Chapter

Extracellular Vesicles as Intercellular Communication Vehicles in Regenerative Medicine

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Abstract

Extracellular vesicles (EVs) represent cell-specific carriers of bioactive cargos that can be of importance in either physiological or pathological processes. Frequently, EVs are seen as intercellular communication vehicles, but it has become more and more evident that their usefulness can vary from circulating biomarkers for an early disease diagnosis to future therapeutic carriers for slowing down the evolution of different afflictions and their ability to restore damaged tissue/organs. Here, we summarize the latest progress of EVs classification, biogenesis, and characteristics. We also briefly discuss their therapeutic potential, with emphasis on their potential application in regenerative medicine.

Keywords: extracellular vesicles, exosomes, microvesicles, intercellular communication, stem cells, regenerative medicine

1. Introduction

Extracellular vesicles (EVs) are cell-derived membranous structures released by a multitude of cell types into the extracellular environment, from where they can enter body fluids and reach distant tissues, releasing their content [1]. Considered an essential pathway for intercellular communication, EVs are non-traditional lipid membrane-enclosed structures, with nanometric sizes [2]. Many studies have shown that EVs are produced by both prokaryotes and eukaryotes, indicating a persistent evolution of their signaling mechanism during a time, giving EVs an increasingly important role in the future [3, 4]. In general, EVs from the human blood are derived from platelets, but they can also be released from leukocytes, erythrocytes, endothelial cells, smooth muscle cells, and even cancer cells [5, 6].

Internal (platelet activation, pH variations, hypoxia, etc.) and external (irradiation, injury, etc.) factors can stimulate cells to produce EVs, that are secreted in lacrimal fluid, breast milk, broncho-alveolar lavage fluid, blood, ascites, urine, faces, etc. [3, 7, 8].

The content of EVs can vary to a great extent (lipids, proteins, nucleic acid species) and depends on the cell of origin [4, 6].

Their main function is represented by intercellular communication [2]. EVs can influence a variety of biological processes, transferring functional molecules (mRNA, microRNAs, and proteins) between cells [6, 9]. Their content is shuttled between cells, making EVs essential for a multitude of physiological, but also pathological processes (**Figure 1**). The various substances contained in the EVs can be taken up by other cells, both from the proximity of the cells of origin, but also from distant locations where they are transported by biofluids, inducing various phenotypic responses [10]. Apparently, this uptake is pH-dependent and can be of significance, especially in the tumor microenvironment [7].

EVs can also be considered as a possible source of biomarkers for early disease diagnosis [6, 11]. The implication of EVs in several diseases, including cancer, infectious diseases, neurodegenerative diseases, and blood diseases amplified the research interest, aiming to discover new possible therapeutic targets. EVs content can provide important leads about the type and stage of cancer, while during oncological treatment, the composition of EVs can change, which can be beneficial for therapeutic evaluation [5, 9, 12–14].

EVs have various physiological and pathological roles. Current evidence points out their involvement in embryonic development, regenerative medicine (tissue regeneration), immunity modulation, angiogenesis, stress response, senescence, cell proliferation and differentiation, the capture of dissipated cancerous cells. [4, 15–17].

Moreover, EVs can be regarded as therapeutic solutions and can act like possible alternatives to stem cell (SC) therapy [4].

Their role was and will continue to be exploited until reaching its maximum. Nowadays, EVs are also regarded as potential drug delivery and gene transport devices [18, 19].

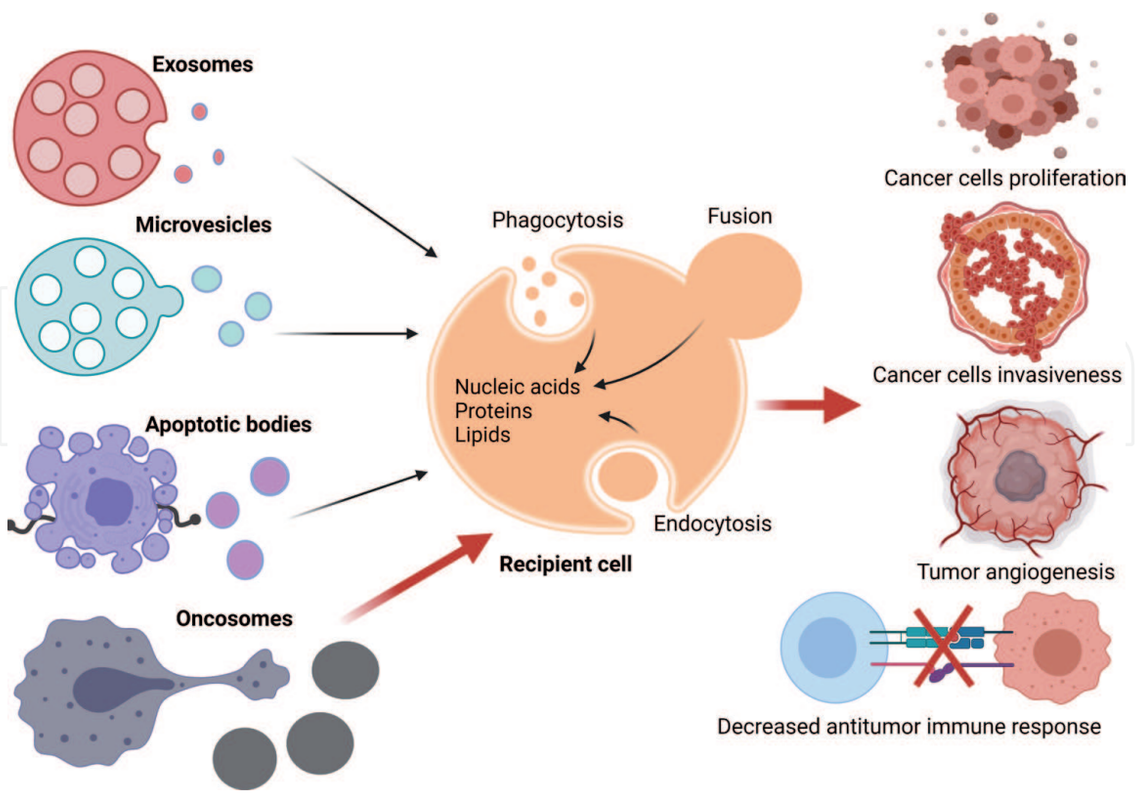

Figure 1.
EVs, produced by different cell types, can be taken up by a recipient cell via phagocytosis, endocytosis, or membrane fusion. Thus, they can determine some biological effects. Oncosomes, a particular type of EVs produced by cancer cells, can stimulate the proliferation and invasiveness of cancer cells and tumor angiogenesis. They can also decrease antitumor immune response. Created with BioRender.com (last accessed on October 26, 2021).

Shortly, EVs are common vehicles between different cell types. Nowadays, their importance has attracted considerable scientific attraction due to their involvement in disease pathogenesis, different therapies, and also in many translational pathways. Extracellular vesicles are certainly a breakthrough in the regenerative medicine field, their involvement in many processes such as apoptosis, cell proliferation, differentiation, migration, angiogenesis, oxidative stress, aging, and inflammation being recently described. Lately, extracellular vesicles were also pointed out as important vehicles for multiple therapies due to their multifaceted roles.

The current chapter will summarize the most up-to-date knowledge about the role of EVs in regenerative medicine and will discuss the effects that EVs may have on tissue regeneration, a phenomenon that was initially focused only on cell therapies or tissue engineering. It will also approach the EVs' significance and crucial role in mediating cell-to-cell communication, especially their relationship with SCs and their biodisponibility in damaged tissue.

2. Extracellular vesicles: definition and main characteristics

Over time, the definition and role of EVs have been strongly questioned. Unanimously, considered as ranging from 20 to 200 nm to 10 μm in diameter, EVs can be differentiated into three major classes: exosomes, microvesicles (MVs), or ectosomes and apoptotic bodies. However, there are a limited number of studies on apoptotic bodies, so frequently the term EVs refers to exosomes and microvesicles [17]. Moreover, recent research underlined the possibility of subdividing EVs, for example, mitochondrial protein-enriched EVs or other categories of exosomes, based on their proteins and RNA profile (such as large or small exosome vesicles) [20–22].

EVs are regularly classified based on biogenesis, release pathway, size, content, and function [1, 6, 23]:

1. *Exosomes* are produced and secreted by all cell types and have a characteristic diameter between 30 and 150 nm [24]. The biogenesis and release pathway begin with early endosomes, deriving from inwardly budding of the plasma membrane of the cell. The same process will be applied then to the limiting membrane of the early endosomes, representing the second phase. The maturation of the early endosomes will lead to multivesicular bodies (MVBs) formation [23]. Both early endosomes and MVBs are participating in performing certain functions related to cellular material (especially proteins), like endocytic and trafficking functions [25]. Finally, MVBs present two possible routes of evolution: one refers to degradation of MVBs by lysosomes, including its components, and the second one to attaching MVBs to the plasma membrane of the cells and releasing its constituents, exosomes, in the extracellular space [6, 26, 27]. Even though the specific factors that regulate these mechanisms are not well known, the most underlined pathway researched by studies is the endosomal sorting complexes required for transport (ESCRT) [28]. Based on the primary mechanism involved in the biogenesis of exosomes, ESCRT proteins, it is obvious that exosomes contain these proteins [29]. However, another mechanism involved independently is based on the sphingomyelinase enzyme, which was studied by researchers because cells without ESCRT mechanism can still produce CD63 positive exosomes, a protein from the tetraspanin family [30]. Exosomes also contain glycoproteins, low levels of proteins associated with endoplasmic reticulum and Golgi apparatus, cholesterol, ceramide, noncoding RNA, mRNA, miRNA, and cytosol [6, 24, 31].

Some well-known functions of exosomes are the facilitation of communication between cells, cell preservation, association with cancer evolution, stimulation of immune response, involvement in the functions of the nervous system (myelination, growth, and survival of the nerve cells, but also the progression of neurological diseases by containing pathogenic proteins, as a beta-amyloid peptide, superoxide dismutase and α-synuclein [24, 32–35]. Because of their constituents, exosomes are becoming more and more attractive for researchers to discover new implications in diseases and potentially new therapeutic methods [24]. For example, as already mentioned, exosomes contain α-synuclein, which is involved in Parkinson's disease [36]. New studies are concentrating on the association with glioblastoma, acute kidney disease, pancreatic or lung cancer, vaccines or other immunological uses, and diminishing tissue injury [37–41].

2. *Microvesicles* are a type of EVs measuring between 100 nm and 1 μm [1]. Their biogenesis and release pathway are still not well known. However, MVs are produced by outward budding of the plasma membrane of the cells, involving cytoskeleton elements (actin and microtubules and other cytoskeletal proteins like ARF6 and RhoA), molecular motors (kinesins and myosins) and fusions machinery (ESCRT, SNAREs, and tethering factors) [1, 42, 43]. The content of the MVs, largely determined by their biogenesis, is represented by proteins associated with cytosol and plasma membrane (especially tetraspanins), cytoskeletal proteins, integrins, glycosylated and phosphorylated proteins, and heat shock proteins [24, 44, 45]. In addition, MVs contain cholesterol, mRNA, miRNA, and cytosol [6]. Other specific markers helping in differentiation between MVs and exosomes need to be further studied [24]. Like exosomes, MVs participate in communication between cells, a particular characteristic being their ability to deliver proteins, lipids, or nucleic acids to another cell [1, 23]. Primarily, this function facilitates communication between healthy cells, but on the other hand, it can be a way to spread cancerous cells in the body, leading to metastasis [46]. That's why future studies must focus on this individuality of MVs, to develop potentially new therapeutic methods in cancer. Other possible purposes of MVs use in the future are, as already noted, the same as with exosomes [24].

A particular type of MVs is represented by *oncosomes*, which are secreted by the shedding of plasma membrane blebs of cancer cells [2, 47]. Even if their main characteristics are still not well known, some experimental studies on glioblastoma and prostate cancer have shown that their biogenesis is linked to serine/threonine kinase 1 (AKT1) and epidermal growth factor receptor (EGFR) pathways [48, 49]. Their size depends on the stage of cancer, reaching up to 1000 nm in the final stages, thus being the largest EVs. The content of oncosomes is represented by elements involved in the evolution of cancer and metastasis, like oncogenic proteins, miRNA, and enzymes for amino acid, glucose, or glutamine metabolism [6, 47].

3. *Apoptotic bodies* are a particular type of EVs measuring between 50 nm and 5000 nm. There are few studies related to apoptotic bodies and thus their characteristics are not well known. Their biogenesis is related to the separation between cytoskeleton and plasma membrane of the cell, because of cell contraction and consequently increased hydrostatic pressure, afterward being released into extracellular space by apoptotic cells [6, 50].

The composition of apoptotic bodies consists of chromatin, low levels of glycosylated proteins, and intact organelles, including proteins associated with the mitochondria, endoplasmic reticulum, Golgi apparatus, and nucleus [6, 51].

The biogenesis and main characteristics of the EVs are summarized in **Table 1** and **Figure 2**.

	Exosomes	**Microvesicles**	**Oncosomes**	**Apoptotic bodies**
Size	30–150 nm [24];	100 nm–1 μm [1];	100–1000 nm [6];	50–5000 nm [24];
Biogenesis	I. Early endosomes [23]; II. Maturation of early endosomes [23]; III. MVBs formation [23];	Direct outward budding of the plasma membrane of the cells [1];	Shedding of plasma membrane blebs of cancer cells serine/threonine kinase 1 (AKT1) and epidermal growth factor receptor (EGFR) pathways [47–49];	I. Cell contraction [24, 50]; II. Increased hydrostatic pressure [24, 50]; III. Separation between cytoskeleton and plasma membrane of the cell [24, 50];
Release pathway	MVBs attach to the plasma membrane of the cells and release their constituents [6, 26, 27];			Released into extracellular space by apoptotic cells [24, 50];
Content	ESCRT proteins, tetraspanin family proteins, glycoproteins, low levels of proteins associated with endoplasmic reticulum and Golgi apparatus, cholesterol, ceramide, noncoding RNA, mRNA, miRNA, and cytosol [6, 24, 29–31];	Proteins associated with cytosolic and plasma membrane (especially tetraspanins) [24, 44]; cytoskeletal proteins, integrins, glycosylated and phosphorylated proteins, and heat shock proteins, cholesterol, mRNA, miRNA, and cytosol [6, 45];	Oncogenic proteins, miRNA, and enzymes for amino acid, glucose or glutamine metabolism [6, 47];	Chromatin, low levels of glycosylated proteins and intact organelles, including proteins associated with mitochondria, endoplasmic reticulum, Golgi apparatus, and nucleus [24, 51];
Function	Intercellular communication, cell preservation, association with cancer evolution, stimulation of immune response, involvement in the functions of the nervous system (myelination, growth, and survival of the nerve cells, but also the progression of neurological diseases) [24, 32–35];	The same as exosomes, with a particular association with metastatic disease [46];	Cancer evolution and metastasis [6];	Insufficiently known [24];

Table 1.
Classification of EVs and their main characteristics.

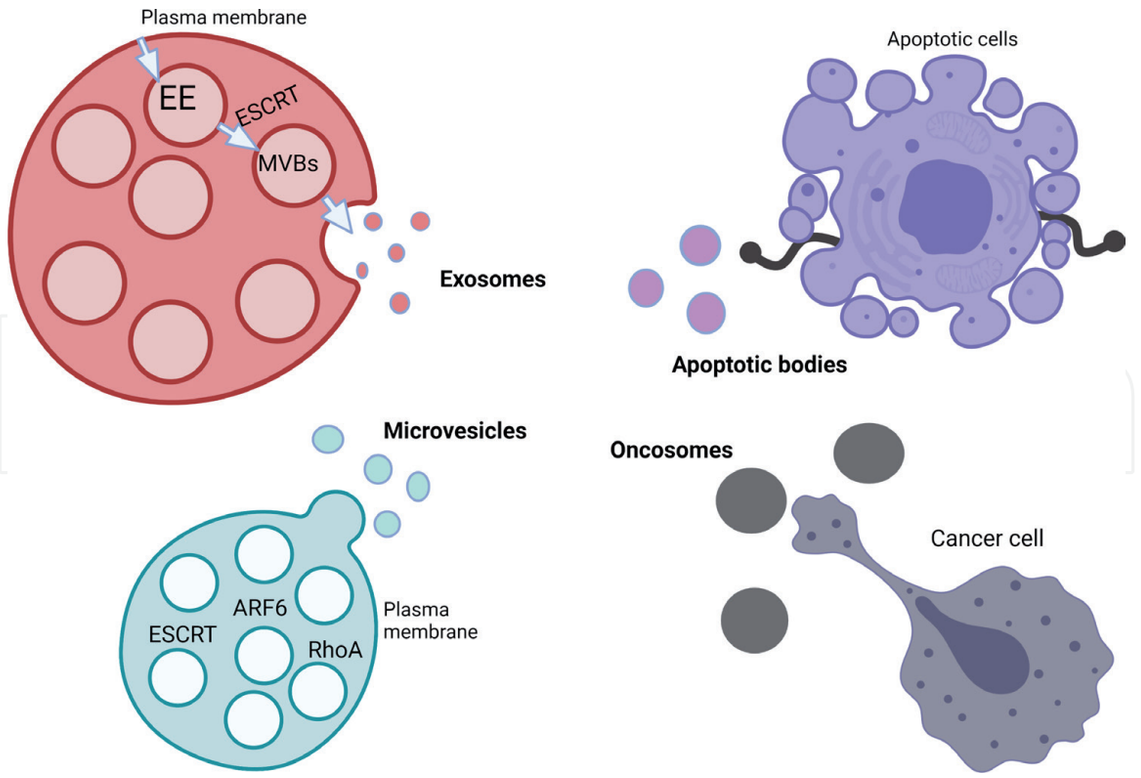

Figure 2.
The biogenesis of EVs. (a) Exosomes are produced from early endosomes (EE) and endosomal pathway (ESCRT) and released into extracellular space by fusion of multivesicular bodies (MVBs) with the plasma membrane; (b) microvesicles are produced by direct outward budding of the plasma membrane, with the involvement of cytoskeleton elements (like ARF6 and RhoA) or ESCRT; (c) apoptotic bodies are released into extracellular space by apoptotic cells; (d) oncosomes are secreted by shedding of plasma membrane blebs of cancer cells. Created with BioRender.com (last accessed on September 21, 2021).

3. Intercellular communication through EVs

EVs can carry a big amount of information within/on their surface to another cell, influencing physiological and pathological pathways [6]. For a better understanding, in this chapter, some of these processes will be exemplified to illustrate the roles of EVs in intercellular communication.

3.1 Implantation and embryonic development

The implantation process refers to the development of the trophoblasts by the embryo, which then will adhere and invade the uterine wall. This is a crucial step in embryonic development, and any inaccuracy can have severe consequences [2]. EVs are secreted by both maternal and embryonic cells. In the first case, studies have shown that endometrial epithelial cells produce EVs that stimulate the activation of focal adhesion kinase (FAK), increasing the adhesion of trophoblasts to the uterine wall [52]. Regarding embryonic production of EVs, recent studies have shown the involvement of MVs. Laminin and fibronectin, two extracellular matrix proteins, found on the surface of MVs, are playing an important role in this case. MVs are transported to trophoblasts, where laminin and fibronectin activate integrins on the surface of the trophoblast, stimulating the activation of c-Jun N-terminal kinase (JNK) and FAK and thus promoting migration of trophoblastic cells and rates of implantation [53]. Embryonic development is influenced by communication between the cells of embryos, through the secretion of factors that are still not well known. Some studies have suggested that EVs could be involved in these processes. For example, a study conducted by P. Qu *et al*. on bovine cells has shown

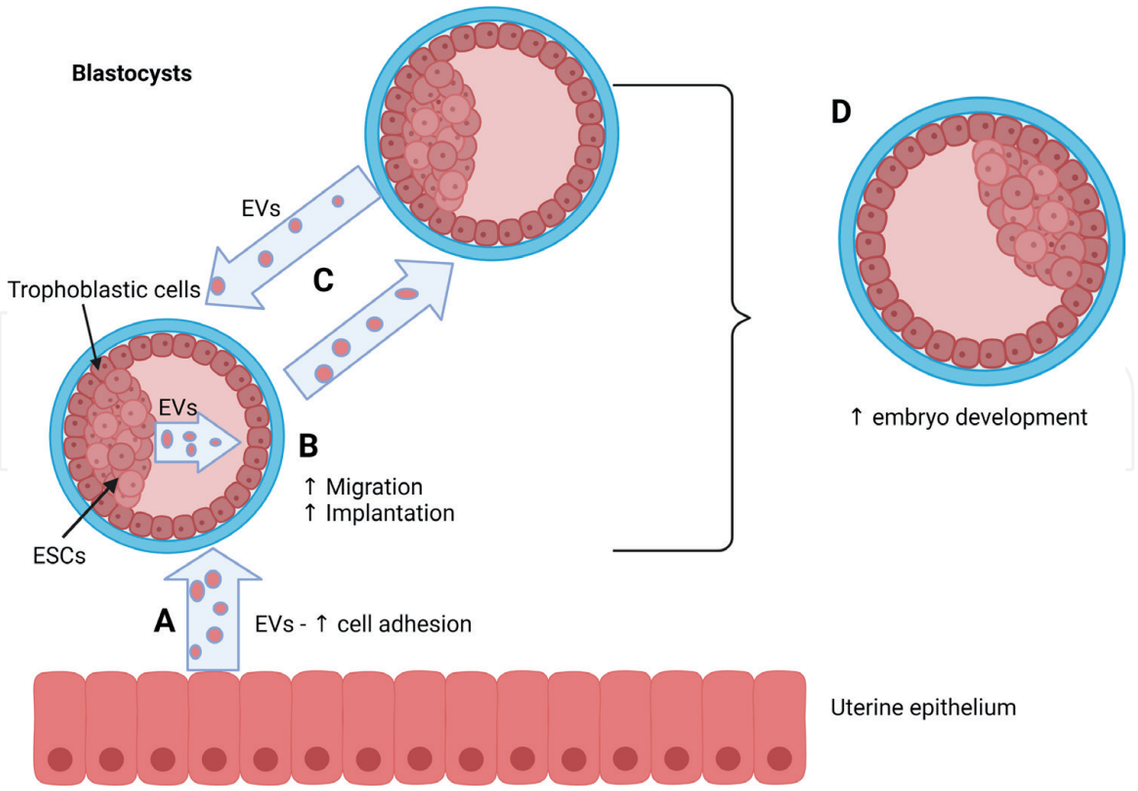

Figure 3.
The roles of the EVs in embryo communication. (A) The uterine epithelium secretes EVs that stimulate the adhesion of trophoblastic cells to the uterus; (B) embryonic stem cells (ESCs) produce EVs that stimulate the trophoblasts to migrate and implant into the uterus; (C) the co-culturing of embryos increases (↑) embryo development (D), mediated by EVs. Created with BioRender.com (last accessed on September 22, 2021).

that embryos without replaced culture medium contain CD9 positive exosomes and have a better chance of a healthy pregnancy [54]. Another study conducted by I.M. Saadeldin *et al*. concluded that EVs are influencing the communication between embryos. They combined cloned embryos with embryos from an unfertilized egg cell and showed that the latter are secreting CD 9 positive exosomes and EVs containing RNA transcripts that encoded some pluripotency genes, improving the features of the cloned embryos if co-cultured [55].

The roles of the EVs in implantation and embryonic development are illustrated in **Figure 3**.

3.2 Cancer development

EVs are produced by stromal cells, which can be found, along with cancerous cells, as components of a tumor mass. In this case, EVs act like a bidirectional transferring mechanism between stromal cells and cancerous cells, influencing tumor evolution [6]. The biogenesis, release pathways, and the contents of EVs will be modified by the tumor microenvironment. Circulating DNA, contained by EVs will be transferred between apoptotic bodies (derived from apoptotic tumor cells) and other cells, leading to increased expression of oncogenes [56]. Tumor-derived EVs play a crucial role in all steps of cancer development, being more and more studied, to discover new treatment methods [57].

For a better understanding of the role of EVs in tumoral processes (cell proliferation, apoptosis resistance, angiogenesis, local invasion and metastasis, therapy resistance, etc.) we will discuss this with respect to some cancer types.

Some studies have shown that exosomes produced and released by ovarian cancer cells can carry RNAs and miRNAs, influencing cell transformation and

tumor evolution. RNA-binding protein LIN 28, a marker of SCs, is associated with an unfavorable outcome when present in malignancies. Ovarian cancer cells which express high LIN 28 levels can secrete exosomes, which can further interact with noncancerous cells, leading to variations of gene expressions and cell behavior. This can lead to consequential amplification of genes responsible for epithelial to mesenchymal transition, human embryonic kidney 293 cells (HEK 293) invasion, and migration [58]. SKOV3, an ovarian cancer cell line, is also involved in cancer development by producing and releasing exosomes that can stimulate the M2 macrophage phenotype and consequently migration and proliferation of cancerous cells [6].

In breast cancer, studies have shown that EVs contain two extracellular matrix proteins, discoidin I-like domains 3 and epidermal growth factor-like repeats, that can activate FAK cascade and, along with an independent mechanism of microRNA biogenesis possessed by EVs, they play a crucial role in cancer development [59, 60].

In glioblastoma, EVs are transferring between cells the protein chloride intracellular channel-1, which stimulates the growth of the recipient cells, and the splicing factor RNA-binding motif protein 11, which increases survival [61, 62]. Moreover, the effect of EVs on angiogenesis, an important process in tumor growth, has been studied on glioma cells and it has been reported that EVs contain factors that promote angiogenesis by stimulating vascular endothelial growth factors [63].

In bladder and gastric cancer and melanoma, EVs are releasing platelet-derived growth factor receptor-beta, which is stimulating PI3K/AKT and MAP/ERK pathways, thus increasing cell proliferation and apoptosis resistance [64, 65].

The role of the EVs in intercellular communication and cancer development occurs not only locally but also remotely, leading to metastatic disease. The most studied components of EVs involved in this process are miRNAs that can influence angiogenesis, local invasion, colonization, immune modulation, etc., and annexin II, a membrane-associated protein, by stimulating angiogenesis [66, 67]. Also, peritoneal metastases of ovarian cancer are accelerated by matrix metalloproteinase-1 from EVs [68].

Therapy response in cancer can be influenced by EVs, until the emergence of multidrug resistance, by transferring some drug resistance traits from cancer cells to recipient cells, like drug efflux pumps (decreasing drug concentrations in the cells by drug efflux), apoptotic regulators (simulating anti-apoptotic pathways), proteins involved in metal ion transportation (decreasing the effect of a metal-based therapy, as cisplatin), but also microRNAs, functional mRNAs and lncRNAs (long non-coding RNAs) [57, 69–71].

3.3 Therapeutic potential of EVs

As already mentioned, EVs have an important role in cell-cell communication and thus in physiological and pathological processes, leading to an increased interest in studying their ability to generate new therapeutic methods. Over time, several studies have tried to demonstrate the involvement of EVs in immunological modulation, tissue regeneration, bioengineering, transportation of therapeutic agents, etc. [4]. One focuses our attention on explaining some other therapeutic potential of EVs, while the role of EVs in tissue regeneration will be separately discussed.

One of the first studied therapeutic potentials of EVs has been in immunotherapy. EVs produced by mesenchymal stromal cells (MSCs), especially exosomes, can induce an M2-like phenotype (anti-inflammatory, regenerative) in monocytes *in vitro* and thus polarization of activated CD4 T-cells to regulatory T-cells [72]. Some experimental studies performed in rats have shown that allograft rejection can be decreased by regulatory T-cells (activated by exosomes) in kidney and intestinal

transplantation in rats and by exosomes derived from immature dendritic cells in cardiac transplantation [73–75]. In ischemic events, MSCs are producing exosomes that are decreasing myocardial inflammation after 24 h, by secreting anti-inflammatory cytokines and MVs that are reducing renal inflammation and fibrosis [74, 75].

4. EVs as drug delivery vehicles

Today's medicine is increasingly focused on personalized treatment methods, on targeted therapies that act at the molecular level. One of the concepts aimed at these aspects is that of theranostics, which aims at diagnosis, treatment, and concomitant follow-up of the response by using very specific drug delivery systems [76]. In this sense, EVs are an extremely useful tool for passive diagnosis (especially in neoplastic pathologies, through the ability to identify the tumor type based on the miRNA, mRNA, and mitochondrial RNA profile of EVs) and active (by associating EVs with advanced imaging methods). Thus, numerous platforms based on EVs technology have been developed for theranostic purposes, namely, transition metal-labeled exosomes, nanoparticle-loaded exosomes, bioluminescently labeled exosomes, nanocluster loaded exosomes, metabolically labeled exosomes. The main applications at present are those in the oncology field, but the lack of uniformity of clear classifications, increased immunogenicity of EVs, and the limited number of drugs that can be loaded at EVs highlight the need for future studies for widespread application of these diagnostic and therapeutic tools [77].

EVs are used as transporters for a variety of substances, ranging from small molecules, small interfering RNA, mRNA, and microRNAs to drugs with suboptimal pharmaceutical effects, carrying active constituents through biological barriers [4, 11].

Exosomal transporters present advantages, as they can travel efficiently between cells, smoothly passing their cargo along the cell membrane, keeping it biologically active, and crossing hard-to-penetrate barriers, such as the blood-brain barrier. Important issues regarding exosome-based drug delivery vehicles are the precise method of exosome loading, without altering the biological characteristics, and the scalable repeatable production of exosome categories [18, 78].

Exosomes tend to have special homing targets, influenced by their cell of origin [18]. Their membrane can be modified, to amplify the targeting of specific cells [18, 79].

The content of EVs can be loaded exogenously (integration of small proteins, RNA, or other molecules) or endogenously (assuring that cells possess the ways to integrate small molecules, proteins or RNA into EVs during their formation) [4, 80]. Exogenous changes of EVs can be done after their collection, incorporating the cargo into EVs through different methods: coincubation (with no modification of vesicle size distribution or integrity, electroporation, and sonication) [79, 80]. The endogenous loading can be obtained through artificial adjustment of the parental cell to overexpress certain proteins or RNA, that can be integrated into secreted EVs afterward [81].

Human MSCs, multipotent adult progenitors, could be an adequate source of exosomes for drug delivery. Their transplantation has been investigated in numerous trials and proved to be safe, MSCs also produce immunologically inert exosomes [18]. As a delivery system, studies have shown that EV derived from MSCs can transfer therapeutic drugs to diseased cells [19]. Their efficiency is based on the adhesion proteins on their surface (like integrins, extracellular matrix proteins, tetraspanins), which facilitate the penetration of the cellular membrane and the accumulation of

EVs in the diseased cells [82]. Other characteristics that make EVs an ideal candidate for a drug delivery system are their decreased toxicity and immunogenicity, as well as their potential to cross the blood-brain barrier [83, 84]. A study conducted by S. Kamerkar *et al*. has shown that EVs produced by MSCs can transfer small interfering RNA targeting the oncogenic KRas (G12D) mutants to pancreatic cancer cells, increasing cells apoptosis and decreasing the risk of metastatic disease [85].

Exosomes have significant immune properties, modulating immunological responses and facilitating antigen presentation [11, 86]. Exosomes derived from dendritic cells can conduct MHC class I/peptide complexes to other dendritic cells for *in vivo* activation of cytotoxic T lymphocytes and promote T cell-dependent antitumor responses *in vivo* [86, 87]. Dendritic cell exosomes have been previously loaded with antigenic peptides, to activate T cell proliferation, with possible use as vaccines against infectious or neoplastic diseases. Due to the immunogenic nature of dendritic cell exosomes, their use as drug delivery vehicles is not ideal. A more suitable choice would be human ESCs-derived mesenchymal cells [86, 88, 89].

Clinical trials with therapies based on EVs are studied in malignancies, such as melanoma, non-small cell lung cancer, colon cancer, metastatic pancreatic cancer, bronchopulmonary dysplasia, malignant ascites, and pleural effusion, but also chronic kidney disease, type 1 diabetes, insulin resistance and chronic inflammation polycystic ovary syndrome, ulcers, and acute ischemic stroke [4]. Exosomes were shown to transport curcumin and chemotherapeutics, such as doxorubicin and paclitaxel [19, 90].

The implication of MSCs was also evaluated in patients with anthracycline-induced cardiomyopathy. Mitochondrial transfer, mediated by large EVs, diminished injury determined by doxorubicin, in patient-specific induced pluripotent SC-derived cardiomyocytes. MSCs could ameliorate cardiac function in anthracycline-induced cardiomyopathy, regardless of regeneration effects [91].

Liposomes possess many favorable characteristics as drug delivery vehicles, being used in the transportation of anti-cancer drugs, anti-fungal medication, and analgesics [92–98]. Liposomes have a phospholipid membrane that helps with the incorporation of hydrophilic or hydrophobic drugs, and they can also deliver the carried drugs to the targeted points through plasma membrane breaching. To diminish the recognition by opsonins and their clearance, liposomes can be covered with polymers (PEG). Their membranes can be adapted, to present ligands or antibody elements, which can interact with specific cells and amplify the targeted drug delivery. Liposomes, with easy-to-control properties, can be loaded with drugs, DNA, diagnostic instruments, enzymes, or peptides. Drugs included in liposomes have attenuated toxicity and do not provoke unwanted toxic reactions [99]. Liposomal drugs have various routes of administration, such as parenteral, oral, topical, and even through aerosols [99].

Synthetic liposomes, although very useful, are overcome by EVs (naturally derived liposomes), which have lower toxicity [19]. Exosomes are considered superior drug delivery vehicles, as an alternative to liposomes. In contrast to the latter, exosomes are usually adequately tolerated by the human body and do not present intrinsic toxicity. They can deliver their content through the plasmatic membrane and protect against its early transformation and elimination [19]. Since exosomes can be found in a variety of biological fluids, such as blood, urine, breast milk the delivered drugs will be well tolerated, less toxic, and with a longer circulating half-life [19, 100].

5. Regenerative medicine and EVS

Regenerative medicine is a relatively new concept and a complex domain that involves the restoration of damaged tissues using multiple techniques (e.g., SCs,

biomaterials, differentiated autologous cells, or combinations of the aforementioned techniques). Regenerative medicine is focusing on repairing, regrowing, or replacing injured, malfunctioning, or missing tissue and addresses many tissular types: skin, heart tissue, cartilage tissue, bone tissue, adipose tissue etc. [101]. Thus, SC research focuses on their properties of repairing damaged tissues, either by producing new tissues by division and differentiation or by their partial repair.

Stem cells represent a highly interesting resource and were considered the ideal choice for regenerative therapies. SCs are defined as non-specialized cells, characterized by an enormous capacity of differentiation, which varies depending on their origin (embryonic, fetal, or adult). SCs are capable of differentiation into adipocytes, osteocytes, chondrocytes, endothelial cells, cardiomyocytes, pericytes, and smooth muscle cells [102–107]. They can also differentiate into neurogenic, cardiovascular, and neovascular pathways [108–113]. Allogeneic transplantation can be used in other applications due to the immunosuppressive properties of SCs [114].

Over the last decades, in an attempt to better understand SCs to use them in the processes of tissue repair and regeneration, multiple classifications have been made, depending on many aspects: the organism of origin (embryo, fetus, infant, adult), the tissue of origin within the adult-origin cells (mesenchymal tissue, hematopoietic, nervous, gastrointestinal, cutaneous, etc.), and the ability to divide (totipotent, pluripotent, multipotent, etc.) [115–117]. The understanding, even partial, of SCs' ability to divide, especially the asymmetric division of adult SCs has opened new horizons in terms of reparative and regenerative medicine [115]. Overpassing the initial idea that considers EVs as cellular debris, nowadays they are seen as tools for intercellular communication and as possible therapeutic vehicles. However, the same cannot be said about SCs. In the last decades, the interest for their properties has gained more and more interest. However, they have been regarded as cells at the origin of many pathologies since 1933, when Sabin *et al.* emphasize the possibility of radioactive damage to lymphoid tissue by affecting SCs [115, 118].

Although at the beginning researchers, scientists, and clinical doctors considered that the success of stem cell transplantation depends on the purity of the transplanted cells, not always the purer means also the better. Over time, it has become increasingly clear that the success of SC therapy depends on EVs and the soluble secreted factors because they play important paracrine roles. Our recent work also demonstrated that some other cellular types, such as the newly discovered telocytes, can act as cellular adjutants participating in the regenerative processes possibly through the released EVs influencing the microenvironment of the stem cell niche [118, 119]. Other additional evidence suggests that EVs can have not only regenerative properties, but also immunomodulatory roles, consequently summing up the therapeutic effects of stem cells. EVs, by contrast to stem cells, are nonimmunogenic and are not able to self-replicate [120]. In addition, EVs display powerful therapeutic potential, with positive outcomes regarding regeneration in many tissues (**Table 2**).

The central point of the pathophysiological mechanisms by which SCs contribute to the tissular repair are EVs that function as carriers of many biomolecules, such as miRNA, mRNA, cytokines, growth factors, differentiating factors with a key role in the main processes involved in tissue regeneration: immunomodulation, angiogenesis, differentiation [2]. Thus, multiple preclinical studies performed *in vitro* or *in vivo* on animal subjects have tried to identify the molecules involved and their role, important advances being made in diseases with high mortality and morbidity such as myocardial infarction, neuronal degeneration, osteoarticular diseases, skin ulcers, corneal damage etc. [121, 133, 134].

The use of SCs, despite promising results, has many disadvantages that require careful control of this procedure and the formation of very specific

Involved tissue	Type of stem cells from which EVs derived	Involved molecules within the EVs	Type of effect on tissue repair	References
Myocardial (myocardial infarction)	MSCs	miRNA 19a, 132, 146-3p, 220, 221 mRNA	Antiapoptotic Proangiogenic Cardioprotective	[121–123]
	CSCs	miRNA 132, 146a, 210 TCA-3 SDF-1 VEGF Eritropoetin bFGF Osteopontin SCF Activin A DKK homolog 1 TGF beta	Antiapoptotic Proangiogenic (stimulates tubules formation in endothelial cells) Improve ejection fraction	[121, 124, 125]
	BM-MSCs	miRNA 22, 126, 130a, 182	Antiapoptotic Proangiogenic Antifibrotic Immunomodulatory Anti-inflammatory	[121–123, 126, 127]
Bone and cartilage (osteoarthritis)	MSCs	miRNAs (92a, 125b, 320) MMP-13	Modulates the immune response Protects the chondrocyte Stimulates regeneration, matrix, and chondrocytes proliferation	[128–130]
Skin (ulcers)	ADSc	IL 2,6,7,9,21 etc. TNF FGF CCL 2,4, 38 etc. BMP 5,7 etc. More than 70 specific miRNAs (204, 210-3p etc.)	Proangiogenic Improve epithelialization Improve epithelial width Decrease scar formation Decrease wound diameter	[131, 132]

MSCs—mesenchymal stem cells; CSCs—cardiac stem cells; BM-MSCs—bone marrow mesenchymal stem cells; ADSc—Adipose tissue stem cells; miRNA—microRNA; TCA-3—T-cell activation gene-3; SDF-1—stromal derived factor 1; VEGF—vascular endothelial growth factor; bFGF—basic fibroblast growth factor; SCF—F box containing complex; DKK 1—Dickkopf-related protein 1; TGF beta—transforming growth factor 1; MMP-13—matrix metalloproteinase; IL—interleukin; TNF—tumor necrosis factor; CCL—CC chemokine ligand; BMP—bone morphogenic protein.

Table 2.
The role of stem cell-derived EVs depending on their content and tissue type.

microenvironments to induce the differentiation of these cells [135]. Among the disadvantages mentioned before, those given by the ethical considerations of embryo use, the risk of uncontrolled differentiation, and the appearance of teratomas and genetic instability (especially those of embryonic origin) are the most important. To these disadvantages, a lower capacity for division and differentiation is added, as well as a laborious procedure for adult SCs acquirement [136, 137].

Among SCs, MSCs secrete growth factors and cytokines, with autocrine and paracrine properties. These substances inhibit the local immune system, fibrosis, and apoptosis, amplifying mitosis and differentiation of tissue-intrinsic reparative

cells. These phenomena are known as trophic effects and differ from the direct differentiation of MSCs for tissue repair [138].

The numerous functions of MSCs in tissue regeneration and implicitly in the possible treatment of many diseases are mainly achieved by the secretion of exosomes loaded with key molecules (cytokines, growth factors, miRNA) and by molecules secreted directly into the extracellular environment with paracrine action [138–141].

However, they cannot produce infinite numbers of exosomes, repetitive isolation of cells being needed. The advantages of MSCs exosomes are their non-immunogenic property, the intrinsic therapeutic capacity of reducing tissue damage, large *ex vivo* expansion, conveniently reachable source, and clinically tested cell source [18].

MSCs are a source of small EVs which can favor angiogenesis and cell proliferation in infarcted myocardium, can inhibit cardiac remodeling, and improve ventricular functions [121, 142, 143].

Moreover, MSCs can repair infarcted myocardium through paracrine interactions. EVs derived from MSCs have a better therapeutic effect than simple MSCs therapy. In animal subjects, suffering from myocardial infarction, exosomes derived from MSCs diminished inflammation, improved cardiac function, stimulated cardiomyocyte H9C2 cell proliferation, inhibited apoptosis induced by H_2O_2 and cardiac fibrosis, and slowed down the transformation of fibroblasts into myofibroblasts mediated by TGF-β [144].

Although macrovascular reperfusion is the gold standard therapy for acute myocardial infarction, heart failure developed due to deficient cardiac remodeling is still a major issue for long-term therapeutic management. Angiogenesis is crucial for tissular regeneration, therefore, interest for therapeutic enhancement of angiogenesis has increased. Preclinical and human clinical trials showed conflicting results, the use of one growth factor not being enough to promote adequate angiogenesis [144–147]. Cell transplantation could be an alternative/another solution [148, 149]. Stem cells were utilized as sources for new cardiac cells production (endothelial progenitor cells, MSCs, cardiac progenitor cells). Paracrine factors secreted by transplanted cells seem to influence endogenous repair of damaged tissues [121].

In vivo studies indicate that small EVs from MSCs that overexpress Akt can amplify neovascularization, ameliorating the left ventricle ejection fraction [150]. The angiogenic involvement is supported by the treatment of renal ischemic reperfusion injury with small EVs derived from umbilical cord MSCs ameliorated capillary density through promoting VEGF up-regulation, independently from HIF-1α [151]. Small EVs can also deliver miRNAs (miR-125a) to endothelial cells, favoring angiogenesis [152]. Comparable outcomes were also obtained from the use of MSCs which overexpress hypoxia-inducible factors (HIF-1α). Injection of exosomes from MSCs, containing Jagged1, and hypoxia-inducible factor—MSCs cultures led to angiogenesis *in vivo* and *in vitro*. Exosomes derived from HIF-1α-overexpressing MSCs have a strong angiogenic function, through an expansion in the packaging of Jagged1 [153]. In addition, the immune system has a big role in the repair of the ischemic myocardium, in the inflammatory and angiogenesis phases. Chemokines, cytokines, and the release of EVs with paracrine actions sustain this restoration. EVs favor tissular regeneration and angiogenesis, therefore, research in this area is of high interest for patients suffering from acute myocardial infarction.

A study evaluating the effect of intracoronary administration of cardiac-derived SCs-secreted small EVs showed a lower number and altered polarization state of CD68+ macrophages in the infarcted myocardium, with elevated expression of anti-inflammatory genes (Arg1, IL4ra, Tgfb1, Vegfa). Macrophages primed with EVs from cardiac-derived SCs displayed high levels of miR-181b, which targets

protein kinase C δ. Therefore, exosomal transfer of miR-181b into macrophages lowered the levels of protein kinase C δ transcript, underlining the cardioprotective properties of stem cell infusion after reperfusion [154].

According to L. Cambier and colleagues, cardiosphere-derived cells proved to reduce myocardial infarction size through secreted EVs-Y RNA fragment, found in generous concentrations in EVs from cardiosphere-derived cells, correlated with the potency of these cells *in vivo*. This fragment can be transferred from cardiac cells to target macrophages through EVs, inducing transcription and secretion of IL-10, offering cardioprotection. *In vivo* injection of EV-Y RNA fragment after reperfusion reduced the infarct size [121, 155].

One of the major issues in diabetic patients is inadequate myocardial angiogenesis, which is responsible for an elevated risk for ischemic heart disease in these patients. Exosomes loaded with miRNAs (miR-320-3p or 320a) derived from diabetic cardiomyocytes proved, they can influence angiogenesis in endothelial cell cultures. Moreover, miR-320-3p, together with the miR-29 family and miR-7a can regulate insulin secretion and its signaling pathways [156, 157].

6. Conclusions

It is becoming increasingly evident that EVs are involved in a multitude of biological processes and can play modulatory and regulatory roles. If one adds their new potential as biomarkers for various diseases and their therapeutic delivery cargo abilities, it is certain that one cannot over neglect their ability to support stem-cell-based therapies. Future avenues are seen at the horizon when further research will probably allow us to use engineered-EVs to support endogenous repair and thus create a modern regenerative medicine.

Conflict of interest

The authors declare no conflict of interest.

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