Extracellular vesicles (EVs), such as exosomes and microvesicles, have over the last 10–15 years been recognized to convey key messages in the molecular communication between cells. Indeed, EVs have the capacity to shuttle proteins, lipids, and nucleotides such as RNA between cells, leading to an array of functional changes in the recipient cells. Importantly, the EV secretome changes significantly in diseased cells and under conditions of cellular stress. More recently, it has become evident that the EV secretome is exceptionally diverse, with many different types of EVs being released by a single cell type, and these EVs can be described in terms of differences in density, molecular cargos, and morphology. This review will discuss the diversity of EVs, will introduce some suggestions for how to categorize them, and will propose how EVs and their subpopulations might be used for very different therapeutic purposes.

1. Introduction

Since the 1940s, when the first indication of the existence of extracellular particles and vesicles was observed in platelet-free serum (Chargaff and West, 1946), a massive number of studies on extracellular vesicles (EVs) have been conducted. Seventy years of research and over 6000 publications illustrate a substantial diversity in isolated vesicles based on differences in isolation protocols, cell origins, and biological functions, all leading up to an overwhelming and sometimes confusing and conflicting nomenclature. Over these years, several types of EVs have been identified and assigned various names based on size, including nanovesicles, microvesicles, virus-like particles, exosome-like vesicles, and microparticles; based on biogenesis, including exosomes, membrane particles, outer membrane vesicles, and shedding membrane vesicles; or based on cell origin or function, including platelet-dust, oncosomes, matrix-vesicles, ectosomes, dexosomes, texosomes, epididymosomes, cardiosomes, prostasomes, rhinosomes, apoptotic bodies, and tolerosomes. It is obvious that there are overlaps between the different subgroups; for example, tolerosomes are vesicles that can induce tolerance in the immune system (Karlsson et al., 2001), and they can be considered nanovesicles based on size or as exosomes if they are formed in the multivesicular body (MVB) pathway. However, it is also clear that within these subpopulations there are even further subpopulations based on vesicle size, density, RNA, protein, and DNA cargo as well as morphology. In this article, we will review how EVs might be subdivided into a vast array of subpopulations, and furthermore we discuss their potential as therapeutics.

2. Current state of the art

Currently, EVs are classified into three broad classes based on their biogenesis as exosomes, microvesicles, and apoptotic bodies. The population with the largest vesicle size, apoptotic bodies, consists of vesicles that are heterogeneous in size, with a diameter range from 200 nm to 5 μm, and they are shed from the plasma membrane of dying cells undergoing programmed cell death. Microvesicles, or ectosomes as they are sometimes referred to, are considered to be shed from the plasma membrane of viable cells and are 100–800 nm in size. Exosomes are 30–150 nm in size and are released into the extracellular space when MVBs fuse with the plasma membrane and release their intraluminal vesicles (Gyorgy et al., 2011; Mathivanan et al., 2010; Thery et al., 2009; van der Pol et al., 2012). However, it has been shown that vesicles that are larger than 100 nm are present within the endosomal pathway (Ronquist and Brody, 1985) and that vesicles smaller than 100 nm...
can bud off directly from the plasma membrane (Booth et al., 2006), which highlights some of the limitations of this decades-old classification. Therefore, totally different approaches to understanding the diversity of extracellular vesicles will be required for future classifications.

Particles of different size have different sedimentation properties, and therefore EVs can be isolated by differential centrifugation (Cvjetkovic et al., 2014; Jeppesen et al., 2014). Apoptotic bodies are released and therefore EVs can be isolated by differential centrifugation. Additionally, EVs can be further purified and separated based on their density. Thus, density gradients and cushions using sucrose or iodixanol have been widely used during EV isolation. Depending on the type of EV-producing cell and type of EV subpopulation isolated, various densities has been reported (Table 1). However, the vesicles isolated in these preparations can be very heterogeneous, probably because of co-isolation of different subpopulations of EVs during the different purification steps. Therefore, these protocols will, at least to some degree, isolate mixed populations of EVs, and it has been suggested that the vesicles isolated in this way should more properly be called 2 K, 10 K, and 100 K vesicles, or possibly large, middle-sized, and small vesicles, or high and low density EVs, because the biogenesis of each vesicle preparation is uncertain and may be independent of their size and density (Kowal et al., 2016).

3. Emerging subpopulations of extracellular vesicles

3.1. Subpopulations of extracellular vesicles based on morphology

For a long time, it was believed that all EVs were spherical in shape and having a very particular and quite limited size distribution. When examined with negative stain by transmission electron microscopy (TEM), they were also described as cup-shaped, with no information on their interior structure. More recently, EVs have also been examined by Cryo-TEM, a technique that uses no fixatives and thus allows the studied vesicles to retain their natural state. It was revealed that the cup-shaped morphology was an artifact from the fixation steps included in the negative stain technique (Gould and Raposo, 2013) and that the morphology of EVs was much more diverse than previously believed.

In a recent publication, we categorized EVs that had been released by a human mast cell line and isolated by floating at approximately at 1.11–1.12 g/cm³. Most of the isolated vesicles (>75%) had a single membrane and were smaller than 100 nm in diameter. However, within this isolate we could in total define nine different categories of vesicles as illustrated in Fig. 1, including one or more vesicles within another vesicle, double membrane vesicles, and various subpopulations of EVs during the different purification steps. Therefore, these protocols will, at least to some degree, isolate mixed populations of EVs, and it has been suggested that the vesicles isolated in this way should more properly be called 2 K, 10 K, and 100 K vesicles, or possibly large, middle-sized, and small vesicles, or high and low density EVs, because the biogenesis of each vesicle preparation is uncertain and may be independent of their size and density (Kowal et al., 2016).

Table 1
List of publications studying subpopulations of EVs based on density.

<table>
<thead>
<tr>
<th>EV source</th>
<th>Isolation protocol</th>
<th>Subpopulations of EVs</th>
<th>Analyses</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human seminal fluid from vasectomized men</td>
<td>3000 × g 10 min; 2 × 10,000 × g 20 min; supernatant top-loaded on density cushion (0.7M/2.0M sucrose), 100,000 × g 75 min; SEC column (Sephacryl S-1000), pooled fractions were loaded onto a sucrose gradient (2.0–0.4M), bottom-loaded, 16 h, 190,000 × g</td>
<td>Low density (~1.09–1.16 g/cm³); High density (~1.23–1.26 g/cm³)</td>
<td>EM, LC-MS/MS, WB</td>
<td>(Aalberts et al., 2012)</td>
</tr>
<tr>
<td>Human seminal fluid from vasectomized men</td>
<td>3000 × g 10 min; 2 × 10,000 × g 20 min; supernatant was loaded onto a SEC column (Sephacryl S-1000), fractions were pooled and loaded onto a sucrose gradient (2.0–0.4M), bottom-loaded, 16 h, 190,000 × g</td>
<td>Low density (~1.13–1.71 g/cm³); High density (~1.24–2.61 g/cm³)</td>
<td>Cryo-TEM, lipid analysis, WB</td>
<td>(Brouwers et al., 2013)</td>
</tr>
<tr>
<td>Mouse melanoma cell line (B16F10)</td>
<td>2000 × g 10 min; 10,000 × g 30 min; 110,000 × g 70 min; 110 K pellet was loaded onto a sucrose gradient (2.5–0.4M), bottom-loaded, 16 h, 200,000 × g</td>
<td>Low density (~1.12–1.19 g/cm³); High density (~1.26–1.29 g/cm³)</td>
<td>EM, nanoLC-MS/MS, RNA size distribution (Bioanalyzer), gene expression analysis in recipient cells, WB</td>
<td>(Willms et al., 2016)</td>
</tr>
<tr>
<td>Human monocyte-derived dendritic cells (from blood samples of healthy donors)</td>
<td>300 × g 10 min; 2000 × g 20 min; 10,000 × g 40 min; 100,000 × g 90 min; 10 K and 100 K pellets were loaded onto an iodixanol gradient (10%, 20%, 30%), bottom-loaded, 1 h, 350,000 × g</td>
<td>F3 10 K pellet (~1.115 g/cm³); F5 10 K pellet (~1.145 g/cm³); F3 100 K pellet (~1.115 g/cm³); F5 100 K pellet (~1.145 g/cm³)</td>
<td>EM, WB, LC-MS/MS</td>
<td>(Kowal et al., 2016)</td>
</tr>
<tr>
<td>Human mast cell line (HMC-1)</td>
<td>300 × g, 10 min; 16,500 × g 20 min; 120,000 × g 70 min; 120 K pellet was loaded onto a sucrose gradient (2.5–0.4M), bottom-loaded, 16 h, 175,000 × g</td>
<td>Low density (~1.09–1.21 g/cm³); High density (~1.24–1.31 g/cm³)</td>
<td>EM, RNA size distribution, miRNA and mRNA microarray, next generation sequencing, LC-MS/MS, WB, Flow cytometry</td>
<td>(Lasser et al., 2017)</td>
</tr>
<tr>
<td>Bone marrow-derived mesenchymal stem cells</td>
<td>1500 × g, 20 min; supernatant concentrated with ultrafiltration (3 KDa); iodixanol gradient (5%, 10%, 20%, 40%), top-loaded, 18 h, 100,000 × g</td>
<td>Low density (~1.01–1.06 g/cm³); Medium density (~1.08–1.15 g/cm³); High density (~1.27–1.27 g/cm³)</td>
<td>EM, miRNA (qRT) PCR, protein array, proliferation in recipient cells</td>
<td>(Collino et al., 2017)</td>
</tr>
</tbody>
</table>

EM, electron microscopy; WB, western blot; SEC, size exclusion chromatography.
oval vesicles, and small and large tubules, to mention a few (Zabeo et al., 2017). Furthermore, we could identify three additional vesicle features, including membrane coatings, filaments, and electron dense areas. These features could be present in any of the nine categories, which further increased the diversity of the isolated EVs. Importantly, even though we isolated EVs with a very specific density, the morphological diversity of the isolated EVs was vast, arguing that vesicles with a certain density probably consist of several subpopulations of vesicles. Additionally, we have suggested that the filaments seen with Cryo-TEM are actin and that these vesicles therefore might be a subpopulation of EVs that possess motile capabilities, especially because we could observe different shapes in vesicles isolated from different sources (Cvjetkovic et al., 2017). Because it is possible that some of the morphological characteristics of cell culture-derived EVs might have been a result of the isolation protocol, we performed another study on unprocessed fresh human ejaculates (Höög and Lötvall, 2015). Most of the observed vesicles (>59%) were single membrane, with an average size of 150 nm. However, a total of 11 categories of membrane vesicles could be identified, including the nine categories that were also found in cell culture media (Höög and Lötvall, 2015; Zabeo et al., 2017) (Fig. 1). Also in the ejaculate we could observe coated, electron-dense, and filamentous vesicles, as well as membranous tubes. Some of these morphological subpopulations were also observed in another study using cryo-TEM to determine the morphology of vesicles that were isolated from ejaculate with a density gradient (Poliakov et al., 2009). The authors of that study also observed vesicles with single or multiple membrane vesicles inside of them; however, in that study the multiple-membrane vesicles were more common than single-membrane vesicles. Elongated vesicles (tubes) and protrusions on the surfaces of the vesicles were also described, presumably equivalent to the coated vesicles that we have described, as well as electron-dense vesicles (Poliakov et al., 2009). However, the percentage of vesicles with protrusions was higher in our study (25–30% vs. 1.5%), which could indicate that this subpopulation of EVs was enriched during the applied isolation procedure (Höög and Lötvall, 2015; Poliakov et al., 2009).

Additionally, EVs in unprocessed platelet-free plasma have been described by cryo-TEM (Arraud et al., 2014). These investigators demonstrated that approximately 50% of the EVs were single spherical vesicles that were 30 nm–1 μm in diameter. Similarly, the investigators also found tubular vesicles in plasma (Arraud et al., 2014). Tubes have also been found in EVs (floating at 1.12–1.18 g/cm³) isolated from breast milk; however, they were not quantified in this study (Zonneveld et al., 2014). However, tubes were much more common in plasma than in the ejaculate and mast cell culture media (50% vs. ~3%) (Arraud et al., 2014; Höög and Lötvall, 2015; Zabeo et al., 2017). Together, these studies show that the morphology of EVs is far beyond that of single-membrane, round, 100 nm vesicles (Fig. 1). Furthermore, they show that one cell type has the capacity to release EVs with many different morphologies and that all of these morphological subpopulations can be found in unprocessed samples and are not artifacts from the isolation method. It is also interesting to note that the morphologies of vesicles that are present inside of mast cell MVBs before they fuse with the plasma membrane can diverge significantly (Zabeo et al., 2017), suggesting that EVs formed and released in a similar fashion can still be morphologically diverse.
3.2. Subpopulations of extracellular vesicles with different densities

For decades, the density of the classical EVs was considered to be in the range of 1.11–1.18 g/cm³, but it was unclear whether EVs with other densities exist (Gyorgy et al., 2011; Mathivanan et al., 2010; Thery et al., 2009; van der Pol et al., 2012). Recently, we and others have characterized subpopulations of EVs distributed at different densities (Table 1), and these different EV preparations have at least partly different protein, lipid, and RNA cargos, as well as different biological functions (Aalberts et al., 2012; Brouwers et al., 2013; Collino et al., 2017; Kowal et al., 2016; Lässer et al., 2017; Willms et al., 2016).

Low-density EVs (1.12–1.20 g/cm³) released by a human mast cell line, a human erythroleukemic cell line, and a mouse skin melanoma cell line all had evidence of 18S and 28S ribosomal RNA subunits and a narrow peak of small RNA below 100 nt in size when analyzed with a Bioanalyzer (Lässer et al., 2017; Willms et al., 2016). The high-density EVs (1.25–1.30 g/cm³) produced by these cells, however, contained small or no ribosomal RNA peaks and instead had peaks for short RNAs of a broader size range up to 1 000 nt (Lässer et al., 2017; Willms et al., 2016). Interestingly, it was also shown that the microRNA (miRNA) and messenger RNA (mRNA) in the exo-dense population were released by a human mast cell line from both human breast cancer cell line correlated with the cellular RNA, while the mRNA and miRNA in the high-density EVs released by those cell lines did not (Lässer et al., 2017; Palma et al., 2012). This suggests that the biogenesis and RNA loading processes are different for these two preparations of EVs with different density. Furthermore, mitochondrial rRNA and tRNA, as well as Y RNA and piRNA were enriched in the low-density EVs released by mast cells compared to the high-density EVs released by these cells, while the high-density EVs were enriched in nuclear associated RNAs such as snRNA, snRNA, and vault RNA (Lässer et al., 2017). It has also been shown that Y RNA and SRP-RNA are associated with low-density vesicles and not the high-density fraction in vesicles derived from co-cultures of dendritic cells and T cells (Nolte-'t Hoen et al., 2012). Additionally, it was shown that the high-density EVs released by the mast cells and skin melanoma cells were enriched in ribosomal proteins (Lässer et al., 2017; Willms et al., 2016), while low-density EVs from the mast cells were associated with mitochondrial proteins, which was in accordance with these EVs also containing mitochondrial RNA (Lässer et al., 2017). The high-density EVs released by a human mast cell line and a mouse skin melanoma cell line, and those found in seminal fluid, were significantly smaller in size compared to the low-density EVs (Aalberts et al., 2012; Brouwers et al., 2013; Lässer et al., 2017; Willms et al., 2016), and the high-density and low-density EVs derived from a mouse skin melanoma cell line differentially affected the gene expression in endothelial cells suggesting that different populations of EVs have distinct biological effects on recipient cells (Willms et al., 2016).

Furthermore, a study on low-density and high-density EVs from human seminal fluid from vasectomized men showed that the high-density EVs were relatively enriched in ceramide, while the low-density EVs were relatively enriched in sphingomyelin (Brouwers et al., 2013), which together with previously mentioned studies (Lässer et al., 2017; Willms et al., 2016) shows that the high-density and low-density subpopulations of EVs are distinct in their RNA, protein, and lipid content.

When different subpopulations of EVs, including 10 K (usually referred to as microvesicles) and 100 K pellets (usually referred to as exosomes), were loaded onto a density gradient, they both generated two separate populations of EVs, one that was recovered at 1.115 g/cm³ (called F3–10 K and F3–100 K) and one at 1.145 g/cm³ (called F5–10 K and F5–100 K) (Kowal et al., 2016). A majority of the proteins were common to all of the subpopulations of EVs, including CD63. However, the different subpopulations of EVs also had unique or enriched proteins. For example, ribosomal proteins were enriched in both 10 K subpopulations (F3–10 K and F5–10 K). However, mitochondrial proteins were enriched in the F5–10 K EV, while proteasome proteins were enriched in the F3–10 K EV. The F3–100 K EV were enriched in proteins associated with the plasma membrane and endosomes (Kowal et al., 2016). Besides showing that exosomes and microvesicles have the same density, this study further argued that the low-density EVs consists of multiple subpopulations.

Few studies have described EVs with a density below 1.1 g/cm³. However, one study suggests that a human colon carcinoma cell line released prominin-1+ and CD63+ vesicles that floated at 1.055 g/cm³ (Marzesco et al., 2005). Interestingly, the same vesicles were not positive for both proteins, indicating that two subpopulations of vesicles were present at this density. Additionally, a recent study identified three subpopulations of EVs released by bone marrow–derived mesenchymal stem cells (MSCs), based on density, including high-density EVs (~1.16–1.28 g/cm³), medium-density EVs (~1.08–1.14 g/cm³), and low-density EVs (~1.01–1.06 g/cm³) (Collino et al., 2017). It was shown that the CD63 and CD81 expression was the strongest in the medium-density EVs (overlapping in density with the low-density EVs described in the studies mentioned above) and that these vesicles were enriched in miRNAs compared to the low-density and high-density subpopulations (Collino et al., 2017).

It has been shown that primary dendritic cell–derived EVs migrate differently in the two most commonly used density gradient media (sucrose and iodixanol (Kowal et al., 2016)), which might at least partly explain the slightly different density distributions of EVs in the studies listed above. Furthermore, it has also been shown that some vesicles found in human seminal fluid as well as vesicles released by mouse skin melanoma cells and a breast cancer cell line have delayed flotation. Thus, if the density gradients were centrifuged for 6–16 h two populations of vesicles could be separated from each other at 1.06–1.16 g/cm³ and 1.25–1.29 g/cm³; however, if the density gradients were centrifuged for 72–90 h, all vesicles were found at 1.06–1.19 g/cm³. This phenomenon is most likely due to the different sizes of the populations because small vesicles move slower through a gradient (Aalberts et al., 2012; Brouwers et al., 2013; Palma et al., 2012; Willms et al., 2016). However, this indicates that there is a subpopulation of vesicles that is fast floating and one that is slow floating, and thus by centrifuging for a shorter time it is possible to separate these from each other for further analysis. This might also explain differences between different studies in which the results might depend on the time the EVs have been allowed to float.

Together these studies show that there are multiple subpopulations of EVs with different densities (Table 1) and that vesicles that have minimal differences in their densities can have very different RNA, protein, and lipid cargos.

3.3. Differences in RNA and DNA cargos in subpopulations of extracellular vesicles

In 2007, we showed that exosomes carry both mRNA and miRNA and that the mRNA was able to produce new proteins when taken up into a recipient cell (Valadi et al., 2007). Subsequently, it was also shown that the miRNA in exosomes can transfer functionality to a recipient cell (Pegtel et al., 2010). The presence of RNA in larger EVs such as apoptotic bodies and microvesicles had been described previously (Biggiogera et al., 1998; Casciola-Rosen et al., 1994; Ratajczak et al., 2006), but the direct biological function of any shuttled cargo RNA was less well described. Subsequently, several studies have investigated the RNA content in different
subpopulations of EVs, but most of the early studies evaluated the RNA cargo only in one EV population, either in an EV pool or in one of the high- or low-density fractions. Direct comparisons between subpopulations of EVs from a single cell type have only been studied in recent years. Recently we have shown that apoptotic bodies, microvesicles, and exosomes have distinct RNA profiles when isolated from several different cell lines (Crescitelli et al., 2013; Lazaro-Ibanez et al., 2017; Lunavat et al., 2015). Apoptotic bodies had a similar RNA profile as cells when analyzed with a Bioanalyzer instrument, carrying peaks for the 18S and 28S rRNA subunits. Microvesicles contained relatively less RNA, but the RNA profile was similar to that of apoptotic bodies. Exosomes, on the other hand, contained no or only small peaks for rRNA and had a broader size distribution of small RNAs (Crescitelli et al., 2013; Lunavat et al., 2015). It was also evident that the small RNA profiles were different in the different subpopulations of EVs released by a human melanoma cell line, and we therefore sequenced the small RNAs in all three subpopulations of EVs that had been separated based on sequential centrifugation (Lunavat et al., 2015). Ro-associated Y RNA was enriched in all subpopulations of EVs compared to the cells, while snRNA and sn1RNA was enriched in cells compared to all three EV subpopulations (Lunavat et al., 2015). Additionally, mitochondrial RNA was only enriched in microvesicles and apoptotic bodies, and not in exosomes, compared to cells (Lunavat et al., 2015). This is interesting because mitochondrial RNA has also been shown to be enriched in the low-density EVs (Lässer et al., 2017), which suggests that the low-density EVs have similarities to microvesicles. Furthermore, the exosomes contained a unique set of miRNA compared to the other EV subpopulations and the cells (Lunavat et al., 2015). Additionally, as mentioned before, low-density EVs contain rRNA, while high-density EVs generally do not (Crescitelli et al., 2013; Lazaro-Ibanez et al., 2017; Lunavat et al., 2015; Lässer et al., 2017; Willms et al., 2016). This highlights that there might be an overlap between several of these different classifications of subpopulations of EVs (Fig. 2).

In contrast to RNA, little is known about DNA in subpopulations of EVs. Several types of DNA such as single-stranded genomic DNA (Balaj et al., 2011), double-stranded genomic DNA (Cai et al., 2013; Lazaro-Ibanez et al., 2014; Ronquist et al., 2011), and mitochondrial DNA (Guescini et al., 2010) have been identified in EVs from several different cell types. Whether single and double-stranded genomic DNA and mitochondrial DNA are present in different subpopulations of EVs or if one subpopulation of EVs contains all the DNA or if all EVs contain DNA needs to be further examined. Additionally, DNA has been suggested to be present both on the inside (Lazaro-Ibanez et al., 2014; Ronquist et al., 2009; Waldenstrom et al., 2012) and on the outside of EVs (Shelke et al., 2016). Whether this is true for different subpopulations of EVs from other cell types has to be examined further.

3.4. Subpopulations of extracellular vesicles defined by surface molecules and protein cargo

Several studies have focused on determining the proteomes of individual subgroups of EVs; however, we have here focused on the publications that isolated and compared several subpopulations of EVs from the same cells or from the same body fluids. For a long time, the field generated lists of proteins that were enriched in the EVs referred to as “exosomes”, which were primarily isolated by sequential high-speed ultracentrifugation, while less was known about the other subpopulations that were pelletted at lower speeds or that floated at different densities. As the volume of proteomics data for other subgroups of EVs increased, it became evident that several of the markers commonly known as “exosome-markers” were also present in the other subpopulations of EVs such as microvesicles and apoptotic bodies (Crescitelli et al., 2013; Cronqvist et al., 2014; Kowal et al., 2016; Tong et al., 2016; Willms et al., 2016). Thus, classical exosome markers such as ALIX, TSG101, CD9, CD81, and CD63 were identified in both high- and low-density EVs (Willms et al., 2016). Additionally, the classical exosome markers CD63, CD9, and CD81 were enriched in the 100,000 × g pellet compared to the 2000 × g and 10,000 × g pellets, but they could still be detected in all three subtypes of EVs (Crescitelli et al., 2013; Kowal et al., 2016). Furthermore, the commonly used exosome markers MHC class II, HSC70, and flotillin were also detected in all three subpopulations of EVs but were not enriched in the 100,000 × g pellet compared to the other two subpopulations, showing that these markers cannot be used to distinguish subpopulations of EVs (Kowal et al., 2016).

In the wake of these studies, new proteins that seem to be unique or enriched in different subpopulations of EVs have been identified. For example, it has been shown that alpha-actinin 4 is enriched in the low-density EVs compared to the high-density EVs (Lässer et al., 2017; Willms et al., 2016). As discussed above, the low-density EVs were significantly larger, similar to the study by Kowal and co-workers that showed that alpha-actinin 4 and alpha-actinin 1 were enriched in larger and middle-sized vesicles isolated at 2000 × g and 10,000 × g compared to the smaller vesicles collected at 100,000 × g (Kowal et al., 2016). Additionally, alpha-actinin 4 is enriched in larger vesicles when size exclusion chromatography is used (Willms et al., 2016). On the other hand, syntenin-1 and ADAM10 are enriched in small vesicles collected at 100,000 × g compared to larger vesicles collected at 2000 × g and 10,000 × g (Kowal et al., 2016). However, ephrin type-A receptor 2 and 5 are enriched in high-density EVs compared to low-density EVs (Collino et al., 2017; Willms et al., 2016).

Besides using density gradients to determine the protein cargo of subpopulations of EVs, immuno-affinity has also been used to separate subpopulations of EVs based on the expression of membrane proteins that might represent differences in biogenesis (Kowal et al., 2016; Tauro et al., 2013). Two subpopulations of EVs released by a human colon carcinoma cell line were isolated using beads coated with anti-A33 and anti-EpCAM antibodies (Tauro et al., 2013). The cells were grown as organoids with polarized cells, and anti-A33 was used to capture EVs released from the basolateral side of the cells, while anti-EpCAM was used to capture the EVs released from the apical side. Interestingly, both subpopulations were similar in size (40–60 nm) and both were positive for ALIX and TSG101. However, when the proteomes were analyzed with LC-MS/MS, it was shown that the EpCAM+ vesicles were exclusively positive or enriched with several tetraspanins such as CD63, CD81, TSPAN3, and TSPAN6. Furthermore, they were carrying several apically expressed proteins such as prominin-1 and MUC13 (Tauro et al., 2013). The A33+ EVs exclusively expressed or were significantly enriched in basolateral trafficking/sorting proteins such as early endosome antigen 1, the Golgi membrane protein ADP-ribosylation factor, and clathrin. Additionally, MHC class I was exclusively expressed in the A33+ vesicles (Tauro et al., 2013). These results suggest that different EV subpopulations might be released from different regions of, in this case, an epithelial cell type and that the expression of surface molecules can be used to separately isolate the EVs because they might have the same size and density and therefore will co-isolate using other techniques.

Additionally, vesicles isolated at 100,000 × g from primary dendritic cells have been further separated by immuno-isolation with beads coated with antibodies against the CD9, CD81, and CD63 tetraspanins (Kowal et al., 2016). Several subpopulations were found to be present, including one that is MHC class II+ but CD9−, CD63−, and CD81− and one that is MHC class II+ and CD9+.
CD63+, and CD81+, as well as one that is CD9+, CD81+, and MHC class II+ but CD63− (Kowal et al., 2016). This again highlights the heterogeneity of small EVs isolated at 100,000 × g and suggests that CD63 is only present on a limited subpopulation of EVs. This was confirmed with mass spectrometry analysis, which showed that the vesicles bound to anti-CD63 beads were distinct from both the vesicles bound to anti-CD9 and those bound to anti-CD81 beads (Kowal et al., 2016).

It can only be speculated how many subpopulations of EVs might actually exist based on protein cargo and surface molecules, and how this is reflected in their biogenesis, and biological functions. Studies have also shown that some EVs contain ATPase and 5′ nucleotidase activity and that they are able to form extracellular adenosine triphosphate (ATP) (Ronquist et al., 2013; Trams et al., 1981), that some EVs have membrane proteins with unconventional membrane orientations (Cvjetkovic et al., 2016), and that some EVs have unique mitochondrial proteomes (Jang et al., 2017). Whether these are truly distinct subpopulations of EVs, and whether additional subpopulations can be identified, will require additional investigations.

3.5. Subpopulations of EVs based on biogenesis

As mentioned above, the current state of art is to consider EV subpopulations based on their biogenesis. Briefly, vesicles are generally called microvesicles if they are shed from the plasma membrane of live cells and are called apoptotic bodies if they are shed from cells undergoing programmed cell death. EVs formed in and released from compartments of the endosomal pathway are generally called exosomes, and most published research has focused on the biogenesis of these. Proteins that are part of the endosomal sorting complex required for transport (ESCRT), Rab GTPases (regulators of intracellular vesicle transport), and soluble NSF attachment protein receptors (SNAREs) have been shown to be involved in the assembly of proteins and lipids, the budding of membranes, and the formation of intraluminal vesicles in MVBs. These proteins are also involved in the transport and fusion of MVBs with the plasma membrane and are therefore considered to play a role in the formation and release of MVB-derived EVs (Colombo et al., 2014; Raposo and Stoorvogel, 2013). However, tetraspanins and lipid-dependent mechanisms have also been suggested to be important for the release of some EVs in an ESCRT-independent manner (Colombo et al., 2014; Raposo and Stoorvogel, 2013). Regarding molecular mechanisms regulating EVs budding from the plasma membrane, less is known. To complicate things, it has been argued that the ESCRT-associated protein TSG101 is also involved in budding of EVs directly from the plasma membrane (Booth et al., 2006; Nabhan et al., 2012). Additionally, increased intracellular concentrations of Ca2+ and lost connections between the plasma membrane and the cytoskeleton lead to the formation of EVs from the plasma membrane (Kalra et al., 2016).

Several studies have shown that the depletion of ESCRT proteins...
results in defects in endosomal maturation, resulting in fewer intra luminal vesicles within MVBS and reduced fusion of MVBS with the plasma membrane (Huotari and Helenius, 2011). It should be noted that relatively few studies have established a direct role for ESCRT proteins in the release of exosomes, with sometimes contradictory results in experiments using different types of cells (Baietti et al., 2012; Colombo et al., 2013; Gross et al., 2012; Hoshino et al., 2013; Jackson et al., 2017; Tamai et al., 2010). The connections between ESCRT proteins and the biogenesis of EVs have therefore primarily been based on the fact that these proteins are commonly present in EVs evaluated by proteomic studies. Given the limited numbers of published studies, the involvement of ESCRT proteins in the release of EVs, and potentially in the release of specific sub-populations of EVs, still requires further investigation.

Similarly, studies have found changes in what has been referred to as “exosome release” when Rab GTases, such as Rab11, Rab27A, Rab27B, and Rab35, have been over-expressed or knocked down (Hsu et al., 2010; Ostrowski et al., 2010; Savina et al., 2002; Zheng et al., 2013). Whether these different Rab GTases are also involved in the release of other subpopulations of EVs, such as microvesicles, and whether they are differentially involved in the release of subpopulations of EVs, remains unknown. Few studies have altered the expression of the different molecules and subsequently determined the concentration of different subpopulations of EVs released from a single cell type. Interestingly it has been shown that Rab27A is involved in the release of ALIX+ , CD63+, Hsc70+, or TSG101+ EVs, but not in the release of CD9+ or Mfge8+ EVs (Robrie et al., 2012). These results indicate that at least two different subpopulations of EVs are present in the 100,000 x g pellet from the mouse breast cancer cell line that was studied. Additionally, one study has determined the role of ceramide in the release of two types of subpopulations of EVs, briefly, the nSMase inhibitor, GW4869, inhibited both the high-density and low-density EVs (Willms et al., 2016), suggesting that both subpopulations are formed through a ceramide-dependent pathway. However, chemicals can have a general effect on membranes and cells and therefore affect several functions in the cell, thus we should be careful with drawing any conclusions about how EV-specific some of these compounds are. For most of these studies, the knockdown of a single molecule does not lead to complete blockage of EV production. This could indicate that subpopulations of EVs are present in the isolated vesicles under study and that the investigated molecule is involved in the biogenesis of only certain subpopulations of the EV secretome.

Besides the overexpression or knockdown of proteins, one of the most common methods used to investigate EV release is the use of different chemicals affecting membranes and lipid rafts, such as methyl-β-cyclodextrin (MBCD) and 5-(N,N-Dimethyl)amiloride hydrochloride (DMA) (Gupta and Knowlton, 2007; Sreekumar et al., 2010; Trajkovic et al., 2008). However, these are blunt tools, just like GW4869 described above, and any observed effect will not necessarily be specific for any EV subpopulation and might instead have a more general effect on membrane integrity that will affect all membrane vesicles.

4. Therapeutic uses of extracellular vesicles

Because of the unique physical and biological properties of EVs, including high biocompatibility and intrinsic targeting activity, EVs have been intensively studied for their use as therapeutics (Gyorgy et al., 2015). Because the most studied subpopulation of EVs are small in size, some being nano-sized (30–150 nm in diameter), it may be possible for them to passively diffuse through tissues. This can at least partly be due to the enhanced permeability of the vasculature of tumors. Furthermore, the proposed ability of some EVs to cross the blood brain barrier, could make them powerful delivery tools for therapeutics to the brain (Alvarez-Erviti et al., 2011). In addition, EVs are prone to be taken-up by recipient cells and to deliver their cargo into the cytosol, which opens up the opportunity to target intracellular molecules, for example, onco-genes. The interaction between EVs and recipient cells is mediated by surface proteins, which can be modulated by engineering the EV-producing cells. In this section, the therapeutic potential of both unmodified and modified EVs will be reviewed and discussed, and the therapeutic activity of different subpopulations of EVs will be proposed. Overwhelming evidence shows that EVs from different cells can evoke totally different responses in recipient cells. As an example of a contrasting function, EVs from one cell type can induce strong angiogenesis, whereas EVs from another cell type can inhibit angiogenesis, and EVs from some other cells might have no effect at all on this biological function (Fig. 3A). To increase the complexity further, subpopulations of EVs from a single cell type, such as the ones discussed in the first section of this review, might also evoke totally different biological functions in recipient cells (Fig. 3B–C). Thus, it has been suggested that from a functional perspective, three different types of EVs exist, those with negative effects, those with positive effects, and those with no effects (Gho and Lee, 2017). When developing EV-based therapeutics, it is therefore ideal to harness any intrinsic effects that the EVs have and to remove any EVs with counterproductive effects or without intrinsic effects. This concept is also considered when EVs are engineered or loaded with specific therapeutic molecules, potentially resulting in therapeutically beneficial synergies between the cargo and the EVs. In the light of the first section of this review, it can also be discussed whether different subpopulations of EVs are differently suitable to engineer or load with therapeutic molecules and whether size, density, biogenesis, morphology, and cargo have to be taken into account when choosing EVs for any specific therapeutic purpose.

4.1. Unmodified extracellular vesicles as therapeutic agents

As described earlier, EVs harbor cargos that at least partly represent their parental cells, suggesting that EVs can be used as an alternative to cell-based therapeutics and might in some contexts even be favorable over cells. For example, stem cells have therapeutic potential in many diseases, including ischemic injury (Hao et al., 2014), multiple sclerosis (Yamout et al., 2010), rheumatoid arthritis (Wang et al., 2013a), autoimmune diseases (Hugle and Daikeler, 2010), and graft versus host disease (Le Blanc et al., 2008). The underlying mechanisms of the therapeutic effects of such cells remain elusive, but paracrine factors such as EVs are emerging as important mediators of these effects and might even be an alternative to cell therapies (Razzafar et al., 2017). In 2009, Bruno et al. described the effects of MSC-derived EVs in treating acute kidney injury (Bruno et al., 2009). Injection of EVs derived from MSCs into SCID mice with acute kidney injury accelerated the morphologic and functional recovery of tubular cells, which was suggested to be mediated by horizontal delivery of mRNA species. The EV-mediated therapeutic effects were comparable to those induced by the MSCs themselves. Similarly, stem cell-derived EVs showed a protective effect against renal injury induced by gentamicin (Reis et al., 2012), cisplatin (Zhou et al., 2013), and ischemia-reperfusion (Catti et al., 2011). In addition to the effects observed in the kidney, ischemia-reperfusion—induced heart injury (Lai et al., 2010), liver fibrosis (Li et al., 2013), liver injury (Tan et al., 2014), endotoxin-induced acute lung injury (Zhu et al., 2014), and allergic airway inflammation (Cruz et al., 2015) have been successfully attenuated by stem cell-derived EVs.

Immunomodulatory activity is another feature of unmodified
EVs. In 1985, one of the pioneering studies showed that EVs derived from tumors have immunosuppressive activity through inhibition of the expression of la antigen (a murine MHC class II antigen) on macrophages (Poutsiaka et al., 1985). In addition, breast milk-derived EVs can induce tolerogenic effects. For example, these EVs were shown to inhibit anti-CD3-induced activation in peripheral blood mononuclear cells, but increased the number of Foxp3+CD4+CD25+ T-regulatory cells (Admyre et al., 2007).

To date, no study has determined and compared the therapeutic potential of different subpopulations of EVs from a single cell type, such as MSCs. However, it is conceivable that EV-based therapeutics could be enhanced if a specific subpopulation of EVs is selected. For example, it is possible that only a small fraction of the EVs from a cell mediate the therapeutic effects, and another EV population could have opposing effects (Fig. 3B–C). This could be true for unmodified EV-based therapeutics as well as for novel modalities of EVs that might be modified or loaded with different types of therapeutic molecules.

4.2. Modified and loaded extracellular vesicles

Although many types of natural EVs have been shown to possess different types of therapeutic potential, the possibility to engineer or modify EVs increases their potential usefulness as therapeutics, including their use as delivery vehicles for different molecules that are otherwise difficult to deliver to diseased cells. Delivering therapeutics specifically to disease sites is essential to maximize their beneficial effects and to minimize their adverse effects. EVs are emerging as delivery vehicles that might improve the therapeutic ratio, and they have been engineered in multiple ways to load and deliver therapeutics such as small molecules, small interfering RNAs (siRNAs), and proteins (Fig. 4). Furthermore, some EVs might have properties that allow them better to penetrate the tissue to reach the target cell, while another subpopulation of EVs might more efficiently deliver the therapeutic cargo to the proper organelle or site after they have entered the cell. By modifying so that one EV contains both properties would increase their effectiveness.

4.2.1. Extracellular vesicles loaded with nucleic acids

Specific gene silencing is a potential therapeutic approach for multiple diseases, especially for targeting mutated oncogenes, and this has been demonstrated by the development of RNA interference (RNAi) techniques using endogenous and exogenous mediators such as miRNAs and siRNAs (Davidson and McCray, 2011). For example, knocking-down of disease-specific genes such as cMyc and KRAS, which are otherwise considered to be un-druggable targets, is a promising anti-cancer therapeutic strategy (Zhang et al., 2013; Zorde Khvalevsky et al., 2013). Because of their poor stability in serum and the potential immunogenic activity of RNAi therapeutics, delivery vehicles such as EVs are crucial for successful therapy (Kim et al., 2016a). Loading of RNAi molecules into EVs has been achieved mostly by electroporation or cell engineering (Fig. 4). Electroporation is the most widely used method to introduce siRNAs both into EVs and onto the surface of EVs. Alvarez-Ervitiz et al. were the first to suggest the functionality of electroporation-based loading of siRNA into EVs. In their work, brain-targeting EVs expressing Lamp2b fused with rabies virus glycoprotein (RVG)-derived peptide on their surface were loaded with BACE1 siRNAs to silence genes related to Alzheimer disease (Alvarez-Ervitiz et al., 2011). More recently, siRNAs against the KRAS G12D mutation were loaded into EVs by electroporation and delivered to pancreatic cancers resulting in successful inhibition of tumor growth (Kamerkar et al., 2017). In that study, the authors suggest that there are significant advantages to using EVs over liposomes to deliver siRNAs by showing the longer circulation time

Fig. 3. The cellular source of extracellular vesicles determines their intrinsic biological function. The subpopulations of EVs from a single cell type will have varying degrees of intrinsic biological function depending on the cell source.
of EVs in the blood due to CD47 expression on the EV surface. One drawback to electroporation is that it might cause the aggregation of a given siRNA which then leads this non-EV associated RNA to co-isolated with the EVs (Kooijmans et al., 2013). This aggregation can be blocked by adding EDTA, but this reduces loading efficiency. Although electroporation is a powerful method, further optimization of the experimental conditions is required before further clinical use of electroporation of nucleic acids into EVs can be implemented.

An alternative way of loading RNAi therapeutics into EVs is by engineering the EV-producing cell to overexpress the RNAi molecule of interest. As a prime example of this, let-7a miRNA was introduced into GE11-expressing HEK293 cells using a lipofection method, and the EVs that were produced from these cells harbored the let-7a miRNA (Ohno et al., 2013). A recent study by Sutaria et al. showed that overexpression of an RNA-interacting peptide fused with LAMP2, a protein located in the membrane of EVs, greatly increased the loading of miR-199a containing a modified RNA loop that binds to the RNA interacting peptide (Sutaria et al., 2017).

More recently, cholesterol-conjugated siRNAs have been utilized to attach the siRNAs to the membranes of EVs because the cholesterol moiety enables rapid incorporation into EV lipid bilayers when the molecules are simply mixed with EVs. Around 1000–3000 cholesterol-tagged siRNAs can be incorporated into a single EV (Didiot et al., 2016), but the retention rate can vary depending on the ratio of EVs to siRNAs (O’Loughlin et al., 2017).

Regardless of loading method, the efficiency of loading might vary for different subpopulations of EVs, and we encourage future studies comparing the loading of EVs with different size or density. Such studies should seek to determine the specific properties of different EV populations that promote the loading of different molecules of interest.

4.2.2. Extracellular vesicles loaded with protein

Recently, cells have been engineered to produce specific protein-protein interaction partners in order to efficiently load EVs with specific proteins (Fig. 4). For example, the photoreceptor cryptochrome 2 (CRY2) can be expressed on the inside of EVs, and this can help load proteins carrying the CRY-interacting basic-helix-loop-helix 1 (CIB1) into the EVs. The interaction between CRY2 and CIB1 is blue light dependent (488 nm) and was originally identified in Arabidopsis Thaliana (Liu et al., 2008). Yim et al. implemented this into their EV protein-loading system, which they have named exosomes for protein loading via optically reversible protein–protein interactions (EXPLORs) (Yim et al., 2016). In their system, CIBN, which is a truncated version of CIB1, was fused with CD9, a protein usually enriched in EVs, and CRY2 was fused with a cargo protein. Under blue light, the cargo protein was recruited to the plasma membrane and eventually secreted as EV cargo (Yim et al., 2016). Another example of protein-protein interaction-mediated protein loading is the WW domain-L domain interaction. L-domains are responsible for MVB formation and for the recruitment of the ESCRT components that are involved in EV biogenesis (Freed, 2002), and they can bind to the WW domain. Sterzenbach et al. demonstrated the loading of Cre recombinase into EVs by tagging it to a WW domain that can bind to the L-domain-containing protein Ndfip1 (Sterzenbach et al., 2017) that has been reported to be an EV cargo protein (Putz et al., 2008). Functional Cre recombinase was delivered via the EVs, and its enzymatic activity could be transferred to recipient cells both in vitro and in vivo (Sterzenbach et al., 2017). Overexpression of therapeutic proteins in EV-producing cells can also result in their loading onto/into EVs. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL), a promising anticancer protein, was loaded on EVs by this overexpression system (Yuan et al., 2017).

In addition, clinical studies have explored the use of dendritic cell-derived, IFN-γ-matured and MHC class I- and class II-restricted cancer antigen-loaded EVs. These conceptual studies have explored the potential of this therapy to enhance T cell and NK function in vivo in non-small cell lung cancer, which was confirmed,
although there was no significant clinical reduction in cancer progression (Besse et al., 2016).

4.2.3. Small molecule-loaded extracellular vesicles

Small molecules are the most commonly used therapeutics in the clinic, but they are often accompanied by a wide range of side effects due to systemic bio-distribution and lack of specificity. Loading of small molecules into EVs can direct their bio-distribution and thereby enhance their therapeutic index (Wiklander et al., 2015). For example, the anti-inflammatory drug curcumin has been encapsulated by EVs by passive incubation (Sun et al., 2010; Zhuang et al., 2011), which increased the solubility, stability, and bioavailability of curcumin, resulting in enhanced its therapeutic efficacy in a mouse lung inflammation model (Sun et al., 2010) and in an experimental autoimmune encephalomyelitis model (Zhuang et al., 2011). Anti-cancer chemotherapeutics, including both hydrophilic and hydrophobic molecules, have also been loaded into EVs by passive diffusion. One of the advantages of EVs is that they can carry and deliver both hydrophilic and hydrophobic molecules because of their membranous structure (Fig. 4). For example, doxorubicin, a hydrophilic drug, was loaded into EVs expressing the tumor-targeting ligand iRGD. These EVs selectively targeted tumor cells and reduced the tumor burden (Tian et al., 2014) and increased the therapeutic index of doxorubicin (Hadla et al., 2016; Srivastava et al., 2016). In addition, paclitaxel, a hydrophobic drug, was loaded into EVs, and this enhanced the therapeutic index of paclitaxel both in vitro and in vivo (Kim et al., 2016b; Pascucci et al., 2014; Saari et al., 2015).

4.2.4. Surface engineered extracellular vesicles

Fusion of proteins of interest with membrane proteins on EVs has been used to decorate the surface of EVs as a way to induce immune modulation and to specifically target diseased cells and organs (Fig. 4). In 2005, Delcayre et al. were the first to demonstrate this EV display technology (Delcayre et al., 2005). The HIV protein Nef was fused with the C1C2 domain of lactadherin, which is localized on the EV membrane, resulting in the presence of Nef on the surface of EVs. Injection of these EVs induced antibody production against Nef and was an early attempt to develop an HIV vaccine (Delcayre et al., 2005). Furthermore, the C1C2 domain of lactadherin has been fused with streptavidin to decorate EVs with biotinylated CpG DNA, a strong immune adjuvant, as a way to deliver both adjuvant and tumor antigen (Morishita et al., 2016). Using a similar strategy, tumor-targeting moieties were fused with EV membrane proteins to achieve specific targeting of tumor cells (Ohno et al., 2013). The GE11 peptide, which binds to the epidermal growth factor (EGF) receptor, was fused with the platelet-derived growth factor receptor transmembrane domain, which located the GE11 peptide to the membrane surface of EVs and resulted in enhanced delivery of exosomes to EGF receptor-positive breast cancer cells (Ohno et al., 2013). Furthermore, EGF was fused with the transferrin receptor transmembrane domain, which also resulted in its location on the surface of EVs and enhanced tumor targeting (Zhao et al., 2016). Additionally, the transmembrane protein Lamp2b is widely expressed on EVs, and it was used for display technology by making a fusion protein. The neuron-specific RVG peptide was fused with Lamp2b, resulting in expression of the RVG peptide on the surface of EVs and seemingly allowing the EVs to penetrate the blood brain barrier (Alvarez-Erviti et al., 2011). In addition, decoration of EVs with vascular stomatitis virus (VSV)-G protein, a fusogenic protein, causes the direct fusion of EVs with cells, and enhances the delivery of integral membrane proteins to the recipient cells (Yang et al., 2017).

The surfaces of EVs have also been functionalized with polymers such as polyethylene glycol (PEG). PEG has been shown to reduce the cellular uptake of particles by the reticuloendothelial system by forming a hydro shell on the surface of the particles, which can prolong their in vivo half-life in circulation (Harris and Chess, 2003; Niidome et al., 2006). When EVs were PEGylated by insertion of phospholipid-conjugated PEG into their membranes, these EVs showed enhanced cell specificity in vitro and increased circulation time in vivo (Kooijmans et al., 2016). In addition, PEG can be functionalized with various types of chemistries that enable the conjugation of different molecules. In the same study, nanobodies specific for the epidermal growth factor receptor were conjugated with PEG to accomplish targeted cellular uptake (Kooijmans et al., 2016).

As we learn more about the protein cargo of specific sub-populations of EVs, it may be possible to increase the effectiveness of loading EVs with proteins as well as altering the expression of EV surface proteins, thereby improving their therapeutic efficacy. When mixtures of subpopulations are used for proteomics studies, the results can be misleading because only a small fraction of the EVs might express a specific protein chosen for further loading experiments. It would also be non-optimal to isolate EVs for clinical use if only a small fraction of the EVs express the therapeutic protein or molecule. To optimize the clinical use of modified EVs, it is important to determine the most suitable subpopulations of EVs for the disease being treated.

4.3. Manufacturing of vesicles from cell membranes

As described in multiple studies, EVs are promising therapeutic and delivery entities. However, low productivity and difficulties with purification are important obstacles for future clinical applications (Lakhal and Wood, 2011; van Dommelen et al., 2012). To overcome this limitation, several artificial EVs, including exosome-mimetic nanovesicles (NVs), nanoghosts, and lipid nanoparticles, have been manufactured from cells (Fig. 5).

Exosome-mimetic NVs are nano-sized lipid-bilayered vesicles that are generated by the manipulation of whole cells (Kim et al., 2017a). NVs have been generated by extrusion of cells through membrane filters with different pore sizes (Jang et al., 2013), by centrifugal force-based filtration (Jo et al., 2014b), by microfluidic fabrication (Jo et al., 2014a), and by cell slicing with 500 nm-thick silicon nitride blades (Yoon et al., 2015). Interestingly, the overall characteristics of NVs such as size, morphology, and protein content are generally similar to those of EVs, but the numbers of NVs that can be produced are approximately 100-fold higher than naturally released EVs. Like EVs, NVs also harbor cargos that originate from the parental cells, which in itself can influence the recipient cell phenotypes. For example, pancreatic beta cell-derived NVs can transform bone marrow cells into insulin-producing cells (Oh et al., 2015), and adipose stem cell-derived NVs can block emphysema in a mouse model of the disease (Kim et al., 2017b). In addition, chemotherapeutics (Goh et al., 2017; Jang et al., 2013) and RNAi molecules (Lunavat et al., 2016; Yang et al., 2016) have been successfully loaded into NVs, resulting in efficient effects in recipient cells both in vitro and in vivo.

Manufactured EVs have also been made from cell membranes of CCR5-expressing cells (Bronstein et al., 2011) and MSCs (Toledano Furman et al., 2013), which are referred to as cell-derived liposomes and nanoghosts, respectively. CCR5 is a receptor for gp120, which is highly expressed on the surface of virions and HIV-infected cells. Bronstein et al. showed that EDTA-loaded CCR5-expressing cell-derived liposomes could be targeted to gp120-expressing cells and induce cell death (Bronstein et al., 2011). In addition, nano-ghosts derived from MSCs were targeted towards tumors and could kill the tumor cells when chemotherapeutics were loaded into the vesicles (Toledano Furman et al., 2013).
Another type of artificial EV can be generated by extracting lipids and re-forming membrane vesicles. Wang et al. developed exosome-likes nanoparticles with lipids that were purified from grapefruit (Wang et al., 2013b). These nanoparticles are similar to EVs in size and can be loaded with different chemotherapeutics but, of course, have few similarities to naturally released EVs in relation to cargo molecules.

By carefully selecting the cells or membranes used for the production or manufacturing of therapeutic vesicles, it is likely that desired molecules and properties can be acquired, resulting in increased efficacy of the developed therapeutic modality.

5. Conclusions and future perspectives

It is apparent that EVs consist of several different subpopulations in terms of morphology, density, and cargo and that this extends far beyond the classic EV subtype classification of apoptotic bodies, microvesicles, and exosomes. Most likely there are subpopulations within these classical subtypes that are generating this vast diversity of EVs. This means that the therapeutic activity seen in EVs so far is a collective effect of all EVs present in an EV isolate, perhaps mediated primarily by one or only a few of the subpopulations that are present. Furthermore, some subpopulations might have much more potent activity than other subpopulations. Therefore, different subpopulations of vesicles could be appropriate for treating different diseases. Additionally, several different uptake mechanisms have been suggested for EVs (Mulcahy et al., 2014), and it is possible that subpopulations of EVs are internalized differently and therefore have a different capacity to deliver their cargo to the location in the cell where any effects will be most pronounced. Therefore, understanding subpopulations of EVs, and determining the activities of these subpopulations, will be essential for obtaining the optimal therapeutic outcome.

By carefully selecting the cells with desired properties to produce unmodified, modified, or manufactured EVs, it is most likely possible that improved therapeutic efficacy can be gained. Unmodified EVs might transfer intrinsic biological effects, while manufactured EVs have great potential as therapeutic delivery vehicles over unmodified EVs because of their scalability and the possibility of overexpressing specific molecules. By working with purer isolates containing only one subpopulation of EVs, and by learning more about the strengths and benefits of different subpopulations of EVs, we should be able to determine which subpopulations show optimal therapeutic activity. Importantly, the ideal cellular source and the best subpopulation might be different depending on the disease to be treated.

Conflicts of interest

JL and SCJ have written several patents in the field of EVs as therapeutics and diagnostics. SCJ is currently an employee of Codiak BioSciences Inc. CL is a co-inventor on a patent using EVs as diagnostic tools in diseases.

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Appendix A. Supplementary data

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