

Extracellular Vesicles for Regenerative Medicine Applications

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Abstract: Tissue engineering and regenerative medicine (TERM) may be defined as a translational discipline focused on the development of novel techniques, devices, and materials to replace or repair injured or diseased tissue and organs. The main approaches typically use cells, scaffolds, and signaling molecules, either alone or in combination, to promote repair and regeneration. Although cells are required to create new functional tissue, the source of cells, either from an exogenous allogeneic or autologous source or through the recruitment of endogenous (autologous) cells, is technically challenging and risks the host rejection of new tissue. Regardless of the cell source, these approaches also require appropriate instruction for proliferation, differentiation, and in vivo spatial organization to create new functional tissue. Such instruction is supplied through the microenvironment where cells reside, environments which largely consist of the extracellular matrix (ECM). The specific components of the ECM, and broadly the extracellular space, responsible for promoting tissue regeneration and repair, are not fully understood, however extracellular vesicles (EVs) found in body fluids and solid phases of ECM have emerged as key mediators of tissue regeneration and repair. Additionally, these EVs might serve as potential cell-free tools in TERM to promote tissue repair and regeneration with minimal risk for host rejection and adverse sequelae. The past two decades have shown a substantial interest in understanding the therapeutic role of EVs and their applications in the context of TERM. Therefore, the purpose of this review is to highlight the fundamental characteristics of EVs, the current pre-clinical and clinical applications of EVs in TERM, and the future of EV-based strategies in TERM.

Keywords: tissue engineering; regenerative medicine; extracellular vesicle



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1. Introduction

Tissue engineering and regenerative medicine (TERM) is a translational discipline in biomedical science focused on developing novel strategies to regenerate and replace damaged tissues with the ultimate goal of restoring native tissue function and physiology [1,2]. Such strategies in TERM implement the use of cells, scaffolds, and signaling molecules—either alone or in combination—that, when implanted into the host, will promote constructive and functional tissue remodeling, integration, and regeneration. Ultimately, cells are required to create new functional tissue, and such cells can be supplied from an exogenous allogeneic or autologous source and injected into the host, or through the implantation of bioactive materials that recruit endogenous stem cells to the site of injury [3,4]. Regardless of the cell source, the relevant cells require appropriate instruction for proliferation, differentiation, and in vivo spatial organization to create new functional tissue. This instruction is supplied through the microenvironment in which cells reside. In mammalian tissues, this microenvironment is largely represented by the extracellular

matrix (ECM) [5,6]. The ECM is a complex, three-dimensional network of proteins, proteoglycans, and glycosaminoglycans that provides cells and tissues with both structural and physiologic support [5]. In addition, the ECM hosts an abundant landscape of sequestered growth factors, morphogens, cryptic peptides, and extracellular vesicles (EVs), all of which function to regulate normal cell physiology and promote tissue regeneration and repair [6].

The specific components of the ECM, and broadly the extracellular space, responsible for promoting tissue regeneration and repair are not fully understood, but EVs found in body fluids and solid phases of ECM have emerged as key mediators of tissue regeneration and repair [7]. Given their potential as therapeutic mediators of tissue repair in a wide variety of pathophysiological conditions [8], EVs are being explored as a cell-free approach to supporting tissue regeneration and repair through influencing host cell phenotype and function entirely independent of a requirement for cell source or scaffold material. This approach could potentially mitigate the issues of cell and scaffold rejection by the host, a major concern for the translational application of TERM therapies [9]. Over the past two decades, there has been substantial interest in understanding the therapeutic role of EVs and their applications in the context of TERM. Therefore, the purpose of this review is to highlight the fundamental characteristics of EVs, the current pre-clinical and clinical applications of EVs in TERM and other disciplines, and the future of EV-based strategies in TERM.

2. Extracellular Vesicles (EVs)

EVs are lipid-bound vesicles produced by cells and secreted into the extracellular space [10]. EVs have been shown to transfer functional cargo (including lipids, proteins, and nucleic acids) to recipient cells whereby they influence cell function and phenotype [11]. EVs have been shown to be involved in development, tissue homeostasis, and wound healing, and have also been shown to be dysregulated during disease progression [12,13]. There are five major subclasses of EVs currently identified: exosomes, microvesicles, apoptotic bodies, biomineralization vesicles, and matrix-bound nanovesicles (Figure 1). The cargo of EVs includes intraluminal proteins and nucleic acids as well as the lipids contained within the membrane of these vesicles. While all five subclasses share overlapping similarities in the types of cargo they contain, the biogenesis, size, specific cargo, and biological function of these subclasses are distinct from each other. The characteristics and functions of these five subclasses are reviewed below.

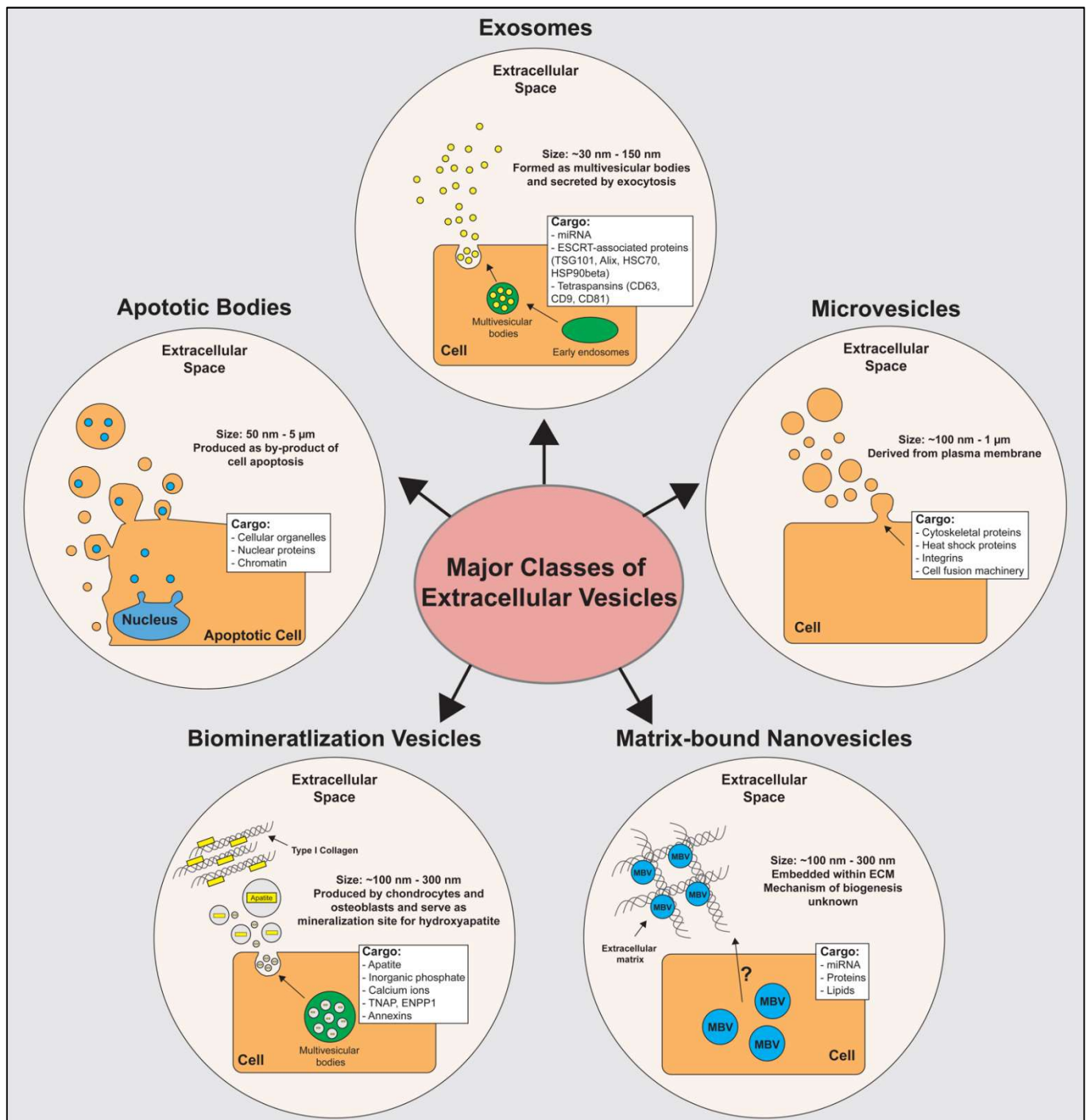


Figure 1. Major subclasses of extracellular vesicles. The five major subclasses of extracellular vesicles used in tissue engineering and regenerative medicine include exosomes, microvesicles, matrix-bound nanovesicles (MBVs), biomineratization vesicles, and apoptotic bodies. Size, cargo, and biogenesis of each vesicle subclass are illustrated. Abbreviations; ESCRT: endosomal sorting complexes required for transport machinery; TSG101: tumor susceptibility gene 101; HSC70: heat shock cognate 71 kDa protein; HSP90beta: heat shock protein 90 kDa beta; CD: cluster of differentiation; TNAP: tissue-nonspecific alkaline phosphatase; ENPP1: ectonucleotide pyrophosphatase/phosphodiesterase; MBV: Matrix-bound Nanovesicles.

2.1. Exosomes

Exosomes are a subclass of EVs secreted by all cell types; they have been identified in plasma, urine, saliva, semen, bronchial lavage fluid, cerebral spinal fluid, amniotic fluid, synovial fluid, tears, lymph, bile, breast milk, and gastric acid [14–27]. Exosomes participate in cell-to-cell communication can induce physiological changes in recipient cells by transferring their molecular cargo including proteins, lipids, and nucleic acids [28]. Exosomes, which range in size from 30–150 nm in diameter, have a biogenesis that occurs via the inward budding of intracellular endosomes [29–33]. Repeated inward budding of the endosomal membrane results in the formation of a multivesicular body. The multivesicular body then fuses with the cell membrane, and the exosomes within are released into the extracellular fluid [29,30,34–36]. Although the molecular cargo of exosomes can vary depending on the cell source, exosome-specific marker proteins have been identified. These include proteins involved in the formation of multivesicular bodies such as ESCRT proteins, ESCRT-accessory proteins (TSG101, Alix, HSC70, and HSP90beta), and select tetraspanins (CD63, CD9, and CD81) [34,37–44].

2.2. Microvesicles

Microvesicles are formed through the outward budding of the cell membrane [45]. Microvesicles range in size from 100 nm to 1 µm in diameter and are thought to be involved in cell-to-cell communication, both locally and systemically [29,31–33,35]. Unlike exosomes that have distinctive marker proteins, there are no specific marker proteins for microvesicles. The biogenesis of microvesicles occurs through the trafficking of molecular cargo to the plasma membrane where contractile machinery induces budding and ultimately the pinching off of these vesicles [46,47]. Since microvesicles are produced from the outward budding of the cell membrane, the proteins found on the surface of microvesicles are similar to the surface proteins of the parent cell. The intracellular cargo of microvesicles is diverse and contains proteins including, but not limited to, cytoskeletal proteins, heat shock proteins, integrins, and proteins involved in cell fusion machinery (SNAREs and tethering proteins) [35,48–54].

2.3. Apoptotic Bodies

Apoptotic bodies are produced as a by-product of cells undergoing programmed cell death [55]. These vesicles are the largest subclass of EVs, ranging from 50 nm to 5000 nm in diameter [30]. The aptly-named apoptotic bodies are formed during apoptosis from the release of cell membrane fragments as cells contract and hydrostatic forces separate pieces of the lipid membrane away from the cytoskeleton of cells [56]. These large EVs can contain much larger cargo than the other EVs reviewed. This cargo can include large, modified proteins, entire cellular organelles, and/or pieces of chromatin. Specifically these bodies are also known to contain components of the nucleus, mitochondria, endoplasmic reticulum, and Golgi apparatus [30,36,57,58].

2.4. Biomineralization Vesicles

Biomineralization vesicles (also known as matrix vesicles) are EVs produced by hypertrophic chondrocytes and mature osteoblasts [59–62]. These biomineralization vesicles range in size from 100–300 nm in diameter and assist in bone formation in both endochondral and membranous ossification [59–62]. While the specific mechanisms for biogenesis are not fully understood, current evidence supports a similar biogenesis mechanism to that of microvesicles which occurs through the outward budding of the plasma membrane [59,63]. These vesicles are thought to bind ECM molecules including collagen and serve as nucleating foci to initiate hydroxyapatite crystallization necessary for bone formation [59–61]. Biomineralization vesicles initiate apatite formation through a combination of luminal and surface proteins. Within these vesicles, orphan phosphatase (PHOSPHO1) and sphingomyelin phosphodiesterase 3 (SMPD3) enzymes are located and function to increase the luminal concentration of inorganic pyrophosphate (Pi) [64]. On the surface

of these vesicles, tissue-nonspecific alkaline phosphatase (TNAP) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) function to increase luminal Pi concentrations [65–67]. Extracellular calcium (Ca^{2+}) is thought to be transported into the vesicles through annexins A5 and A6 [68]. The combination of elevated Pi and Ca^{2+} within the vesicles causes intraluminal apatite to crystalize, and these apatite crystals are necessary for bone mineralization and the formation of hydroxyapatite in bone [59].

2.5. Matrix-Bound Nanovesicles (MBVs)

MBVs are a subtype of EVs embedded within the extracellular matrix (ECM). Ranging in size from 100–300 nm in diameter, MBVs are composed of a lipid membrane that stores and transports a diverse portfolio of signaling molecules. These molecules include proteins, miRNAs, and bioactive lipids [69–72].

MBVs are secreted by tissue-resident cells and are integrated into the solid-phase, fibrillar structure of the ECM [70]. This solid-phase localization of MBVs to the ECM, rather than to the surrounding extracellular fluid, suggests a unique mechanism for MBV synthesis, cellular export, and matrix association that, when taken cumulatively, differs from the mechanisms of synthesis and the release of other EVs [70]. Although MBVs have been shown to be produced by multiple cell types, the mechanisms for the biogenesis of the other EV subtypes are better understood; the mechanisms for the release and matrix association of MBVs remain unknown [73]. MBVs can be isolated from a wide variety of ECM source materials through enzymatic digestion [69–71,74]. While MBVs are similar in size and morphology to exosomes secreted into body fluids or cell culture supernatant, recent evidence show that MBVs contain a distinctive protein, miRNA, and lipid signature compared to liquid-phase exosomes produced from the same cell type [70]. Furthermore, in contrast to liquid-phase EVs, MBVs possess a marked reduction in CD63, CD81, and CD9, with all three of these protein markers being used to identify and characterize liquid-phase EVs (the fourth marker being Hsp70, which has not shown any differences between EVs and MBVs) [69,71,75].

3. Pre-Clinical Applications of EVs in Tissue Engineering and Regenerative Medicine

Given the broad diversity and biologic functions of EVs, there has been a growing interest in the implementation of both naturally derived and bioengineered EV therapies in TERM. Examples of potential regenerative medicine applications of EV-based therapy ranging across a variety of organ systems are reviewed below.

3.1. Cardiovascular System

The cardiovascular system, specifically the heart, represents a challenge in the field of TERM, as the inherent regenerative capacity of the heart cannot compensate for extensive cardiomyocyte damage and death [76,77]. Therefore, there is great interest in developing novel treatment strategies to promote cardiac tissue regeneration and repair. One specific approach is the implementation of EV-based therapies for cardiac regeneration.

There has been notable success in implementing EV-based therapies in animal models of cardiac injury, including myocardial infarction (MI), ischemia-reperfusion injury, and heart transplantation. Exosomes derived from mesenchymal stem cells (MSCs) have been shown to reduce disease progression in models of myocardial infarction and ischemia-reperfusion injury, with an end result of decreased cardiac fibrosis and increased blood flow to the site of injury [8,78–82]. In a separate study, placental MSC-derived exosomes were shown to promote angiogenesis in a model of an ischemic cerebral vascular accident [83]. In addition to unaltered, MSC-derived exosomes, exosomes released from CD34+ human cells engineered to contain Sonic Hedgehog (Shh), a potent mediator of angiogenesis, have shown great promise in reducing infarct size, increasing capillary density, and improving cardiac function in a model of MI [84].

In addition to applications related to MI and ischemia-reperfusion injury, EV-based therapies for promoting tissue transplantation have also been investigated. Specifically,

local delivery of MBVs containing IL-33 in a rodent model of cardiac transplantation have been shown as a promising chronic graft rejection prophylaxis via the immunomodulation of pro-inflammatory macrophages towards an anti-inflammatory phenotype [85].

3.2. Respiratory System

Major challenges for TERM in the context of the respiratory system involve inhibiting the pro-fibrotic and pro-pulmonary hypertension pathways central to the healing of damaged lung tissue. MSC- and lung-derived EVs have been considered as potential therapies for the mitigation of these adverse healing pathways. Specifically, MSC-derived exosomes have been shown to inhibit pulmonary hypertension and suppress hyperproliferative and fibrotic pathways [86]. In addition, lung-derived microvesicles have been shown to be particularly useful in TERM as there is evidence that these EVs can reprogram marrow cells into a pulmonary epithelial phenotype which could help provide a stable cell source for lung engineering [87,88].

3.3. Renal System

While EV research regarding the renal system has primarily focused on EV-based diagnostics, there have been a few recent examples of EV-based therapies in TERM. Specifically, there is evidence supporting the use of microvesicles from both endothelial progenitor cells and MSCs to protect kidney epithelial cells in models of acute kidney injury as well as an ischemia-reperfusion injury of the kidney [89,90]. These microvesicles have great promise in aiding tissue regeneration and cellular survival of bio-engineered kidney devices.

3.4. Nervous System

A major focus in the field of TERM has been on the regeneration and repair of injuries to the central and peripheral nervous system. Like the cardiovascular system, the innate regenerative capacity of the nervous system is limited; consequently, there is a growing interest to implement novel strategies such as EV-based therapies to promote neural regeneration.

There have been several studies showing the promise of EV-based therapy for neural regeneration in both central and peripheral nerve injury. In the central nervous system (CNS), exosomes derived from bone marrow MSC and stromal MSC containing miR-133b have been shown to promote neurite outgrowth and the re-myelination of CNS nerve fibers after injury [91,92]. Exosomes derived from MSC are also understood to inhibit pericyte migration after spinal cord injury and, as a result, these exosomes improve motor function [8,93]. Intravitreal administration of MBVs derived from porcine urinary bladder ECM protect against ischemia-induced retinal ganglion cell axon degeneration and death, as well as preserved visual function in an in vivo rodent model of retinal damage [94]. In the peripheral nervous system (PNS), Schwann cell-derived exosomes have been shown to promote axon growth and regeneration in a peripheral nerve injury model [95]. Broadly, there is a growing body of literature supporting the use of EVs in peripheral nerve repair and showing pre-clinical promise [96].

3.5. Liver

Unlike previously reviewed organ systems, the liver possesses a far greater innate ability for regeneration and repair. However, this regenerative capacity is not without limitations as liver disease and failure represent a significant burden on worldwide health. EVs represent a complementary strategy to the extensive approaches previously implemented in liver TERM. Applications of EVs in liver TERM have focused primarily on the use of hepatocyte and liver stem-cell derived EVs. Hepatocyte-derived EVs have been shown to increase hepatocyte proliferation, viability, and regeneration through the activation of stellate cells in the liver [97–99]. In addition, microvesicles derived from human liver stem cells have been shown to accelerate liver regeneration in hepatectomized rats through the means of EV-mediated RNA transfer to target hepatocytes [100].

3.6. Skin and Wound Healing

The application of TERM in skin and topical wound healing has been extensively studied; the result is that there is an extensive number of products available and in development for this purpose. EVs are a logical supplement to these TERM approaches to skin and wound healing and have shown initial promise in pre-clinical studies. Across various applications of EVs in skin and wound healing, results have consistently shown that EVs derived from a variety of cell sources promote skin and topical wound healing through promoting re-epithelialization, angiogenesis, ECM remodeling, and the migration of keratinocytes and fibroblasts to the wound region [101–110]. Specific applications of note include adipose MSC-derived exosomes and gingival MSC-derived exosomes embedded in a hydrogel formulation [109,110]. In addition, there has been extensive interest in using EVs derived from adipose-derived stem cells for application in skin regeneration and hair follicle regeneration [111].

3.7. Bone and Cartilage Regeneration

The regeneration of bone and cartilage are active research interests in TERM and the utility of EVs in enhancing bone and cartilage regeneration has been shown as promising in pre-clinical results. EV-based therapies derived from MSC sources have been shown to promote cartilage regeneration in vitro and in vivo through increasing type II collagen production and inhibiting cartilage degradation [112–115]. Furthermore, MSC-derived exosomes have been shown to promote cartilage regeneration in vivo when printed into hydrogel scaffolds [116]. In bone regeneration, osteoblast-produced EVs and periodontal-ligament stem cell-derived EVs, and specifically exosomes, have been shown to promote bone regeneration and facilitate osteoclast activation [117–121]. Biomineralization vesicles (matrix vesicles) have also shown promise in the engineering of bone tissue through promoting bone formation and the calcification of bone matrix [60,62,122,123].

Moreover, there has been extensive research focused on the therapeutic efficacy of EVs in the axial skeleton, with particular emphasis on treating intervertebral disc degeneration. Specifically, exosomes derived from MSC sources, such as bone marrow [124–129], human urine [130,131] adipose [132], human placenta [133], and human umbilical cord [134], have all shown promise in reducing various aspects of inflammation and damage in intervertebral disc degeneration. In addition to MSC-derived exosomes, exosomes isolated from nucleus pulposus cells [135–137], endplate chondrocytes [138–140], annulus fibrosus cells [141], platelet-rich plasma [142], and notochordal cells [143] have shown similar early promise in the treatment of intervertebral disc degeneration.

In addition to regeneration of bone and cartilage, EVs have also been explored as a therapy for pathophysiological conditions that affect the joint such as rheumatoid arthritis (RA); for example, bone marrow-derived dendritic cells can be genetically modified to produce exosomes enriched with anti-inflammatory molecules, such as IL-10 [144,145] and IL-4, [146] or pro-apoptotic molecules, such as FasL [147], or immune checkpoint inhibitors, such as CTLA4-Ig. [148] EV have also been isolated from the plasma of antigen-immunized mice [149]. All these approaches have shown pre-clinical efficacy in RA through the immunomodulation of innate and adaptive immune cell populations [144–149]. In addition, MBVs have been shown in vivo to mitigate RA through the immunomodulation of macrophage phenotypes locally and systemically in a rat model [150]. MBVs, a distinct population of extracellular vesicles, represent an alternative to these fluid-phase exosome therapies, with minimal processing and manipulation in comparison [70,74].

4. Clinical Development of EVs

4.1. Companies Developing EV Products

Despite the relatively early stage of EV therapy and diagnostics, EV technology has generated considerable traction in the pharmaceutical industry over the past decade. For example, more than 2000 patents related to EVs (Google Patents, Query in June 2022) have been filed since 2010. The companies involved in the development of EV products represent

small- to medium-sized enterprises. These companies are generally spin-offs from larger companies and the majority are currently in the early process of raising funds, developing technology, and working toward becoming leaders in the growing market (Table 1). In addition, several multinational pharmaceutical companies such as Pfizer, AstraZeneca, Roche, and Merck are also expanding their research and development portfolios to focus on EV therapies. Although no leaders have established market dominance in this growing industry, several companies have been making considerable progress.

Table 1. Companies developing EV-based products.

Company	Technology and Therapeutic Target	Business Status	Headquarters
Capricor Therapeutics	Exosomes for genetic diseases and vaccines	Preclinical development	San Diego, CA, USA
Codiak Biosciences	IL-12 and STING loaded exosomes for immune activation in cancer	Clinical trials—Phases I–II/III	Cambridge, MA, USA
Evox Therapeutics	RNA-loaded exosomes for gene therapy	Preclinical development	Oxford, United Kingdom
Exostemtech	Adipose-derived exosomes for osteoarthritis	Clinical Trials—Phase I	Ansan, South Korea
Florica Therapeutics	Exosome-based therapeutics for neurodegenerative applications	Preclinical development	Livermore, CA, USA
ILIAS	Free-load exosomes for drug delivery	Preclinical development	Daejeon, South Korea
Mantra Bio	Engineered exosomes for targeted drug delivery	Preclinical development	San Francisco, CA, USA
Puretech	Cow's milk exosomes for gene therapy	Preclinical development	Phoenix, AZ, USA

Codiak Bioscience (Cambridge, MA, USA) is a company dedicated to EV-based diagnostics and therapeutics. Specifically, the company is interested in using cell culture-derived EVs for immune activation in the treatment of cancer. Codiak Biosciences filed 11 patents for exosome manufacturing and purification in 2021 and has experienced an exponential growth in revenue over the last few years, reaching a total of \$22.9 million in 2021 (Pitchbook, June 2022). Evox Therapeutics (Oxford, UK) has developed modified EVs for drug delivery with a special interest in siRNA and mRNA delivery for protein replacement or gene therapy. This company has accrued \$155 million in venture capital support. ExoCoBio (Seoul, South Korea) has developed EV-based therapies for cosmetic and skin regeneration applications and has submitted five patents related to this technology in 2021. Other companies like Aegle Therapeutics (Woburn, MA, USA), Panacea Pharmaceuticals (New Delhi, India), and Exopharm (Melbourne, Australia) are in early funding stages and are following similar strategies to the companies mentioned above through an approach to protecting intellectual property and evaluating various potential applications for their own EV technology [151].

4.2. Clinical Trials Testing EV Therapies

Currently, there are 20 active clinical trials investigating EV therapies and six clinical trials that have been concluded. Given the early stage of EV technology, most of these trials or studies are Phase I/II clinical trials focused on evaluating the safety and early efficacy of EV therapies for a wide range of clinical indications, directly and indirectly related to the traditional scope of TERM. Recently, the SARS-nCOVID-19 pandemic has accelerated the clinical translation of EV therapies in general, and these trials have been primarily

directed toward testing EV-based therapies in the context of inflammatory conditions with a particular focus on EV-based therapies for SARS-nCOVID-19 [152–154]. Targeted cancer therapies are also common application of EVs in clinical trials, with these trials using modified EVs to deliver drugs to specific cellular targets as well as to mitigate the effects of aggressive therapies such as chemotherapy and radiation. Table 2 summarizes completed or active clinical trials for EV therapies. While not all of the trials reviewed herein were developed specifically to address questions in TERM, these studies are worth highlighting as the success of EV-based therapies across a wide variety of clinical indications will only help to facilitate the growth and translation of EV-based approaches in TERM.

Table 2. Clinical trials investigating EV-based therapies.

NCT Number	Study Description	Status	Patients
<i>Phase I</i>			
NCT01668849	Edible plant exosome ability to prevent oral mucositis associated with chemoradiation treatment of head and neck cancer	Active, not recruiting	60
NCT04276987	Pilot clinical study investigating inhalation of MSC-derived exosomes for the treatment of severe COVID-19 pneumonia	Completed	24
NCT04313647	Clinical study assessing tolerance of aerosolized inhalation of MSC-derived exosomes in healthy volunteers	Completed	24
NCT04134676	Study assessing the therapeutic potential of stem cell conditioned medium on chronic ulcer wounds.	Completed	38
NCT04849429	Investigation of intra-discal injection of platelet-rich plasma (PRP) enriched with exosomes in chronic low back pain	Recruiting	30
NCT01294072	Study investigating the ability of plant exosomes to deliver curcumin to normal and colon cancer tissue	Recruiting	35
NCT04747574	Evaluation of the safety of CD24-derived exosomes in patients with COVID-19 infection	Recruiting	35
NCT03608631	Exosomes in treating participants with metastatic pancreas cancer with KRAS G1D mutation	Recruiting	28
NCT05387278	Safety and efficacy of placental-derived exosomes and umbilical cord mesenchymal stem cells (UMSC) in moderate to severe acute respiratory distress syndrome (ARDS) associated with COVID-19 infection	Recruiting	20
NCT05375604	A study of exoASO-STAT6 (CDK-004) in patients with advanced hepatocellular carcinoma (HCC) and patients with liver metastases from primary gastric cancer and colorectal cancer (CRC)	Recruiting	30
<i>Phase I/II</i>			
NCT04384445	Zofin (organicell flow) for patients with COVID-19	Active, not recruiting	20
NCT04491240	Evaluation of safety and efficacy of exosome inhalation in COVID-19-associated pneumonia.	Completed	30
NCT05402748	Safety and efficacy of human placenta MSC derived exosomes for the treatment of complex anal fistulas	Recruiting	80
NCT03384433	Assessment of using allogenic mesenchymal stem cell derived exosomes in patients with acute ischemic stroke	Recruiting	5

Table 2. Cont.

NCT Number	Study Description	Status	Patients
NCT04544215	A clinical study of nebulized mesenchymal progenitor cell exosomes for the treatment of pulmonary infections	Recruiting	60
NCT04213248	Assessment of the efficacy of UMSC-derived exosomes on dry eye in patients with chronic Graft Vs Host Disease (GVHD)	Recruiting	27
NCT04602104	A clinical study of nebulized MSC-derived exosomes for the treatment of ARDS	Recruiting	169
NCT04388982	Evaluation of the safety and the efficacy of allogenic adipose MSC-derived exosomes in patients with Alzheimer's disease	Recruiting	9
<i>Phase II</i>			
NCT04969172	A phase II randomized, double-blind, placebo-controlled study to evaluate the safety and efficacy of exosomes overexpressing CD24 to prevent clinical deterioration in patients with moderate or severe COVID-19 infection	Active, not recruiting	155
NCT04493242	Extracellular vesicle infusion treatment for COVID-19-associated ARDS	Completed	120
NCT03228277	Olmotinib trial in t790m (+) non-small cell lung cancer (NSCLC) patients detected by liquid biopsy using bronchoalveolar lavage (BALF) extracellular vesicular DNA	Completed	25
NCT04602442	Assessment of the safety and efficacy of exosome inhalation in COVID-19 associated pneumonia	Enrolling by invitation	90
NCT05261360	Clinical efficacy of MSC-derived exosomes in degenerative meniscal injury	Recruiting	30
NCT04902183	Safety and efficacy of exosomes overexpressing CD24 in two doses for patients with moderate or severe COVID-19	Recruiting	90
<i>Phase III</i>			
NCT05216562	Efficacy and safety of MSC-derived exosome therapy to reduce hyper-inflammation in moderate COVID-19 patients	Recruiting	60

4.3. Future Challenges

The translation of EV-based therapies has grown rapidly over the past two decades and has shown early potential for applications in TERM. However, there remain important challenges that must be addressed to facilitate the translation of these technologies into the clinic. These challenges primarily concern issues of large-scale production, understanding the biodistribution of EVs, and the absence of a thorough characterization of EVs to ensure safe and efficacious clinical translation.

While the production of EVs shares similarities with the manufacturing of traditional biologic products (i.e., industrial production of antibodies and recombinant proteins), the optimal cell source and culture conditions for the large-scale production of EVs have not yet been established. MBVs are a potential solution to this limitation since they are isolated from readily available tissue. However, the preparation of MBVs also requires additional tissue decellularization and ECM solubilization steps that may affect their properties [74]. With regard to the collection, isolation, and purification of EV-based therapies, several studies have established a consensus that standardized protocols and outcome measurements are

necessary for large-scale translation [155–158]. To address concerns in EV isolation and purity, the manufacturing trends are shifting from techniques including ultracentrifugation (which is time consuming and can produce contaminants) towards isolation techniques potentially capable of large-scale manufacturing such as tangential flow filtration (TFF) or size-exclusion chromatography (SEC) [74,157–159]. However, these techniques still have their limitations and further studies are necessary to determine the optimal technique for the isolation and purification of EVs at the large-scale, commercial level. In addition to concerns regarding the manufacturing of EV therapeutics, the long-term storage and stability of EV therapies pose a significant hurdle to clinical translation. The effects of various preservation techniques on EV stability and bioactivity are not well understood [157,160]. Initial studies suggested that cryopreservation at -80°C was superior to storage at 4°C and would preserve EV structure and composition. However, storage at -80°C comes with increased costs and technical challenges. The lyophilization of EVs might address this concern for long-term storage as long as the optimal concentration of cryoprotectants is used to protect the biologic activity of the EVs through this process [156,160]. Altogether, the optimal conditions for the preservation of EVs depend on multiple factors including, but not limited to, the EV source, isolation and purification technique, and the route of administration.

The functional and structural characterization of EVs represent an additional and unavoidable hurdle for the translation of EV-based therapies, and fundamental understanding of the heterogeneity and specificity of each kind of EV is critical. Relevant properties, such as size, shape, molecular cargo (i.e., sRNA, miRNA, cytokines, proteins, or DNA), membrane composition, or surface markers, vary not only between different EV kinds or cell sources but also within EVs derived from the same source [70,155,159,161,162]. Technical limitations for separating EV subpopulations and the lack of specific markers for EV subtypes obstruct the understanding of how these subpopulations affect bioactivity and safety [156,157,159]. Further research and technical innovations are necessary to overcome this hurdle.

The safety of EV-based therapies remains a major barrier to clinical translation. Many pre-clinical and early clinical studies have shown that EV therapies are generally biocompatible and have a low risk of immunogenicity in humans [156,163–165]. However, these clinical studies seldom discuss the safety of repeated administrations as well as the safety of a wide variety of administration routes [156]. Several pre-clinical studies have assessed the biodistribution of EVs in rodents and have shown that EVs are rapidly cleared from the circulatory system [166–168]. However, the accumulation of EVs in the liver or kidneys, and the EV-mediated effects on these organs, are unknown and therefore must be investigated in future studies. Moreover, the relatively rapid clearance of EVs from the systemic circulation could affect the specificity of EV therapies and increase the frequency of off-target, non-specific effects. To address these non-specific, off-target effects, EV membrane decorating techniques have been developed and have shown early potential for more focused, targeted therapy as seen in specific models of cancer [156,168]. In addition to the off-target effects, there remain safety concerns including the possibility of viral vector contaminants in EV preparation. These viral contaminants cannot be overlooked as they present similar characteristics to EVs and are often capable of surpassing filtration systems during the harvest of EVs [155–157].

5. Conclusions

During the past two decades of growing research and interest in EVs, it is clear that EVs represent a promising therapy applicable across a wide variety of TERM applications. It is evident from extensive preclinical and clinical research as well as the rapid growth of companies developing EV-based therapies that there exists a reproducible proof-of-concept model for using EVs in TERM. However, future studies will be necessary to support the development and clinical translation of these therapies. Major hurdles remain in the full biologic and functional characterization of EV subtypes, as well as in establishing cost-effective, safe, and reproducible preparation techniques at the commercial scale. Finally,

future regulatory concerns exist regarding how to define the efficacy, consistency, and safety of EVs, as these EV-based therapies are inherently multimodal in their mechanisms of action due to compositional heterogeneity. Nevertheless, the field of EV-based therapies in TERM is promising and the potentially wide-reaching applications of these therapies continue to inspire the important work of researchers, innovators, and investors alike.

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