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Plastic Surgery Update on the Mesenchymal Stem-Cell Derived Extracellular Vesicles towards Cell-Free Therapeutic Applications

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Abstract

In the biogenesis of extracellular vesicles (EVs), exosomes and other lipid-lined vesicles are released upon fusion of multivesicular bodies with the cell membrane of stem cells. EVs contain a diverse number of growth factors, cytokines and bioactive molecules of proteins, lipids, microRNA, and mRNA that mediate cell-cell communications for homeostasis, immune signaling, angiogenesis, anti-inflammation, senescence, proliferation, and differentiation. To further explore its potential usages, plastic surgeons are beginning to show an increased interest in this novel cell-free therapy to partially explain the paracrine effects of cell-based therapies on cell repair, tissue engineering, and aesthetic rejuvenation. The burgeoning preclinical and clinical experience appears to be promising, but current *in vitro* studies, translational research, and IRB-registered investigations emphasize the need to clarify product identification/purity, attributed biologic functions, standardized protocols, and applications to advance basic science findings and provide beneficial safe clinical outcomes. Since the specialty of Plastic Surgery is committed to advancing evidence-based stem cell studies in compliance with FDA regulations, an updated review of EVs is timely to provide insights to achieve these goals.

Subject Areas

Surgery & Surgical Specialties

Keywords

Regenerative Medicine, Extracellular Vesicles, Mesenchymal Stem Cells

1. Introduction

The goals of Plastic Surgery and Regenerative Medicine are often intertwined because of similar aims to functionally restore injured, malfunctioning and absent tissue by cell, tissue, organ-based therapies [1]-[6] or by tissue engineering combining cells with natural or synthetic scaffolds [7] [8] [9] [10] [11]. For these therapies to succeed, however, the presence of intercellular communications becomes indispensable to direct tissue repair, growth and development and is believed to be mediated through either direct cell-cell contact (juxtacrine signaling) and/or by secreting soluble molecules, such as hormones, growth factors, cytokines, chemokines and neurotransmitters with specific membrane receptors (secretome signaling) [12]. These soluble factors can act on the cell itself (autocrine) or have an impact on both adjacent (paracrine) and distant cells (endocrine).

From the inception of our specialty, plastic surgeons have recognized the value of autogenous tissue as a practical replacement for point-of-care correction of reconstructive and aesthetic purposes. Subsequent investigators later determined that mesenchymal stem cells (MSCs) are self-renewing, multipotent progenitors involved in multilineage differentiation, tissue repair, anti-inflammation, immunosuppression and neuroprotection [13]-[18]. Although the underlying mechanisms that regulate these biological functions are not precisely known, earlier investigations favored the concept that MSCs homed to injured tissue and replenished damaged cells or apoptotic cell populations along their particular cell differentiation pathways. Current studies, however, give more weight to the importance of cell signaling molecules, released by MSCs, than cell replication, to account for tissue recovery emphasizing their paracrine functions of cellular repair by angiogenesis and suppression of inflammation by the host-derived cells [19] [20] [21] [22] [23]. In plastic surgery, these adult stem cells are isolated primarily from adipose tissues and bone marrow because of their *in vivo* expansion capability and ethical acceptability [24] [25] [26]. However, cell therapies have been limited because of immunologic incompatibilities that prevent allogeneic usages for clinical potential. Furthermore, the current regulatory environment restricts the use of cell therapies because of limited long-term controlled studies that demonstrate safety and efficiency issues associated with undesirable differentiation and de-differentiation, senescence-induced genetic instability and cell survival [27] [28] [29] [30] [31].

In the past three decades, a third mechanism by which mesenchymal stem cells act in a paracrine fashion has emerged that involves intercellular transfer of extracellular vesicles (EVs), also known as exosomes [32]. Although other subtypes of EVs have been identified, such as microvesicles (MVs), membrane particles, and apoptotic bodies, this chapter will focus mainly on the current knowledge on the composition, functions and isolation strategies of MSC-derived exosomes. As a cell-free alternative to stem-cell-based strategies, EVs may be adoptable to other tissue regenerative applications that may be unhampered

from allogeneic constraints and still remain under the strict regulatory guidelines as a biologic product in the practice of plastic surgery.

2. Literature Review

Prokaryotic and eukaryotic cells have conserved an evolutionary remnant for cell-cell functional communications during physiological and pathological processes through the secretion of EVs. Initially, the release of EVs was believed to represent a waste disposal mechanism for cells to eliminate unwanted molecular material in its extra or intra-cellular environment. Although Chargaff and West (1946) [33] observed platelet-derived particles, that was later referred to as "platelet dusts" by Wolf (1967) [34], Pan and Johnstone (1983) [35] clearly described them as membrane-contained vesicles of endosomal origin secreted from sheep reticulocytes. In 1987 Johnstone [36] coined the term "exosomes" to describe the vesicle formation and release from the reticulocyte's cell membrane. Twenty years later, Viladi [37] discovered that EVs shuttled mRNA and micro-RNA strands of genetic material between cells as mediators of normal and pathological cell-to-cell communications.

Each human cell type is capable of secreting different subspecies of EVs that possess specific physiologic properties, content, and functions for their own waste management and recycling of membrane proteins and lipids, as well as for targeting adjacent recipient cells to influence their immune modulations, senescence profile, angiogenesis, and cellular proliferation and differentiation [38] [39] [40] [41]. Although EVs have been isolated in all body fluids, including blood, urine, saliva, breast milk, pleural effusions, bronchioalveolar lavage, synovial fluid, amniotic fluid, ascites, cerebrospinal fluid, bile and semen [42], a major ongoing challenge remains the establishment of standardized methods that can distinguish amongst the different isolated subtypes on the basis of their size, morphology, density, composition, main protein markers, and subcellular origin (plasma membrane vs intracellular compartment). In the past, vesicular nomenclature was primarily based on the tissue of origin. More recently, the EV community has shifted towards a terminology based on mechanism of generation of these vesicles. As a consequence of their cell of origin, the molecular compositions of EVs are diverse containing a variety of intracellular cytosolic proteins [43] (endosome-associated RAB GTPase, SNAREs, Annexins, and flotillin) and cytoskeletal proteins (actins, cofilin, tubulin), extracellular membrane adhesion proteins [44] [45] (adhesion domains of integrins and tetraspanins [CD^{63}, CD^{81}, CD^{82}, CD^{53}, CD^{37}], lipids [46] (cholesterol, sphingomyelin, phosphotidylserine, and hexosylceramides), RNA [47] [48] (mRNA, microRNA), and DNA fragments [49]. Although the above-mentioned molecular compositions of EVs have been reported, none of them is described as a distinct marker that identifies subsets of EVs. The database ExoCarta (http://www.exocarta.org) and Vesiclepedia continue to update and identify these novel components. Currently, there remains no consensus about the nomenclature or classification of

cell-derived EVs. Members of the International Society of Extracellular Vesicles continue efforts to define equivalent and standardized protocols for isolation and characterization of EVs.

3. Current Limitations of Isolation and Characterization of EVs

The inter-related complexities of detection, recovery and characterization of EVs have been hampered by the complex nature of biological fluids, heterogenous and overlapping vesicular sizes, densities and shapes, which are summarized in **Table 1**. The use of variable g-forces and other sophisticated separation techniques during differential centrifugation processes [50] [51] [52] also contribute to dilution, fragmentation, fusion, and contamination of pellets with cellular components. That being said, centrifugal and ultra-centrifugal forces of 200 - 1500 g are useful to segregate discarded cells and cellular debris, while greater forces of 10,000 - 20,000 g isolate vesicles larger than 100 nm, and 100,000 - 200,000 g collect vesicles smaller than 100 nm. Since 2011, efforts to develop new technologies [53] are being coordinated with the International Society for Extracellular Vesicles to unify the nomenclature and methodologies of EVs (www.journalofextracellularvesicles.net).

3.1. Size and Morphology

Although Transmission Electron Microscopy (TEM) [54] [55] has been the preferred technique for determining the size and morphology of EVs, the vacuum, fixation and dehydration processes create artifacts that can display appearances ranging from a cup-like to round configurations. Since most differential size determinations are based ideally on the number of vesicles per unit particle size and suspension volume, the associated variables of TEM or fluorescence microscopy, super-resolution microscopy, and nanoparticle tracking devices probably do not convey the natural sized population of EVs. However, quantitative analysis of multiple EVs in many samples underscore the heterogenous diameter measurements of exosomes [56] (30 - 150 nm), microvesicles [57] [58] [59] (20 - 1000 nm), membrane particles [60] (50 - 80 nm, 600 nm), and apoptotic vesicles [61] (1000 - 5000 nm).

3.2. Density

One of the most defining characteristics that distinguish different extracellular vesicles is their ability to equilibrate at different levels by sucrose density gradient centrifugation [62]. Like all lipid vesicles, EVs equilibrate at densities ranging from 1.13 to 1.19 g/mL from pooled fractional analyses [50] [54]. Although subtypes of EVs can be separated by buoyant velocity centrifugation in a sucrose gradient for varying lengths of time [63], density studies emphasize that EVs are heterogenous and, therefore, will require new technologies to distinguish their densities in both small and large vesicles.

Table 1. Characteristics of different subtypes of extracellular vesicles.

Features	Exosomes	Microvesicles	Apoptotic Bodies
Diameter	30 - 150 nm	50 - 1000 nm	1000 - 5000 nm
Appearance	cup - shape	cup - round shapes	variable shapes
Density in (Sucrose Gradient)	1.13 - 1.19 g/mL	1.04 - 1.07 g/mL	1.16 - 1.28 g/mL
Sedimentation	100,000 g	10,000 g	16,000 g
Marker Proteins	tetraspanins, Alix, TSG101	integrins, selectins, CD40 ligand	histones
Origin	Endosome budding into MVBs, fusion of MVB with cell membrane	Outward budding of cell membrane	Outward blebbing of apoptotic cell membrane
Composition	Protein, lipids, coding RNA, Noncoding RNA, DNA	Protein, lipids, cell organelles, coding RNA, noncoding RNA, DNA	Cell organelles, proteins, nuclear fragments, coding RNA, noncoding RNA, DNA

3.3. Molecular Composition

Most studies on the biochemical composition of EVs are based on protein, lipid and nucleic acid analysis of the vesicles' intracellular or extracellular membrane origins by a number of techniques such as differential ultracentrifugation, western blotting and mass spectroscopy of *total* populations or by fluorescent flow cytometry of *single* vesicles. Currently, the exact composition of each subtype has not been defined. The database ExoCarta [64] (http://www.exocarta.org) and the updated compendium Vesiclepeida [65] continue to catalogue proteins, lipids and RNA and purification procedures from different groups with equivalent and standardized EV isolation protocols.

3.3.1. Proteins

Proteomic investigations demonstrated that exosomes are composed of a specific subset of extracellular and cellular proteins of the cell type that secretes them but also composed of nonspecific intracellular proteins from endosomes, plasma membrane, and cytosol that are common to all cell types. Of interest, exosomes are usually lacking proteins found in their nucleus, mitochondria, endoplasmic reticulum and the Golgi apparatus suggesting that protein sources are selected from specific subcellular compartments and not obtained from other randomly available protein entities. Columbo *et al.* [66] have proposed a schematic representation of a canonical exosome depicting a vesicle enclosed by a bi-lipid layer that is populated with spatially specific intracellular domains of 1) lipid raft-bound fusion proteins of annexins, flotillins, RABs, and ARFs and 2) other intracellular domains of cytosolic histones, ribosomal proteins, and proteasomes, as well as extracellular domains of transmembrane-bound proteins of LSMPSs, and TfRs, and other adhesion molecules of tetraspanins and integrins. Although the protein content of exosomes has been extensively investigated, refinements of purification techniques are continuing to clarify their functional roles in each EV subgroup.

3.3.2. Lipids

The characteristic composition of the bi-lipid layer around exosomes is made up of specific families of sphingomyelin, phosphatidylserine, cholesterol, and ceramide with lipid-raft domains of proteins that are distributed differently within EV subtypes [67] [68]. Lipids are not randomly included into EVs but, similar to other biomolecules, they are specifically sorted to provide structural rigidity to EVs [69] and possibly to be involved in their formation and release [70]. It remains unclear whether the makeup of portions of the bi-lipid layer of the cell's plasma membrane (PM) that contribute to the sorting complex remain the same throughout the transformative process of endosomes (IEs) to intraluminal vesicles (ILVs) housed within multi-vesicle bodies (MVBs). Studies show that exosomes differ from the secreting cells in terms of lipid composition suggesting the presence of sorting mechanisms for these specific lipid species into vesicles (see Biogenesis of Extracellular Vesicles Biogenesis, below). Further studies are required to elucidate the roles of lipids in the biogenesis and biological functions of EVs.

3.3.3. Ribonucleic Acids (RNAs)

Since the 2006-2007 discoveries of RNAs in murine-derived EVs [37] [71], subsequent studies found the presence of intact mRNA [72], mRNA fragments [73], long non-coding RNA [74], miRNA [48], piwi-interacting RNA [74], and fragments of tRNA [75]. Most studies reported absence or minor presence of ribosomal 18S and 28S in EVs [76]. Evidence suggest that certain populations of RNAs are loaded and enriched in specific subtypes of EVs and excluded in others by active sorting mechanisms [77] [78]. By horizontal transfer, loaded EVs were observed to release specific mRNAs that regulated gene expression in the parental cells [79], increased protein expression in other cells [71], triggered neoangiogenesis in endothelial cells [80], enhanced cell survival and repair tissue under stressful conditions [81] [82], and promote cell differentiation, proliferation, and immune regulation [82]. EV-mediated transfer of mRNAs from adipocytes has also been shown to stimulate lipid synthesis [83] and secreted into the blood circulation for other extracellular physiological roles [84] [85] [86] [87]. However, the precise individual RNA's contributions to regulate neighboring or distant cellular effects remain difficult to assess [74].

3.3.4. Deoxyribonucleic Acids (DNAs)

The presence of oncogenic DNAs, mitochondrial DNAs, single and double-stranded DNAs [88] [89] have been detected in EVs targeting fibroblasts [90] and in tumor cells [91]. Currently, the biological significance of DNA cargo in EVs is unknown.

3.4. Biogenesis of Extracellular Vesicles

Although microenvironmental factors such as hypoxia [92] and inflammation [93] have been proposed as initiators for MSCs to commence biogenesis and se-

cretion of EVs, it is likely that both extracellular factors and intracellular demands play specific roles in the regulation and sorting of EVs for intracellular requirements and for cell-cell communications [94].

3.4.1. Exosome Formation

In the "classic endocytic pathway", the process begins in the parent cell membrane by the initiation of curvature-induction mechanism(s) that sort membrane constituents such as lectins [95], lipid-membrane protein rafts [96] [97], tetraspanin-enriched microdomains [98] [99] [100] for shaping, sizing, composing, and inward budding of the vesicle. Both the protein Endosomal Sorting Complex Required for Transport (ESCRT), located at the neck region of the emerging bud, and the clathrin-dependent or caveolae-dependent membrane pathways, are believed to play significant roles in the fission of the membrane's bud to form early endosomes.

Figure 1 shows the pathway of Extracellular Vesicles (EVs). Biogenesis, degradation and secretion of EVs generally follows the classic endolysosomal pathway that orchestrates Endocytosis, MultiVesicle Body (MVB) formation, Exosome (E) fusion and secretion, or autophagy induction and lysosomal cargo degradation and subsequent recycling, preserving cellular homeostasis. Alternatively, a second immediate pathway involves the release of Microvesicles (MV) directly from the plasma membrane often during cell stress. Different sorting pathways exist directing EVs predestined for either secretion or degradation. During apoptosis, membrane blebbing precedes the release of larger sized apoptotic vesicles. EVs deliver their cargo of proteins, lipids and RNAs by the interaction of transmembrane proteins on EVs with receptors on cell membranes through several pathways such as phagocytosis, fusion or endocytosis.

By the inward budding of the endosome membrane into their lumen, early endosomes developed into mature endosomes that accumulate intraluminal vesicles (ILVs) that are generally referred to as multivesicle bodies (MVBs). Although a number of investigations [101] [102] demonstrated the presence of ESCRT-independent mechanisms, other studies [103] [104] [105] found that ESCRT-dependent mechanisms were necessary for exosome biogenesis through the sorted binding of microdomains of ubiquitinilated proteins and lipid rafts for ILVs' sequestering of selective proteins, lipids and cytosols. Further investigations [106] [107] [108] suggest that incorporation of a given protein into ILVs may predestine the endosomal vesicles to follow one of two pathways. For example, subpopulations of MVBs may follow the classic intracellular pathway leading to either cargo degradation (lysosomal pathway for recycling) after ubiquitination of transmembrane proteins [109] or secretion indirectly as released exosomes when the MVB's membrane fuses with the cell's membrane [110]. A second pathway exists for other vesicular species that follow a direct pathway for their release from the cell membrane as microvesicles that are for the most part indistinguishable from exosomes in that they are capable to transfer functional genomic and proteomic content to recipient cells [71] [72] [109] [110] [111]. In

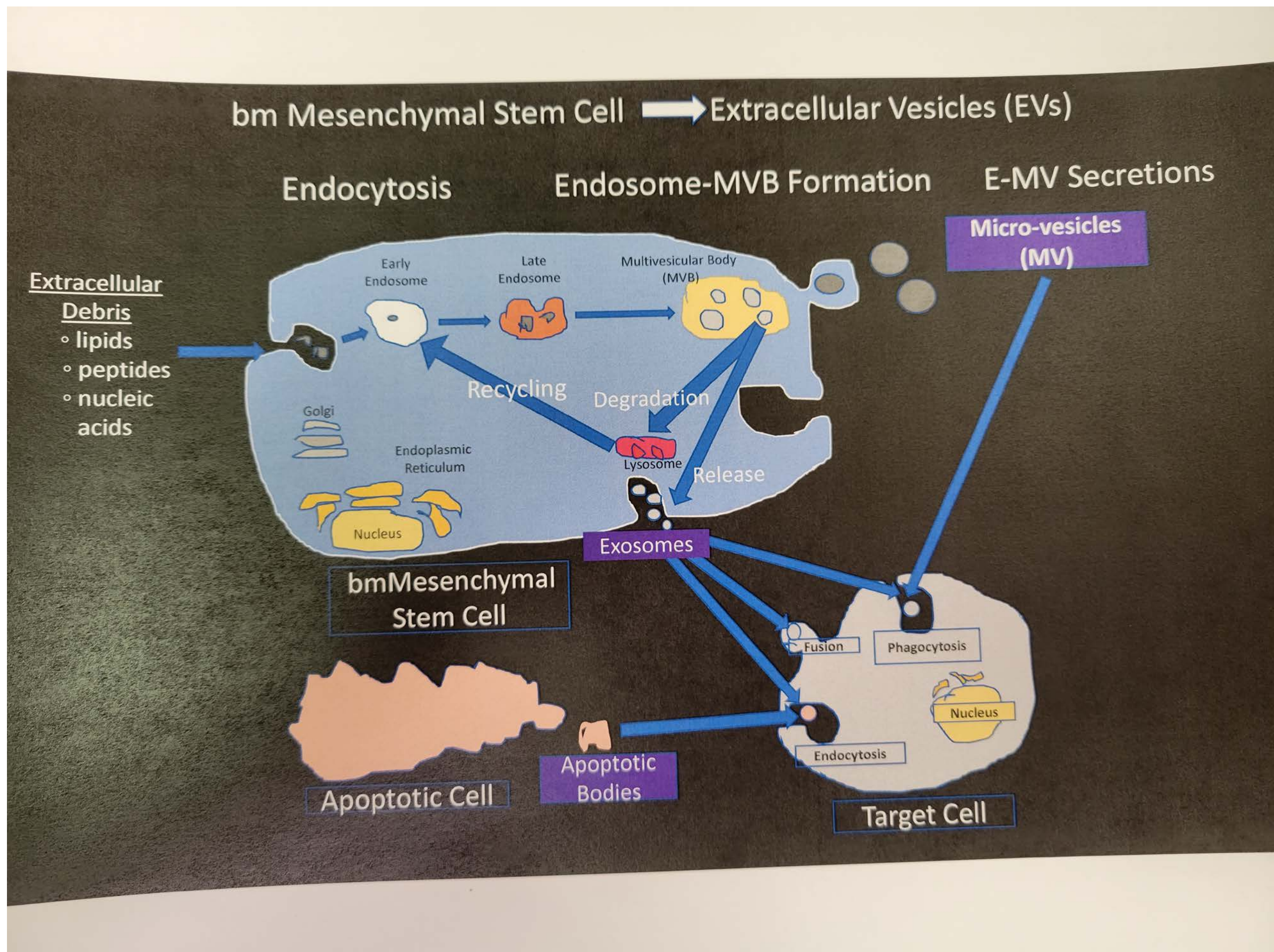

Figure 1. Extravesicle pathway.

most cells, different subpopulations of MVBs coexist with the majority destined to fuse with lysosomes to ensure degradation of their cargo, while others are designated for exocytosis as exosomes or microvesicles.

Apoptotic vesicles [59] [61] originate at the cell membrane in a process referred to as "membrane blebbing" as the cell undergoes death. The major differences between apoptotic vesicles and other cell-derived vesicles are their large sizes, variable shapes, and the presence of a specific histone marker [112]. Since platelets and apoptotic vesicles undergo similar blebbing process from their parent cell, they may be indistinguishable based on their similar ranges in size.

3.4.2. EVs Secretion, Docking, and Endocytosis

The processes involved with scission, release and docking of EVS from the cell membrane of the parent cell to the recipient cell's membrane surface are still being defined. These mechanisms are believed to involve the cytoskeleton (actin and micro-tubules), associated molecular motors (kinesins and myosins), molecular switches (small GTPases), the fusion apparatus (SNAREs and tethering factors) and the Rab proteins [113]. The RAB family of over 60 GTPases has garnered attention because they are believed to be essential regulators of intra-

cellular vesicle transport between different compartments and also interactions between vesicle budding, mobility through cytoskeleton network, secretion release, and fusion on the acceptor cell membrane [114] [115].

The binding of EVs to the cell membrane is believed to involve the targeting of several specific ligand/receptor pairs on both EVs and the recipient's plasma membrane [116]. The first binding step of EVs to recipient cells might be sufficient to induce physiological responses in the recipient cells [117]. In other circumstances, EVs contents of proteins, lipids, and RNAs must be transferred through endocytotic mediated-pathways [118]. After internalization has occurred, the vesicles can be degraded and their contents recycled for usage or transferred as exosomes to recipient cells [119].

3.4.3. EVs in Regenerative Medicine (Plastic Surgery)

Since Owen [120] and Caplan [121] introduced respectively, the terms stromal stem cell or mesenchymal stem cells to the scientific literature over thirty years ago, MSCs have established themselves with *in vitro* and *in vivo* credentials with traits of self-renewal and multipotent differentiation. Under the criteria of the International Society of Cellular Therapy, MSCs are characterized by a combination of culture properties, phenotype markers, multi-lineage differentiation capacity and identification of tissue origin [122] [123]. MSCs exhibited intense paracrine activity by secreting a number of bioactive molecules (growth factors, cytokines) directly or releasing others moieties via the intracellular EVs pathways with trophic [124] [125], angiogenetic [126], immunomodulatory [127] and immunosuppressive [128] capacities to reduce apoptosis, inflammation, fibrosis, and promote angiogenesis and proliferation. In fact, most of these secreted bioactive molecules consisted of soluble factors, exosomes and microvesicles [129]. As of 2012, open hMSCs clinical trials registered at *clinicaltrials.gov* include the treatment of graft-versus-host diseases, autoimmune disorders, bone and cartilage repair, cardiac, neurological and renal diseases [130] [131] [132] [133] [134].

For over a century, adipose tissue has been the preferred autologous tissue replacement for many reconstructive and aesthetic procedures in plastic surgery [135]. Besides its attributes as an autogenous graft material, adipose tissue is recognized as a more attractive supplier of adult adipose-MSCs [136]. Within the past two decades, two separate autologous adipose-derived stem cell therapies emerged as promising methods to improve fat grafting. Of the two, the combination of adipose tissue either with adipose stem cells (ASCs), derived enzymatically from stromal vascular fraction (SVF), has been the most controversial strategy because of regulatory concerns and varied results [137]. Surgeons reported both encouraging [138] [139] [140] [141] [142] and equivocal [143] [144] outcomes with SFV cell-assisted lipo-transfer. A second less contentious clinical strategy is represented by combining adipose tissue with either mechanically-derived adipose mesenchymal stem cells [145]-[150] or with platelet-rich plasma (PRP) [142] [151] [152] [153] [154]. However, a critical and comprehen-

sive reading of the positive published outcomes with either mechanically-derived adipose-stem cells or platelet-enriched plasma leads to the conclusion that designed randomized controlled trials are needed to demonstrate unambiguous long-term results. Stem-cell-based therapies are based on autologous harvesting and transplantation. As examples of cellular therapy, adipose-stem cells and platelets require oversight as biologic products by the Federal Drug Administration (FDA) under the Public Health Service Act and the Federal Food Drug and Cosmetic Act [137] [155].

As a cell-free therapy, the potential applications for the use of EVs in plastic surgery are being investigated through clinical trials because the diverse released bioactive constituents from exosomes and microvesicles display similar paracrine effects as growth factors and cytokines discharged from MSCs and activated platelets. The use of EVs biochemical products over cell-based therapies are the following: alternative to cell-based therapies, allogenic usage, superior safety profile, less immunogenicity, "off-the-shelf" strategy, stability and scalability [156] [157] [158]. The data obtained thus far has established EVs as novel players in mediated-horizontal transfer of a cargo of growth factors, cytokines, chemokines, proteins, lipids and nucleic acids for gene regulation, immune evasion, disease formation, tissue healing, growth and development.

The potential therapeutic benefits and mechanisms of action of MSC-derived EVs are being recognized to-date in some of the following pre-clinical studies and early clinical applications for plastic surgery. The finding that vesicles facilitate intercellular exchange of biomolecules may have important implications for the development a new class of therapeutics and regulatory pathways [159].

1) Vascular Biology: One of the best characterized roles of EVs is their capacity to enhance hemostasis, angiogenesis, and suppress endothelial cell senescence [160] [161] [162] [163].

2) Immunology: The diverse immunomodulatory properties of MSC-EVs include the paracrine messaging as pro-inflammatory mediators in infections, sepsis, and chronic inflammatory diseases [164] [165] [166].

3) Wound Healing: MSC-EVs have been demonstrated improve wound repair and to increase re-epithelialization, promote skin cell proliferation and inhibit apoptosis [167]-[172].

4) Biofilm: Bacterial outer membrane vesicles (OMVs) have been shown to contain proteins and other biomolecules that play a role in the formation and maintenance of the extracellular matrix contributing to biofilm formation and stability [173] [174].

5) Adipose Tissue: MSC-EVs have been shown to increase fat graft survival and browning from white cells [175] [176] [177].

6) Cartilage and Bone Healing: MSc-EVs have been found to increase chondrocyte growth and bone regeneration after degenerative disease and trauma [178] [179] [180] [181] [182].

7) Hair Growth: EVs have resulted in increased hair growth density, shaft diameters and pigmentation [183] [184] [185] [186] [187].

4. Summary and Future Directions

Stem cell-based research and therapies are currently being conducted worldwide with the underlying mechanisms for cellular proliferation, differentiation and homeostasis significantly achieved through paracrine functions of lipids, proteins, mRNAs, microRNAs, and bioactive molecules (growth factors, cytokines). In recent years, increasing attention has been given to the roles of extracellular vehicles not only in causing diseases but also in promoting normal physiological homeostasis and regenerative repair. Although the applications of cell-free therapy over cell therapy have been discussed, there exist major hurdles in both fields that include lack of standardization, molecular characterization, purity and reproducibility. Regarding clinical applications for safety and effectiveness, the active biological agents or cells used in both regenerative therapies are not FDA-approved and therefore under strict FDA regulation for their isolation, quality of production, and clinical usage. Future EVs studies and clinical experience in Plastic Surgery should focus on mechanisms by which EVs ameliorate diseases and aging, and, through evidence-based research, develop protocols and guidelines for the different use of stem-cell-derived EVs, while working within the current regulatory pathways.

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Conflicts of Interest

The author declares no conflicts of interest.

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