Behind the Scenes of Extracellular Vesicle Therapy for Skin Injuries and Disorders

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Significance: Skin wounds and disorders compromise the protective functions of skin and patient quality of life. Although accessible on the surface, they are challenging to address due to paucity of effective therapies. Exogenous extracellular vesicles (EVs) and cell-free derivatives of adult multipotent stromal cells (MSCs) are developing as a treatment modality. Knowledge of origin MSCs, EV processing, and mode of action is necessary for directed use of EVs in preclinical studies and methodical translation.

Recent Advances: Nanoscale to microscale EVs, although from nonskin cells, induce functional responses in cutaneous wound cellular milieu. EVs allow a shift from cell-based to cell-free/derived modalities by carrying the MSC beneficial factors but eliminating risks associated with MSC transplantation. EVs have demonstrated striking efficacy in resolution of preclinical wound models, specifically within the complexity of skin structure and wound pathology.

Critical Issues: To facilitate comparison across studies, tissue sources and processing of MSCs, culture conditions, isolation and preparations of EVs, and vesicle sizes require standardization as these criteria influence EV types and contents, and potentially determine the induced biological responses. Procedural parameters for all steps preceding the actual therapeutic administration may be the key to generating EVs that demonstrate consistent efficacy through known mechanisms. We provide a comprehensive review of such parameters and the subsequent tissue, cellular and molecular impact of the derived EVs in different skin wounds/disorders.

Future Directions: We will gain more complete knowledge of EV-induced effects in skin, and specificity for different wounds/conditions. The safety and efficacy of current preclinical xenogenic applications will favor translation into allogenic clinical applications of EVs as a biologic.

Keywords: extracellular vesicle, multipotent stromal cells, exosome, preclinical study, therapy

SCOPE AND SIGNIFICANCE

EXOGENOUS EXTRACELLULAR vesicles (EVs), particularly from cultured and bioengineered multipotent stromal cells (MSCs) as the focus of this review, have gained momentum as safe and effective therapy for skin wounds/disorders, even with the challenging composition of pathological mammalian skin. However, the extent to

which variations in MSC or source cell fitness, culture conditions, and physical EV isolation techniques are comparable across studies, and whether that impacts the biological response in the destination skin cells needs analysis and forms the core of our discussion in this study.

This review is geared toward a wide audience in wound care research, from bench scientists to clinical staff to aid in making informed decisions about experimental approaches, details, and assessment of preclinical data.

TRANSLATIONAL RELEVANCE

Exogenous EV treatments benefit from physiological uptake and demonstrate conspicuous efficacy in resolving skin injuries/disorders. EVs provide noteworthy advantages over their source cells, including that EVs negate tumorigenic potential as they are non-nucleated and potentially have less specialized storage requirements.[1,2] For evidence-based translation, knowledge of the EV-induced molecular landscape in the wound cellular milieu is necessary. To that end, first, we need accurate and comparable details of laboratory-based EV generation, isolation, and preparation as these criteria dictate the downstream activities induced in skin for tissue-level repair and regeneration outcomes. Our goal in this study is acknowledging and summarizing the wide array of parameters, including conditions of source tissues and cells, EV isolation measures and sizes, storage, and modes of administration in studies of skin wound healing.

CLINICAL RELEVANCE

The socioeconomic costs of skin-related conditions, especially chronic wounds,[3,4] highlight the urgent need for development of feasible therapeutic options. Worldwide, we are witnessing progress toward clinical trials of EV therapy for a large range of skin wounds/disorders, with potential for improving treatment duration and patient quality of life. As EVs are a biologic treatment, which are generated from cells, characterization of every procedural step is critical to ensure that EVs from comparable cell types carry comparable molecular cargoes and subsequently induce predictable outcomes. The impact of human cell-derived EV topical/local therapy on the preclinical skin wound/disorder models discussed in this study will outline the outcome expectations in future clinical studies.

BACKGROUND

As the largest, most visible organ of the human body and first layer of defense against the outside environment, skin injuries or disorders are conspicuous and can compromise a person's health. By the same token, treatment options can include noninvasive measures and any sign of improvement is obvious. EVs, cell-derived vesicles, have captured the attention of researchers as treatment for cutaneous disorders like chronic wounds, surgical wounds, ischemic flaps, photoaging, and eczema.

All cell types, from embryonic stem to aged cells, secrete EVs.[5] The study of cell-derived membrane-bound vesicles, under classification of EVs, has gained traction in recent years. The rise of EV research societies around the world demonstrates the need for dedicated platforms and leaders in the development of the EV field. EVs are appreciated as a significant addition to knowledge of molecular communication mediators. EVs are often referred to interchangeably with "exosomes" when discussing eukaryotic EVs, but exosomes are just a subtype of EVs. Pertinent to skin wound applications, the other major subtypes studied are microvesicles and apoptotic bodies. Microvesicles bud off from the plasma membrane and carry cytoplasmic components.[5,6] Apoptotic bodies are subcellular fragments that result from plasma membrane blebbing during physiological programmed cell death.

Contrary to expectations of cellular messages from cell death, apoptotic bodies have roles in intercellular communication, in addition to clearance of cell remains.[7,8] They can carry parts from fragmented nuclei and organelles and, similar to other EVs, interact with cells to induce signaling pathways and affect survival, tissue remodeling, and immune modulation.[9–11]

Identifying correct EV subtype is important for consistency and specificity in our work in this field. Numerous reviews have extensively discussed and clarified the distinctions in excellent detail and we will not delve into EV biogenesis pathways in this study, except to summarize that EV classification spans size, route of biogenesis, cargo, and release pathways.[5,6,12] At our current stage in the wound healing field, we do not yet have published data comparing the differential biological activity among the EV subtypes. While the roles of EVs in physiological and pathological processes are still challenging to study, and room remains for innovation and investigation, the most studied avenue is as diagnostic and prognostic biomarkers for diseases, such as cancer and liver, lung, kidney, and cardiovascular disease.[5]

Another popular avenue is as therapeutics, which can be further split into two prominent routes of use. First, EVs themselves as biologic therapeutics and at the core of this review, specifically for skin injuries and disorders.

Our focus is largely on EVs derived from monolayer cultured cells, as these cells are highly accessible sources.[1] Second, EVs as delivery vehicles where they are not the active therapeutic, but offer advantages of stability and physiological uptake mechanisms.[13] Both routes are built on using exogenous EVs from cultured cells for outcomes in tissues/organs with completely different tissue identity than the source cells.

Within the biologics route, one of the largest applications is in preclinical studies in regenerative medicine. A simple PubMed search for EVs or vesicles for tissue repair returns over 5,000 results, although the last decade has seen the greatest surge in this type of research. The cutaneous healing field has been an eager adapter of EVs as therapy to address skin disorders. Mammalian skin is a challenging tissue to treat with distinct compartments composed of cells with distinct identities and behavior, woven through with blood vessels, lymphatics, nerves, and immune cells. Injury and physiologic wound repair are multiphased processes with complex cellular and molecular milieu, which can be confounded further by manifestation of age, diabetes, or venous disease.[14] Single cytokine approaches have not proven as successful in the same skin conditions.[15]

To restore dermal integrity and prevent further complications, we need therapeutic options that are multitargeted. Studies to date strongly suggest that EVs, due to their protein, lipid, and nucleic acid content, may be affecting the necessary interactive networks of signaling cascades. Studies also report success following EV application in preclinical animal cutaneous wound models, but with very broad range of variables such as cell sources, culture conditions, storage practices, and administration regimens. The impact of sources and culture conditions has just begun to be explored and is critical for all downstream applications.[16]

MSCs are the broadest category of cells used to derive EVs for skin repair.[1] MSCs, also referred to as mesenchymal stem or mesenchymal stromal cells, have been investigated for their functions and potential roles in tissue regeneration. Large numbers of studies focused on administering MSCs for the treatment of skin conditions, with initial assumptions that MSCs could find new niches in skin, disparate from their source tissues, and differentiate to give rise to cell types relevant in the wound bed cellular milieu. Despite consistent impact on tissue repair and regeneration in *in vitro* assays and in preclinical models, no evidence exists for MSC engraftment or typical stem/progenitor properties *in vivo*.[17,18]

The lack of engraftment, or the "hit and run" mechanism of MSCs[19] led to investigations into whether the beneficial effects are potentially mediated through paracrine, communicative factors and/or adaptive mechanisms such as cyto/chemokines and EVs. We are focusing on EVs as the paracrine and adaptive mediators, as these biologics can recapture the functional impact of administering whole MSCs in skin condition studies, such as in skin ulcers, psoriasis, and eczema. Use of EVs, not MSCs, removes possibilities of vascular occlusion and eliminates tumorigenic potential as they lack replication capacity.[2,20] Easy storage at ultra-low temperatures is another practical advantage of EVs when compared to therapeutic MSCs, as the latter requires specialized handling for cryopreservation and thawing.

Several other cell sources are now published in preclinical skin repair studies and the common intriguing theme is an absence of eliciting adverse immunologic reactions, even in allogenic or xenogenic applications.[21] No adverse reaction has been reported yet with the use of xenogenic EVs in preclinical studies.[22] Although clinical data regarding safety of allogeneic EVs are still mostly unpublished, few small-scale studies have reported safety information following systemic administration.[22–24]

EVs can be isolated from cells with relatively common and accessible equipment, such as cell culture incubators and ultracentrifuges, making EVs a pragmatic therapeutic option globally. Wide access is uncommon for a promising treatment modality and EV-based options are not restricted only to regions and countries with financial access. Coupled with efficacy, despite flexible culture conditions, storage, and quantities administered, MSC-derived EVs are being actively investigated for their promise in addressing clinical hurdles. The diverse contribution of ideas, innovation, and preclinical data is an enriching factor for this field and is evident in the rapidly evolving technology to match our EV isolation and characterization needs.

Among the demonstrated efficacy in promoting tissue repair and/or regeneration, an important discussion of details of the methods associated has been missing. Tissue of origin, cell integrity and fitness, passage number, cell culture media composition, isolation protocols, treatment regimens, and storage/banking parameters are critical, and require discussion as an area that requires standards and rigor.

Our goal with this review is to provide our colleagues across academia, medicine, industry, and regulation with parameters to facilitate comparisons across peer-reviewed studies to date that

detail use of exogenous EVs for cutaneous therapies. Our focus is on the conditions of source tissues, cells, and EV isolation measures, storage parameters, and modes of administration, as those criteria dictate variations in EV contents and induced outcomes in skin. The same human cells that are xenogenic EV sources in preclinical studies will form the allogeneic sources during clinical translation. Despite variations in human to human molecular expressions, procedural parameters may be the key to generating EVs that demonstrate consistent efficacy. This approach will help identify the most suitable EV subtypes and modifications targeted for different skin wounds and disorders, as well as ensure consistent outcomes and advance translational use of these biologics.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

EV classification and nomenclature

For this review, we categorized EVs reported in published studies per the criteria and standards advised by the International Society of Extracellular Vesicles (ISEV) as the Minimal Information for Study of EVs in 2018 (MISEV2018).[25,26] Determination of the biological origin of EVs, such as endosomal or plasma membrane derived, in large-scale isolations from even one cell type demands extensive high-resolution characterization. While innovative methods are being developed to profile EVs and assign identities, results are undermined by our limited knowledge of specific EV markers and variation in biogenesis events.[27–32]

Published literature dedicated to isolation and characterization of EVs, and placing them in context of cellular biology, mostly do not have results from downstream application yet for inclusion in this review. For EV biogenesis pathways, once again, we refer the reader to elegant reviews written by leaders in the EV field across the globe as well as to resources hosted by the ISEV such as EV-TRACK.[5,6,33,34]

We assessed the EV subtype of each study discussed here based on available information regarding size, biochemical characterization, tissue and cell of origin, cell fitness, priming conditions, and/or manipulation, together with author-provided descriptors such as "exosomes," "microvesicles," and "apoptotic bodies." The guidelines for reporting of EVs have been updated every few years to reflect our evolving knowledge and technology, and to build reproducible and comparable methods accompanied by specific EV-associated functional activity readouts. We strongly support the need for benchmarks for rigor and reliability of all EV-associated research, particularly for translational studies focused on treatment of skin injuries/conditions.

As per MISEV guidelines on size-based characterization, we refer to EVs <200 nm diameter as small EVs (sEVs), and those larger than 200 nm as medium/large EVs (mlEVs).[26] The sEVs are most commonly referred to as exosomes, while mlEVs include both microvesicles and apoptotic bodies. When consensus features are not available in the publication, we use "extracellular particle" or EP terminology as suggested by MISEV2018.[26]

For *in vivo* therapeutic use and translational studies, the isolation method and specificity of EVs are critical.[35] Specificity, yield, isolation techniques, and downstream applications are inherently completely interdependent. Reporting details of the whole process, even in studies that focus on the outcomes of EV application as therapy, is essential, as additions such as antibodies, polymers, and beads may interfere with functional studies and prevent replicability and adaptation by other laboratories. Vigilance of all cell source, isolation technique, and characterization variables is necessary for unbiased interpretation and associating function or molecular impact of each EV preparation administered as therapy for skin injuries/conditions.

Source cells for EV generation

Studies assessing impact of EV application in skin injury/conditions include EVs, primarily sEVs, from numerous sources—bone marrow stromal cells, umbilical tissue, amniotic fluid, saliva, and urine, to name a few (Fig. 1 and Table 1). However, the prevalent choice are MSCs, which, according to Society for Cellular Therapy (ISCT) guidelines, are adherent heterogeneous cell populations with only *in vitro* demonstration of self-renewal and trilineage differentiation along osteogenic, adipogenic, and chondrogenic lineages.[36,37] For this review, we refer to these adult mesenchymal lineage cells as MSCs based on their *in vitro* differentiation capacities, and will specify the source, such as bone marrow or adipose tissue.

The reasons behind widespread use of MSCs for EV harvests are multifactorial, mainly relating to easy access, optimized and scalable culture requirements, and lack of immunogenicity.[38] The important factor in MSC cultures is attention to regular cell culture practices to ensure the primary cells are not overgrown and/or stressed, as that could impact EV generation and EV content. Research into other cell types lacking multipotency, but relevant to skin, such as epithelial and endothelial cells or fibroblasts, is underway and is

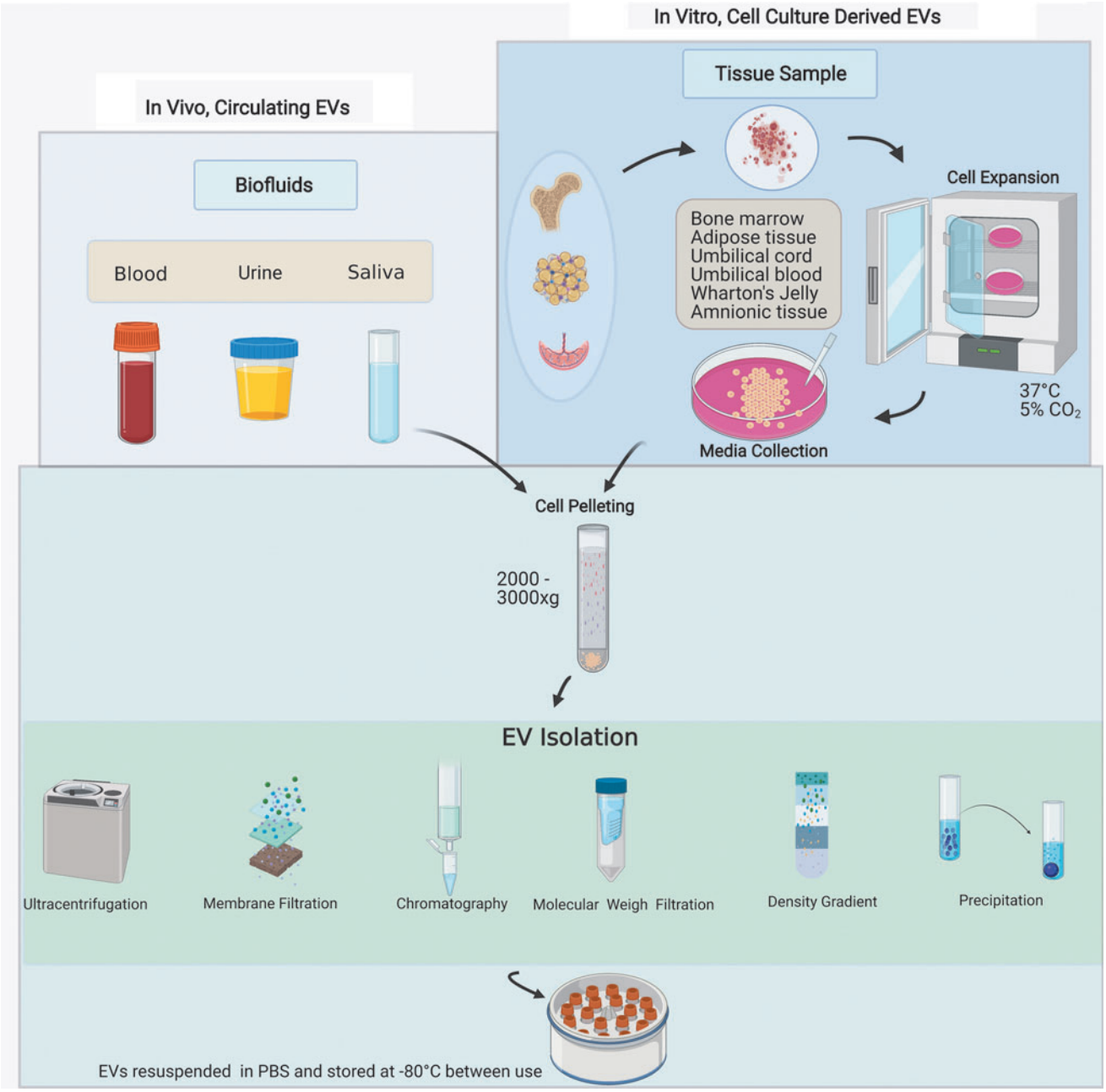

Figure 1. EV sources and isolation methods. EVs used for wound healing can be derived from biofluids (*in vivo* produced) or harvested from cell cultures (*in vitro* produced). *In vitro* production has the advantage of scaling up EV supply, and cells can be easily maintained in hypoxic environment at 37°C using common laboratory protocols and equipment. EV harvest involves first removing cell debris, and then isolating the desired EV by methods that select for size or biochemical properties. Following isolation, storage of EVs at –80°C protects from loss of their wound healing potential. EV, extracellular vesicle.

described in this section. However, whether EVs from a specific cell source or with certain characteristics dictate healing outcomes depending on wound type remains to be determined. Majority of studies use xenogenic EVs in mouse or rat rodent cutaneous injury models, indicative of the potential for safe, future clinical allogenic EV application.

Restriction to autologous use would severely limit patient access and eligibility for such treatment. A preclinical wound study compared the therapeutic effect of EVs from autologous versus allogeneic sources, but found no difference in outcomes.[39]

Bone marrow. Adult bone marrow stromal cells are the emblematic MSCs and were first reported in the late 60s-early 70s, as non-hematopoietic colony-forming fibroblasts capable of osteogenic differentiation.[40,41] They were later iden-

Table 1. *Source tissue and cells for extracellular vesicles used as treatment in skin wounds/conditions*

Species	*Tissue Source*	*Type of Cell*	*Author EV Descriptor*	*MISEV Nomenclature*	*References*
Human	Bone marrow	MSC	Exosomes	sEV	47,52,53,96,121,125,141,142,169
	Adipose tissue	ADSC	Exosomes	sEV	56–60,97,99,102,103,105,133,134,150,161,163,170–172
	Adipose tissue	ADSC	Microvesicles	mlEV	107,136
	Umbilical cord	MSC	Exosomes	sEV	69,99,103,131,135,143,144,153,154,158,159,162,173,174
	Umbilical cord blood	MSC/endothelial progenitor cells	Exosomes	sEV	100,130,151,152
	Umbilical cord blood	Mononuclear cells	Small EV	sEV	70
	—	HUVECs	Exosomes		72
	Amniotic membranes	Amniotic epithelial cells (hAECs)	Exosomes	sEV	73,175
	—	Amniotic MSC	Exosomes	sEV	147
	Wharton's jelly	MSC	Exosomes	sEV	176
	Acellular gelatinous Wharton's jelly	—	Exosomes	sEV	71
	Whole blood	—	Exosomes	sEV	84,177
	Urine	MSC like/urine stem cell	Exosomes	sEV	81
	Saliva	—	Exosomes	sEV	85
	—	Embryonic stem cells	Exosomes	sEV	98
	Induced pluripotent stem cells line	MSC	Exosomes	sEV	148
	Leukapheresis packs (leukopaks)	Human fibrocytes	Exosomes	sEV	117
	Synovial membrane	MSC	Exosomes	sEV	101
	—	Epidermal stem cells	Exosomes	sEV	146
	Dorsal skin of fetal samples	Fetal dermal mesenchymal stem cells	Exosomes	sEV	145
	—	Dermal fibroblast	Exosomes	EP	178
	Endometrial tissue	Endometrial stem cell	Exosomes	sEV	179
	Gingival connective tissue	MSC	Exosomes	sEV	76
Mouse	Bone marrow	MSC	Exosomes	sEV	48,49
	Bone marrow	MSC	Apoptotic bodies	mlEV	108
	—	Stromal cell line	EV	sEV	109
	Bone marrow	Endothelial progenitor cells	Exosomes	sEV	180
	—	iPSC	Microvesicles	mlEV	106
Rat	Adipose tissue	ADSC	Exosomes	EP	104
Rabbit	Adipose tissue (inguinal fat pads)	ADSC	EV	EP	39
	Bone marrow	MSC	EV	EP	39
Canine	Bone marrow	MSC	MV/exosomes	EP	50
Monkey	Skin	Skin fibroblast iPSC	Exosome	sEV	181

ADSC, adipose tissue-derived MSC; EP, extracellular particle; EV, extracellular vesicle; HUVEC, human umbilical vein endothelial cell; MISEV, Minimal Information for Study of Extracellular Vesicles; mlEV, medium/large extracellular vesicle; MSC, multipotent stromal cell; sEV, small extracellular vesicle.

tified as an MSC source with proof of *in vitro* differentiation into multiple mesenchymal identities—such as fat and cartilage along with bone—in high-impact articles, with the coining of the terms "marrow stromal cell" and "mesenchymal stem cell."[42–44]

Adult human bone marrow MSCs (BM-MSCs), primarily from the iliac crest, are a main source of xenogenic EVs for skin injury and wound healing applications in rodent models. Human BM-MSCs can be obtained from commercial sources with well-characterized parameters,[45] including expression of cell surface markers CD73, CD90, and CD105, and absence of negative markers, such as CD45, CD34, CD14, CD11b, CD19, CD79a, and HLA-DR.[37]

Age of the BM donor is critical as the MSC numbers and cell fitness diminish sharply in adults.[46] Conveniently, BM-MSCs can be cryopreserved between uses without loss of integrity.[47] EV studies using murine BM-MSCs tend to flush bones like the femur and tibia.[48,49] A few studies utilizing allogenic BM-MSC EV and wound models used rabbit and canine iliac crests.[39,50]

The expansion capacity of primary adult BM-MSCs has been a decisive factor in their use for cellular therapy and EV generation in regenerative medicine studies. Although these cells constitute <0.1% of the BM population, hundreds of millions of cells can be cultured from one pass worth of bone marrow aspirate.[44,51]

BM-MSCs from the sources discussed above are maintained in culture in α-Minimum Essential Medium α or Dulbecco's Modified Eagle Medium, supplemented with fetal bovine serum (FBS), glutamine, and penicillin/streptomycin, under standard 5% CO_2, 37°C, humidified conditions for 2–8 passages without loss in quality of EVs generated.[47,49,50,52,53] Before collection of conditioned media, the regular FBS is substituted with EV-depleted FBS for 48 h to remove or minimize bovine serum-EVs, while ensuring cell proliferation proceeds unimpeded.[54,55]

To maximize cell expansion, Shabbir *et al.* cultured human BM-MSCs in five-layer 875 cm^2 multiflasks and attained an impressive number of

$\sim 17\times10^6$ to 22×10^6 cells per flask at 60–80% confluence.[47] We have been able to isolate 1×10^{11} sEVs from early passaged human BM-MSCs after beginning with only 1×10^6 cells (unpublished). With the scalability of MSC culture as demonstrated by Shabbir *et al.*,[47] increase in EV generation will be exponential as well.

Adipose tissue. EVs isolated from human adipose tissue-derived MSCs (ADSCs) are abundantly used in cutaneous wound studies, arguably due to the ease of access from clinical lipoaspirations, including during planned caesarean sections.[56–60] Initially adipose-tissue derived cells were under the same umbrella of MSCs,[61,62] but now are specified as "adipose derived," rather than the blanket MSC terminology.

ADSCs share majority of cell surface markers as BM-MSCs, but with the distinguishing addition of CD36.[63] MSCs can be harvested in much larger quantities from human adipose tissue compared to other sources, and at a typical yield of between 3 and 5 million cells per 100 mL of fat aspirate, adipose tissue produces about 40 times more MSCs than BM.[64,65]

Whether ADSCs decline with age in healthy donors, as for BM-MSCs, remains to be seen, but most studies report that their donors for ADSC-EV isolations were between 18 and 30 years old. A study also obtained EVs from murine inguinal pads and rabbit adipose tissue, but did not report species-specific ADSC properties or yield discrepancy.[39]

Umbilical cord, blood, and Wharton's jelly. Umbilical tissue is another MSC EV source with growing appeal due to availability as a medical waste specimen. Previous studies found that conditioned media from umbilical progenitor cells influence keratinocyte migration and credited the effect to EVs.[66,67] Similar to BM-MSCs and ADSCs, umbilical cord MSCs (UC-MSCs) are isolated based on plastic adherence of umbilical tissue homogenates and multilineage differential potential of adherent cells[68,69] Further phenotyping, such as with cell surface and proliferation markers, and functional assays would confirm MSC enrichment in these adherent cultures. Recently, Cardoso *et al.* described the use of umbilical cord blood mononuclear cell-derived EVs to stimulate comparable wound healing effects as UC-MSC-EVs.[70]

In a break from cell culture-derived EV isolation, Wharton's jelly (WJ), the acellular connective tissue in umbilical cords, can also serve as an innate biological niche for harvesting EVs.[71] This study gives credence to the supposition that both *in vivo* and *in vitro* produced EVs can influence tissue repair and regeneration. Human umbilical vein endothelial cells (HUVECs), which are ubiquitous in bench research, can also be an EV source.[72] Results from this study indicated that EVs from commercially and widely available HUVECs promote similar cell proliferation and migration effect when applied in skin wounds as other umbilical tissue sources.[72]

Amniotic tissues. One benefit of using amniotic fluid-derived MSCs (AF-MSCs) for studies is that donors have gone through genetic and infectious disease screening during pregnancy monitoring.[73] As a result, these cells are already partially quality controlled. Tissue processing first involves separation from the placenta, followed by trypsinization for cell isolation.[74] These cells are easily maintained following general MSC culturing protocols, but produced 1.3 times more sEVs per 1 million cells compared to BM-MSCs.[73,75] The authors found that AF-MSCs were easier to expand and store through cryopreservation in comparison to BM-MSCs.[75]

Other sources. Gingival MSCs, isolated from the connective tissue underlying the oral mucosal epithelium on the gums, are gathering interest as a source cell type in wound healing studies.[76] These MSCs thus far maintain self-renewal and proliferation potential at higher passages compared to BM-MSCs.[77] Given that the oral mucosa has comparable histology to outer skin, but has propensity for faster healing,[78,79] gingival MSC EVs are relevant to skin repair and regeneration studies.

Less typical EV sources used for preclinical skin wound studies include blood, urine, and saliva. One explanation for the desirability of these sources is that they are obtained noninvasively. Dalirfardouei *et al.* isolated MSCs from human menstrual blood for culture before EV harvest, even though EVs can and have been isolated from plasma.[80]

Adherent MSCs can be cultured from pelleted urine samples for EV isolation as well.[81] To warrant sterility of the samples and all downstream products, urine was collected in tubes with antibiotic-antimycotics.[81] EVs can also be efficiently retrieved directly from biological fluids to avoid the cell expansion steps and collect exclusively *in vivo* produced EVs.[82–84] This notion is also true for EVs from saliva.[85] While *in vivo* sources, not based in cell culture, limit the EV yield, wide availability and accessibility make them viable options for therapeutic use.

EVs from cell types that constitute skin, such as keratinocytes and fibroblasts, as well as from cells

associated with skin wound inflammation, such as lymphocytic lineages, myeloid lineages, and neutrophils, are emerging as potential therapy for skin wounds as well and are described in an elegant review.[86] A handful of studies report the effect induced by this contingent of skin-relevant EVs on preclinical wound models[87,88] and we anticipate a rise in such studies as tissue EV isolation and characterization become more feasible.

Isolation and identification of EVs

Most EV isolation techniques are based on the original protocol by Théry *et al*.[89] but recent publications use the increasingly available proprietary commercial reagents (Fig. 1 and Table 2). One outstanding caveat is obtaining homogenous populations of EVs of interest, limited by our knowledge of heterogeneity of cultured MSCs, of biogenesis factors determining EV sizes and content/markers, and of technology tailored for individual EV analysis, also discussed in EV Classification and Nomenclature section.

While most wound healing studies do not comment on extensive MSC characterization or EV sample purity, awareness exists that different protocols may enrich for different EV subtypes from different cells. Even lipoproteins, which are considered undesired co-isolates, may reflect proteins that are specific to certain EV subtypes.[90]

Co-isolates or heteregenous EV preps do not discredit the impact of EV therapy on wound healing results, but we need to be aware that efficacy may not be due to EVs alone. Studies need to pursue technical controls and additional methods for verification when parsing out EV-induced mechanisms induced in a wound bed.[26] Researchers are constantly seeking advances to optimize their protocols to obtain more specific, enriched EV batches and draw links to the observed wound healing outcomes. In the future, we may be able to choose the isolation protocol based on the study question and type of EV.

Western blotting and flow cytometry are the most common methods of phenotyping EVs. The most relevant of these markers are the tetraspanins CD81, CD63, and CD9, as well as the proteins Alix, flottilin-1, AGO2, and TSG101.[5,6]

Transmission electron microscopy is also used to verify the presence of unlabeled, isolated EVs and their renowned cup-shape artifact morphology resulting from fixation.[89,91,92] Unlabeled EVs applied to skin wounds can also be analyzed by nanoparticle tracking analysis (NTA) to resolve diameter size-based distribution of EVs in an isolate.[93] Techniques such as surface plasmon resonance with Raman spectroscopy are evolving for single EV resolution, and have the potential to contribute significant information about EVs and their contents in relation to their source cells.[94,95]

sEV isolation from cells and biofluids. The landmark article by Théry *et al*. described successive differential centrifugation to isolate a pellet enriched in EVs, whether from conditioned media or biofluids.[89] Some laboratories have amended their ultracentrifugation speeds, but adhering to the original Théry protocol.[69,96,97] NTA often demonstrates that, while majority of vesicles isolated are in the putative sEV or exosome size range, ultracentrifugation retains nonsignificant levels of mlEVs over 200 nm in diameter.[98,99]

For additional size-based purity, some studies run their EVs through a sucrose cushion gradient and isolate only the appropriate fraction with sEVs.[89,100,101] Filtration through 0.1–0.22 μm pore low-protein binding membranes, 100,000 Da molecular weight cutoff centrifugal units, and gravitational size exclusion columns also adds to size-based purity of EV preps.[96,101–104]

Proprietary immunoaffinity-based ExoCap™ and EV precipitation solutions like Exo-Quick-TC are often used as time-saving approaches, although size-specific yields are orders of magnitude below the other methods discussed above.[59,105] The commercial products are undergoing perpetual upgrades. For example, the Exo-Quick-LP kit now contains a lipoprotein clearing proprietary reagent (System Biosciences).

mlEV isolation. Very few studies report specific use of mlEVs for skin wound therapy, but often use the descriptors microvesicle or ectosomes to describe their EV isolates that have modal diameter larger than 200 nm.

Similar to sEVs, isolation is carried out by differential centrifugation, but using a pellet from a lower speed than that for sEVs.[89] Yan *et al*. used the affinity-based ExoEasy Maxi Kit (Qiagen), which selects for specific epitopes, and allowed them to harvest vesicles with a modal diameter of 214.6 nm.[106] Trinh *et al*. used flow cytometric analysis following the typical ultracentrifugation steps and reported mlEVs carrying CD105 and CD90 markers.[107] Some of the studies in Table 2 performed an intermediate molecular weight cutoff centrifugal filtration step after a 13,000 *g* spin, but before ultracentrifugation to enrich for mlEVs.

Apoptotic bodies and other EV isolation. Liu *et al*. described specifically isolating apoptotic bodies by first inducing chemical death of mouse

Table 2. *Range of skin wounds and disorders treated with extracellular vesicles*

Skin Disorder	*References*	*EV Type*	*Mode of Administration*	*Dosage*
Acute wounds	74	sEV	Local injection	25, 50, 100 μg/mL
	147	sEV	Local injections	Not reported
	69	sEV	Local injection	100 μg/mL
	59	sEV	Intravenous vs. local injection	200 μg/200 μL
	134	sEVs	Local injection	200 μg/100 μL
	170	sEVs	Local injection	200 μg/mL
	182	sEVs	Local injection	Not reported
	133	sEVs	Local injection	100 μg/100 μL
	136	mIEVs	Local injection	1.0 μg/μL
	60	sEVs	Not reported	Not reported
	104	EP	Oxband dressing	500 μg/mL
	163	EP	Local injection	126.48 μg/mL
	108	mIEV (apoptotic bodies)	Topical	50 μg/100 μL
	141	EP	Local injection	100 μg/100 μL
	53	EP	Subcutaneous injection	250 μg
	145	sEV	Subcutaneous injection	200 μg/200 μL
	142	sEV	Intravenous injection	200 μg
	49	sEV	Intradermal/local injection	100 μg/100 μL
	143	sEV	Subcutaneous injection	200 μg/100 μL
	131	sEV	Intravenous injection	100 μg/100 μL
	103	sEV	Subcutaneous injection	100 μg/100 μL
	146	sEV	Local injection	100 μg/mL
	144	EP	Subcutaneous injection	100 μg/100 μL
	71	EP	Matrigel topical	Not reported
	85	sEV	Subcutaneous injection	100 μg/100 μL
	148	EP	Subcutaneous injection	40 μg
Diabetic wounds	48	EP	Local injection	Not reported
	125	Deferoxamine: sEV, Control: EP	Subcutaneous injection	100 μg/100 μL
	121	EP	Subcutaneous injection around pouch and excision	Not reported
	117	sEV	Injection and topical	5 μg/200 μL, 50 μg/200 μL
	129	sEV	Topical	Not reported
	101	sEV	Topical	Not reported
	102	EP	Topical	Not reported
	150	EP	Subcutaneous injection	200 μg/200 μL
	84	sEV	Topical	Not reported
	130	EP	Topical	1,080 μg
	83	EP	Subcutaneous injection	50 μg/mL
	151	EP	Intramuscular injection	5×10^{10}/mL
	99	EP	Local injection	100 μg/100 μL
	73	sEV	Not indicated	1,000 μg/mL
	100	sEV	Injection	2×10^{10}/200 μL, 1×10^{11}/200 μL
	152	EP	Subcutaneous injection	100 μg
	80	sEV	Intradermal injection	10 μg/100 μL
Surgical wounds	56	sEVs	Subcutaneous injection	100 μg/200 μL
	111	EP	Surgical mesh	4,000 μg
	107	mIEV	Subcutaneous injection	Not reported
Photo-aging	155	EP	Injection	Not reported
	153	sEV	Culture	20×10^{8}/mL
	154	EP	Intracutaneous injection	0.02 μg/μL (2 μg per day for 5 days)
Aging	98	sEVs	Local/pipette drop	1×10^{10}/100 μL
Burn	174	sEV	Injection	1 mg/200 μL
	157	mIEV	Topical	Not reported
	159	sEV	Intravenous injection	800 μg/mL
	106	mIEV	Subcutaneous injection	Not reported
	158	sEV	Subcutaneous injection	200 μg/200 μL
Other	161	sEV	Subcutaneous injection	1, 3, 10 μg/mouse (100 μL each)
	162	sEV	Topical	15, 60 μg/mL
	109	EP	Percutaneous/gel	Not reported
In vitro	57	sEV	Culture media	100 μg/mL
	47	EP	Culture media	0.1, 1.0, 10 μg/mL
	165	sEV	Culture media	10^{5}–10^{8}/50 μL
	52	EP, $CD63^{+}$	Culture media	1.0 μg/mL
	97	sEV	Culture media	5, 10 μg/mL
	68	sEV, EP	Culture media	50, 75, 150 μg/mL
	183	sEV	Culture media	Not reported
	164	sEV	Culture media	Not reported

BM-MSCs in culture.[108] They carried out two-step centrifugation, first at 1,000 *g* to remove cells, and then at 16,000 *g* to pellet the apoptotic bodies. Dynamic light scattering analysis confirmed that particles isolated were in the 400–2,000 nm diameter range, with a mean size of 955.41 nm.[108]

Several works used the general terminology "EVs" for their isolated particles to account for insignificant, but still present larger particles, without any further characterization steps. Berger *et al.*, for instance, admittedly carried out a 110,000 *g* spin, but did not acknowledge any further steps to allow for size exclusion and reported 70–500 nm particles, with the majority being under 200 nm.[109]

Another study relied on ultrafiltration followed by size exclusion to obtain an EV prep where 80% of contents were 50–200 nm in diameter, but had no further characterization regarding origin of the EVs.[70]

Conditioning

Attempts at priming EV source cells, for increased efficacy or specific repair phenotypes in recipient tissue wound beds, are in their infancy. Standardization of cell culture conditions for specificity of EV production is still a work in progress, because priming conditions during culture potentially alter a cell's fitness, metabolism, endosomal processing, and expression of molecules associated with EV biogenesis and content determination.[6]

The very limitations of our knowledge of EV content determination are the exciting avenues for upcoming discoveries. The microenvironment ought to influence source cell function and subsequently, EV contents (Fig. 2). The details discussed for standardization of MSC cultures for initial cell therapy approaches are still relevant when isolating EVs from these cells.[110]

For preservation of EVs following isolation, most of the works in this study stated that the particles were stored at –80°C, without compromising downstream applications.[96] One exception stored their isolated exosomes at –20°C, although the shelf life at this temperature was not reported.[111]

The studies that used preserved EVs do not comment on structural integrity after thawing, however. Non-wound application studies confirmed that –80°C conserves EV features and function for at least a month, but temperatures above –20°C were severely detrimental even in the short term.[112,113] Additional specifications to improve EV storage are emerging, including the use of siliconized tubes or addition of dispersion agents to prevent aggregation of EV suspensions.[26,114–116] The functional efficacy of EVs stored in such manner remains to be evaluated in *in vivo* cutaneous wound models.

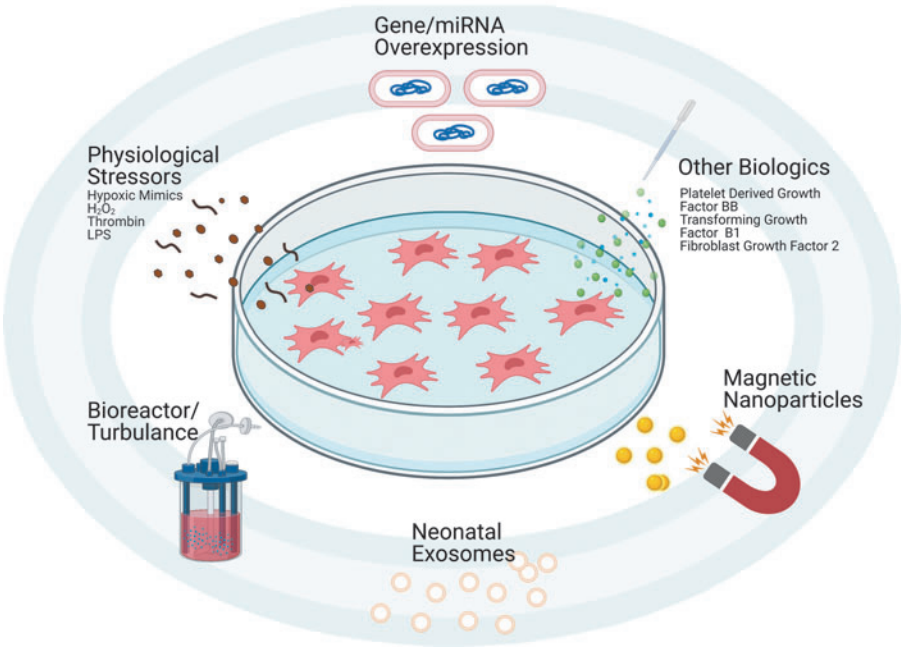

Figure 2. Preconditioning parameters improve EV wound healing potential. EV properties can be adjusted by stimulation of their source cells. Physical and chemical methods precondition source cells and thus the cargo of their derived EVs, resulting in phenotypic variations of the vesicles. Cells can be preconditioned by (1) transfection of nucleic acids, (2) physiological stressors, (3) nanoparticles, (4) other EVs, (5) mechanical stressors, and (6) other biologics.

Improving EV yield. The majority of studies discussed in this review relied on conventional cell culturing and EV isolation methods, testing the effect of manipulation-free EVs on animal wound models. To scale up EV yield, a study used bioreactors to support three-dimensional (3D) cultures that are adhered to microcarrier beads and showed that steady turbulence of the bioreactor spinner flask induced increased EV secretion, and granted them a yield of 10^{13} EVs from a 1 L bioreactor, which is 2–3 orders of magnitudes of total EVs above all other reported studies.[109]

Cultured fibrocytes responded to stimulation with platelet-derived growth factor-BB (PDGF-BB), transforming growth factor-β1 (TGF-β1), and fibroblast growth factor 2 (FGF2), by increasing proliferation and EV generation (concentration normalized to cell numbers), compared to unstimulated cells.[117]

Similarly, human UC-MSCs increased EV yield per cell when exposed to thrombin, hypoxia, lipopolysaccharide (LPS), or hydrogen peroxide (H_2O_2), with thrombin quadrupling the EV output compared to untreated cells.[118] Of note, the UC-MSCs responded by upregulating EV production to an extent, as cell death occurred at the highest concentrations used in the study.

Controlled level of stressors potentially benefit EV generation as part and parcel of inducing homeostatic mechanisms in source cells. BM-MSCs, for example, are capable of responding to hypoxia and oxidative stress through cytoprotective pathways.[119] An elegant study used a proteomics approach to demonstrate that controlled serum starvation of UC-MSCs benefits sEV yield, cargo, and function.[120]

Another pertinent observation was that unlike in UC-MSCs, serum deprivation significantly reduces sEV yield in ADSCs and BM-MSCs. While serum starvation does not affect mlEV quantity, it negatively affects capacity to transport nucleic acids. These reports circle back to the need for appreciation that different protocols may enrich for different EV subtypes from different cells.

Priming EVs for the wound environment. When priming EV source cells for use in wound treatment, common themes include pathways and mediators of inflammation and cell growth and motility. For example, pretreatment of human BM-MSCs with melatonin due to its putative role in mediating inflammatory responses or priming HaCaT cells with the fibrinolytic drug mesoglycan, both generate EVs that increase mobilization of fibroblasts and endothelial cells.[121,122]

Chemical-induced senescence during culture in a study altered the content of ADSC-EVs.[103] BM-MSC studies often use hypoxic incubators or chemically induce hypoxia-associated signaling to mimic the physiological BM environment,[119,123] maintain stemness, and upregulate immunomodulatory pathways that play roles in wound repair.[124–126] Priming periods reported are very short and the new conditions are induced just before EV harvest setup.

As discussed in the previous section, source cells may be neutralizing the imposed environmental cue and in the process generating EVs that are better suited to the stress conditions of wound tissues. When cultured in thrombin, hypoxia or H_2O_2 UC-MSC EVs carry higher angiogenic cargo.[118] Conditioning MSCs with LPS induces inflammation resolution responses in the source cells[127] and the molecular changes are reflected in EVs as well. Priming adult BM-MSCs with neonatal serum potentially results in EVs with enhanced cell growth cargo, harnessing parts of a neonatal system that has to support large scales of tissue growth and maturation vesicles.[49]

Improving delivery. EVs are physiological delivery vehicles, a role fundamental to their success as a therapeutic option for cutaneous wounds. Yet delivery vehicles are necessary to facilitate administration into skin wounds, with focus on localization and penetrance in the wound tissue (Fig. 3).

Conventional wound care often involves the application of a "dressing" to protect the damaged skin through maintenance of sterility and physical support. Biological polymers like chitosan are candidates for such dressings based on their sustained medication release profiles and individual beneficial effects.[128] Gingival MSC EVs delivered in such a chitosan/silk hydrogel sponge promoted diabetic excisional wound closure faster than phosphate-buffered saline (PBS)-treated gauze covered or PBS-hydrogel controls.[76] Hydroxyapatite/chitosan hydrogel dressings alone promote wound closure and when loaded with EVs, facilitate sustained release of EVs over 6 days to further accelerate diabetic wound closure.[129]

Another dressing, OxOBand, consists of ADSC sEVs dispersed within a polyurethane-based cryogel with antioxidant properties from ascorbic acid and was targeted for the oxidative stress in diabetic wounds.[104] Combination of UC-MSC EP in biomaterial carriers like polyvinyl alcohol (PVA)/alginate (Alg) nanohydrogel or pluronic F-127 hydrogel similarly accelerated excisional wound closure in type 1 diabetic rats.[99,130]

In the treatment of incisional hernia, surgical fibrin mesh provided a physical scaffold for EV

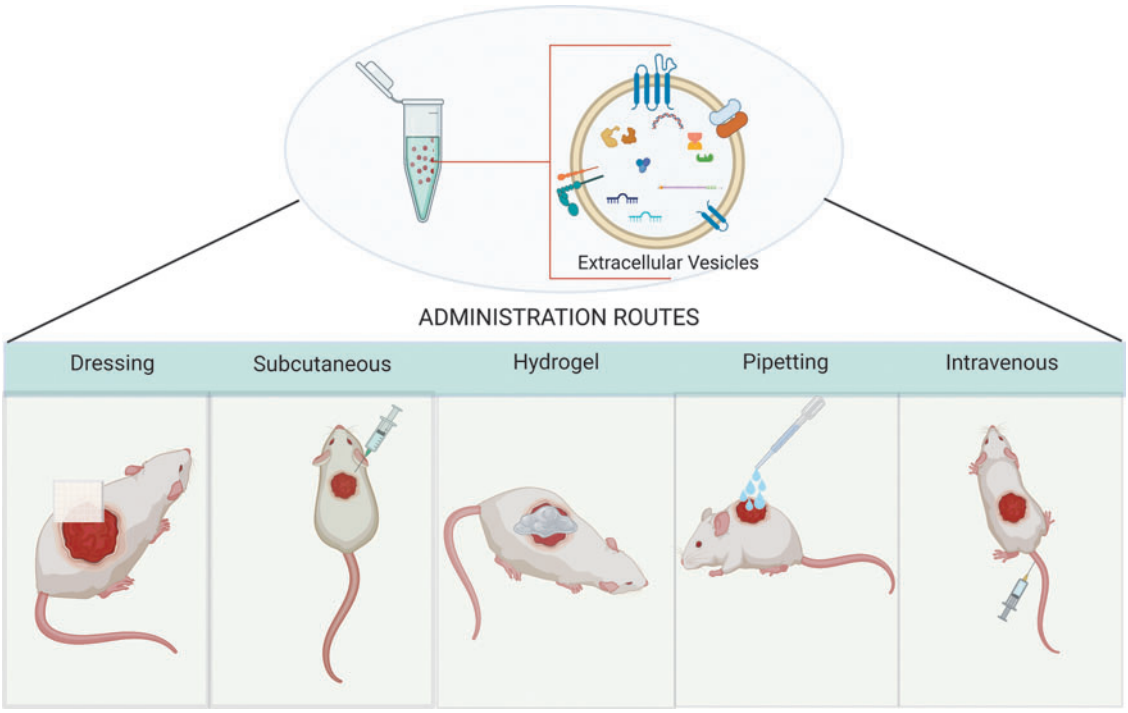

Figure 3. EV administration routes. Various administration routes have been investigated in preclinical wound models to seek out the modes that allow for improved treatment pharmacodynamics and pharmacokinetics.

delivery and led to improved tensile strength of the healed incision.[111] For improved targeting of EV treatments, UC-MSCs were incubated with superparamagnetic iron oxide nanoparticles that accumulate within sEVs, such that the labeled EVs could be guided to the wound site with a magnetic field.[131] An important observation is that combination with the vehicles discussed does not hinder the impact of EVs on accelerating wound resolution.

EV therapy outcomes for cutaneous wounds and disorders

The urgent nature of skin disorders has driven the expansion of EV preparation-based therapies. The accessibility of EV preps and lack of toxicity in preclinical studies, regardless of specificity and purity, describe promising results in the context of treating skin disorders from eczema to chronic wound models. Majority of studies use xenogenic EVs in rodent models and demonstrate efficacy in promoting measurable resolution of the disorders (Fig. 4), as well as indicate potential success in allogenic clinical applications.

Cutaneous wounds. Cutaneous wound healing is by far the largest preclinical area assessing EV therapy efficacy. The complexity of the cellular milieu and signaling in skin wound healing, both spatially and temporally, is challenging to address in this field. EVs, with their wide range of cargo, demonstrate reliable efficacy in pushing the limits of even physiologic healing in healthy animal models. Most studies employ the excisional or full-thickness wounds on rodents, both unstented and stented-humanized[132] models, to assess efficacy of xenogenic EVs, notably without an adverse reaction.

Hu *et al.* performed a necessary comparison of local versus intravenous injection of ADSC-sEVs to assess their efficacy in acute cutaneous wounds and found fastest closure in the local cohort.[59] Local injection of EVs prompted earlier expression of collagen III in wound tissue sections than the other two groups, to lead to faster closure and demonstrating the efficacy of this method.

Studies inhibited EV secretion in ADSCs, but processed the conditioned media as usual, then applied the preparation onto excisional wounds and reported no impact on wound closure acceleration.[99,133] ADSC-EV-treated wounds expressed microRNA 19b (miR-19b, a noncoding regulatory RNA) and reepithelialized faster than controls, but this finding was negated with secretion inhibition in the ADSCs.[133] These studies demonstrated that the EV contents and uptake are necessary for the efficacy in promoting wound closure.

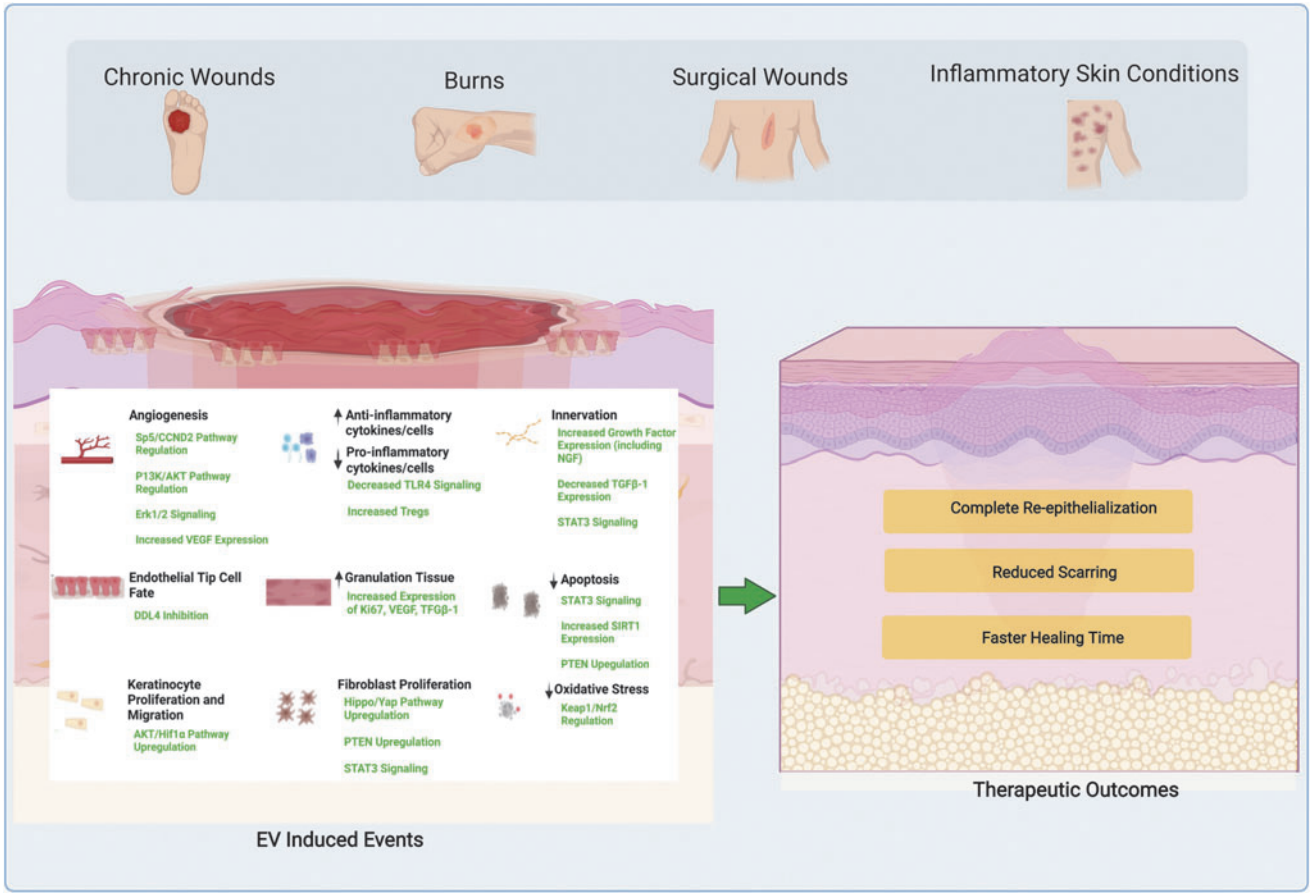

Figure 4. Events promoted by EV therapy for resolution of cutaneous wounds. Regardless of type, skin wounds are complex, requiring a number of molecular events to resolve pathology, and to be considered "healed." Prominently, multiple cutaneous and immune cell types are mobilized to replace damaged tissue, and provide necessary immune intervention. EV therapy can provide these functions and other molecular support to enable complete wound reepithelialization, faster closure time, and reduced scarring.

Analysis of excisional wounds treated with ADSC-EVs consistently shows proliferation and migration of keratinocytes for reepithelialization, and that of fibroblasts and endothelial cells for promotion of collagen-laden well-vascularized granulation tissue, and factors involved in immune modulation.[99,133–136]

Current level of evidence indicates that microRNA (miRNA) content of EVs is a driver of these phenotypes; miR-486-5p in sEVs regulates the Sp5/CCND2 pathway to promote angiogenesis in excisional wounds and miR-19b affects levels of CCL1 and TGF-β1.[133,134] miR-21 in ADSC-sEVs affects the PI3K/Akt pathway to affect expression of matrix metalloproteases and angiogenic proteins.[99] Zhang *et al.* found modulation of AKT/Hif1a in their incisional wound and ADSC-sEV treatment model.[135] A study using ADSC-microvesicles (mlEVs) on excisional wounds also reported the expected faster reduction in wound area.[136]

Senescent ADSC-sEVs with differential expression of miR-146a were not effective in enhancing wound repair, unlike young ADSC-sEVs, suggesting that treatment with exogenous EVs is dissimilar to senescence-associated vesicle secretion.[103] EVs and vesicles from senescence-associated secretory phenotype have been compared, as senescent cells secrete larger quantities of vesicles compared to healthy cells, and these vesicles have even been known to affect the phenotype of surrounding cells.[137,138] However, the regenerative potential of senescence-associated EVs is debatable, as senescence alters their cargo for prosenescent, proinflammatory paracrine effects.[137,139,140]

BM-MSCs are the second most used source of EVs for skin injury studies. Studies typically use a single administration of BM-MSC EVs in excisional wound models in adult mice or rats for consistent efficacy in acceleration of wound closure, or reduction of wound area at the time points studied,

as well as fibroblast proliferation and extensive neovascularization compared to untreated/vehicle-treated wounds.[49,53,108,141,142] Liu *et al.* used apoptotic bodies as a treatment based on their observation that BM-MSCs undergo apoptosis within a short time frame of transplantation into mouse skin wound models.[108]

To answer their question of whether apoptotic bodies are part of the therapeutic effect, multiple doses of BM-MSC apoptotic bodies within the first week of wounding promoted increased numbers of proresolution macrophages and accelerated healing. Wu *et al.* used BM-MSCs cultured with iron oxide under a static magnetic field to find enrichment for miR-21-5p that inhibits SPRY2 to activate PI3K/AKT and ERK1/2 signaling pathways in the wound bed.[141]

In another approach, Qiu *et al.* exposed adult mouse BM-MSCs to neonatal serum EVs and reported faster wound closure following treatment with EVs from the "educated" versus naive BM-MSCs.[49] Due to the technical challenges of locating EVs within specific cells in wound tissue, several studies supplement the *in vivo* wound closure data with *in vitro* setups to distil and analyze the effects of EV treatment on critical wound cell types, such as macrophages, keratinocyte lines, dermal fibroblasts, and endothelial cells.

Application of EVs isolated from human umbilical cord-MSCs or human Wharton's Jelly-MSCs onto excisional wounds on mice promoted faster wound closure without excessive scarring compared to control treatments.[69,71,143,144] The miRNA content of these EVs, such as miR-23a, miR-125b, and miR-27b, potentially attenuate differentiation of myofibroblasts through the TGF-β/SMAD signaling pathways.

One study found the suppression of apoptosis-induced factor (AIF) nuclear translocation and PARP1 activation in wound bed cells.[144] Treatment with nonmesenchymal cell EVs, isolated through similar protocols as ADSCs, BM-MSCs, UC-MSCs, or Wharton's jelly-derived multipotent stromal cells (WJ-MSCs), affects excisional wound healing timelines as well, in comparison to untreated/sham-treated wounds.

In addition, fetal dental dermal MSCs, epidermal stem/progenitor cells, amniotic membrane MSCs, amniotic epithelial cells, and saliva and induced pluripotent stem cells-derived MSCs have been used to isolate EVs for testing efficacy in wound closure studies.[74,85,145–148]

Duan *et al.* postulated that miRNA in epidermal stem/progenitor cell EVs blocks TGF-β1 signaling,[146] while Mi *et al.* reported that transcripts for *UBE2O* (ubiquitin-conjugating enzyme E2O) are a main component of salivary EVs and may be responsible for the observed effects of rapid wound closure. Gao *et al.* reported that EVs from amniotic membrane MSCs with forced expression of miR-135a modulate cell migration proteins in the wound bed.[147] Whether the same miRNAs are present across EVs from varying tissue sources with the same culture conditions is yet unknown.

Diabetic wounds. Both type 1 and type 2 diabetes models are used widely to assess efficacy and activity of EVs in resolution of healing delays within the complex microenvironment of diabetic wounds. These wound models demonstrate delayed healing, but not the chronicity associated with human diabetic ulcers. Streptozotocin (STZ) administration is the most popular means to induce a type 1 phenotype in both mice and rats. The $Lepr^{db/db}$ mice on the Black Kallis background are one of the most widely used type 2 diabetic models. Some studies use a combination of STZ and high-fat Western diet (HFD) to promote hyperglycemia and phenotypes associated with type 2 diabetes.

The blood glucose content is always a vital data point when assessing a prediabetic or diabetic wound model, as a prediabetic animal (100–125 mg/dL [5.6–6.9 mM]) will not reflect the uncoupled mechanisms and delays associated with diabetes (≥126 mg/dL [7 mM]).[149]

A noteworthy fact is that studies that utilize EVs to therapeutically address diabetic wounds tend to utilize source cell preconditioning as discussed earlier, unlike studies of acute wounds that focus on accelerating healing within physiologic limits. As the pathology and manifestation of diabetes in skin wounds are multifactorial and complex, investigators have shifted their focus to upstream mechanisms of molecular regulation, primarily noncoding RNAs like miRNA, over messenger RNA (mRNA) expression, to attempt to address a wider range of molecules and pathways in the wound bed. EVs offer advantage over singular pathway-targeted approaches as the diverse nature of their contents affect multiple molecular networks.

The source cell preconditioning trend is geared toward generating EV preps that can override or switch the pathological pathways within diabetic wound tissues. This approach is supported by a study that discovered overexpression of miR-20b-5p in serum-EP of patients with diabetes compared to those from a nondiabetic healthy cohort.[83] Following injections around wounds on STZ/HFD mice, the diabetic serum-EP did not promote healing in contrast to the nondiabetic serum-EP. The result indi-

cates that the choice of EV and conditions of the source cell culture dictate outcomes in the complex diabetic wound bed. This priming is distinct from short-term priming in cell culture discussed previously, as it captures the long-term chronic changes in EVs induced by diabetes.

Given the importance of miRNA from source cells in EV functionality in wound healing, other regulatory RNAs such as chromatin and gene modulating long noncoding RNAs (lncRNAs) may be just as relevant. Li *et al.* demonstrated that forced overexpression of lncRNA H19 in mouse BM-MSCs resulted in the EP packaging H19.[48] In a STZ and high-fat diet combination diabetic model, injections of these EP led to faster reduction in open wound area, compared to untreated diabetic wounds. The overexpressed H19 targeted miR-152-3p in cells of the wound bed, which in turn targets PTEN, to ultimately affect fibroblast proliferation, migration, and apoptosis.

Other conditioning such as melatonin treatment of human BM-MSCs, where subsequent EP administered to type 1 diabetic excisional wounds in rats, led to faster closure compared to sham treatments, and by targeting PTEN.[121]

Human BM-MSCs, which were preconditioned with deferoxamine to mimic hypoxia, produced sEVs carrying miR-126, which when injected to type 1 diabetic rat wounds activates the PI3K/AKT pathways in endothelial cells, to affect wound closure time.[125] At this time, knowledge of the distribution of overexpressed RNAs among the different sized EVs in an EP of each study or differential uptake in a wound bed is unknown, but the impact on accelerating diabetic wound closure is remarkable nonetheless.

Topical application of sEVs from human synovial fluid, overexpressing miR-126 or miR-126-3p, led to accelerated excisional wound closure in type 1 diabetic rats, with pronounced angiogenesis in a dose-dependent manner, as well as fibroblast proliferation and collagen maturity compared to the vehicle gels alone.[101,129]

EP from human ADSCs that were transfected with stable miR-21-5p mimics or circular RNA (circRNA) *mmu_circ_0000250* (another non-coding RNA) promoted faster reduction in open wound area in type 1 diabetic rats after topical application and subcutaneous injection, respectively.[102,150] The circRNA-carrying EP promoted SIRT1 expression by miR-128-3p inhibition in the diabetic wound bed, to alleviate apoptosis and expansion of endothelial cells.

Pretreating UC-MSCs with TNFα, IL-6, and VCAM1 altered the size distribution and miRNA constitution of the EVs secreted, with miR-21-5p being differentially regulated.[151] When the UC-MSC-EP with knockdown and overexpression of miR-21-5p were locally injected around a type 1 diabetic rat excisional wound model, wounds closed faster with higher levels of the specific miRNA.[151]

Among the less conventional sources for EVs in diabetic wound studies is platelet-rich plasma (PrP). A mixture of sEVs from PrP from healthy donors and sodium alginate when applied topically to type 1 diabetic excisional wounds on rats induced faster reduction in wound area compared to the vehicle alone.[84] The PrP sEV treatment promoted fibroblast proliferation and migration modulated by the Hippo/YAP signaling pathway, as well as collagen remodeling to produce a well-organized matrix, much like that of normal unwounded skin.

A study using amniotic epithelial cells as the source for sEVs found that the PI3K/AKT/mTOR pathway plays a crucial role in promoting rapid closure of excisional wounds in the $Lepr^{db/db}$ type 2 diabetic mouse model.[73]

In the same type 2 diabetic mouse model, a study successfully utilized sEVs from human circulating fibrocytes, which are $CD34^{+}$ bone marrow-derived progenitor cells, to promote dose-dependent faster wound closure.[117] The fibrocytes were conditioned with PDGF-BB and TGF-β1 before sEV isolation and found to package proangiogenic and anti-inflammatory miRNA in the EV cargo.

Local injections of EP or sEV isolated from another less conventional source, endothelial progenitor cells of umbilical cord blood, also promoted wound closure in excisional wounds in STZ-diabetic rats.[100,152] Reepithelialization, collagen maturity, vascular density, and scar dimensions were all reduced in a dose-dependent manner. The endothelial response in particular may be mediated through the ERK1/2 signaling pathway.

Surgical wounds. Surgical wounds are another potential growth direction for EV-based therapies. In a study looking at peninsular flap survival in mice, subcutaneous injections of human ADSC EP reduced necrotic area, and promoted angiogenesis and survival of the flap epidermis.[107] Bai *et al.* had similar findings in a rat model of abdominal superior inferior epigastric artery flap, where they injected human ADSC sEVs circumferentially.[56]

The sEVs were harvested from hypoxia-conditioned ADSCs, customized for this ischemia-reperfusion injury model, and promoted flap recovery and vascular expansion by reducing apoptosis of endothelial cells. In a mouse incisional hernia model, bone marrow EVs were applied with the surgical mesh that is standard of care and

resulted in less aggressive inflammation with more pro-repair immune phenotype cells present in the tissues.[111] Unlike most studies in skin disorders that adapt a xenogenic EV approach in rodent models, Blázquez *et al.* used mouse bone marrow as their source cells, but saw similar efficacy.[111]

Aging and photoaging. Aging skin often presents with cellular dysfunction, such as microvascular impairment, which are seen in other skin disorders like chronic wounds, along with senescence, oxidative stress, and loss of integrity. Chen *et al.* explored the use of sEVs from human embryonic stem cells (ESCs) in the treatment of pressure ulcers that are frequent in aging skin.[98] Pipetting the sEVs onto the ulcers on aged mouse skin reduced senescence and dysfunction of vascular endothelial cells. Particularly, they found that the ESC-sEVs transfer miR200a, which inhibits Keap1 and drives transcriptional activity of the factor Nrf2 to alleviate oxidative stress in the ulcers, and promote healing.

Other studies using umbilical cord EVs intradermally in ultraviolet-aged mouse skin and on human keratinocytes also reported Nrf2 activity upon treatment, which reduced oxidative stress, relieved apoptosis, reduced fibrosis and dermal thickening, and lowered levels of inflammatory cytokines.[153,154] EP preparations from ADSCs, although from a different source cell type, also show similar reduction of skin thickness in photo-aged rat skin models with just a single injected dose and promote maturation of collagen, to reduce the fibrotic phenotype.[155]

Burns. Burns or thermal injuries could benefit from EV-based therapies as new options, or as supplement to current clinical approaches that use autologous skin grafts.[156] Topical co-transplantation of human WJ-MSCs with skin-derived mlEVs onto third-degree burns on mouse dorsum promoted faster tissue repair, increased fibroblasts and collagen abundance, vascularization, and obvious epidermal differentiation, including a basal layer, spinous, granular and horny layers.[157]

Another study using UC-MSC sEVs, which carry Wnt4, demonstrated similar enhanced healing of a rat burn model.[158] UC-MSC EVs upregulated miR-181c and inhibited TLR4 signaling in the burn tissue within the first 24 h after intravenous administration, offering a molecular mechanism that can affect the excessive inflammation associated with burns, and thus alter the healing prognosis.[159]

Even mlEVs from a less traditional source, iPSCs cells, promoted faster healing in a second-degree burn model in mice, potentially through the abundant miR-16-5p content of the EVs.[106] The common thread among the reports on EV treatments of burns is promotion of keratinocyte migration and reepithelialization, along with neovascularization and fibrogenesis.

Other skin conditions. Considering the range of skin disorders, very few other conditions than the ones mentioned above have been studied for possible EV therapy. One limiting factor is the lack of preclinical models.

In an oxazolone-induced atopic dermatitis model in mice, subcutaneous local treatment with ADSCs-EVs showed a dose-dependent mitigation of inflammatory cytokines and reduction of mast cell-induced allergic inflammation, concomitant with expansion of lamellar bodies between the granular layer and stratum corneum to enhance the skin's barrier function, while reducing abnormal skin thickness.[160,161] Unlike the clinical standard topical dexamethasone treatment for atopic dermatitis, sEV application did not have adverse systemic effects such as weight loss in the mice.

Atopic dermatitis has multifactorial pathology and ADSC-sEVs have a network of impact to ameliorate the symptoms in mouse models. In a mouse eczema model, human UC-MSC sEVs gave rise to epidermal and dermal expansion, reduced peripheral blood mononuclear cell proliferation, and increased Tregs, with reduced scarring at the lesion sites.[162]

For both atopic dermatitis and eczema, the standards of care are based on topical steroids (which cannot have prolonged use) and sometimes systemic immune modulators. EVs offer a much-needed promising alternative.

A very recent report also describes the potential of local EV treatment for postsurgical colocutaneous fistulas in a rat model that fuses the cecum to the skin, joining two types of epithelium.[109] The authors administered xenogenic mouse EP percutaneously into the fistulas and observed significant reduction in fistula output and orifice diameter, as well as reduced fibrosis and increased neovascularization, compared to vehicle controls. Given that postsurgical colocutaneous fistulas are associated with high morbidity and often require resurgical intervention, this approach with local EV application is promising.

Finally, in a rabbit ear scar model, weekly treatment with human ADSC-EP prevented formation of a hypertrophic scar through suppression of myofibroblast aggregation and collagen deposition, in sharp contrast form the control.[163]

In vitro *models.* *In vitro* models are useful to investigate the events following EV uptake and horizontal transfer of information in recipient cell types in cutaneous wounds, namely keratinocytes, fibroblasts, and endothelial cells. Bakhtyar *et al.* elegantly demonstrate that exogenous EV uptake and interaction with recipient cells is a necessary physiological event for the beneficial effects in skin disorder treatment, as addition of lysed exosomes and components onto fibroblasts does not induce any effect.[71]

Shabbir *et al.* show that bone marrow EP induces dose-dependent proliferation and migration of fibroblasts from both human healthy and chronic wounds by activating intracellular Akt-, ERK1-/2-, and STAT3-mediated pathways in the recipient cells, and affecting transcription factors relevant to wound healing, such as HGF, IGF1, NGF, SDF1, and IL-6.[47] HUVECs responded to both BM and ADSC EVs by tube formation.[47,57]

One study inferred that transfer of miR-125a in EVs inhibits DLL4 to increase endothelial tip cell fate and enhances higher branching *in vitro.*[57] A few studies demonstrate the activation of canonical Wnt signaling in EV-receiving cells and suggest Wnt ligands may be on the exterior of EVs.[52,164] These reports raise questions of how long EV-induced effects are sustained, as prolonged Wnt activation is detrimental to wound healing.

Two studies have demonstrated the preferential packing of regulatory RNAs in EVs from MSCs.[97,142] A study using 3D culture of MSCs to harvest sEVs demonstrated that even with a different culture system, EVs affect fibroblast behavior, but without the dose-dependent trends seen in other studies cited above.[165] Hu *et al.* honed in on fibroblast to myofibroblast differentiation, a critical event in cutaneous wound healing.

They showed that EVs prevent the transition even in the presence of TGF-β1, which drives fibroblast to myofibroblast differentiation[68] evidenced by reduced expression of collagen I, collagen III, α-SMA, Smad2/3, and Smad2/3 phosphorylation. While the *in vitro* model-based studies cannot provide a conclusion about resolution of a skin disorder, they can reveal essential molecular and cellular mechanisms that potentially underlie the success of EV cutaneous therapies.

FUTURE DIRECTIONS

The relationships among choice of source cell, EV contents, and molecular/cellular impact in a wound site are critical to determine whether EVs from specific tissues and cells are better suited for preclinical efficacy in specific wounds. In this regard, multiomics bioinformatics approaches will be important allies for discovering and deciphering genomic, proteomic, and lipidomic profiles of exogenous EVs, as well as how the content of specific EV types affects signaling networks and ultimately wound resolution.

Most publications discuss promotion or enhancement of beneficial events, such as cell motility and division. At a mechanistic level, we are yet to learn about the balance of EV-induced promoting and inhibitory responses that enable the desired wound resolution outcome. As tools and technology evolve to characterize EVs at a higher resolution,[94,95] multiomics data could help determine whether specificity of EV isolates, based on size, content, and source cell, benefits certain wound healing phases.

Parts of the puzzle at large for exogenous EV-based skin wound treatment are the factors affecting uptake and whether differential uptake by recipient cells underlies the wound healing outcomes. In the same vein, modifications that can address precision of delivery and sufficient cellular uptake will be important. Pseudotyped EVs carrying wound site cell-specific membrane proteins could target EVs to wounds, concentrate and retain EVs, and enhance uptake.[166–168]

Optimization of EV treatments will increasingly incorporate biomaterials. Considering the interactions of these adjuncts with the wound environment and the impact on pharmacodynamics will be equally critical. We anticipate that larger animal models, such as swine that bear most similarity with human skin, will be the next step in investigating efficacy and safety of EV-based therapies.

SUMMARY

Current clinical approaches to cutaneous wounds and disorders involve management, but lack therapeutics with proven efficacy. Local and limited administration of exogenous EVs from human MSCs or other adherent self-renewing cells are emerging as a biologic therapeutic option with demonstrated safety and efficacy in preclinical studies conducted worldwide. *In vitro* self-renewal capacity is a requirement for the source cells, as EVs from committed dermal fibroblasts that are cultured and isolated in parallel to MSC EVs do not replicate the wound healing impact of the latter.[159]

Condition of MSC source tissues, cell culture, and isolation protocols bear on EV quality, efficacy, and the molecular mechanisms initiated once the EVs are administered locally in preclinical models. Current analysis focuses on molecular and pheno-

typic effects on critical cell types of skin wounds—keratinocytes, fibroblasts, and endothelial cells.

Data sharing and transparency on a worldwide scale are necessary to enable replication of methods and optimization of EV purity and specificity for different skin wounds and disorders. This review provides a comprehensive look at the procedural details, which are fundamental in dictating characteristics and impact of these exogenous, xenogenic EVs on preclinical mouse skin wound and disorder models. This is a rapidly evolving field, with research being carried out to advance technology for analysis of EVs and content, seek improved efficacy in cutaneous wound resolution, and enhance usability for eventual clinical applications.

TAKE-HOME MESSAGES

- This review provides readers with an overview of the state of cell-free EV-based therapies for skin wounds and disorders. The procedural steps leading up to application of EVs onto skin are critical for the desired biological response in the treated tissue. Advocates for EV therapy should consider the EV generation details when reading and assessing the literature.
- EVs, commonly from MSCs, can improve skin conditions from chronic wounds to eczema.
- Exogenous EV therapy is safe and effective in resolving wound closure and healing through local, noninvasive topical, and painless administration.
- Limited and controlled EV administration show robust efficacy in promoting cutaneous wound resolution preclinical models. The lack of toxicity and adverse reactions thus far from initial clinical trials, where EV administration is systemic,[22–24] are possible indicators of safety with local or topical routes for skin conditions in the future. EVs are a promising therapy for translation into the clinic.
- EV preparations for cutaneous wound and disorder therapy are accessible around the world due to relatively simple isolation techniques.

AUTHORS' CONTRIBUTIONS

B.S.S., M.K., A.V., S.S., and P.S.R. identified, read, and summarized publications discussed in the review. B.S.S. and P.S.R. composed, wrote, and reviewed the article. All authors have reviewed and approved the article.

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Abbreviations and Acronyms

3D = three dimensional
ADSC = adipose tissue-derived MSC
AF-MSC = amniotic fluid-derived multipotent stromal cell
BM-MSC = bone marrow multipotent stromal cell
circRNA = circular RNA
EP = extracellular particle
ESC = embryonic stem cell
EV = extracellular vesicle
FBS = fetal bovine serum
H_2O_2 = hydrogen peroxide
HFD = high fat Western diet
HUVECs = human umbilical vein endothelial cells
ISEV = International Society of Extracellular Vesicles
lncRNA = long noncoding RNA
LPS = lipopolysaccharide
miR/miRNA = microRNA
MISEV = Minimal Information for Study of Extracellular Vesicles
mIEV = medium/large extracellular vesicle
MSC = multipotent stromal cell
NTA = nanoparticle tracking analysis
PBS = phosphate-buffered saline
PDGF-BB = platelet-derived growth factor-BB
PrP = platelet-rich plasma
sEV = small extracellular vesicle
STZ = streptozotocin
TGF-β1 = transforming growth factor-β1
UC-MSC = umbilical cord multipotent stromal cell
WJ = Wharton's Jelly
WJ-MSCs = Wharton's Jelly-derived multipotent stromal cells