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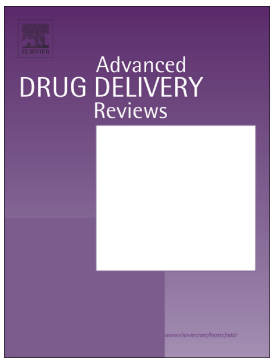

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Extracellular vesicles as modulators of wound healing

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Abstract

Impaired healing of cutaneous wounds and ulcers continues to have a major impact on the quality of life of millions of people. In recent years, the capacity for stem and progenitor cells to promote wound repair has been investigated with evidence that secreted factors are responsible for the observed therapeutic benefits. This review addresses current evidence in support of stem/progenitor cell-derived extracellular vesicles (EVs) as a regenerative therapy for acceleration of wound healing. Encouraging results for local or systemic administration of EVs have been reported in a range of clinically-relevant animal models of cutaneous wounds. Furthermore, a number of plausible mechanisms involving EV-mediated transfer of proteins and RNAs that trigger pro-repair pathways in target cells have been demonstrated experimentally. However, for successful clinical translation in the coming years, further emphasis on standardized experimental protocols, detailed methodological reporting and clear definition of EV-based therapeutic products will be required.

Keywords: exosomes, microvesicles, inflammation, miRNA, intercellular communication, proliferation, angiogenesis, scar formation

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1. Introduction

Our skin protects us from environmental challenges such as extremes of temperature and from invading pathogens. However, the skin is frequently damaged due to trauma (acute wounds) or may fail to heal properly following injury, particularly in the setting of medical conditions such as diabetes mellitus (chronic wounds or ulcers). Rapid and efficient closure of wounds is paramount for the skin to maintain its integrity and prevent systemic invasion by infectious agents. Impaired wound healing is a major issue for our societies as it confers pain, suffering, psychological stress and loss of quality of life on many millions of people and is associated with huge costs to health care providers and insurers [1]. It is estimated that the global wound care market will reach over $22 Billion by 2020 [2, 3]. Despite tremendous progress made over the last decades to improve wound healing, there are still major challenges to be addressed, in particular related to the healing of chronic wounds.

Currently, one of the areas of intense investigation to promote and improve wound healing is the application of stem cells or stem cell-related products. Recent advances in wound healing therapy have shown great potential for the application of mesenchymal stem/stromal cells (MSC) or pluripotent stem cells [4-6]. Clinical trials are currently exploring the possibility of using MSC as a cellular therapy for a wide range of diseases involving acute and chronic tissue injury with evidence accumulating for clinical efficacy from early-phase clinical trials [7-12], However, there have also been reports of equivocal or negative efficacy signals and early trial terminations for indications including multiple sclerosis, acute myocardial infarction, acute kidney injury, critical limb ischemia and stroke [8, 13, 14]. Several reasons for the failure of MSC clinical trials have been discussed in the literature. Firstly, culture expansion and cryopreservation of MSC may reduce therapeutic efficacy by inducing immunogenicity [15]. Secondly, when MSC are injected intravenously they are generally trapped in the lungs, limiting direct access to primary disease sites [16]. Thirdly, limited knowledge on the mechanism of action results in a lack of specific potency assays for individual disease indications. Interestingly, it has been repeatedly demonstrated that the beneficial effects of MSC result – at least in part – from their secreted products in a paracrine fashion, which could be mediated via extracellular vesicles (EVs) [17-20].

Recent studies of the application of EVs derived from MSC and other cell types for acceleration of the wound healing process have shown promising results [21-33], albeit with varying levels of stringency in the reporting of EV isolation and characterization. The aims of this article are to summarize and critically appraise the existing knowledge related to the

potential clinical translation of EVs as a cell-free delivery vehicle for wound healing and tissue repair and to highlight the most important avenues of investigation for maximizing the future therapeutic benefits of EV-based therapies.

2. Wound healing

Wound healing is a complex and dynamic physiological process that has been sub-divided into four well-orchestrated, sequential and overlapping phases – haemostatic, inflammatory, proliferative and remodelling [34-37]. In the haemostatic phase, the clot formed protects the wound site from environmental contaminants and provides matrix and soluble factors [e.g. transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF)] that facilitate adherence and act as chemoattractants for the various cell lineages involved in the healing process [34-36, 38, 39]. The subsequent inflammatory phase, characterized by infiltration of bone marrow-derived immune cells, contributes to the healing process by clearing the wound site of invading pathogens, apoptotic cells, cellular debris and damaged matrix in preparation for resolution of the injury [35, 36]. Microbial pathogens are removed by neutrophils which undergo apoptosis [35, 40, 41] and are, in turn, phagocytosed by macrophages along with other apoptotic cells and cellular debris [38, 41, 42]. Lymphocyte infiltration also occurs but its precise role in wound healing is less clear. Some reported observations, such as decreased wound-breaking strength following T-cell depletion, suggest an important role for T-cells in promoting wound resolution while others, such as the absence of scar formation in immunodeficient mice, suggest a detrimental T-cell effect [43-45]. These discrepancies may reflect divergent effects of different T-cell subsets on wound healing. Specifically,$CD4^+$ T-cells have been associated with pro-healing effects while $CD8^+$ T-cells have been observed to negatively impact wound repair [44]. Interestingly, a skin-resident gamma-delta T-cell subset has been identified as playing important roles in wound healing by modulating tissue architecture and inflammation as well as protecting against infection [46, 47]. These inflammatory events culminate in the transition of macrophages towards an alternatively-activated phenotype that promotes tissue regeneration by modulating keratinocyte, fibroblast and endothelial cell activation [39, 48]. This triggers the proliferative phase of wound healing which is characterized by extensive fibroblast proliferation and production of extracellular matrix (ECM) components that provide support for re-epithelization to occur. The intense proliferative activity results in decreased oxygen tension which, when combined with high lactate levels and acidic pH

found at the wound bed, promotes angiogenesis [6, 35, 36, 49, 50]. Finally, in the remodelling phase, collagen breakdown and structural adjustment of the newly produced ECM results in decreased wound thickness [35, 36, 51]. During this phase, the majority of the newly-formed capillaries regress so that the vascularity of the tissue is normalized [35] and the margins of the wound are drawn together by contraction of the underlying connective tissue [6, 36].

The complexity and tight orchestration of the wound healing process present a significant challenge to the development of new therapies given that these have to affect, in the correct sequence, the coordinated activities of numerous cell types, each of which produce various soluble factors. Since EV cargo has the potential to target a range of molecular processes and recipient cells, EV are emerging as a promising delivery vehicle for improving wound healing.

3. Extracellular vesicles

Extracellular vesicles are lipid bilayer membrane-enclosed subcellular structures which are usually spherical in shape with sizes ranging from several nanometres (nm) to a few micrometres (μm). These are produced by virtually every cell type in the body and have been isolated from most body fluids (from breast milk to urine samples) as well as from dissociated tissues and cell culture supernatants [52-65]. Over the last decade, research on EVs has flourished and has progressed dramatically from the initial notion of "garbage bags" for cellular waste disposal to an emerging consensus that they operate as a highly-regulated mode of communication between and among cells and tissues.

Current understanding of EVs allows for their classification into three main subtypes, according to their mode of biogenesis (Figure 1) – exosomes, ectosomes (also known as microvesicles) and apoptotic bodies [53, 54, 66, 67]. Exosomes (30-150 nm) are of endocytic origin and their formation begins with inward budding of the cell membrane and the creation of an early endosome. Endosomes can bud again to form so called multivesicular bodies (MVBs). These MVBs can then undergo successive inward budding events which create multiple intraluminal vesicles and ultimately follow one of two fates. Firstly, they may fuse with lysosomes and have their contents degraded. Secondly, they may fuse with the plasma membrane, leading to the release of their contents, the exosomes, into the extracellular space (Figure 1A) [68-71]. Ectosomes (100-1000 nm), also known as shedding microvesicles,

originate from the outward budding and enzymatic fission of small protrusions of the plasma membrane which occur at points where the interaction between cytoskeletal proteins and the plasma membrane has been lost. This process is highly promoted by increased cytosolic calcium levels [72-74]. Even though the precise mechanism by which ectosomes are formed is yet to be fully elucidated, it is believed to be triggered by the redistribution of the phospholipid contents of the plasma membrane [70, 75]. This occurs through the combined activities of multiple enzymes which flip the lipid molecules from the inner to the outer leaflet (or the inverse) of the plasma membrane and constitute a signal for the vesicles to be formed [70, 76, 77]. Ectosome biogenesis requires cytoskeletal protein contraction to be completed and this occurs through the modulation of actin and myosin interactions (Figure 1B) [78, 79]. Apoptotic bodies (50-5000 nm) are created when cells are undergoing apoptosis through outward blebbing of the plasma membrane, being largely destined for phagocytic clearance [80-82]. Apoptotic bodies are composed of cytoplasm from the parent cell and can often contain nuclear fragments and functional organelles which can have functional effects during intercellular communication events (Figure 1C) [82-86].

Despite having unique biogenesis pathways, the different EV subtypes display overlapping composition, density and size. These shared properties are highly problematic for the investigation of the biological roles of the individual EV subsets [67, 87]. However, although unique distinctive features are yet to be described, some proteins such as programmed cell death 6 interacting protein (Alix), tumor susceptibility gene 101 (TSG101), heat shock protein 70 (HSP70) and the tetraspanins CD9, CD63 and CD81 are believed to be specifically enriched within the cargo of exosomes and have been used, along with the size range difference, for the isolation of EV samples which are predominantly composed of exosomes [69, 71].

The cargo of these vesicles is reflective of their cellular origin, is modulated by the surrounding environmental stimuli and consists of various biomolecules (proteins, lipids, DNA, mRNA and miRNA) [53, 66, 88]. Over the years, information from multiple studies on EV composition has been compiled into databases which are regularly updated – Exocarta, Vesiclepedia and EVpedia [74, 89, 90]. The functional relevance of EVs is derived from their involvement in the transport of biomolecules and signals to other cells through intercellular communication events [53, 66, 91]. Their composition and surface molecules provide EVs with specific mechanisms of interaction with target cells (e.g. through ligand-receptor interaction, internalization or direct membrane fusion [Figure 2]). As a result of these

interactions, EVs may induce intracellular signaling events and changes in cellular structure and function [53, 92-99]. They can also participate in various physiological processes including hemostasis and thrombosis, inflammation, immune interactions and angiogenesis [53, 100]. Their importance is not exclusive for normal physiological processes as EVs have been implicated in the development and progression of cancer, autoimmune diseases and bacterial and viral infections [52, 53, 101-110]. Therefore, there is increasing interest within the scientific community in exploring the potential application of EV-based assays as biomarkers in various diseases as well as in the adaptation of EVs as therapeutic tools in multiple clinical settings including wound healing [6, 54, 100, 111].

In the following sections, we will discuss current research progress regarding the potential therapeutic value of EVs for wound healing/tissue repair and the extent to which the published reports support clinical translation. Emphasis is placed primarily on work that has been reported during the past three years. Importantly, as we will subsequently highlight, there is substantial methodological variability across the literature and not all of the studies in this area provide sufficiently detailed information to allow for definitive conclusions regarding EV efficacy and mechanism of action.

4. EVs for cutaneous wound healing

Recent work has provided increasing evidence for a potential therapeutic role of EVs for cutaneous wound healing [21-33]. A detailed summary of these studies, grouped by animal model used, is provided in Tables 1 to 4. As is clear from the Tables, several different rodent cutaneous wound models have been studied in order to gather evidence for the potential clinical translation of EV treatment and, in general, the experimental results from these studies have demonstrated that EV administration results in accelerated wound healing and reduced scar formation. Among these published reports, varying degrees of observational detail are presented regarding specific aspects of wound healing such as re-epithelization, neovascularization/blood vessel maturation, increased collagen deposition and development of hair follicles and sebaceous glands. Furthermore, some omitted potentially important controls or were unclear relatively to experimental group size [21, 25, 32, 33]. It is also important to consider that negative studies may not have been published resulting in a publication bias toward positive results. Moreover, apart from having in common the fact that EVs were isolated from cell culture conditioned media, the published animal model studies in

this area vary greatly in multiple aspects including the cellular origin of the EV preparations (from MSCs to amniotic epithelial cells), the protocols used for EV isolation, the quantification method used to determine dosage for administration and the administration method employed.

For example, the reported isolation methods differ greatly between different studies. Even though all the isolation protocols used incorporate initial steps of differential centrifugation and filtration, these are followed either by PureExo® Exosome Isolation Kit [26], ultracentrifugation alone [21, 23, 27, 31, 33], ultracentrifugation followed by ultrafiltration [28], density gradient ultracentrifugation with sucrose cushion [22, 24, 25], ultrafiltration followed by density gradient ultracentrifugation [29, 30] or ultrafiltration followed by ExoQuick-TC exosome precipitation solution [32]. This is likely to have resulted in widely varying proportions of different EV subtypes and contributions of protein and lipid contaminants [66, 71, 87, 112-114]. Similarly, EV characterisations and quantification are highly variable among the published wound healing studies. Some provide very limited characterisation of the EVs used [21, 23, 27, 28] and most do not conform closely to the emerging standards in the field [67, 87, 115]. This lack of standardization, in particular, results in wide diversity in the units used to describe EV dosage as well as variations among studies employing the same quantification method. Another level of variability arises from the EV administration techniques used in animal models of wound healing. The reported studies have utilised both local and systemic (intravenous) administration. Locally applied EVs have been delivered by direct topical application [21, 31] , injection into or around the wound [22-25, 27, 28, 30, 32, 33] and integration into biomaterials [29]. In addition, most reported studies have focussed on very early administration which may not be of clinical relevance to established or chronic wounds. Among the other specific issues of high relevance to clinical translation that are likely to require further animal model-based research are the potential mechanistic differences between EV preparations from different cell types and the donor-to donor variability associated with EVs isolated from primary progenitor cells such as MSC and EPC-derived human EVs. *Thus, while inherently promising and broadly consistent, the current pre-clinical literature regarding EV-mediated enhancement of wound healing may yet be insufficient to robustly guide effective translation into clinical protocols.*

Despite these limitations that the divergent experimental parameters imply, the studies summarized in Tables 1-4 do provide a substantial amount of valuable evidence that researchers in the field can build upon to drive the clinical translation of EV administration

for wound healing in the coming years. In particular, clues regarding cellular and molecular mechanism of action of EVs in the setting of cutaneous wounds are likely to be of great value. At the cellular level, EV administration has been found to enhance endothelial cell proliferation, migration and tube formation, resulting in the promotion of angiogenesis and blood vessel maturation *in vivo* [21, 22, 25, 27-31, 96]. The cellular origin of the human EVs tested included induced pluripotent stem cell (iPSC)-derived MSC, bone marrow-derived MSC, umbilical cord-derived MSC (uMSC), urine-derived stem cells, umbilical cord blood-derived endothelial progenitor cells (EPC), synovium MSC and fibrocytes [21, 22, 25, 27-31, 96]. In the case of fibroblasts, EV treatment has been shown in several studies to exert a dose-dependent enhancing effect on proliferation and migration [21, 23, 24, 31-33]. These effects on fibroblasts were observed with EVs isolated from iPSC-derived MSC, uMSC, adipose tissue-derived MSC, amniotic epithelial cells and fibrocytes. Although most of the studies included in this review also reported increased collagen synthesis in association with accelerated wound healing [21, 24, 31-33], Zhao et al. observed an inhibitory effect of EVs on collagen expression by fibroblasts, which they interpreted as being relevant to the prevention of excessive scaring [23]. This discrepancy may reflect differential timing of the analyses during the healing process [32]. In *in vitro* and *in vivo* experiments, dermal and epidermal cells have been found to be increased in numbers following treatment with EVs (both of uMSC and fibrocyte origin) due to enhanced cell proliferation and reduced heat stress-induced apoptosis [24, 25, 31]. Administration of EVs isolated from uMSC has also been found to modulate inflammation by reducing neutrophil and macrophage recruitment to the injury site and by promoting macrophage polarisation towards an anti-inflammatory (M2) phenotype [26, 27].

At the molecular level, EV administration effects have been shown to be mediated by multiple bioactive molecules present within the EV cargo and to modulate a number of intracellular signalling pathways within the recipient cells. Among the signalling pathways with a recognized role in wound healing, EVs isolated from bone marrow-derived MSC have been found to induce the activation of the attenuation of protein kinase B (AKT), signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase-1/2 (ERK1/2) signalling pathways which lead to transcriptional changes associated with extracellular matrix remodelling, cell proliferation, migration and angiogenesis [96]. A substantial body of evidence now demonstrates a critical anti-inflammatory role for epithelial and endothelial cell STAT3 in tissue injury and wound healing [116-118]. Using endothelial

cell conditional STAT3 null models, it was shown that STAT3 activation is important in reducing tissue damage during would healing, and its absence results in prolonged pro-inflammatory cytokine secretion in infiltrating immune cells [118]. STAT3 has also been shown to regulate growth factor dependent keratinocyte migration but not proliferation during wound healing [117, 119]. Importantly, IL-6 induced activation of STAT3 limits STAT1 activity and prevents an IFN-γ-like pro- inflammatory response, indicating the critical role for STAT3 in regulating IFN-γ and IL-6 induced cellular responses [120]. The analysis of the kinetics of AKT, STAT3 and ERK1/2 activation in specific cell types during the wound healing process following EV treatment could be useful to better refine the therapeutic potential of MSC-derived exosomes in wound repair. In fact, prevention of apoptosis in association with activation of AKT signalling by uMSC-derived EV treatment has been demonstrated in one of the most comprehensive studies in this area [24]. In addition, endothelial cell proliferation, migration and tube formation have also been described to be a result of AKT and ERK1/2 signalling activation mediated by EVs isolated from synovium MSC and umbilical cord blood-derived EPC [29, 30]. Analyses of fibrocyte-derived EV cargo have identified the presence of several molecules with potential to modulate the wound healing process including HSP90a, p-STAT3 and several miRNAs which have been described to be pro-angiogenic (miR-126, miR-130a, miR-132), anti-inflammatory (miR124a, miR-125b) or to modulate collagen secretion and deposition (miR-21) [31]. In the studies of Zhang et al., uMSC-derived EV-delivered Wnt4 and consequent activation of the Wnt/β-catenin signalling pathway was shown to underlie observations of enhanced cell proliferation and migration after EV administration [24, 25]. Increased β-catenin stability and nuclear accumulation in endothelial cells after EV treatment was observed to be accompanied by increased expression of N-cadherin, proliferating cell nuclear antigen (PCNA) and cyclin D3 along with reduced expression of E-cadherin [25]. Furthermore, pharmacological inhibition of β-catenin signalling was found to abrogate EV-mediated enhancement of blood vessel formation [25]. These findings are in accordance with the recognized role for Wnt in angiogenesis [121, 122] and, along with other studies cited here, *provide convincing evidence the components of biomolecular cargo of EVs from MSC and other cell types have the capacity to specifically and potently modulate intra-cellular signalling pathways that regulate cutaneous wound healing*.

From a different mechanistic perspective, EV administration has also been described to control the inflammatory response by reducing the number of leukocytes and the serum

concentrations of pro-inflammatory cytokines (tumour necrosis factor alpha [TNF-α] and IL-1β) while increasing the concentration of the anti-inflammatory cytokine IL-10 [26]. In the study of Li et al., the anti-inflammatory effects of uMSC-derived EVs in a cutaneous burn model were shown to be associated with downregulation of Toll-like receptor 4 (TLR4) signalling. This effect was mediated by the transfer of miR-181c, which inhibits the translation of TLR4 from its mRNA. In complementary *in vitro* experiments, uMSC-derived EVs were shown to inhibit lipopolysaccharide (LPS)-induced macrophage activation by modulating TLR4 signalling through miR-181c transfer [26]. Previous studies have indicated a role for TLR4 expression and function in keratinocytes and innate immune cells during the process of wound healing, and suggest a time-dependent effect of TLR4 as a critical regulator in wound inflammation [123]. However, it is important to highlight the fact that inflammatory patterns in wounds are complex and may be modulated by numerous ligands and receptors beyond TLR4. In addition to direct modulation of pro-inflammatory signalling pathways, macrophage polarization towards an anti-inflammatory (M2) phenotype has been found to be associated with LPS pre-conditioned uMSC-derived EV-mediated transport of let-7b, miR-1180, miR-183, miR-550b, and miR-133a. In particular, let-7b was increased and TLR4/NF-κB/STAT3/AKT signalling was modulated in THP-1 cells following their uptake of EVs derived from LPS pre-conditioned uMSC [27]. Consistent with these findings, protein expression studies from EV-treated wounds demonstrated reduced TLR4 and phosphorylated (p)-P65 along with increased p-STAT3 and p-AKT [27]. *Thus, it is now quite convincingly established that, in addition to acting directly upon epithelial cells, endothelial cells and fibroblasts during wound healing, EVs and their biomolecular cargo also influence the healing process by modulating inflammatory mediators.*

Finally, reduced scar formation has been shown to result from the transfer of uMSC-derived EV-contained miRNAs (miR-21, miR-23a, miR-125b, and miR-145) which prevent TGF-β/ Sma and Mad related protein 2 (SMAD2) pathway activation, thus inhibiting myofibroblast differentiation and collagen production [33]. Altogether, these studies demonstrate that EVs isolated from multiple cell types have the ability to promote wound healing by interfering with the proliferation and activity of the cells in the vicinity of the wound as well as by modulating signalling pathways and inflammatory mediators that play critical roles in the different stages of the wound healing process.

5. Challenges for the clinical application of EVs for wound healing

Overall, the studies performed over the past few years and described in the preceding section provide evidence that local or systemic EV administration holds promise as a novel therapeutic approach for wound healing. Importantly, the field has moved beyond observational research to include convincing and plausible mechanistic details. Nevertheless, caution should also be taken when interpreting the results to date from a clinical translational perspective and some specific technical challenges should be acknowledged and considered if EV treatment is to be established as a widely-available therapy for acute and chronic cutaneous wounds.

Firstly, the current state-of-the-art in EV research is yet lacking broadly applicable methods for the isolation and validation of homogeneous samples of the individual EV subtypes. The techniques available result in isolates that, to varying extents, consist of multiple EV subtypes and may also contain non-EV constituents such as lipoproteins, proteins, viruses and bacteria, depending upon the nature of the initial biological fluid [66, 71, 87, 112-114, 124]. Moreover, lack of standardization of the isolation protocols and failure to adapt protocols to the equipment available at individual laboratories (rotor type, centrifugation time and applied g forces) can result in further discrepancies and inconsistencies. This variability presents a substantial problem for comparing results obtained from different studies [66, 125, 126]. Similarly, the techniques available for detection and characterization of EVs are also varied and differ in their accuracy and precision as well as in the specific parameters that are measured (e.g. structure, size, molecular content, buoyant density, optical properties and zeta potential) [127-129]. The techniques applied for phenotyping of EV samples range from biochemical assays to imaging modalities including a wide range of different microscopic and flow cytometric approaches which are in continuous evolution [127-129]. Recently, imaging flow cytometry has emerged as a potentially powerful tool for EV analysis as it combines the high-throughput analytical abilities of a flow cytometer with the resolution power of a microscope. This technique may prove to be particularly valuable for the integrated analysis of EV biogenesis, phenotype and functional interactions with recipient cells [130, 131]. Finally, vesicle concentration is also currently determined by various methods which range from simple protein quantification assays to nanoparticle tracking analysis. As a result, quantitative analysis of EV preparations may be expressed in a variety of units representative of the biomolecular content, the particle number or the mass of the isolated EV sample [128, 129].

Based on these considerations, it is clear that the rapidly growing field of EV research would benefit from standardization of the sample collection methods as well as of the techniques applied for the isolation and characterization of the vesicles. This would greatly facilitate the collection of reliable and reproducible data across different laboratories and fields of research. Going forward, scientists entering the EV research field need be conscious of the challenges and benchmark their experimental procedures against guidelines such as those recently proposed by the International Society for Extracellular Vesicles (ISEV) [67, 87]. Moreover, transparent and detailed reporting of the experimental methods is crucial for accurate data interpretation and reproducibility. In support of this, a freely accessible knowledgebase has recently created for the EV-related research community [115]. Given that the field is yet to identify and establish gold standard protocols for the isolation and characterization of EVs, researchers should search the literature to ascertain which isolation method yields best results for their specific starting biological material. Moreover, the characterization of EV isolates should include the application of multiple different techniques such as nanoparticle tracking analysis, transmission electron microscopy, Western blotting and flow cytometry which, when combined, provide sufficient information so that the resulting experimental work can be directly compared with that being performed elsewhere. Ultimately, the development and regulatory approval of good manufacturing process (GMP)-compliant protocols for the manufacture and definition of clinical-grade EVs derived from MSC or other cell types will be essential for clinical trials of EVs in wound healing and other conditions [132]. As the field progresses in this direction, well-performed animal model experiments will also play a critical role by providing pre-clinical data in support of the safety, efficacy and pharmacokinetics of GMP-manufactured EVs.

6. Future perspectives for engineering enhanced EVs

Although the previous sections describe how EVs from various progenitor cells have been found to be efficacious in both *in vitro* and *in vivo* wound models, there may well be a need to further improve the therapeutic efficacy. Engineering EVs to enhance efficacy has become a hot topic in the literature and has recently been extensively reviewed by others [133, 134]. Nonetheless, as EV engineering for enhanced therapeutic effect has not yet been extensively pursued in the area of wound healing, we will briefly summarize some of the key principles here. Currently, there are three approaches that have been utilized to further improve EV

efficacy: (i) treatment of EV donor cells with therapeutic drugs, (ii) genetic manipulation of EV donor cells and (iii) direct manipulation of EVs.

Pre-treatment of EV donor cells with specific drugs has the potential to alter EV cargo profile and, thereby, modulate their therapeutic properties. Recently, Lu and colleagues reported that pre-conditioning or "licencing" of adipose-derived MSCs with TNF-α increased Wnt3a content in the released EVs resulting in the promotion of osteogenic gene expression. The authors proposed that TNF-α preconditioning offers a promising approach to replace stem cell transplantation for bone repair [135]. A recent study by Harting and colleagues, has demonstrated that TNF-α and IFN-γ stimulation of hMSCs enhances the anti-inflammatory properties of isolated EVs from these cells, giving further weight to the potential for enhancing therapeutic efficacy of EVs [136]. Genetic engineering of cells prior to EV isolation with the aim to modulate the cargo appears to be a very promising strategy. Many different technologies are available to introduce genetic material into target cells including recombinant viral vectors (lentivirus, adenovirus, adeno-associated virus (AAV)) or non-viral transfection technologies such as liposomal gene transfer or electroporation [137, 138]. Depending on the therapeutic target, overexpression or silencing of specific mRNA or miRNAs can be applied to engineer specific alterations to EV cargo prior to isolation and application. As already described, overexpression of miRNA-126 in human SMSC cells resulted in the production of EVs with enhanced ability to promote proliferation, migration and tube formation of human dermal microvascular endothelial cells (HMEC-1) and to enhance cutaneous wound healing in a diabetic rat model [29]. However, the genetic manipulation of donor cells prior to EV isolation may not only increase or reduce the content of a specific mRNA/miRNA but may also alter the expression of other molecules present in the EV cargo with potential for unanticipated adverse effects. It is suggested, therefore, that a careful analysis of the cargo of genetically engineered EVs must be carried out prior to therapeutic application. Finally, the direct manipulation of EVs has been promoted as a strategy which would eliminate the requirement for pharmacological or genetic manipulation of the donor cells. Methods of choice for directly manipulating EVs include treatment with liposomes, direct incubation with a drug or biomolecule or electroporation [133]. Clearly, in the case of wound healing, the selection of molecules (such as miRNAs) to be manipulated in EVs for therapeutic purposes must be driven by a continued growth in knowledge of the cellular constituents and pathways involved in wound healing.

7. Conclusion

In conclusion, a significant amount of compelling evidence has recently emerged in support of the development of EVs as a novel therapeutic tool for clinical conditions in which wound healing is compromised. The various studies described in this review demonstrate the ability of EVs to target physiological processes and intracellular pathways involved in the haemostatic, inflammatory, proliferative and remodelling phases of wound healing. Although most of these published studies have focussed on exploring the pro-healing effects of EVs derived from various adult stem/progenitor cells, it remains to be determined whether one cell type is clearly superior to another for this purpose. Through the modulation of gene expression levels and signalling pathway activation, EVs have the capacity to variously regulate proliferation, migration, angiogenesis, collagen synthesis and extracellular matrix remodelling by epithelial cells, endothelial cells and fibroblasts. From a molecular perspective, evidence from a limited number of high quality studies suggests that EV transfer of miRNAs or growth factors may be of particular importance. It is to be hoped that further understanding of these and other relevant mechanisms coupled with the development of improved, standardized methods for EV purification and characterization will eventually lead to highly effective EV-based therapeutic products for acute and chronic cutaneous wounds. However, as we have also highlighted, there is significant potential for over-interpretation of animal model-based research results in this area as well as substantial challenges related to methodological limitations and variability that are currently inherent in experimental studies involving EVs. Thus, full realization of the promise of EV-based therapy in wound healing will be dependent upon a strong commitment from the research community to adopting emerging best practice standards in the field and to consistently reporting experimental methodology in a detailed, reproducible and clinically relevant manner.

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Figure Legends

Figure 1: Extracellular vesicle biogenesis. A) Exosomes are of endosomal origin and are released into the extracellular space upon fusion of multivesicular bodies (MVBs) with the plasma membrane. B) Ectosomes (also known as microvesicles) result from the outward budding of the plasma membrane. C) Apoptotic bodies are originated from the degradation of cells which are undergoing apoptosis so that their clearance by phagocytic cells is facilitated.

Figure 2: Extracellular vesicle interaction with recipient cells. While there is overlap among the different EV subtypes, the expression levels of some molecules are distinct across the major EV subtypes, allowing for the differential expression of some proteins and lipids to be used as candidate markers for the identification of EV subtypes. Molecules which have been found to be differentially present in the different EV subtypes are listed in the lower left box. EV interaction with recipient cells can take place through 1) ligand-receptor interaction; 2) internalization; and 3) direct fusion with the plasma membrane which culminate with the transfer of the biochemically-active EV cargo to the recipient cells. This results in the regulation of signalling pathway activation and modulation of physiological processes such as proliferation, migration and differentiation which ultimate modulate the recipient cell physiological state (mid right boxes). Abbreviations: AKT - attenuation of protein kinase B; Alix - programmed cell death 6 interacting protein; CD – cluster of differentiation; ERK - extracellular signal-regulated kinase; HSP70 - heat shock protein 70; IL – interleukin; LPS – lipopolysaccharide; NF-κB - nuclear factor kappa-light-chain-enhancer of activated B-cells; SMA - smooth muscle actin; SMAD - Sma and Mad related protein; STAT - signal transducer and activator of transcription; TGF-β - transforming growth factor β; TLR - Toll-like receptor; TNF - tumour necrosis factor; TSG101 - tumor susceptibility gene 101; Wnt4 - wingless-type MMTV integration site family, member 4

Table 1: Rat skin wound model. Summary of details on EV isolation and quantification methods, dosage and timing of EV administration and resulting effects.

Isolation method	Quantification method	Dosage/ Administration time	*In vivo* treatment groups	Characterization	EV cellular origin	Observations	Ref
Rat Skin wound model							
DC, F, U	BCA	***In vitro*****:** 0; 50; 100 µg/ml ***In vivo*****:** 160 µg + 40 µg (160 µg local injection at 4 sites around the wound plus 40 µg applied to the wound bed) T=Unclear	• PBS • MesenGro hMSC medium • iPSC-MSC-EV N=Unclear	**TEM** (30-100 nm) **WB:** CD81/ CD9 & CD63	Human iPSC-derived MSC cultured for 48 hours in serum free media (80% confluent)	<u>*in vivo*</u> • increased re-epithelization, collagen deposition, sebaceous gland and hair follicle formation, and angiogenesis • increased density and maturity of blood vessels • accelerated wound closure • reduced scaring <u>*in vitro*</u> • dose-dependent promotion of fibroblast proliferation, migration and collagen synthesis • increased endothelial cell proliferation, migration and tube formation	(21)
DC, F, DGU	NTA	***In vitro*****:** 0; $2x10^{10}$; $1x10^{11}$ particles/ml ***In vivo:*** 0; $2x10^{10}$; $1x10^{11}$ particles (sc injection)	• PBS • EV N=8	**TRPS** (size range) **NTA (qNano)** **WB:** CD81/CD9/CD63	Human Urine-derived stem cells cultured for 48 hours in serum free media (80% confluent)	<u>*in vivo*</u> • improved re-epithelialisation and neovascularization • accelerated wound closure • reduced scar formation <u>*in vitro*</u> • increased endothelial cell proliferation, migration and tube formation	(22)

		T=Unclear					
DC, F, U	BCA	***In vitro*:** 0; 2.5; 5;10 µg ***In vivo:*** 0; 2.5; 5;10 µg (sc injection around the wound) T=Unclear	• PBS • EV N=6	**TEM** **WB:**CD9/ CD63/ Alix & TSG101 **FCM**: CD9/ CD63/ CD81 & HLA-G	Human amniotic epithelial cells cultured for 48 hours in serum free media (80% confluent)	*in vivo* • dose-dependent promoting effect on wound healing and re-epithelization • reduced scar formation *in vitro* • dose-dependent promotion of fibroblast proliferation and migration	(23)

Abbreviations: Alix - programmed cell death 6 interacting protein; BCA - bicinchoninic acid assay; CD – cluster of differentiation; DC – differential centrifugation; DGU – density gradient ultracentrifugation (with sucrose cushion); F – 0.22-µm filtration; FCM – flow cytometry; hMSC – human mesenchymal stromal cells; iPSC - induced pluripotent stem cell; NTA – nanoparticle tracking analysis; PBS - phosphate buffered saline; TEM – transmission electron microscopy; TRPS - tunable resistive pulse sensing; TSG101 - tumor susceptibility gene 101; U – ultracentrifugation; WB – western blot.

Table 2: Severe burn rat model. Summary of details on EV isolation and quantification methods, dosage and timing of EV administration and resulting effects.

Isolation method	Quantification method	Dosage/ Administration time	*In vivo* treatment groups	Characterization	EV cellular origin	Observations	Ref
Severe Burn Rat model							
DC, F, DGU	BCA	***In vitro*:** Unclear ***In vivo*:** 200 µg (sc injection at 3 sites) T=unclear	• No treatment • PBS • 1x10^6 uMSCs/HFL1 • uMSC-EV/ HFL1-EV • +/- β-catenin/ AKT inhibitor • Wnt4-shRNA-EV N=6	**TEM** **NanoSight LM10** (size & concentration) **WB:** CD81/ CD9 & CD63	Human Umbilical cord-derived MSC cultured for 48 hours in serum free media	*<u>in vivo</u>* • enhanced dermal and epidermal cell proliferation • enhanced repair of the burned skin with complete re-epithelization of the wound area • reduced scar formation associated with enhanced collagen deposition *<u>in vitro</u>* • promotion of human keratinocytes and dermal fibroblasts proliferation and migration (Wnt/β-catenin signalling activation) • prevention of heat stress-induced apoptosis (AKT signalling activation)	(24)
DC, F, DGU	BCA	***In vitro*:** 80; 160 µg/ml ***In vivo*:** 200 µg (sc injection at 3	• PBS • uMSC-EV/ HFL1-EV • +/- β-catenin inhibitor	**TEM** **NanoSight LM10** (size & concentration) **WB:** CD9/ HSP70	Human Umbilical cord-derived MSC cultured for 48 hours in serum free media	*<u>in vivo</u>* • enhanced frequency of both epidermal and dermal cells at the burned sites • enhanced endothelial cell	(25)

		sites) T=Unclear	• Wnt4-shRNA-EV N= Unclear			 • accelerated wound healing *in vitro* • dose-dependent promoting effect on endothelial cell proliferation, migration and tube formation • enhanced Wnt/β-catenin pathway activation	function and angiogenesis
DC, F, PureExo® Exosome Isolation Kit	RNA	***In vitro*:** 500 µg RNA concentration ***In vivo:*** 800 µg RNA concentration (tail injection) T=Unclear	• PBS • uMSC-EV • human skin fibroblast-EV N=5/6	**TEM** **NTA** (size range) **Genechip** **WB**: CD9 & CD63 **FCM:** CD44/CD90/ CD105/HLA-I	Human Umbilical cord-derived MSC cultured for 48 hours in serum free media	*in vivo* • anti-inflammatory effects • reduced number of white blood cells; reduced TNF-α and IL-1β protein levels; increased IL-10 in the serum • reduce neutrophil and macrophage infiltration • inhibition of TLR4 signalling pathway by transfer of miR-181c *in vitro* • inhibition of LPS-induced macrophage activation by modulation of TLR4 signalling pathway through miR-181c	(26)

Abbreviations: AKT - attenuation of protein kinase B; BCA - bicinchoninic acid assay; CD – cluster of differentiation; DC – differential centrifugation; DGU – density gradient ultracentrifugation (with sucrose cushion); F – 0.22-µm filtration; FCM – flow cytometry; HFL1 - from human lung fibroblasts; HSP70 - heat shock protein 70; IL – interleukin; NTA – nanoparticle tracking analysis; PBS - phosphate buffered saline;

TEM – transmission electron microscopy; TLR - Toll-like receptor; TNF - tumour necrosis factor; uMSC – umbilical cord-derived mesenchymal stromal cells; WB – western blot; Wnt4 - wingless-type MMTV integration site family, member 4.

Table 3: Cutaneous wound models in diabetic rodents. Summary of details on EV isolation and quantification methods, dosage and timing of EV administration and resulting effects.

Isolation method	Quantification method	Dosage/ Administration time	*In vivo* treatment groups	Characterization	EV cellular origin	Observations	Ref
Diabetic (Type I) Rat wound model							
DC, F, U	BCA	***In vitro*:** 10; 20 µg/ml ***In vivo*:** 60 µg local injection T=Unclear	• Normal • Diabetic • Diabetic + untreated MSC-EV • Diabetic + LPS treated MSC-EV N=6	**TEM** **WB:** CD81/ CD9 & CD63	Human Umbilical cord-derived MSC (+/- LPS preconditioning) cultured for 48 hours in serum free media (70-80% confluent)	<u>*in vivo*</u> • reduced inflammation • enhanced angiogenesis • promotion of a M2 macrophage phenotype • improved wound healing <u>*in vitro*</u> • promotion of macrophage polarization towards a M2 phenotype • increased Let-7b levels which correlate with modulation of macrophage plasticity through the regulation of TLR4/NF-κB/STAT3/AKT signalling	(27)
DC, F, U, UF	BCA	***In vitro*:** 100 µg/ml ***In vivo:*** 100 µg (injection around the wound at 4 sites) – 100 µg X 4? T=Unclear	• PBS • EV N=8	**TEM** **WB:** CD81/ CD9 & CD63	Human Umbilical cord blood-derived endothelial progenitor cells cultured for 24 hours in serum free media (80% confluent)	<u>*in vivo*</u> • accelerated wound healing • reduced scar formation <u>*in vitro*</u> • enhanced vascular endothelial cell proliferation, migration and tube formation	(28)

	DC, F, UF, DGU	CD63 ExoELISA kit	Unclear Chitosan Hydrogel Loaded With Exosomes T=Unclear	Chitosan dressing loaded with • PBS • EV • miR-126-EV N=6	**TEM** **NTA** **WB:** CD81/ CD9/ CD63/ TSG101/Alix	Human Synovium MSCs (with/without miR-126-3p overexpression) cultured for 48 hours in serum free media (50-60% confluent)	*in vivo* • improved neovascularisation and maturation of new blood vessels • enhanced re-epithelisation, collagen deposition and development of hair follicles and sebaceous glands at the wound site. • accelerated wound closure *in vitro* • enhanced promotion of human dermal microvascular endothelial cells proliferation, migration and tube formation	(29)
	DC, F, UF, DGU	NTA	***In vitro*:** 0; $2x10^{10}$; $1x10^{11}$ particles/ml ***In vivo:*** 0; $2x10^{10}$; $1x10^{11}$ particles (sc injection around the wound 4 sites) T=Unclear	• PBS • EV N=6	**TRPS** (size range) **NTA (qNano)** **WB:** CD81/ CD9/ CD63	Human Umbilical cord blood-derived endothelial progenitor cells cultured for 24 hours in serum free media (80-90% confluent)	*in vivo* • improved re-epithelialisation and collagen deposition • increased number and maturity of blood vessels • accelerated wound closure • reduced scar formation *in vitro* • enhanced human microvascular endothelial cell proliferation, migration and tube formation (ERK1/2 signalling pathway activation)	(30)

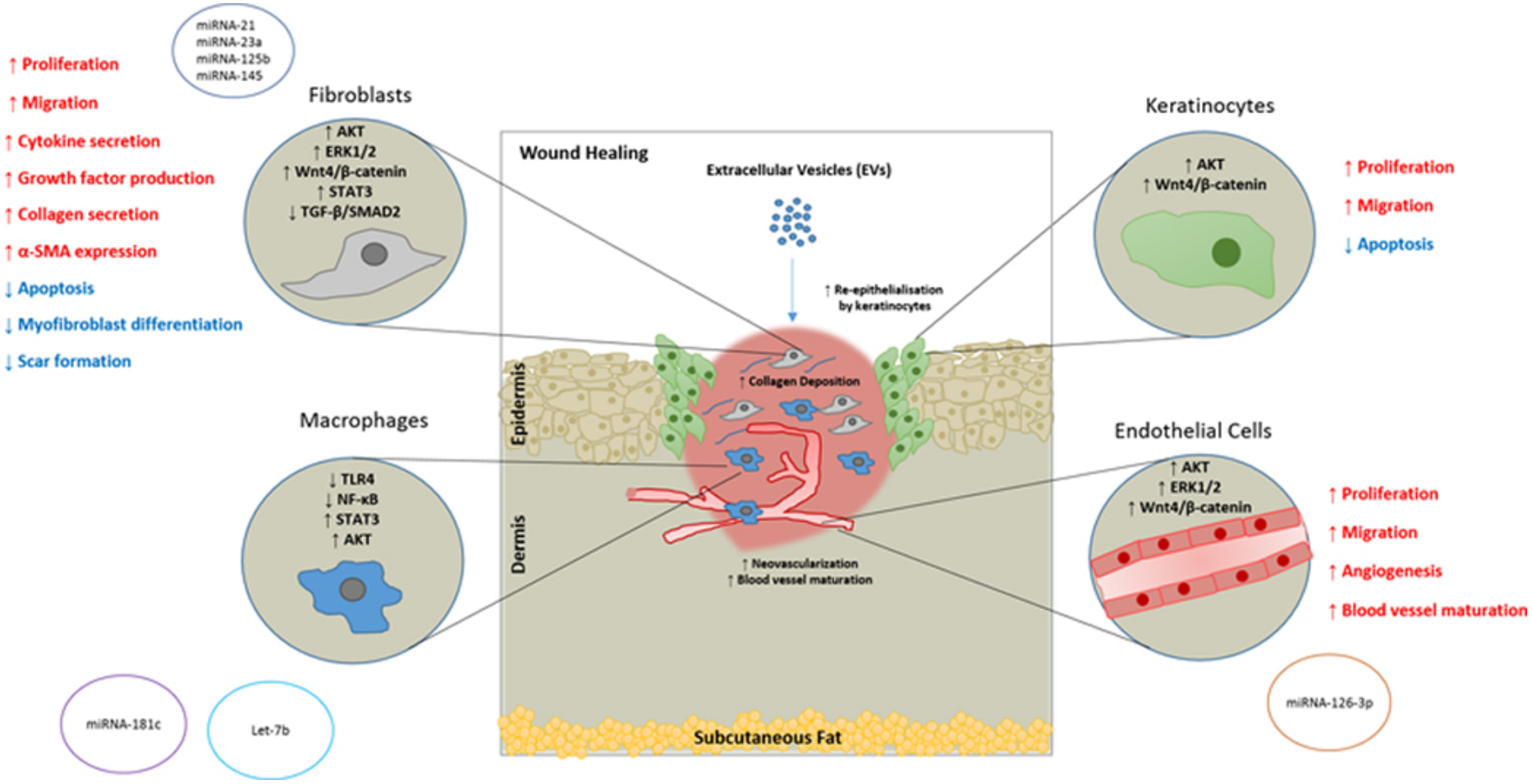
Full-thickness skin defect type 2 diabetic mouse model							
DC, F, U	NTA BCA	***In vitro*****:** 0; $7x10^8$; $7x10^9$ particles/ml ***In vivo:*** 0; $3.5x10^9$; $3.5x10^{10}$ particles/ml (40µl topical and 4x 40µl sc injections) T=Unclear	• PBS • EV N=5-7	**TEM** **NanoSight NS500** (size & concentration) **FCM:** CD9/CD63/CD81/MHC-I/MHC-II/CD80/CD86 **WB:** TSG101/Flotilin-1	Human fibrocyte cultured for 48 hours in EV-depleted serum-containing media	<u>*in vivo*</u> • modulation of endothelial and epithelial cells in the vicinity of the wound • enhanced collagen deposition • accelerated wound closure <u>*in vitro*</u> • promotion of endothelial cell tube formation/ angiogenesis • increased migration and proliferation of human diabetic keratinocytes • enhanced cell division, collagen secretion and α-SMA expression by human dermal fibroblasts	(31)

Abbreviations: AKT - attenuation of protein kinase B; Alix - programmed cell death 6 interacting protein; BCA - bicinchoninic acid assay; CD - cluster of differentiation; DC - differential centrifugation; DGU - density gradient ultracentrifugation (with sucrose cushion); ERK - extracellular signal-regulated kinase; F– 0.22-µm filtration; FCM – flow cytometry; LPS – lipopolysaccharide; NF-κB - nuclear factor kappa-light-chain-enhancer of activated B-cells; NTA – nanoparticle tracking analysis; PBS - phosphate buffered saline; SMA - smooth muscle actin; STAT - signal transducer and activator of transcription; TEM – transmission electron microscopy; TLR - Toll-like receptor; TRPS – tunable resistive pulse sensing; TSG101 - tumor susceptibility gene 101; U – ultracentrifugation; UF – ultrafiltration; WB – western blot.

Table 4: Mouse skin wound model. Summary of details on EV isolation and quantification methods, dosage and timing of EV administration and resulting effects.

Isolation method	Quantification method	Dosage/ Administration time	*In vivo* treatment groups	Characterization	EV cellular origin	Observations	Ref
Mouse skin wound model							
DC, F, UF, ExoQuick-TC exosome precipitation solution	NTA BCA	***In vitro*:** 0; 25; 50; 100 µg/ml ***In vivo:*** 200 µg sc or iv T=Unclear	• No treatment • PBS • EV sc injection • EV iv injection N=Unclear	**TEM** **NanoSight LM10** (size & concentration) **WB**: CD9 & CD63	Human Adipose tissue-derived MSC cultured for 24 hours in serum free media	*in vivo* • accelerated wound healing • enhanced collagen deposition *in vitro* • enhanced human dermal fibroblasts migration, proliferation and collagen synthesis	(32)
DC, F, U	BCA	Unclear 100 µg/ml Injected around the wound T=48H	• PBS • uMSC-EV • HEK-293-EV • dCM N=Unclear	**NanoSight NS300** (detection) **WB**: CD81 & CD63 **Laser Vertriebsgesellschaft** for assessment of particle size distribution	Human Umbilical cord-derived MSC cultured for 48 hours in EV-depleted serum-containing media	*in vivo* • Reduced myofibroblast differentiation • accelerated wound healing • reduced scar formation *in vitro* • prevention of myofibroblast generation • TGF-β/SMAD2 pathway inhibition • Promotion of fibroblast proliferation and migration	(33)

Abbreviations: AKT - attenuation of protein kinase B; Alix - programmed cell death 6 interacting protein; BCA - bicinchoninic acid assay; CD – cluster of differentiation; DC – differential centrifugation; dCM – EV depleted uMSC-derived conditioned media; DGU – density gradient ultracentrifugation (with sucrose cushion); ERK - extracellular signal-regulated kinase; F – 0.22-μm filtration; FCM – flow cytometry; HFL1 - from human lung fibroblasts; hMSC – human mesenchymal stromal cells; PBS - phosphate buffered saline; SMAD - Sma and Mad related protein; TEM – transmission electron microscopy; TGF-β - transforming growth factor β; U – ultracentrifugation; UF – ultrafiltration; uMSC – umbilical cord-derived mesenchymal stromal cells; WB – western blot.



Graphics Abstract

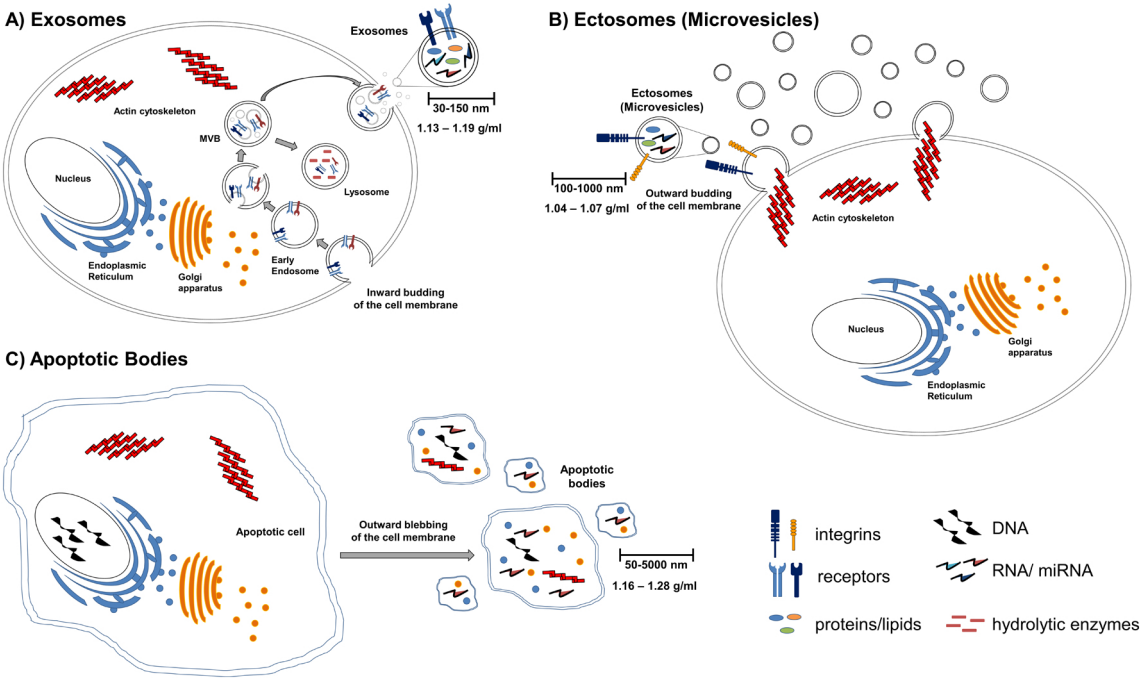
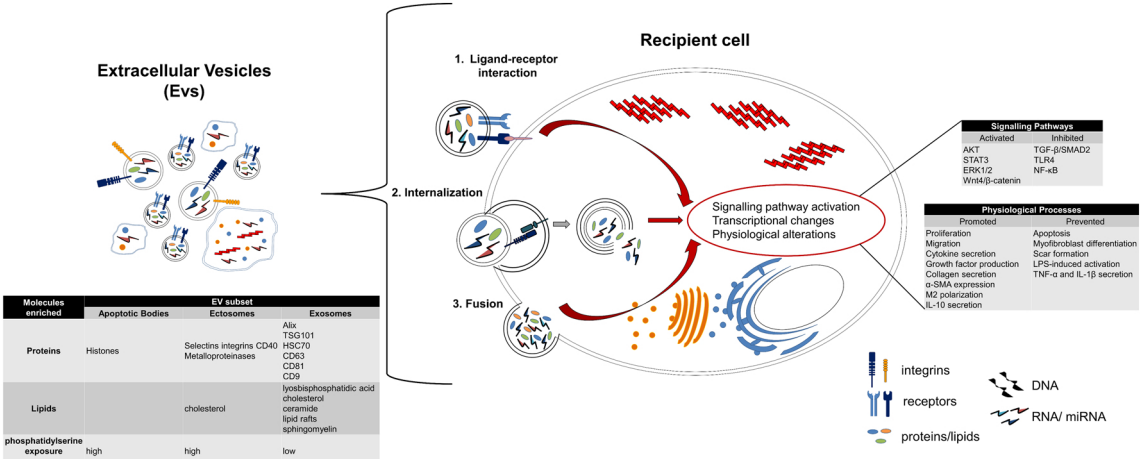

A) Exosomes
Exosomes
30-150 nm
1.13 – 1.19 g/ml
Actin cytoskeleton
MVB
Lysosome
Nucleus
Endoplasmic Reticulum
Golgi apparatus
Early Endosome
Inward budding of the cell membrane
B) Ectosomes (Microvesicles)
Ectosomes (Microvesicles)
100-1000 nm
1.04 – 1.07 g/ml
Outward budding of the cell membrane
Actin cytoskeleton
Nucleus
Golgi apparatus
Endoplasmic Reticulum
C) Apoptotic Bodies
Apoptotic cell
Outward blebbing of the cell membrane
Apoptotic bodies
50-5000 nm
1.16 – 1.28 g/ml
integrins
receptors
proteins/lipids
DNA
RNA/ miRNA
hydrolytic enzymes

Figure 1



Molecules enriched	EV subset		
	Apoptotic Bodies	Ectosomes	Exosomes
Proteins	Histones	Selectins integrins CD40 Metalloproteinases	Alix TSG101 HSC70 CD63 CD81 CD9
Lipids		cholesterol	lyosbisphosphatidic acid cholesterol ceramide lipid rafts sphingomyelin
phosphatidylserine exposure	high	high	low

Figure 2