (Fuller et al. 1998).

Osteocyte-Osteoblast Communication

A growing body of evidence now supports the concept that the osteocytes are multifunctional cells capable of orchestrating virtually every aspect of skeletal

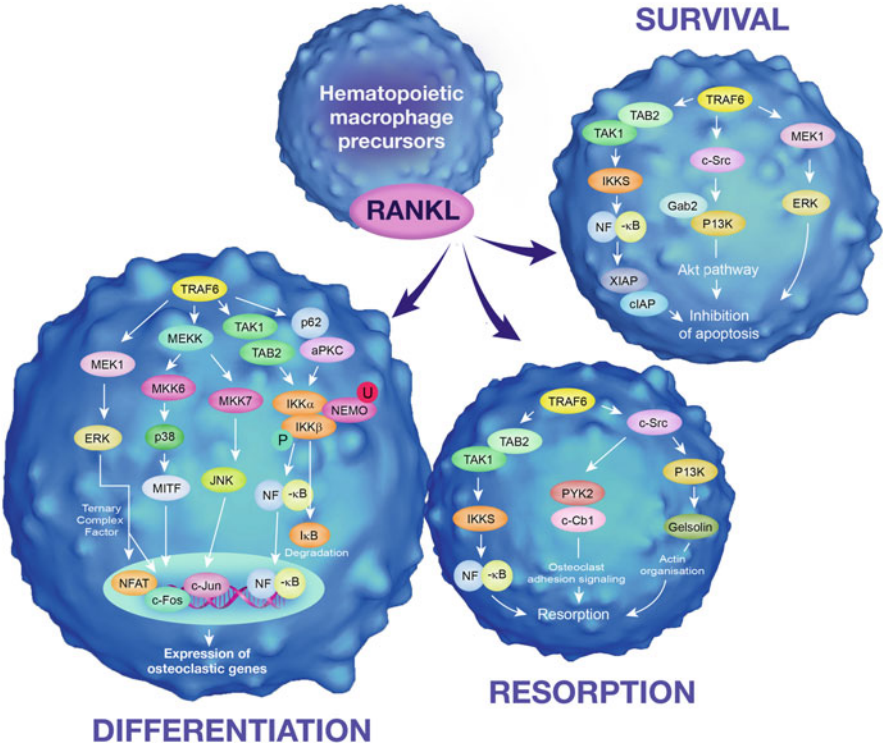

Fig. 12.5 RANKL signaling via TRAF6 in osteoclasts. The RANKL-activated pathways converge at the level of TRAF adapter proteins. In the early stage of osteoclastogenesis, the signal initiated by RANKL binding to RANK is transduced by the recruitment and activation of adapter molecules belonging to the TRAF family, majorly TRAF6. Activated TRAF6 induces AP-1 (not shown) and NF-κB activity by activating the IKK complex, either via the scaffolding protein p62 and aPKC or TAK1-dependent phosphorylation. Interaction between the TRAF-bound p62 and aPKC results in the formation of a multimeric protein complex that regulates IKKβ whereas the interaction between TRAF6 and TAK1 is required for IKKγ (also called NEMO) ubiquitination to achieve optimal activation of the IKK complex. These TRAF6 complexes formed with TAK1-TABs or TAK1-RACK1-MKK6 also facilitate the activation of MAPKs such as p38, JNK, and ERK. The recruitment and activation of Gab2 and PLCγ2 are also required for NF-κB activation. In the intermediate stage of signaling, activated NF-κB and MAPKs induce c-Fos that subsequently activate the AP-1 transcription factor complex (Park et al. 2017). The activated NF-κB also amplifies the expression of NFATc1 which then translocates into the nucleus to activate and induce osteoclast-specific genes that encode proteins related to multi-nucleation and bone resorption function of osteoclasts. The mobilization of NF-κB, c-Fos/c-Jun/AP-1, and NFATc1 is considered as one of the important events downstream of the RANKL/RANK interaction (Zhang et al. 2001). The role of TRAF6 in survival and bone resorption functions of osteoclasts are also shown (image inspired from (Bhattacharyya et al. 2008)). Abbreviations: TRAF6, TNF Receptor-Associated Factor 6; aPKC, atypical Protein Kinase C; PLCγ2, Phospholipase Cγ2; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; RACK1, Receptor for Activated C Kinase 1; ROS, Reactive Oxygen Species; PYK2, Proline-rich kinase2; XIAP, X-linked Inhibitor of Apoptosis; cIAP, cellular Inhibitor of Apoptosis Proteins; NFATc1 Nuclear Factor of Activated T cells, cytoplasmic 1; and NOX1, Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase 1

activity from wholesale tissue modeling/remodeling to global mineral homeostasis. In recent years, conceptual and technological advances have demonstrated an extensive regulatory sphere of influence of osteocytes, encompassing not only local (osteocytic osteolysis whereby osteocytes can remove the perilacunar matrix) and regional (modeling and remodeling) processes but also systemic (endocrine effects in the parathyroid, kidney, and cardiac muscle) processes (Dallas et al. 2013).

Bone has historically been recognized as a mechanosensitive tissue. Investigations of cellular mechanisms underlying the bone mechanobiology have identified the critical regulatory role of osteocytes in mechanosensation and mechanotransduction (Schaffler et al. 2014). Osteocytes function as an extensive 3-dimensional network of sensor cells, or 'syncytium' that sense and integrate mechanical and chemical signals from their environment to regulate both bone formation and resorption. When mechanical forces are exerted on bone, the osteocyte and its network are exposed to a variety of stimuli which include the physical deformation of the perilacunar matrix, such as glucocorticoid-induced hypomineralization (Lane et al. 2006); fluid flow shear stress generated by the load-induced flow of canalicular fluid through the lacuno-canalicular network; or electrical streaming potentials that are generated from the flow of ionic canalicular fluid solution over the charged surfaces of the lacuno-canalicular walls and/or cell membrane (Dallas et al. 2013). Studies have established that the sensitivity of osteocytes to mechanical stimuli is directly coupled to their distinctive anatomy, in particular, its rigid dendritic processes and the structural features of its pericellular environment. Insights into the mechanisms of osteocyte mechanotransduction have identified the importance of osteocyte network for effective integration of both local and global stimuli as well as communication with target effector cells (osteoblasts and osteoclasts) to regulate appropriate modeling or remodeling responses. Osteocytes have been shown to communicate with each other and other bone cells directly via gap junctions, or indirectly via delivery of small (short-range) metabolites [such as Nitric Oxide (NO), PGE_2, and Adenosine triphosphate (ATP)] or macromolecules (such as sclerostin, IGF-1, Fibroblast Growth Factor FGF-23, and RANKL) through hemichannels or pannexin channels (Schaffler et al. 2014).

Studies on paracrine signaling from osteocytes have focused on the anabolic signals produced by the osteocytes in response to mechanical demand. In response to extracellular calcium or mechanical stimulation induced by the fluid flow shear stress, signaling molecules such as NO, PGE_2, and ATP are rapidly released by osteocytes that affect bone phenotype and directly regulate osteoblast activity. These molecules also act as key effectors of load-related osteogenic responses such as in osteocyte gap junction function and communication, osteocyte viability, and bone integrity (Dallas et al. 2013).

In vivo, NO released by osteocytes promotes bone formation in response to an acute increase in mechanical loading (Watanuki et al. 2002). In vitro, osteocytes inhibit the proliferation of early osteoblasts and promote osteoblast differentiation via soluble factors released upon the activation of the NO pathway in response to Pulsating Fluid Flow (PFF) stimulation (Vezeridis et al. 2006). NO signaling via

osteocytes has also been shown to influence bone mass by increasing bone formation while at the same time inhibiting osteoclastic bone resorption (Tan et al. 2007).

PGE_2 has been determined as one of the key effectors of load-related osteogenic responses. So far, in vitro studies have suggested a stimulatory role of PGE_2 on bone formation (Raisz et al. 1993) and osteoblastic differentiation of primary calvarial cell cultures (Nagata et al. 1994). It also stimulates osteoblastic recruitment and activity that enhance bone formation at all bone surfaces (Jee et al. 1990).

Recent studies have drawn attention to the role of one of the most exciting osteocyte-specific proteins, sclerostin, in suppressing the activity and viability of osteoblasts (Rochefort et al. 2010). Sclerostin, the protein product of the SOST gene, has been implicated to regulate differentiation of late osteoblast to pre-osteocyte through regulation of endopeptidases on the X chromosome (PHEX) and matrix extracellular phosphoglycoprotein (MEPE) (Dallas et al. 2013). Binding to the Wnt co-receptors—lipoprotein-related receptors 5 and 6 (LRP5/6), sclerostin has been identified to down-regulate bone formation by antagonizing the Wnt/β-catenin signaling pathway (Li et al. 2005). Lara-Castillo et al. have postulated that the mechanical load initially activates β-catenin signaling in osteocytes and that this activation is connected to the decreased expression of the LRP5/6 and Wnt ligand inhibitors, e.g., sclerostin and Dickkopf-related protein 1 (DKK1) (Lara-Castillo et al. 2015). It has been suggested that downregulation of DKK1 and sclerostin and local Wnt signaling are required for the osteogenic response to mechanical loading, leading to enhanced bone formation (Tu et al. 2012). On the other hand, mechanical unloading of wildtype mice decreases the Wnt/beta-catenin signaling activity accompanied by the upregulation of sclerostin (Lin et al. 2009). Taking all these studies together, it appears that osteocytes affect osteoblasts twofold: in response to mechanical stimuli, (i) osteocytes positively regulate osteoblasts through the production of signaling messengers, such as NO and PGE_2, and (ii) osteocytes negatively regulate osteoblasts by expressing several modulators of Wnt signaling, such as sclerostin and DKK1 (Rochefort et al. 2010).

In addition to their role in mediating adaptive responses to mechanical loading, it is now clear that osteocytes also sense hormonal signals generated by PTH, a regulator of bone resorption and formation (Tian et al. 2011), or gonadal estrogen, an inhibitor of osteocyte apoptosis (Khosla et al. 2012; Rhee et al. 2013). The co-expression of Parathyroid Hormone-related Protein (PTHrP) and PTH receptor 1 (PTHR1) detected on the surface of osteocytes has been attributed to the anabolic actions of PTH in the skeleton, at least partly, due to the downregulation of osteocyte sclerostin expression (Bellido et al. 2005; Keller and Kneissel 2005). When tested in vivo, constitutive activation of the PTHR1, under the control of DMP1 promoter, inhibits SOST expression and dramatically increases bone formation via increased Wnt signaling (O'Brien et al. 2008). Like PHEX and MEPE, DMP1 is known to regulate phosphate homeostasis and mineralization of the bone matrix (Dallas et al. 2013). Cumulatively, osteocytes integrate diverse signals in triggering appropriate responses from effector cells and providing a mechanism for adapting the skeleton to mechanical loading and systemic hormones (For a detailed understanding of other

mechanosensitive bone cells and the mechanical regulation of bone signaling, refer (Thompson et al. 2012)).

Osteocyte-Osteoclast Communication

Osteocytes regulate bone resorption by relaying the sensory inputs to osteoclasts (and their progenitors) through paracrine signals involving macromolecules (Schaffler et al. 2014). The osteocyte-secreted macromolecules are transported to the target cells by mechanical loading induced-interstitial fluid flow (Schaffler et al. 2014). Several reports have provided insights into the potential contributory role of osteocyte-derived RANKL to promote osteoclastogenesis in adult bone remodeling. Co-culture studies performed on osteocyte-like MLO-Y4 cells have shown that osteocytes can stimulate and support osteoclast formation and activation even in the absence of $1,25(OH)_2D$ or any other exogenous osteotropic factor (Zhao et al. 2002). The MLO-Y4 cells expressed mRNA for both secreted and membrane-bound M-CSF, suggesting that M-CSF could be a secretory factor made by osteocytes to signal and support proliferation and differentiation of osteoclast precursors. The MLO-Y4 cell support of osteoclast formation was also shown to be dependent on RANKL expressed as a surface molecule or along with their exposed dendritic processes. Like osteocytes, MLO-Y4 cells also express OPG, a gene encoding a decoy receptor for RANKL. The role of osteocytes in regulating osteoclastic activity was further delineated by Nakashima and colleagues who showed that purified osteocytes, expressing the higher amount of RANKL, have a greater capacity to support osteoclastogenesis in vitro than osteoblasts and bone marrow stromal cells (Nakashima et al. 2011). Additional evidence for osteocyte regulation of bone homeostasis was provided using mice models in which RANKL was selectively ablated in osteocytes. These mice developed an osteoclast-poor osteopetrotic (increased bone density) phenotype, substantiating the role of osteocyte-derived RANKL for normal bone remodeling in adult mice. Intriguingly, this contradicts the prevailing paradigm, based primarily on in vitro experiments (Takahashi et al. 1988), that osteoblasts or their progenitors are a major source of RANKL responsible for osteoclast generation (Xiong et al. 2011).

While the traditional osteoanabolic role of PTHrP, signaling through the osteocyte PTHR1, in enhancing osteoblastogenesis and/or osteoblast survival is clear, studies have also uncovered its catabolic effects on osteoclastogenesis and bone resorption. During lactation, under the influence of PTHR1 signaling, osteocytes induce the expression of a subset of osteoclast-specific genes including, TRAcP and catK (Qing et al. 2012). Induced TRAcP activity and catK expression, together with constitutively active PTHR1 on osteocytes, was found concurrent with osteocytic perilacunar/canalicular remodeling whereby osteocytes, like osteoclasts, remove their surrounding mineralized matrix (Qing et al. 2012). Besides, evidence that PTHR1 signaling in osteocytes regulates RANKL-mediated stimulation of bone resorption in the adult skeleton further potentiates the key role of osteocytes in

mediating the effects of PTH on osteoclast-mediated bone resorption (Ben-awadh et al. 2014).

Interestingly, an obligate link between osteocyte death (specifically apoptotic, or regulated death) and the initiation of bone resorption is recognized. Apoptotic osteocytes serve as beacons for targeted osteoclast remodeling. It has been hypothesized that dying osteocytes stimulate osteoclast formation through inflammatory signaling molecules such as the alarmins (i.e., high mobility group box protein 1, HMGB-1) (Bidwell et al. 2008). However, the most widely accepted theory states that the dying osteocytes induce expression of RANKL by neighboring healthy osteocytes and thus mediate RANKL-stimulated osteoclast resorption (Schaffler et al. 2014). Mechanisms that mediate communication between apoptotic osteocytes and neighboring osteocytes, the exact nature of apoptosis-driven signals that stimulate the upregulation of RANKL, and its propagation through the osteocytic network remain largely unknown.

Extracellular Vesicles: Novel Regulators of Bone Remodeling and Skeletal Metabolism

Bone homeostasis is of critical importance and relies on cellular communication between osteoclasts, osteoblasts, and osteocytes, and the coupling of bone resorption to bone formation (Tamma and Zallone 2012). Cells in the osteoblast and osteoclast lineages generally adopt any one of the three modes of communication which involve secretion of diffusible paracrine regulatory factors, direct cell-cell physical engagement via gap junctional connections, and cell-bone matrix interaction (Tamma and Zallone 2012). In recent years, identification of a new mode of cell-cell communication mediated by EVs has added a potential piece of evidence to the complex puzzle of bone cellular crosstalk (Cappariello et al. 2018).

The Characteristics and Contents of Bone-Derived Extracellular Vesicles

Accumulating evidence has specified the secretion of EVs by virtually all bone cell types, such as bone marrow stromal cells, osteoclasts, osteoblasts, and osteocytes (Liu et al. 2017). Bone-derived EVs contain multifarious proteins, lipids, and nucleic acids, whose distribution and packaging vary dynamically according to cell types, microenvironments, as well as pathological and physiological conditions (Lyu et al. 2020).

Characterization of EVs secreted by mature osteoclasts and their precursors have shown similarities in size and morphology as well as the expression of highly specific surface markers such as Epithelial Cell Adhesion Molecule (EpCAM) (Runz et al. 2007) and tetraspanin Cluster of Differentiation (CD) 63 (Pols and Klumperman 2009). Proteomic analysis of osteoclast-derived EVs has shown that

the EVs are particularly enriched with ephrinA2 protein (Sun et al. 2016). Ephrin A2 is a glycosylphosphatidylinositol-anchored protein that interacts with another membrane-bound protein, EphA2. This EphrinA2/EphA2 bi-directional signaling within osteoclast precursors or between osteoclast and osteoblast precursors facilitates the initiation phase of bone remodeling by enhancing osteoclastogenesis while inhibiting osteoblast differentiation (Irie et al. 2009). Specifically, in bone remodeling, ephrinA2 has been postulated to act as a coupling inhibitor. In that case, forward signaling through the EphA2 receptor expressed on osteoblasts inhibit both osteoblastic bone formation and mineralization whereas reverse signaling through ephrinA2 into osteoclasts enhances osteoclastogenesis (Sun et al. 2016). In addition, osteoclast-derived EVs manifest differential expression of RANK; a subset of EVs isolated from mature osteoclasts exhibit higher levels of RANK than the EVs isolated from their precursors (Huynh et al. 2016).

Isolation and characterization studies of osteoblast-derived EVs have revealed their characteristics typical to that of small EVs: diameter between 30 and 100 nm, round shape with cup-like concavity, and expression of transmembrane markers Tumor Susceptibility Gene (TSG) 101 and flotillin (Flot) 1. The osteoblast-derived EVs have been demonstrated to show marked ex vivo intrinsic osteotropism and integration and in vivo bone biodistribution (Cappariello et al. 2018). Evidence indicates that osteoblasts secrete EVs at different stages of osteoblast differentiation under both nonmineralizing and mineralizing conditions (Morhayim et al. 2015). Proteomic profiling has identified a total of 1536 proteins contained within the osteoblast-derived small EVs. A majority of these proteins were located in the plasma membrane and cytosol and predominantly involved in protein localization and intracellular signaling. Furthermore, proteins were mapped to the Eukaryotic Initiation Factor (EIF)2, integrin, Bone Morphogenetic Protein (BMP), Wnt, and Transforming Growth Factor-Beta (TGF-β) signaling pathways (Ge et al. 2015). A total of 23 proteins, including EIF family members, Protein Phosphatase 1 (PP1C) and Poly(A)-Binding Protein (PABP), were mapped to the EIF2 signaling pathway which plays an important role in osteogenesis (Ge et al. 2015). Advanced high throughput RNA profiling using microarrays and sequencing technologies have greatly facilitated the characterization of distinct EV-mRNA patterns in human osteoblasts. Studies have identified 254 mRNAs contained within the osteoblast-derived EVs with specific osteoblast-related and EV-mediated function (Morhayim et al. 2017). Specifically, enriched osteoblast-EVs-mRNAs are linked to cellular processes, such as RNA post-transcriptional modification, gene expression, and cell-to-cell signaling and interaction (Morhayim et al. 2017). Intriguingly, in one study, comparisons between EV and cellular transcriptomic profiles did not reveal selective sorting or enrichment of mRNAs in EVs, thus, hinting at a possibility that the osteoblast-derived EVs mirror the transcript profile of their donor cells (Cappariello et al. 2018; Zomer et al. 2010). This is at variance with observations made in other cells that display selective packaging of molecules within the secreted EVs.

Emerging evidence has implicated the mediatory role of EVs (Rani et al. 2015) in bone-protective functions of BMSCs. The BMSC-derived EVs display well-established tetraspanin markers CD9, CD63, and CD81 (Pethő et al. 2018) as well

as other markers including Hsp90, Hsp70, and Flot-1 (Wang et al. 2014). Differential expression of miRNAs is detected in the EVs derived from of BMSCs, including miRNAs that are functionally associated with bone remodeling (Xu et al. 2014) (Table 12.2). seven mRNAs (RPS2, DGKA, ACIN1, DKK2, Xsox17, DDX6, and Lsm2) are also differentially expressed over time in differentiated BMSC-derived EVs (Xu et al. 2014). Furthermore, osteogenic differentiated BMSC-derived EVs show dysregulated expression of two NF-κB-related genes, A Disintegrin And Metalloproteinase metallopeptidase domain 17 (ADAM17) and Nuclear Factor-Kappa B1 (NF-κB1) (Xu et al 2014).

Role of Bone-Derived Extracellular Vesicles in Bone Homeostasis

The past decade has witnessed significant progress in delineating the network of regulatory activities of EVs in bone homeostasis. Compelling evidence has emerged to prove the functions of bone-derived EVs and their bioactive cargo in bone remodeling via mechanisms involving paracrine/autocrine signaling or in the regulatory processes of differentiation and communication of osteoclasts, osteoblasts, and other cell types (Table 12.2).

Role of Osteoclast-Derived Extracellular Vesicles

1. **Regulation of osteoclastogenesis**

It is evident that bone remodeling and osteoclast formation are closely regulated by the RANKL-RANK-OPG axis and the coordinated activities of osteoblasts and osteoclasts (Boyce and Xing 2008; Hofbauer et al. 2000). Besides the well-documented regulatory mechanism of osteoblast-osteoclast communication via cytokines such as RANKL and RANK and soluble factors in bone remodeling, osteoclast-derived EVs have been reported to regulate osteoclastogenesis and hence, bone remodeling (Yuan et al. 2018). Huynh and the team have recently reported the paracrine regulation of osteoclastogenesis by osteoclast-derived EVs (Huynh et al. 2016). In response to systemic signals, such as $1,25(OH)_2D_3$, RANK-rich EVs derived from the osteoclast precursors enhance $1,25(OH)_2D_3$-dependent osteoclast formation in mouse marrow cultures whereas EVs from mature osteoclasts inhibit $1,25(OH)_2D_3$-stimulated osteoclastogenesis (Huynh et al. 2016). RANK-containing osteoclast-derived EVs were found to inhibit osteoclastogenesis competitively by binding to RANKL (in the manner of OPG) as a trimer forming a high-affinity heterohexameric RANKL-RANK complex that prevents stimulation of the RANK-signaling pathway in osteoclasts (Liu et al. 2010). This data implies the capacity of osteoclasts-derived EVs to regulate $1,25(OH)_2D_3$-stimulated osteoclast formation and in general, the anti-osteoclastogenic activity of EVs in osteoclasts.

Table 12.2 A summary of functions of bone-derived extracellular vesicles and their associated cargo in bone remodeling

EV-derived cargo	Source	Markers	Pathways/mechanisms involved	Role	Refs.
Proteins Rank	Osteoclast precursors	EpCAM CD63	RANK-signaling pathway	Stimulates differentiation of osteoclasts and osteoblasts	Huynh et al. (2016)
	Mature osteoclasts			Inhibits osteoclastogenesis and osteoblastic bone formation	Huynh et al. (2016)
miRNAs miR-21			• Downregulates PDCD4 protein levels and • Forms a positive feedback loop of c-Fos/miR-21/PDCD4	Induces osteoclastogenesis	Sugatani et al. (2011)
miR-148a			Represses protein expression of MAFB	Promotes osteoclast differentiation	Cheng et al. (2013)
miR-214			Targets PTEN and activates PI3 kinase/Akt/NFATc1 pathway		Zhao et al. (2015)
miR-214-3p			• Inhibits EphrinA2/EphA2-mediated bidirectional osteoclast-osteoblast interactions	Inhibits osteoblast functions	Sun et al. (2013)
Proteins EIF family members, PP1C, PABP	Osteoblasts	TSG 101 Flotillin-1 and 2	EIF2 signaling pathway	Induces osteoblast differentiation and bone formation	Antonyak et al. (2011); Ge et al. (2015); Xie et al. (2017)
RANKL, TRAcP			RANK/RANKL signaling pathway	Stimulates osteoclast formation	Xie et al. (2017); Solberg et al. (2015)
OPG			RANKL/RANK/OPG pathway	Inhibits osteoclast differentiation	

(continued)

Table 12.2 (continued)

EV-derived cargo	Source	Markers	Pathways/mechanisms involved	Role	Refs.
Decreased ADAM17 and NF-κB1	Osteoblast precursors	CD13 CD29 CD44 CD73 CD105	TNFα-signaling pathway	Involved in BMSC osteogenic differentiation	Xu et al. (2014); Araya et al. (2018)
BMP9			• BMP signaling pathway • Molecular crosstalk with the Wnt/ β-catenin, IGF, and retinoid signaling pathways	Induces the osteogenic differentiation of MSCs	Luther et al. (2011); Narayanan et al. (2016)
TGF-β1			• Increased TAZ • SMAD2/3 signaling pathway		Zhao et al. (2010); Narayanan et al. (2016)
miRNAs Increased Let-7			• Targets Axin-2 • Represses HMGA2	Promotes osteogenic differentiation of MSCs, and enhances bone formation	Wei et al. (2014); Egea et al. (2012)
Increased miR-378			Negatively regulates Caspase 3 and inhibits CytC, Apaf-1 and Bax proteins via the PI3K/Akt pathway	Induces osteogenic differentiation and restores osteoblast viability	You et al. (2014)
Increased miR-199b and miR-218 Decreased miR-181a			• Increased expression of Runx2 • Stimulates Wnt pathway; down-regulates three Wnt signaling inhibitors: Sclerostin, DKK2, and SFRP2 • Targets TGF-βi, and TβR-I/Alk5	Enhances osteoblast differentiation	Xu et al. (2014); Bhushan et al. (2013)
Decreased miR-885-5p			• Represses Runx2	Inhibits osteogenic differentiation of BMSCs	Xu et al. (2014)
Increased miR-135b			• Decreases SMAD5 expression	Negatively regulates osteogenic differentiation in BMSCs	Xu et al. (2013, 2014)

Increased miR-667-3p, miR-6769b-5p, miR-7044-5p, miR-7668-3p, and miR-874-3p	Mineralizing osteoblasts		• Targets Axin-1 • Increases β-catenin expression	Stimulates osteogenic differentiation of BMSCs	Cui et al. (2016)
Increased miR-335-5p	Osteoblasts		• Targets DKKI • Regulates Wnt signaling	Induces osteogenic differentiation	Zhang et al. (2011)
Increased miR-503-3p			Targets RANK	Inhibits RANKL-induced osteoclast differentiation	Chen et al. (2014)
Proteins Decreased Sclerostin RANKL OPG	Osteocytes	LAMP1	RANKL/RANK/OPG pathway	Enhances bone formation and regulates bone metabolism	Lu et al. (2012); Morrell et al. (2018)

Abbreviations: *TAZ* Transcriptional co-activator with PDZ-binding motif

Advances in understanding the molecular mechanism and function of miRNA-mediated intercellular communication between bone cells have guided the in-depth characterization of a series of EV-associated miRNAs that are secreted by human osteoclasts. Osteoclast-derived miRNAs, secreted via EVs, represents a new class of osteoclast-released coupling factor with a potential role in osteoclastogenesis (Zhu et al. 2018). Recent studies on the miR signature of RANKL-induced osteoclastogenesis have identified significant upregulation of miR-148, miR-21, miR-214, and miR-183a-5p in osteoclast-derived EVs (Sun et al. 2016). Among these upregulated miRNAs, to date, only miR-21 and miR-148a have been identified to promote osteoclastogenesis. In vivo, miR-148a has been shown to repress the protein expression of V-maf Musculoaponeurotic Fibrosarcoma oncogene homolog B (MAFB) and promote osteoclast differentiation. MAFB is a transcription factor that negatively regulates RANKL-induced osteoclastogenesis (Cheng et al. 2013). In vitro, miR-21 has been found to mediate RANKL-induced osteoclastogenesis by down-regulating the protein levels of Programmed Cell Death 4 (PDCD4) and forming a positive feedback loop of c-Fos/miR-21/PDCD4, thus regulating osteoclastogenesis (Sugatani et al. 2011). Further investigations to decipher the bioactivity of several identified osteoclast-derived miRNAs have shown that miR-214 plays a critical role in osteoclastogenesis and affects osteoclast activity by regulating the expression of osteoclast functional genes (Zhao et al. 2015). It has been suggested that miR-214 promotes osteoclast differentiation by targeting PTEN and activating the Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (Akt)/NFATc1 pathway (Zhao et al. 2015). Also, overexpression of miR-183-5p in Bone Marrow-derived Macrophages (BMMs) has been shown to facilitate osteoclastogenesis through inhibiting Heme Oxygenase-1 (HO-1) expression (Ke et al. 2015). Cumulatively, these results highlight crucial miRNAs that control osteoclasts and regulate osteoclastogenesis.

2. Communication between osteoclasts and osteoblasts

Recent research focusing on the crosstalk and coupling mechanisms of bone formation to the preceding resorption activity has led to the identification of coupling factors that are produced by the osteoclasts to influence osteoblast activity in the BMU (Zhu et al. 2018). These osteoclast-derived coupling factors can either enhance or inhibit osteoblast migration and differentiation through paracrine and juxtacrine mechanisms and therefore may have a central role in the coupling of bone formation to preceding resorption (Zhu et al. 2018). To date, four major classes of osteoclast products have been reported that may function as potential coupling factors. These include osteoclast membrane-bound molecules, osteoclast-secreted factors, matrix-derived factors released during osteoclastic bone resorption, and osteoclast-derived vesicular miRNAs (Zhu et al. 2018).

Recently, Li and group examined the expression of miRNAs, potentially participating in mediating osteoclast-to-osteoblast communication, in bone specimens and serum EVs from elderly women with bone-fractures as well as from aging ovariectomized (OVX) mice (Li et al. 2016). When compared to 12 miRNAs, the group found an age-related increase in miR-214-3p level in whole serum, serum EVs, and

bone tissues. They reported a positive correlation between increased intraosseous miR-214-3p and serum vesicular miR-214-3p levels in elderly women with low-energy fractures and between intra-osteoclast miR-214-3p level and serum vesicular miR-214-3p level in OVX mice, respectively. MiR-214-3p has been shown to target osterix (Osx), an osteoblast-specific transcription factor and thereby, suppress osteogenic differentiation of C2C12 myoblast cells (Shi et al. 2013). It also targets Activating Transcription Factor 4 (ATF4), an important osteogenic transcriptional factor, to suppress bone formation (Wang et al. 2013). In line with these observations, both in vitro and in vivo studies have demonstrated the negative impact of osteoclast-derived vesicular miR-214-3p on osteoblastic bone formation (Li et al. 2016). Consistently, another group has demonstrated that EV-containing miR-214-3p acts as an osteoclast-derived inhibitor of osteoblast differentiation and bone formation by targeting EphrinA2/EphA2-mediated bidirectional osteoclast-osteoblast interactions (Sun et al. 2016). Thus, osteoclasts-derived vesicular miR-214 may have multiple functional implications in osteoblastogenesis. Taken together, this data points out the role of vesicular miRNA-mediated osteoclast-to-osteoblast communication in the homeostasis mechanism of the local bone environment.

Role of Osteoblast-Derived Extracellular Vesicles

1. **Regulation of osteogenesis**

Investigations into exploring the mechanisms by which osteoblasts deliver their specific regulatory message has shown that EVs, isolated from bona fide primary osteoblasts, contain a set of mRNA transcripts related to osteoblast activity (Cappariello et al. 2018). These EVs have been demonstrated to regulate the transcriptional expression of osteoblast-specific genes such as Osx, osteopontin, osteonectin, and osteocalcin, associated with bone metabolism and osteoblast function. Osteocalcin is one of the most abundant noncollagenous proteins in the mineralized bone matrix (Komori 2010) and has an important endocrine function, outside bone, regulating energy metabolism, neural development, male fertility, and muscle function (Li et al. 2016; Karsenty and Oury 2014; Mera et al. 2016). The modulation of transcriptional expression of osteocalcin by osteoblast-derived EVs also implies the role of EVs in exerting control over the osteoblast endocrine function.

A growing body of studies also suggests the role of miRNAs, associated with EVs produced from osteoblast lineage cells, in regulating osteoblast activities via an autocrine mode of action. For example, the dynamic temporal expression pattern of members of lethal-7 (let-7) miRNA family has been identified in EVs isolated from both osteoblast precursors and mineralized osteoblasts (MOBs) (Wei et al. 2014). Let-7 is a documented positive regulator of bone development which can promote osteogenic differentiation, suppress adipogenesis of human adipose-derived

mesenchymal stem cells (hADSCs) in vitro, and enhance bone formation in vivo. Subsequent studies have demonstrated that let-7 represses high-mobility group AT-hook 2 (HMGA2) to regulate the balance of osteogenesis and adipogenesis of MSCs (Wei et al. 2014). These observations validate that osteoblasts gain the competence to participate in the selective autocrine regulation of osteoblast gene expression via the secretion of EVs.

Proteomic profiling of EVs, secreted by nonmineralizing osteoblasts (NMOBs) and MOBs, have shown selectively packaging of not only the commonly known vesicle proteins but also of certain proteins that are unique to the mineralization and differentiation status of the osteoblasts. These proteins contribute to a wide array of vesicle-related molecular functions and biological processes linked to skeletal development, mesenchymal differentiation, calcium ion binding, and phosphatase activity (Morhayim et al. 2015). Specifically, the proteomic cargo has been implicated in four cardinal osteogenesis-related pathways, namely, Rho GTPase binding, integrin, mammalian Target of Rapamycin (mTOR), and EIF2 signaling (Ge et al. 2015).

In vitro culture studies aimed at characterizing the function of MOB-derived EVs and their ability to mediate cellular osteoblast differentiation have demonstrated the upregulation of functionally confirmed osteo-miRNAs (miR-1192, miR-680, and miR-302a), together with miR-30d-5p, miR-133b-3p, miR-140-3p, miR-335-3p, miR-378b, and miR-677-3p in mineralizing pre-osteoblast MC3T3-E1-derived EVs (Cui et al. 2016). When co-cultured with mouse bone marrow-derived stromal cells (ST2), mineralizing MC3T3-E1 EVs significantly promoted ST2 cell osteogenic differentiation and matrix mineralization, suggesting a potential amplification loop for enhancing osteogenesis (Cui et al. 2016). This pro-osteogenic effect was found to be manifested via the miRNA-mediated cooperative regulation of a network of important pathways that play pivotal roles in the differentiation (Wnt signaling pathway, insulin signaling pathway, and TGF-β signaling pathway) and function (calcium signaling pathway) of osteoblasts. For example, miR-335-5p regulates the Wnt signaling by downregulating the expression of DKK1 at various stages of osteoblast differentiation (Zhang et al. 2011). Activation of the Wnt signaling pathway by MOB EV-induced upregulated miRNAs (specifically miR-667-3p, miR-6769b-5p, miR-7044-5p, miR-7668-3p, and miR-874-3p) in recipient ST2 cells has been determined. to be potentiated by co-targeting Axin1, an important negative regulator of the Wnt signaling pathway, and increasing β-catenin expression to promote ST2 cell osteogenic differentiation (Cui et al. 2016).

The capacity of miR-378 to restore viability and osteogenic differentiation has been determined in the high glucose culture of pre-osteoblastic MC3T3-E1 cells. High glucose conditions have shown to suppress osteogenic differentiation and osteoblast viability by inducing high levels of pro-apoptotic Cytochrome C (CytC), Apoptotic protease activating factor 1 (Apaf-1), and Bax proteins. Under hyperglycemic conditions, miR-378 negatively regulates target gene Caspase 3 (CASP3) and inhibits CytC, Apaf-1, and Bax proteins via the PI3K/Akt pathway. These positive effects of miR-378 on osteogenic differentiation makes it an

interesting therapeutic candidate against hyperglycemia-associated osteoporosis and bone fracture in diabetes mellitus patients (You et al. 2014).

Intriguingly, a panel of miRNAs was downregulated by the MOB-derived EVs (Cui et al. 2016). Gene ontology and pathway network analyses revealed that the co-target genes of these down-regulated miRNAs were significantly enriched in the insulin signaling pathway, Mitogen-Activated Protein Kinase (MAPK), and PI3K/Akt pathways, that are crucial in osteoblast differentiation. Such unanticipated finding proposes the capacity of EVs to activate several important osteogenesis pathways in recipient cells even by inhibiting the osteo-miRNAs (Cui et al. 2016).

Qin et al. have shown that EVs isolated from BMSCs are efficiently internalized by target osteoblast cells and enhance the expression of osteogenic genes and osteoblastic differentiation (Qin et al. 2016). Studies analyzing the regulatory mechanism by which osteogenic differentiated BMSC-derived EVs promote osteogenic differentiation have revealed alterations in the EV-associated miRNA profile at different time points (Xu et al. 2014). While a number of miRNAs (let-7a, miR-199b, miR-218, miR-148a, and miR-135b,) were up-regulated in EVs during BMSCs osteogenic differentiation, a separate set of miRNAs (miR-221, miR-155, miR-181a, miR-320c, and miR-885-5p) were significantly under-expressed in individual EV samples (Xu et al. 2014). miR-885-5p negatively regulates osteogenic differentiation of BSMCs by repressing Runx2 and thereby inhibiting the expression of osteoblast-related genes (Xu et al. 2014). Analysis of the role of potent osteogenic miRNAs in anabolic pathways for bone formation has demonstrated the regulatory role of miR-199b in controlling osteoblast differentiation via Runx2 (Xu et al. 2014). A signal-amplification circuit between miR-218 and Wnt/β-catenin signal has been determined to induce commitment and osteogenic differentiation of hADSCs (Zhang et al. 2014). In a positive regulatory Wnt signaling loop, miR-218 enhances Wnt/β-catenin signaling activity by down-regulating three Wnt signaling inhibitors during the process of osteogenesis: sclerostin, Dickkopf-related protein 2 (DKK2), and Secreted Frizzled-Related Protein 2 (SFRP2) (Hassan et al. 2012). A crucial functional link between miR-181a and BMP-induced osteoblastic differentiation has also been established (Bhushan et al. 2013). In C2C12 and MC3T3 cells, miR-181a represses TGF-β signaling molecules by targeting the negative regulators of osteoblastic differentiation, TGF-β-induced (TGF-βi) and TGF-β types I receptor (TβRI)/Alk5, to promote osteoblastic differentiation (Bhushan et al. 2013). Collectively, these studies indicate that osteoblastic differentiation is controlled by the complex interplay of several signaling pathways and associated key transcription factors, as well as by varied expression profiles of EV-associated miRNAs.

2. **Communication between osteoclasts and osteoblasts**

The majority of investigations into the osteoinductive effects of osteoblast-derived vesicles have been focused on EVs bound within the Extracellular Matrix (ECM) (Davies et al. 2017). Deng and team have reported osteoblast-derived EV-mediated intercellular communication between osteoblasts and osteoclasts, during bone modeling and remodeling, via RANKL (Deng et al. 2015). Osteoblast-derived EVs have been shown to interact with target osteoclast precursors through

the RANKL-RANK interface to facilitate osteoclast formation. However, the authors reported that EV internalization was noted even when the specific receptor-mediated recognition between osteoblast-derived EVs and target cells could not be achieved. Another study on primary mouse osteoblast-derived EVs has confirmed the role of osteoblast-derived EVs, enriched with membrane-bound RANKL, in improving osteoclastic variables and increasing osteoclast size and number of nuclei/cell, indicative of their direct in vivo osteoclastogenic potential (Cappariello et al. 2018).

Characterization of mRNA content of osteoblast-derived EVs has indicated the abundance of mRNAs encoding functional proteins that are involved in communication with the cells in the osteoblastic microenvironment as well as regulatory proteins that affect the fate and biological functions of target neighboring cells (Morhayim et al. 2017). Of note, the osteoblast-EVs abundantly express RAB13 mRNA and are co-packaged with its encoded protein RAB13. RAB13, a member of the Rab family of small GTPases, is involved in vesicular trafficking events during osteoclast differentiation that is unrelated to bone resorption (Hirvonen et al. 2012). It is speculated that putative transfer of RAB13 to recipient immature osteoclasts via osteoblast-derived EVs alters the osteoclast phenotype and provokes osteoclast-mediated secondary extracellular signals.

Besides the synergistic effect of osteoblast-derived EVs on osteoclast activities, evidence also highlights the antagonistic functions of osteoblast EVs. Evidence indicates that osteoblast lineage cell-secreted EVs and their associated miRNAs can regulate osteoclast activity via a paracrine mechanism, independent from coupling effects (Zhu et al. 2018). For example, osteoblast-derived EVs-associated miR-503-3p has been shown to target RANK and inhibit its regulation in osteoclastogenesis (Chen et al. 2014). Furthermore, miR-503 influences bone mass by regulating bone resorption in OVX mice, predominantly through its effect on RANK (Chen et al. 2014).

In summary, osteoblast-derived EVs participate in the paracrine signaling system which coordinates intra- and intercellular communication and contribute to the pro- or anti-osteoclastic effect. From a clinical standpoint, it provides a proof-of-concept for the relevance of osteoblast-derived EVs as biological tools for the delivery of active molecules to cleverly target bone cells (Cappariello et al. 2018).

Role of Osteocyte-Derived Extracellular Vesicles

Regardless of the cumulative evidence on the influence of osteocytes in bone metabolic activity, the mechanisms of osteocyte mechanotransduction remain poorly understood. It has been determined that osteocyte mechanosensitivity is encoded through unique intracellular Ca2+ dynamics. However, the downstream consequences of mechanosensitive Ca2+ signaling and its link to mechanotransduction have only been recently delineated (Morrell et al. 2018).

In response to increased mechanical loading magnitudes, tissue strain, and shear stress in the lacuno-canalicular system, osteocytes, in their native bone matrix environment, display actomyosin contractions immediately after intracellular Ca2+ oscillations, demonstrating a mechanism by which the cortical actin network respond and restructure during mechanical loading (Morrell et al. 2018). These Ca2+ oscillations in osteocytes are dependent on extracellular Ca2+ and the second messenger, ATP as well on the P2 purinergic receptor (P2R)/Phospholipase C (PLC)/inositol trisphosphate/Endoplasmic Reticulum (ER) pathway (Jing et al. 2014). Actin networks have been implicated in osteoblast differentiation into osteocytes as well as in mechanically induced protein responses in osteocytes (Morrell et al. 2018; McGarry et al. 2005). Importantly, actomyosin contractions have also been linked to the Ca2 + −dependent release of EVs that deliver bone regulatory proteins, upon mechanical stimulation of osteocytes (Morrell et al. 2018).

Morrell and team have extensively characterized Ca2 + −dependent contractile behavior in osteocytes and consequent EV release in a mechanosensitive context (Morrell et al. 2018). In vivo testing of the functional role of Ca2 + −mediated mechanosensation in anabolic bone formation responses in C57BL6/J mice revealed a load-related increase in expression of secretory vesicle marker Lysosomal-Associated Membrane Protein 1 (LAMP1) and expected downregulation of sclerostin, a negative regulator of the bone-forming osteoblasts. Increased immunostaining for LAMP1, suggestive of the enhanced EV production, confirmed that osteocyte mechanosensation via Ca2+ oscillations modulates levels of EV production. Secretion of sclerostin-containing EVs, downregulation of intracellular sclerostin protein expression in osteocytes, and significant load-related increases in both trabecular bone volume fraction and cortical thickness further validated the role of mechanically induced Ca2+ oscillations in osteocytes in regulating bone metabolism (Morrell et al. 2018). These observations suggest the plausibility of osteocyte EV release as a mechanoresponsive mechanism by which osteocytes impact tissue-level adaptive responses to mechanical loading.

Prostate Cancer Bone Metastasis

Distant organ metastasis is an ominous feature of most malignant tumors and involves an orderly sequence of steps classified as local invasion, intravasation, survival in the circulation, extravasation, and colonization (Nguyen et al. 2009). Irrespective of this common framework, striking disparities exist in the natural progression of metastasis in different cancers such as differences in the infiltration and colonization competence of cancer cells to distant organs, diverse temporal course (relapse within a range of organs) of metastasis, and the distinct kinetics (time to relapse/recur) of metastatic progression between two tumor types (Nguyen et al. 2009). Progress made into metastasis research has by far clarified that both the organ microenvironment and the oncogenetic background play important parts in

dictating the temporal course of metastasis, the kinetics of disease progression, and the organ-selective evolution of metastatic cell populations (Nguyen et al. 2009).

The organ-specific colonization functions have been well-documented in lung, brain, and liver parenchyma, however, the precise role of the bone microenvironment in conferring the specific functions remains intriguing. There is ample evidence to indicate that the anatomy of the bone alone does not explain the organ-specific pattern of metastasis. Rather, bone serves as a congenial turf for cancer cell priming to metastasis, owing to its large repository for EMT inducers, growth factors, neovascularization factors, cytokines, and chemokines (Bussard et al. 2008). Physical properties of the bone matrix, including low oxygen content, acidic pH, and high extracellular calcium concentration, also potentiate tumor metastasis (Kingsley et al. 2007). Furthermore, mounting evidence has implicated that the differential rigidity of the mineralized bone microenvironment stimulates the transition of quiescent tumor cells to a bone-destructive phenotype (Page et al. 2015). This transition to the osteolytic phenotype is regulated in an Iβ3/TGF-β-dependent manner, together with an increased expression of PTHrP (Page et al. 2015). Still, the proclivity of certain primary tumors to spawn metastatic outgrowths in bone, despite the similarities in circulatory patterns and the infiltration of latent disseminated tumor cells (DTCs; identified as tumor cells that reside in the permissive target tissues of distant organs and show metastatic propensity) in the bone matrix, is not well-characterized. Even within cancer types with a strong predilection to form bone metastasis, different clinical subtypes manifest bone metastasis to vastly different degrees, thereby showing the complexity of predicting the mechanisms of bone metastasis.

Research into investigating the biology of bone metastasis has attributed its complexity to the intricately organized bone microenvironment which involves jointly regulated but spatially restricted stages of hematopoiesis, osteogenesis, and osteolysis (Esposito et al. 2018). One of the features of bone metastasis is hijacking the physiological mechanisms of HSC homing in the bone marrow (Muller et al. 2001; Taichman et al. 2002). In the marrow, HSCs reside in a unique microenvironment (or niche) (Taichman 2005) that supports their homing, quiescence, and self-renewal (Wilson and Trumpp 2006; Yin and Li 2006). HSC homing is primarily regulated by the chemokine gradients (CXCL12/CXCR4 axis) in the marrow and adhesion molecules [Vascular Cell Adhesion protein 1 (VCAM-1) and annexin II (Anxa2)] expressed by the HSC niche (Taichman 2005). Once disseminating into the marrow, DTCs are thought to directly compete with HSCs for occupancy and hijack the homing mechanisms to establish footholds in the bone marrow (Muller et al. 2001; Taichman et al. 2002). In vivo studies have shown that metastatic cells also usurp the HSC mobilization protocols to egress out of the niche and get back into the circulation (Shiozawa et al. 2011). This data suggests that the bone marrow HSC niche serves as a direct target for metastatic cell dissemination and hence, in bone metastasis. Another feature of bone metastasis is the subversion of the biological processes that regulate the structure of bone, both osteolysis, and osteogenesis (Esposito et al. 2018).

Research into the molecular mechanisms of bone metastasis has focused on three mechanisms that have been identified as the minimum essential pre-requisites for

forming bone metastasis: (1) seeding (infiltration) to the bone, (2) survival via dormancy, and (3) eventual outgrowth into osteolytic or -genic tumors (Esposito et al. 2018). Similar to the infiltration in the lungs, liver, and brain, infiltration into the bone marrow is influenced by the structural features of capillary walls and, in part, by circulation patterns. The capillaries in the bone marrow, called sinusoids, are lined with fenestrated endothelia that facilitate the trafficking of hematopoietic cells (Kopp et al. 2005). This fenestrated structure renders the bone marrow more permissive to tumor cell infiltration as compared to the contiguous structure of lung capillary walls. The metastatic infiltration to distant organs is generally supported by the specialized extravasation functions that are required for tumor cell passage through capillary walls and survival in the newly invaded parenchyma. These functions are thought to be acquired through distinct genetic or epigenetic alterations, mediated by a distinct class of 'metastasis progression genes' prominently expressed in the primary tumor, which endow circulating tumor cells (CTCs; identified as tumor cells that dissociate from the primary tumor and enter the circulation) with the competence to infiltrate and extravasate distant organs (Esposito et al. 2018). However, it has been noted that during bone metastasis, the infiltration of CTCs occurs in lieu of any primary tumor gene expression event that confers specialized extravasation functions to the tumor cells entering the bone marrow (Nguyen et al. 2009).

Once infiltrating into the bone microenvironment, DTCs encounter different selective pressures from those at the primary site. These selective pressures arise from a balance of growth-promoting and death signals in the newly infiltrated stroma (Nguyen et al. 2009). Studies indicate that this balance of signals affects DTC turnover and overt metastatic colonization (Nguyen et al. 2009). Owing to the restrictive forces of the host microenvironment, some DTCs undergo apoptosis due to their inability to establish productive interactions with the newly infiltrated environment. Alternatively, DTCs with appropriate genetic and epigenetic makeup can acquire advantages from newly encountered survival cues. Depending on how DTCs respond to local signals, a population of DTCs enters a state of metastatic latency which is defined as the time between primary tumor diagnosis and clinically detectable metastatic outgrowths (Nguyen et al. 2009). During this time, DTCs can either exit the proliferative cycle and enter the state of dormancy (a state of cellular quiescence in the G0 phase of the cell cycle) or grow indolently as micrometastatic colonies due to the balanced phase of proliferation and apoptosis (Fig. 12.6a). Studies have shown that the bone marrow provides survival signals that sustain the survival and viability of DTCs. Notably, SDF1 and CXCR4 are the principal mediators of survival of latent DTCs in the bone marrow (Nguyen et al. 2009). Considerable evidence suggests that EMT/Mesenchymal-to-Epithelial Transition (MET) and stemness are also essential to early DTC survival (Kang and Pantel 2013; Malladi et al. 2016).

The mechanisms of tumor cell dormancy have been substantially studied in PCa models. Several groups have recognized the contributory role of osteoblasts in the endosteal niche in tumor cell dormancy. The endosteal niche is a complex structure that interposes between bone and bone marrow; it principally includes osteoblasts,

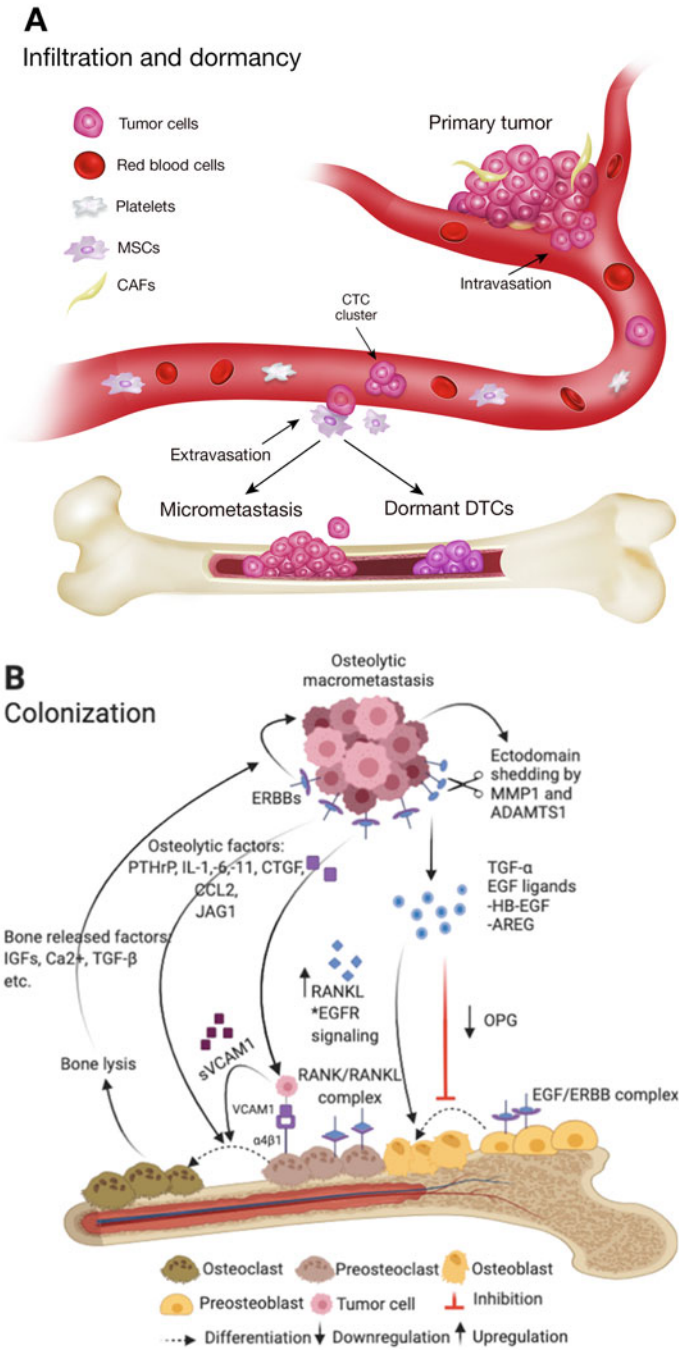

Fig. 12.6 A model of survival, bone homing, and colonization of disseminated tumor cells during osteolytic bone metastasis. (**a**) Facilitated by EMT and the abilities to surmount the natural barriers against metastasis, invasive tumor cells may disperse through the lymphatic or blood vessels either as CTCs or CTC clusters. Only CTCs that survive and carry the necessary functions for

osteoclasts, and primitive but potent Hematopoietic Stem and Progenitor Cells (HSPCs), growth factors, stromal cells, and ECM molecules that together participate in the regulation of hematopoiesis (Tamma and Ribatti 2017). Taking clues from HSCs-niche interactions, two groups have found that like HSCs, Growth Arrest-Specific 6 (GAS6)/Axl axis regulates DTC dormancy and tumor development (Jung et al. 2012; Shiozawa et al. 2010). Using a xenograft model of tumor dormancy, an association between high expression of Axl, one of the tyrosine kinase receptors for Gas6, and PCa cell dormancy in the marrow has been established, confirming the role of Axl signaling in inducing dormant phenotype (Taichman et al. 2013). The same group also explored the impact of the loss of Axl in PCa cellular dormancy when co-cultured with the pre-osteoblastic cell line, MC3T3-E1. Although MC3T3-E1 dramatically enhances the dormancy signature of PCa cells [elevated expression of both Transforming Growth Factor-Beta isoform 2 (TGF-β2) and its receptor, Transforming Growth Factor Beta Receptor 2 (TGFBR2)], loss of Axl expression limits the induction of dormant phenotype (Yumoto et al. 2016). Diminished Axl expression impairs the TGF-β2-mediated growth suppression of PCa cells. Taken together, these results underscore the critical crosstalk between TGF-β and Gas6/Axl signaling pathways in regulating osteoblast-mediated PCa cell dormancy: binding of Gas6 produced by osteoblasts to its tyrosine kinase receptor Axl expressed by disseminated PCa cells elevates expression of both TGF-β ligands [Transforming Growth Factor-Beta isoform 1 (TGF-β1) and TGF-β2] and their receptors [TGFBR2 and Transforming Growth Factor Beta Receptor 3 (TGFBR3)] in PCa cells. The autocrine and paracrine TGF-β signaling contributes to the dormancy induction of PCa cells (Yumoto et al. 2016). TGF-β2 and another osteoblast-derived factor GDF10 has also been recently shown to induce dormancy of metastatic PCa in the bone via activation of TGFBR3 and phospho-p38MAPK at the novel N-terminal S249/T252 sites (Yu-Lee et al. 2018). Furthermore, BMP7, secreted by osteoblasts and mesenchymal stromal cells, have been demonstrated to induce dormancy in PCa

Fig. 12.6 (continued) extravasation gain the capacity to infiltrate distant organs. In the bone marrow, the fenestrated structure of sinusoid capillaries allows more unrestricted infiltration of cancer cells. Once infiltrating into the bone marrow, DTCs encounter different selective pressures from those at the primary site. These selective pressures arise from a balance of growth-promoting and death signals in the newly infiltrated stroma. Owing to these restrictive forces, DTCs may enter a dormant state or show indolent micrometastatic growth. (**b**) Eventual colonization of the bone marrow by the surviving DTCs could involve the acquisition of specific genetic or epigenetic traits that yield organ-specific metastatic phenotypes. Gene expression profiling of bone metastatic cancer variants have shown the upregulation of several candidate bone metastasis genes, such as PTHrP, IL-11, IL-6, TNF-α, CTGF, CCL2, MMP-1, ADAMTS1, and JAG1 that initiate osteoclastogenesis and osteolytic bone metastasis. Tumor-derived sVCAM1 stimulates chemoattraction, cell fusion and differentiation of pre-osteoclasts, and osteolytic expansion of indolent micrometastasis (Lu et al. 2011). The subsequent bone resorption causes the release of Ca^{2+} and cytokines that are normally stored in the bone matrix such as BMPs and IGFs. These factors in turn act on the cancer cells promoting their proliferation and perpetuate a cycle of macrometastasis outgrowth. Abbreviations: sVCAM1, soluble Vascular Cell Adhesion Molecule 1; and ERBB, Erythroblastic Leukemia Viral Oncogene Homologue

stem-like cells (CSCs) by activating p38 MAPK and increasing expression of the cell cycle inhibitor, p21, and the metastasis suppressor gene, N-myc downstream-regulated gene 1 (NDRG1) (Kobayashi et al. 2011). Collectively, it is proven that cells of osteoblast lineage contribute to tumor cell dormancy occurring during initial PCa cell bone dissemination. Yet, the particular contribution of MSCs, bone-lining cells, or terminally differentiated osteoblasts in vivo remains unclear. Equally, other myeloid or endothelial populations are also assumed to play a role in the initial stages of tumor engraftment and dormancy.

For subsequent macrometastatic outgrowth, DTCs shall not only survive during latency but also exit dormancy and gain the ability to initiate metastasis when the conditions are favorable, often termed as 'tumor-propagating phenotype' or 'cancer stem cell phenotype' (Nguyen et al. 2009). Successful emergence of surviving DTCs from dormancy and their ability to aggressively colonize is believed to be influenced by the random genetic and epigenetic variations in the latent DTC population, systemic or local changes in the microenvironment as well as newly established interactions between DTCs and the target organ microenvironment. These events ultimately give rise to new metastatic populations that are optimally adapted to the host microenvironment and equipped with full metastatic competence; the process known as 'metastatic speciation' (Nguyen et al. 2009).

The exact molecular mechanisms behind dormant tumor cell reactivation in bone are not well known, but it is influenced by many factors, including secretion of tumor-promoting signals, osteoblast-derived factors, osteoclast activation, and ECM remodeling (Shupp et al. 2018). From the limited work that has been performed, 3 major developmental signaling programs are implicated in dormancy exit: TGF-β, Wnt, and Notch (Esposito et al. 2018). Of these 3 signaling pathways, Notch activation is a critical regulator of early colonization events as well as advanced osteolysis (Sethi et al. 2011; Zayzafoon et al. 2004). In the case of PCa, studies indicate that osteoblast-derived factors including RANKL, TGF-β, Gas6, and BMP7 regulate dormant PCa cell reactivation in the bone. Decreased osteoblast expression of TGF-β and Gas6 has been documented to release PC3 and DU145 PCa cells from dormancy (Yumoto et al. 2016). Furthermore, Kobayashi and colleagues have demonstrated that withdrawal of BMP7, secreted from bone stromal cells, induces tumor recurrence of PCa stem-like cells in vivo (Kobayashi et al. 2011). Consistent with this data, a significant inverse correlation has been observed between the expression of a BMP7 receptor, BMPR2, and bone metastasis of PCa patients (Kobayashi et al. 2011). These results further validate the role of BMP7 in maintaining a balance between dormancy and recurrence of CSCs in bone metastasis of PCa. Given the typically high levels of BMP in bone and its role in osteoblast differentiation, it's evident that BMP signaling mediated by osteoblasts in the bone may elicit dormant PCa cell reactivation (Kobayashi et al. 2011). However, it is hypothesized that there may be subsets of dormant tumor cells that are not responsive to BMP-mediated reactivation suggesting the complexity of mechanisms governing dormant tumor cell re-activation (Kobayashi et al. 2011).

All these studies markedly highlight an intriguing fact that osteoblasts in the bone govern tumor cell dormancy, reactivation, tumor progression, and metastasis;

however, no appealing data exists to describe the events that drive their transition from 'dormancy-promoting' to 'metastasis-promoting'.

Altered Bone Remodeling and the Underlying Bone-Tumor Cell Crosstalk in Bone Metastasis

Studies analyzing the mechanisms underlying skeletal pathology in metastatic cancers have largely credited the DTCs, with unique infiltrative and colonization functions, as being the master regulators of bone metastasis. However, in-depth research has refined and redirected the focus from studying the properties of DTCs to determining their crosstalk with bone cells (osteoblasts and osteoclasts) and the bone matrix. This dynamic crosstalk involves a self-sustaining reciprocal stimulation between tumor and bone cells that results in the perturbation of homeostatic bone remodeling through enhanced osteoblast and osteoclast activity and release of tumor-promoting growth factors. These perturbations result in mixed lesions that are characterized by the areas of extensive osteogenesis (bone formation) and osteolysis (bone degradation).

Osteolytic Metastasis

The classic model of osteolytic metastasis, as proposed by Guise (Guise et al. 1996) and Mundy (Mundy 1997; Mundy 2002), has been well described in breast cancer. Osteolytic metastasis is typified by sustained bone degradation which occurs by the virtue of constitutive osteoclast activation and altered osteoblast adhesion and differentiation (Mercer et al. 2004). Osteolysis has also been demonstrated, although rarely, in PCa, despite being a prototypic osteoblastic (also known as sclerotic or osseous) tumor, generally occurring in the initial stages of metastatic establishment that allows expansion of the metastatic site (Oades et al. 2002; Karlsson et al. 2016). It has been established that PCa has a strong resorptive component in addition to the element of bone formation.

During typical osteolytic metastasis (as seen in breast cancer), DTCs secretenumerous growth factors and cytokines, such as RANKL, PTHrP, IL-11, IL-6, TNF-α, Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Vascular Endothelial Growth Factor (VEGF)-A, Hepatocyte Growth Factor (HGF), and Jagged 1 (JAG1) (Nguyen et al. 2009; Sethi et al. 2011). RANKL and GM-CSF has been shown to directly promote osteoclastogenesis whereas PTHrP, IL-6, IL-11, VEGF-A, HGF, and TNF-α stimulate osteoblasts to promote the secretion of RANKL and M-CSF, suppress the release of OPG, and initiate RANKL-mediated osteoclastogenesis in the bone metastatic tumor microenvironment. PTHrP has been identified as one of the bioactive prostate factors that participate in PCa pathogenesis and progression through its regulatory interactions

with other bioactive prostate cell products (Deftos 2000). In prostate to bone metastasis, tumor cell-derived PTHrP drives the bone destructive cascade by enhancing the osteoclastic and endothelial cell activity in the bone marrow via upregulating Monocyte chemoattractant protein 1 (MCP-1 or CCL2) expression (Li et al. 2009). MCP-1, derived from both tumor cells and the stroma, mediates co-operative interactions between tumor-derived factors and host-derived chemokines to promote skeletal metastasis (Li et al. 2009). The stimulatory effects of HGF and VEGF-A on M-CSF and RANKL expression in osteoblasts is mediated by c-Met and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) activation, respectively (Lee et al. 2018). Numerous publications have supported the role of the HGF/c-Met axis and VEGF-A/VEGFR2 axis in PCa bone metastasis. This explains the therapeutic efficacy of Cabozantinib, a dual kinase inhibitor of c-Met and VEGFR-2, in reducing PCa growth in bone, with evidence for suppressing tumor-induced osteolysis (Lee et al. 2018). Furthermore, JAG1 has been shown to interact with both osteoblasts and pre-osteoclasts in the bone niche to drive sustained bone resorption (Kokabu and Rosen 2017). Specifically, JAG1 binds to the Notch receptor on pre-osteoclasts, leading to increased osteoclastogenesis and the formation of mature osteoclasts capable of resorbing bone (Sethi et al. 2011). In addition to binding Notch on pre-osteoclasts, JAG1 has also shown to bind the Notch receptor on osteoblasts and stimulate the production of tumor-promoting IL-6 from osteoblasts, leading to more bone destruction (Tat et al. 2006). Activated osteoclasts ultimately degrade the bone matrix via secreted catK and other cysteine proteinases into the resorption pits, thus releasing cytokines that are normally stored in the bone matrix: TGF-β, BMPs, MMPs, HGFs, and IGF-1. These factors, in turn, further enrich the local milieu, promote cancer cell proliferation, enable continued expression of osteoclast initiating factors, and eventually perpetuate a cycle of macrometastasis outgrowth (Fig. 12.6b).

In addition to the tumor-derived factors, proteases have also been implicated in the osteolytic cascade. Although the perceived functions of metalloproteinases in metastasis have been associated mainly with their proteolytic ability to degrade ECM, tumor invasion, and cytokine mobilization (Egeblad and Werb 2002; Overall and Lopez-Otin 2002; Page-McCaw et al. 2007), their functional role in bone metastasis is not well-defined. Lu and co-workers have demonstrated the involvement of two distinct metalloproteinases, MMP-1 and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS1) in the pro-osteolysis signaling cascade that modulates the bone microenvironment in favor of osteoclastogenesis and bone metastasis (Lu et al. 2009). These two proteases have been shown to proteolytically release Epidermal Growth Factor (EGF)-like ligands, such as Amphiregulin (AREG), Heparin-binding EGF (HB-EGF), as well as Transforming Growth Factor-alpha (TGF-α), from tumor cells. These EGF ligands signal through the Epidermal Growth Factor Receptor (EGFR) pathway in osteoblasts to reduce osteoblast production of OPG in the local bone microenvironment and subsequently potentiate osteoclast differentiation. This study not only established the specific functional mechanism of metalloproteinases in promoting

bone metastasis but also provided a molecular basis to place EGF family ligands in the feed-forward loop mechanism of bone metastasis.

Similarly, certain ECM modifying enzymes have been implicated in the activation of the osteolytic process. For example, studies have specified the importance of lysyl oxidase (LOX) enzyme in the early events of osteolytic lesion formation (Cox et al. 2015). LOX enzymes belong to a family of copper-dependent amino oxidases that participate in ECM remodeling, due to their capacity to cross-link collagen and elastin (Grau-Bove et al. 2015). In bone microenvironment and metastasis, LOX mediates NFATc1-driven osteoclastogenesis that disrupts normal bone homeostasis and leads to the formation of focal pre-metastatic lesions. These lesions, in combination with elevated expression of LOX, subsequently provide a platform for DTCs to colonize and form bone metastasis (Cox et al. 2015). Studies in PCa models have identified a stimulatory role for lysyl oxidase propeptide (LOX-PP) in coupling interactions between osteoclasts and osteoblasts in vitro, and exacerbation of tumor cell modification of bone in vivo. In PCa-associated bone remodeling, LOX-PP shifts the balance towards resorption by stimulating the Connective Tissue Growth Factor (CTGF)- and RANKL-mediated osteoclast differentiation and fusion (Alsulaiman et al. 2016).

Histologic studies in PCa have shown an increase in the biochemical indices of bone resorption and a greater fraction of eroded trabecular surfaces, together with the expected rise of bone-forming surfaces and active osteoblast numbers in skeletal sites adjacent to the tumor tissue (Urwin et al. 1985). The fraction of eroded surfaces is associated with increased bone volume and is consistently high within metastasis.

Clinical studies have also provided considerable evidence of osteoclastic activity in metastatic PCa. In PCa patients with active bone metastasis, higher urinary excretion of biochemical markers of bone resorption—pyridinoline and deoxypyridinoline is noted in comparison to patients with benign prostatic hyperplasia (BPH), patients with localized disease and patients with bone metastasis under hormonal therapy (Ikeda et al. 1996). Higher urinary levels of these collagen breakdown compounds correlated with the extent of bone metastasis in new and reactivated cases of PCa with bone metastasis. There are a few case reports in the medical literature that present clinical evidence of pure osteolytic metastasis in PCa. Rajendiran et al. have reported a case of a 65-year-old African American man with metastatic prostate adenocarcinoma, high Prostate-Specific Antigen (PSA) level of 7242 ng/mL, and extensive osteolytic metastasis in the entire spine, ribs, and pelvis (Fig. 12.7a) (Rajendiran et al. 2011). Another case report reveals the presence of diffuse osteolytic lesions involving the femur, sacral and pelvic bones, the entire spine and the humerus in a 65-year-old Namibian man with infiltrating (Gleason score 9) prostate adenocarcinoma (Segamwenge et al. 2012). The extremely rare presence of solitary osteolytic radial head metastasis (Ansari et al. 2003) and solitary metastasis to the right hip (Agheli et al. 2009) has also been reported. It is worth noting that the rarity of such osteolytic metastatic presentation in PCa and its coexistence with other bone metastatic cancers, especially, multiple myeloma (MM), often results in mis- or delayed diagnosis. A few reported cases have described the synchronous occurrence of MM and prostate adenocarcinoma

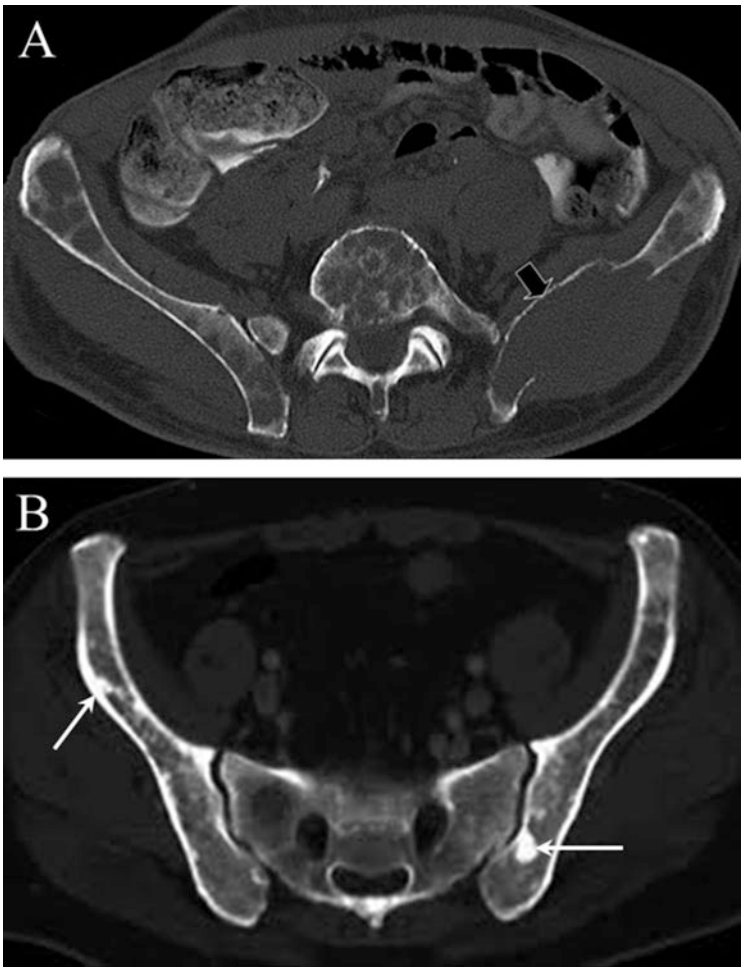

Fig. 12.7 Computed tomography (CT) scan of pelvis showing (**a**) an isolated expansile osteolytic lesion in the left iliac bone (indicated by a black arrow) (Rajendiran et al. 2011) and (**b**) multiple osteoblastic lesions at iliac and sacral bones (indicated by white arrows) (Se Kyoung et al. 2014)

metastasis within the same bone lesion (Adrianzen Herrera et al. 2018). Such unusual clinical phenomenon emphasizes proper radiographic imaging assessment and interpretation to guide precise diagnosis, early detection, and initiation of supportive treatment aimed at attenuating skeletal complications, including bisphosphonates, radiopharmaceuticals, radiotherapy, surgery, vertebroplasty, and kyphoplasty (Smith et al. 2009).

Osteoblastic Metastasis

In comparison to the well-researched mechanisms of osteolytic metastasis, the biology of osteoblastic metastasis remains less understood. The majority of insights into the pathogenesis of osteoblastic bone metastasis have been gained from PCa which presents characteristic osteoblastic lesions (Fig. 12.7b). The preferential homing of PCa cells to osteoblast-rich regions of the bone as well as the autocatalytic cycle of bone metastasis has been shown to promote both osteoblast proliferation and differentiation and metastatic PCa cell proliferation (Shupp et al. 2018). Prior to the development of bone metastasis, PCa cells home to the endosteal niche of the metaphysis region of long bones (Pedersen et al. 2012; Shiozawa et al. 2011). Osteoblastic bone formation predominantly occurs on lateral endocortical bone surfaces with abundant osteoblast lineage cells (Frost 2001) and therefore, during very early stages of bone metastasis, PCa cells preferentially traffic to these areas. This preferential homing of PCa cells is mediated by the osteoblast-derived SDF-1/CXCR4 axis (Wang et al. 2014). High expression of SDF-1 mRNA has been observed in the long bones near the endosteal surfaces covered by osteoblastic and lining cells, underlining the importance of skeletal localization of SDF-1 in PCa bone metastasis (Sun et al. 2005).

The classic osteoblastic bone lesions are attributed to the aftermath of the uncoupling of normal bone resorption and deposition. This uncoupling shifts the balance towards new matrix formation and mineralization (Oades et al. 2002). Generally, bone resorption precedes bone formation in the development of osteoblastic metastasis; however, it is still unclear whether bone resorption naturally precedes during osteoblastic metastasis or is a consequence of the increased bone formation (Roodman 2004).

Another strategy adopted by PCa cells to fully exploit the bone marrow niche and osteoblast functions is the ability to resemble osteoblasts, also called as osteomimicry (Scimeca et al. 2018). In osteomimicry, PCa cells express bone matrix proteins, such as osteocalcin, bone sialoprotein, OPG, and RANKL (Jadaan et al. 2015) and osteoblast-related factors including ALP and Runx2, a crucial transcription factor in the acquisition of osteomimicry (Brubaker et al. 2003). These factors have been implicated in osteoblastic lesions, either by directly influencing the osteoblast function, or indirectly influencing bone formation through modification of the bone matrix or microenvironment. Traditionally, a feedback loop prototype (also widely known as the vicious cycle paradigm) describing the dynamic crosstalk between metastatic PCa cells, cellular components of the bone marrow microenvironment, and the bone matrix has been used to model the co-option of homeostatic bone remodeling by PCa to drive prostate to bone metastasis (Cook et al. 2014) (Fig. 12.8). On establishing foothold in the bone, PCa cells adapt to, and modify, the surrounding microenvironment by secreting active molecules such as ET-1, Wnts, TGF-β, Endothelin (ET)-1, Platelet-derived Growth Factor (PDGF), urokinase-type Plasminogen Activator (uPA), IGF-1, FGFs, PTHrP, and BMPs that induce the dysfunctional osteoblastic phenotype and promote osteoblast differentiation and

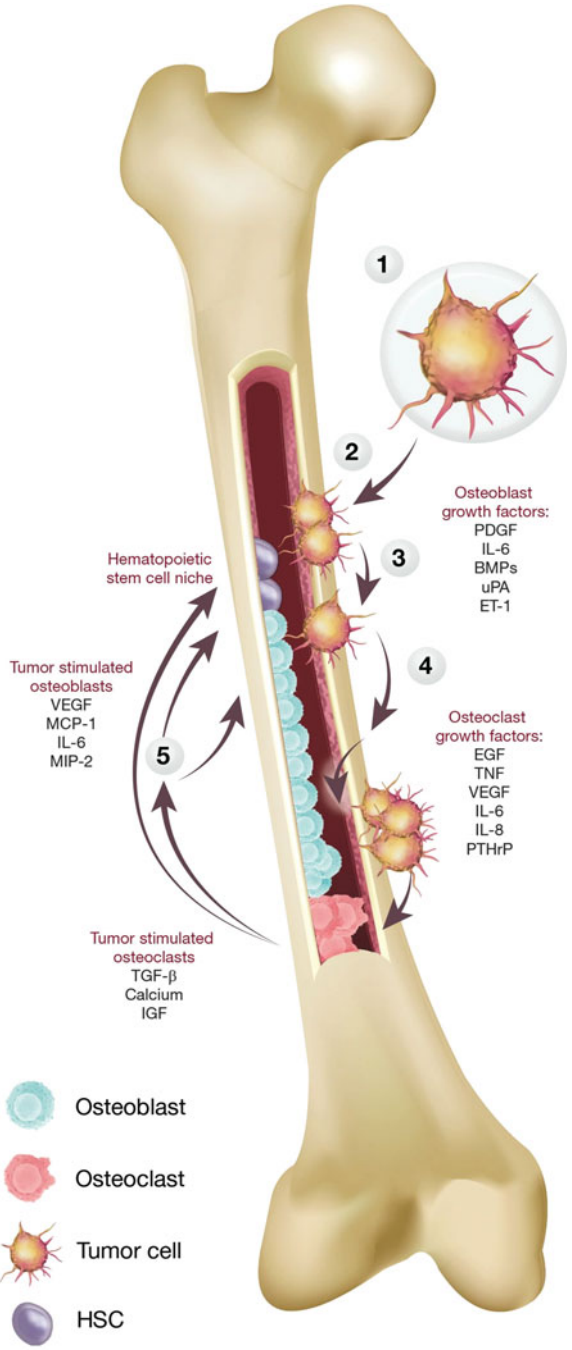

Fig. 12.8 Biology of bone metastasis in prostate cancer. (1) The CTCs arrive in the bone via the vasculature. They preferentially adhere to bone marrow endothelial cells and then migrate through the endothelial layer, both mediated in part by many integrin-ECM interactions. (2) Tumor cells preferentially home to regions rich in osteoblast-lineage cells via the CXCL12/CXCR4 axis and colonize the stem cell niche. (3) The proliferation of the stem cell niche reactivates tumor cell dormancy. (4) Tumor cells secrete osteoblast and osteoclast stimulating factors. (5) Activated osteoblasts and osteoclasts secrete factors that stimulate tumor cell proliferation and growth in bone. Abbreviations: MIP-2, Macrophage Inflammatory Protein 2

activity (Quiroz-Munoz et al. 2019). Besides the osteoclastic manipulation, PCa-derived PTHrP actively governs osteoblastic skeletal progression by stimulating osteoblast progenitor cell proliferation and inducing early osteoblast differentiation (Liao et al. 2008). The differentiated osteoblasts, in turn, stimulate matrix mineralization and formation of new bone. Additionally, activated osteoblasts produce factors including IL-6, MCP-1, VEGF, and MIP-2, that induce invasion and colonization of PCa cells in the bone. Osteoblast-secreted TGF-β1 has been demonstrated to modulate the secretion of urokinase-type Plasminogen Activator (uPA) and activation of MMP-9 that enhance PCa cell chemotaxis and invasive activity by increasing proteolytically active areas of the cell surface (Shupp et al. 2018; Liao et al. 2008; Festuccia et al. 2000; Festuccia et al. 1999).

Secretion of ET-1, an osteoblast mitogenic factor, by PCa cells has shown to stimulate osteoblast activity by reducing autocrine production of DKK1, activate Wnt signaling, and contribute to osteoblast-mediated bone anabolic response (Clines et al. 2007). Similarly, the cancer cell-derived PDGF exerts its mitogenic effects on pre-osteoblasts resulting in an increased number of osteoblasts that are capable of forming new bone (Canalis 2013). Another osteoblast master transcription factor that is aberrantly expressed by PCa cells is Runx2 (Baniwal et al. 2010). The transcriptional regulation of Runx2 during osteoblastogenesis is well-known, however, in bone metastasis, it exerts pleiotropic roles necessary for the metastatic process. Runx2 has been shown to upregulate a multitude of genes with prominent pro-metastatic functions such as EMT, tissue invasion, as well as homing and attachment to bone (Baniwal et al. 2010). Elevated expression of osteoblast-cadherin (also known as cadherin-11) has also been reported in acinar PCa (propensity to metastasize to bone) specimens (Chu et al. 2008). Cadherin-11 is a hemophilic cell adhesion molecule highly expressed in primary osteoblasts (Okazaki et al. 1994) and is associated with osteoblast differentiation and maturation (Kawaguchi et al. 2001). Being an adhesion molecule, it is hypothesized that the high expression of cadherin-11 might augment the binding of cancer cells to osteoblasts in the skeleton and thus increase the potential for PCa to metastasize to bone (Chu et al. 2008). This enhanced binding could then facilitate paracrine molecular interactions between cancer cells and the osteoblasts to aid the survival and growth of PCa cells in bone (Chu et al. 2008).

In aggregate, these results show the complex nature of PCa in bone tissue and the differential disturbance of bone formation and resorption within metastasis and in the bone surrounding micrometastasis (Clarke et al. 1991).

Role of Extracellular Vesicles in Prostate Cancer Bone Metastasis

In the last 5 years, several models have been formulated which recapitulate the current paradigm of the bone metastasis and shed light on the multifaceted interplay

between heterogeneous tumor cells and the bone microenvironment. Discoveries on the mechanisms of bone metastasis, for example, EMT and osteomimicry, extends past the evolutionary aspects typically considered in the molecular mechanisms underlying osteoblast-mediated bone metastasis (Ibrahim et al. 2010). Recently, studies have implicated EV-mediated intricacies of interactions between PCa cells and the bone microenvironment in conferring the 'tumor propagating phenotype' (Fig. 12.9). While this implication of EVs has been well ascertained, their role in bone metastasis development is still an open field and only recently has garnered voluminous attention.

Itoh and co-workers have presented first evidence on mechanisms underlying the stimulation of osteoblast differentiation by PCa derived-EVs (Itoh et al. 2012). Exposure of murine pre-osteoblast MC3T3-E1 cells to EVs released from the PCa cell line PC3 facilitate osteoblast differentiation, primarily, through the delivery of osteoblast differentiation-related transcription factor, v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1). Tumor-derived Ets1 regulates the expression of MMP-1, −3, −9, and uPA (Sementchenko and Watson 2000) along with certain osteogenic proteins such as osteopontin, tenascin-C, and procollagen (Raouf and Seth 2000; Sato et al. 1998), making it a candidate inducer of osteoblast differentiation. These results not only label the PCa-derived EVs delivery system as a reasonable transfer model for Ets1 but also as a cell-to-cell communication tool in osteoblastic metastasis. In line with this, a proof-of-principle study by Probert and group has further provided critical evidence of the importance of PCa-derived EVs in changing osteoblast behavior to support PCa cell proliferation (Probert et al. 2019). In vitro, EVs isolated from PCa cells with a bone-metastatic propensity induce changes in osteoblast viability and create a supportive growth environment for PCa cells when grown in co-culture with EV-treated osteoblasts.

The increase of invasive capability of PCa cells after Osteoblast-derived Conditioned Media (OBCM) stimulation has been reported (Festuccia et al. 1999). Co-culturing PC3 cells with serum-free OBCM stimulates in vitro chemotaxis of cancer cells and invasion. Also, OBCM stimulates the secretion of uPA and MMP-9 in PCa, while at the same time increasing the rate of PCa proliferation (Festuccia et al. 1999). The plasmin cascade driven by uPA is associated with ECM degradation and implicated in modulating the properties of PCa cells (Quax et al. 1997). Ultrastructural morphological analysis of these osteoblast-conditioned PC3 cells has demonstrated increased membrane activity and stimulated membrane vesicle shedding into the extracellular area in comparison with unstimulated cells (Millimaggi et al. 2006). Analysis of the protease expression in the shed vesicles has shown elevated expression of proenzymatic and active forms of gelatinase A (MMP-2) and gelatinase B (MMP-9) and that of High Molecular Weight urokinase Plasminogen Activator (HMWuPA). The addition of HMWuPA-containing EVs to cultures of the poorly invasive PCa cell line LNCaPhave been shown to enhance the adhesive and invasive capabilities of the LNCaP cells, confirming a mechanism involving vesicle-associated uPA in substrate recognition, degradation, and invasion in PCa (Angelucci et al. 2000). Given the role of vesicle-associated lytic enzymes in distant proteolysis to aid tumor implantation in target organ, this modulation of

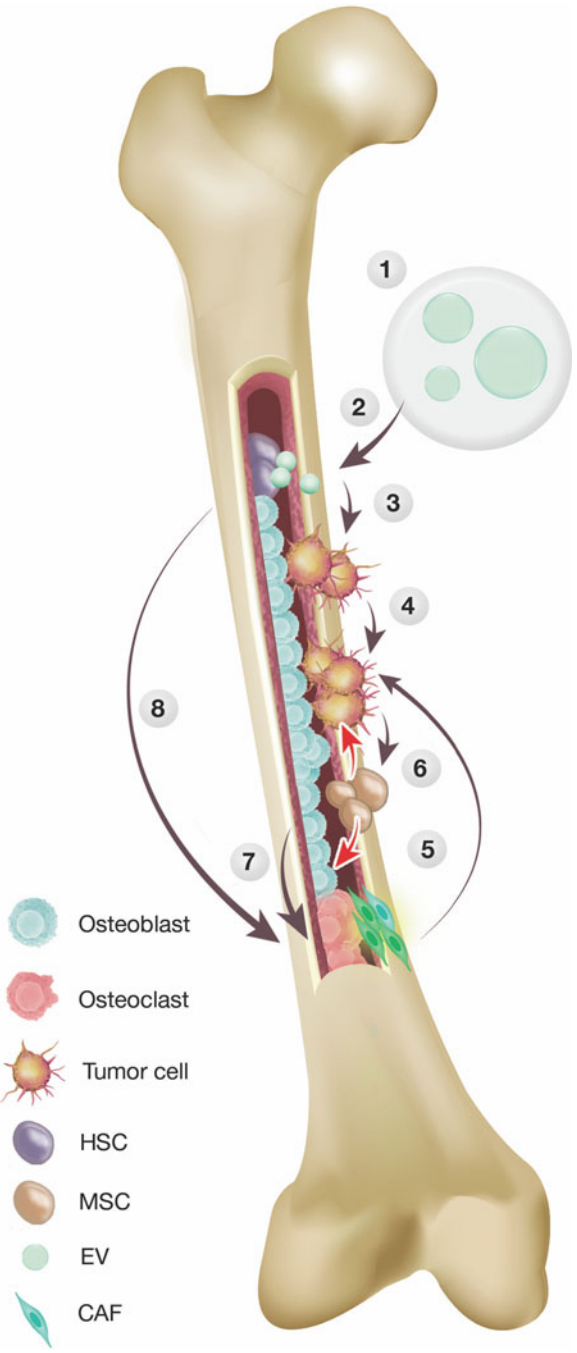

Fig. 12.9 Role of extracellular vesicles in the establishment of the pre-metastatic niche and metastasis in prostate cancer. (1,2) Prior to the metastatic outgrowths in bone, tumor-derived EVs provide various autocrine and paracrine signaling cues that contribute to the reprogramming of primary tumor microenvironment through processes such as EMT, vascular remodeling, coagulation, ECM remodeling, and modulation of immune responses. (3,4) With enhanced invasiveness and motility of neoplastic cells, activated metastasis is established in bone via the expression of VCAM-2 and the recruitment of osteoclast precursors. (5,6) Tumor-derived osteoblast differentiation-related transcription factors such as Ets1 as well as EV-associated miRNAs can promote recruitment and differentiation of MSCs into CAFs or osteoblasts. (7) Enhanced osteoblast activity and the osteoblast stromal cell expression of RANKL may, in turn, drive osteoclastogenesis and contribute to osteoclast-mediated osteolysis. (8) Osteoclast-mediated osteolysis may be further enhanced by the osteoclastogenic differentiation of HSCs. Abbreviations: VCAM-2, Vascular cell adhesion protein 2

vesicle shedding from bone-metastatic PCa cells by the paracrine factors released by osteogenic cells could potentially contribute to the bone tissue remodeling by PCa and local growth in PCa metastasis (Millimaggi et al. 2006). Similarly, the role of NMOBs and MOBs human osteoblast-secreted EVs in promoting PC3 cells in vitro through an array of mineralization stage-specific proteins has been reported (Morhayim et al. 2015). It has been demonstrated that osteoblast EV proteomes and EV-regulated PCa gene expression profiles converge on pathways involved in cell survival and growth.

EVs are enriched in tetraspanins, two of which (CD9 and CD151) show altered expression patterns (decreased CD9 levels and increased CD151 levels) in metastatic PCa (Wang et al. 2007; Ang et al. 2004). Tetraspanins together with their partner molecules, including integrins, members of the immunoglobulin superfamily, and MMPs, form tetraspanin-enriched microdomains or the 'tetraspanin web' on the cell surface. These microdomains act as signaling platforms that facilitate tetraspanins to influence cellular functions such as cellular motility and migration and also dissemination and metastasis of tumors (Brzozowski et al. 2018) (For a detailed review on tetraspanins and cancer metastasis, refer (Detchokul et al. 2014)). Considering the relationship between altered CD9 and CD151 expression with the metastatic phenotype in PCa, the influence of tetraspanins on EV cargo recruitment, and the potential for EVs to affect metastatic functions, it has been determined that the altered expression patterns of CD9 and CD151 alter the proteome of EVs which eventually influence cellular behavior and increase the migratory and invasive capabilities of non-tumorigenic PCa population (Brzozowski et al. 2018). Soekmadji and her team have demonstrated that CD9-enriched EVs mediate paracrine signaling in PCa and modulate cellular proliferation in the absence of androgens (Soekmadji et al. 2016). The co-localization of CD9 and its novel partner, the 75-kDa protein HSPA9B—also known as mortalin—induces mitotic 'catastrophe' in PCa (Zvereff et al. 2007). Further analyses have implicated the role of CD9+ EVs, distinct from Tumor Susceptibility Gene (TSG)101- and Alix-positive EVs, regardless of the activation of the androgen receptor (AR) signaling axis (Soekmadji et al. 2016). Furthermore, elevated levels of CD9+ EVs have been discovered in plasma derived from CTC positive metastatic PCa cohort as compared to CTC negative patients, thus emphasizing the role of CD9+ EVs in advanced metastatic PCa (Soekmadji et al. 2016). These observations state that cellular tetraspanins should be recognized as crucial metastasis-inducing molecules in addition to being widely recognized as EV-specific markers.

A study by Karlsson et al. has illustrated the rapid uptake of the PCa-derived EVs by osteoclast progenitor cells and their consequent anti-osteoclastogenic properties (Karlsson et al. 2016). The uptake resulted in their decreased proliferation and de-differentiation of RANKL-induced osteoclastogenesis of monocytic osteoclast precursors to mature, multinucleated osteoclasts. Further, the inhibitory effects of these EVs on osteoclast formation and activity was confirmed by the decreased mRNA expression of cell fusion markers such as Dendritic Cell-Specific Transmembrane Protein (DC-STAMP), several markers for osteoclast differentiation and activity, including TRAcP, and the proteinases catK and MMP-9. The anti-

osteoclastogenic effects of PCa cell-derived EVs are well in line with the osteoblastic phenotype of skeletal metastasis formed by disseminated tumor cells in patients with metastatic PCa. All this data points out to the fact that tumor-derived EVs isolated from PCa cells contribute to the osteoblastic phenotype of skeletal metastasis by impairing osteoclast formation and thereby, bone degradation in metastatic sites (Karlsson et al. 2016).

The Involvement of Extracellular Vesicle RNA Cargo in Prostate Cancer Bone Metastasis

Studies employing in vitro models and novel RNA-tracking techniques have yielded important information on the role of RNA cargoes of PCa-derived EVs in mediating the interaction between PCa cells and osteoblasts. Characterization of the EV-miRNA cargo derived from the bone-metastatic PCa cell line PC3 and the subsequent comparative biological pathway analysis has shown significant enrichment of genes relating to cell surface signaling, cell-cell interaction, and protein translation (Probert et al. 2019). PCa cells have been demonstrated to interact with osteoblasts and increase their viability and activity via the EV-mediated transfer of miR-21 and miR-375 (Probert et al. 2019; Li et al. 2019). Similar to the study of EV-miRNAs, analysis of mRNA transcripts in osteoblasts exposed to PC3-derived EVs has indicated a significant abundance of the Colony-Stimulating Factor 1 (CSF-1), Ephrin A3 (EFNA3), VEGF-A, CCL2, Runx2, and FGF transcripts with relative roles in osteoblast cell-cell interaction, osteoclastogenesis, and osteoblastic bone formation (Probert et al. 2019). Transfection with differentially expressed miRNAs in EVs from PCa populations such as miR-30 and miR-218 have been shown to regulate osteoblast differentiation and augment the expression of RANKL that is crucial for osteoclastogenesis (Sánchez et al. 2016). Analysis of target genes regulated by differential miRNAs overexpressed in EVs revealed that miRNAs have a common set of targets, out of which BMPR2 and Heterogeneous Nuclear Ribonucleoprotein U (HNRNPU) are related with osteoblast differentiation that could be involved in the preparation of the premetastatic niche. Furthermore, in vivo, miR-141-3p, associated with PCa cell-derived EVs, show bone-target specificity and regulate the bone microenvironment to promote osteoblastic metastasis and osteogenesis damage. Upon uptake by osteoblasts, miR-141-3p promotes osteoblast activity by increasing the OPG expression. It also suppresses the protein levels of its target gene Deleted in Liver cancer 1 (DLC1) prompting the activation of p38MAPK signaling to further promote bone formation (Ye et al. 2017). Another miRNA, hsa-miR-940, expressed in EVs from osteoblastic phenotype-inducing PCa cell lines, has been shown to significantly promote the osteogenic differentiation of human MSCs. In vitro, hsa-miR-940-targets Rho GTPase Activating Protein 1 (ARHGAP1) and Family With Sequence Similarity 134 Member A (FAM134A) and induces extensive osteoblastic lesions (Hashimoto et al. 2018). Cumulatively, this study supports the contribution of the RNA element of the PCa EV cargo as a potential novel route to mediate osteoblastic metastasis. Table 12.3 summarises the

Table 12.3 Functional relevance of extracellular vesicles in prostate cancer bone metastasis

EV-derived cargo	Targets	Functions	Refs.
miR-21	TGF- β RANKL MMPs	• Promotes osteoblast viability • Mediates distant bone marrow colonization • Promotes osteolysis	Bonci and Maria (2015); Sanchez et al. (2016); Probert et al. (2019)
miR-141-3p	OPG DLC1	• Bone-target specificity • Regulates bone microenvironment to promote osteoblastic metastasis • Activates p38-MAPK signaling pathway, increasing OPG/RANKL expression leading to increased osteoblast activity	Ye et al. (2017)
hsa-miR-940	ARHGAP1 FAM134A	• Promotes osteogenic differentiation of MSCs resulting in osteoblastic phenotype in the bone metastatic microenvironment	Hashimoto et al. (2018)
miR-375	OPN OPG Runx2 BSP ALP	• Increases osteoblast differentiation, proliferation, and activity	Li et al. (2019)
CSF-1		• Promotes osteoblast- mediated osteoclastogenesis and a supportive growth environment	Yao et al. (2002); Probert et al. (2019)
EFNA3		• Promotes osteoblast cell-cell interaction and osteoblastic bone formation	Matsuo and Otaki (2012); Probert et al. (2019)
CCL2 (MCP-1)		• Mediates recruitment and differentiation of osteoclast monocyte precursors • Facilitates osteoclastogenesis via RANK/RANKL axis	Li et al. (2007); Probert et al. (2019)
FGF2		• Regulates osteoblast replication and differentiation • Promotes bone formation	Montero et al. (2000); Probert et al. (2019)
Runx2		• Regulates osteoblastogenesis	Prince et al. (2001); Probert et al. (2019)
Ets1		• Enhances osteoblast differentiation	Itoh et al. (2012)

role of EVs and their bioactive cargo in mediating the interaction between PCa cells and bone cells.

Considering the increased levels of miR-409-3p/−5p, miR-379, and miR-154* in the circulating EVs of patients with PCa (Nguyen et al. 2013), studies on these miRNAs, located within the delta-like one homolog-deiodinase, iodothyronine 3 (DLK1-DIO3) imprinted region located on human chromosome 14, have substantiated their tumor-inductive effects (Gururajan et al. 2014). In the setting of PCa, the

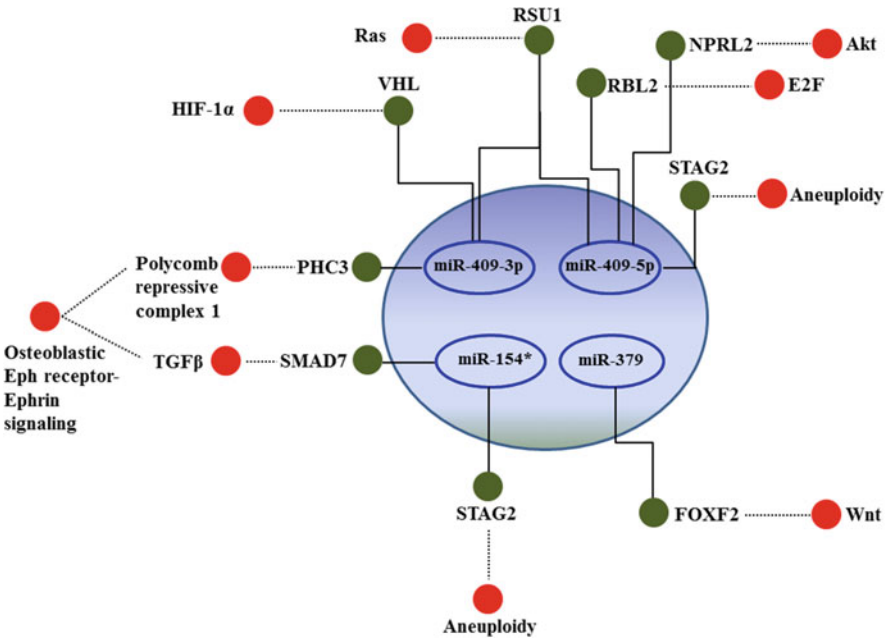

Fig. 12.10 Analyses of target genes and oncogenic pathways regulated by miR-409-3p/−5p, miR-154*, and miR-379 in the DLK1-DIO3 miRNA mega-cluster in prostate cancer. Cytoscape map of the human cancer pathways regulated by the DLK1-DIO3 miRNAs show targeting of Ras-integrin linked kinase signaling, HIFα pathway, and osteoblastic pathways by miR-409-3p; aneuploidy, E2F pathway, Ras signaling, and Akt pathway by miR-409-5p, aneuploidy and osteoblastic Eph-receptor-Ephrin signaling by miR-154*, and Wnt signaling pathway by miR-379. Green circles represent the activation of oncogenic pathways and red circles represent inhibition of tumor suppressor genes (Figure modified with changes from (Gururajan et al. 2014). Abbreviations: STAG2, Stromal Antigen 2; HIF-1α, Hypoxia-Inducible Factor-1α; RSU1, Ras suppressor protein 1; RBL2, Retinoblastoma-like 2; PHC3, Polyhomeotic homolog 3; NPRL2, Nitrogen permease regulator-like 2; FOXF2, Forkhead box F2; and VHL, von Hippel-Lindau

DLK1-DIO3 cluster miRNAs promote PCa growth, EMT, and bone metastasis. (Gururajan et al. 2014). Elevated expression of miR-409-3p/−5p, miR-154*, and miR-379 has been reported in bone metastatic PCa cell lines and human PCa tissues with higher Gleason scores with a causal correlation between miR-409-3p/−5p and miR-379 expression and progression-free survival of patients (Gururajan et al. 2014; Josson et al. 2014). These findings dictate the translational importance of the DLK1-DIO3 cluster miRNAs as potential biomarkers and possible therapeutic targets for bone metastatic PCa. Figure 12.10 illustrates the cytoscape analyses of target genes and signaling pathways altered by the DLK1-DIO3 cluster miRNAs: miR-154*, miR-409-3p/−5p, and miR-379 in PCa.

Understanding Vesicle Cargo Sorting and Selection of Metastasis-Inducing Molecules

It is becoming increasingly clear that elucidation of the spectrum of molecular mechanisms regulating EV secretion and cargo selection of metastasis-inducing molecules is instrumental in understanding PCa metastasis. Recently, a study was performed by Lázaro-Ibáñez and group to systematically address the uptake and functional properties of EVs released by PCa cells with varying degrees of malignancy (Lazaro-Ibanez et al. 2017). The team showed that the metastatic state and cell cycle phase of parent tumor cells influence the efficiency of uptake/internalization and functionality of PCa cell-derived EVs. Analyses of functional outcomes and mechanisms of Cavin-1 (also known as polymerase I and transcript release factor; PTRF) expression on PCa cell-derived vesicles have shown that Cavin-1 reduce the EV levels of a subset of structural and functional proteins such as cystatin B, collagen VI, and fibronectin and miRNA such as miR-148a (Inder et al. 2014). This Cavin-1-mediated regulation on the recruitment of functional EV cargo was concomitant with attenuated EV-mediated osteoclastogenesis and osteoblast proliferation, reduced xenograft tumor growth, and metastasis (Inder et al. 2014). Nonetheless, Cavin-1 expression did not alter EV release or in vivo distribution. These results suggest that Cavin-1/PTRF is important for vesicle cargo sorting and internalization to attenuate vesicle-mediated osteoclastogenesis and osteoblast proliferation in advanced metastatic PCa cells.

Collectively these studies underscore the role of EVs in delivering a pro-tumorigenic signal and inducing phenotypes of metastasis in the bone environment in PCa. Importantly, it draws attention to the importance of EV-mediated crosstalk between cancer cells and the resident effector bone cells in tumor progression to metastasis. Delineating the specific mechanisms underlying vesicle-mediated cancer-to-bone cell communication will provide new diagnostic, prognostic, and therapeutic ideas to combat bone metastasis and skeletal-related events in advance metastatic PCa.

Conclusions

Skeletal metastatic cancers are typically lethal and therefore, research efforts are directed towards identifying the intrinsic and extrinsic factors that contribute to tumor colonization of the skeleton. Multiple mechanisms have been identified that increase the preferential colonization of tumor cells in the bone, including the intrinsic properties of the tumor cells, complex interactions between DTCs and the bone/bone marrow microenvironment, and the bone microenvironment itself (Roodman 2018). DTCs from various common malignancies such as prostate, lung, and breast and hematological malignancy such as myeloma are uniquely primed to subvert the bone remodeling processes to form pathological osteolytic

or osteoblastic lesions or both. However, recent developments focused on re-evaluating traditional assumptions about the pathophysiology of bone metastasis have been revolutionized by the discovery of EVs and their diverse metastatic functions.

EVs have emerged as multifunctional nano-vesicles that carry a prodigious amount of guidance cues that enable reprogramming of tumor microenvironment to provide a tumor-supportive ecosystem for cancer progression and metastasis. They have also been implicated in the etiology of organotropic metastasis owing to their affinity to specific recipient cells in target organs. In bone metastatic PCa, EVs have gradually emerged as critical regulators of homeostatic bone remodeling. Considering the gravity of metastasis in PCa, several pertinent questions need to be addressed and intricate sequences of coordinated events need to be delineated such as (1) How do EVs secreted by different cell types contribute to the formation of metastasis and what are their unique vesicle cargo? (2) Do heterogeneous populations of EVs target specific cell types and facilitate the initiation/evolution of the pre-metastatic niche and the metastatic environment through different but overlapping mechanisms? (3) What are the somatic molecular and genetic determinants of EVs dysregulation in solid cancers? (4) What is the relative contribution of complex interactions between EVs, responding cells, and environmental factors in the seemingly controversial anti-tumorigenic or pro-tumorigenic effects of EVs? (Syn et al. 2016; Becker et al. 2016). The essential pre-requisites in explaining these questions are an in-depth knowledge of EV binding, uptake, binding-initiated signal transduction, and uptake-promoted target cell reprogramming. Efforts are currently underway to optimize isolation, purification, and characterization procedures for different EV subtypes and find modulators of their composition, secretion, and targeting.

Currently, a limited number of models are available that can fully recapitulate the development of bone metastasis or accurately replicate the natural history of clinical progression. Efforts to delineate steps in the tumor cell dissemination/metastatic cascade has been made possible by novel engineered organotypic models (Ghajar et al. 2013) and extended culture bioreactors (Sosnoski et al. 2015); however, assembly of such models is challenging due to the technical hurdles and complexity of the bone microenvironment (Chen et al. 2012). Moreover, advances in studying the molecular mechanisms of bone metastasis have indicated the significance of the control of and emergence from dormancy in predicting metastatic relapse; yet there are no good models to investigate cancer cell dormancy from multiple vantage points and thus no therapies to target dormancy per se. These challenges emphasize the need to develop efficient models of bone metastasis seeding, dormancy, and exit to further our understanding of the exact molecular basis of dormancy and metastatic cancer cell progression in bone.

Considering the urgency to decrease the overall incidence of bone metastasis, the convergence of experimental studies with clinical research has become a necessity. Given the critical and pharmacokinetically specific tumor environment, tumor heterogeneity, intricately organized bone microenvironment, the complex bone metastasis cascade, and significant bone metastasis-related deaths in PCa, the idea of

finding a more complex panel of biomarkers is appealing. Several studies have already reported the prognostic value of bone-related parameters, including bone turnover markers such as urinary N-telopeptide and bone-specific ALP, for overall survival in men with bone metastasis from CRPC (Fizazi et al. 2015). Still, new biomarkers that can identify high-risk patients and faithfully predict future long-term outcomes are needed. Research efforts have been directed to isolate and characterize the bone metastasis-derived EVs and improve their sensitivity as biomarkers. Researchers are testing the possibility of predicting incipient bone pathology by determining the contents of EVs isolated from the patients' biofluids and thus their use as ex vivo biomarker in predictive oncology. Such investigations into biomarker profiling of EVs may provide better diagnostic and prognostic biomarkers for bone metastatic PCa.

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Chapter 13
Extracellular Vesicles Contain Putative Cancer Biomarkers

Sai V. Chitti and Christina Nedeva

Abstract Emerging evidences have implicated extracellular vesicles (EVs), nanoparticles secreted by cells, in regulating cancer progression. Several seminal studies on EVs have added an additional layer to the previously unanswered questions in understanding the complexity of diseases such as cancer. It has been observed that EV content is highly heterogenous and it likely reflects the dynamic state of the parent cell. Hence, these nano-sized vesicles have been proposed as reservoirs of cancer biomarkers for diagnostic and prognostic purposes. Due to their presence in almost all biological fluids, ability to display membrane, and sometimes cytosolic, cargo of its host cell and increase in their number during disease states has supported the potential utility of EVs as an alternative to current methods of cancer diagnosis. The following chapter will discuss the use of cancer cell-derived EVs as a resource of tumor specific biomarkers for the early diagnosis of disease. In addition, EVs could also be used in personalised medicine as a resource of predictive biomarkers to understand a patient's response to therapy. Overall, EVs could be exploited as a source of cancer biomarkers and could aid in treatment and stratification options to improve patient survival and quality of life.

Keywords Extracellular vesicles · Biomarkers · Cancer · Tumor biomarkers · Disease diagnosis

Introduction

Knowledge gained from the human genome project and advances in technology has reformed clinical practises, leading to the development of vaccines, drugs and targeted therapies (Weatherall et al. 2006; Collins 1999; Ullal et al. 2014; van Buggenum et al. 2018; Brofelth et al. 2020). Though cancer as a disease is extremely

S. V. Chitti (✉) · C. Nedeva
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia
e-mail: S.Chitti@latrobe.edu.au

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complex and drugs that could treat the disease effectively are limited, increase in the number of molecular and genetic approaches along with powerful screening technologies has accelerated drug discovery and development timelines (Biomarkers Definitions Working 2001). Clinical response to such therapeutic interventions could be better achieved by analytical tools which measure the biological parameters known as biological marker or biomarker (Alizadeh et al. 2000; Biomarkers Definitions Working 2001). According to the National Institute of Health, a biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working 2001).

The last few decades have seen the expansion of powerful tools and technologies such as microarray, mass spectrometry, single-cell RNA sequencing, whole exome sequencing and high throughput screening which not only assisted in understanding disease biology but also identification of biomarkers for diagnosis of various diseases (Hoffman et al. 2020; Kim et al. 2020; Bennett and Devarajan 2011; Beltran et al. 2015). Biomarker development also offers a valuable tool for diagnostics, for staging and for disease prognosis. For example, discovery of cancer antigen 125 (CA125) as an ovarian tumor marker, not only aided in disease diagnosis but also corelated with ovarian cancer progression, status and response to therapies (Colakovic et al. 2000). Hence, several studies aim to identify reliable biomarkers for various types and stages of cancer (Mathivanan 2012; Mathivanan et al. 2012). However, to be ideal candidates for disease detection, biomarkers should possess the following characteristics: (1) should reliably discriminate between diseased and healthy individuals during early stages of disease, (2) should be non-invasive, in-expensive and produce rapid results, (3) should be present in biological fluids that can be easily obtained with minimal patient discomfort and intervention, (4) must have clinical value – they can be validated in larger populations and large prospective clinical trials (Duffy 2013). Despite this, there are very few biomarkers that possess all the ideal characteristics and hence alternative means to identify biomarkers are routinely investigated.

EVs Are Reservoirs of Cancer Biomarker Candidates

Most cell types, including healthy and disease cells, secrete phospholipid bilayer-bound nanoparticles that are present in the bodily fluids and are collectively known as extracellular vesicles (EVs) (Buzas et al. 2014; Gangoda et al. 2015; Boukouris and Mathivanan 2015). These small structures were once thought to be merely extracellular debris, however, it is now known that EVs are one of the key players in intercellular communication (Gangoda et al. 2015; Mathivanan et al. 2010). There are several subtypes of EVs based on their biogenesis (Gould and Raposo 2013). These include exosomes (30–150 nm), ectosomes or shedding microvesicles (100–1000 nm), large oncosomes (1000–10,000 nm), apoptotic bodies (50–5000 nm), exomeres (<50 nm) and migrasomes (500–3000 nm) (Kalra et al.

2016; Zijlstra and Di Vizio 2018). It was observed that EV content is highly heterogenous and it likely reflects the dynamic state of the originating cell. The varying proteins, lipids, metabolites, messenger RNAs (mRNAs), micro RNAs (miRNAs) (Samanta et al. 2018), genomic DNA (Thakur et al. 2014) and mitochondrial DNA (Guescini et al. 2010) are derived from the host cells of origin (Castillo et al. 2018; Pathan et al. 2019). The cargo contained in the EVs are protected from harsh degrading conditions and are highly stable due to the presence of the lipid bilayer. These features of EVs make them a reservoir of potential biomarkers. Another interesting aspect for employing EVs as a biomarker is less signal to noise ratio. For example, blood contains an abundance of proteins that masks the identification of molecules of interest which are normally of low abundance. However, isolation of EVs from blood depletes the high abundant proteins and hence aids in the detection of disease specific molecules (Vallabhajosyula et al. 2017; Boukouris and Mathivanan 2015).

Several studies have shed light on the importance of tumor-derived EVs in cancer diagnosis, progression, organotropism, metastasis, chemoresistance, cancer associated weight loss and treatment outcome (Costa-Silva et al. 2015; Chen et al. 2017; Au Yeung et al. 2016). For example, recent reports have shown that tumor-derived EV surface proteins have shown to drive metastasis, organotropism (Hoshino et al. 2015) and cancer associated weight loss (Chitti et al. 2018). Metastasis, which is a complex hallmark of cancer, is shown to be regulated by the various integrins that are associated with tumor-derived EVs (Armacki et al. 2020). EVs are released into the circulation, transfer to the distant sites and establish pre-metastatic niche to facilitate cancer colonization and metastatic tumor growth. Few interesting studies have reported that tumor derived EVs expressing heat shock protein (Hsp) 70/90 (Zhang et al. 2017) and adrenomedullin (Sagar et al. 2016) have shown to induce muscle atrophy and lipolysis, respectively, and contribute to the tumor associated wasting. Overall, despite understanding of EV heterogenicity and selective cargo sorting mechanisms still in their infancy, these nano-sized vesicles have attracted significant attention and are considered as promising source of cancer biomarkers.

Tumor-Derived EVs as Source of Cancer Biomarkers

Screening for cancers, in its early stages, has a potential to provide the best chance for successful treatment of the disease (Duffy 2010). According to the National Cancer Institute, the following are the most common diagnostic and screening tests for various cancers. These include (1) physical examination, that checks for signs of cancers like skin and breast (2) laboratory-based tests, such as screening of biological fluids or genetic test, (3) Imaging tests, include ultrasound, computed tomography, magnetic resonance imaging, X-ray and nuclear medicine scans, the specific imaging tests is performed depending upon the tumor type and site, (4) biopsy and cytology studies include testing tissue biopsy and cytology specimens for certain

cancers. Furthermore, laboratory-based approaches detect tumor markers in the patient biological fluids, which are commonly taken from the blood and urine.

Tumor and various cells in the tumor microenvironment release a diverge population of secretory proteins, cytokines, signalling molecules, transmitters and EVs in the biological fluids (Sharma et al. 2017) and these can serve as excellent candidates for biomarker development. Among all the factors released by cancer and its associated cells, EVs have attracted significant attention as several molecules which are native to the tumor tissues are also detected in EVs. Recent studies also support the hypothesis that cancer cells release more EVs than healthy cells (Fig. 13.1) (Kreger et al. 2016; Bandari et al. 2018). Various cargo in circulating EVs have been proposed as potential biomarkers (Fig. 13.2) for several cancer types including breast cancer (miR-1246) (Hannafon et al. 2016), colon cancer (exosomal tetraspanin 1) (Lee et al. 2018), hepatocellular carcinoma (miR-10b-5p, miR-17-5p, miR-29a, miR-106a, miR-122, miR-125b, miR-145, miR-192, and miR-194) (Xue et al. 2019; Cho et al. 2020), ovarian cancer (CA125, miR-21, phosphatidylserine) (Taylor and Gercel-Taylor 2008; Lea et al. 2017), pancreatic cancer (CA19-9, glypican-1) (Melo et al. 2015) and lung cancer (CD151, CD171, and tetraspanin 8) (Sandfeld-Paulsen et al. 2016).

CA125/MUC16 is the first biomarker identified for the detection of ovarian cancer (OC) (Bast et al. 1983). Studies have reported that markers such as CA125, claudin 4, EpCAM, CD24, miR-21, miR-141, miR-200, miR-214 and miR-30a-5p are reported to be present in soluble fractions, and in EVs isolated from biological fluids of the OC patients (Yokoi et al. 2017; Im et al. 2014; Li et al. 2009; Taylor and Gercel-Taylor 2008; Vaksman et al. 2014; Peng et al. 2011; Zhou et al. 2015). Several studies have demonstrated that the expression level of these proteins and miRNAs were high in OC patient-derived EVs rather than in soluble form, and their expression was reported to be altered according to the OC type (benign or malignant), stage and response to treatment (Bouanene and Miled 2009; Rustin and Tuxen 1996; Carollo et al. 2019). Li J et al., observed the presence of claudin 4 containing EVs in 32 of the 63 OC patients whereas only one out of 50 plasma samples taken from healthy individuals contained claudin 4 in EVs. The study also reported that the dual measurement of CA125 and claudin 4 in EVs increased the specificity and sensitivity and hence could be informative for disease diagnosis and response to treatment (Li et al. 2009). In addition to proteins, it was highlighted that EVs were characteristically different amongst malignant and benign OC patients with the display of significantly higher phosphatidylserine (PS) on EVs. The malignant group of patient-derived plasma EVs had a higher expression of PS compared to the benign group (Lea et al. 2017).

Recently, Hydbring and colleagues have shown that EV RNA profiling of pleural effusions could differentiate between lung adenocarcinoma from patients with benign inflammatory diseases. EVs isolated from 36 pleural effusions (18 healthy and 18 adenocarcinoma) revealed differential expression of 17 miRNAs and 71 mRNA profiles. Overall, there was increased expression of miR-200b, miR-200c, miR-141, miR-375 and lipocalin-2 in lung cancer pleural effusions compared to benign lung inflammation (Hydbring et al. 2018). Similarly, Guillherme

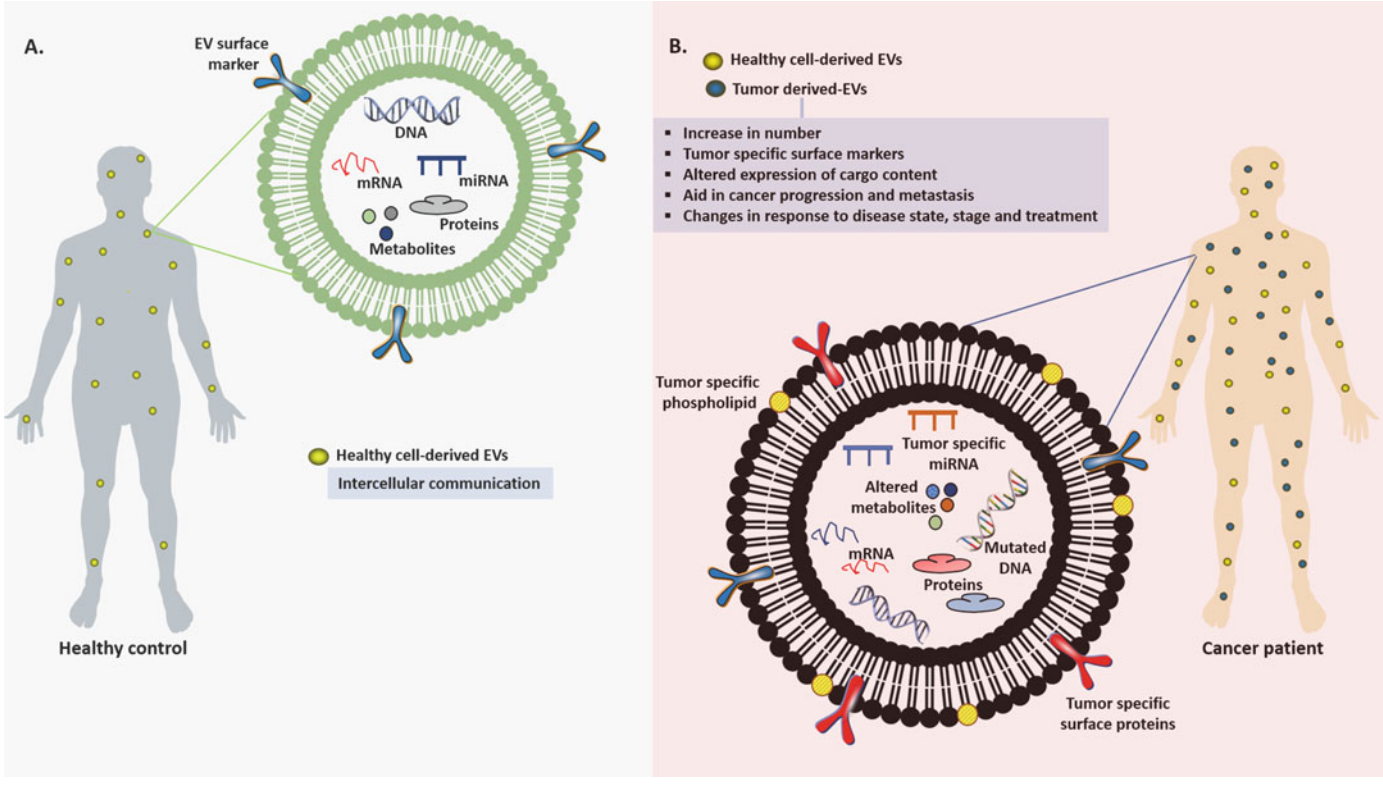

Fig. 13.1 EV molecular profile in healthy and cancer patients. (**a**) In healthy individuals, EVs aid in intracellular communication. EV content include DNA, mRNA, mi-RNA, proteins, lipids and metabolites. (**b**) Cancer patients have EVs that are derived from various organs including the tumor. Tumor derived EVs display mutated DNA, altered mi-RNA, mRNA, proteins, metabolites and lipids

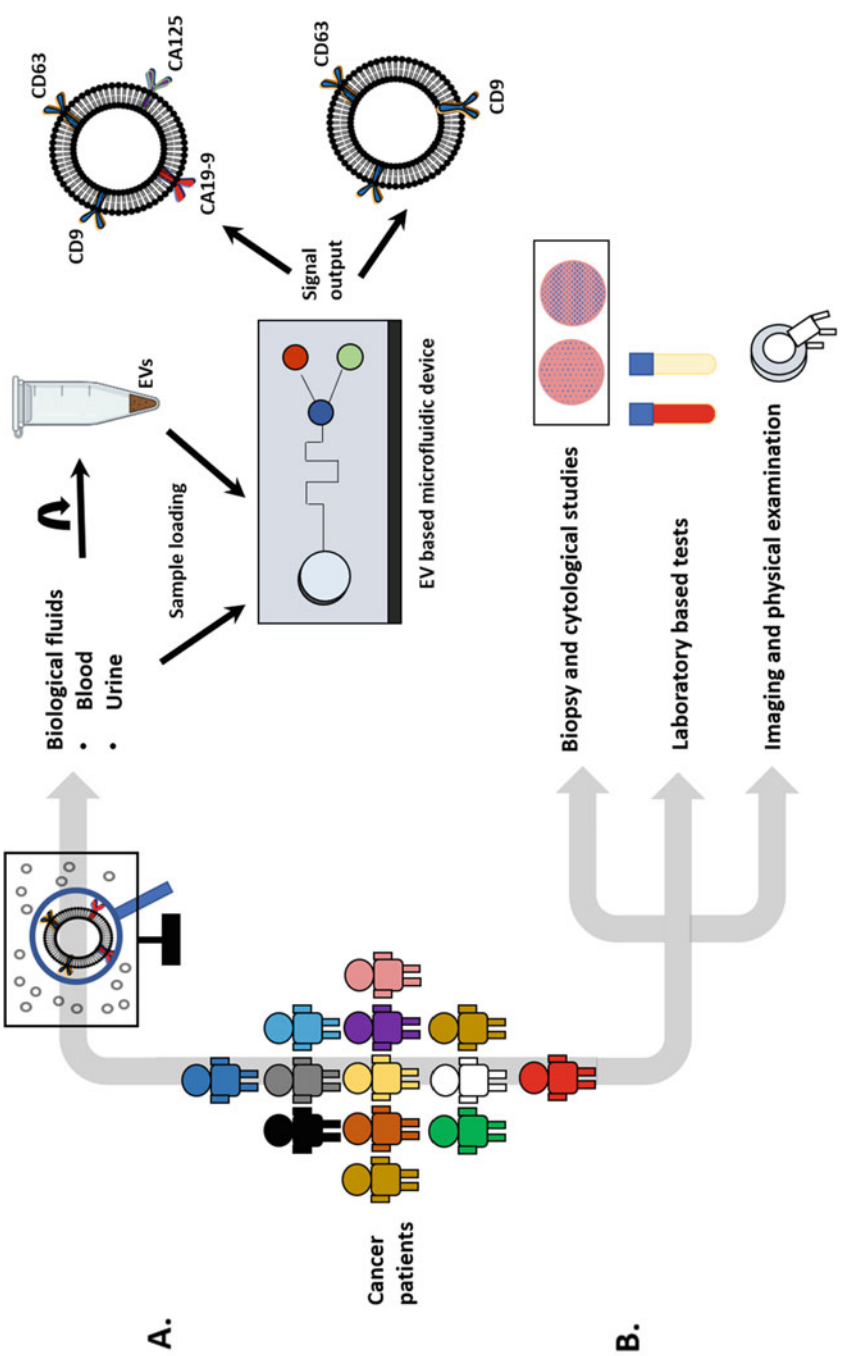

Fig. 13.2 EVs are promising non-invasive diagnostic tools. (**a**) Schematic representation of EV based rapid diagnostic approach. EVs could be captured based on microfluidic device, which detects EV marker along with the tumor marker from the small volumes of body fluids. (**b**) Common cancer diagnosis and screening methods which includes biopsy and cytological studies, laboratory-based body fluid tests, imaging and physical examination

and colleagues showed that specific miRNAs such as miR-17-3p, miR-21, miR-146, miR-155, miR-191, miR-203, miR-205, and miR-214 were present in lung cancer patient plasma-derived EVs but were not detected in plasma-derived EVs from healthy controls (Rabinowits et al. 2009). Proteomics studies conducted by Paulsen and colleagues, revealed that plasma-derived EV protein profiles from 581 patients (431 with lung cancer and 150 healthy controls) showed differential expression of various EV proteins including CD151, CD171 and tetraspanin 8 (Sandfeld-Paulsen et al. 2016). It was also observed that 80% of the EVs, purified from the lung cancer biopsies, contained epidermal growth factor receptor (EGFR), which was only present in 2% of the chronic lung inflammation biopsies (Huang et al. 2013).

Currently, imaging, invasive surgery and evaluation of serum cancer antigen 19-9 (CA 19-9) is routinely performed for pancreatic cancer (PC) detection (Ballehaninna and Chamberlain 2011). Recent study by Sonia and colleagues have proposed glypican-1 circulating EVs in the serum of the PC patients as a potential biomarker. It was also reported that the circulating levels of serum EV glypican-1 was higher in late stage PC patients compared to the early stage PC patients (Melo et al. 2015; Herreros-Villanueva and Bujanda 2016). These observations offer a new insight of EVs as future cancer biomarkers enabling early detection and might offer precision or personalised medicine options.

Conclusion

In 2018, there were 17 million new cases and 9.6 million deaths accredited to cancer globally (Bray et al. 2018). According to UK worldwide cancer statistics, it is estimated that there will be 27.5 million new cases of cancer each year by 2040. This large increase in the number of cancer cases could pose a significant challenge and burden to health care system, along with economic implications. Early diagnosis of cancer could offer the advantages of appropriate clinical care, positive impact on the survival, quality of life and the cost involved with the treatment. In recent years, several studies have focused on EVs as a source of cancer biomarkers. Various properties of EVs such as abundance in biological fluids, increase in their number in systemic circulation of cancer patients, presentation of tumor specific surface antigens, alterations in the cargo content based on the disease stage and response to treatment make these nano-sized vesicles ideal for cancer diagnostic applications. Currently, there are more than 36 clinical trials underway testing EV diagnostic efficacy for various diseases (Makler and Asghar 2020). Despite the advances in understanding the role of EVs on all those fronts, purity in isolation of EVs is one serious challenge in EV based biomarker development for clinical application. Nevertheless, latest advancement in immunoaffinity and membrane particle precipitation-based isolation of EVs, alongside EV based microfluidic design might streamline the whole process of EV based biomarker development and validation. Furthermore, EVs can also be utilised as a resource of predictive biomarkers for response to therapy in personalised medicine where cancer patients can

be stratified based on their response to therapy and alternate treatment options can be utilised.

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Chapter 14
Engineering Extracellular Vesicles for Cancer Therapy

Christina Nedeva and Suresh Mathivanan

Abstract Extracellular vesicles (EVs) are lipid bilayer containing nanovesicles that have a predominant role in intercellular communication and cargo delivery. EVs have recently been used as a means for drug delivery and have been depicted to elicit no or minimal immune response in vivo. The stability, biocompatibility and manipulatable tumour homing capabilities of these biological vessels make them an attractive target for the packaging and delivery of drugs and molecules to treat various diseases including cancer. The following chapter will summarise current EV engineering techniques for the purpose of delivering putative drugs and therapeutic molecules for the treatment of cancer. The relevance of EV source will be discussed, as well as the specific modifications required to manufacture them into suitable vehicles for molecular drug delivery. Furthermore, methods of EV cargo encapsulation will be evaluated with emphasis on intercellular coordination to allow for the effective emptying of therapeutic contents into target cells. While EVs possess properties making them naturally suitable nanocarriers for drugs and molecules, many challenges with clinical translation of EV-based platforms remain. These issues need to be addressed in order to harness the true potential of the EV-based therapeutic avenue.

Keywords Extracellular vesicles · Drug delivery · Pathology · Cancer therapy · Exosome therapy · Cargo loading · Extracellular vesicles sources · Disease · Clinical trials

C. Nedeva (✉) · S. Mathivanan
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia
e-mail: C.Nedev@latrobe.edu.au

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Introduction

Several decades of extensive research has been conducted to identify targets and to develop suitable therapies to treat cancer (Cully 2018; Bushweller 2019). Standard approaches such as surgery, chemotherapy, radiotherapy and hormone therapy remain as the mainstay treatments to combat this disease. However, as our understanding of cancer pathogenesis improves, it has been realised that more combinatorial and comprehensive therapies are required to treat the disease. Even though some of the traditional therapies improve survival benefit with certain cancers, the proportion of curative effects are relatively small and are often most beneficial in early stage cancer patients. Therefore, several therapies including targeted therapies are being developed to efficiently manage cancer as well as reduce the undesirable side effects with low doses of the drug. One type of targeted therapy employs the use of engineered nanoparticles, which are now being utilised as viable drug carriers to bypass issues associated with conventional cancer therapies (Schroeder et al. 2011). Drug carriers are mainly produced using synthetic materials including polymers, metal particles, lipids and carbon nanotubes (De Jong and Borm 2008).

Although the use of synthetically produced nanoparticles as drug delivery vehicles has gained popularity in the past decade, this strategy has encountered multiple limitations (Arms et al. 2018). The major pitfalls associated with the use of synthetic nanoparticles in biological systems include cytotoxicity and immunogenicity (Loureiro et al. 2020). For instance, mice administered with copper nanoparticles exhibited organ deterioration (Chen et al. 2006). Hence, various naturally available drug delivery vehicles are being investigated. An alternative biological nanoparticle that has been exploited in drug delivery are extracellular vesicles (EVs). These nanosized membrane-bound particles are released by cells and act as communicative vehicles that deliver messages throughout the system of several organisms. Based on their biogenesis, many types of EVs exist in nature including ecotosomes or shedding microvesicles, apoptotic bodies, large oncosomes, migrasomes and exosomes (Zaborowski et al. 2015; Kalra et al. 2016). Exosomes in particular are small EVs ranging from 30 to 150 nm in size and are intrinsically produced and secreted by a variety of cells (Batrakova and Kim 2015; Luan et al. 2017; Kalra et al. 2019). Upon their secretion, exosomes can transfer protein, nucleic acids and lipid cargo between cells (Luan et al. 2017; Anand et al. 2019). The cargo present in exosomes can induce various signalling pathways in the recipient cells (Fig. 14.1). To perform these functions, exosomes contain and shield a range of molecules such as mRNAs and proteins, transporting this cargo through the system virtually undetected by the immune system (Luan et al. 2017). The ability to transfer material, coupled with low immunogenicity and toxicity, makes exosomes an attractive target for putative drug delivery and therapy. In this chapter, we have used the terminology EVs to refer to the general population of the vesicles and exosomes for studies where the authors have specifically mentioned the term.

In order to successfully use EVs as drug delivery vehicles, their properties need to be comprehensively assessed. This requires an understanding of source, size,

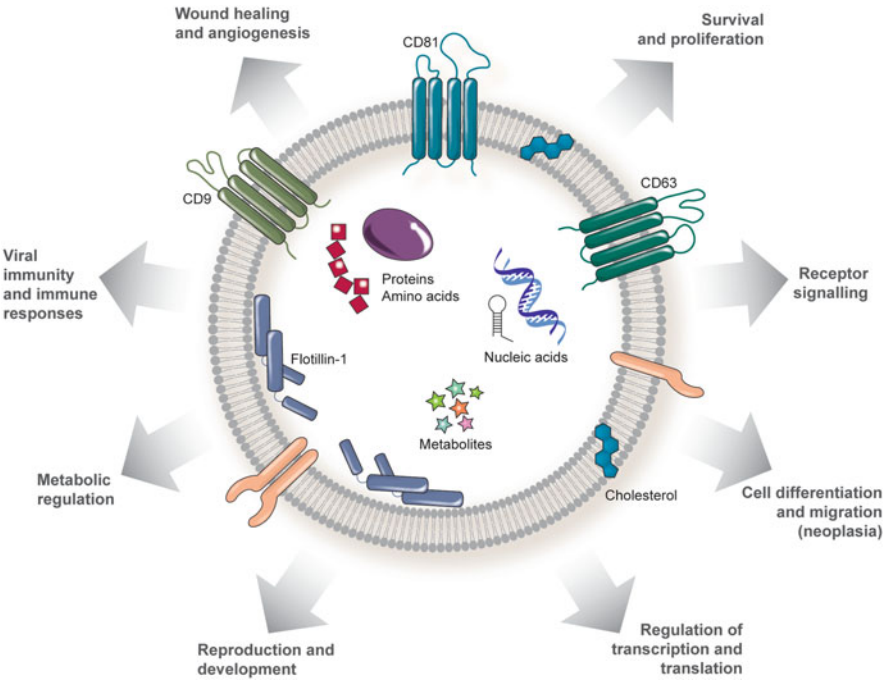

Fig. 14.1 EVs are vital communicative transit vessels within the body that can elicit various functions on recipient cells. Exosomes, a subtype of EVs, are derived from multiple cell sources and carry various cargo such a proteins, nucleic acids and metabolites. These biological vessels mediate intercellular communication for the maintenance of homoeostasis or contribute to disease states such as cancer

trafficking, targeting strategy, biodistribution, dissemination, aptitude for tumour homing and clearance. To recognize and study the properties of EVs, a variety of in vitro cell lines have been utilised to produce and isolate quantifiable amounts of EVs (Kalra et al. 2016; Pathan et al. 2019). The use of in vitro methods for EV production allows for amenability to customised modification as opposed to those produced under natural conditions, i.e. isolation of EVs from bodily fluids (Vader et al. 2016). Additionally, having a broad range of cellular sources facilitates EV heterogeneity permitting different types of cellular based diseases to be targeted for therapy. With careful evaluation and optimised isolation and purification methods in place, the fundamental goal and most challenging would be the mass production of drug bearing EVs which effectively neutralise diseased cells with limited toxicity to the host. Nevertheless, multiple strategies will be discussed in following text to address the merits and limitations associated with EV-based drug therapy for the treatment of cancer.

Functions and Sources of EVs for Therapeutic Applications

Exosomes originate from endosomes and are secreted by many cell types under a variety of conditions within the body. They are stable in different fluid environments such as tissue culture medium, breast milk, saliva, blood, urine, and joint fluid (Gonzalez-Begne et al. 2009; Muller et al. 2014; Kolhe et al. 2017; Gheinani et al. 2018; Baxter et al. 2019; Fonseka et al. 2019; Mirza et al. 2019). Markers or proteins enriched in exosomes include TSG101, Alix and tetraspanins (Soltani et al. 2015). Due to a variety of surface markers, several immunoaffinity-based exosome isolation methods have been developed and optimised (Mathivanan et al. 2010; Kowal et al. 2016). Most popular approaches for the separation of exosomes include ultracentrifugation, density gradient centrifugation, immunoprecipitation, size exclusion chromatography, micro-fluidic based isolation and PEG-mediated precipitation (Thery et al. 2006; Tauro et al. 2012; Kalra et al. 2016).

Additional to their use for identification and separation, the molecules present on the surface of EVs possess vital functional properties. These molecules serve as a tool to navigate exosomes to target cells where they can deliver contents with a direct influence on the recipient (Lopez-Leal et al. 2020). Upon reaching their destination, receptors on recipient cells are activated allowing for long range communication (Paolicelli et al. 2019). Inadvertently, cancer cells can also hijack this valuable communicative system to become highly metastatic, giving rise to aggressive tumours. Patients and laboratory animals with cancer have substantially higher levels of EVs in the bodily fluids, indicating that EVs play a critical role in the progression of cancer (Benameur et al. 2013). Studies have shown that colon cancer cells secrete EVs containing proteins and RNAs directly into the bloodstream for signal transmission to recipient cells, facilitating metastasis and tumorigenesis (Ren et al. 2019). Colorectal cancer cells have also been shown to acquire resistance to chemotherapeutic agents through the EV-based transfer of resistance inducing proteins (Zhang et al. 2019). The availability of these resources through the establishment of an effective EV-educated system facilitates tumour growth and survival amongst a wide range of distinct cancers. These findings emphasize the importance of EVs as liaisons for cell-to-cell communication not only under homeostatic conditions but also under pathological incidences. The fact that cancer cells indeed have an affinity to produce and accept EVs for various purposes, further supports the use of EVs as drug delivery vehicles for the targeted treatment of cancer.

To produce an effective vesicular-based drug delivery system for the treatment of cancer requires exploration of different sources of EVs. The reason for this is the protein and lipid composition of EVs can directly influence their ability to interact with target cells, hence restricting the intended effects and potentially leading to toxicity (Romagnoli et al. 2014; Frydrychowicz et al. 2015). Careful study and consideration of the biology and properties of EV sources is therefore required to evaluate the benefits and weaknesses for applications in therapy. With this considered, multiple groups have explored the use of tumour-derived EVs for the transportation of anticancer and chemotherapy agents (Tian et al. 2014; Kim et al. 2016).

EVs-derived from tumours packaged with vaccines and drugs are gaining interest in the field of immunotherapy. The high concentration of tumour-derived EVs, existing in malignant pleural effusions, have been shown to contain tumour-associated antigens in addition to MHC class I molecules. These EVs can be successfully repurposed to deliver tumorigenic antigens to dendritic cells, in-turn priming T-cell responses against the primary tumour (Wolfers et al. 2001). The presence of tetraspanins specifically in tumour EVs is similarly of value, as various tissues preferentially interact with these ligands, hence allowing for selective targeting and delivery (Rana et al. 2012). However, lesser metastatic cancer cells have shown to become aggravated by these EVs, leading to enhanced tumour growth (Zomer et al. 2015). Tumour-derived EVs have also shown to contain adhesion molecules, such as vimentin and annexin A1 and cathepsins, which have potential to enhance tumour-cell invasion (Harris et al. 2015).

A drawback of using tumour-derived EVs is that they can inhibit effector T-cells through expression of ligands such as TRAIL and FasL. This ability to induce apoptosis of cytotoxic T-cells leads to an impairment in monocyte maturation culminating in a state of immune suppression (Tomihari et al. 2010). Among some of the potential positive uses for tumour-derived EVs for drug delivery, many aversive problems associated with their use exist. Therefore, alternate EV sources such as plants and animal products including milk-derived EVs have been investigated to mitigate these issues (Wang et al. 2015; Sanwlani et al. 2020). Investigators have used grapefruit plants to generate large quantities of EV-like particles for drug-delivery purposes. The EVs were modified additionally and shown to traffic preferentially to sites of increased inflammation in which tumours favourably reside (Wang et al. 2015). Bovine milk is another source of EVs which has recently gained popularity due to safety, scalability and reliability of the product. Studies have shown bovine EVs can be effectively loaded with small molecules and chemotherapeutic agents and delivered to lung cancer cells in vitro and in xenograft models. Although these natural methods of EV-derivation are relatively safe, they lack immunotherapy applications (Munagala et al. 2016). To address this aspect, studies have directed focus on the use of immune cell derived EVs. Unlike other EVs, exosomes produced by monocytes have optimum immune evasion capacity, allowing them to circulate for longer in-turn making them more efficacious (Tang et al. 2012). Nevertheless, the most promising vesicular drug-vehicle currently studied is exosomes derived from dendritic cells (DCs). Proteome analysis of DC exosomes found marked expression of MHC class I and II upon there surface, suggesting a potential to stimulate $CD8^+$ and $CD4^+$ T-cells, respectively, as well as other costimulatory molecules (Thery et al. 2001). DC-derived exosomes also possess integrin-α and ICAM-1, an immunoglobulin family member, which allow for targeting and subsequent docking upon target cells. The abundance of tangible surface markers for targeting specificity and immune-stimulatory effects of DC-exosomes makes them one of the most attractive targets for EV-based therapy.

Specific Targeting and Modification of EVs for Cancer Therapy

Many characteristics of EVs make them ideal vehicles for drug delivery in a biological environment. The biocompatible and biodegradable properties of EVs are among those natural features lacked by synthetic nanoparticles. The specificity of EVs, to their respective target, can also be further increased by introducing additional modifications to them, either by genetic manipulation or by chemical modification (Fig. 14.2). To further increase specificity using genetic approaches, donor cells have been engineered to produce EVs with modified receptors to increase cellular recognition (Rana et al. 2012). For instance, mammalian cells can be exploited to generate EVs with surface proteins such as LAMP2b and tetraspanins fused to ligands or targeting signals (Ohno et al. 2013; Stickney et al. 2016). This robust system allowed for the continuous production, secretion and uptake of

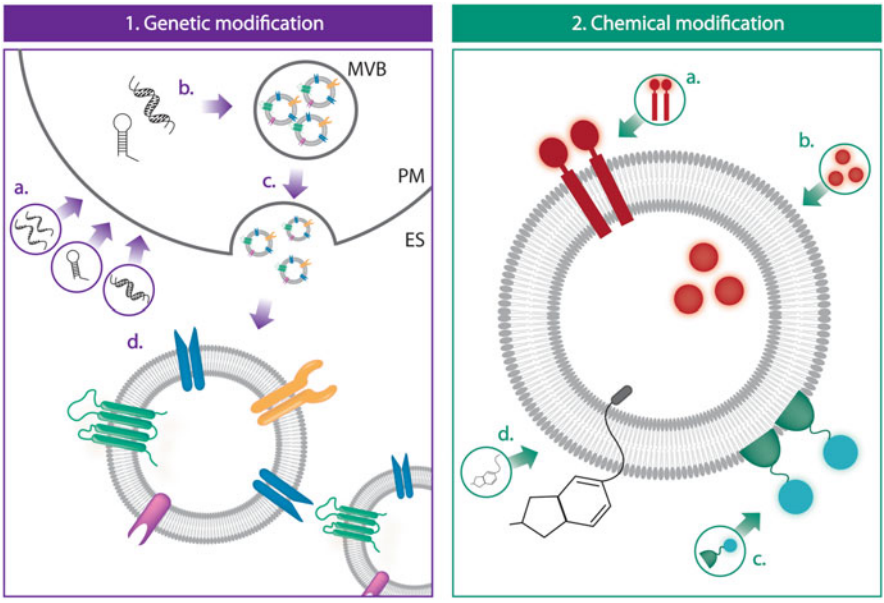

Fig. 14.2 EVs can be modified using various techniques to increase target specificity. (**1a**) Genetic modification involves the introduction of exogenous genetic material into cells. (**1b**) Genetic material can be translated and used by the cells to modify EVs. Following modification, intraluminal vesicles are packaged into multivesicular bodies (MVBs). (**1c**) MVBs then fuse with the plasma membrane (PM) (**1d**) and release modified exosomes into the extracellular space (ES). Chemical modification involves the modification of the EVs directly. (**2a**) EV membrane proteins can be covalently attached to proteins or specific ligands. (**2b**) Metal particles, such as magnetic iron, can be directly inserted into EVs to aid in delivery. (**2c**) GPI anchored nanobodies. (**2d**) Metabolic-labelling can be performed to incorporate functional groups into EVs, allowing for bio-orthogonal reactions. Both genetically and chemically modified exosomes can be purified and used to increase target specificity of EVs for therapeutic purposes

embedded EVs by specific target cells (Stickney et al. 2016). The display of functional proteins on the surface of EVs is a technique also adapted for use with DCs. Here, DCs were engineered to produce exosomes with LAMP2b and iRGD integrin specific peptide which vastly increased the efficiency of drug delivery to αγ integrin positive breast cancer bearing mice (Tian et al. 2014).

The restrictive penetrability of the blood-brain barrier makes it virtually impossible for many drugs to enter and exert the appropriate therapeutic effects in the brain environment. However, a recent study successfully accomplished neuronal delivery of engineered exosomes for the treatment of brain cancer in zebrafish (Yang et al. 2015). Genetic modification to generate cells lines that secrete EVs with desired target specificity has also been used to modify HEK293T cells to produce exosomes that overexpress SIRPα. This protein, once expressed on the surface of exosomes, acts to disrupt the CD47-SIRPα interface between macrophages and tumour cells, in-turn waning the tumour cells ability to resist phagocytosis (Koh et al. 2017). Additionally, recombinant techniques have been utilised to modify HEK293 cells to secrete GE11 containing exosomes, which is a peptide that specifically binds EGFR, thus promoting exosomes interactions with EGFR-positive breast cancer cells in mice (Ohno et al. 2013). A study investigating trastuzumab resistance in HER2-positive breast cancer developed a modified DC line which released exosomes expressing ovalbumin (OVA). OVA expressing exosomes were able to stimulate $CD8^+$ T cell responses via MHC I complex leading to cancer eradication (Wang et al. 2013). Production of exosomes with specific modifications for effective targeting is one of many aspects required for successful drug delivery to diseased recipient cells. Drug-loaded EVs also require critical protection against liver clearance to be available for treatment applications. Hence, a study used blockers to inhibit scavenger receptors on exosomes, derived from macrophages, to reduce liver clearance and increase exosomal concentration in the blood (Watson et al. 2016). Increasing EV stability and limiting clearance is key for the bioavailability of the modified EVs and allowing for timely systemic distribution.

Covalent chemical modification is a technique that has similarly been used to increase functionality and surface molecule stability of exosomes. A recent study, using this method, isolated reticulocyte-derived exosomes and coated them, in situ, with synthesised superparamagnetic nanoparticles—conjugated with transferrin. These decorated exosomes were additionally loaded with chemotherapeutic drugs and subcutaneously injected into mice with H22 established cancer. Using a magnet to direct the particles to the tumor site allowed for a significant accumulation of drug at the tumour (Qi et al. 2016). Another group transfected donor cells with engineered nano-body bearing anti-EGFR and a GPI-anchor fusion protein to generate exosomes displaying nano-bodies. These vesicular-nano-body complexes were effectively targeted to EGFR-positive cancer cells (Kooijmans et al. 2016). With chemical modification methods, it is important to consider and elude to the possibility of bio-incompatibility and toxicity toward the chemical particles or molecules associated with the EVs. With the correct source and ideal modifications, EVs—in particular exosomes—have shown to be one of the most suitable vehicles in a biological system.

Methods for Loading Therapeutic Agents and Molecules into EVs

EVs are essentially lipid membrane structures that are topically embedded with ligands and receptors inherited from source cells (Frydrychowicz et al. 2015). To manipulate EVs for therapeutic purposes requires comprehensive knowledge of their structural make-up. With this understanding, therapeutic agents and molecules have been successfully encapsulated into EVs using a variety of active and passive approaches (Table 14.1). Each approach has its own advantages and limitations over the other with variations in efficiency and subsequent stability of the agents within the exosomes. The passive method of drug loading simply entails the incubation of drugs with EVs, where a concentration gradient allows drugs to diffuse freely into the particles. Here, the hydrophobic properties of a drug determine loading efficiency (Luan et al. 2017). For instance, hydrophobic drugs such as curcumin, which interact with vesicular lipid membranes, can be incubated with exosomes in situ to achieve effective loading (Sun et al. 2010; Aqil et al. 2017b). Bovine milk-derived EVs have also been used for passive loading with chemotherapeutic agent paclitaxel (Agrawal et al. 2017). Another study used EVs recovered from RAW264.7 cells and incubated with a large tetramer protein, however, loading volume was quite low with this method (Haney et al. 2015). Passive loading approaches require simplistic protocols, although they are strictly limited to hydrophobic cargo and mostly result in low packaging efficiency.

Energy driven or active processes of cargo-loading involve tedious methods of drug incorporation into EVs, yet the range of drugs and molecules introduced in this way is far broader. Hydrophilic molecules such as miRNA and siRNA can be introduced actively via electroporation (Wahlgren et al. 2012). One group tested various electroporation conditions for the loading of miRNA-155 into B cell-derived exosomes and found that higher voltages lead to improved loading capacities (Momen-Heravi et al. 2014). Similarly, Tian et al., showed successful incorporation of chemotherapeutic agent doxorubicin into modified exosomes, with the ability to efficiently target breast cancer cells (Tian et al. 2014). Pomatto et al., showed that low pulsation of anti-tumour miRNAs into plasma-derived EVs increased encapsulation ability whilst preserving native vesicle cargo and EV integrity. These loaded-EVs showed to effectively promote apoptosis of hepatocellular carcinoma cells (Pomatto et al. 2019). Electroporation also allows for hydrophilic molecule loading such as TMP which gives EVs a photodynamic effect thereby permitting biodistribution and uptake monitoring in biological systems (Fuhrmann et al. 2015). Voltage based techniques are widely used for siRNA, shRNA and miRNA loading, however, without optimisation of the appropriate buffer conditions, RNA may aggregate leading to EV instability (Wahlgren et al. 2012). Another technique which causes temporary compromise of the EV membrane, for assisted drug entry, is sonication. Paclitaxel is an antimitotic drug that can be loaded into EVs using this technique (Liu et al. 2015; Kim et al. 2016). One study showed that EV encapsulated drug offers a 50-fold increase in cancer cell cytotoxicity compared to free drug (Liu

Table 14.1 Representative studies utilising therapeutic cargo loaded into EVs derived from various sources for cancer therapy

Cargo	Source	Cancer target	Method of loading	References
miR-143	Mesenchymal stem cells	Osteosarcoma	Transfection	Shimbo et al. (2014)
miR-335-5p	Hepatic stellate cells	Hepatocellular carcinoma	Transfection	Wang et al. (2018)
miR-146b	Marrow stromal cells	Glioma	Electroporation	Katakowski et al. (2013)
miR-21 and 5-FU	HEK293T cells	Colon cancer	Electroporation	Liang et al. (2020)
siRNA (S100A4)	Breast cancer cells	Metastatic breast cancer	Extrusion	Zhao et al. (2020)
siRNA, shRNA ($KRAS^{G12D}$)	HEK293	Pancreatic Cancer	Electroporation	Kamerkar et al. (2017)
siRNA (c-Myc)	Monocytes	Lymphoma	Electroporation	Lunavat et al. (2016)
PTEN mRNA	Mouse embryonic fibroblasts and DCs	Glioma	Nanoporation	Yang et al. (2020a)
SIRPα protein	HEK293T	Colon carcinoma	Transfection of cells	Koh et al. (2017)
TRAIL protein	K562 cells	Myeloma	Transfection of cells	Rivoltini et al. (2016)
MAGE peptides	Dendritic cells	Non-small cell lung cancer	Direct/indirect	Wahlund et al. (2017)
HSP70 protein	J558 myeloma cells	Myeloma	Transfection	Xie et al. (2010)
Anthocyanidins	Bovine milk	Ovarian cancer	Passive	Aqil et al. (2017a)
Cisplatin	Hepatocarcinoma/ ovarian cancer cells	Hepatocarcinoma/ ovarian cancer	Passive	Tang et al. (2012)
Doxorubicin	Dendritic cells	Breast cancer	Electroporation	Tian et al. (2014)
Paclitaxel	Bovine milk	Lung cancer	Passive	Agrawal et al. (2017)
Curcumin	Bovine milk	Lung cancer	Passive	Aqil et al. (2017b)

et al. 2015). Additionally, EV encapsulating paclitaxel have shown to block lung metastasis in mouse tumour models (Kim et al. 2016). Although sonication increases encapsulation yield of drugs exponentially, distortion of the lipid membrane can cause drugs to attach to the outer surface of the EV resulting in a dual phase release of the drug. This can potentially lead to a decrease in drug target delivery and therefore undesirable cytotoxicity (Kim et al. 2016).

Delivery of siRNA has also been achieved using the extrusion method, though the mechanics of how membrane disruption occurs, to allow molecules to gain entry into

the EVs remains unknown. Using the extrusion method for drug loading of porphyrin into EVs, studies have reported effective cytotoxicity of breast cancer MDA-MB231 cells. Interestingly, preparation of exosomes loaded with porphyrin using other methods rendered these exosomes less cytotoxic (Fuhrmann et al. 2015). An alternative method to extrusion, used to a lesser extent, involves freeze and thaw cycles for drug encapsulation. Here, EVs are incubated with the desired drug or molecule at room temperature before rapid freezing in liquid NO_2, then followed by a thaw cycle at room temperature (Costa et al. 2014). Studies performed by Sato et al., demonstrated that exosomes, isolated from macrophages in vitro, combined with liposomes can be fused using the freeze and thaw method, in-turn enhancing cell membrane binding and uptake efficiency of exosomes. However, FRET analysis of exosome-liposome complexes revealed that exceeding freeze-thaw cycles of these particles disrupted their formation (Sato et al. 2016). These findings exemplify the importance of using precise drug encapsulation methods to maximise cargo loading and, importantly, maintain exosome integrity in order to deliver effectual drugs to the anticipated target.

EVs are also utilised as delivery vehicles for peptides and macromolecules such as tumour antigens and cell death-inducing proteins (Cho et al. 2005; Haney et al. 2015). Genetic engineering of donor cells is a common method used to deliver therapeutic proteins to exosomes. Aspe et al., utilised isolated exosomes from a genetically engineered melanoma cell line for the treatment of pancreatic adenocarcinoma cells. Dominant-negative Survivin-T34A packaged in these melanoma-derived exosomes showed to effectively block wild-type pro-survival protein Survivin in recipient cells. Consequently, this blockade induced caspase activation followed by apoptosis of the target cancer cells (Aspe et al. 2014). Rivoltini and colleagues, on the other hand, exploited a lenti-viral system to stably integrate *TRAIL* into K562 cells to produce $TRAIL^+$ exosomes. These exosomes armed with soluble TRAIL induced apoptosis of target myeloma cells, in vivo, dramatically slowing tumour progression (Rivoltini et al. 2016). Using donor cells to process and package transfected material seems like the feasible approach for delivering apoptotic proteins to exosomes, however, cytotoxicity with this method needs to be considered. With the expression of cytotoxic proteins, the possibility of inhibition or induction of death of the donor cells cannot be ignored. Moreover, as well as having desirable effects, delivering apoptotic proteins packaged in EVs, in vivo, can lead to promiscuous interactions causing cell devastation of non-targets (Rivoltini et al. 2016).

Clinical Applications of EVs for the Treatment of Cancer: Advancements and Limitations

In agreement of the promising potential benefits of EVs in disease therapy, several clinical trials have been initiated using them as drug delivery vehicles and as vaccines. Notably, a few of these trials have demonstrated viability of applied

Table 14.2 Current and ongoing human clinical trials using EVs for cancer therapy

Cohort	Phase	Cargo	Trail result	References
NSCLC	I	Antigenic tumor peptides	Well tolerated, disease stability in two patients	Morse et al. (2005)
Advanced NSCLC	II	Antigenic tumor peptides	Disease stabilisation in a third of patients, boost in NK call immunity	Besse et al. (2016)
Metastatic melanoma	I	MAGE peptides	Well tolerated, non-toxic, five patient responders at varying degrees	Escudier et al. (2005)
Malignant glioma	I	Antigenic tumor peptides (vaccine)	No serious adverse effects, two patients partially responded	Kikuchi et al. (2001)
Colon cancer	I	Curcumin	Ongoing/in progress	NCT01294072
Head and neck cancer	I	Grape extract	Ongoing/in progress	NCT01668849

techniques (Table 14.2). A completed phase I clinical trial found that release of tumour EVs, likely bearing tumorigenic antigens, were capable of stimulating immune responses in glioma patients, supporting the clearance of tumour cells post-resection (Andrews 2000). One group, specifically, isolated plant-derived EVs for the delivery of drugs for therapeutic applications (NCT01294072). Several phase I trials have shown that DC exosomes are safe and well-tolerated (Morse et al. 2005). Encouragingly, DC-derived exosomes bearing tumorigenic antigens, acting as vaccines in amalgamation with chemotherapeutic drugs, have shown promise against non-small cell lung cancer (NSCLC) in progressive phase II trials. Additional evidence revealed a boost in NK cell mediated immunity in these patients with DC-exosome treatment (Besse et al. 2016). Of note, DCs utilised in this study were pre-stimulated with LPS or INF-γ to enhance stimulatory properties. With continuous investigation and revelations in this emerging field, EV-based therapy can become an adaptable solution for the treatment of disease.

Despite its immense potential, barriers with the reproducibility and scalability of EV therapies in the clinic remain (Nordin et al. 2015). The stimulatory effects of DC-exosomes necessitate further enhancement beyond prophylactic priming to be effectual in the clinic. Engineering of DCs to enhance activity such as upregulation of co-stimulatory molecules (i.e. GM-CSF) and downregulation of certain checkpoints (i.e. PDL1) could be of use (Yaddanapudi et al. 2019; Daassi et al. 2020). Moreover, combinatorial use of drugs and better suited tumour-associated antigens used for encapsulation could offer favourable outcomes with EV treatment. Trials performed by Dai et al., solidify the prospect of correct tumour-antigen use for enhancement of DC responses (Dai et al. 2008). Adaption of adjuvants could also be utilised for T-cell priming to enhance responses and tumour killing capability (Chaput et al. 2004). The matters associated with scalability of EV-based therapy have continued to challenge researchers in the field. However, various groups have taken on the task of solving "up-scaling" barriers with the adaption of routine

procedures or even with new inventive approaches, such as use of hollow fibre bioreactors (Yan et al. 2018; Yang et al. 2020a, b). Some have addressed scaling issues by effectively engineering mesenchymal stem cells (MSCs), which are a preferred exosome source from specific applications, to address technical issues with the secretion of EVs. Studies utilising these cells have successfully amplified vesicle secretion from MSCs with the use of a dynamic 3D-bioprocessing method. However, for clinical utility, GMP grade large quantity of EVs are needed and certain groups and commercial institutions have successfully established large scale setups. Nevertheless, approaches such as these, facilitate diversity in EV-based applications for therapy and will eventually take us from bench to bedside (Cha et al. 2018).

Prospects and Concluding Remarks

EVs have immense potential as drug delivery vehicles over other synthetic nanocarriers for treating diseases such as cancer. This can be accredited to their biocompatibility, which makes them highly suitable for clinical applications. However, with the advent of any therapeutic delivery system, caution should be taken as undesired side-effects may arise, such as immune suppression and reversion of tumorigenesis (Lee et al. 2018). Hence, persistent search for innocuous and effectual EV formulations are needed in the creation of robust and adaptable EV-based platforms. Despite many advancements in the field of EV-based cancer therapy, many issues need to be solved before these vehicles become equipped for mainstream use in a clinical setting. Technology associated with quality control and large-scale production of EVs remains in its infancy. Smaller scale production is currently conceivable, however, a consensus for broader production and reproducibility has yet to be reached. Moreover, storage and stability of EVs continue to be a confounding factor, as freeze-thaw cycles has shown to cause deformation and aggregation of EVs (Maroto et al. 2017). All of these issues need to be rectified in order to engineer an EV for cancer therapy. Nevertheless, the concepts surrounding the use of EVs as a clinically suitable nanoplatform remain attractive and promising.

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Chapter 15
Extracellular Vesicles in Metabolism and Metabolic Diseases

Akbar L. Marzan, Christina Nedeva, and Suresh Mathivanan

Abstract As living organisms constantly need energy to maintain and perform cellular functions, metabolism plays a vital role in producing the required energy to execute these processes. Hence, various metabolic pathways are highly regulated and disruption in critical pathways can result in the onset of multiple disorders such as hypertension, diabetes, obesity, and dyslipidaemia. Extracellular vesicles (EVs) are membrane-bound nanosized vesicles that are known to be secreted by various cell types into their respective extracellular environment. EVs have been implicated in cell-to-cell communication via mediating cellular signaling and can functionally impact recipient cells with the transport of bioactive proteins, nucleic acids, lipids and cellular metabolites. Recently, several studies have highlighted the role of EVs in metabolism. Alterations in the plasma derived EV concentration and their cargo in patients with metabolic disorders have been reported by multiple studies, further proposing EVs as a potential source of disease biomarkers. The following chapter will discuss the functional significance of EVs in metabolic diseases and the processes by which EVs act as cellular messengers to reprogram the metabolic machinery in recipient cells.

Keywords Metabolism · Metabolites · Extracellular vesicles · Exosomes

Introduction

The term metabolism refers to the sum of all the chemical reactions that occur within each cell of an organism. It ultimately provides the body with the energy necessary to carry out all the vital processes (Bing 1971; Rodwell et al. 2015). As living organisms have a constant demand for energy to maintain cellular functions, metabolism plays a crucial role in fulfilling this essential energy requirement in order to

A. L. Marzan · C. Nedeva (✉) · S. Mathivanan (✉)
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia
e-mail: C.Nedeva@latrobe.edu.au; S.Mathivanan@latrobe.edu.au

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sustain life (Patel and Harris 2016). Due to its indispensable importance in the survival of an organism, disruption of metabolic processes can result in the onset of multiple metabolic disorders (Knight 2011). Therefore, having a better understanding of the complex regulatory mechanisms of metabolism in living organisms is essential to prevent the development and progression of diseases. Multiple organs are known to work together to contribute and regulate the metabolic homeostasis. Of note, communication between the organs is considered to be crucial for the maintenance of body's metabolic balance. Different circulating factors including hormones, growth factors, cytokines, chemokines, and extracellular vesicles (EVs) are known to participate in this inter-organ cross-talk (Guay and Regazzi 2017). Over the past decade, EVs have gained much attention due to their role in regulating different physiological and pathological conditions (Boukouris and Mathivanan 2015; Gangoda et al. 2015). EVs are also known to contain a variety of biomolecules such as nucleic acids, proteins, lipids, and metabolites (Pathan et al. 2019). With these findings, several questions arise regarding the function of these biomolecules packaged within EVs and their role in metabolic pathways in recipient cells in the surrounding environment. Given the ubiquitous nature of EVs, it is of no surprise that they could be influential players in the regulation of complex metabolic pathways in an organism (Kalra et al. 2016). Therefore, the focus of this chapter will be to review how EVs and their cargo mediate cell-to-cell communication and regulate metabolic pathways. Furthermore, how these cellular messengers can alter different metabolic pathways in the recipient cells and contribute to different metabolic diseases will also be discussed. Additionally, this chapter will further explore how the release and uptake of tumour derived-EV cargo affects cancer metabolism and progression.

Overview of Metabolism and Metabolic Pathways

Chemical reactions of metabolic processes result in the production of chemical energy in the form of adenosine triphosphate (ATP), which is essential for a cell to continue functioning. Metabolism can be mainly divided into two parts; catabolism and anabolism (Schutz and Deurenberg 1996; Stenesh 1998; Voet et al. 2016). The breakdown of compounds or nutrients to produce energy is referred to as catabolism. This exergonic, oxidative process uses coenzymes such as Nicotinamide adenine dinucleotide (NAD^+) as electron acceptors to transfer phosphate groups to adenosine diphosphate (ADP) consequentially producing ATP (Voet et al. 2016). On the other hand, the endergonic synthesis process of new biomolecules is known as anabolism. This process requires energy in form of ATP, which is produced by catabolic reactions. Anabolic reactions are reducing in nature and use nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) as a donor for H^+ ions (Blanco and Blanco 2017a, b). Under normal homeostatic conditions, equilibrium is maintained between catabolic and anabolic reactions (Metallo and Vander Heiden 2013).

Metabolic pathways or cycles are enzymatically regulated by a sequence of catalytic events whereby products of one reaction can act as the substrate or intermediate for the proceeding reaction (Werner et al. 2016). The reactants and products of such chemical reactions make up the metabolome and are referred to as metabolites (Yang 2016). They consist of low molecular weight (<2000 Da) or intermediate products of cellular processes and metabolic reactions. These products can also be classified based on their functional groups which include sugars, ethyl alcohol, carboxylic acid, amino acids and amides (Hadacek and Bachmann 2015). In addition, a separately considered class or a second class of metabolites includes lipids and their derivatives (Fahy et al. 2011).

The central metabolic pathways in the human cells include glycolysis, Krebs' cycle, oxidative phosphorylation, pentose phosphate, fatty acid β-oxidation, urea cycle and gluconeogenesis (Werner et al. 2016). Glycolysis is the process of intracellular breakdown of glucose to produce pyruvate and ATP. Acetyl coenzyme A (acetyl CoA), the oxidized derivative of pyruvate generated during glycolysis, initiates the next stage of intracellular glucose metabolism known as krebs' cycle or citric acid cycle, in order to obtain guanosine triphosphate (GTP) and other important derivatives (Blanco and Blanco 2017a, b; Kumari 2018a, b). This pathway is followed by oxidative phosphorylation (OXPHOS), which is the final stage of cellular respiration. Here, five transmembrane complexes drive the disposal of electrons produced during glycolysis and krebs' cycle. Electrons are transferred from one molecule to the next through a series of redox reactions known as the electron transport chain. Energy released from this process is stored as ATP. One molecule of glucose can yield up to 38 ATP by OXPHOS (Cardol et al. 2009; Engelking 2015; Blanco and Blanco 2017a, b). The alternative pathways that branch out of glycolysis lead into the pentose phosphate pathway. Glucose-6-phosphate produced during glycolysis is repurposed by pentose phosphate pathways to produce NADPH, which is necessary for anabolic reactions or, more precisely, required to synthesize nucleotides such as DNA and RNA. Furthermore, fatty acid beta-oxidation refers to a process by which fatty acids are broken down to acetyl CoA, where these products then gain the ability to enter krebs' cycle and produce NADH and $FADH_2$ (Kumari 2018a, b). Other pathways include gluconeogenesis, which involves the synthesis of glucose from lactate, glycerol and amino acids (Komoda and Matsunaga 2015; Voet et al. 2016; Werner et al. 2016).

Extracellular Vesicles: Composition and Functions

EVs are membrane-bound nanosized vesicles that are known to be secreted by various cell types into the extracellular space (Sanwlani et al. 2020). EVs are composed of a lipid bilayer membrane, which encompasses biomolecular content such as proteins, nucleic acids, lipids and metabolites (Cossetti et al. 2014; Kalra et al. 2016; Samuel et al. 2017). More recently they have been referred to as 'intercellular communicasomes' as they mediate cell-to-cell communication by

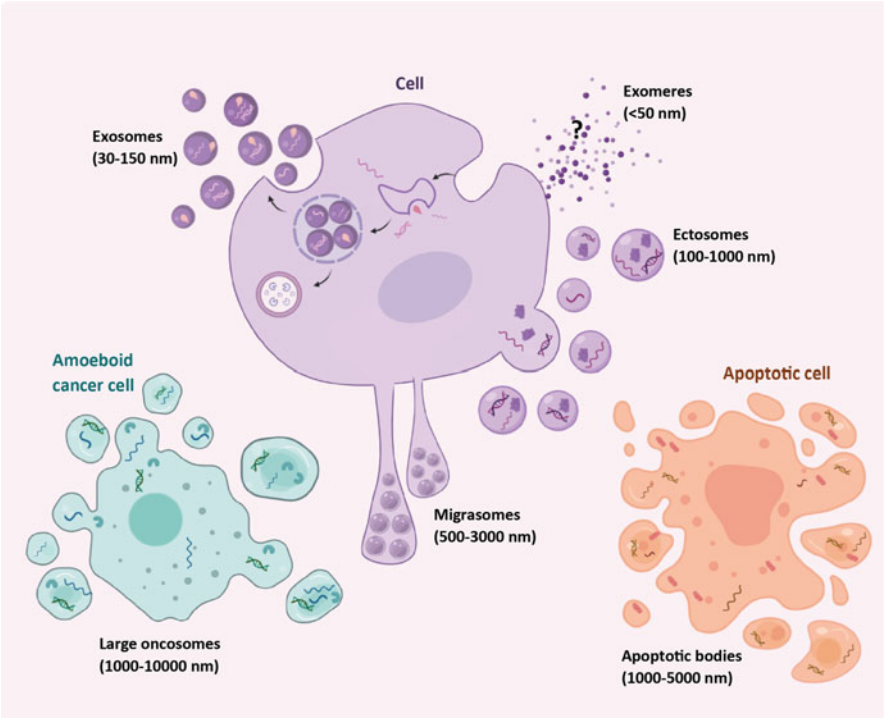

Fig. 15.1 EVs come in all shapes and sizes. EVs can be categorised into different subtypes based on their size and origin of secretion which include exomeres, exosomes, ectosomes, migrasomes, oncosomes and apoptotic bodies. Various biological processes determine the release of these vesicles into the extracellular space. Exosomes are generated from multivesicular bodies (MVBs), while ectosomes, apoptotic bodies and large oncosomes are released via the external budding of the plasma membrane of live, dying and amoeboid cancer cells, respectively. Migrasomes are generated from the tip of the retraction fibres by the migrating cells. The biogenesis and secretion of non-membranous nanoparticles, exomeres remains elusive. EE: Early endosomes, MVB: Multivesicular bodies. Created with BioRender.com

transferring functional biomolecules between cells and tissues (Palviainen et al. 2019). The blanket term "EV" can be categorised into different subtypes based on their size and mode of secretion (Mathivanan et al. 2010; Zijlstra and Di Vizio 2018) (Fig. 15.1). The most recent addition to the subtypes of EVs is termed exomeres (Zhang et al. 2018). However, exomeres are non-membranous nano-particles that are less than 50 nm in diameter. The biogenesis and secretion of exomeres are unclear and hence warrants further research (Chuo et al. 2018). Exosomes are small vesicles ranging from 30 to 150 nm in diameter, formed by inward budding of endosomes and are secreted through exocytosis (Gangoda et al. 2017), the process of fusion of the endosome with the cell membrane. Ectosomes or shedding microvesicles (SMVs), on the other hand, are large vesicles, 100–1000 nm in diameter, which are released from the plasma membrane (PM) through outward budding (Cocucci

et al. 2009; Keerthikumar et al. 2015). During apoptosis or programmed cell death, dying cells release histone rich heterogenous vesicles, referred to as apoptotic bodies. They range from 100 to 5000 nm in diameter and are secreted directly from the PM to the extracellular matrix (Kalra et al. 2016; Caruso and Poon 2018; Anand et al. 2019). Migrasomes emerge from the tailing edge of the migrating cell by detaching from the retraction fibres, tubular actin rich filament projections. They are found to harbour multiple smaller vesicles within its lumen and it ranges from 500 to 3000 nm in diameter (da Rocha-Azevedo and Schmid 2015; Ma et al. 2015). Large oncosomes on the other hand are secreted from amoeboid cancer cells and range from 1000 to 10,000 nm in diameter (Di Vizio et al. 2012). The vesicles will be collectively referred to as EVs from here on in this chapter.

EVs have gained significant interest in recent times due to their important role in cell-to-cell communication. The biomolecular cargo not only provides key information on the origin of the EVs but also dictates the biogenesis of EVs and the apparent crosstalk mechanisms between cells (Cossetti et al. 2014; Liem et al. 2017). Studies focusing on the cargo of EVs mostly analyse proteins and nucleic acids, therefore, knowledge on the metabolite content of EVs remains rather limited (Keerthikumar et al. 2016). Nevertheless, the metabolome of EVs have vested the interest of researchers due to the abundance of clinical information provided regarding the disease states and treatment responses of patients with metabolomic aberrations (Zhang et al. 2012; Sharma et al. 2016). Mass spectrometry (MS)-based studies have disclosed information on the metabolite content within EVs. The content consisted of glycerophospholipids and sphingolipids, alcohol, fatty acids, amides, amino acids, steroids, phenols, peptides, nucleotides, and their derivatives (Lydic et al. 2015). Furthermore, sugars, carboxylic acid, aromatic compounds have also been detected, but in less abundance (Altadill et al. 2016; Puhka et al. 2017; Clos-Garcia et al. 2018; Luo et al. 2018). However, further metabolome-orientated studies performed on EVs, obtained from bodily fluids, is essential to gain significant information to allow for the discovery of new biomarkers for disease monitoring, detection and therapy in clinical settings.

EVs as Metabolic Messengers

The metabolic function carried out by individual tissues is highly regulated and specialized. It is often modulated by specific molecules released by one tissue then taken up and utilised by other tissue (Sullivan 2017). As EVs are considered as a critical mode for intercellular communication, it is expected that EVs may media intercellular transfer of metabolites and metabolic enzymes and hence might also have a major role in the maintenance of metabolic homeostasis and alteration of metabolic phenotype of both nearby and distant tissues (Whitham et al. 2018; Palviainen et al. 2019; Sanwlani et al. 2020) (Fig. 15.2). In an effort to ascertain if the unique cargo of EVs alone could modify metabolites or contribute to metabolism, a consumption/release assay was conducted by Pluchino and colleagues (Iraci

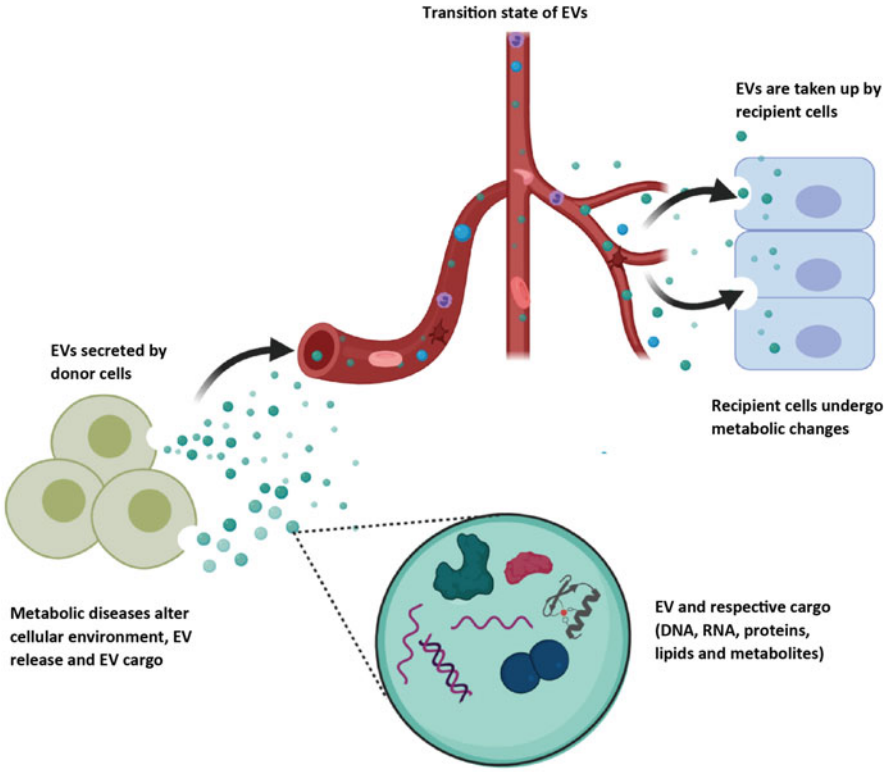

Fig. 15.2 Depiction of how EVs behave as metabolic messengers to alter the metabolic profile of recipient cells. Specific cargo released by donor cells are packaged into EVs which are then transferred as metabolic messages to the target cells. Upon uptake of EV content, the recipient cells decode the received messages and alter their phenotype accordingly. Created with BioRender.com

et al. 2017). Commercial cell culture media was incubated with EVs isolated from neural stem/progenitor cells (NSC) and the relative levels of metabolites pre- and post-incubation were determined using liquid chromatography, coupled with mass spectrometry (LC-MS). Interestingly, this assay revealed that EVs alone were sufficient in altering the levels of metabolites. Particularly, levels of glutamate and aspartate were found to be increased, while a reduction in the levels of asparagine was observed. This phenomenon of consumption of asparagine and the production of aspartate was striking, as it indicated that EVs derived from NSCs contained asparaginase enzyme, which is thought to be absent in most mammals (Iraci et al. 2017). Additional biochemical characterization was carried out to demonstrate whether asparaginase was indeed trafficked into EVs. This finding was further validated when isotope-labelled asparagine was converted to labelled aspartate by NSC derived EVs, confirming the presence of asparaginase activity. This data

suggests that EVs could independently communicate and change the levels of metabolites within their interactive environment.

Another study showed that energetic stress, such as glucose starvation, not only resulted in enhanced production and secretion of EVs by cardiomyocytes but also increased the trafficking of glycolytic enzymes and glucose in EVs derived from cardiomyocytes (Garcia et al. 2015). As these EVs are internalized, they transfer glucose transporters and glycolytic enzymes to the engulfing endothelial cells. Evidence from this study showed that the absence of glucose resulted in the upregulation of genes associated with the fusion of MVBs to plasma membrane and proteins involved with glucose trafficking (Glut4). Consequently, this energetic stress increased the production of EVs harbouring Glut, by assisting with the convergence of the Glut trafficking pathway with the EV secretion pathway. In addition, an increase in other glucose metabolism associated enzymes were observed, particularly lactate dehydrogenase (LDH). LDH is known to catalyse the interconversion of the two metabolites, lactate and pyruvate. This process is important for maintaining homeostasis in heart by acting as a checkpoint of gluconeogenesis. Endothelial cells depend on aerobic glycolysis for their growth through lactate production and glucose consumption. Thus, exposure to glucose-starved cardiomyocyte-derived EVs leads to the increase of uptake and pyruvate synthesis in endothelial cells as a response of cardiomyocyte's metabolic demand. Given the low energetic capacity of endothelial cells, pyruvate can diffuse to local cardiomyocytes and be oxidized by Krebs cycle, therefore supporting the nourishment of cardiomyocytes. This cardio-endothelial communication is therefore considered to have an association with metabolic regulation (Garcia et al. 2016).

Like most cells, hepatocytes secrete EVs packed with signalling biomolecules into their extracellular environment. Whilst the role of EVs in intercellular communication is well established, their metabolic functions are beginning to emerge. Hence, to analyse the global effect of hepatocyte released EVs on the metabolite composition of serum, a comprehensive metabolomics approach was utilized. Serum samples taken from rat incubated with or without hepatocyte derived EVs were analysed to identify differentially expressed metabolites. Interestingly, the levels of purine-related compound (deoxyinosine), lipids (glycerophosphocholine and lysophosphatidylcholine), and amino acids with its related compounds (arginine, N-methyl-L-arginine, ornithine, citrulline, glutamate, 3-methyl-L-histidine, glutamyl-alanine, and L-anserine) were found to be altered. The levels of purine nucleoside, deoxyinosine, were found to be increased approximately by fourfold, in serum samples exposed to EVs-derived from hepatocytes. Mammalian cells are known to use purine nucleoside as an energy source. Therefore, it was proposed that hepatic EVs, harbouring deoxyinosine, generate energy substrate through the pentose phosphate pathway and via glycolysis, within their extracellular environment, in response to injury or during stress conditions to exert protective effects (Royo et al. 2017).

EVs in Metabolic Diseases

In order to maximize the efficiency of the metabolic processes, crosstalk amongst organs is essential. Communication between principle metabolic tissues such as liver, adipose tissue and skeletal muscle is crucial for maintaining metabolic homeostasis (Palviainen et al. 2019). Hence, dysregulation within the system can result in the onset of metabolic diseases. The rise of metabolic disease world-wide is equating to a serious public health issue, that warrants immediate attention (Priest and Tontonoz 2019). Metabolic disease is referred to a cluster of several interconnected health conditions that increase the risk of cardiovascular diseases, diabetes, and cancer, to name a few. Increasing evidence suggests that EVs circulating in the system, carrying proteins and metabolites, have the potential to target and modify both the local and distant cellular environments, thus having a role to play in the onset of metabolic diseases (Huang-Doran et al. 2017; Martínez and Andriantsitohaina 2017). EVs derived from patients with metabolic diseases exhibit altered cargo that have differential effects on their respective recipient cells (Lakhter and Sims 2015). Studies have shown that obesity, diabetes and inflammation increases EV secretion into the serum (Stepanian et al. 2013; Freeman et al. 2018). When circulating EVs were profiled from 265 patients with hypertension, diabetes, and metabolic syndrome, it was revealed that cluster of unique microRNAs (miRNAs) in EVs were associated with different metabolic diseases. Among the dysregulated miRNAs that were identified, miR-103 showed to associate with PANK genes, which participate in lipid metabolism. Additionally, miR-130a, miR-195, and miR-92a were observed to be distinct to hypertension and metabolic syndrome. Beside these, altered expression of miR-195, miR-197, miR-23a and miR-509-5p were found to positively correlate with blood pressure (Santovito et al. 2014). Moreover, differential expression of miRNAs (miR-192, −27a, −320a, and −375) were observed to be upregulated in both Type-2 diabetes and metabolic syndrome. Overall, this plethora of information indicates that aberrant expression of miRNAs could be implicated as risk factors in manifesting metabolic disorders. Nevertheless, extensive future studies are imperative to elucidate the potential of miRNAs as therapeutic targets and biomarkers for metabolic syndromes.

Adipose tissue is one of the critical players in systemic inflammation that mediate insulin resistance through secreting various factors such as adipocytokines, hormones, fatty acids and EVs, responsible for modulating different metabolic pathways (Tilg and Moschen 2006; Delarue and Magnan 2007; Gao et al. 2017). EVs secreted from adipose tissues were identified as chemoattractants for monocytes to induce inflammation in both insulin resistant animal models and humans (Wadey et al. 2019). Moreover, studies suggest that metabolic signals carried by EVs tend to activate adipose tissue resident macrophages and endothelial cells, which in turn, promote remodelling of adipose tissue and induce systemic inflammation, the characteristic feature of obesity (Deng et al. 2009, Kranendonk et al. 2014a, b, Eguchi et al. 2015, Crewe et al. 2018). Adipocytes release inflammatory cytokines including tumour necrosis factor-alpha (TNFα) and interleukin-6 (IL-6) that aid in

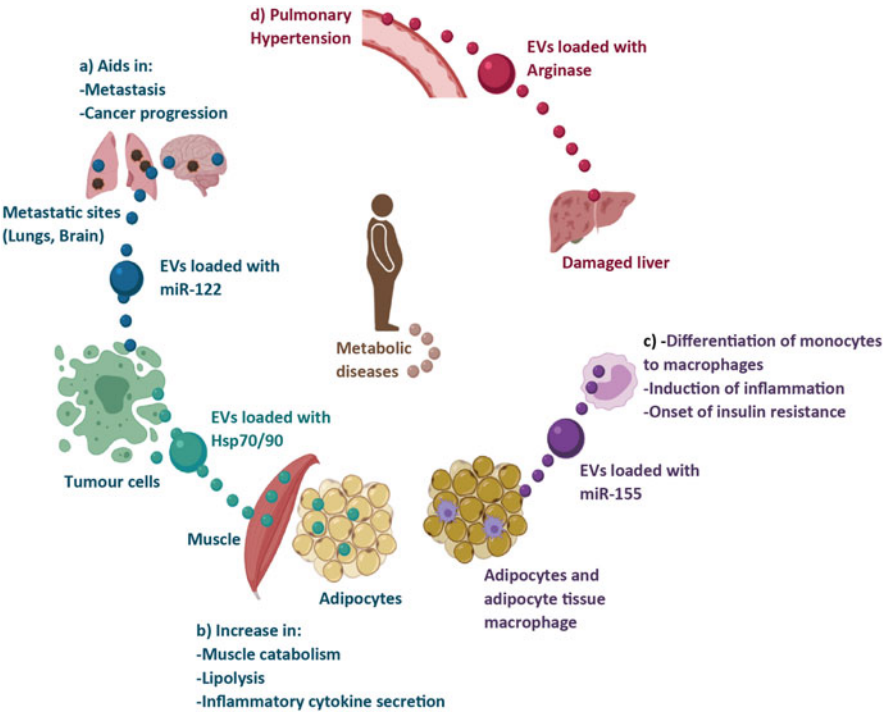

Fig. 15.3 EVs mediate intercellular/interorgan crosstalk which can contribute to metabolic disease. (**a**) Breast cancer cell derived EVs packed with miR-122 can alter the metabolism of recipient cells and aid in cancer metastasis and progression; (**b**) Cachectic tumour derived EVs are enriched with Hsp70/90 that contributes to cachexia by inducing muscle wasting and systemic inflammation; (**c**) Metabolic signals shuttled by adipocyte-derived EVs not only aid in the differentiation of adipose tissue resident monocytes but also induce inflammation and insulin resistance. In addition, EVs derived from adipose tissue macrophages tend to carry miR-155, that is associated with alteration of glucose homeostasis and insulin sensitivity; (**d**) EVs secreted by damaged hepatocytes are loaded with arginase, when taken up by the vascular endothelium, leading to pulmonary hypertension. Created with BioRender.com

the development of insulin resistance (Coelho et al. 2013). Adipocyte derived EVs were not only found to interfere with insulin signalling in adipose tissue, but were also associated with altered signalling in muscle cells and liver (Kranendonk et al. 2014a, b). These studies suggest that secretion of EVs under obesity conditions contribute to systemic inflammation to develop insulin resistance and therefore, represent a mode of communication for the regulation of blood glucose homeostasis (Fig. 15.3c). Studies by Deng et al., showed that adipose tissue released increased amounts of EVs when mice were fed a high fat diet. Moreover, these EVs were found to be enriched with RBP4 protein, known to activate the TLR4/NFκB pathway. Also, EVs isolated from adipose tissue of high fat diet mice were readily taken up by the monocytes, when injected in mice of healthy weight. This combined data shows that the EV cargo not only aided with the differentiation of monocytes to

macrophages, but also enhanced the production of cytokines (IL-6 and TNFα) from macrophages and prompted the development of insulin resistance that can lead to diabetes. Furthermore, incubation of monocytes and human adipose tissue, with EVs derived from adipocytes of high fat diet mice, resulted in a reduction of AKT phosphorylation, in response to insulin. Taken together, these observations demonstrate that EVs derived from adipocytes act as mediators of obesity and the development of inflammatory states associated with insulin resistance and diabetes (Deng et al. 2009). Furthermore, EVs secreted by macrophages residing in adipose tissue, were found to have the capability to modulate glucose tolerance, and insulin signalling. When EVs derived from obese adipose tissue macrophages were injected into lean mice, they displayed glucose intolerance and insulin resistance. However, EVs from lean adipose tissue macrophages normalized glucose tolerance and improved insulin resistance in obese mice. To investigate if EV miRNAs are responsible for such deviations in metabolic phenotype, EV miRNAs form both lean and obese adipose tissue macrophages were sequenced. Upon analysis of all differentially expressed miRNAs, miR-155 appeared to be most abundant. This study further showed that KO of miR-155 in mice displayed systemic insulin sensitivity and glucose tolerance compared to control mice. Hence, the study suggested that EV miRNA cargo, derived from adipose tissue macrophages, can be taken up by insulin dependent target cells and alter glucose homeostasis and insulin sensitivity (Fig. 15.3c) (Ortega et al. 2015).

It has been postulated that the secretion of hepatocyte-derived EVs increases after liver damage (Brodsky et al. 2008). There is also growing evidence that these circulating EVs might alter the serum metabolome. One such important change is the transformation of arginine into ornithine by arginase, as the arginase activity was found to be increased in EVs secreted by damaged hepatocytes. Arginine is a substrate or precursor of nitric oxide synthase. In the vascular endothelium, nitric oxide is necessary for normal vasoconstriction of blood vessels. Therefore, the increase in arginase activity would result in the depletion of arginine, in the serum, which in turn could have consequences in vascular dynamics, as the synthesis and activity of nitric oxide would be compromised. In vitro observations have revealed that EVs released from damaged hepatocytes could impair vascular relaxation of pulmonary arteries. Upon incubation with EVs secreted by the damaged or injured liver, the relaxant response of the pulmonary artery to acetylcholine was inhibited. This effect was completely blocked by arginase inhibitor. Further, the relaxant response to acetylcholine, was abolished with endothelial nitric oxide inhibitor, indicating a dependence of nitric oxide for vascular endothelial relaxation. Quite possibly, depletion of arginine by EV-derived arginase, which leads to nitric oxide deficiency, might contribute to the pulmonary hypertension observed in patients with liver disease (Fig. 15.3d) (Royo et al. 2017).

Cachexia is another metabolic syndrome that is characterized by loss of skeletal muscle and adipose tissue. It is common in patients with advanced cancers, kidney diseases, heart failure and chronic infections like HIV (Tisdale 2002; Fearon et al. 2012). In the advanced stage, most cancer patients suffer from some sort of cachexia irrespective of the type of cancer (Tisdale 2002; von Haehling and Anker 2014).

Cancer cachexia is estimated to contribute to one third of all cancer related deaths (Fearon et al. 2011, 2012; von Haehling et al. 2016). Cancer cell derived pro-inflammatory and pro-cachectic factors are known to drive wasting of specific tissues by disturbing the balance between the anabolism and catabolism of proteins in muscle and fats in adipocytes, tipping the balance to catabolism. However, the exact mechanism of tumour-induced muscle atrophy remains elusive. It is speculated that EV-induced intracellular cross talk might play a key role in transporting bioactive molecules to distally located skeletal muscle and adipose tissue sites, away from the primary tumour burden (Fig. 15.3b). Furthermore, tumour-derived EVs have been reported to stimulate muscle wasting and cachexia (Chitti et al. 2018; Yang et al. 2019). EVs released by cancer cells were found to be enriched with heat shock protein 70/90 (Hsp70/90), which activates TLR4, assisting in muscle wasting. Activation of TLR4 results in increased synthesis and secretion of inflammatory cytokines, which are known to mediate cachexia. Along with inflammatory factors, TLR4 activation mediates muscle catabolism through the p38-mitogen-activated protein kinase (p38-MAPK) pathway and through upregulation of ubiquitin ligase, atrogin 1 transcription. Of note, Hsp70/90 encapsulated within EVs also takes part in degradation of myofibrillar proteins, by activating autophagy-lysosomal pathway (Zhang et al. 2017). Similarly, it has been demonstrated that pancreatic cancer cell-derived EVs promote lipolysis via polypeptide adrenomedullin (AM). When AM binds to AM receptor, it activates p38-MAPK pathway which induces lipolysis, by phosphorylating hormone sensitive lipases (Sagar et al. 2016). Collectively, these findings advocate for the role of tumour cell-derived EVs in promoting catabolism in distant skeletal muscle and adipose tissue.

EVs in Cancer Metabolism and Progression

Cancer cells have a greater glucose requirement to meet bioenergetic and biosynthetic demands compared to normal healthy cells (DeBerardinis et al. 2008). Therefore, to satisfy this need, glycolysis is uncoupled from Krebs cycle and is directed towards anerobic glycolysis. This metabolic phenomenon is known as Warburg effect (Koppenol et al. 2011; Lu et al. 2015). Indeed, several studies have demonstrated that the production of various cytokines, chemokines, growth factors, and EVs by the tumour cells can affect the behaviour of cancer associated fibroblasts, endothelial cells, and immune cells which in turn can reprogram metabolism of cancer cells (Peinado et al. 2012; Brauer et al. 2013; Hoshino et al. 2015).

To fulfill nutritional demand, cancer cells are known to adapt different strategies to enhance the availability of glucose, such as exploitation of angiogenesis to obtain nutrients from blood. Fong and colleagues have demonstrated an interesting observation where cancer cells favour themselves by diminishing the nutrient consumption of other non-tumour cells. For instance, high levels of miR-122 detected in the circulation of breast cancer patients has previously been associated with metastasis (Wu et al. 2012). This observation was further strengthened by Fong et al., where

they showed that breast cancer cell-derived EVs are packed with miR-122, that can alter the metabolism of recipient cells and aid in proliferation and metastasis (Fig. 15.3a). Mechanistically, in both in vitro and in vivo conditions, breast cancer EVs, packed with miR-122, enhanced the availability of glucose to cancer cells, in the pre-metastatic niche, by downregulating pyruvate kinase M2 (PKM2). In addition, when miR-122 was inhibited, in vivo, restoration of glucose uptake in distant organs such as brain and lungs was apparent with a reduction in the occurrence of metastasis. These results indicate that cancer cell-derived EVs can reprogram systemic metabolic processes within recipient cells to promote cancer progression (Fong et al. 2015).

Cancer-associated fibroblasts (CAFs) are one of the key stromal cell components in the tumour microenvironment. A study conducted by Zhao et al., portrayed that EVs secreted by patient-derived CAFs have the capability to modify the metabolic machinery in recipient pancreatic and prostate cancer cells. Uptake of EVs secreted by CAFs were found to direct cellular metabolism of the prostate cancer cells towards glycolysis by inhibiting mitochondrial oxidative phosphorylation. Furthermore, the metabolite content of CAF-EVs were identified by experimental intra-EV metabolomics using 13C-labelled isotope. Analysis of metabolic data showed that EV metabolic cargo is preferentially trafficked to nutrient-deprived cancer cell to facilitate growth of the tumour (Zhao et al. 2016).

Large oncosomes are cancer-derived EVs ranging from 1 to 10 μm in diameter and are associated with advanced disease (Minciacchi et al. 2015). Quantitative proteomics analysis of EVs secreted by prostate cancer cells revealed that the unique EV content has the potential to reprogram the metabolic machinery of both cells within the tumour and the tumour microenvironment. Minciacchi and colleagues identified an enriched enzyme called GOT1 in large oncosomes, which when taken up by recipient cells could promote glutamine metabolism. In addition, another cytoplasmic enzyme, glutaminase (GLS), that converts glutamine to glutamate, was also found in large EVs (Minciacchi et al. 2015). Furthermore, a protein known as HSPA5 (GRP78) was also identified to be enriched in large EVs that affects glutamine metabolism by inducing c-Myc-mediated glutamine flux, aiding in the proliferation of cancer cells (Li et al. 2014). Altered glutamine metabolism is often observed in aggressive cancers such as glioma, melanoma and pancreatic cancer (Wise et al. 2008; Long et al. 2013; Son et al. 2013). Taken together, the unique cargo of large oncosomes was implicated with the alteration of glutamine metabolism in tumour microenvironment. Perhaps, these observations indicate that this distinct EV type and its cargo helps in promoting cancer cell proliferation (Minciacchi et al. 2015). The release and uptake of EV content by cancer cells alters both the behaviour and metabolism of recipient cells. This intercellular communication plays a major role in creating a niche for invasion, metastasis, and the progression of cancer. Therefore, decoding the cross-talk mediated by tumour derived EVs would take us a step closer to harnessing the mechanism and improving the standard of care for cancer patients (Becker et al. 2016).

Concluding Remarks

The role of EVs in metabolism is a nascent field of study that holds huge potential. The numerate studies currently conducted to gain better understanding of the function of EVs in metabolism, in both physiological and pathological contexts, is continuously evolving and rapidly growing. However, further studies are essential to evaluate the function of EVs and their respective metabolic cargo. It is still unclear how cargo consisting of specific proteins, RNAs and metabolites are selectively encapsulated in EVs. Undoubtedly, the metabolome of EVs can serve as a goldmine for biomarker detection with respect to a variety of diseases. However, there is a need for better knowledge and understanding of how the biogenesis of EVs is regulated during both heathy and metabolic disease conditions and how metabolic disease state can alter and/ or influence not only the secretion of EVs but also the EV cargo. Therefore, additional studies will provide valuable insights into our understanding of the role of EVs in metabolism. Deciphering the messages carried by EVs secreted by normal cells, cancer cells and in metabolic disease state would help to decode different disease conditions. Further, the knowledge may provide insights into developing therapeutic strategies to neutralize EV signalling and to maintain metabolic homeostasis in humans.

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Chapter 16
Extracellular Vesicles in Neurological Disorders

Alex Mazurskyy and Jason Howitt

Abstract The role of extracellular vesicles (EVs) in the central nervous system, and in particular the brain, is a rapidly growing research area. Importantly, the role for EVs in the nervous system spans from early development through to old age, with EVs being associated with several different neurological disorders. To date, researchers have been studying the function of EVs in the nervous system in three major areas: (i) the role of EVs in promoting disease pathways, (ii) the ability of EVs to be used as a diagnostic tool to identify cellular changes in the nervous system, and (iii) the potential use of EVs as therapeutic tools for the delivery of biomolecules or drugs to the nervous system. In each of these settings the analysis and use of EVs performs a different function, highlighting the breadth of areas in which the EV field is applicable. A key aspect of EV biology is the ability of vesicles to cross biological barriers, in particular the blood brain barrier. This allows for the measurement of serum EVs that contain information about cells in the brain, or alternatively, allows for the delivery of biomolecules that are packaged within EVs for therapeutic use.

Keywords Exosomes · Autism (ASD) · Brain development · Mental health · Glioblastoma · Neurodegeneration

Introduction

Neurological disorders can affect the brain or central nervous system (CNS) at any age across our lifespan. This includes during the early developmental years, adolescence to adulthood, through to old age. Each disorder also affects individuals

A. Mazurskyy
Swinburne University of Technology, Hawthorn, VIC, Australia

J. Howitt (✉)
Swinburne University of Technology, Hawthorn, VIC, Australia

Florey Institute of Neuroscience and Mental Health, Parkville, Australia
e-mail: jhowitt@swin.edu.au

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differently, with divergences occurring in both symptoms and the mechanisms behind the disorder's development. This variety presents researchers with a plethora of issues to explore, as understanding these mechanisms will reveal the aetiology of neurological disorders and lead to discovery of methods to improve the quality of life for affected individuals. The most common neurological disorders across our lifespan include autism spectrum disorder (ASD) from early development, epilepsy, which can develop in early childhood or later in life after brain injury, schizophrenia during adolescence, brain tumours in mid-life, through to neurodegenerative diseases which are prevalent in the older population. While the literature surrounding the development and progression of these disorders is vast, the purpose of this chapter will be to introduce and elaborate on the involvement of EVs in neurological disorders.

Within the body, cells communicate with each other through different mechanisms, including direct cell-to-cell contact or through the secretion of molecules into the extracellular space (Mathieu et al. 2019). EVs are used by cells as both a communication pathway, and as a method to remove molecules from the cell. EVs encapsulate cargo of interest within a lipid-bilayer membrane prior to releasing them from the cell (Mathieu et al. 2019; Johnstone et al. 1987). This process protects cargo from being degraded by enzymes and immune cells found in the extracellular space, providing an efficient means for the transfer of molecules. It is because of EVs ability to transport cargo that they have become of increasing interest to researchers for both disease mechanisms as well as a diagnostic tool. Thus far, studies have been able to link the involvement of EVs to both early-life brain development, as well as in disease mechanisms of several neurological disorders and neurodegenerative diseases.

Importantly, EVs are generated by different pathways and can contain surface markers unique to the cells from where they originate. Through the characterization of these markers and the pathways they are relevant to, researchers can uncover new information about the pathogenesis of neurological diseases. It is for the same reasons that EVs offer a novel approach towards the early detection and treatment of diseases, overall contributing to improving health outcomes for affected individuals.

Extracellular Vesicles in Brain Development and Developmental Disorders

Extracellular vesicles have been demonstrated in multiple studies to be an important component of cell-to-cell communication. EVs can communicate over short distances when proximally located to other cells and can signal over significantly longer distances through transportation within the cerebral spinal fluid (CSF) or bloodstream. The communication capacity of EVs has been shown to help coordinate the development of pre- and postsynaptic elements, as well as regulate gene expression (Korkut et al. 2009, 2013). While the role of EVs has been widely explored in neurological disorders, little is currently known about the role of EVs in brain development.

As our understanding behind the involvement of EVs in neurodegenerative diseases has increased, studies have begun to explore their role in early life development. A study published by Sharma et al. in 2019 approached this gap with both in vitro and in vivo experimentation. The authors observed that the application of exosomes (small EVs originating from multivesicular bodies inside the cell) derived from pluripotent stem cells onto primary neural cultures resulted in increased proliferation of neural progenitors, neuronal differentiation and circuit connectivity (Sharma et al. 2019). This first observation was an indicator towards the role of EVs in developmental pathways within the brain. Due to exosomes native involvement in cellular communication, this result highlighted the potential that exosome signalling can carry components relevant to neuronal development. To further explore this, the authors injected purified rodent exosomes into the CNS of early post-natal mice. After 24 h, an increase in proliferation of the granule cell layer of the dentate gyrus was observed when compared to controls, indicating a role for EVs in signalling during brain development (Sharma et al. 2019). Following these observations, the group hypothesised that deficits in exosome signalling pathways would ultimately disrupt normal neuron circuit development. To test this hypothesis, Sharma et al. assessed the ability of exosomes derived from cells with a defect in the X-linked Methyl-CpG-binding protein 2 (MECP2) to promote neuronal development. This genetic disorder is most commonly recognised in people diagnosed with Rett syndrome, with patients presenting fewer neuronal synapses, reduced spine density, smaller soma size and altered calcium signalling when compared to normal neurons (Marchetto et al. 2010). Patients diagnosed with Rett syndrome present with neurodevelopmental deficits and often have language and coordination issues as well as reduced head growth. The experiment by Sharma utilised two different cell lines, isogenic rescue control neural cultures and human induced pluripotent stem cells (hiPSC)-derived neural cultures which displayed the MECP2 loss of function (MECP2-LOF) trait seen in Rett syndrome patients. The application of exosomes isolated from control cells into hiPSC-derived neural cultures displaying the MECP2-LOF saw an improvement in the cell proliferation rate, while the application of exosomes from MECP2-LOF cells onto the controls yielded no differences (Sharma et al. 2019).

Analysis of exosomal contents found that the controls contained a 1.5-fold increase in 237 different signalling proteins. Further proteomic analysis found that many of these proteins are involved in neurogenesis pathways (Sharma et al. 2019). Together, these results highlight the role that EVs can play in the developing brain. However, further research is required to understand how these communication pathways affect neuronal development in the brain. Importantly, the results presented by Sharma et al. do not show that EVs play a role in neurodevelopmental disorders, rather that EVs contain factors that can promote neuronal development. Taken together, the results by Sharma et al. provide an exciting glimpse into the role of EVs in neuronal development, indicating a potential for the use of EVs as a therapeutic agent.

Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a predominantly heritable, multistage disorder that results from changes occurring during brain development. These changes impact a person's ability to perceive and react to social information and may also contribute to motor learning deficits. It is now clear that ASD is clinically detectable and can be diagnosed by 14 months of age (Pierce et al. 2019). Recent advances have identified hundreds of genetic risk factors, including common and rare genetic variants, which can increase the likelihood of ASD (Ronemus et al. 2014). Many autism susceptibility genes are known to have important roles in the brain, with functions ranging from synaptic transmission to RNA processing and neurogenesis (Gilman et al. 2011; O'Roak et al. 2012; De Rubeis et al. 2014). Over the past 3 years researchers have begun to investigate the role that EVs may play in ASD, identifying potential roles in the disorder and also pathways for the development of therapeutics.

Previously, neuroinflammation and microglial activation has been observed in ASD brains (Vargas et al. 2005; Rodriguez and Kern 2011), suggesting that it may play a role in the pathogenesis of the disorder. Tsilioni and Theoharides compared EVs from the serum of both control and ASD patients and observed that total EV-associated protein was increased in the ASD samples (Tsilioni and Theoharides 2018). The authors interpreted this as an increase in total EVs, however it is also possible that EVs from the ASD serum contain higher protein levels per vesicle. Importantly, they went on to show that the ASD serum EVs were able to stimulate the secretion of the pro-inflammatory cytokine IL-1β from immortalized microglia in cell culture experiments. Suggesting that the ASD derived EVs have a potential role in inflammation in the brain.

Many individuals with ASD have food selectivity and restricted food interests due to the texture, smell, or colour of specific foods, and food intolerance (Marí-Bauset et al. 2014; Bryant-Waugh et al. 2010). This limited food intake behaviour in individuals with ASD leads to nutrition imbalance and gastrointestinal (GI) symptoms, such as diarrhoea and constipation (Kral et al. 2013; Hyman et al. 2012). In concordance with this, ASD individuals have been shown to have altered gut microbiota (Wang et al. 2011a; Adams et al. 2011; Mulle et al. 2013). To further understand differences in gut microbiota between ASD and controls Lee et al. investigated DNA that was isolated from bacteria-derived EVs in the urine of participants (Lee et al. 2017). Metagenome analysis of the bacteria EV derived DNA indicated altered microbiota profiles at the levels of the phylum, class, order, family, and genus in ASD individuals relative to control subjects. As such, this study highlighted a method for the rapid assessment of microbiota changes in ASD individuals through the sampling of urine, providing a simple method of sample collection in a population that can be difficult to obtain clinical samples.

More recently EVs have been investigated as potential therapeutic agents in animal models of ASD. Such studies are somewhat controversial in the ASD community given that many people believe that ASD does not require therapeutic intervention. Nevertheless, there are many forms of ASD that are debilitating to both

the individual and their caregivers, as such it remains an active area of interest. To date, a single laboratory has published in this area using EVs derived from mesenchymal stem cells (MSC). Importantly, these studies have used two different mouse models of ASD, the first being BTBR mice, an inbred mouse line that has multiple behavioural phenotypes thought relevant to some symptoms of autism. The second model used was the SHANK3 knockout mice, SHANK3 has been identified as a monogenic cause for ASD. SHANK3 functions as a scaffolding protein found in the postsynaptic density of excitatory neurons and genetic alterations in SHANK3 have been shown to affect dendritic spine development.

In these studies, intranasal delivery was used to deliver EVs to each of the mouse models of ASD. In the case of BTBR mice the authors delivered EVs at 4 of weeks of age and observed a change in behavioural deficits in both ultrasonic vocalisations and a reduction of repetitive behaviours (Perets et al. 2018). For the SHANK3 knockout mice the authors delivered EVs at 8-10 weeks of age and observed improvements in social behaviour deficits, increased ultrasonic vocalization, and a reduction in repetitive behaviours (Perets et al. 2020). The timing of the EV delivery in these studies was after the pre- and post-natal periods known to be critical in mouse brain development (the time point when ASD is thought to develop). As such, the authors therefore investigated a number of potential pathways that could alter behavioural phenotypes. In the SHANK3 knockout mouse model they observed changes in GABA subunits, suggesting that GABA-mediated pathways in the prefrontal cortex may be attenuated by nasal delivery of MSC EVs. Supporting this, it was observed that after nasal delivery there was an increase in delivered MSC EVs in the frontal cortex and cerebellum of SHANK3 knockout brains compared to wild-type control mice. Results from these studies suggest that EVs may be a potential pathway to deliver therapeutic targets for the treatment of ASD.

Epilepsy

Epilepsy is a chronic neurological disorder that causes recurring seizures. Epileptic episodes are believed to occur due to an imbalance of neuronal excitation and inhibition, which ultimately leads to seizures and chronic neuroinflammation (Stafstrom and Carmant 2015). A proportion of individuals with epilepsy can experience “status epilepticus”, which is defined either by having a single seizure with a duration of over 30 min, or a series of recurring seizures between which the patient does not regain normal mental function (Cherian and Thomas 2009). Individuals who experience status epilepticus have significant morbidities and high mortality rates (Pitkänen et al. 2007). Current literature suggests that a variety of different triggers can lead to epilepsy. A high number of individuals with epilepsy develop the disorder after an injury, with studies showing that traumatic brain injuries (TBI), strokes and neuronal infections are capable of inducing pathogenesis of epilepsy (Pitkänen et al. 2007). The remainder of people affected by epilepsy develop it either through inheritance or via sporadic genetic mutations. In recent

times, EVs have become of increasing interest to researchers in the epilepsy field. This is due to the discovery that EVs are capable of internalising and transporting microRNA (miRNA) between cells. This finding is significant as miRNA are able to regulate the translation and degradation of their messenger RNA (mRNA) targets, giving them the ability to regulate cellular phenotypes (Henshall 2014). While these exact processes are currently poorly understood within the scope of epilepsy pathogenesis, it is believed that the dysregulation of miRNA is important. This is supported by a study published by Henshall et al. in 2016, which described the ability of miRNA to target complementary sequences of mRNA, thereby affecting the mRNA stability and translation inhibition (Henshall et al. 2016). Further studies assessing exosomal content with focus on miRNA have found that the expression of miRNA differs in patients with epilepsy when compared to samples from healthy individuals and from patients with other neurological diseases (Batool et al. 2020).

Several studies have explored the mechanisms initiated following a TBI event, with a focus on the effects on EVs and miRNA. In vivo experiments replicated a TBI event through different methods, including controlled cortical impact injury (CCI) or fluid-percussion injury (FPI). A study published by Harrison et al. (2016) using the CCI method, found that several miRNAs within exosomes were upregulated following injury. These included the upregulation of miR-21, miR-146, miR-7a and miR-7b, and the downregulation of miR-212 (Harrison et al. 2016). The upregulation of miR-146 and the miR-7a/b was associated with anti-inflammatory and neuroprotective effects, respectively (Harrison et al. 2016). However, the opinions regarding the upregulation of miR-21 differ, as studies have reported both neuroprotective and neurotoxic roles for miR-21. For example, miR-21 was reported to reduce glial scar formation and hypertrophy of astrocytes in spinal cord injuries (Bhalala et al. 2012) as well as reducing microglia-mediated neuronal death following experimentally induced stroke (Zhang et al. 2012). Meanwhile other studies have observed that increased expression of miR-21 contributes to neuronal dysfunction by dysregulating potassium channel activity (Yelamanchili et al. 2010) and promotes neurotoxicity by binding to TLR-7 of the innate immune pathway (Yelamanchili et al. 2015). Based on the reported differences in miR-21 function, more research is currently needed to elucidate the function of miR-21 in disease. The impact of the downregulation of miR-212 is unknown, however has previously been reported to be associated with other neurodegenerative diseases, including schizophrenia, Alzheimer's disease and anencephaly (Kim et al. 2010; Wang et al. 2011b; Zhang et al. 2010).

A study by Zhao et al. (2018), using the FPI method assessed the impacts of TBI on noncoding RNA molecules called circRNA. These circRNA have the ability to regulate miRNA expression and are involved in the onset of diseases such as Alzheimer's disease and Parkinson's disease (Haque and Harries 2017). Zhao reported that FPI injured brain tissue released an increased concentration of exosomes, which also differed greatly in content from controls. In total, the study reported that there was a difference in 231 exosomal circRNA post-FPI, with 155 being upregulated and 76 being downregulated (Zhao et al. 2018). Follow-up analysis into the impact of the upregulated circRNA determined that these were

involved in pathways relevant to the growth and repair of damaged neurons. However, it was also noted that pathways associated with calcium regulation were impacted (Zhao et al. 2018). Calcium is normally involved in modulating cellular pathways and is therefore highly regulated. However, dyshomeostasis of calcium has been linked to many neurological diseases and has also been shown to impact the development of epilepsy (Kovac et al. 2017).

Other clinical studies assessed this effect through analysing EVs contained within the CSF and plasma of patients who had experienced a TBI. TBI was reported to cause significant increases in the volume of EVs secreted from neurons. Following injury, EV concentrations would reach their peak within the first 24 h, and gradually return to normal over a prolonged period of time (Hazelton et al. 2017). Other studies have also assessed EV contents with focus on miRNA, during the periods of elevated EV levels. These studies reported that miRNA contained within exosomes differed greatly during the period of recovery, when compared to controls. For example, a study by Patz et al. in 2013 found that EVs within the CSF of patients post severe TBI, contained 81 different forms of miRNA. However, only two miRNA, miR-9 and miR-451, were observed within CSF samples collected post-injury (Patz et al. 2013). Further studies on the functions of miR-9 and miR-451 found that this upregulation was consistent with neuronal and vascular regeneration, and did not contribute to the pathogenesis of epilepsy (Patz et al. 2013). Meanwhile, a study published by Yan et al. assessed the EV content of patients with epilepsy caused by hippocampal sclerosis. Here, it was reported that there were 50 differently expressed EV-associated miRNA in epilepsy patients compared to the controls, with two miRNA upregulated and 48 downregulated (Yan et al. 2017).

A study by Batool et al. in 2020 assessed if events of status epilepticus had an impact on the biogenesis of exosomes as well as the miRNA contents. It was reported that ESCRT-dependent and independent complexes were upregulated, with the increases still detected 2 weeks after initial insult (Batool et al. 2020). The study also reported that status epilepticus upregulated several miRNA, a result consistent with other studies exploring miRNA in experimental and human epilepsy. MiR-21 was upregulated to a significantly high degree post-seizure, leading to the suggestion that miR-21 could be used as a biomarker for diagnosing status epilepticus and as a tool to uncover further mechanisms behind the pathogenesis of epilepsy (Batool et al. 2020). While the overall involvement of miRNA and exosomes in the pathogenesis of epilepsy is not well defined, currently available data implicates both as important elements in the process.

Considering the involvement of EVs in cellular communication and signal transfer, several studies have experimented with the use of EVs as therapeutic agents for the treatment of brain injury. Studies assessing the beneficial roles of EVs on TBI treatment have found several positive outcomes in animal models. The application of EVs derived from mesenchymal stem cells to mice with a CCI induced injury led to the promotion of vascular density, increased neuronal growth, and reduced brain inflammation (Zhang et al. 2017). Other studies have found that application of EVs from stem cells was able to reduce the concentration of proinflammatory cytokines, such as IL-1β, in CCI mice and promoted cognitive function after recovery (Kim

et al. 2016). A TBI swine model published by Williams et al. in 2018 observed similar beneficial outcomes and reported faster recovery rates (Williams et al. 2019). Thus far, few studies have assessed the ability of EVs to specifically treat epilepsy. However, a study published by Long et al. found that intranasal administration of EVs derived from stem cells was able to reach the hippocampus within a relatively short time. The effects of this was an increase of anti-inflammatory cytokines, which demonstrated reduced neuronal loss 4 days after status epilepticus (Long et al. 2017).

Schizophrenia

Schizophrenia is a serious mental disorder in which people interpret reality abnormally, usually involving hallucinations, delusions, and disordered thinking that impairs daily functioning. The first study to investigate a role of EVs in schizophrenia focused on differences in exosomal miRNAs derived from post-mortem prefrontal cortices (Banigan et al. 2013). The authors observed differences in schizophrenia exosomal miRNA in comparison to controls, in particular miR-497 was shown to have significantly increased expression in schizophrenia derived EVs.

A subsequent study by Du et al. also investigated differences in EV miRNAs from schizophrenia patients compared to controls (Du et al. 2019). In contrast to the Banigan study, Du and colleagues analysed serum derived EVs rather than samples from post-mortem studies. Importantly, in this study samples were from first-episode, drug free schizophrenia patients, limiting the potential confounding effects of drug treatments over time that occurs in post-mortem studies. Using genome-wide miRNA expression profiling, they observed that hsa-miR-206 was the most upregulated miRNA in schizophrenia patients. Previously, hsa-miR-206 has been reported to regulate brain-derived neurotrophic factor expression, reducing mRNA and protein levels in the blood of schizophrenia patients. This study also investigated the use of EV miRNA profiles as a method to classify schizophrenia patents from controls, showing an accuracy of approximately 75% on naive blood samples. These findings suggest that it may be possible to use EV profiles as a method to diagnose the disorder.

Tan et al. also investigated EV samples from blood collection, this time looking at circRNA expression (Tan et al. 2020). A total of 44 plasma EV circRNAs were differentially expressed (38 upregulated, 6 downregulated) between schizophrenia patients and matched healthy controls. Bioinformatics analysis indicated that the differentially expressed circRNAs could play potential roles in the pathogenesis of schizophrenia through changes to metabolism, stress response, and histone ubiquitination.

Outside of studying alterations in EV RNA levels in schizophrenia, Kapogiannis et al. investigated if EVs were involved in glucose metabolism impairments in drug-naive first-episode schizophrenia patients (Kapogiannis et al. 2019). Brain energy metabolism and insulin signalling is linked to schizophrenia through the control of brain development and maturation. In particular, the mTOR pathway is associated

with dendritic branching and synaptic plasticity. The team investigated peripheral blood EVs enriched for neuronal origin using L1 neural cell adhesion molecule (L1CAM) immunoprecipitation. The isolated EVs were then tested for the activation of various molecules including AKT, GSK3β, mTOR and p70S6K, it was found that for all except AKT, that phosphorylation levels were reduced in schizophrenia samples. These findings support the notion that neuronal insulin resistance occurs in early stages of schizophrenia, however the small sample size of the study indicates that larger cohorts will be required to confirm the findings.

Cancers

Cancer is one of the leading causes of death worldwide and can affect any human system. Glioblastoma multiforme is the most common neurological cancer, affecting both the brain and the spinal cord, recent evidence now suggests that EVs are part of the diseases process (Cumba Garcia et al. 2019). Glioblastoma multiforme often presents as malignant and aggressive, with poor prognosis for affected patients. As is widely known, cancer is the result of uncontrolled cellular growth following mutations within the genetic code. As has already been discussed, EVs possess the ability to carry genetic information in the form of circRNA, miRNA and DNA. For this reason, the involvement of EVs in the progression of cancers has been a topic of much research. Thus far, the crosstalk between cancerous cells, made possible by EV communication has been shown to promote primary tumour growth (Skog et al. 2008). Meanwhile, EV communication between cancerous and healthy cells has been shown to initiate secondary tumour growth within the brain (Pavlyukov et al. 2018).

One of the first major studies to demonstrate the ability of EVs to transfer genetic information in a cancer setting was published by Skog et al. in 2008. This study assessed the contents of glioblastoma-derived microvesicles. Skog et al. focused on mRNA relevant to angiogenesis, as cell proliferation, immune response, cell migration and histone modification are all aspects commonly represented by enhanced tumour growth. It was reported that mRNA known to potentiate these factors were expressed at significantly increased levels when compared to microvesicles derived from serum of healthy controls (Skog et al. 2008). Furthermore, Skog's study demonstrated that microvesicle-delivered pathogenic mRNA was translated by recipient cells. The uptake of EVs containing pathogenic mRNA not only stimulated further proliferation of existing tumours, but it was also able to modify the translation profile of nearby healthy cells (Skog et al. 2008). In doing so, healthy cells adopted a new angiogenic phenotype. Notably, the study also explored the presence of epidermal growth factor receptor (EGFR) within EVs, as some mutant variants are specific to subtypes of glioma (Nishikawa et al. 2004). EGFR variant 3 was detected within microvesicles of samples derived from glioblastoma patients but was not detected within the serum of healthy controls (Skog et al. 2008).

Since this discovery by Skog et al., other groups have explored the significance of EVs in the development of glioblastoma. Building upon this research, a study by Guescini et al. in 2010 was able to demonstrate that glioblastoma and astrocyte cells are able to release mitochondrial DNA (mtDNA) within exosomes. With respect to mtDNA, other studies have been able to demonstrate that pathogenic mtDNA is able to migrate between cells by using tunnelling nanotubules, thereby affecting the phenotype of recipient cells (Gerdes et al. 2007; Schapira 2006). Guescini's study provides evidence for an alternative mode of mtDNA transmission, via EVs. The study reported that mtDNA was protected from degradation and was able to gain access to target cells. However, the impact on phenotype was not assessed in this study (Guescini et al. 2010).

A later study by Pavlukov et al. in 2018, demonstrated that apoptotic glioblastoma cells secrete EVs containing spliceosomes. According to this study, apoptotic cells make up 70% of an entire tumour's cell population, which can communicate with surrounding cells by secreting EVs (Pavlyukov et al. 2018). Splicing factor RBM11 from tumour cells was transmitted between cells and was resistant to lysosomal degradation upon internalisation. In addition, these spliceosomes were transported towards the nuclei of recipient cells, which resulted in increased glycolysis, a hallmark of malignant cancers (Pavlyukov et al. 2018). It was also noted that upon the internalisation of EVs containing spliceosomes, recipient cells began to display aggressive migratory phenotypes and were also resistance to treatments (Pavlyukov et al. 2018). The evidence presented by these studies support the involvement of EVs in promoting further tumour growth and secondary malignancies.

A silver lining is also presented, wherein theoretically EVs can be assayed for biomarker purposes. Current research is exploring the possibility of using EVs and their contents as biomarkers for the early detection of cancer. The detection of cancer specific miRNA in the plasma or CSF of patients would allow physicians to begin treatments earlier. A study by Akers et al. in 2013 explored the possibility of using the abundance of miR-21, a miRNA over-expressed in glioblastoma, as a biomarker. This study reported that glioblastoma cells secrete miR-21 within EVs. It was noted that the CSF of glioblastoma patients contained on average a tenfold higher concentration of EVs containing miR-21 when compared to controls ($p < 0.05$) (Akers et al. 2013). Another study by Goldvaser et al. in 2017 found that human telomerase reverse transcriptase transcript (hTERT) mRNA can be found within exosomes secreted from fibroblasts. This mRNA was found in 67.5% of patients tested across all cancer types, which was a stark contrast to none detected in healthy controls (Goldvaser et al. 2017). As is demonstrated here, the use of EVs as biomarkers for the early detection of cancers in the brain is promising, however much more research is required before this practice can be widely incorporated.

Prion Diseases

Extracellular vesicles, namely exosomes, have been reported to contribute to the pathophysiology of several neurodegenerative diseases. The most characterised of these is Creutzfeldt Jakob disease (CJD), one of the few prion diseases. Other commonly known prion diseases include the human associated Kuru, bovine spongiform encephalopathy (mad cow disease) in cattle, and scrapie in sheep. Prion diseases in particular have been shown to be highly infectious, with some possessing the ability to be transmissible between species (Geschwind 2015). Overall, prion diseases cause rapid deterioration in affected individuals (Geschwind 2015), which is a significant difference from other neurodegenerative diseases, most of which take decades to manifest completely.

The discovery and analysis of the mechanisms involved in prion diseases have been highly instructive towards how we understand many neurodegenerative diseases today. Prion diseases are caused by the accumulation of misfolded proteins within affected cells and propagate through a unique 'protein-only' mechanism. First demonstrated by Stanley B. Prusiner, the 'protein-only' hypothesis explores the role of proteins as infectious agents in spongiform encephalopathies. According to this theory, some natively occurring proteins are susceptible to misfolding when exposed to abnormal cellular environments (Prusiner et al. 1998). In this form, the misfolded proteins adopt a primarily β-sheet structure, causing them to become insoluble and resistant to degradation (Laurent 1996). In addition, a shift towards this abnormal isoform causes these susceptible proteins to lose their normal function and instead gain a toxic function (Prusiner et al. 1998). The most unique aspect of prion diseases is their mode of propagation, which lives up to the 'protein-only' name. Typically, protein production and function results from the transcription and translation of a cell's genetic information. However, prion proteins replicate in a template dependent manner (Acquatella-Tran Van Ba et al. 2013). Misfolded and pathogenic proteins in prion diseases begin to act as a template, affecting the folding of nascent or existing proteins within the cell (Acquatella-Tran Van Ba et al. 2013). As such, prion proteins replicate by inducing the misfolding of proteins without affecting a cell's genetic information.

Normally, when cells detect the presence of irregular proteins, they designate them to be degraded by the ubiquitin proteasome system (UPS) or the autophagy-lysosome pathway (ALP) (Lecker et al. 2006). The prompt polyubiquitination of toxic proteins signals for the cells to transport them to sites of degradation, where they are broken down (Lecker et al. 2006). However, the template-regulated change into a primarily β-sheet structure causes the prion proteins to be resistant to this process (Ciechanover and Kwon 2015). In addition to being resistant to degradation, prion proteins are also said to utilize native cellular communication methods, including cell-to-cell contact, tunneling nanotubules and exosomes. Studies have been able to demonstrate the ability of exosomes to encapsulate prion proteins and assist in transferring prion infectivity (Guo et al. 2016). Through this use of exosomes to cross the extracellular space unimpeded, the prion proteins gain access

to nearby cells and thereby 'seed' them (Fevrier et al. 2004). Through this use of EVs, pathogenic proteins gain access to naïve cells and begin to exhibit their influence over the newly infected cells through the template dependent manner and promote the further production of misfolded, prion proteins (Acquatella-Tran Van Ba et al. 2013). This combination of the template mechanism and seeding of naïve cells describes the propagation processes behind prion proteins. These processes act in a continuous cycle, eventually resulting in the death of affected individuals.

Support for this mode of propagation by prion proteins has been expressed both by in vitro and in vivo analysis. Cell culture experiments have been able to establish the ability for prion proteins to be contained within exosomes. This was achieved through the isolation of exosomes using differential ultracentrifugation of cell culture media and analysis with Western blotting for the prion protein. Further analysis of the markers present on EVs surface, such as TSG101 and flotillin-1, distinguished between microvesicles and exosomes, thereby indicating that prion proteins were in fact encapsulated within exosomes (Fevrier et al. 2004). Having identified prion proteins were in exosomes, further assays were performed to access whether the intercellular communication via exosomes that contain prion proteins was able to infect naïve cells. The comparison was performed by assessing the infectivity between control exosomes containing normal endogenous proteins against exosomes with prion protein isoforms. The observed results demonstrated that exosomes containing prion proteins were able to transmit the protein to naïve cells, as the concentration of prion proteins in cell lysates increased significantly (Fevrier et al. 2004). Meanwhile, cells treated with exosomes containing endogenous proteins showed no signs of misfolded forms of the protein (Fevrier et al. 2004). In a similar fashion, exosomes extracted from infected cells were administered into healthy mice. Following an incubation period, brain tissue analysis of the newly infected mice presented with protein aggregates away from the initial site of administration (Vella et al. 2007). Together, these findings demonstrate the ability for exosomes to assist in the propagation of prion diseases.

Parkinson's Disease

Parkinson's disease (PD) is the fastest growing neurodegenerative disease in the world, affecting approximately 1% of the world's population over the age of 65 (Pagano et al. 2016). Patients affected by PD typically present with a variety of symptoms, including but not limited to the slowing of movement (bradykinesia), tremors and speech difficulties (Politis et al. 2010). These symptoms occur due to the gradual destruction of dopaminergic neurons within the substantia nigra of the brain. The loss of these essential neurons results in a deficiency of dopamine within the central nervous system (CNS), a neurotransmitter essential for smooth muscle control (Politis et al. 2010). As the neurodegeneration progresses, the symptoms eventually extend into inability to move then death. While these are the symptoms

which patients present with, this is the culmination of decades of internal mechanisms that result in the accumulation of misfolded proteins that prevent normal cellular homeostasis. In fact, as we have come to understand more about the mechanisms behind the development of PD, symptoms such as loss of smell (anosmia) and constipation are becoming recognized as prodromal factors in the disease process (Pellicano et al. 2007). The loss of smell and bowel issues are indicative of the involvement of the olfactory bulb and the gastrointestinal tract, which is supported by the resistance of these areas to anti-parkinsonian treatments, suggesting a higher degree of damage.

Lewy bodies are currently considered as the hallmark pathology of PD. These bodies are aggregates comprised of a variety of proteins, with α-synuclein being the primary constituent (Spillantini et al. 1997). Lewy bodies appear in almost all cases of PD, and it is for this reason that α-synuclein is the key protein implicated in the pathogenesis of PD. Alpha-synuclein itself is a natively found protein within the human body and has been described to be associated with synapse plasticity, neurotransmitter release, vesicle trafficking and even in directing immune responses within the CNS and the peripheral nervous system (PNS) (Lashuel et al. 2013). However, α-synuclein is vulnerable to misfolding in a similar manner as the previously described prion proteins (Collier et al. 2016). The misfolding of α-synuclein also results in a structural shift towards a primarily β-sheet structure, making the protein toxic and resistant to degradation by the UPS and ALP systems (Ciechanover and Kwon 2015). Furthermore, current literature supports the premise that α-synuclein also replicates and propagates with the use of the prion template dependent manner, including transmission through exosomes (Goedert et al. 2010). However, despite the mechanical similarities between PD and prion diseases, no infectivity between humans or animals has been observed for α-synuclein. For this reason, the propagation of α-synuclein is labelled as 'prion-like' or more recently 'prionoid' (Hafner Bratkovič 2017).

The prion-like propagation for PD was proposed by Heiko Braak and his team in 2003, when they detected that Lewy body pathology originated within the olfactory bulb and the brain stem (Braak et al. 2003). From these locations, it was proposed that Lewy body pathology spreads throughout the CNS in a caudo-rostral direction, eventually reaching the midbrain where majority of the damage in PD patients occurs (Braak et al. 2003). This transmission of Lewy body pathology was later observed in patients who received grafted mesencephalic neurons as part of an experimental cell replacement treatment for PD (Kordower et al. 2008). Whilst these studies were unable to establish a clear clinical benefit to patients, many individuals who received the treatment showed short-term improvement (Kordower et al. 2008). However, the most important observation from these studies came years later, as post-mortem analysis of the grafted brain tissue from patients who underwent this treatment demonstrated the presence of the hallmark Lewy bodies (Kordower et al. 2008). This pathology was present within grafted neurons even though the cells were too young to have developed Lewy bodies themselves (Kordower et al. 2008). Significantly, this study also highlighted that misfolded α-synuclein was contained in Lewy bodies found in the grafted neurons, indicating a

possible transmission of the misfolded protein. While this observation did not prove the ability of α-synuclein to propagate between cells, it did provide substantial evidence towards the hypothesis that the protein could be transmitted between cells in the brain.

Since this discovery, both unstructured and aggregated forms of α-synuclein have been detected within many human fluids, including blood, plasma, cerebrospinal fluid (CSF) and even saliva (Lee et al. 2005). Furthermore, the release of α-synuclein has been reported to occur through EV biogenesis pathways, such as the ESCRT dependent and ESCRT-independent, and through EV-independent pathways, which have no interactions with the endoplasmic reticulum and Golgi apparatus (Lee et al. 2005). However, the precise mechanisms behind the release of non-vesicle associated α-synuclein and the implicated physiological roles are still widely unknown.

Alpha-synuclein secreted via EVs is believed to occur due to the build-up of misfolded α-synuclein within cells, followed by the inability of the UPS and ALP systems to degrade the toxic proteins. This is further supported by studies which demonstrated increased secretion of misfolded α-synuclein within exosomes following the inhibition of the UPS/ ALP systems through genetic or pharmacological manipulation (Fussi et al. 2018). The same study assessing the inhibition of the ALP system also exposed cell lines to GW4869 used to inhibit exosome formation. GW4869 is a non-competitive inhibitor of neutral sphingomyelinase, a vital enzyme in the generation of ceramide which is required for the generation of exosomes. This approach resulted in a significant decrease in the secretion of α-synuclein contained within exosomes and exacerbated α-synuclein induced cell death (Fussi et al. 2018). The lipid environment of exosomes has also been shown experimentally to affect the aggregation kinetics of α-synuclein. Grey et al. found that the association of α-synuclein within exosomes can catalyze the misfolding process. This is primarily dependent on the lipid makeup of exosomes, with this study reporting that exosomes associated with phospholipids inhibited further aggregation of α-synuclein, while the presence of ganglioside lipids such as GM1 and GM3 accelerated the process (Grey et al. 2011).

The association of pathogenic α-synuclein with EVs provides several avenues which theoretically assist with the propagation of PD pathology. Through being encapsulated within EVs, the misfolding kinetics of α-synuclein are accelerated, pathogenic α-synuclein is protected from degradation by extracellular factors and pathogenic α-synuclein is more readily taken-up and over longer distances by naïve cells. Together, these factors demonstrate the significance of EVs in PD development. However, the involvement of EVs in the development of PD simultaneously means that EVs can be utilized in detecting and diagnosing PD, prior to the onset of motor symptoms. Current research is focusing on determining whether EVs derived from the blood or CSF of PD patients can be efficiently utilized as a biomarker. A study by Shi et al. in 2014 reported that they were able to detect EV-contained α-synuclein in PD patient plasma expressing the L1 adhesion molecule, which is specific to the CNS (Shi et al. 2014). Furthermore, it was also reported that EV associated α-synuclein was significantly higher in patients, compared to controls (Shi et al. 2014). Outside of CSF, blood and plasma, a recent study by Cao et al.

reported that pathogenic α-synuclein levels are significantly higher within the saliva of PD patients, when compared to healthy controls (Cao et al. 2019). While the results that are demonstrated in these studies are promising, much more research is required to develop assays, which are efficient and consistent at detecting biomarkers indicative of the eventual onset of PD.

Alzheimer's Disease

Alzheimer's disease (AD) is currently the most prevalent neurodegenerative disease in the world and is a major cause of dementia in the elderly population. The hallmark pathology of AD is the accumulation of extracellular amyloid-β (Aβ) plaques and the formation of neurofibrillary tangles caused by the abnormal phosphorylation of tau (Mandelkow and Mandelkow 1998; Thal et al. 2002). It is thought that plaques cause neuronal cell death through enhancing inflammation and oxidative stress. Both components in AD plaques, Aβ and tau, have been demonstrated through in vitro and in vivo experiments to associate with exosomes.

Natively, Aβ has several functions, including antimicrobial activity, tumour suppression and promoting recovery from brain injury (Brothers et al. 2018). The proteolytic cleavage of the amyloid precursor protein (APP), a type 1 cell-surface protein, is responsible for the production of Aβ (Rajendran et al. 2006). Despite APP being a protein generated within the cytosol and possessing a transmembrane domain, it was found to be associated with the early endosome system and internalised within exosomes (Rajendran et al. 2006). A study by Rajendran et al. discovered that the mechanical cleavage of APP occurs at the early endosome by β-secretase. This association with the early endosome eluded to the ability of Aβ to be internalised within exosomes, as the invagination of endosomes into multivesicular bodies (MVBs) results in the creation of intraluminal vesicles (re-designated as exosomes upon release into the extracellular space). This discovery first highlighted the ability of exosomes to assist in the propagation of AD pathology. Furthermore, proteases responsible for the further processing of APP are also found within exosomes, suggesting that other alterations to APP may occur within exosomes (Sharples et al. 2008).

Similarly to what is observed with α-synuclein in PD, cells of AD patients are thought to secrete Aβ oligomers to prevent build-up. In addition, an initial protective mechanism is observed following the secretion of Aβ oligomers from the cell. Some studies have proposed that the association of Aβ with exosomes derived from N2a neuroblastoma and BV-2 microglial cells can influence the isoform of Aβ into degradable non-toxic fibrils (Yuyama et al. 2012). Following this, exosomes containing the amyloid fibrils can be degraded through insulin-degrading enzymes. However, while the protective effect is observed initially, the system is thought to eventually be overwhelmed and instead promotes further propagation of Aβ oligomers throughout the CNS in a prionoid manner (Yuyama et al. 2012). A study published by Sinha et al. in 2018 assessed the ability of exosomes containing Aβ

oligomers to spread the pathology to nearby cells. This study supported the involvement of exosomes with AD pathology through analysis of post-mortem AD brains and through modulating exosomal secretion pathways in an AD model (Sardar Sinha et al. 2018). The post-mortem analysis of AD patient brains observed that exosomes contained significantly higher concentration of oligomeric Aβ when compared to controls (Sardar Sinha et al. 2018). A follow-up in vitro experiment was able to confirm the ability of exosomes to potentiate the spread of oligomeric Aβ. The application of exosomes derived from AD brains onto hiPSCs and dSH-SY5Y cells saw that the exosomes were readily taken up (Sardar Sinha et al. 2018). Furthermore, following an incubation period of 48 h the cell medium was assessed for the release of lactate dehydrogenase, demonstrating the levels of toxicity. The exposure of cells to exosomal Aβ resulted in significant toxicity when compared to controls (Sardar Sinha et al. 2018). Finally, this study also assessed whether inhibition of exosome formation would reduce or prevent the spread of exosomal Aβ. They accomplished this by knocking down several ESCRT complexes essential for the generation of exosomes. This inhibition in a transwell model utilising the same hiPSCs and dSH-SY5Y cells found that almost no oligomeric Aβ was transmitted (Sardar Sinha et al. 2018).

Tau is another protein which contributes to the neurodegeneration observed in AD. Physiologically, tau assists in cell signalling, synaptic plasticity and regulating genomic stability and stabilizes microtubule structure (Guo et al. 2017). However, the abnormal phosphorylation of tau has been demonstrated to cause the formation of neurofibrillary tangles, which lead to neuronal loss and cognitive decline (Götz et al. 2001). Natively, endogenous tau assists in the formation of microtubules, an important aspect of the cellular cytoskeleton for the functions of mitosis, cell motility, intracellular transport, and maintenance of cell shape (Kolarova et al. 2012). Tau has also been shown to propagate in a template-dependent manner, similar to α-synuclein pathology. Several studies have been able to demonstrate the ability for tau, aggregated and free form, to being secreted from the cellular environment into the extracellular space by exosomes. One study in particular by Asai et al. in 2015 made mention of the ability for microglia, the main phagocyte in the brain, to secrete exosomes containing pathogenic tau. The study speculated that microglia are attracted towards neurons containing toxic forms of tau and phagocytize them. However, following this internalization, microglia secrete tau within exosomes into the extracellular space thereby potentiating further spread (Asai et al. 2015). A later mouse experiment utilized GW4869 and found a significant decrease in tau propagation within the dentate gyrus but not within the entorhinal cortex, where phosphorylated tau pathology is thought to initiate (Asai et al. 2015). This observation perhaps suggested that the propagation of pathogenic tau is more dependent on exosomes within the dentate gyrus than within the entorhinal cortex. Ectosomes (another type of microvesicle), have also been shown to potentiate the spread of pathogenic tau. A study by Dujardin et al. in 2014 proposed the secretion of tau within EVs that is independent of exosomes. This study suggested that within AD pathophysiology, the secretion of tau is primarily dependent on ectosomes

(Dujardin et al. 2014). It is the over accumulation of phosphorylated tau which contributes to the shift in secretion towards the exosome-dependent system.

Similar to the proposed use of EVs in the early detection of PD, AD research is working towards developing reliable assays for the early detection of AD via EV analysis. Current methods for AD diagnosis include the assessment of the CSF for Aβ and hyperphosphorylated tau (Mattsson et al. 2009). However, this method is used to differentiate between AD and other dementias, after the symptoms have already been established. Current studies are working on refining the detection processes, in an attempt to detect the disease prior to major neurological damage. A study by Fiandaca et al. explored the accuracy of assessing for varying levels of Aβ1-42, P-T181-tau and P-S396-tau (phosphorylated variants of Aβ and tau) within the CSF of AD and frontotemporal dementia (FTD) patients. This study reported that they had a 96.4% and 87.5% success rate in identifying AD and FTD patients, respectively, based on the exosomal levels of Aβ1-42, P-T181-tau and P-S396-tau (Fiandaca et al. 2015). Following this success, the study suggested that assaying for a combination of biomarkers can potentially help predict the onset of AD by as much as 10 years (Fiandaca et al. 2015).

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in the world (Ferrara et al. 2018). This degenerative disorder primarily targets motor neurons located within the motor cortex, brainstem, and spinal cord. Progressive damage of these neurons results in reduced electrical signal transduction, leading to the inability to initiate and control voluntary movement. To date, 12 different pathogenic proteins have been tied to ALS. Of these, superoxide dismutase one (SOD1), RNA-binding protein (TDP-43) and fused in sarcoma (FUS) have been shown to be associated within exosomes (Ferrara et al. 2018). Within the cellular environment, SOD1 functions as an enzyme which converts superoxide molecules into hydrogen peroxide and dioxygen, effectively removing free radicals. TDP-43 assists in regulating RNA splicing, stability, and transport, and FUS is a nuclear DNA/RNA-binding protein that regulates gene expression (Ferrara et al. 2018).

Mutations in these proteins results in loss off function and their subsequent movement towards the cytosol, where the proteins have been observed to aggregate (Ferrara et al. 2018). Each of these proteins have been shown to self-replicate in the prion-like manner with the use of EVs. A study by Grad et al. in 2014 demonstrated this effect in vitro with SOD1. The study observed the ability of SOD1 fibrils to transfer between HEK293 cells by using exosomes, effectively seeding recipient cells (Grad et al. 2014). A later in vivo study by Iguchi et al. in 2016 assessed the propagation of TDP-43. This group reported that the release of TDP-43 by cells acted as a protective mechanism, using exosomes to prevent build-up within the cytoplasm (Iguchi et al. 2016). However, this release resulted in the propagation of pathogenic TDP-43 towards naïve recipient cells (Iguchi et al. 2016). Furthermore,

the role of exosomes in this process was supported by inhibiting EV release through the administration of GW4869, or by silencing RAB27A. The inhibition of exosome generation through these methods resulted in a faster build-up rate of cellular TDP in affected mice (Iguchi et al. 2016). Regarding FUS, a study by Kamelgarn et al. in 2016, assessed its involvement with exosomes. Different forms of mutated and pathogenic FUS exist, including $R_{521}G$ and $R_{495}X$ (Kamelgarn et al. 2016). Kamelgarn confirmed that both WT and mutant forms of FUS were found within exosomes, noting that there was a significantly higher concentration of $R_{495}X$ within exosomes of ALS cell culture medium. Further support for the involvement of EVs in the movement of each of these proteins was provided by Sproviero et al. in 2018. The study analysed the concentrations of SOD1, TDP-43 and FUS within microvesicles and exosomes of ALS patient plasma and healthy controls. This study found that each of SOD1, TDP-43 and FUS were at higher concentrations from ALS patient plasma when compared to controls (Sproviero et al. 2018). Sproviero et al. also aimed to characterise whether cells selectively preferred to release SOD1, TDP-43 and FUS within microvesicles or exosomes. Here, it was reported that SOD1 was generally enriched within exosomes when compared to microvesicles, while TDP-43 and FUS were more concentrated in microvesicles (Sproviero et al. 2018). It was proposed that cells preferentially secrete pathological proteins within microvesicles during disease, to accommodate for bulk clearance from the cellular environment (Sproviero et al. 2018). Meanwhile, exosomes are used to scavenge unfolded proteins.

As is evidence by these studies, EVs may play a role in the pathogenesis and progression of ALS in a similar manner as with AD and PD. However, more research is needed to understand the mechanisms underlying the aggregation and transmission of SOD1, TDP-43 and FUS.

Concluding Remarks

The role of EVs in neurological disease is a growing field with mounting evidence to now suggest that EVs play an important role in a number of disease states. Importantly, EVs have now been observed to play a role in disease pathogenesis, methods for diagnosis and also as a potential therapy (Fig. 16.1). A central field highlighting the function of EVs in the brain is neurodegenerative diseases, where they have been shown to have a role in the transmission of aggregation prone proteins within the brain. It should be noted however that the mechanisms behind the loading of neurodegenerative proteins into EVs has not been identified for any of the age-related diseases. Future work will aim to identify such pathways in an effort to understand and treat these diseases given our increasing aging population and the rising rates of neurodegeneration.

One emerging area is the role of EVs in developmental biology and the nervous system. Currently there is only limited research being conducted, but fields such as communication pathways between the mother and foetus, as well as into

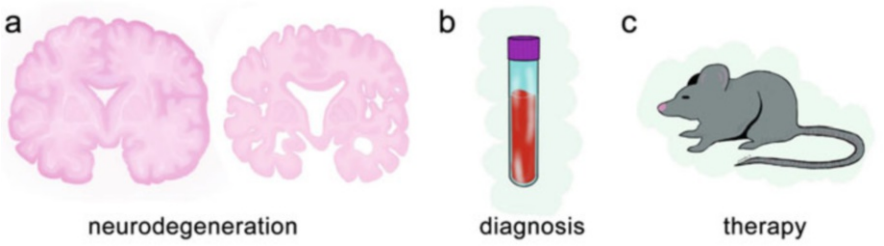

Fig. 16.1 The function of EVs in neurological disorders. (**a**) EVs have been shown to play a role in the transmission of aggregation prone proteins in the brain, resulting in neurodegeneration. Currently nearly all proteins linked to brain aggregates found in neurodegeneration have been discovered in EVs. (**b**) EVs have been used as a biomarker in multiple neurological disorders, central to this is the ability of EVs to cross the blood brain barrier, allowing for blood samples to be used for diagnostic means for events happening in the brain. (**c**) An emerging field is the use of EVs for the delivery of therapeutic agents. Current in vivo experiments for neurological disorders have been limited to animal models, but the increasing number of clinical trials in the EV field support the pathway for future neurological EV applications

neurodevelopmental disorders such as ASD, show that there is a rich line of EV biology to be understood. Future research will undoubtedly start to understand the role of EVs in brain development providing new pathways for therapeutic targeting.

Currently a highly important aspect of EVs in neurological disorders is their use as a biomarker. The ability of EVs to cross the blood brain barrier has allowed for neuronal specific studies on EVs from serum to be performed. The majority of these studies have focused on immunoprecipitation techniques using the L1 CAM epitope, it will be interesting to see in the future if other epitope markers will show different EV profiles given the large heterogeneity of EVs in the brain. It should be noted that a number of studies have shown differences in multiple different RNA profiles in EVs between control and disease states. Further research is required to understand what these differences mean in relation to the diseases or disorders being studied. Not all biomarkers have to have a direct association to the disease state, but it will be important moving forward if we want to understand disease mechanisms. Care should also be taken in that some studies do not have sufficient sample sizes to efficiently represent the populations being investigated, this may be due to the difficulty in obtaining samples for analysis, but should be remembered when interpreting findings.

An exciting research area for EVs is their use as a therapeutic in neurological disorders, initial animal studies have shown this potential. In particular, EVs derived from MSC cells appear show functional improvements in behavioural tests in animal models of ASD. With current developments of engineering EVs to target specific cell types or deliver cargos, it is clear that there will be a host of new research around the use of EVs to therapeutically target neurological diseases.

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Chapter 17
Emerging Roles of Extracellular Vesicles Derived Non-Coding RNAs in the Cardiovascular System

Ramasamy Subbiah, Divya Sridharan, Karthika Duairaj, K. Shanmugha Rajan, Mahmood Khan, and Venkata Naga Srikanth Garikipati

Abstract Cardiovascular disease is the leading cause of morbidity and mortality all over the world. Emerging evidence emphasize the importance of extracellular vesicles (EVs) in the cell to cell communication in the cardiovascular system which is majorly mediated through non-coding RNA cargo. Advancement in sequencing technologies revealed a major proportion of human genome is composed of non-coding RNAs viz., miRNAs, lncRNAs, tRNAs, snoRNAs, piRNAs and rRNAs. However, our understanding of the role of ncRNAs-containing EVs in cardiovascular health and disease is still in its infancy. This book chapter provides a comprehensive update on our understanding on the role of EVs derived ncRNAs in the cardiovascular pathophysiology and their therapeutic potential.

Keywords Extracellular Vesicles · Cardiovascular disease · miRNA · lncRNA · circular RNA

R. Subbiah · K. Duairaj
Cardiac Hypertrophy Laboratory, Department of Molecular Biology, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India

D. Sridharan · V. N. S. Garikipati (✉)
Department of Emergency Medicine, Dorothy M. Davis Heart Lung and Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH, USA
e-mail: venkata.garikipati@osumc.edu

K. S. Rajan
The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel

M. Khan
Department of Emergency Medicine, Dorothy M. Davis Heart Lung and Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH, USA

Department of Physiology and Cell Biology, The Ohio State University Wexner Medical Center, Columbus, OH, USA

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Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality all over the world. Over the years, although pharmacological treatments have improved survival in heart failure patients, prognosis in these patients, however, still remains poor (Thum 2012). Therefore, there is a burning need for future research on understanding novel mechanisms and for developing effective and innovative therapies for CVDs. In this perspective, using next generation sequencing technologies, growing evidence emphasize the importance of EVs in the cell-cell communication within the cardiovascular system, majorly mediated by their non-coding RNA (ncRNA) cargo (Cheng et al. 2014; Garikipati et al. 2015, 2017, 2018, 2019; Garikipati and Kishore 2018; Joladarashi et al. 2015; Kishore et al. 2016, 2020; Ramasamy et al. 2018; Yue et al. 2017b, 2020). EVs are biological entities, secreted by cells, with a lipid bilayer and constitutive expression of tetraspanins, heat shock proteins, Rab GTPases, flotillin, Alix, and TSG101 (Kowal et al. 2016; Yoshioka et al. 2013). Depending on their size, EVs can be largely classified into two groups: exosomes and microvesicles. Exosomes are released by multi-vesicular bodies (MVBs) through exocytosis. Their size ranges between 30–120 nm. On the other hand, microvesicles are formed by budding from the plasma membrane and range between 0.1 and 1 μm in size (Thery et al. 2018) (Fig. 17.1).

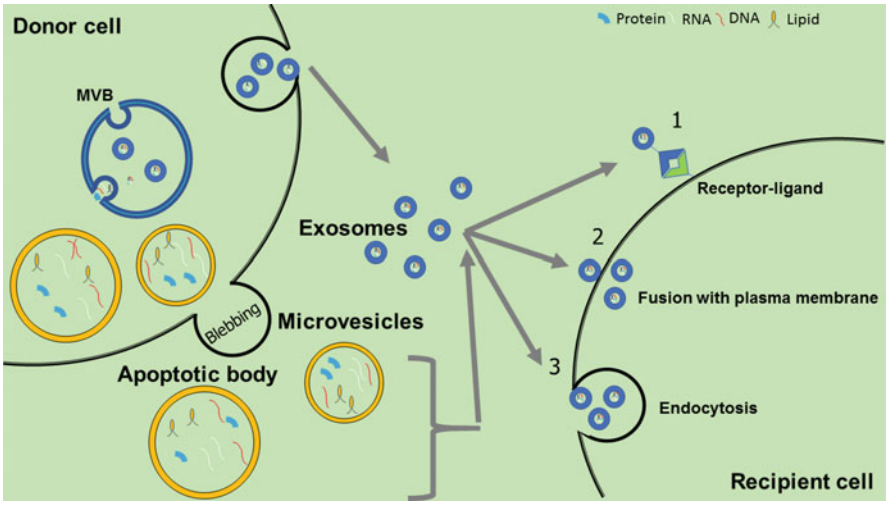

Fig. 17.1 Exosome biogenesis. Inward budding of membrane forms early endosome, which further accumulates as intraluminal vesicles comprising of bioactive molecules, such as DNA, RNA, lipids and proteins, within multi-vesicular bodies. Conversely, microvesicles are formed by the outward budding of the plasma membrane. Exosomes and/or microvesicles transfer their cargo to recipient cells by fusing with the plasma membrane or receptor ligand-mediated transfer or through endocytosis. *MVB* multi-vesicular bodies; *DNA* deoxyribose nucleic acid; *RNA* ribonucleic acid

The composition of EVs' cargo is heterogeneous and contains DNA, RNA, proteins and lipids. Moreover, EV cargo depends on the physiological or pathological state of a given source (Yue et al. 2017b, 2020). Over the last decade, has identified a treasure of non-coding (nc) RNA species in the EVs secreted by various cell types. These ncRNAs can be broadly characterized into small and long non-coding RNAs based on their size. NcRNAs < 200 nucleotides in size (miRNA, tRNA, snoRNA, piRNA, rRNA) are classified as small ncRNAs, while ncRNAs > 200 nucleotides (lncRNA, circular RNA) are classified as long ncRNAs. Of all the ncRNAs, miRNAs are the most thoroughly characterized exosomal RNAs having a functional effect on recipient cells. Sequencing of the RNA content from cardiovascular cells revealed the presence of tRNA, snoRNA, piRNA, rRNA and lncRNAs in their EVs (Yue et al. 2017a; Lee et al. 2017). Our understanding of EV cargo in terms of ncRNAs in the cardiovascular system is still in its infancy. This book chapter provides a comprehensive update on our understanding on the role of EVs derived ncRNAs including miRNA, lncRNA and circular RNA in the cardiovascular pathophysiology and their therapeutic potential (Table 17.1).

Role of EV Derived Small RNAs in the Cardiovascular System

MicroRNAs (miRNA) are small non-coding RNAs of 21–25 nucleotides. MiRNAs are transcribed to a hairpin-structured pri-miRNA and processed to pre-miRNA with 70 nucleotides. Further, in the cytoplasm DICER cleaves pre-miRNA into a mature miRNA. Mature miRNA regulates gene expression by mRNA degradation or repression of translation (Beermann et al. 2016). We and others have previously shown a critical role of miRNA in cardiovascular physiology and pathology (Ramasamy et al. 2018; Yue et al. 2017a; Garikipati et al. 2017; Joladarashi et al. 2015).

Role of EV Derived miRNAs in Cardiovascular Cells

The heart is a muscular pump and is made up of major cell types including myocytes, fibroblasts cells, endothelial cells (ECs), inflammatory cells and resident stem/progenitor cells(Gray et al. 2018). Recent evidence implicate the role of cross-talk between different cardiac cells via EVs in pathophysiological conditions. This section provides literature on the role ncRNA in the cargo of EVs derived from aforementioned cells and their functional relevance in the physiological/pathological conditions.

Cardiomyocyte Derived EV-miRNA Cardiomyocytes are important cells for contractile force generation in the heart (Bergmann et al. 2015). Upon an ischemic event,

Table 17.1 Cardiovascular cells and their EV associated ncRNA

EV-donor	ncRNA	EV-recipient cell/animal model	Function	References
Cardiomyocytes (healthy and Goto-Kakizaki diabetic)	miR-320	Endothelial cells	Vascular dysfunction	Wang et al. (2014)
Cardiomyocytes	miR-145	Fibroblasts	Enhanced fibrosis	Morelli et al. (2019)
Diabetic cardiomyocytes	miR-29 and miR-455	NA	Increased fibrosis	Chaturvedi et al. (2015)
Ischemic cardiomyocytes	miR-1, miR-208, miR-499	Bone marrow cells	Migrate to sites of injury as a physiological response	Cheng et al. (2019)
Endothelial cells	miR-214	Endothelial cells	Enhanced endothelial cell function	van Balkom et al. (2013)
Endothelial cells	146a	Endothelial cells and cardiomyocytes	Anti-angiogenesis and impair cardiac function	Halkein et al. (2013)
Endothelial progenitor cells	miR-375	Endothelial	Endothelial cell survival	Yue et al. (2017b)
Fibroblasts	miR-21	Cardiomyocyte	Cardiomyocyte hypertrophy	Bang et al. (2014)
Macrophages	miR-155	Cardiac fibroblasts	Fibrosis and inflammation	Wang et al. (2017)
Rat MSC	miR-19	MI	Reduction in infarct size and improved cardiac function	Yu et al. (2015)
Mouse MSC	miR-223	MI	Anti-apoptotic and anti-inflammatory	Feng et al. (2014)
Mouse CPC	miR-451	MI	Suppression of apoptosis	Vrijsen et al. (2016)
Human CDC	miR-146a	MI	Inhibit cardiomyocyte apoptosis, enhance neovascularization, cardiomyocyte proliferation	Ibrahim et al. (2014)
Human CDC	miR-181b	I/R injury	Cardio protection	de Couto et al. (2017)
Human CPC	Cluster of miRNAs	MI	Improved cardiac function	Agarwal et al. (2016)

(continued)

Table 17.1 (continued)

EV-donor	ncRNA	EV-recipient cell/animal model	Function	References
Human CPC	miR-210, miR-132, and miR-146a-3p	MI	Reduced apoptosis, enhanced angiogenesis and improved cardiac function	Vrijsen et al. (2016)
ESC	miR-294	MI	Improved cardiac function. Enhanced angiogenesis and cardiomyocyte survival and stimulation of the survival and proliferation of CPCs	Khan et al. (2015)
iPSC	miR-21 and miR 210	MI	Cardiomyocyte survival	Wang et al. (2015)
CDC	YRNA-YF-1	Cardiac I/R injury mouse model	Enhanced IL-10 secretion and improved cardiac function	Cambier et al. (2017)
ESC/iPSC	lnCRNA	MI	Cardio protective	Lee et al. (2017)
MSC	lncRNA-NEAT1	Cardiomyocytes	Inhibit cardiomyocyte apoptosis and improvement in cardiac function	Chen et al. (2020)
Cardiomyocytes	lncRNA-NEAT1	Fibroblasts	Pro-fibrotic phenotype	Kenneweg et al. (2019)
Cardiomyocytes	lncRNA-AK139128	Fibroblasts	Fibroblasts proliferation	Wang and Zhang (2020)
Cardiomyocyte	circRNA-HIPK3	Cardiac microvascular endothelial cells	Endothelial dysfunction	Wang et al. (2020)
Coronary artery disease patients plasma	hsa_circ_0005540	NA	Elevated and could be a potential biomarker	Wu et al. (2020)

ncRNA non-coding RNA; *ESC* embryonic stem cell; *iPSC* inducible pluripotent stem cell; *CPC* cardiac progenitor cell; *HSP* heat shock protein; *MSC* mesenchymal stem cell; *CDC* cardiosphere derived cell; *MI* myocardial infarction; *I/R* ischemia/reperfusion injury; *miR* microRNA; *lncRNA* long non-coding RNA; *circRNA* circular RNA; *NA* not applicable

huge cardiomyocytes loss leads to enlargement of existing cardiomyocytes as a compensatory mechanism. However prolonged enlargement of cardiomyocytes causes irreversible pathological hypertrophy and heart failure. A recent study demonstrated transfer of miR-145 from ischemic cardiomyocyte-derived exosomes isolated from mouse MI hearts to cardiac fibroblasts, thereby promoting

myofibroblast formation (Morelli et al. 2019). Wang et al. demonstrated that cardiomyocytes were subjected to hyperglycemia-induced endothelial cell dysfunction in vitro via transfer of exosomal miR-320/GF-1, Hsp20 and Ets2 (Wang et al. 2014) pathways. Additionally, diabetic mice subjected to exercise revealed elevated miR-29 and miR-455 levels in the cardiomyocytes targeting MMP9 to reduce cardiac fibrosis compared to sedentary control diabetic mice (Chaturvedi et al. 2015). A recent study demonstrated release of cardiomyocyte specific Exo-miRs (miR-1, miR-208, miR-499) induces mobilization of stem cells from the bone marrow as a physiological response after MI in mice (Cheng et al. 2019).

Endothelial Cells Derived EV-miRNA Endothelial cells line blood vessels and inside of the heart. Endothelial dysfunction is an important factor in predicting cardiac disease (He et al. 2017). Along these lines, a recent report showed that EC-derived exosomal-miR-214 promotes endothelial cell function via ataxia telangiectasia mutated protein (van Balkom et al. 2013). Another report identified miR-146a as a novel mediator of EC-derived exosomes in post-partum cardiomyopathy. In this study, authors have shown that EC-derived exosomal miR-146a impairs cardiomyocyte metabolism by targeting erb-b2 receptor tyrosine kinase 4 (ERBB4)/NOTCH1/interleukin-1 receptor-associated kinase 1 (IRAK1) signaling axis and in vivo experiments using miR-146a antagomiRs revealed improved post-partum cardiomyopathy phenotype in mice. These results suggest modulation of miRs involved in disease progression could be a potential therapeutic strategy for treating post-partum cardiomyopathy.

Cardiac Fibroblasts Derived EV-miRNA A recent study demonstrated the induction of hypertrophy in cardiomyocytes by cardiac fibroblast-derived EV-miR-21. The study identified cardiac fibroblast-derived EV- miR-21* to be a key pathway in inducing hypertrophic response in cardiomyocytes via targeting sorbin and SH3 domain-containing protein 2 (SORBS2)/PDZ/LIM domain 5 (PDLIM5). Importantly, modulation of miR21* levels rescued hypertrophy of the hearts in a mouse model of Ang-II induced cardiac hypertrophy (Bang et al. 2014). These studies identified targeted deletion of miRs in fibroblasts as a novel therapeutic approach to treating cardiac hypertrophy.

Macrophage Derived EV-miRNA Macrophages are crucial mediators of inflammation in CVDs. To understand the cross-talk between macrophages and fibroblasts via macrophage-derived exosomes, Lyu et al. demonstrated that macrophage-derived exosomes after MI in mice show elevated miR-155 levels and induced fibroblast proliferation and inflammation by targeting Son of Sevenless 1 expression suppressor of cytokine signaling-1 expression respectively (Wang et al. 2017). These results highlight the understanding of novel mechanisms and for developing novel macrophage targeted therapeutic regimens for MI.

Pericardial Fluid-Derived EV-miRs It is well known that pericardial fluid (PF) harbors hormones crucial in regulating cardiac function (Watanabe et al. 2009). However, role of PF derived exosomes in cardiovascular health and disease is a new research avenue. In these lines, Cristina *et al.* demonstrated that PF exosomes from

aortic valve replacement (AVR) patients possess exosomes and upon PF-derived exosomes treatment to endothelial cells in vitro improved endothelial cell function via exosomal let-7b-5p. Interestingly, using a hind limb ischemia model authors have shown that intramuscular injection of PF-derived exosomes improved partial blood flow recovery and neovascularization via regulation of the expression of let-7b-5p target, transforming growth factor beta receptor-1 (TGFBR1) (Beltrami et al. 2017). Overall, these data strongly show that cardiovascular cells cross talk with each other through EVs, thus highlighting the importance of ncRNAs both in healthy and diseased hearts.

Stem Cell-Derived EV-miRs

Stem/progenitor cells demonstrated a great promise in cardiac repair/regeneration after an injury (Garikipati et al. 2015; Krishnamurthy et al. 2011; Joladarashi et al. 2015; Rajasingh et al. 2007, 2008; Cheng et al. 2010; Mohsin et al. 2012; Williams et al. 2013a, b; Hare et al. 2012; Bolli et al. 2011; Makkar et al. 2012; Smits et al. 2003; Quyyumi et al. 2017). Even though stem/progenitor cells in preclinical trials demonstrated huge success, their efficacy in clinical trials remained modest. This has mainly been attributed to low retention, the survival of transplanted stem cells in the hostile microenvironment of the diseased heart. Importantly co-morbidities such as aging, diabetes, hypertension etc. remain unsolved questions in the cell therapy research (Vrtovec et al. 2013; Garikipati et al. 2015; Krishnamurthy et al. 2011). As it is well established that stem cells majorly act through paracrine mechanisms, cell free products as EVs can circumvent the issues associated with cell therapy and a paradigm shift towards cell-free therapy for cardiac repair/regeneration (Khan et al. 2015; Cambier et al. 2017; Ibrahim et al. 2014; Agarwal et al. 2016; Mackie et al. 2012; Mathiyalagan et al. 2017; Sahoo et al. 2011).

Endothelial Progenitor Cell-Derived EV-miRs in Cardiac Repair

We and others have previously shown the role of EPCs in neovascularization, and ischemic tissue repair (Garikipati et al. 2015; Krishnamurthy et al. 2011; Joladarashi et al. 2015; Yue et al. 2017b) and increasing evidence suggest EPCs impart beneficial effects via paracrine signaling (Sahoo et al. 2011). A recent study demonstrated that human CD34-Exo- miR-126 augments neovascularization targeting SPRED1 after an ischemic injury (Mathiyalagan et al. 2017). Exosome content is mirrored by the genetic and or physiological state of the parent cell. Until recently it was not clear if stem cell-derived EVs under stress conditions were functional. In this perspective, we have shown utilizing an inflammatory stimulus model IL-10 KO mice that EPC

exosomes exhibited elevated miR-375 levels. Our subsequent experiments modulating miR-375 levels using antagomiRs inhibited endothelial cell dysfunction (Garikipati et al. 2019). These results suggest modulation of exosomal content may address concerns related to modest or no functional benefits of stem cell transplantation with patients with co-morbidities.

Mesenchymal Stem/Stromal Cell-Derived EV-miR in Cardiac Repair

Mesenchymal stem cells (MSC) are the most studied cell types in terms of paracrine mechanisms (Matsushita and Dzau 2017; Gnecchi et al. 2006; Heldman et al. 2014). Cardiac stem cells preconditioned with MSC-Exo improved neovascularization and cardiac function post-MI in the mice. Subsequent experiments related to MSC cargo demonstrated several miRs viz. miR-147, let-7i-3p, miR-362-3p and miR-503-5p involved in angiogenesis and cell proliferation (Zhang et al. 2016). Furthermore, a recent study demonstrated MSC exosomes derived miR-223 exhibit protective responses in a murine sepsis model. Interestingly MSC-exo-miR-223 reduced inflammation in macrophages and cardio myocyte survival via targeting semaphorin 3A and signal transducer and activator of transcription-3 (STAT-3), respectively.

Role of Cardiac Stem/Progenitor Cells Derived EV-miR in Cardiac Repair

Owing to their source, cardiac stem cells gained a lot of attention and exhibited immense potential in cardiovascular regenerative medicine. Recent reports demonstrated exosomes derived from cardio sphere derived cells or cardiac stem cells exhibited cardio protection. In these lines, Ibrahim et al. have shown that CDC-exosomes promote cardio myocyte proliferation and angiogenesis in a mouse model of MI via miR-146 and blockage of exosome secretion from these cells completely nullified the beneficial effects. The same group using a cardiac I/R model demonstrated that CDC-exo-miR-181b regulates inflammatory macrophage phenotype via PKCδ (de Couto et al. 2017) to improve cardiac function (de Couto et al. 2017). Whereas utilizing human CPC exosomes, Barlie et al. has shown that CPC-exosomes enriched with miR-210, miR-132, and miR-146a-3p led to improved angiogenesis and cardiac function after MI in mice (Barile et al. 2014). On the whole, both CDC and CPC-derived EVs improved cardiac tissue repair/regeneration.

Role of Pluripotent Stem Cells Derived EV-miRs in Cardiac Repair

Pluripotent cells are exciting cells to work on due to their ability to proliferate and differentiate to all the major cardiovascular cell types, especially into cardiomyocytes. Cardio-myocytes offers new possibilities for cardiac tissue repair. A recent study demonstrated iPSC exosome therapy after I/R injury in rats exhibited cardio protective role through exosome transfer of miR-21 and miR 210 targeting Nanog and HIF-1α, respectively (Wang et al. 2015). In similar lines, Adamiak et al. demonstrated mouse iPSC-EVs more superior in augmenting cardiac repair after myocardial I/R injury in mice via transfer of multiple cardio protective such as miR-145, let-7 family and miR-302a-5p, miR-290-295, miR-19b, miR-20a, miR-126-3p, miR-130a-3p, miR-210-3p and miR-17-92. All the aforementioned miRs in this study have been shown to have a role in self-renewal, pluripotency, cell proliferation, angiogenesis, apoptosis etc. These reports highlight the potential of iPSC-EVs for future clinical applications for the treatment of cardiovascular diseases. We have previously shown that ESC exosomes enriched with embryonal miRs including miR-294 enhances cardiac progenitor cells proliferation and cardio myocyte cell cycle re-entry thereby improving cardiac function after MI in mice (Khan et al. 2015). All the above studies point out to EVs derived from pluripotent stem cells do not show teratogenicity unlike their parental cells and circumvent the issues associated with adult stem cells offering new possibilities to understand these highly versatile cells (Yoshida and Yamanaka 2017).

Role of EV-Derived Y-RNA in the Cardiovascular System

Y RNAs are small noncoding RNA transcripts ranging from 83–112 nucleotides. In humans there are four Y RNAs hY1, hY3, hY4 and hY5 reported. They have polyuridine tail, lower stem domain, an upper stem and a loop domain (Hizir et al. 2017). The functional role of Y RNA is not well established. However, a recent report identified abundant YRNA fraction in cardio-sphere derived cells EVs and upon transplantation of CDC exosomes these YRNA fragments induced IL-10 expression mediated cardiac protection post-MI in rats (Cambier et al. 2017). This study highlights a novel role of YRNA as functional units of EV in cardiac repair post-MI.

Role of EV-Derived lncRNA in the Cardiovascular System

Long non-coding RNAs (lncRNA) are transcripts >200 nt generally does not code for functional proteins, with some exceptions. LncRNAs are categorized into five classes including (a) stand-alone RNAs, (b) antisense transcripts, (c) pseudogenes containing lncRNA; (d) long intronic ncRNAs, and (e) other lncRNAs containing divergent transcripts. Increasing evidence suggests that lncRNAs exert their function via interacting with proteins, miRNA or mRNA (Hobuss et al. 2019; Lorenzen and Thum 2016). A recent report identified macrophage inhibitory factor pretreated MSC exosomes via lncRNA-NEAT1 reduces cardiomyocyte apoptosis. Further authors have shown that MSC exosomes derived lncRNA-NEAT1 sequesters miR-142, there by regulating FOXO1 levels to protect cardiomyocyte apoptosis (Chen et al. 2020). Hypoxic cardiomyocytes derived EVs secrete Neat1 to induce a pro-fibrotic phenotype in recipient cardiac fibroblasts both in vitro and in vivo (Kenneweg et al. 2019). In similar lines, Wang et al. identified exosomal AK139128 derived from hypoxic CMs to modulate cardiac fibroblasts proliferation and apoptosis (Wang and Zhang 2020). Further, a recent study also proposed exosomes carrying lncRNAs in iPSC and ESC derived exosomes exhibited reparative abilities post myocardial ischemia in mice (Lee et al. 2017). Overall, these results highlight the importance of lncRNAs as novel regulators and can serve as potential therapeutic targets.

Role of EV-Derived circRNA in the Cardiovascular System

CircRNA are a class of lncRNAs >200 nt. circRNA are covalently circularized single strand RNA loops, where pre-mRNA splicing machinery back splices to join a downstream 5′ splice site to an upstream 3′ splice site. They can be transcribed from exonic, intronic, exon-intron, or intergenic DNA (reviewed in Kishore et al) (Kishore et al. 2020). Due to their circular nature, they exhibit more stability. As the field is still emerging to understand functional role of circRNAs, we and others have reported their regulatory role via interaction with proteins, miRNA and mRNA (Kishore et al. 2020; Garikipati et al. 2019; Santer et al. 2019).

A recent report by Wang et al. (2020) demonstrated cardiac microvascular endothelial cell dysfunction is mediated via transfer of exosomal circHIPK3 derived from cardio myocytes subjected to hypoxia stress. Subsequent experiments demonstrated exo-circHIPK3 acts as miR-29a sponge in regulating IGF-1 levels in cardiac microvascular endothelial cell. Further et al. reported differential expression (119 down-regulated and 66 up-regulated) of circRNA in the cardiac EVs after ischemia/reperfusion injury in mice (Ge et al. 2019). In these lines, circRNA expression profiling of plasma exosomes from coronary artery disease patients revealed elevated hsa_circ_0005540 compared to healthy controls (Wu et al. 2020). Overall, these studies warrant future studies on EVs derived circRNA role

in cardiac physiology/pathology and most importantly their potential role as biomarkers for cardiovascular disease diagnosis.

Conclusions

Although EV's exhibited immense potential in understanding complex inter cellular communication, future studies on generating cell specific genetic models to study de novo or induced EVs in cardiovascular health and disease may enrich our knowledge. Furthermore, challenges with EVs isolation in vivo and heterogeneity associated with EVs pose hurdles to identify which EV sub population is important/functional. In this scenario, future studies on improving isolation strategies are warranted (Lener et al. 2015; Adamiak et al. 2017). Intriguingly, a recent study on stoichiometric and quantitative analysis revealed a minimum of 100 exosomes are required to fuse and transfer their cargo in to the recipient cell highlighting an important point that, there needs to be a significant amount of EV and/or its cargo to have a functional relevance (Chevillet et al. 2014). Also, it is important to examine if one or more ncRNAs are abundant and critical for EV function? Additionally, comparison and integration of the growing number of ncRNA data sets from different sources in the cardiovascular system will help identify critical regulators of cardiovascular health and disease.

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Chapter 18
Extracellular Vesicles and Preeclampsia: Current Knowledge and Future Research Directions

Carlos Palma, Jessica Jellins, Andrew Lai, Alexis Salas, America Campos, Shayna Sharma, Gregory Duncombe, Jon Hyett, and Carlos Salomon

Abstract Preeclampsia (PE) is associated with long-term morbidity in mothers and lifelong morbidities for their children, ranging from cerebral palsy and cognitive delay in preterm infants, to hypertension, diabetes and obesity in adolescents and young adults. There are several processes that are critical for development of materno-fetal exchange, including establishing adequate perfusion of the placenta by maternal blood, and the formation of the placental villous vascular tree. Recent studies provide persuasive evidence that placenta-derived extracellular vesicles (EVs) represent a significant intercellular communication pathway, and that they may play an important role in placental and endothelial cell (both fetal and maternal) function. These functions are known to be altered in PE. EVs can carry and transport

C. Palma · A. Lai · A. Campos · S. Sharma
Faculty of Health Sciences, Exosome Biology Laboratory, Centre for Clinical Diagnostics, University of Queensland Centre for Clinical Research, Royal Brisbane and Women's Hospital, The University of Queensland, Brisbane, Australia

J. Jellins · J. Hyett
Royal Prince Alfred Hospital Sydney, University of Sydney, Sydney, NSW, Australia

A. Salas
Faculty of Biological Sciences, Department of Pharmacology, University of Concepcion, Concepción, Chile

G. Duncombe
Faculty of Health Sciences, Exosome Biology Laboratory, Centre for Clinical Diagnostics, University of Queensland Centre for Clinical Research, Royal Brisbane and Women's Hospital, The University of Queensland, Brisbane, Australia

Royal Brisbane and Women's Hospital, Brisbane, QLD, Australia

C. Salomon (✉)
Faculty of Health Sciences, Exosome Biology Laboratory, Centre for Clinical Diagnostics, University of Queensland Centre for Clinical Research, Royal Brisbane and Women's Hospital, The University of Queensland, Brisbane, Australia

Faculty of Biological Sciences, Department of Pharmacology, University of Concepcion, Concepción, Chile
e-mail: c.salomongallo@uq.edu.au; http://www.uqccr.uq.edu.au/

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a wide range of bioactive molescules that have potential to be used as biomarkers and therapeutic delivery tools for PE. EV content is often parent cell specific, thus providing an insight or "thumbprint" of the intracellular environment of the originating cell (e.g., human placenta). EV have been identified in plasma under both normal and pathological conditions, including PE. The concentration of EVs and their content in plasma has been reported to increase in association with disease severity and/or progression. Placenta-derived EVs have been identified in maternal plasma during normal pregnancy and PE pregnancies. They contain placenta-specific proteins and miRNAs and, as such, may be differentiated from maternally-derived EVs. The aim of this review, thus, is to describe the potential roles of EVs in preecmpatic pregnancies, focussing on EVs secreted from placental cells. The biogenesis, specificity of placental EVs, and methods used to characterise EVs in the context of PE pregnancies will be also discussed.

Keywords Pregnancy · Placentation · Preeclampsia · Exosomes · Extracellular vesicles

Introduction

A successful pregnancy requires involvement of a series of processes, commencing from fertilization to establishment of complex maternal and placental vascular connections with the fetus. Adequate placentation is one of the prerequisites for maintenance of a normal healthy pregnancy. Placentation involves migration and invasion of the extra villous trophoblast (EVT) cells into the walls of the uterine arteries. Here, they interact with the cells of the maternal circulatory immune system and mediate the remodelling of the uterine spiral arteries supplying the placenta. This process is then followed by deportation of aggregates containing transcriptive materials (Burton and Jauniaux 2015). Any deviation in these events may lead to pathological pregnancies, including those affected by preeclampsia (PE). Physical signs of PE are hypertension, proteinuria, renal insufficiency, haemolysis, reduced platelet count, and/or increased platelet activation (Nwanodi 2016). Prevalence of PE is variable between different countries, and is associated with high morbidity and mortality in affected mothers and children (Helou et al. 2016). Apart from the immediate risks and complications for mother and fetus, each additionally carries an elevated lifelong risk for neurological and cardiovascular disorders (Helou et al. 2016). A large body of evidence indicates that placental hypoxia and incomplete vascularization are important initial events in the onset of PE. Interestingly, hypoxia and oxidative stress can induce the secretion of extracellular vesicles (EVs) from placental cells. EVs are involved in cell-to-cell communication and can transport a wide range of bioactive molecules which can interact with target cells to modify their phenotype and cell function. To date, there is a paucity of data defining changes in the release, role and diagnostic utility of placenta-derived EVs in pregnancies complicated by preeclampsia. Placental EVs may engage in paracellular interactions

(i.e. local cell-to-cell communication between the cell constituents of the placenta and contiguous maternal tissues) and/or distal interactions (i.e. involving the release of placental EVs into biological fluids and their transport to a remote site of action).

Pathophysiology of PE

Preeclampsia is defined as new onset of hypertension after 20 weeks of gestation with fetal and/or, renal, hepatic, haematologic or neurological involvement. Pre-eclampsia is a serious complication of pregnancy affecting ~7.6% of pregnancies globally, and is associated with high morbidity and mortality in affected mothers and children (Helou et al. 2016). It is a lifelong disorder for both the mother and offspring, with increased risk of cardiovascular disease (McDonald et al. 2008), neonatal and child morbidity, mortality, and health risks continuing into adulthood (Helou et al. 2016). PE contributes significantly to the incidence of intrauterine fetal growth retardation (IUGR), and spontaneous and iatrogenic preterm birth. Nearly 5%–10% of women develop hypertension during pregnancy, and pregnancy-induced hypertension (PIH) is one of the most prevalent risk factors for PE (Pai et al. 2016).

Pre-eclampsia and other hypertensive diseases of pregnancy are associated with systemic endothelial dysfunction. These diseases occur de novo (that is, in patients who did not present with hypertensive symptoms before gestation), and they worsen during the course of pregnancy. The hypertension in PE is partnered with coexistent proteinuria. In the most severe cases of PE, pre-term delivery of the fetus is required due to maternal and/or fetal disease status (Bakhti and Vaiman 2011). Clinical risk factors for developing PE assessed before 16 weeks of gestation include nulliparity, advanced maternal age, prior history of PE, chronic hypertension, family history of PE, pre-pregnancy BMI > 30 kg/m^2, use of assisted reproductive technology, multiple gestation, race and ethnicity, and co-morbidities such as pregestational diabetes and autoimmune conditions (Poon et al. 2019). The present management of patients with PE depends on symptom severity and although there are drugs available to treat symptoms and prolong pregnancies in mild to moderate PE (McCoy and Baldwin 2009), delivery of the placenta is the only management that allows progressive resolution of the symptoms and signs of preeclampsia.

In PE, there is defective implantation and placentation due to inadequate trophoblast invasion and partial failure of uterine arterial remodelling. This leads to a high resistance and low capacitance vascular supply to the placenta and fetus (Gilani et al. 2016). Studies have shown that during a normal healthy pregnancy, there is loss of smooth muscle and the elastic lamina of the maternal uterine vessels. Dilation at the vessel mouth occurs and this facilitates the entrance of the trophoblast cells. When conversion of the maternal uterine vessels does not occur, there is retention of smooth muscle cells which contributes to an increased viscosity of maternal blood. Nonetheless, maternal blood enters into the intervillous space as a turbulent jet which increases the risk of spontaneous vasoconstriction and ischemia-reperfusion

injury, generating hypertension and oxidative stress in maternal circulation (Burton et al. 2009). Consequently, there is less blood and oxygen perfusion in the placenta with secondary effects to the fetus.

The underlying pathophysiological processes of PE includes evidence of uteroplacental insufficiency, uteroplacental underperfusion, chronic hypoxia, and altered placental histology, leading to abnormal ultrasound indices and biochemical markers in maternal blood. The placental pathology involves villous infarcts, spiral artery abnormalities, mural hypertrophy and fibrin depositions in the PE placentas (Friedman and Cleary 2014). The potential mechanisms inducing poor trophoblastic invasion in PE involve imbalance of angiogenic and antiangiogenic factors—vascular endothelial growth factor (VEGF), soluble endoglin, soluble fms- like tyrosine kinase-1 receptors (s Flt-1), and placental growth factor (PlGF). Abnormal production of these factors are closely associated with PE and intrauterine growth restriction (Friedman and Cleary 2014). In normal pregnancies, from about 8 to 17 weeks, trophoblasts invade the uterine spiral arteries, replacing the smooth muscle and endothelium. The remodelled arteries have increased blood flow and reduced resistance.

Following placentation, residual syncytiotrophoblastic material (derived from placental shedding and/or from placental microparticles), release vasoactive substances into the maternal circulation (Armant et al. 2006; Redman and Sargent 2009). These, in turn, affect the maternal endothelium throughout the body. When these processes fail, there is inadequate perfusion, placental damage and an imbalance of thromboxane-prostacyclin with consequent platelet aggregation (Cuckle et al. 2013), key factors involved in PE. Several proteomic studies have identified specific alterations of protein content in the mother's serum in PE (Blumenstein et al. 2009; Auer et al. 2010). Pro-inflammatory molecules such as C-reactive protein are increased in maternal serum, along with coagulation factors such as gamma fibrinogen, various SERPINs of the A and C classes (some are synthesized by the placenta) (Chelbi et al. 2007) and lipid transport molecules such as apolipoproteins.

Defective placentation may lead to focal regions of hypoxia that are responsible for modifying the production of growth factors, cytokines (Granger et al. 2002), lipid peroxides (Lyall and Myatt 2002), and prostaglandins by placental trophoblasts (Granger et al. 2002). Elevated placental levels of inflammatory cytokines, such as tumour necrosis factor-α, interleukin (IL)-1α, IL-1β, and IL-6, are generally considered unfavourable to pregnancy (Lockwood et al. 2008). Moreover, clinical studies have shown changes in the levels of cytokines and prostaglandins in women with PE (Benyo et al. 2001; Raghupathy 2013). Maternal circulatory neutrophils are activated in pregnancy and further stimulated in PE, acting as the source of oxidative stress by generating reactive oxygen species such as hydrogen peroxide and superoxide anion. These molecules cause damage to proteins, lipids and nucleic acids (Vaughan et al. 2006). Neutrophil activation is initiated in the intervillous space by increased secretion of lipid peroxides by the placenta, which is abnormally increased in PE. This stimulates phospholipase A2 and cyclooxygenase enzymes to increase the production of thromboxane. Thromboxane is implicated in monocyte activation

responses and plays a role in mediating TNF-α production by neutrophils in response to oxidative stress (Vaughan et al. 2006).

The balance between thromboxane A2 (TXA2) and prostaglandin I2 (PGI2) is altered in women with PE, and high levels of TXA2 metabolite have been detected in the circulation of these patients (Walsh 1985, 2004). TXA2 and PGI2, derivatives of arachidonic acid, are functional antagonists. TXA2 stimulates platelet activation and aggregation, vessel constriction, and proliferation and mitogenesis of vascular smooth muscle cells, whereas PGI2 is an inhibitor of platelet aggregation and a vasodilator (Reslan and Khalil 2010). The placental ischemia/hypoxia is caused by aberrant implantation, where PGI2 synthesis is downregulated and TXA2 synthesis is upregulated. The release of TXA2 from basal trophoblasts is increased in placentas affected by PE (Zhao et al. 2008) and high levels of TXA2 may play a role in placental cell apoptosis, which may contribute to maternal hypertension (Yusuf et al. 2001). Abnormally elevated TXA2 levels are also known to induce thrombosis (Ally and Horrobin 1980). Thus, elevated TXA2 level plays a significant role in the production of the major clinical symptoms of PIH and PE, such as hypertension, platelet aggregation, and reduced uteroplacental blood flow (Sellers and Stallone 2008; Gilbert et al. 2008). Platelet count is reduced in PE due to platelet activation and aggregation under the effect of elevated levels of TXA2 Synthase (Pai et al. 2016). In addition, there is an apparent imbalance between free circulating anti-angiogenic factors and pro-angiogenic factors in early-onset PE. This angiogenic imbalance has been proposed to be the cause of PE (Cuckle et al. 2013).

Furthermore, biochemical changes in the maternal circulatory system, including an increase in phosphodiesterase-5 (Downing et al. 2004), thromboxane synthase (Pai et al. 2016) and an elevated hCG level (Euser et al. 2016), are also characteristic of PE. There is rise in anti β_2 - glycoprotein I antibodies which are related to aberrant implantation (Di Simone et al. 2007). Current predictive biomarkers for PE include the use of placental biomarkers (PAPP-A, PLGF, s-FLT-1), Free HbF, Alpha 1 Macroglobulin, Uterine Artery Doppler Pulsatility Index (Anderson et al. 2015) and possibly low concentrations of placental protein 13 (PP 13) (Meiri et al. 2014). However, studies involving placental protein 13 levels are inconsistent, with other studies failing to demonstrate an association with PE (Seravalli et al. 2016). Furthermore, these markers are unable to identify women at risk of developing pre-eclampsia, and thus other markers have been proposed. These include measurements of total cell free DNA and the fetal fraction in maternal plasma at 11–13 and 20–24 weeks of gestation. However, these have not been found to be strongly predictive of PE (Rolnik et al. 2015). Thus, there is a clinical need for minimally invasive biomarkers to predict women that are at risk of developing preeclampsia, as well as an urgent requirement to unravel the mechanisms underlying the preeclamptic pathophysiology. Recent studies have proposed that small extracellular vesicles, released by the placenta, may be key to understanding these pathophysiological developments.

EVs Heterogeneity and Biogenesis

Extracellular vesicles (EV) is a broad term that encompasses a heterogeneous group of membrane bound vesicles, released from all living cells and found in biological fluids. There are multiple subtypes, and these can be classified based on their characteristics. A study of the transferrin receptor during reticulocyte maturation described these vesicles as structures of globular shape. This study used the term "exosome" to refer to a population of vesicles formed by invagination of the plasma membrane (Pan and Johnstone 1983; Pan et al. 1985; Johnstone et al. 1987). Formation of these vesicles begins with an inward budding of the cellular plasma membrane which results in the formation of early endosomes, which later mature to become late endosomes. Inward invaginations of the endosomal membrane leads to the formation of 30–100 nm vesicles, known as intraluminal vesicles (ILVs) (Colombo et al. 2014). During this process, several proteins and other nucleic acid species are packed into the ILVs. These endosomes containing ILVs are then termed multivesicular bodies (MVBs). These MVBs then fuse with the plasma membrane to release the ILVs, which once in circulation, are termed small extracellular vesicles or exosomes (Kowal et al. 2014). However, exosomes are not the only type of particles or EVs found in circulation. Microvesicles and apoptotic bodies are other types of EVs that are present in biological fluids. There are several differences between the different types of EVs, including size, where microvesicles are usually over 200 nm and apoptotic bodies are larger vesicles of up to 5000 nm. Microvesicles, also called microparticles or ectosomes, were previously described as products of direct fission and segments of the plasma membrane (Chargaff and West 1946; Colombo et al. 2014; Caruso and Poon 2018; Poutsiaka et al. 1985). Apoptotic bodies, on the other hand, are recognised as remnants of the programmed cell death process (Schwartzman and Cidlowski 1993; Jan and Chaudhry 2019).

The different populations of EVs can also be distinguished from each other in terms of biogenesis process, however, the obtainment of a "pure" fraction of EVs is unrealistic in practice due to an overlap in structural characteristics or functionality. To address this problem, the International Society of Extracellular Vesicles (ISEV), a collaborative group, has set recommendations for isolation and characterization of different EV subtypes, based on their physical and biochemical features. In addition to size, the identification of proteins on the vesicle membrane can also help distinguish between the different subpopulations. Tetraspanins and components of the ESCRT machinery are useful to separate small EVs, integrins and selectins allow the identification of microvesicles, and annexin V can indicate the presence of apoptotic bodies. Although cells release a mixture of these vesicles, EVs have received significant attention, as owing to their biogenesis, they are able to capture both membranous and cytosolic molecules. EVs are released under physiological conditions, however, studies have shown that their release is altered in pathological conditions such as PE (Salomon et al. 2017).

PE is associated with changes in the concentration and bioactivity of EVs in the maternal circulation. Interestingly, plasma and serum are the most commonly used

source for EVs, although they are also present in other biological fluids (Table 18.1). In addition, there are several methods used to isolate or enrich EVs from biological fluids and cell-conditioned media, including differential centrifugation and/or ultracentrifugation, which is the most commonly used isolation technique (Zarovni et al. 2015). However, there are several other techniques that have been described in literature, with several studies utilising these techniques to examine EVs obtained from biological fluids in the context of PE.

Profiling EVs in Biological Fluids

EVs concentration has been shown to be significantly higher in PE compared to normal pregnancies, across gestation (Salomon et al. 2017). Knight et al., established that the levels of EVs (called microparticles in the study) is higher in maternal venous plasma obtained from women with PE in the third trimester of pregnancy, compared to values observed in women with normal pregnancies (Knight et al. 1998). Interestingly, the levels of placental EVs found in the maternal circulation, also known as Syncytiotrophoblast-derived microvesicles/microparticles (STBM), were higher in PE compared to normal pregnancy.

Furthermore, STBM levels are higher in early-onset preeclampsia but not in normotensive intrauterine growth restriction cases. Late-onset preeclampsia and normotensive intrauterine growth restriction, however, showed no difference against normal controls (Goswami et al. 2006). Circulating EVs present in the maternal circulation may have originated from a varied range of sources, and therefore, it is possible to detect an endothelium-origin subpopulation of microparticles, by evaluating surface membrane markers such as $CD31^{+}/42b^{-}$. $CD31^{+}/42b^{-}$ corresponds to endothelial-derived microparticles, and it was seen that women with normotensive, preeclamptic and gestational hypertensive pregnancies in the third trimester had an increase in the concentration of this subpopulation in preeclampsia patients compared to control and gestational hypertension (González-Quintero et al. 2004). Endothelium-derived microparticles were evaluated in plasma samples from women with normal development of pregnancy and women who developed preeclampsia. The samples were collected after 36 weeks of gestation, and additionally, a second and third set of samples were collected 48 h postpartum and 1-week postpartum, respectively. Findings of this study indicated that at 38 weeks, there was an elevated number of endothelial-derived microparticles in the preeclampsia condition compared with control. The use of $CD31^{+}/42^{-}$, $CD105^{+}$, and $CD62E^{+}$ markers gave evidence of activation of the endothelium as well as the apoptotic state of it. The concentration of microparticles positive for CD31 (negative for CD42) and CD105 correlated with sFlt1:PlGF, which is a current high predictive index for the diagnosis and the prediction of preeclampsia.

Microparticles have also been isolated and evaluated in normotensive and preeclamptic plasma samples from women across gestation, and postpartum. Results showed a decrease at 12 weeks gestation, however, the levels returned to normal

Table 18.1 Preeclampsia studies using biological fluids as a source of EVs

Extracellular vesicles	Source of EVs	Isolation method	References
Syncytiotrophoblast microparticles	Maternal plasma and uterine plasma (third trimester)	Ultracentrifugation	Knight et al. (1998)
Microparticles	Plasma samples (third trimester)	Centrifugation	Vanwijk et al. (2002), Meziani et al. (2006), Biró et al. (2007), Lok et al. (2006, 2007, 2008a, 2012)
Endothelial-derived microparticles	Plasma (third trimester)	Ultrafiltration	González-Quintero et al. (2004)
Syncytiotrophoblast microparticles	Maternal plasma (early-onset pre-eclampsia <34 weeks, late-onset pre-eclampsia ≥34 weeks)	Ultracentrifugation	(Goswami et al. 2006)
Microparticles, monocytic MPs, erythrocyte MPs, placental MPs	Maternal plasma (across gestation and postpartum)	Centrifugation	Lok et al. (2008b)
Leukocyte-derived microparticles	Maternal plasma (third trimester)	Centrifugation	Lok et al. (2009)
Endothelial- and platelet-derived microparticles	Maternal plasma (second trimester)	Centrifugation	Salomon et al. (2009)
Microparticles	Maternal plasma (total blood was also used)	Centrifugation	Alijotas-Reig et al. (2012)
Syncytiotrophoblast microparticles	Maternal plasma (<34 weeks, ≥34 weeks, 36 weeks), human umbilical vein endothelial cells	Ultracentrifugation	Chen et al. (2012)
Platelet, endothelial, erythrocyte and leucocyte-derived microparticles	Maternal plasma (third trimester)	Centrifugation	Marques et al. (2012)
Endothelial-derived microparticles	Maternal plasma (48 h postpartum and 1-week postpartum)	Centrifugation	Petrozella et al. (2012)
Extracellular vesicles	Maternal plasma (third trimester)	Immunoisolation	Tan et al. (2014)
Leukocyte-derived microparticles	Maternal plasma (third trimester)	Centrifugation	Mikhailova et al. (2014)
Lympho-monocyte and platelet-derived microparticles	Maternal plasma (in the beginning and at the end of pregnancy)	Centrifugation	Boisramé-Helms et al. (2015)
Microparticles	Maternal plasma, human umbilical cord blood	Centrifugation	Campello et al. (2015)

(continued)

Table 18.1 (continued)

Extracellular vesicles	Source of EVs	Isolation method	References
Endothelial- and platelet-derived microparticles	Maternal plasma (third trimester)	Centrifugation	Salem et al. (2015), Sokolov et al. (2016)
Exosomes	Human umbilical cord blood (at delivery)	Centrifugation, ultrafiltration	Jia et al. (2015)
$CD63^+$ extracellular vesicles	Saliva and gingival crevicular fluid (third trimester)	Ultracentrifugation	Chaparro et al. (2016)
Apoptotic bodies, microvesicles and exosomes	Maternal plasma	Centrifugation and ultrafiltration	Sandrim et al. (2016)
Blood-borne microvesicles	Plasma (postmenopausal women)	Centrifugation	Miller et al. (2016)
Urinary extracellular vesicles	Maternal urine (at delivery)	Centrifugation or unprocessed	Gilani et al. (2017)
Exosomes	Maternal plasma (first, second and third trimester)	Centrifugation, ultracentrifugation, density gradient	Salomon et al. (2017)
Microparticles	Maternal plasma and serum (first trimester)	Centrifugation	Jadli et al. (2017)
Syncytiotrophoblast microvesicles and Syncytiotrophoblast exosomes	Maternal plasma (third trimester)	Centrifugation	Motta-Mejia et al. (2017)
Exosomes	Maternal plasma (third trimester) and human umbilical cord blood	Precipitation (commercial kit)	Chang et al. (2018)
Urinary exosomes	Maternal morning urine (third trimester)	Ultracentrifugation	Hu et al. (2018), Ellis et al. (2019)
Exosomes	Maternal plasma (early-onset pre-eclampsia <34 weeks, late-onset pre-eclampsia ≥34 weeks)	Centrifugation, ultrafiltration, precipitation	Pillay et al. (2019)
Syncytiotrophoblast-derived microvesicles and syncytiotrophoblast-derived exosomes	Placenta and maternal plasma (third trimester)	Centrifugation, ultracentrifugation, size exclusion and ultrafiltration	Gill et al. (2019)
Placenta-derived extracellular vesicles	Maternal plasma (third trimester)	Not mentioned	Han et al. (2019)
Total and Syncytiotrophoblast extracellular microvesicle	Maternal plasma (third trimester)	Ultrafiltration and ultracentrifugation	Levine et al. (2020)
Extracellular vesicles	Placenta explant (first and second trimester)	Centrifugation	O'Brien et al. (2017)

(continued)

Table 18.1 (continued)

Extracellular vesicles	Source of EVs	Isolation method	References
Nanovesicles	Placenta explant (first trimester and term)	Centrifugation	Tong et al. (2017a)
Placental macrovesicles and placental debris	Placenta explant (first trimester)	Centrifugation	Zhao et al. (2017)
Macro-, micro- and nano-extracellular vesicles	Placenta explant and maternal serum (first trimester)	Centrifugation and ultracentrifugation	Tong et al. (2017b)
Syncytiotrophoblast-derived extracellular vesicles	Placental perfusate (at delivery)	Centrifugation and ultracentrifugation	Sammar et al. (2018)
Placenta-derived extracellular vesicles	Placenta explant (first trimester)	Centrifugation and ultracentrifugation	Tang et al. (2020)
Extracellular vesicles	Mouse endothelial cells and human-derived human umbilical vein endothelial cells	Centrifugation	Kohli et al. (2016)
Microparticles	Trophoblast primary culture (at delivery)	2-steps centrifugation	Xu et al. (2017)
Exosomes	Placental mesenchymal stem cells culture conditioned media and peripheral blood	Ultracentrifugation	Motawi et al. (2018)
Exosomes	Plasma (third trimester) and HTR-8/SVneo cell culture conditioned media	Membrane affinity spin column method	Biró et al. (2019)
Exosomes	Human umbilical cord mesenchymal stem cells	Flow-cytometry	Moro et al. (2016), Xiong et al. (2018)
Small extracellular vesicles	HTR-8/SVneo cell line	Centrifugation, ultracentrifugation and size exclusion chromatography	Dutta et al. (2020)

when evaluated at 6 weeks postpartum. Placenta-derived microparticles increased in pregnancy and preeclampsia, and interestingly, there was a correlation between placental microparticles and systolic pressure. These variations in the different subpopulations can be evidence of systemic inflammation, however, it also exposed an intricate balance between different cell populations and their influence on the vascular system through microparticles (Lok et al. 2008b). Early- or late-onset PE are associated with different origins, but both are associated with placental dysfunction. Interestingly, in early-onset PE, STMB were significantly greater than the late-onset PE group and the control group (Chen et al. 2012). Using a commercial isolation kit to isolate small EVs, Pillay et al., identified that the early-onset subgroup had a greater concentration of vesicles than the late-onset PE group. A specific subpopulation of placental alkaline phosphatase (PLAP) positive EVs was

significantly higher in early onset-preeclampsia than controls, however, late-onset-preeclampsia exhibited a lower concentration of PLAP-associated vesicles compared to the controls (Pillay et al. 2019).

Leukocyte-derived microparticles were isolated from plasma samples from the third trimester of gestation. From this subpopulation, it is possible to evaluate subsets such as monocyte-, Cytotoxic T-cell-, and granulocyte- derived microparticles. It has been found that microparticles with a monocytic origin were raised in preeclamptic patients compared with normotensive pregnant women, whereas cytotoxic T-cell- and granulocyte-derived microparticles showed greater levels compared with non-pregnant women. These results are an indication that preeclampsia involves activation of the immune system, and that this activation can be observed in the subpopulations of microparticles circulating in the blood (Lok et al. 2009). $CD41^+$ and $CD31^+$ microparticles were used to differentiate platelet- and endothelial- origin subpopulation of microparticles, respectively. These microparticles were quantified in maternal plasma samples from pregnancies at the second trimester of gestation. However, a correlation between the abundance of these microparticles and the development of late pregnancy complications such as preeclampsia, intrauterine growth restriction, pregnancy-induced hypertension and small for gestational age infants (Salomon et al. 2009), was not established. In another study, analysis of maternal plasma at 33 weeks of gestation was performed in a group with severe preeclampsia, and an increase in the total number of microparticles present in plasma from preeclamptic patients compared to normotensive or non-pregnant women was identified. Further details evidenced an association between platelet count and microparticles with platelet origin in preeclampsia. Also, there was a positive correlation between the level of endothelial-derived microparticles and microparticles originating from platelets, neutrophils, leukocytes and lymphocytes. The findings in this study suggested endothelium activation in severe cases of preeclampsia (Marques et al. 2012).

A panel of 10 leukocytic markers was evaluated in microparticles isolated from non-pregnant women, third-trimester healthy pregnant women and women suffering from preeclampsia. The microparticles expressing the aforementioned markers showed greater counts in healthy pregnancies compared to non-pregnant women, however, healthy pregnancies exhibited no differences compared to preeclampsia cases. The preeclamptic women presented with fewer NK cells-derived $CD45^+CD16^+CD56^+$ particles. Conversely, other leukocyte phenotypes, $CD45^+CD16^-CD56^+$ and $CD45^+CD16^+CD56^-$, were found to be higher compared with normotensive patients. The complete profile found in this study proved a neutrophilic and monocytic activation but also demonstrated key changes in NK cell population, which can be related to an inability to maintain tolerance, observed in preeclampsia (Mikhailova et al. 2014). Total microparticles (Annexin V^+), activated platelet-derived (P-$Selectin^+$), leukocyte-derived microparticles, and Tissue factor-bearing microparticles were greater in preeclamptic pregnancies compared to normotensive women. Platelet-derived microparticles ($CD61^+$) levels were lower in preeclampsia than in healthy women and no difference was found in endothelial-derived microparticle levels between the two groups. The same subpopulations were

analysed in umbilical cord blood obtained at delivery. A comparison between umbilical cord blood obtained from normal pregnancies and preeclampsia patients showed that microparticles levels are higher compared to maternal blood from preeclamptic patients. Additionally, the phospholipid-dependent procoagulant activity of the microparticles was measured in the blood samples. It was found that they present shorter coagulation time compared to controls. These discoveries establish the existence of a hypercoagulable and pro-inflammatory environment in patients with preeclampsia (Campello et al. 2015).

Interestingly, women with a history of preeclampsia expressed a different range of coronary artery calcification (CAC), and two risk factors for metabolic syndrome evaluated in this study showed elevated levels. Also, it was found that CD117 positive microparticles differed between normal pregnancies and preeclampsia groups. Furthermore, there is an association between CAC in preeclampsia and microparticles expressing tissue factor, ICAM-1, stem cells, and adipocytes (P16-set) antigens (Miller et al. 2016).

Finally, analysis of PLAP associated with EVs in gingival crevicular fluid from preeclamptic patients showed a higher number of $CD63^+$ EVs than the control group. In addition, PLAP/$CD63^+$ ratio as a measure of the percentage of vesicles originating from the placenta compared to the total CD63+ve vesicles significantly increased in gingival crevicular fluid from the same patients. This study gave evidence that non-invasive sample collection techniques are useful due to the significantly higher expression of placental and angiogenic factors in patients with PE, and showed promising results as an early predictor of the disease (Chaparro et al. 2016).

The EV field has grown exponentially in the last 15 years, mainly due to the capacity of EVs to carry bioactive molecules such as proteins and miRNAs that can be used as biomarkers for a wide range of diseases, and due to the capacity of EVs too deliver these molecules to target cells to induce changes in the cell function. Thus, we next look at studies that have examined the content of EVs.

Content of EVs from PE Compared to Normal

EVs can express different molecules on their membrane including proteins involved in multiple signalling pathways. Non-pregnant and normal pregnant women present with C-reactive protein bound to the membrane of microparticles, and this has been associated with an activation of the classical complement pathway. Preeclamptic microparticles have been shown to express serum amyloid P component and IgG, contributing to the complement component C1q binding, however, the activation of the classical pathway was unclear. Furthermore, the levels of microparticles binding C-reactive protein are higher in preeclampsia although it does not correlate with an increase in activation. It is known that preeclampsia exhibits generalized vascular dysfunction, however, even though microparticles expressing CRP are increased; the

mechanism to support an association with complement activation through the classical pathway (Biró et al. 2007), is yet to be identified.

In PE, there is platelet activation which is mirrored by high levels of P-selectin expressed in platelets, but also in its soluble form. It has been proposed that one fraction of this soluble form corresponds to platelet-derived microparticles associated with P-selectin.Flow-cytometric analysis of second-trimester samples has shown that a small fraction of P-selectin is associated with microparticles. Furthermore, this fraction is increased in preeclamptic women, and an increase was observed in normal pregnancies compared to non-pregnant levels. Additionally, this study found that P-selectin microparticles also expressed CD61, which is proof of platelet activation. These findings suggest that preeclamptic patients present an altered endothelium, where the P-selectin microparticles interact and contribute to leukocyte recruitment, leading to the development of inflammation, and activation of the coagulation process. (Lok et al. 2007). Another factor that has been detected in higher concentrations in preeclamptic patients is Fms Related Receptor Tyrosine Kinase 1 (Flt-1), which was measured in circulation and associated with microparticles. It was noted that 5% of Flt-1 was microparticles-associated and that this fraction was elevated in preeclamptic women compared to normotensive patients. The microparticles associated with full-length Flt-1 were related to platelet and placental origin. The presence of this non-cell bound form of Flt-1 may explain changes in bioactivity and the participation in the development of preeclampsia by modifying the interaction with other molecules in the vascular system (Lok et al. 2008a).

Furthermore, based on studies which proposed that circulating vesicles could be differentiated by their membrane phospholipid composition, specifically GM1 gangliosides and phosphatidylserines, plasma membrane vesicles were separated by their affinity with cholera toxin B chain (CTB), which binds GM1 ganglioside, and annexin V (AV), which binds phosphatidylserines. After isolation, 15 different proposed biomarkers for preeclampsia were evaluated. Plasma membrane vesicles isolated using CTB presented with higher levels of CD105, interleukin-6, placental growth factor, tissue inhibitor of metallopeptidase 1, and atrial natriuretic peptide. Considering both CTB and AV, preeclamptic women showed higher levels of plasminogen activator inhibitor-1, pro-calcitonin, S100b, tumour growth factor β, vascular endothelial growth factor receptor 1, brain natriuretic peptide, and placental growth factor. Interestingly, proteomic analysis of the content revealed that combining CTB- and AV-binding vesicles, there were 24 proteins expressed exclusively in preeclampsia cases, and 67 found only in healthy pregnant women. The existence of specific content in samples with the condition suggested that these different molecules can be considered as biomarkers and as potential diagnostic tools (Tan et al. 2014).

Another study looked at small extracellular vesicles isolated at delivery from umbilical cord blood samples. There was an alteration in the protein profile of vesicles that were obtained from patients with preeclampsia. 221 proteins were found to be expressed in these small vesicles. The profile showed an upregulation of 14 proteins and downregulation of 15 other proteins in preeclampsia compared to

control. Interestingly, proteins which showed variation between control and condition are part of the complement and coagulation cascades (Jia et al. 2015). In an another study, EVs were isolated from urine samples from normotensive and preeclamptic women at delivery. A higher ratio of podocin/nephrin positive urinary extracellular vesicles was observed in preeclamptic women. This expression seems to be associated with podocyte injury which is possibly related to the exposure of the cells to cell-free fetal hemoglobin (Gilani et al. 2017).

First-trimester microparticles were analysed after isolation from maternal serum samples. This study determined that preeclamptic patients presented with higher levels of serum copeptin, and annexin V than controls, whereas placental growth factor levels were lower. This result suggests that a set of serum markers combined with plasma microparticles can be used as a predictor and diagnostic tool for preeclampsia and for other pathological conditions (Jadli et al. 2017). Interestingly, lower levels of endothelial nitric oxide synthase (eNOS) in vesicles coming from preeclamptic women compared to normal pregnancy were noted, and this might contribute to the decreased NO bioavailability observed in PE pregnancies (Motta-Mejia et al. 2017).

Urinary EV proteins were analysed in samples before 20 weeks of gestation and phosphorylation of the Na-K-Cl cotransporter was noted. This implied an increase in activity which would enhance sodium reabsorption in preeclamptic cases compared to normal pregnancies. In preeclampsia, decreased phosphorylation of the sodium-chloride transporter was also noted, compared to normal pregnancies. The Epithelial sodium channel, on the other hand, expressed larger forms of the subunit α. These results may indicate higher sodium reabsorption in preeclampsia and may give evidence to associate it with hypertension (Hu et al. 2018). Another study examined early morning urine samples from women between 30 and 33 weeks of gestation. The main findings of this study showed that urine exosomes have a higher expression of 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 2 in preeclamptic women compared to normal pregnancies, as well as the phosphorylation at Ser483. The phosphorylation of residues Ser466/Ser461 present in isoforms 2/3 was increased in preeclamptic cases. Isoform 3 was the most common isoform expressed in preeclampsia. Moreover, isoform 4 was also found to be increased in preeclampsia. The differences found in these regulatory proteins propose the existence of renal glycolysis in preeclampsia (Ellis et al. 2019). Evaluation of plasma samples using a BeWo cell model was performed. Ultrafiltration and ultracentrifugation were used for the isolation of total and syncytiotrophoblast extracellular microvesicles. Total plasma extracellular vesicles showed no difference between normal and preeclamptic patients. However, lower expression of syncytin-1 was noted in samples from patients with preeclampsia (Levine et al. 2020).

Another study examined placental syncytiotrophoblast-derived microvesicles and placental syncytiotrophoblast-derived EVs, which were isolated from samples at 34–40 weeks (placenta) and at 35–36 weeks (plasma). Comparison of normal and preeclamptic cases revealed that placental microvesicles and exosomes from preeclamptic patients expressed a higher level of Neprilysin (membrane-bound metalloprotease) which is active and inhibited by thiorphan. It was also found that

microvesicles from plasma showed higher expression of neprilysin compared to samples from normal pregnancy, although exosomes from preeclamptic women presented greater neprilysin activity. It is known that excessive neprilysin activity can lead to hypertension, heart failure and amyloid deposition, all features of preeclampsia (Gill et al. 2019). Therefore, these studies have established that proteins associated with EVs have a significant role in preeclampsia, however, there are several questions that remain unanswered. Thus, the field has expanded to include the analysis of other vesicle content, including miRNAs. Several studies have examined the miRNA profile of EVs, including a recent study that examined the miRNA content in the different populations of EVs.

Apoptotic bodies, microvesicles and exosomes were isolated from maternal plasma of normotensive and preeclamptic patients. miR-376c-3p, miR-19a-3p, and miR-19b-3p were identified as being downregulated, whereas miR-885-5p was upregulated in preeclampsia,. Furthermore, expression of miR-885-5p was shown to be associated with exosomes (Sandrim et al. 2016). Another study used a retrospective stratified study design to quantify the levels of miRNAs across PE pregnancies, and over 300 microRNAs were identified as being significantly differentially expressed in PE compared to normal pregnancies in small EVs across gestation. Hsa-miR-486-1-5p and hsa-miR-486-2-5p were identified as the major contributors to the differences (Salomon et al. 2017). Interestingly, EVs isolated from umbilical cord mesenchymal stem cells (UCMSCs) from PE are enriched with miRNAs 136, 494 and 495 compared with EVs from UCMSCs from normal pregnancies (Motawi et al. 2018). Hypoxia (i.e. deprived of adequate oxygen supply) is a common characteristic of PE placentae. Interestingly, higher levels of hsa-miR-210 have been observed in placental cell lines exposed to hypoxia, and preeclamptic placentas presented high levels of hsa-miR-210 (Biró et al. 2019).

Thus, specific changes in the content of EVs in PE has been observed in many studies, suggesting that circulating EVs might participate in the onset and development of PE through the delivery of specific molecules to target cells. In the next section, we will describe the effect of EVs from PE pregnancies on target cells.

Bioactivity of EVs Compared to Normal

The bioactivity of STBMs has been tested in endothelial cell culture and early findings showed that STBMs from preeclamptic patients are able to inhibit endothelial cell proliferation and induce endothelial dysfunction, which is a characteristic of PE (Knight et al. 1998). A wire myography system showed that total microparticles from PE women can also abolish the relaxation of isolated myometrial arteries produced by bradykinin, causing endothelial dysfunction (Vanwijk et al. 2002). Interestingly, further evidence in this study showed that microparticles from healthy patients as well as incubation with the whole plasma did not have the same effect. This study evidenced the existence of plasma constituents with a protective role, which is lost in pathophysiological conditions (Vanwijk et al. 2002).

PE plasma contains an increasing number of endothelial-derived microparticles compared to control and gestational hypertension. Intriguingly, in vitro assays using renal microvascular endothelial cells have given proof that plasma from preeclamptic women can induce an increase in $CD31^+$ and $CD62E^+$ endothelial microparticle levels (González-Quintero et al. 2004). Incubation of human umbilical vein endothelial cells with total microparticles from non-pregnant, normotensive and preeclamptic pregnancies induced changes in the RNA expression of inflammation-related genes, whereas genes encoding adhesion receptors were not affected (Lok et al. 2006). Preeclamptic microparticles induced vascular hyporeactivity to serotonin in human omental arteries and in ex vivo study using mouse aorta (Meziani et al. 2006). Microparticles from preeclamptic cases also abolished the serotonin-induced contraction inhibited by COX-2. Preeclampsia induced upregulation of inducible nitric oxide synthase (iNOS) and COX2 expression (Meziani et al. 2006).

The bioactivity of microparticles isolated from samples from the third trimester was tested in co-culture with human umbilical vein endothelial cells or acute monocytic leukaemia derived cell line (MM6). In this study, it was established that microparticles from preeclamptic women could induce the expression of ICAM-1 in both cases. Additionally, this induction was also observed during incubation with microparticles from normal pregnancies, however, this occurred at a lower level (Lok et al. 2012). Early and late-onset preeclampsia STBMs presented higher levels of active caspase-3, and therefore led to apoptosis, compared to controls. Moreover, these microparticles inhibited HUVEC proliferation. This evidence proposes the existence of different mechanisms underlying the pathogenesis (Chen et al. 2012). In addition, preeclamptic microparticles induced hyporeactivity in omental arteries when treated with phenylephrine and serotonin, regardless of the concentration of microparticles. N-(3-(Aminomethyl)benzyl)acetamidine restored the contraction, supporting the participation of iNOS in the process. Microparticles from preeclamptic women also induced the production of vasoconstricting molecules by inducing the expression of cyclooxygenase-2. It was possible to establish an increase of NF-κβ expression, activation of which leads to a vascular inflammatory process (Boisramé-Helms et al. 2015). These results reveal a potential role of preeclamptic microparticles and the induction of vascular inflammation and expression of oxidative markers, so greater oxidative stress and arterial dysfunction.

The bioactivity of microparticles from non-pregnant, normal pregnancy and preeclampsia were studied by testing their effect on TPH-1 cell line. Microparticles from healthy pregnant women increased CD18, CD54, and integrin b7 expressions and decreased CD11a and CD29 expressions (Sokolov et al. 2016). Microparticles from preeclamptic women, on the other hand, decreased CD18 expression on tumour necrosis factor α (TNF- α)-activated TOR-1 cells. EVs from PE expressed higher levels of the soluble fms-like tyrosine kinase-1 and soluble endoglin (Chang et al. 2018). Using human umbilical vein endothelial cells, the negative effect of preeclamptic EVs on proliferation, migration and endothelial tube formation was noted. Thus, this data suggests that the development of vascular dysfunction in women with preeclampsia may be facilitated by the transfer of sFlt-1 and sEng to endothelial

cells. The bioactivity of placenta-derived EVs from third-trimester gestation was determined using a murine model (Han et al. 2019). Interestingly, mice developed hypertension and proteinuria only after being infused with placenta-derived EVs purified from the injured placenta (Han et al. 2019).

Current evidence suggests that EVs derived from placenta obtained from PE can induce endothelial cells dysfunction and inflammation, which are characteristic of PE pregnancies. However, there is an ongoing debate about the specificity of the PLAP protein as a placental marker, and this is important as several studies use PLAP to isolate placental vesicles. Studies using PLAP to isolate and/or quantify EVs originating from the human placenta are based on the idea that PLAP is only expressed in the placenta, however, there are several issues that need to be taken into consideration. Some of these issues are briefly discussed in the next section.

Placental Alkaline Phosphatase (PLAP)

Since EVs are released from most cells in the body, their cellular origin post-isolation from sources such as biological fluids can be difficult to establish. Given the heterogeneous nature of EVs, there has been a great interest in isolating/studying a subpopulation of EVs from different cellular origins. In the context of pregnancy, placental alkaline phosphatase (PLAP) has been used as a surrogate protein marker of EVs originating from the placenta, that can be identified in maternal circulation (Dragovic et al. 2015). However, the specificity of PLAP for the human placenta is unclear, including the uniqueness of the peptide sequencing and structure.

Alkaline phosphatases (APs) belong to the enzyme superfamily (EC 3.1.3.1) with a similar structural and catalytic site containing a metal ion (Bairoch 2000). In humans, there are 4 AP isozymes with distinct tissue expression: placental (PLAP, UniProt:P05187), germ cell (GCAP, UniProt:P10696), intestinal (IAP, UniProt: P09923) and tissue non-specific (TNAP, UniProt:P05186) (Rader 2017). They can be found attached to the surface of the cellular membrane via a glycosylphosphatidylinositol anchor (Paulick and Bertozzi 2008). The alignment of the primary amino acid sequences showed a high degree of similarity, with a difference of only 13 amino acids between PLAP and GCAP with 96.3% sequence identity (Fig. 18.1). PLAP and IAP had 86% followed by IAP and TNAP at 55% sequence identity, respectively.

The refinement of the model in membrane and water in a dynamic molecular simulation, allows us to evaluate the stability of the established model, and to refine the surface accessible to the solvent in the simulation time. With this background, the surface accessible to the solvent can be analyzed. In addition, the three-dimensional arrangement of the three selected regions which are used to design selective peptides of the alkaline phosphatase isoforms can be visualized (Fig. 18.1). By analyzing the entropy of the alignment in conjunction with the solvent accessible surface, the selected regions can be evaluated to maximize entropy and accessibility. In other words, the selected peptides are required to be in highly different regions between

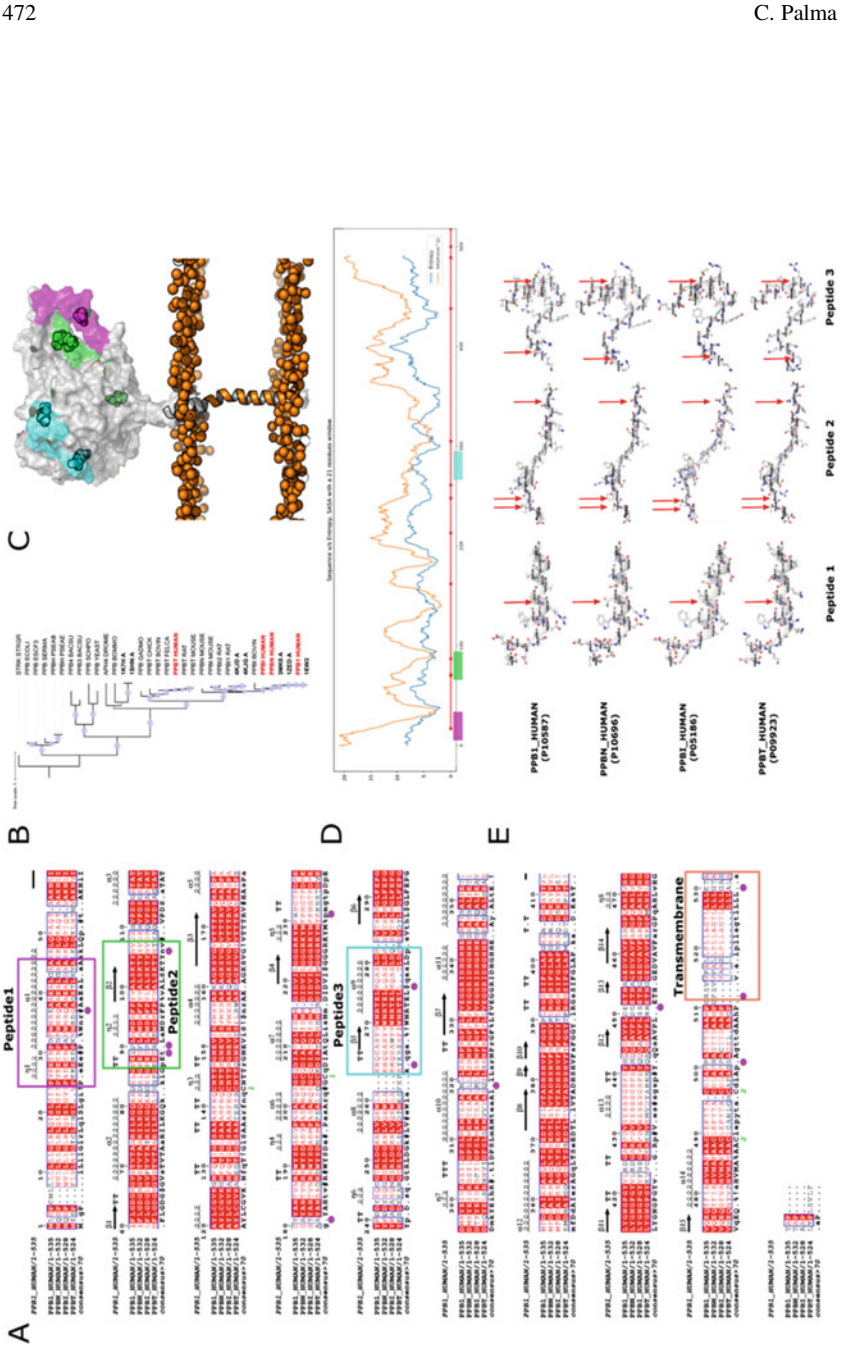

Fig. 18.1 Peptide design for immune assay detection. (**a**) Human PLAP sequences and peptide selection. Four human PLAP sequences (sp codes P10587, P10696, P09923, and P05186) were aligned and contain the secondary structure from crystallized PLAP protein (PDB code 1EW2) of human origin. Conserved regions are shown in red, the peptide is enclosed in a purple square, the transmembrane region is labelled in orange, and the purple dots indicate 13 unique

Fig. 18.1 (continued) residues which can distinguish PPB1 from PPBN. (**b**) Phylogeny analysis of placental PLAP. The phylogenetic tree from BLASTp search using P05187 (PPB1_HUMAN) sequences was performed with RAxML. The tree was re-rooted in STRK_STRGR so as to show human sequences in red and the PDB sequences in bold. The bootstrap sphere over 70% is shown and the length branch is in scale from the top left. (**c**) PLAP model and peptide selection. A complete model of the PLAP protein (sp code P01587) was established with Modeller software and embedded into a POPC lipids membrane. Molecular dynamics simulation was performed in which a value of 200 ns at 300 K was obtained. The surface protein shows peptides 1, 2, and 3 which are colored in magenta, green, and cyan, respectively. The only variant residues between the PPB1 and PPBN are represented by filled spheres. (**d**) PLAP alignment entropy and surface accessibility plot. The Shannon entropy is calculated from the alignment of the 4 PLAP sequences. The Shrake-Rupley surface is accessible to the solvent (SASA in nm^2). Both are projected into the PLAP sequence with a sum of values that leads to obtain a "window" sequence of 21 residues, in which entropy is colored in blue, and SASA is colored in orange. Red dots along the red line represent 13 unique and distinct residues of PLAP. Rectangles under the red line represent peptides 1, 2 and 3 which are colored in magenta, green, and cyan, respectively. (**e**) Peptide structure details and isoform variances. A complete model was established using the 4 PLAP sequences in Modeller. Peptides 1, 2 and 3 are evaluated and shown in ball and stick aminoacid structure indicating secondary structures as transparent cartoons. Red arrows show distinct residue variants between the isoforms

the sequences and at the same time, accessible to the solvent. In order to accomplish the latter, a sequence of 21 amino acids was used to add these values. The three peptides are found in regions of high entropy and with accessibility greater than 7 nm^2. Interestingly, Peptide 3 had less entropy and accessibility. Finally, an atomic detail of the selected regions shows the minimal differences between the residues from the four isoforms of the protein found in humans, and shows the conformation in the protein for each selected region (Fig. 18.1).

Several studies have primarily used antibodies for PLAP for the quantification of placenta-derived EVs. The PLAP $^{+ve}$ EVs are firstly captured onto antibody-coated substrates, and are subsequently quantified (Kobayashi et al. 2014; Pillay et al. 2016). Alternatively, the PLAP $^{+ve}$ EVs can be assessed directly using flow cytometry or fluorescence Nanoparticle tracking analysis (Dragovic et al. 2015; Elfeky et al. 2017). Regardless of the method, the anti-PLAP antibody plays a critical role in the specific selection of PLAP $^{+ve}$ EVs and not to one of the highly similar AP isoforms. This potentially raises the question of antibody selectivity and also reproducibility, which has been an ongoing issue for antibody-based assays (Baker 2015). We suggest that the approach to isolate or quantify EVs from placental origin using PLAP or even other placenta-specific antibody needs to incorporate positive (e.g. PLAP recombinant protein), and negative (e.g., EV from non pregnant women) controls.

Profiling EVs Using In Vitro and Ex Vivo Systems

In the context of PE, there are several systems used to identify changes in the concentration or bioactivity of EVs, including in vitro (e.g., primary cells or cell lines), and ex vivo (e.g., placenta perfusion system) models. For example, a study examined embryonic survival using a murine model, and interestingly noted that EVs treatment led to less developed embryos as well as placental mal-perfusion. They also noted renal dysfunction with elevated blood pressure and increased plasma sFlt-1 (Kohli et al. 2016). These effects were only observed in pregnant mice and can be defined as a PE-like phenotype. Inflammasome markers in placental tissue were likewise increased after procoagulant EV injections. Interestingly, the PE-like phenotype required the activation of the inflammasome. Furthermore, there was inflammasome activation in trophoblast cells of women with preeclampsia and the phenotype can be abolished by genetic or pharmacological inhibition, as well as by inhibition of platelet activation (Kohli et al. 2016). This data highlighted the relevance of inflammasome activation in the development of preeclampsia and elucidated an important mechanism related to it.

PE is also associated with failure in the spiral artery remodelling and extravillous trophoblast (EVT) migration and interaction with endothelial cells, which plays a central role in maintaining a normal pregnancy. In this regard, using HTR-8/SVneo cells as a model of EVT, the effect of low oxygen tension on the EV secretion and protein content was studied (Dutta et al. 2020). Interestingly, a specific protein

profile within EV associated with hypoxia was identified. Moreover, EVs from EVTs cultured under low oxygen tensions increased the levels of proinflammatory cytokines such as GM-CSF, IL-6, IL-8, and VEGF compared to normoxic EVs from endothelial cells. Finally, In vivo injection of hypoxic EVs in a murine model revealed raised mean arterial pressure, with increases in systolic and diastolic blood pressures. Thus, hypoxia induces specific packaging of proteins in EVs from placental cells, which induces a proinflammatory environment during gestation and might be involved in the physiopathology of PE. EVs isolated from first-trimester placental explants exposed to antiphospholipid antibodies induced activation of endothelial cells (Tong et al. 2017b). Transthyretin protein which is produced by the placenta is crucial for fetal development in the first trimester, and is specifically packaged into EVs in preeclampsia (Tong et al. 2017a). On the other hand, placental protein-13 is decreased in EVs from PE compared to normal pregnancies, which might be associated with several signalling pathways, and may affect processes such as immune tolerance, inflammation, and fetal growth restriction, among others (Sammar et al. 2018).

In summary, several experimental approaches have been used to study the role of EVs in the physiopathology of PE, including isolation and characterisation of EVs from biological fluids, cell-conditioned media and placental perfusion systems using samples from normal and preeclamptic pregnancies. In addition, in order to mimic the pathophysiological conditions associated with PE, primary placental cells, and placental cell lines have been cultured under several conditions like hypoxia, and EVs have been isolated from the cell-conditioned media.

Summary and Future Directions

Overall, pregnancy is a complex physiological state which requires an exquisite system of intercellular communication. In this context, extracellular vesicles have shown to be fundamental elements in the modulation of numerous processes in pregnancy. In addition, studies related to PE have also shown a more specific role in immunological modulation, protection from insults, balancing of oxidative state, and multi-level adaptation in several signalling pathways, among others. Currently, several potential biomarkers for PE have been identified through profiling the cargo of extracellular vesicles. Although early PE markers are not yet well established, the differential release of subpopulations of extracellular vesicles in PE is promising evidence in the search for cargo biomolecules with a predictive role. Also, further understanding of how EV release is regulated and how their cargo is modified in response to external factors opens a new set of strategies for early diagnosis of PE and an understanding of the underlying pathophysiology (Fig. 18.2).

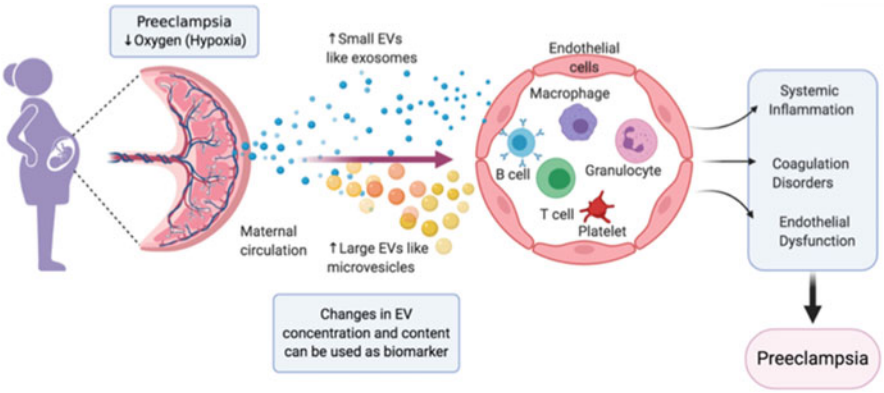

Fig. 18.2 EVs secreted from placenta are involved in several biological processes associated with PE. We are only beginning to develop an understanding of the role of placenta-derived EVs in early pregnancy events and, in particular, how they might affect the function of key cell types involved in the development of the placenta, and its vascular communication with both mother and fetus. Placental cells can sense changes in the microenvironment milieu, and increase the secretion of EVs into the maternal circulation. EVs secreted from the placenta can be identified in maternal circulation, and the concentration and bioactivity of placental-derived EVs seem to be specific under preeclamptic conditions. Placental EVs can interact with maternal organs and cells, and induce endothelial cell dysfunction, systemic inflammation, and coagulation disorders

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Conflict of Interest The authors have nothing to declare.

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Chapter 19
The Role of Extracellular Vesicles in Sperm Function and Male Fertility

Natalie J. Foot and Sharad Kumar

Abstract Within the reproductive tract, distinct cell types must have precisely controlled communication for complex processes such as gamete production, fertilisation and implantation. Intercellular communication in many physiological processes involves extracellular vesicles (EVs). In reproductive systems, EVs have been implicated in many aspects, from gamete maturation to embryo development. Sperm develop within the testis and then exit into the epididymis in an immature form, lacking motility and fertilising capabilities. Due to their small size, compact nature of the nucleus and the lack of specific organelles, sperm are unable to perform de novo protein synthesis, and thus rely on extrinsic signals delivered from the external milieu to gain full function. Mounting evidence points to EVs as being a major provider of these signals, not just within the male reproductive tract but also within the female as the sperm make their way through a seemingly hostile environment to the oocyte. In this chapter, we review the current knowledge on EVs as mediators of sperm maturation and function and highlight their potential roles in male fertility.

Keywords Sperm maturation · Extracellular vesicle · Epididymosome · Prostasome · Uterosome · Oviductosome · Male fertility

Introduction

The mature spermatozoon is an elaborate, highly specialised cell (Fig. 19.1). Within the testis, stem cells undergo prolific mitotic and meiotic divisions to generate the haploid spermatids, and extensive remodelling to package the chromosomes for effective delivery to the oocyte (Johnson 2013). During this remodelling phase, or spermiogenesis, major physical changes occur: a tail is generated for forward

N. J. Foot (✉) · S. Kumar
Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, SA, Australia
e-mail: Natalie.foot@unisa.edu.au

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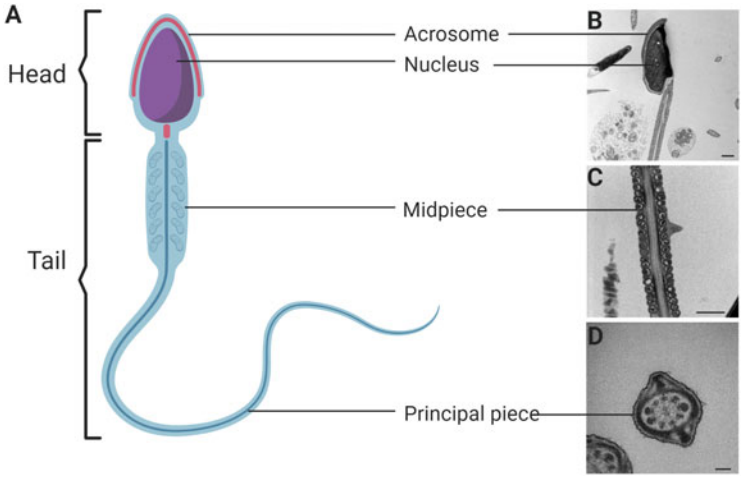

Fig. 19.1 Sperm structure. (**a**) Upon leaving the testis, sperm have fully developed into its complex cellular structure. (**b**) The head region contains the condensed nuclear material to be transferred to the oocyte, and the acrosome, an enzyme-rich structure derived from the Golgi apparatus required for cumulus cell and zona pellucida penetration upon fertilisation. (**c**) This is connected to the midpiece of the sperm tail, which contains all the mitochondria necessary for energy production. (**d**) The remainder of the tail contains the principle and end pieces, consisting of the axoneme in the typical 9 + 2 array of microtubules found in flagella. The principle piece is also thought to be the site of glycolytic energy production, with the contribution of glycolysis and oxidative phosphorylation to energy production varying between species (Tourmente et al. 2015). TEM images from wild type mouse sperm. Scale bar, 2 μm (**b**), 1 μm (**c**) and 100 nm (**d**). Created using Biorender.com

propulsion, the midpiece contains all the cell's mitochondria for energy production, the acrosome is formed to enable penetration of the oocyte and the nucleus contains the highly compacted haploid chromosomes (Fig. 19.1). All other organelles and cytoplasmic material are discarded in the residual body, which is phagocytosed by the Sertoli cells, resulting in the fully formed spermatozoa.

To reach the site of fertilisation, the ampulla of the oviduct, sperm must travel a substantial distance through the male and female reproductive tract (Fig. 19.2); the epididymis alone is extremely long, varying in length from 1 m in mice (Takano et al. 1981), 3 m in rats (Turner et al. 1990), 3 to 6 m in humans (Kim and Goldstein 2010), and up to 80 m in horses (Nickel et al. 1973). Sperm first functionally mature as they travel through the length of the epididymis before being stored in the distal segment (cauda) prior to ejaculation (Robaire et al. 2006). During the ejaculation process, sperm travel through the vas deferens to be deposited in the female reproductive tract where they must then travel through the cervix and uterus to reach the oviduct. In the oviduct, sperm must then bind and penetrate the cumulus cells and zona pellucida for successful fertilisation. To successfully complete this long and arduous journey, sperm cells must be both highly mobile and possess specific factors which allow the guiding of sperm to an oocyte, as well as appropriate binding and penetration (Topfer-Petersen et al. 2000). These functions are primarily

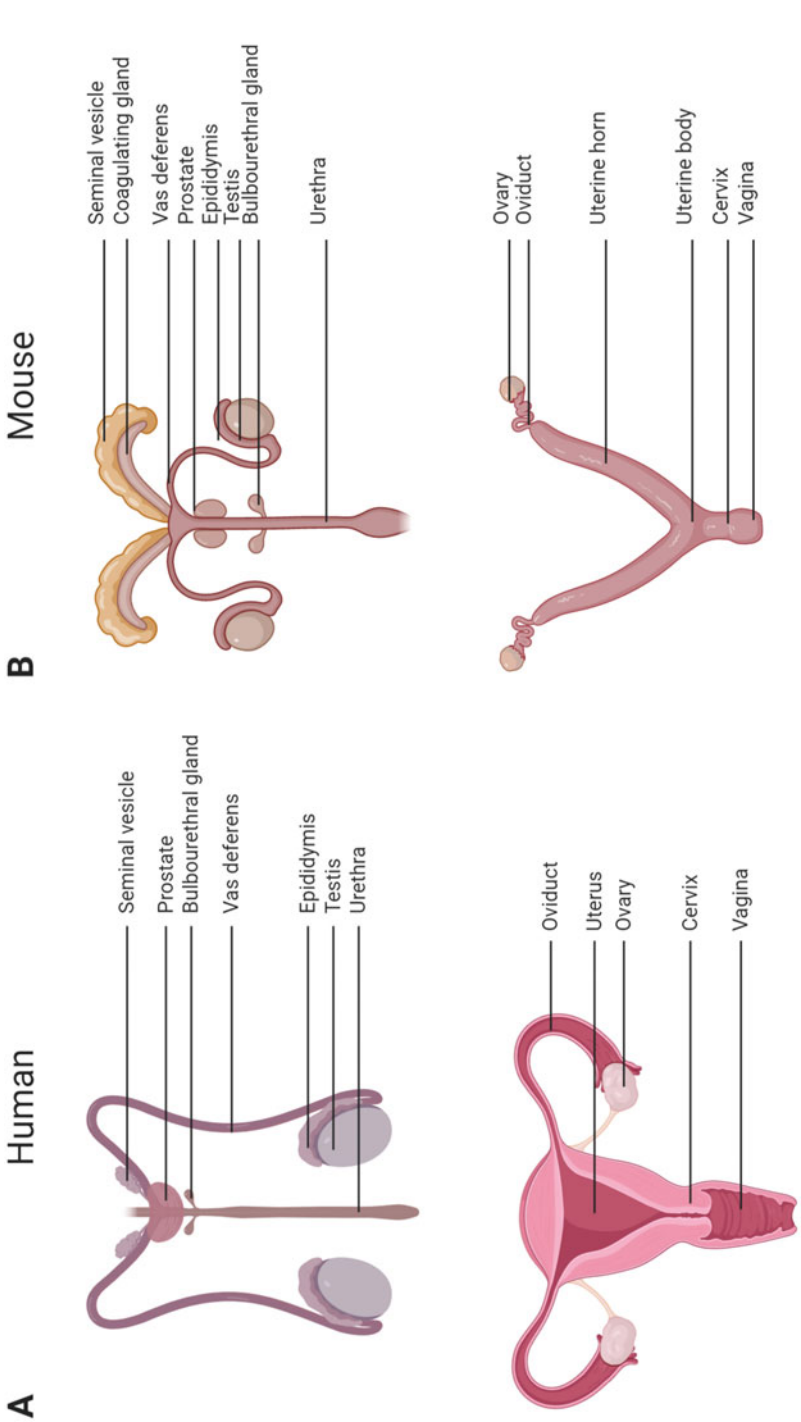

Fig. 19.2 Anatomy of the male and female reproductive tract. Sperm must travel through an extensive network of tissues in the male and female to reach its final destination, the oviduct. Figure shows the (**a**) human and (**b**) mouse reproductive tracts to demonstrate differences in structures between species. Created using Biorender.com

acquired by sperm during transit of the male reproductive tract, largely via the uptake of EVs released into the seminal fluid by the epididymis and prostate (Gervasi and Visconti 2017; Topfer-Petersen et al. 2000). Moreover, in the female reproductive tract, sperm undergo the final processes necessary for the development of sperm competency. These include hyper-motility, activation of some signalling pathways and destabilisation of the acrosomal region of the sperm head resulting in an increased capacity for fusion to the egg (Yeste et al. 2017; James et al. 2020). Sperm also need to survive the highly acidic vaginal environment and evade maternal immune surveillance (Suarez and Pacey 2006). Sperm released from the testis are in an immature state, being incapable of progressive movement and cannot attach to and fertilise an oocyte. In order to acquire these abilities, the sperm must rely on the delivery of essential macromolecules from the external environment, as they lack the requisite cellular machinery to produce their own. These processes are primarily mediated by extracellular vesicles (EVs) contained within the seminal fluid, as well as those secreted by the female organs.

EVs are small, membrane-bound vesicles secreted by cells, both constitutively and in response to stimuli. EVs contain a wide variety of proteins, genetic material, lipids and metabolites which are transferred to neighbouring cells to facilitate cell-cell communication (see other chapters in this book for more in-depth discussion of EV characteristics). EVs are primarily formed through two main routes—by the release of intraluminal vesicles from multivesicular bodies when they fuse with the plasma membrane (termed exosomes), or by budding directly from the plasma membrane (often termed microvesicles or ectosomes) (Anand et al. 2019; Mathieu et al. 2019). However, in the reproductive tract, apocrine secretion is considered to be the major secretory pathway, and has been described in the prostate (Fullwood et al. 2019), seminal vesicles (Aumuller et al. 1997), coagulating gland (Groos et al. 1999), vas deferens (Manin et al. 1995) and epididymis (Hermo and Jacks 2002; Rejraji et al. 2006) of males, as well as in the uterus (Griffiths et al. 2008a) and oviduct (Bathala et al. 2018) of females (Fig. 19.3). Apocrine secretion is the result of blebbing at the apical pole of the secretory cells, which detach from the cell and their content released into the extracellular compartment when the blebs disintegrate (Farkas 2015) In the reproductive tract, these apocrine blebs contain small, membranous vesicles which are shed into the extracellular compartment upon disintegration (Rejraji et al. 2006; Bathala et al. 2018). These vesicles are of similar size to canonical EVs and contain typical EV markers such as HSC70, CD9 and CD63 (Rejraji et al. 2006; Bathala et al. 2018; Al-Dossary et al. 2013; Ng et al. 2013), and thus are considered to be a form of non-classical EV secretion.

In this chapter, we provide an overview of the current knowledge on reproductive system EVs and their known roles in mediating sperm function and describe accumulating evidence for their use as biomarkers and therapeutics for male infertility and disease.

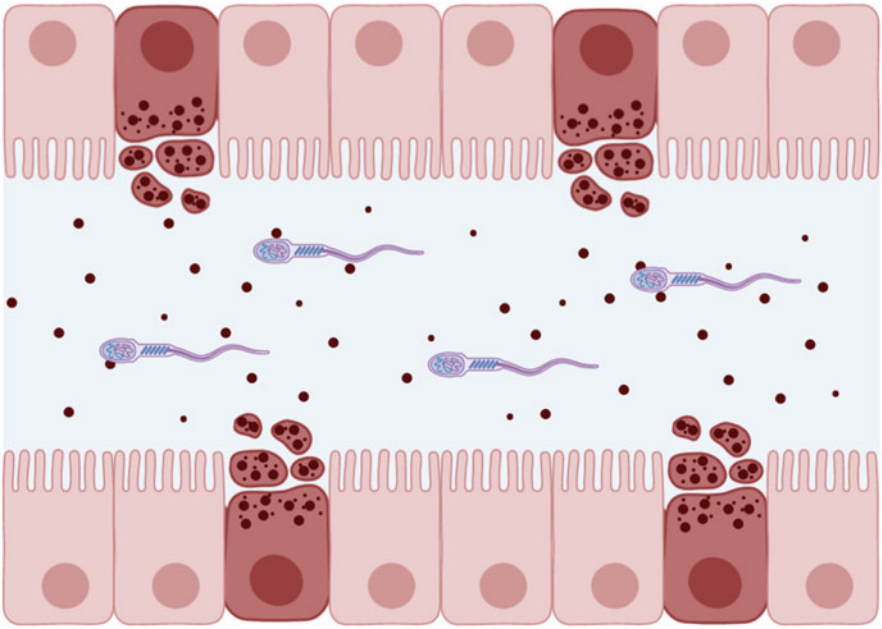

Fig. 19.3 Apocrine secretion of EVs in reproductive tract. The epithelial cells lining the lumen of the reproductive tissues pinch off their apical regions through an apocrine secretory mechanism. These apocrine buds contain EVs which are released into the extracellular space when the outer membrane is dissolved. Figure adapted from James et al. (2020) and created using Biorender.com

Role of EVs in the Male Reproductive Tract on Sperm Function

Epididymis

After being released from the testis, sperm gain functional maturity as they traverse through the epididymis, a highly convoluted and specialized duct distal to the testis (Jones and Lopez 2013). This occurs via direct uptake of contents from the epididymal luminal environment. Epididymosomes are EVs released by the epithelial cells of the epididymis (Martin-DeLeon 2015; Sullivan 2016), and transfer many of these functional components to sperm. Epididymosomes have been identified in many mammalian species including hamsters, rats, mice, rams, bulls, horses and humans, and have recently been discovered in non-mammalian species such as turtles (Waqas et al. 2017). Each segment of the epididymis provides region-specific content due to the varied cell composition of the epithelium and changes in hormonal regulation (Cornwall 2009). The variation in both protein and genetic content of epididymosomes from the different epididymal segments is well documented (Frenette et al. 2010; Girouard et al. 2011; Reilly et al. 2016; Nixon et al. 2019; Twenter et al. 2020; Belleannee et al. 2013; Girouard et al. 2009; Twenter et al.

2017). In the bull, proteomic profiling of epididymosomes identified 324 and 207 proteins expressed exclusively in the caput and cauda, respectively (Girouard et al. 2011), the majority of which were enzymes and transporter molecules. Results from mice showed similar profile, with the majority of identified proteins associating with transport and metabolic processes (Nixon et al. 2019). miRNAs are also differentially expressed in epididymosomes. Stallion epididymosomes contain over 300 miRNAs, with several being unique to the individual epididymal regions (Twenter et al. 2017, 2020). Pathway enrichment analysis predicted roles for these miRNAs in processes related to the function of the region. Caput epididymosomes contained miRNAs predicted to target estrogen signalling and calcium reabsorption responsible for fluid reabsorption (Twenter et al. 2017). Caudal epididymosomes contained miRNAs predicted to target the forkhead box O (FoxO) signalling pathway which regulates apoptosis, oxidative stress resistance and longevity, and also the oxytocin signalling pathway which regulates tissue contractions required for sperm expulsion (Twenter et al. 2017). In the bull, several miRNA sequences were selectively identified in epididymosomes from either the caput or cauda regions, and interestingly these profiles were distinct from the epithelial tissue of the corresponding region, suggesting a selective secretion pathway rather than passive release by the epithelial cells (Belleannee et al. 2013).

Few studies have described the mechanism of epididymosome uptake in sperm. Earlier work demonstrated that the transfer of epididymosomes to sperm was partly dependent on the tetraspanin CD26 (Caballero et al. 2013). In this study, epididysmosomes extracted from cauda epididymal fluid from bulls could be taken up by sperm, and this uptake was inhibited by the addition of CD26 antibodies, but not CD9 or CD224 (Caballero et al. 2013). A more recent study demonstrated that the post-acrosomal domain of the sperm head was the primary site for epididymosome protein uptake, with adhesion of epididymosomes to sperm being observed in as little as 1 min of co-incubation, transfer of contents occurring after 5 min and spread of proteins throughout the sperm head and tail being observed between 1 and 3 h (Zhou et al. 2019). The epididymosome adherence to the sperm was shown to trigger a relocation of Dynamin 1 to the docking site to aid in the redistribution of cargo throughout the sperm head and tail, which could be inhibited by a Dynamin inhibitor (Zhou et al. 2019).

While there are numerous studies describing the transfer of molecules to sperm via epididymosomes and their possible role in sperm function (Suryawanshi et al. 2012; Twenter et al. 2020; Wu et al. 2017; Belleannee et al. 2013; D'Amours et al. 2012; Frenette et al. 2010; Girouard et al. 2009; Patel et al. 2013; Taylor et al. 2013; Frenette and Sullivan 2001), very few demonstrate a true functional role for these transferred cargo. In the cat, cauda epididymosomes enhanced motility and sustained forward progressive motility in immature caput sperm that normally have limited motility, but this was not attributed to a specific cargo (Rowlison et al. 2018). The kinase cSrc was shown to be transferred to sperm by caudal epididymosomes and in cSrc deficient mice sperm forward motility and the ability to fertilise oocytes in vitro were severely impaired, as well as caudal epididymis tissue size and expression of key transport proteins in the epithelial cells significantly reduced (Krapf et al. 2012).

Epididymosomes are also important for facilitating long range signalling from the epididymis. Blocking Notch signalling using the Notch inhibitor DAPT disrupted the expression of Notch pathway proteins in epididymosomes and sperm and significantly decreased sperm motility without affecting fertilising capabilities (Murta et al. 2016). Wnt signalling is also robustly induced in sperm by epididymosomes isolated from epididymal luminal fluid, and this is blocked is mice lacking the Wnt regulator Ccnyl1 (Koch et al. 2015).

Alterations in the transfer of genetic material by epididymosomes can also affect sperm function. In mice, small RNAs are transferred to sperm through epididymosomes to modify the sperm epigenome (Sharma et al. 2018). The small RNA payload in epididymosomes is altered by protein abundance in the paternal diet, thus changing the sperm epigenome and affecting preimplantation gene regulation in embryos (Sharma et al. 2016).

Prostate

The prostate is an exocrine gland of the male reproductive system. It consists of multiple lobes or zones surrounding the urethra (Standring 2016). The prostate secretes an alkaline fluid which contributes approximately 30% of the volume of semen (Huggins et al. 1942) and is rich in metabolites, inorganic ions and enzymes (Grayhack et al. 1980; Huggins et al. 1942). The prostatic fluid also contains extracellular vesicles termed prostasomes after their tissue of origin (Brody et al. 1983). Like epididymosomes, prostasomes have been shown to contain a large number of proteins and genetic material that can be transferred to sperm (Ronquist et al. 2009; Jones et al. 2010; Olsson and Ronquist 1990; Arienti et al. 1997b, c, d). The role of prostasomes on sperm function was first identified by the discovery that isolated prostasomes could promote forward motility of immotile sperm suspended in an isotonic buffer (Stegmayr and Ronquist 1982), and that the addition of prostasomes increased the percentage of sperm displaying progressive motility and improved the recovery of hyperactive sperm in human samples (Fabiani et al. 1994, 1995; Carlsson et al. 1997; Wang et al. 2001).

The role of prostasomes in the acrosome reaction is still under some debate. To initiate fertilisation, the calcium-dependent acrosome reaction allows the contents (primarily digestive enzymes) of the acrosome to be released to enable the sperm to penetrate the zona pellucida of the oocyte and permitting the transfer of nuclear material. Prostasomes have been shown to prevent the acrosome reaction in response to progesterone in vitro (Cross and Mahasreshti 1997), and to inhibit spontaneous acrosome reaction (Pons-Rejraji et al. 2011). However, other studies have demonstrated that the addition of prostasomes stimulated the acrosome reaction (Palmerini et al. 2003; Siciliano et al. 2008; Park et al. 2011), possibly by promoting an increase in sperm intracellular calcium levels (Palmerini et al. 1999; Arienti et al. 2002). Prostasomes also transfer calcium signalling molecules to sperm such as progesterone receptors, cyclic adenosine diphosphoribose (cADPR)-synthesizing enzymes

and ryanodine receptors (Park et al. 2011), and the addition of ryanodine receptor-depleted prostasomes to sperm results in low fertilisation rates and reduced sperm motility (ref). However, in this study the acrosome reaction appeared to be independent of cADPR-mediated calcium signalling, suggesting distinct calcium signalling pathways in the regulation of motility and the acrosome reaction.

Unlike epididymosomes, which bind to the sperm and mediate their function within the male reproductive tract, prostasomes appear to exert their functions within the female reproductive tract. Fusion of prostasomes to sperm appears to require an acidic pH, with prostasome cargo transfer occurring a pH 5–7, whereas there was no measurable transfer at pH 8 (Arienti et al. 1997a). The normal pH of seminal fluid is between 7 and 8, which suggests prostasomes cannot fuse with sperm in this medium. The pH of the vagina is normally between 3.8 and 4.5, which when combined with the alkaline seminal fluid, provides a more favourable environment for sperm/prostasome fusion. Highly purified prostasomes have also been shown to bind live sperm only after capacitation has been initiated (Aalberts et al. 2013), an event that occurs within the female reproductive tract. Prostasomes also contain high levels of tissue factor antigen, a blood clotting agent (Fernandez et al. 1997). Seminal fluid has very potent blood clotting activity, and it is hypothesised that in the event of abrasion and bleeding during intercourse, rapid blood clotting at lesion sites would prevent sperm and seminal components from entering the female blood stream and generating deleterious anti-sperm antibodies, a common cause of infertility. This provides further evidence that prostasomes act within the female reproductive tract.

Role of EVs in the Female Reproductive Tract on Sperm Function

Uterus and Vagina

Compared with EVs in the male reproductive system, EVs in the female tract have only recently been identified and their possible roles in sperm functions are still being elucidated. The existence of uterine nanoparticles was first reported in mice. Sperm were able to take up uterine EVs (termed uterosomes) and acquire their protein content (Griffiths et al. 2008a). Since then, uterine EVs have been identified in several species including sheep (Burns et al. 2014), cattle (Qiao et al. 2018) and humans (Ng et al. 2013; Franchi et al. 2016), as well as non-mammalian species (Riou et al. 2020). Unlike prostasomes, which appear to require an acidic pH for binding to sperm, uterosomes are taken up by mature caudal epididymal sperm under neutral pH conditions in vitro (Griffiths et al. 2008a; Al-Dossary et al. 2013), reflective of the neutral to slightly alkaline uterine environment. In humans, sperm were able to take up EVs from uterine fluid in as little as 15 min of incubation which

lead to an increase in capacitation as indicated by an increase in phosphotyrosine levels and stimulation of the acrosome reaction (Franchi et al. 2016).

Only one study thus far has identified EVs in vaginal fluid (Fereshteh et al. 2019). In this study, murine vaginal EVs were able to transfer protein cargo to sperm, stimulate capacitation and improve acrosome reaction rates in sperm stimulated with progesterone. In mammals, semen is deposited into one of two sites: the vagina (e.g. humans, rabbits, rodents) or the uterus (e.g. horses, pigs) (Suarez and Pacey 2006). Vaginal EVs may therefore provide different functions depending on the species, with a role in sperm function only necessary once the sperm has been deposited in the vagina. Further studies on the comparison of vaginal EVs between species may answer this question.

Oviduct

In mammals, the oviduct is the site of sperm storage and fertilisation. Like in other reproductive tissues, oviductal EVs (or oviductosomes) have been identified in many species including sheep, pigs, mice, cats, dogs and humans. They have also been shown to transfer their content to sperm. The calcium pumps PMCA1 and PMCA4, and SPAM1, a hyaluronidase required for penetration of the cumulus cells and zona pellucida binding, are transferred to sperm via oviductal EVs (or oviductosomes) (Al-Dossary et al. 2013, 2015; Griffiths et al. 2008b; Bathala et al. 2018). miR-34c-5p, essential for zygotic cleavage and solely derived from sperm, is also transferred to sperm via oviductosomes (Fereshteh et al. 2018).

Several recent studies support the idea that interaction between oviductal EVs and sperm is important for fertilisation. Oviductosomes have been shown to improve survival of cryopreserved sperm, as well as stimulate capacitation signalling events and increase progesterone-induced calcium levels and acrosome reaction in bull sperm (Franchi et al. 2020). In pigs, co-incubation of sperm with porcine oviductosomes increased fresh and frozen sperm survival, but reduced the total and progressive sperm motility (Alcantara-Neto et al. 2020), suggesting a role of oviductosomes in maintaining the oviduct sperm reservoir. In cats, incubating sperm with oviductosomes improved motility and fertilizing capacity and prevented the acrosome reaction (Ferraz et al. 2019). The differences seen in sperm motility after incubation with oviductosomes may reflect differences between species. In pigs, sperm is primarily stored in the oviduct (Hunter 1981), whereas in cats, the sperm is initially stored in the uterotubal junction and uterine crypts before ovulation and only transported to the oviduct at the time of ovulation (Chatdarong et al. 2004), suggesting that cat sperm may require a higher motility to reach the site of fertilisation at the appropriate time. Further work is required to determine the true role of oviductosomes on sperm motility in species with varying reproductive systems.

EVs in Male Infertility

Due to their non-invasive nature and ease of collection, EVs have been highly touted as ideal sources of biomarkers for a large range of diseases (see other Chapters in this book). This is also the case for infertility diagnoses. There have been several studies that have compared EV content between healthy and infertile seminal fluid. In early studies, EVs in seminal fluid from healthy donors were compared with successfully vasectomised men to identify potential biomarkers of azoospermia (absence of sperm in semen). A total of 84 miRNAs and 995 piRNAs were identified to be upregulated in healthy samples compared with vasectomised men (Hu et al. 2014), suggesting that this may be a valid approach for assessing azoospermia. Further work identified several miRNAs contained within seminal fluid EVs as markers of spermatic reserve in the testis, by comparing patients with azoospermia as a result of an obstruction in the genital tract and azoospermia caused by spermatogenic failure (Barcelo et al. 2018). Here, they showed that miR-31-5p, miR-539-5p and miR-941 had potential as sensitive and specific biomarkers for predicting the presence of testicular sperm in azoospermic individuals (Barcelo et al. 2018). The miRNA expression profile of seminal fluid EVs was also found to be altered in oligoasthenozoospermic patients (reduced sperm motility and low sperm count), with a significantly higher expression of miR-765 and miR-1275 and lower expression of miR-15a compared with healthy donors (Abu-Halima et al. 2016). Long noncoding RNAs and their regulatory networks have also been analysed in patients with asthenozoospermia (low sperm motility) (Lu et al. 2020; Zhang et al. 2019). Differential expression of several lncRNA-mRNA regulatory networks was discovered in these patients including *IGF2, MGAT1, CLPP* and *LRRC8A,* all known to be associated with male infertility (Boissonnas et al. 2010; Batista et al. 2012; Gispert et al. 2013; Bao et al. 2018).

Seminal plasma EVs from non-normozoospermic patients also show an altered proteome, with a decrease in levels of proteins involved in energy production pathways and sperm activity (Garcia-Rodriguez et al. 2018), although in this study the diagnosis of patients was not specified. A more in-depth study revealed a significant upregulation of ANXA and downregulation of KIF5B in infertile patients with unilateral varicocele and suggested that these two proteins could be utilised as potential biomarkers of infertility (Panner Selvam et al. 2019). Patients with asthenozoospermia were also shown to have an altered proteome (Murdica et al. 2019a), with 52 proteins enriched in EVs of asthenozoospermic patients and 37 being downregulated. A similar study by Lin et al. (2019) identified 11 upregulated and 80 downregulated proteins in EVs of asthenozoospermic patients, however there were no shared targets identified by both studies, thus limiting their potential use as biomarkers. As differences in the techniques used for mass spectrometry and the limited number of patients used in each study could account for these inconsistencies, more work is required to determine whether specific proteins in seminal EVs could be considered as biomarkers for specific sub-types of infertility.

Currently, studies into the restorative role of EVs in male fertility are only just beginning and so our knowledge in this area is still lacking. Work done in our laboratory has demonstrated that male mice deficient in Arrdc4, an α-arrestin protein important for EV biogenesis (Mackenzie et al. 2016), are sub-fertile, primarily due to a lack of sperm maturation though the epididymis (Foot et al., manuscript submitted). *Arrdc4*$^{-/-}$ sperm incubated with epididymal EVs from wild type mice can restore sperm fertilisation capacity in vitro as evidenced by an increase in zona pellucida binding and production of 2-cell embryos. In a mouse model of chemically induced nonobstructive azoospermia, EVs from urine-derived stem cells transplanted into the interstitial space in the testes could restore spermatogenesis 36 days after busulfan treatment by upregulating spermatogenic gene (Pou5f1, Prm1, SYCP3, and DAZL) and protein (UCHL1) expression (Deng et al. 2019). Only one study has demonstrated a functional role for EVs in human patients. Murdica et al. isolated EVs from normozoospermic and asthenozoospermic individuals and demonstrated that normozoospermic EVs were able to improve motility and trigger capacitation of sperm, whereas asthenozoospermic EVs were not, and that this phenomenon may in part be due to the transfer of CRISP1 (Murdica et al. 2019b). The use of EVs as a potential treatment option for male infertility or sub-fertility is an exciting prospect and more work into this area could uncover previously unexplored avenues for discovery.

Concluding Remarks

It is clear from the numerous studies performed that EVs play an important role in many aspects of sperm function. Some EVs have well defined functions, for example epididymosomes are essential for sperm maturation after leaving the testis, whereas others are less clear such as the role of prostasomes in the acrosome reaction. Controversies in the role of EVs in sperm function need further analysis and resolution as differences in isolation techniques, contamination by EVs from different tissue sources, sperm source (epididymal or ejaculate) could all contribute to the differences observed between studies. While many of the studies reported here describe components of EVs and their transfer to sperm, direct evidence for a role of EVs and their components in sperm function is still rare and needs further investigation.

Our understanding of EVs in reproduction and their potential roles as therapeutics is currently in its infancy. Here we have discussed several reports describing the presence and contents of EVs in the fluids of various reproductive tissues and their potential effects on sperm (e.g. epididymis, prostate, uterus, oviducts). EVs have attracted significant interest for their untapped potential as disease biomarkers. However, while the current studies demonstrate that EVs have the potential to contain useful diagnostic and prognostic markers, there has as yet been no consensus in the proteins or genetic material identified and thus definitive biomarkers are yet to be determined.

Currently there are relatively few reliable diagnostic tools to guide infertility treatment, and this often results in multiple failed cycles and poor mental health and economic outcomes for patients. The use of different additives in culture medium to improve gamete or embryo fitness have met with little improvement in birth rates after assisted reproduction, with many experts arguing this is due to the lack of signalling molecules and other factors otherwise present in the female reproductive tract during the periconception period. Because of their ease of collection, their remarkable stability in biological fluids and their ability to be taken up by all cell types, EVs may provide a "shield" for fragile or short-lived additives such as proteins or miRNA, which may facilitate the transfer of these essential components to target cells, and are perfect candidates to solve this question.

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Chapter 20
Extracellular Vesicles and Cerebral Malaria

Georges Emile Raymond Grau and Elham Hosseini-Beheshti

Abstract Cerebral malaria (CM) remains a major problem of public health at the world level (Idro et al. 2010; WHO 2009), in spite of numerous efforts from various disciplines to improve our knowledge of disease mechanisms (Hunt and Grau 2003; Schofield and Grau 2005; van der Heyde et al. 2006). Our approach to a better understanding of CM pathogenesis has involved the dissection of immunopathological pathways which, in addition to direct changes caused by malaria parasite-infected erythrocytes (IE), lead to neurovascular lesions. We posited that immunopathology is important in CM because a role for cells and soluble mediators of the immune system has been widely recognised as contributing to the complications of viral, bacterial, fungal and many parasitic infections. As detailed earlier, it would be extraordinary if malaria did not conform to this general pattern. As a matter of fact, there now is strong evidence to support immune mechanisms in malarial pathogenesis (Grau and Hunt 2014).

Extracellular vesicles (EV) and their subtypes have been described and reviewed by a number of investigators (Hosseini-Beheshti and Grau 2018, 2019; Raposo and Stahl 2019; Witwer et al. 2017; Zijlstra and Di Vizio 2018) and in others chapters of the present book.

Keywords Extracellular vesicles · Exosomes · Microvesicles · Immunopathology · Neuro-immunlogy · Malaria

Cerebral malaria (CM) remains a major problem of public health at the world level (Idro et al. 2010; WHO 2009), in spite of numerous efforts from various disciplines to improve our knowledge of disease mechanisms (Hunt and Grau 2003; Schofield and Grau 2005; van der Heyde et al. 2006). Our approach to a better understanding

G. E. R. Grau (✉) · E. Hosseini-Beheshti
Vascular Immunology Unit, Discipline of Pathology, School of Medical Sciences; Marie Bashir Institute and The University of Sydney Nano Institute (Sydney Nano), The University of Sydney, Camperdown, NSW, Australia
e-mail: ggrau@med.usyd.edu.au; georges.grau@sydney.edu.au

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of CM pathogenesis has involved the dissection of immunopathological pathways which, in addition to direct changes caused by malaria parasite-infected erythrocytes (IE), lead to neurovascular lesions. We posited that immunopathology is important in CM because a role for cells and soluble mediators of the immune system has been widely recognised as contributing to the complications of viral, bacterial, fungal and many parasitic infections. As detailed earlier, it would be extraordinary if malaria did not conform to this general pattern. As a matter of fact, there now is strong evidence to support immune mechanisms in malarial pathogenesis (Grau and Hunt 2014).

Extracellular vesicles (EV) and their subtypes have been described and reviewed by a number of investigators (Hosseini-Beheshti and Grau 2018, 2019; Raposo and Stahl 2019; Witwer et al. 2017; Zijlstra and Di Vizio 2018) and in others chapters of the present book.

Identification of MV as Important Players in CM

It is the importance of cytokines in CM immunopathology (Hunt and Grau 2003) and the demonstration that tumor necrosis factor (TNF) can enhance endothelial EV release (Combes et al. 1999) that led us to investigate a potential role of these EV in CM pathogenesis. In view of high levels of plasma TNF, we and others had found in CM patients (Grau et al. 1989; Kern et al. 1989; Kwiatkowski et al. 1990), we reasoned that circulating levels of microvesicles (MV, formerly called microparticles) could be elevated in these patients. Our first study in malaria-infected Malawian children confirmed this and demonstrated that only patients with the neurological complications had massively elevated MV plasma levels; indeed, children with severe malarial anaemia had MV plasma levels comparable to those seen in non-infected subjects (Combes et al. 2004). Moreover, these elevated MV levels on admission were significantly correlated with plasma TNF concentrations, and returned to normal at convalescence (Combes et al. 2004). Further studies in malaria patients from other epidemiological settings, Cameroon (Pankoui Mfonkeu et al. 2010) and Vietnam (Nantakomol et al. 2011), confirmed that high MV levels were associated with severity and that these MV originated from diverse cellular sources, including the erythrocytes (Nantakomol et al. 2011).

Immune-Related Properties of MV

These clinical observations prompted us to analyse the effects of MV. To this end, we set out to model CM in vitro by using platelet derived MV as a source and IE and brain endothelial cells (EC) as targets. In this system, platelet derived MV bind to IE via CD31 and CD36, and transfer some of their antigens onto the IE surface membrane (Faille et al. 2009). More importantly, we were able to show that platelet derived MV are taken up by brain EC and internalised in various compartments of their cytoplasm.

This leads to profound modifications of brain endothelial cell surface phenotypes, and dramatically enhanced their adhesive properties: platelet derived MV therefore play an important role in the phenomenon of IE cytoadherence, a hallmark of CM pathogenesis (Wassmer et al. 2011a).

Interestingly, brain EC themselves appeared to play a role in the determinism of CM. After studies performed in the mouse model for CM, in which we demonstrated that brain EC isolated from CM-susceptible mice have a higher reactivity to TNF than their counterparts from CM-resistant mice (Lou et al. 1998), we were able to show that EC cultured from patients with cerebral or uncomplicated malaria also exhibit a differential reactivity to TNF, notably in that EC from CM patients produced significantly more MV than EC from patients with uncomplicated malaria or healthy controls, in response to the cytokine (Wassmer et al. 2011b).

After MV released by host cells during malaria infection, we turned our interest on vesicles produced by the parasite itself, after it invaded erythrocytes. We first found that malarial-induced MV could trigger a significant macrophage activation, while Lipopolysaccharide(LPS)-induced MV could not (Couper et al. 2010). This major effect of IE-derived MV in mice was shown to be MyD88/TLR-4-dependent and mediated by a CD40/CD154 interaction, opening the possibility that MV in infected patients may have a significant bearing on the development of severe malaria complications by generating previously unexplored adaptive immune responses (Couper et al. 2010). Indeed, the inflammatory cascade that is likely to follow IE-derived MV release will likely amplify the sequestration and activation of immune cells in the microvasculature, particularly in brain and lungs, where malarial pathology is prominent (vide infra).

The capability of MV to transfer antigens was further investigated in the context of T cell activation, an important feature of the immune disturbances in CM (Schofield and Grau 2005). After we showed that endothelial MV express molecules required for antigen presentation and T cell co-stimulation, including β2-microglobulin, MHC II, CD40 and ICOSL (Wheway et al. 2014), we asked the question of a possible involvement of MV in the well characterised T cell hyperactivation in malaria. Fluorescently-labelled endothelial MV derived from resting or cytokine-stimulated human brain EC readily formed stable conjugates with CD4+ and CD8+ T cells within in vitro co-cultures, very much like the conjugates that occur between antigen-presenting cells and T cells. This binding between T cells and endothelial MV derived from cytokine-stimulated human brain EC was shown to occur in a VCAM-1- and ICAM-1-dependent manner. Endothelial MV were further demonstrated to induce the proliferation of $CD4^+$ and $CD8^+$ T cells in vitro, and this occurred both in the absence of exogenous stimuli, and in the presence of T cell mitogens (Wheway et al. 2014). The observed interactions between endothelial MV and T cells may therefore represent a previously unrecognized pathway for T cell activation, and potentially antigen presentation, in the context of neuropathological conditions such as CM (Wheway et al. 2014).

Various facets of the interplay between EV and add immune disturbances in malaria have been recently assess, with particular attention to the mounting evidence for innate immune memory, leading to “trained” increased or tolerised responses and

to the modulation of immune cell function through metabolism, that could shed light on why some patients develop this life-threatening condition whilst many do not (Sierro and Grau 2019).

Therapeutic Efficacy of MV Inhibition in CM

The first hint that MV inhibition could lead to prevention or cure of CM was provided by the observation that ABCA1 knock-out mice were fully resistant to the development of the neurological syndrome, in spite of parasitaemia levels that remained identical to those seen in wild-type mice (Combes et al. 2005). Interestingly, both platelets and monocytes derived from ABCA-1 KO mice were unable to produce MV upon in vitro restimulation by adequate agonists, ADP and lipopolysaccharide, respectively (Combes et al. 2005).

This led to several attempts to reproduce pharmacologically those beneficial effects of ABCA-1 gene deletion. In vivo, in the mouse model, pantethine was shown to be able to not only prevent but also cure CM (Penet et al. 2008). In vitro, inhibitors such as the endothelial activation inhibiting peptide, LMP-420 (Wassmer et al. 2005), and diannexin, a homodimer of annexin V created by the late Anthony Allison, were able to potently downregulated TNF-induced MV overproduction (Combes et al. 2016).

Another original way to block CM also turned out to inhibit MV production: we studied the effects of artificial, exogenous microparticles. Our investigations in vivo in the mouse model led us to identify a novel subset of monocytes that has particular pathogenic relevance in the neurovascular pathology. We identified $Ly6C^{lo}$ monocytes as the major accumulating subset in the brain and the lung microvessels during severe malaria. These cells are derived from $Ly6C^{hi}$ monocytes from the bone marrow, and can therefore specifically be targeted with immune-modifying particles (IMP). Treatment of PbA-infected mice with IMP alone led to a remarkable 50% protection, in an otherwise fully lethal syndrome (Niewold et al. 2018). Administration of IMP in combination with the anti-parasitic drug, artesunate, at the onset of clinical CM signs markedly reduced Ly6Clo monocyte accumulation in the brain and lung, resulting in 88% survival in a lethal preclinical model. This is a significant improvement over the 55% survival with artesunate treatment alone. Moreover, this treatment did not interfere with the generation of immunity, which remains robust for more than a year post infection (Niewold et al. 2018).

In our murine CM model, high-dimensional computational analysis allowed us for the first time to separate microglia and Ly6Clo monocytes in the brain by flow cytometry, thereby enabling us to identify the latter as the major leucocyte population sequestering in microvessels during CM. This reveals a target for directed immune-modifying treatment in CM. Furthermore, we show IMP are also a viable adjunctive treatment for the pathology of malarial-induced acute respiratory distress syndrome, where anti-parasitic treatment alone has more limited success. Thus, our findings indicate that targeted immune modulation with IMP is an effective

adjunctive treatment for anti-parasitic compounds, dramatically improving efficacy in late stage disease. Remarkably, the marked IMP efficacy against CM-associated mortality was correlated with a significant reduction in MV levels, bringing them back to the level seen in non-infected mice (Niewold et al. 2018).

Results of these inhibition experiments support a pathogenic role for MV in CM. This was further substantiated by the demonstration that the adoptive transfer of MV can transfer CM pathology, particularly brain haemorrhages and infiltration of alveolar septa (El-Assaad et al. 2014), thereby reproducing partly but significantly the cerebral and pulmonary lesions that are hallmarks of CM.

Based on these pieces of evidence suggesting that MV are important pathogenic elements in CM, the notion that their plasma levels could serve as biomarkers of severity emerged (Pankoui Mfonkeu et al. 2010) and was investigated further in the field of proteomics (Tiberti et al. 2016) and miRNA (Cohen et al. 2018) profiles.

Numerous fascinating aspects of EV involvement in malarial pathophysiology outside CM have been reviewed recently (Babatunde et al. 2020; Debs et al. 2019; Sampaio et al. 2018).

Exosomes in Malaria Parasite Biology

While studied less extensively than MV in CM specifically, exosome overproduction has been identified during both human and murine malaria, both from host cells and from the pathogen. As early as 2013, Mantel's group showed the in vitro production of a subset of exosomes by erythrocytes (reticulocytes) infected with *P. falciparum* (Mantel et al. 2013) and subsequently demonstrated that such exosomes can activate host monocytes and neutrophils in vitro (Mantel et al. 2016). These exosomes contain parasite material, including proteins associated with erythrocyte invasion, so their increased production during *P. falciparum* malaria may represent an important pathway of enhanced parasite transmission (Schorey and Harding 2016). Exosomes derived from reticulocytes may also play a significant role in the modulation of host immunity to malaria, as shown by Hernando del Portillo's group in *P. yoelii*-infected mice (Martin-Jaular et al. 2011). Results from this group indicate that, rather than pathogenesis, exosomes appear to be involved in the development of protective immunity (Martin-Jaular et al. 2016). Not only the adaptive, but also the innate immune cells, can be affected by exosomes: a subset of parasite-derived exosomes containing *P. falciparum* DNA can be internalized by monocytes, thereby releasing malarial DNA into the host cell cytosol to facilitate STING (i.e. an innate immune cytosolic adaptor)-dependent DNA sensing. *P. falciparum* may utilise STING as a decoy mechanism to disseminate pathogenic material that could promote parasite virulence (Sisquella et al. 2017). Beyond immunopathology, exosomes produced during malaria also can modulate angiogenesis: exosomes derived from the plasma of *P. yoelii*-infected mice have the potential to inhibit tumour angiogenesis in a murine model of lung cancer (Yang et al. 2017).

Aside from these effects on host immune cells, exosomes have a remarkable regulatory role on parasite gametocytogenesis, as elegantly identified by Neta

Regev's group. Exosomes or exosome-like vesicles (~70 nm in diameter) carrying a protein specific to *P. falciparum*, referred to as PfPTP2, were shown to promote gametocytogenesis (i.e. sexual differentiation) of a subset of parasites in vitro (Regev-Rudzki et al. 2013).

Conclusions

Overall, the findings discussed here provide some insight into EV-mediated pathways of communication in malaria, including the transfer of genetic information, modulation of host immunity, and dissemination of pathogenic material. EV produced during malaria are important players in the pathogenesis of CM, potent regulators of innate and adaptive immunity, and essential modulators of parasite biology. While a number of significant discoveries have been made through decades of research in uncovering severe malaria pathogenesis and its complications, the study of different subtypes of EV in the development and progression of this disease is still in its infancy. Although significant progress has been made in the field, it is still unclear whether there are specific signatures/cargo that direct EV to different targets, how recipient cells discriminate among EV, how host and parasite derived EV influence the finer mechanisms in development of the disease and how they escape the immune response. Similarly, although a considerable body of experimental research has highlighted important physiological processes contributing to the complex pathogenesis of CM, the multifactorial nature of the disease in the context of humans compels further inquiry.

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Chapter 21
Are Dietary Extracellular Vesicles Bioavailable and Functional in Consuming Organisms?

Rahul Sanwlani, Pamali Fonseka, and Suresh Mathivanan

Abstract It has been well established that diet influences the health status of the consuming organism. Recently, extracellular vesicles (EVs) present in dietary sources are proposed to be involved in cross-species and kingdom communication. As EVs contain a lipid bilayer and carry bioactive cargo of proteins and nucleic acids, they are proposed to survive harsh degrading conditions of the gut and enter systemic circulation. Following the bioavailability, several studies have supported the functional role of dietary EVs in various tissues of the consuming organism. Simultaneously, multiple studies have refuted the possibility that dietary EVs mediate cross-species communication and hence the topic is controversial. The feasibility of the concept remains under scrutiny primarily owing to the lack of significant in vivo evidence to complement the in vitro speculations. Concerns surrounding EV stability in the harsh degrading gut environment, lack of mechanism explaining intestinal uptake and bioavailability in systemic circulation have impeded the acceptance of their functional role. This chapter discusses the current evidences that support dietary EV-based cross species communication and enlists several issues that need to be addressed in this field.

Keywords Extracellular vesicles · Exosomes · Dietary EVs · Milk EVs · Food-derived EVs · Inter-kingdom communication · Species crosstalk

Extracellular Vesicles

Extracellular vesicles (EVs) are nanosized membrane bound vesicles shed by all cell types (Kalra et al. 2016). EV secretion has been reported to occur in cells from organisms belonging to all domains of life including prokaryotes, eukaryotes and archaea (Prangishvili et al. 2000; Jan 2017; Pathan et al. 2019). Distinct biogenesis,

R. Sanwlani · P. Fonseka · S. Mathivanan (✉)
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia
e-mail: S.Mathivanan@latrobe.edu.au

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size distribution and cargo content render EVs highly heterogenous in nature. Thus, based on these characteristics, eukaryotic EVs have been classified broadly into several subtypes; exosomes, ecotosomes or shedding microvesicles and apoptotic bodies (van Niel et al. 2018; Raposo and Stahl 2019). Exosomes are of endocytic origin, formed by inward budding of endosomes to form intraluminal vesicles, ranging from 30 to 150 nm in diameter (Pan et al. 1985; Keerthikumar et al. 2015). In contrast, ectosomes and apoptotic bodies are shed *via* outward budding of the plasma membrane. Ectosomes range from 100 to 1000 nm in diameter. Both exosomes and ectosomes are actively shed by live cells (Kalra et al. 2016; van Niel et al. 2018). Whereas, apoptotic bodies, as the name suggests, are shed by apoptotic cells and range in diameter from 1000 to 5000 nm (Hristov et al. 2004; Atkin-Smith et al. 2015). In addition other subtypes of EVs such as large oncosomes, migrasomes and exomeres have also been described (Minciacchi et al. 2015; Zijlstra and Di Vizio 2018). EVs sequester a diverse functional cargo comprised of proteins, lipids, nucleic acids and other bioactive compounds (Pathan et al. 2019). Over the years, EVs have emerged as sophisticated vectors of signalling molecules capable of manifesting varying phenotypes in recipient cells and their diverse role in pathophysiology are beginning to unravel (Raposo and Stahl 2019; Kalluri and LeBleu 2020).

Dietary EVs in Cross-Species and Kingdom Communication

Since the initial discovery that EVs have a immunomodulatory role and mediate intercellular communication (Raposo et al. 1996), several studies have implicated EVs in a plethora of biological processes regulating the state of health and wellbeing in an individual (Yuana et al. 2013; Kalluri and LeBleu 2020). EVs may not only function by crossing barriers within a system to connect distant cells but overcome the confines of one individual and mediate inter-species and trans-kingdom crosstalk (Munir et al. 2019; Woith et al. 2019; Sanwlani et al. 2020). In this regard, uptake of dietary biomolecules resulting in phenotypic manifestation was first reported in subjects fed with rice as their main diet. The study demonstrated the presence of rice exogenous miRNA (xenomiRs) in serum and peripheral tissue of mice and human subjects. Further, the xenomiR was observed to induce metabolic phenotypes by altering serum lipoprotein levels in the consumers (Zhang et al. 2012). Subsequent study with Chinese herb honeysuckle led to similar observations where xenomiRs exhibited therapeutic implications by inhibiting viral replication both in vitro and in vivo (Zhou et al. 2015). However, in order to ensure efficient uptake upon oral administration and elicit phenotypic response at target site, dietary biomolecules need to be maintained in native state and survive the harsh and degrading gut environment (Dickinson et al. 2013; Snow et al. 2013). Thus, packaging of the xenomiRs in EVs was proposed in these studies to explain their survival in gut and subsequent intestinal uptake into systemic circulation (Zhang et al. 2012; Zhou et al. 2015).

Initial observations indicating a functional role of rice xenomiRs in altering the recipient's physiology sparked considerable interest in the field. Ever since, numerous studies have probed the idea of plant bioactive RNAs and EVs as vectors that mediate species crosstalk (Otsuka et al. 2018; Akuma et al. 2019; Munir et al. 2019). Corroborating with this, EVs have been isolated from multiple plant based dietary sources including grapefruit (Zhuang et al. 2016), ginger (Zhang et al. 2016a), lemon (Raimondo et al. 2015) and grapes (Ju et al. 2013) among others (Munir et al. 2019). Plant EVs have been characterized to present features similar to eukaryotic EVs, thus also referred as EV-like nanoparticles (ELNs) (Ju et al. 2013; Chen and Yu 2019). Further, their application as natural therapeutics as well as nanovectors for in vivo drug delivery has also been probed (Zhang et al. 2016b, c; Akuma et al. 2019; Munir et al. 2019). For instance, ginger-derived ELNs (GELN) target intestinal epithelial cells (IECs) in vitro and demonstrated anti-inflammatory therapeutic potential in vivo by promoting intestinal repair and preventing colitis and associated cancers (Zhang et al. 2016a). Similarly, another independent study confirmed GELN anti-inflammatory function in vivo by demonstrating uptake by macrophages resulting in inhibition of aberrant activation of inflammasomes with implications in Alzheimer's disease and diabetes (Chen and Yu 2019).

Anti-inflammatory mode of action has also been associated to EVs isolated from broccoli and citrus fruits such as grapes and grapefruit with immense therapeutic benefits *via* mediating intestinal repair, IEC proliferation and preventing colitis in mice models, similar to GELNs (Ju et al. 2013; Mu et al. 2014; Deng et al. 2017). Overall, the ability to facilitate inter-kingdom communication and a vast untapped therapeutic potential of dietary EVs laden with bioactive compounds is apparent. However, majority of these studies indicate a localised activity of dietary EVs limited to the gut rather than peripheral tissues (Munir et al. 2019). Furthermore, lemon EVs exhibited in vivo anti-tumor activity by inducing tumor cell apoptosis in various xenograft models. Yet, concrete evidence suggesting oral bioactivity and bioavailability due to EVs entering systemic circulation and reaching peripheral tissues could not be obtained as lemon EVs were administered intraperitoneally (Raimondo et al. 2015). Interestingly, another study with GELNs demonstrated oral bioavailability and bioactivity by preventing alcohol induced liver damage, highlighting the ability of dietary EVs to reach peripheral tissues upon oral administration (Zhuang et al. 2015).

Milk as a neonatal diet is enriched with a variety of biomolecules including proteins, casein fragments, amino acids, cytokines, antibodies, antimicrobials and miRNA. These bioactive factors may aid in regulating pathophysiological processes in recipients accounting for the immense benefits such as early growth and development in infants (German et al. 2002; Ballard and Morrow 2013; Hosseini et al. 2014; Le Doare et al. 2018; Benmoussa and Provost 2019). In addition, milk from various mammalian sources has been found to contain abundant quantities of milk-derived EVs (MEVs) that are enriched with protein and miRNA cargo implicated in immunomodulation and regulating growth and development (van Herwijnen et al. 2016; Chen et al. 2017; Samuel et al. 2017; Sedykh et al. 2017). MEVs have been

shown to promote proliferation and survival of IECs, thus ensuring gut development and integrity in infants (Chen et al. 2016; Martin et al. 2018).

MEVs have been demonstrated to withstand intense conditions of acidification, boiling and enzymatic activity in vitro (Izumi et al. 2015; Pieters et al. 2015; Benmoussa et al. 2016; Rahman et al. 2019). Furthermore, MEVs have also been found to survive processing steps in pasteurization, ultra-heat treatment and prolonged periods of freezing (Howard et al. 2015; Manca et al. 2018; Melnik and Schmitz 2019). Hence, the remarkable stability of MEVs, at least in vitro, has led to the speculation that MEVs could also survive gut and be bioavailable in consuming organism. Further, in vivo studies have also suggested a cross-species activity of bovine MEVs by promoting myogenesis and osteogenesis in rat and mice models, respectively (Oliveira et al. 2016; Parry et al. 2019). Similar to plant ELNs, MEVs have been studied for their immunomodulatory and anti-inflammatory mode of action, influencing recipient's gut and systemic immunity (Sanwlani et al. 2020). Human MEVs induced an increased number of regulatory T cells in vitro, associated with tolerance induction and thus lower risk of atopy and autoimmunity upon milk consumption (Admyre et al. 2007; Melnik et al. 2014). Similarly, MEVs from commercial milk too have been shown to induce differentiation of immune cells in vitro (Pieters et al. 2015). Bovine MEVs also have displayed therapeutic benefit and have been demonstrated to be effective in preventing onset of rheumatoid arthritis (Arntz et al. 2015). Corroborating with this, other studies have demonstrated MEV efficiency in treatment of colitis and osteoporosis in vivo (Benmoussa et al. 2019; Yun et al. 2020). Similarly, bovine MEVs have been demonstrated to work efficiently as oral drug-delivery vectors by encapsulating chemotherapeutics to target tumor tissues in vivo (Munagala et al. 2016). Overall, these studies suggest a functional role for dietary EVs in mediating species crosstalk and controlling recipient's pathophysiology.

Pitfalls and Limitations of Dietary EV Studies

The premise of dietary EVs in inter-species crosstalk has led to a paradigm shift, igniting considerable interest to elucidate their functional role (Akuma et al. 2019; Munir et al. 2019; Zempleni et al. 2019; Sanwlani et al. 2020). However, many studies in an attempt to validate the delivery and function of dietary biomolecules in vivo suggested a lack or ineffective intestinal uptake and phenotypic manifestation (Dickinson et al. 2013; Snow et al. 2013; Laubier et al. 2015; Title et al. 2015; Auerbach et al. 2016). Further, the amount of dietary miRNA detected in serum upon PCR amplification in one such study was rendered physiologically irrelevant (Witwer et al. 2013). Non-specific amplification of host sequences instead of dietary miRNA has also been implicated (Witwer 2018). Collectively, these studies have argued against the bioavailability of dietary biomolecules and attributed the functional effect to nutrition over bioavailable EVs or RNA. Furthermore, current studies suggesting a role for dietary EVs in species crosstalk do not provide detailed

mechanistic insights to explain the intestinal uptake of EVs and entry into systemic circulation from the gut. As discussed, these studies have demonstrated the feasibility of IEC uptake of dietary EVs accounting for in vivo function. Although few studies with plant ELNs and MEVs have claimed that dietary EVs reach peripheral tissues and even cross blood-brain barrier upon oral administration, they have not been traced or detected in systemic circulation (Zhuang et al. 2015; Munagala et al. 2016; Manca et al. 2018). Whereas, other studies demonstrating dietary EV activity at peripheral tissue sites used alternative administration routes such as intraperitoneal or intravenous (Raimondo et al. 2015; Wang et al. 2015; Munagala et al. 2016; Manca et al. 2018). Most studies suggesting oral bioavailability of dietary EVs in peripheral tissues have hypothesized in vivo uptake to occur *via* paracellular translocation or transendocytosis (Wolf et al. 2015; Kusuma et al. 2016; Sanwlani et al. 2020). Presence of dietary exogenous biomolecules in serum of colon cancer or ulcerative colitis patients, possibly due to compromised gut integrity, adds merit to such hypothesis (Wang et al. 2012). However, it is noteworthy that none of the functional studies have been successfully able to demonstrate either of these hypothesized mechanisms of EV uptake in vivo (Fig. 21.1).

Furthermore, it is unclear whether dietary EVs can survive the harsh degrading conditions of the gut. Though several studies have suggested that dietary EVs may be intrinsically suited to survive the gut environment, this has only been observed in vitro by simulating the intestinal conditions. Thus, unequivocal proof of EV stability in gut in vivo is not understood (Fig. 21.1). In this regard, there is an apparent need to demonstrate the presence of dietary EVs in circulation in the recipient to unequivocally state that dietary EVs are bioavailable upon oral administration. Even though several studies have demonstrated dietary EVs labelled with lipophilic dyes to reach peripheral tissues, such methods are not completely reliable (Munagala et al. 2016; Manca et al. 2018). Lipophilic dyes, such as DiR and PKH for instance are known to modify the EV properties upon binding. Further, they may be able to diffuse freely into circulation upon administration thus rendering the results in dietary EV bioavailability studies unreliable (Takov et al. 2017; Simonsen 2019). Hence, orthogonal experiments are needed to confirm the bioavailability of dietary EVs.

Extrapolating the role of dietary EVs in species crosstalk, studies have reported their feasibility and efficiency as drug-delivery vectors (Wang et al. 2013; Munagala et al. 2016; Zhuang et al. 2016; Iravani and Varma 2019). However, mechanisms underlying the ability of dietary EVs to target specific cell types and tissues remain ambiguous. In addition, heterogeneity of dietary EVs and varying cargo composition due to presence of multiple subtypes in isolated fractions may render it challenging to attribute the function to a particular subtype of EVs (Zonneveld et al. 2014). Furthermore, varying composition of EVs may lead to contradictory results in terms of phenotype observed (Fig. 21.1). Lastly, dietary EVs have been shown to interact with gut microflora and induce changes in bacterial populations and regulate their metabolic behaviours (Teng et al. 2018; Benmoussa et al. 2019; Zhou et al. 2019). This may indicate that the functional effects upon oral administration of dietary EVs could be indirect or secondary and be regulated by their effects on gut microbiome.

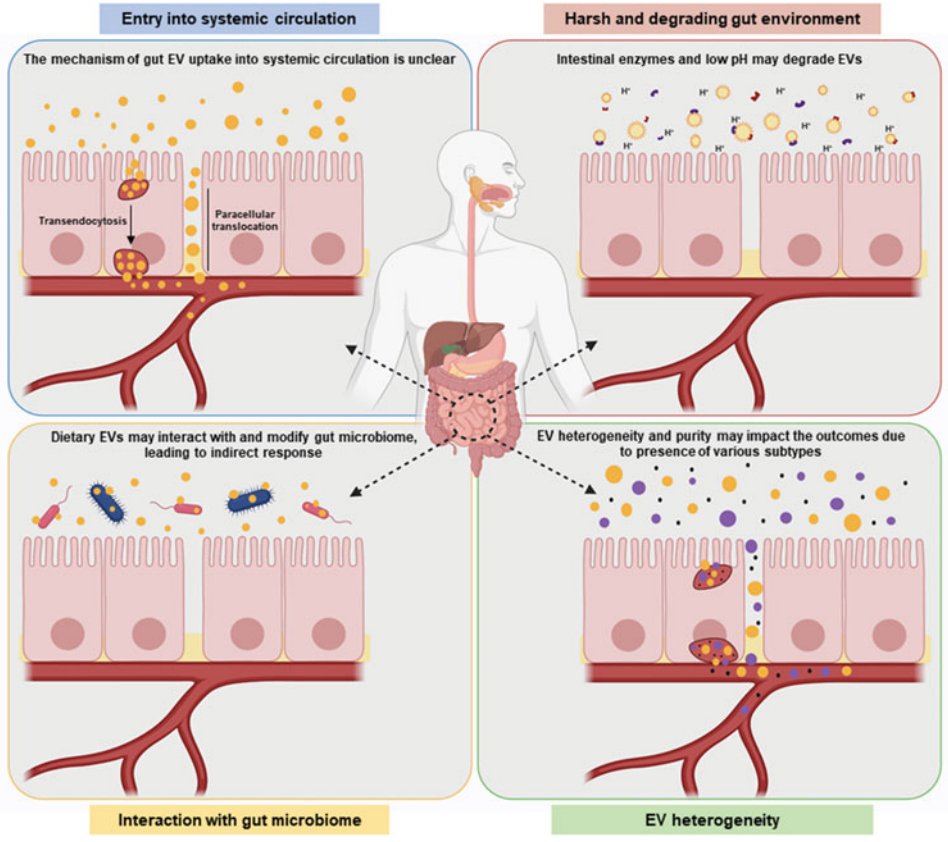

Fig. 21.1 Challenges facing oral bioactivity and bioavailability of dietary EVs. Schematic illustration to demonstrate four major challenges to oral bioactivity and bioavailability of dietary EVs as current drawbacks to accept their role in cross-species and kingdom communication. (**a**) Paracellular translocation and transendocytosis are hypothesized mechanisms to explain intestinal uptake of dietary EVs upon oral delivery. However, neither of these mechanisms has been

successfully demonstrated in vivo to account for intestinal EV uptake. (**b**) In vitro assays have determined inherent stability of dietary EVs. However, simulated intestinal conditions in vitro do not guarantee similar stability in vivo. (**c**) Dietary EVs have been shown to interact with gut microbiome and alter its composition. Thus, the observed effects in vivo could be indirectly accounted to dietary EV-gut microflora interaction. (**d**) EV heterogeneity may account for varying composition and purity upon isolation with different methods reflecting in varying phenotypes. Figure generated with Biorender.com

Overall, there are several limitations to assigning the functional roles of dietary EVs to be a direct consequence of their activity at the target site (Fig. 21.1). Nevertheless, the functional effect of dietary EVs upon oral administration, direct or indirect, has been claimed by multiple studies. The concerns surrounding their oral bioavailability and mechanistic insights of intestinal uptake need to be addressed in more detail prior to accepting their indisputable role in species crosstalk.

Concluding Remarks

Current studies have demonstrated the ability of dietary EVs in mediating cross species and kingdom communication. However, scepticism surrounds the idea challenging the rationality of the concept (Witwer and Zhang 2017). Uptake and functional role of dietary EVs has been largely proposed on basis of in vitro observations which is a proof-of-concept of their plausible in vivo function at best. Lack of experimental evidence demonstrating in vivo stability, uptake and function of dietary EVs hinders the wider acceptance of the phenomenon by scientific community. Furthermore, lack of mechanistic insights explaining intestinal EV uptake coupled with contradictory findings by several groups claiming either no functional role or an indirect role of dietary EVs have led to two schools of thought (Yang et al. 2015, 2016; Witwer and Zhang 2017; Zempleni et al. 2019). Current endeavours with clinical applications of dietary EVs have successfully utilised them in disease models as novel therapeutics and drug-delivery vehicles advocating their possible role in species crosstalk (Arntz et al. 2015; Raimondo et al. 2015; Munagala et al. 2016; Zhang et al. 2016a). Nevertheless, the functional ability of dietary EVs, direct or indirect, cannot be ignored. Overall, improvements in EV isolation methods coupled with strategies to study their bioavailability in systemic circulation and peripheral tissues will be critical to progress the field of cross-species communication and for the wider acceptance of the concept among researchers.

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