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Review

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Review

# Extracellular vesicles modulate key signalling pathways in refractory wound healing

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## Abstract

Chronic wounds are wounds that cannot heal properly due to various factors, such as underlying diseases, infection or reinjury, and improper healing of skin wounds and ulcers can cause a serious economic burden. Numerous studies have shown that extracellular vesicles (EVs) derived from stem/progenitor cells promote wound healing, reduce scar formation and have significant advantages over traditional treatment methods. EVs are membranous particles that carry various bioactive molecules from their cellular origins, such as cytokines, nucleic acids, enzymes, lipids and proteins. EVs can mediate cell-to-cell communication and modulate various physiological processes, such as cell differentiation, angiogenesis, immune response and tissue remodelling. In this review, we summarize the recent advances in EV-based wound healing, focusing on the signalling pathways that are regulated by EVs and their cargos. We discuss how EVs derived from different types of stem/progenitor cells can promote wound healing and reduce scar formation by modulating the Wnt/$\beta$-catenin, phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin, vascular endothelial growth factor, transforming growth factor $\beta$ and JAK–STAT pathways. Moreover, we also highlight the challenges and opportunities for engineering or modifying EVs to enhance their efficacy and specificity for wound healing.

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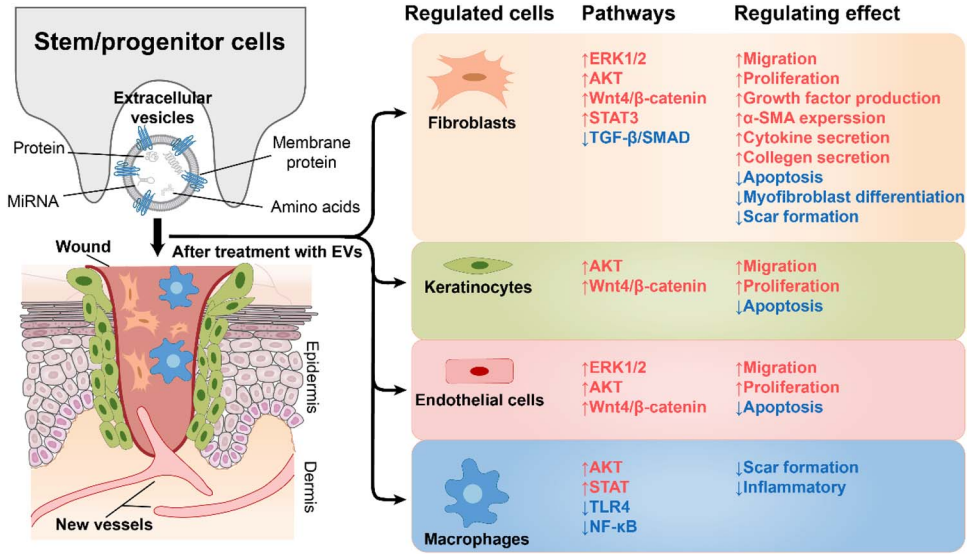
## Graphical Abstract




**Key words:** Extracellular vesicles, Refractory wound, Stem/progenitor cells, Scar formation, Engineered extracellular vesicles, Wound healing, Scar, Signalling pathway

## Highlights

- EVs derived from stem/progenitor cells can enhance wound healing and reduce scar formation by modulating various signalling pathways and inflammatory mediators.
- EVs modulate various signalling pathways, such as Wnt/$\beta$-catenin, PI3K/AKT/mTOR and VEGF, to regulate inflammation, angiogenesis, cell proliferation and migration, and extracellular matrix remodelling.
- Engineered EVs can optimize their therapeutic potential and target specific aspects of wound healing.

## Background

Wound healing refers to the healing process of skin and other tissues after being broken or damaged by external forces, and includes the complex combination of regeneration of various tissues, granulation tissue hyperplasia and scar tissue formation [1, 2]. Wound care is one of the fastest growing segments of the modern health care market, with the global wound care market valued at US $17.49 billion in 2021. The market is projected to grow from USD 18.51 Billion in 2022 to USD 28.23 billion in 2029, progressing at a compound annual growth rate (CAGR) of 6.2% during the forecast period. The types of wounds have also changed. Wounds can be divided into acute or chronic wounds [3, 4]. At present, refractory chronic wounds have surpassed acute wounds, and have become a widespread medical burden worldwide, bringing pain, psychological pressure and loss of quality of life to millions of people [5]. The common clinical chronic wounds mainly manifest as pressure ulcers, diabetic foot ulcers and venous ulcers of the lower limbs. The global epidemic of chronic underlying diseases, such as ageing, heart disease and intractable infections, contributes to the development of chronic wounds [6, 7]. Traditional treatment options for chronic wound healing are now divided into nonsurgical and surgical treatments. Nonsurgical treatments include physical therapy, negative pressure wound therapy, gene therapy, new surgical dressing and application of growth factors [8–13]. Refractory wounds have multiple aetiologies and pathologies, but the clinical symptoms are quite similar, making diagnosis and proper treatment difficult [14]. How to prevent scar formation caused by refractory wounds is also a tricky problem and new treatment options are needed [15].

Extracellular vesicles (EVs) can be used in the diagnosis and treatment of many wounds and diseases and are a promising new therapy [16]. EVs are 30 nm–10 $\mu$m membranous particles that can be derived from almost all cell types, and that transfer various bioactive molecules between cells, thereby mediating a variety of physiological processes, such as cell differentiation and proliferation, blood vessel formation, stress response and immune response [17]. EVs are natural, nanotransport particles with biocompatibility, circulatory stability, low toxicity and low immunogenicity, so they can be used as efficient carriers of molecular cargo and are ideal therapeutic candidates for regenerative medicine [18]. In addition, compared with synthetic transporters, EVs are more stable, simpler to produce and lower in cost. Several studies have demonstrated that stem cell therapy has a significant effect in the treatment of trauma [19]. Recently, it has been found that stem cells exert their effects mainly

through EVs, and EVs can bypass the potential tumourigenic risk of stem cells, adverse immune response and poor survival of stem cells in wounds [20]. In other words, EVs are more promising because they have better therapeutic effects than stem cells and can avoid many disadvantages of cell therapy. EVs derived from stem/progenitor cells have shown great potential in the treatment of refractory wounds and have been investigated for skin beauty treatment, relief of chronic skin wounds caused by diabetes and bedsores [21, 22]. There are even teams investigating the efficacy of EVs for many diseases, such as epidermolysis bullosa, dry eye, cardiovascular diseases, cancer, neurodegenerative diseases and COVID-19, showing the powerful generalizability of the therapeutic effects of EVs [23].

An engineered EV is a type of EV that has been modified or enhanced to improve its therapeutic potential for wound healing. Engineered EVs can be derived from different cell sources and loaded with various molecules, including growth factors, cytokines or drugs, to target specific signalling pathways or processes in wound healing. The use of engineered EVs in wound treatment is an emerging and promising field that has the potential to overcome some of the limitations of natural EVs or other regenerative therapies. More research is needed to optimize the engineering methods, characterization techniques, standardization protocols, delivery systems and safety assessments of engineered EVs for clinical applications. In this review, we discuss how EVs derived from different sources can regulate the Wnt/$\beta$-catenin, phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-$\beta$ and JAK–STAT signalling pathways to promote wound healing and reduce scar formation in refractory wounds. We also highlight the recent advances in engineering and modifying EVs to control their biotherapeutic performance and enhance their application prospects.

## Review

### Wound healing processes and challenges

**Four phases of wound healing** (1) Haemostasis phase: within seconds of trauma, the blood and the fibrous proteins in the wound are quickly solidified into a clot, some of which dry to form scabs, and the clots and scabs play a role in the protection of the wound, preventing further blood loss and pathogen invasion [24]. Platelets secrete a variety of growth factors, such as fibroblast growth factor (FGF), platelet-derived growth factor and TGF, which recruit inflammatory cells to the wound, triggering the inflammatory phase of wound healing [25].

(2) Inflammatory phase: inflammation often occurs 24–36 h after injury, manifested as congestion, serous exudation and leukocyte swim out, resulting in local redness and swelling [26]. The main leukocytes involved are neutrophils, which change to macrophages after 3 days [27]. Neutrophils infiltrate the wound and secrete proteases and antimicrobials (such as reactive oxygen species) to remove damaged extracellular matrix (ECM) and pathogens [28, 29]. Released products such as nuclear factor-$\kappa$B (NF-$\kappa$B) and Toll-like receptor (TLR), which are required for cytokine transcription and pathogen recognition, respectively, attract macrophages and lymphocytes to the wound and activate them [30, 31]. Macrophages can initiate or end inflammation through phenotypic transformation. In the early stage of inflammation, classically activated macrophages (M1-type) are recruited at the wound site, expressing a broad spectrum of proinflammatory cytokines, such as interleukin-1$\beta$ (IL-1$\beta$) [32]. In the later stage of inflammation, macrophages change to the M2 type, which mediates anti-inflammatory effects. By activating keratinocytes, fibroblasts and endothelial cells, M2-type macrophages promote tissue regeneration and initiate the proliferative phase of wound healing [33].

(3) Proliferation phase: this phase mainly depends on the activity of keratinocytes and fibroblasts to form new tissue and blood vessels in the wound area [34]. Keratinocytes are the main proliferating cells of the epidermis and can differentiate and replace ageing epidermal cells under physiological conditions [35]. The process of re-epithelialization of a wound begins with the proliferation of keratinocytes at the edge of the wound [36]. Fibroblasts also play a significant role, initiating extensive proliferation to secrete a variety of substances to form new ECM, including collagen type-III matrix, mucins, fibronectin and proteoglycans (such as hyaluronic acid) [37, 38]. The newly formed ECM promotes the formation of granulation tissue, and the damaged ECM is degraded by several enzymes, including matrix metalloenzymes (MMPs) and plasminogen activators [39–41]. Granulation tissue is composed mainly of fibroblasts, new blood vessels and type-III collagen, which will mature to form a scar [1]. Fibroblasts can also differentiate into $\alpha$-smooth muscle actin ($\alpha$-SMA)-rich myofibroblasts, which have a contractile function and pull the edges of the wound together [40]. Increased levels of growth factors such as VEGF, hepatocyte growth factor and FGF stimulate the intact blood vessels around the wound and degrade the partial basement membrane of blood vessels, thus making the vascular endothelial cells in this area proliferate and migrate to rebuild new blood vessels [42–45].

(4) Reconstruction phase: the delicate process of scar tissue remodelling, which involves the reconstruction of new blood vessels, cells and tissue, can take months or years to manifest itself, much longer than other stages of wound healing [46, 47]. Collagen decomposes, and type-III collagen, which constitutes immature ECM, is replaced by stronger mature type-I collagen, leading to ECM structure adjustment and wound-thickness reduction [48, 49]. Most new cells undergo apoptosis, leading to a decrease in dermal cells and blood vessel density [50]. Most of the newly formed capillaries recede, and a small part rebuilds to form a new network of blood vessels [51]. The connective tissue underneath the wound continues to contract, pulling the edges closer together [52].

## Chronic wound aetiology and pathology

Chronic wounds fail to achieve anatomical and functional integrity through a normal, orderly and timely process of repair [53]. Clinically, the term mostly refers to wounds that cannot heal for more than 1 month and have no tendency to heal [54]. Here, the limit of 1 month is not absolute [55]. However, due to the complex aetiology of chronic wounds, it is difficult to define them simply, so there is no unified definition of chronic trauma at present [56]. The correct sequence, timing and regulation of each of the four phases of wound healing are critical, and any adverse effects of disease or complications can lead to chronic ulceration where the wound does not heal properly and enters a persistent state of inflammation [57]. Chronic wounds usually encompass venous insufficiency ulcers, peripheral arterial ulcers, diabetic ulcers, pressure ulcers, infectious ulcers, neoplastic ulcers and refractory wounds caused by connective tissue diseases such as leprosy [58–60]. The aetiology of chronic wounds is complex and various mechanisms have been proposed in existing research. (1) At the cellular level, increased levels of proinflammatory factors secreted by neutrophils make matrix metalloproteinases hyperactive, promote ECM breakdown and impair cell migration [61, 62]. Dysfunction of the transformation of fibroblasts into myofibroblasts leads to fibrous disease, hypertrophic scars and keloid formation [63]. The formation of scars on the skin, especially on the face, neck and hands, will increase the patient's psychological stress [64]. (2) Underlying diseases, such as diabetes can lead to dysregulation of insulin levels, and excessive blood glucose can hinder macrophage polarization, resulting in macrophages maintaining a proinflammatory (M1-type) state and causing inflammation and cellular stress, which are difficult to terminate [65]. Moreover, due to the lack of polarization, macrophages cannot be transformed into the M2 type, resulting in stagnation of the healing process in the inflammatory phase and difficulty in entering the proliferative phase, making diabetic patients more prone to chronic skin injury [66]. Dehydration and malnutrition can also greatly affect cell proliferation and angiogenesis. Ageing and heart disease are common underlying conditions leading to the development of chronic wounds in an ageing society [67]. Loss of certain types of collagen, such as epidermolysis bullosa due to loss of collagen type VII, can lead to weak skin that is prone to blisters and nonhealing ulcers that must be remedied by artificial treatments [68]. (3) Reinjury or infection is a problem, and if there is persistent pathogen infection or wound reinjury at the wound site, this will hurt new cells and ECM in the process of proliferation, which easily leads to serious scar formation [69].

## EVs as therapeutic agents

### Definition, origin, composition, structure and function of EVs

EVs contain three main isoforms, i.e. exosomes, ectosomes and apoptotic bodies [70]. In fact, there is still research constantly refining the categories of EVs. Therefore, the EVs mentioned in many articles are generally understood as generalized EVs. To avoid confusion, EVs are used to refer to all vesicles secreted by cells in this review. EVs are formed by fusion of multivesicular bodies (MVBs) with plasma membranes. During endocytosis, the cell membrane buds inwards to form endosomes, which then bud inwards to form MVBs [71]. MVBs are assembled by either an endosomal sorting complex required for transport (ESCRT)-independent transport pathway or an ESCRT-dependent endosomal sorting complex [72]. In the ESCRT-independent pathway, sphingomyelin (present in endosomal membranes within lipid rafts) is converted into ceramide by sphingomyelinase [73]. Ceramide binds to form microdomains that drive intraluminal vesicles (ILVs) to form MVBs [74]. The ESCRT-dependent pathway requires ESCRT-protein complexes (specifically ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III) and related proteins [such as Vps4, Vta1 and programmed cell death 6 interacting protein (ALIX)] that contribute to ILV production [66, 75]. ESCRTs works together to twist the endosomal membrane and selectively transport the components that form ILVs. ESCRT-III component proteins are aggregated by ESCRT-II or ALIX, and the polymerization of these components results in neck contraction and cleavage to form ILVs [76]. Vsp4, an ATPase, dismantles ESCRTs and allows them to be recycled [76].

Farnesyltransferase is required for Ras protein activation, and Ras proteins and their downstream effectors, including Raf and the extracellular signal-regulating kinases RhoA and ADP-ribosylation factors 1 and 6 (ARF1 and ARF6), increase myosin contractility by phosphorylating myosin light chains, thereby encouraging MVB release [66, 77, 78]. Some of the formed MVBs bind to lysosomes and are degraded by the contained hydrolases [79]. Other MVBs do not fuse with lysosomes and are transported to the cell membrane with the help of cytoskeletal actin and microtubules [79]. This process is regulated by several proteins, such as the Rab protein, and cholesterol can also affect this process [80]. The contents of EVs are quite abundant, including various proteins, enzymes, transcription factors, lipids, ECM proteins, receptors, metabolites, nucleic acids and especially a wide variety of microRNAs (miRNAs) [81]. These contained bioactive molecules enable them to perform functions ranging from targeting/adhesion to metabolism to inflammation regulation. Some of these proteins are specifically enriched in EVs and can be used as markers for EV identification or isolation, such as ALIX, tetramer CD9, CD63, CD81, CD82 and tumor susceptibility gene 101 [82] (Figure 1). Exocarta (http://exocarta.org/index.html); The Urinary EV Protein Database (http://dir.nhlbi.nih.gov/papers/lkem/exosome/); and Vesiclepedia (http://www.microvesicles.org/) [83].

## Mechanisms of EV cargo selection

Research has shown that the type and content of EVs are highly dependent on specific conditions [84]. Stressors, drug treatments or the type of stem cells from which they are derived can significantly alter EV yield, size and composition, which are dependent on EV cargo sorting

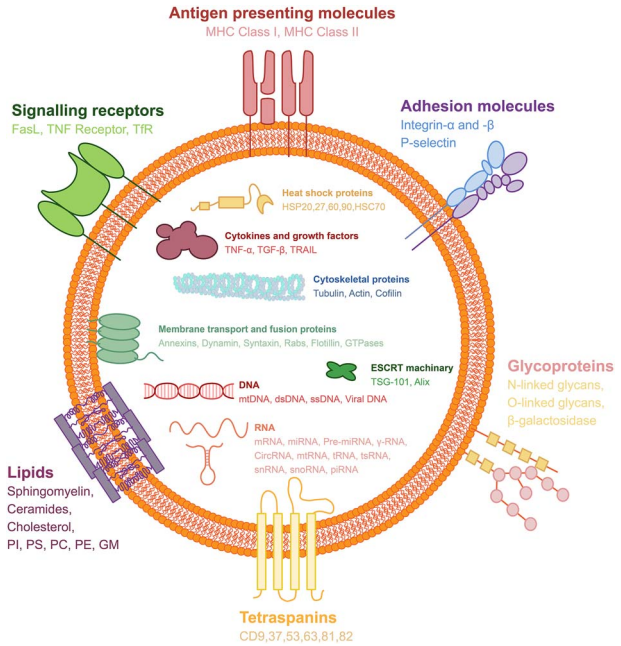



**Figure 1.** Schematic diagram of cellular EVs, their surface molecules and their contents. The surface of EVs is decorated with different types of molecules that play important roles in their biogenesis, trafficking and function. These molecules include: (1) antigen-presenting molecules, such as major histocompatibility complex (MHC) class I and II, which are involved in immune recognition and modulation; (2) adhesion molecules, such as integrin-$\alpha$ and $\beta$, and P-selectin, which mediate EV binding and uptake by target cells; (3) signalling receptors, such as Fas ligand (FasL), tumor necrosis factor (TNF) receptor and transferrin receptor (TfR), which trigger intracellular signalling pathways upon EV interaction; (4) glycoproteins, such as N-linked glycans, O-linked glycans and beta-galactosidase, which modulate EV stability, solubility and bioactivity; (5) lipids, such as sphingomyelin, ceramides, cholesterol, phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and ganglioside GM1, which affect EV membrane fluidity, curvature and charge; and (6) tetraspanins, such as CD9, CD37, CD53, CD63, CD81 and CD82, which are the most abundant and characteristic proteins on EV surfaces and participate in EV formation, sorting and targeting. The contents of EVs are enriched with different types of molecules that reflect the origin and function of the EV. These molecules include: (1) heat shock proteins, such as HSP20, HSP27, HSP60, HSP90 and HSC70, which are involved in protein folding, stability and protection from stress; (2) cytokines and growth factors, such as tumor necrosis factor-alpha (TNF-$\alpha$), transforming growth factor-beta (TGF-$\beta$) and TNF-related apoptosis-inducing ligand (TRAIL), which modulate immune responses, inflammation and cell survival; (3) cytoskeletal proteins, such as tubulin, actin and cofilin, which regulate EV shape, size and release; (4) membrane transport and fusion proteins, such as annexins, dynamin, syntaxin, Rabs, flotillin and GTPases, which facilitate EV budding, trafficking and fusion with target membranes; (5) endosomal sorting complex required for transport (ESCRT) machinery, such as tumor susceptibility gene 101 (TSG-101) and ALIX, which mediate EV formation and sorting of cargo molecules; (6) DNA, such as mitochondrial DNA (mtDNA), double-stranded DNA (dsDNA), single-stranded DNA (ssDNA) and viral DNA, which can transfer genetic information and induce immune responses; and (7) RNA, such as messenger RNA (mRNA), microRNA (miRNA), precursor miRNA (pre-miRNA), gamma-RNA ($\gamma$-RNA), circular RNA (circRNA), mitochondrial RNA (mtRNA), transfer RNA (tRNA), transfer strand RNA (tsRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and piwi-interacting RNA (piRNA), which can regulate gene expression and cellular functions in target cells. *EVs* extracellular vesicles

mechanisms, including ESCRT, tetraspanins and lipid-dependent mechanisms [80, 83]. For instance, mesenchymal stem cells (MSCs) cultured under serum-free priming conditions or hypoxia (1% $O_2$) generate EVs rich in specific metabolites such as cholesterol, arginine, aspartic acid, adenosine, palmitate and isoleucine [85]. These molecules have anti-inflammatory activity, promote M2-macrophage polarization and regulate lymphocyte function [86]. The MSC-EVs obtained under these conditions can further reduce the formation of scars compared with those obtained under general conditions. As another example, treatment of MSCs with the stressor lipopolysaccharide induced the stem cells to be in an inflammatory state [87]. The resulting MSC-EVs after this treatment are found to contain higher levels of anti-inflammatory signalling factors such as IL-10, TGF-$\beta$ and cyclooxygenase-2 (COX-2) (important in the bioproduction of prostaglandin E2) and are shown to be more effective in the treatment of chronic trauma [87]. An attractive research project is achieving the artificial regulation of EV generation and content [88]. The molecular composition of EVs is derived from cells, but differs from cells in that EVs contain a variety of specific proteins, lipids and RNAs and do not contain some of those present in the cytoplasm [89, 90]. This suggests that specific sorting mechanisms exist to control the entry of specific molecules into EVs. Research has shown that the sorting of specific proteins and RNA in outer secreted bodies is controlled in a variety of ways, although most ways are not fully understood. Through existing sorting methods, we can manipulate the contents of EVs according to different purposes to achieve a better therapeutic effect with fewer side effects.

## EV-mediated modulation of wound healing

Commonly used EVs derived from various stem cells and their rich inclusions can be involved in different phases of wound healing. The therapeutic effect of EVs is mainly achieved by surface proteins and contents, including a variety of cytokines and miRNAs, and they play a key role in different phases of the wound healing process by regulating multiple signalling pathways and inflammatory mediators [23, 91]. The proliferation and migration of endothelial cells, angiogenesis and maturation, and the development of hair follicles and sebaceous glands are observed after EVs are injected into the wound [92–94]. EVs can also enhance the proliferation and migration of fibroblasts in a dose-dependent manner and increase collagen deposition [21]. Importantly, in general, the stronger the collagen synthesis ability of fibroblasts at the wound site, the more likely scar formation is to occur, which affects the skin appearance and is detrimental to the normal function of the skin, increasing the psychological burden of patients [95]. However, EVs can promote the proliferation and secretion of fibroblasts at the beginning of wound healing, inhibit the deposition of collagen at the end of healing and mediate the structural remodelling of newly formed tissue, achieving the ideal therapeutic effect of both promoting wound healing and inhibiting scar formation [96, 97]. Therefore, EV therapy has significant advantages and potential over traditional methods.

The sequence of wound healing steps is strict and explicit, and numerous signalling pathways are involved. Different pathways focus on different functions. For instance, Wnt/$\beta$-catenin has a variety of pathways to regulate collagen deposition; the PI3K/AKT/mTOR pathway is more inclined to activate endothelial cell, keratinocyte and fibroblast functions; the VEGF pathway focuses on promoting angiogenesis; and the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and JAK–STAT pathways focus on inhibiting excessive inflammatory responses in wounds [98–102]. The TGF-$\beta$ pathway is mainly activated in the second phase of wound healing and has a strong ability to promote inflammation and collagen deposition [103]. In addition, detection of the degree of activation of different signalling pathways can be helpful in refining the classification of wounds and selecting a more ideal treatment plan. For instance, kinetic analysis of AKT, STAT-3, ERK1/2, Wnt, and $\beta$-catenin in cells at the site of injury can be performed to determine more appropriate targeted treatment.

**Modulation of Wnt/$\beta$-catenin pathway: a dual regulator of wound healing and scar formation** The Wnt/$\beta$-catenin signalling pathway is a highly conserved pathway that regulates various aspects of development, stem cell maintenance and wound healing [104]. The activation of this pathway involves the binding of Wnt ligands to their receptors, which inhibits the degradation of $\beta$-catenin and allows it to translocate to the nucleus and activate Wnt-responsive genes [104]. EVs, which are membrane-bound particles released by various cell types, can modulate the Wnt/$\beta$-catenin pathway in wound healing by delivering Wnt ligands or other factors that affect $\beta$-catenin stability or activity [105]. EVs can act as both positive and negative regulators of the Wnt/$\beta$-catenin pathway depending on the stage of wound healing and the source of EVs [106, 107]. In this section, we will review how EVs derived from different stem/progenitor cells can influence the Wnt/$\beta$-catenin pathway in wound healing and discuss the potential applications and challenges of EV-based therapy for wound healing (Figure 2).

In the early stage of wound healing, EVs derived from human umbilical cord mesenchymal stem cells (HUCMSC-EVs) or adipose mesenchymal stem cells can promote re-epithelialization, angiogenesis and tissue regeneration by delivering Wnt4 and activating the Wnt/$\beta$-catenin pathway in endothelial cells, keratinocytes and fibroblasts [108–110]. These EVs can increase the expression of genes related to cell proliferation, migration and differentiation, such as N-cadherin, collagen I, CK19 and proliferating cell nuclear antigen (PCNA), while decreasing the expression of E-cadherin, which is associated with cell–cell adhesion and epithelial integrity [111, 112]. These effects can be reversed by treatment with a $\beta$-catenin inhibitor (ICG001), indicating that the therapeutic effect of EVs is mediated by the Wnt/$\beta$-catenin pathway [113]. *In vivo* experiments also demonstrated that adipose mesenchymal stem cell-derived EVs can home to the site of skin incision and significantly promote skin wound healing in mice [114].

In contrast, in the late stage of wound healing, HUCMSC-EVs or endothelial progenitor cell-derived EVs (EPC-EVs) inhibit excessive cell proliferation and collagen deposition by delivering 14–3-3$\zeta$ protein, which regulates the phosphorylation of YAP and its interaction with phosphorylated-LATS, resulting in the inhibition of the Wnt/$\beta$-catenin pathway in skin cells [107, 115, 116]. YAP is a transcriptional coactivator that interacts with TCF family transcription factors and enhances Wnt-responsive gene expression. The phosphorylation of YAP by p-LATS leads to its cytoplasmic retention and degradation, thus inhibiting the Wnt/$\beta$-catenin pathway. By regulating YAP or other factors, EVs can fine-tune the activity of $\beta$-catenin to balance wound healing and scar formation [117].

EVs have emerged as a promising tool for wound healing therapy because they can deliver various bioactive molecules to modulate key signalling pathways involved in wound healing. However, there remain challenges and limitations that need to be addressed before EV-based therapy can be translated into clinical practice. Some of these challenges include: (1) standardization of EV isolation and characterization methods; (2) optimization of EV dosage and administration routes; (3) evaluation of EV safety and immunogenicity; (4) identification of specific biomarkers for EV tracking and monitoring; and (5) engineering of EVs to enhance their targeting and loading capacity. Further research is needed to overcome these challenges and explore the full potential of EVs for wound healing [106].

**Modulation of PI3K/AKT/mTOR pathway: a key driver of cell proliferation, migration and angiogenesis** The PI3K/AKT/mTOR signalling pathway is a key pathway that regulates cell growth, survival, migration and angiogenesis, which are essential for wound healing (Figure 3). This pathway can be activated by various cytokines and miRNAs that are delivered by EVs derived from different stem/progenitor cells [118]. The activation of this pathway can: stimulate the expression of growth factors such as VEGF, FGF and epidermal growth factor (EGF), which promote angiogenesis and tissue regeneration; enhance the proliferation and migration of endothelial cells, keratinocytes and fibroblasts, which are involved in re-epithelialization and granulation tissue formation [119]; and inhibit excessive inflammation by downregulating proinflammatory factors such as tumor necrosis factor alpha (TNF-$\alpha$), IL-1$\beta$ and IL-6 and upregulating anti-inflammatory factors such as IL-10 and signal transducer and activator of transcription 3 (STAT3) [120–123].

(1) Synovial mesenchymal stem cell EVs can deliver miR-222, miR-21 and miR-let7a, which can activate the Wnt/$\beta$-catenin and AKT/ERK/STAT3 pathways to promote angiogenesis [124]. (2) EPC-EVs can deliver VEGF-A and VEGF receptor 2 (VEGFR-2), which can activate the VEGF/PI3K/AKT/endothelial nitric oxide synthase (eNOS)

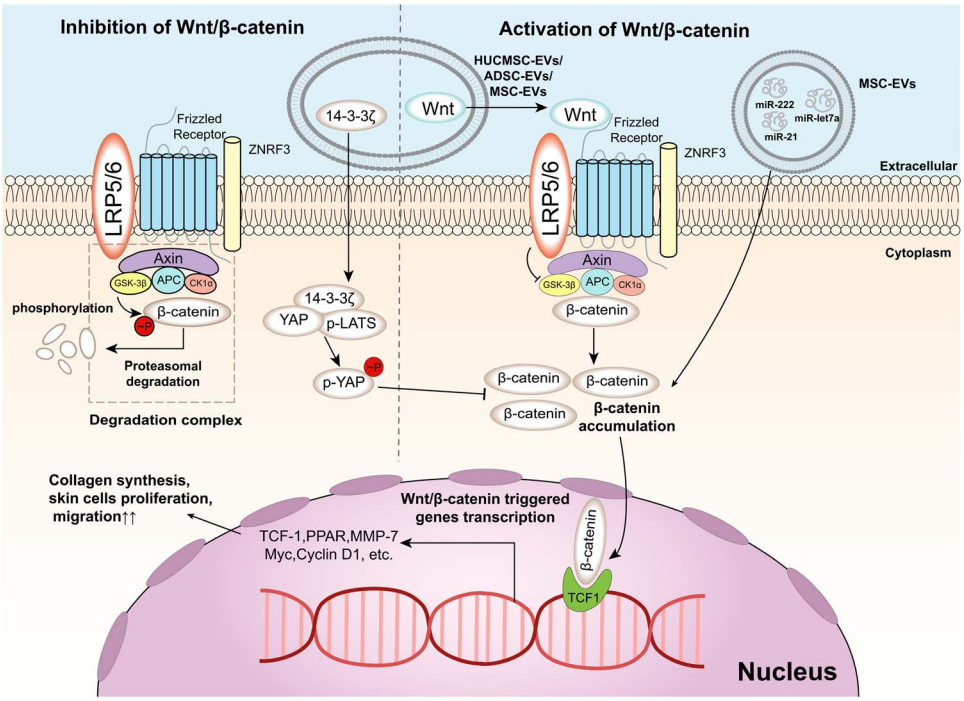



**Figure 2.** Wnt/$\beta$-catenin signalling pathway and its regulation by EVs in wound healing. The Wnt/$\beta$-catenin signalling pathway is a key pathway that regulates cell proliferation, differentiation and survival. The pathway is activated by the binding of Wnt ligands to Frizzled receptors and LRP5/6 co-receptors on the cell membrane, which inhibits the degradation of $\beta$-catenin by the destruction complex composed of Axin, APC and GSK3$\beta$. The stabilized $\beta$-catenin translocates to the nucleus and interacts with TCF1 to activate the transcription of target genes, such as MMP-7, Myc and Cyclin D1. EVs are small membrane-bound vesicles that can modulate the Wnt/$\beta$-catenin signalling pathway by delivering various molecules to target cells. For example, EVs can carry Wnt ligands or 14–3-3$\zeta$ protein to activate or inhibit the pathway, respectively. EVs can also deliver microRNAs (miRNAs) such as miR-let7a, miR-21 and miR-222 to activate the pathway by targeting its different components. EVs can also affect the crosstalk between the Wnt/$\beta$-catenin signalling pathway and other pathways, such as the Hippo/YAP pathway. The Hippo/YAP pathway is a negative regulator of the Wnt/$\beta$-catenin signalling pathway by phosphorylating yes-associated protein (YAP) and preventing its interaction with $\beta$-catenin. EVs can deliver phosphorylated YAP (p-YAP) or phosphorylated large tumor suppressor kinaseLATS (p-LATS) to target cells and suppress the Wnt/$\beta$-catenin signalling pathway. The regulation of the Wnt/$\beta$-catenin signalling pathway by EVs is diverse and dynamic in wound healing. For example, in the early stage of wound healing, EVs contain a dominant Wnt effect, activating the Wnt/$\beta$-catenin signalling pathway to promote collagen synthesis, skin cell proliferation and migration. In the late stage of wound healing, 14–3-3$\zeta$ contained in EVs plays an important role in inhibiting the Wnt/$\beta$-catenin signalling pathway, preventing excessive secretion and deposition of collagen causing scar formation. *EVs* extracellular vesicles

pathway to promote angiogenesis [125]. (3) HUCMSC-EVs can deliver VEGF-A, IL-15 and EGF, which can activate the PI3K/AKT and mTOR pathways to regulate the expression of IGF-1 in DETCs that is beneficial to wound healing in diabetic mice [126]. (4) Human amniotic epithelial cell derived EVs can deliver miR-21-5p and miR-181a-5p, which can target phosphatase and tensin homolog (PTEN) and increase AKT phosphorylation in keratinocytes and fibroblasts [127]. (5) MSC-EVs can deliver miR-126-3p and miR-210-3p, which can target sprouty-related EVH1 domain-containing protein 1 (SPRED1) and protein tyrosine phosphatase 1B (PTP1B) and increase AKT phosphorylation in keratinocytes and fibroblasts [128–130].

These EVs can also increase YAP nuclear accumulation in keratinocytes and fibroblasts by delivering YAP mRNA or inhibiting LATS1/2 phosphorylation. YAP is a transcriptional coactivator that interacts with TEA domain transcription factor (TEAD) family transcription factors and enhances cell proliferation and migration gene expression [131, 132]. HUCMSC-EVs or MSC-EVs can also inhibit excessive inflammation by modulating the PI3K/AKT/mTOR pathway in macrophages or other immune cells by delivering various miRNAs and proteins that are involved in inflammation regulation [133]. For example, HUCMSC-EVs can deliver miR-181c, miR-1180, miR-183, miR-550b and miR-133a, which can induce M2 macrophage polarization and reduce proinflammatory cytokine production [134, 135]. MSC-EVs can deliver: miR-let7b and miR-181c, which can upregulate AKT and STAT3 phosphorylation and downregulate TLR4 expression in macrophages or other immune cells [136, 137]; heat shock protein (HSP) 90a protein, which can inhibit NF-kB activation and reduce proinflammatory cytokine production; and anti-inflammatory miRNAs such as miR-124a, miR-125b, miR-126 and miR-let7b, which can target various inflammatory mediators such as NF-kB, MAPK, TNF-$\alpha$, IL-1$\beta$ and IL-6 [138, 139].

**Modulation of VEGF pathway: a potent stimulator of angiogenesis and tissue regeneration** The VEGF signalling pathway is a key pathway that regulates angiogenesis, which is the formation of new blood vessels from pre-existing ones. Angiogenesis is essential for wound healing, as it provides

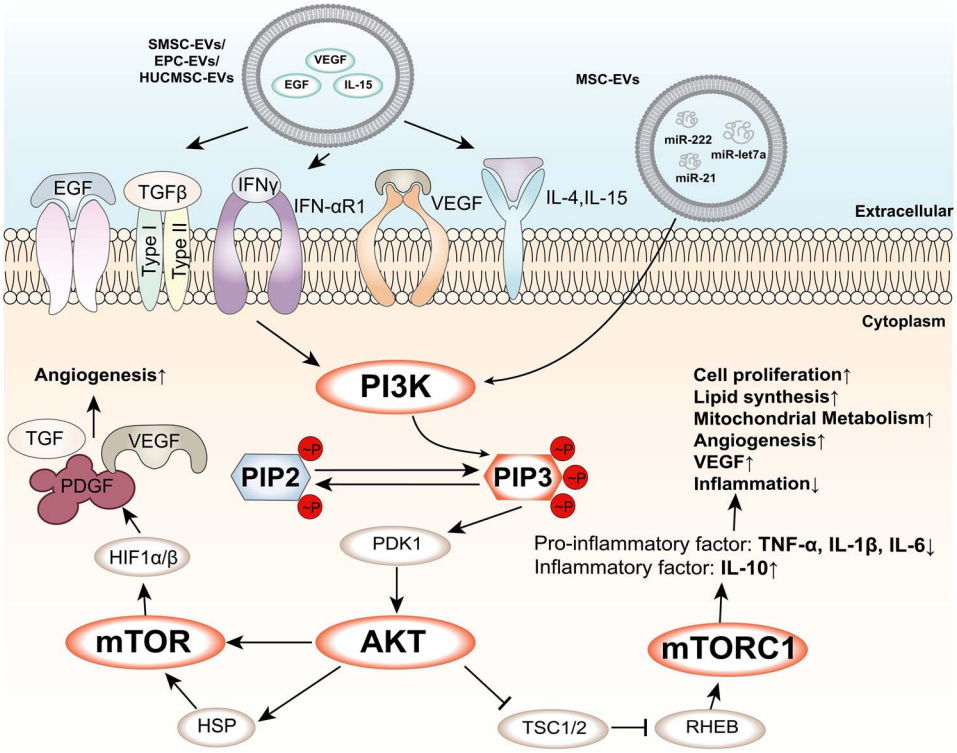



**Figure 3.** PI3K/AKT/mTOR signalling pathway and its regulation by EVs. The PI3K/AKT/mTOR signalling pathway is a central pathway that regulates cell growth, metabolism and survival. The pathway is activated by various growth factors and cytokines, such as epidermal growth factor (EGF), transforming growth factor-beta (TGF-$\beta$), interferon-gamma (IFN-$\gamma$), vascular endothelial growth factor (VEGF), interleukin-4 (IL-4) and IL-15, which bind to their respective receptors on the cell membrane and trigger the activation of phosphatidylinositol 3-kinase (PI3K). PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3), which recruits and activates protein kinase B (AKT) and phosphoinositide-dependent kinase 1 (PDK1). AKT phosphorylates and inhibits tuberous sclerosis complex 1 and 2 (TSC1/2), which releases the inhibition of Ras homologue enriched in brain (RHEB). RHEB activates mammalian target of rapamycin complex 1 (mTORC1), which regulates various cellular processes, such as protein synthesis, lipid synthesis, mitochondrial metabolism, angiogenesis and inflammatory response. mTORC1 also activates hypoxia-inducible factor 1-alpha (HIF1$\alpha$), which forms a heterodimer with HIF1$\beta$ and induces the expression of target genes, such as TGF-$\beta$, platelet-derived growth factor (PDGF), and VEGF. EVs are small membrane-bound vesicles that can modulate the PI3K/AKT/mTOR signalling pathway by delivering various molecules to target cells. For example, EVs can carry EGF, IL-15 or VEGF to activate the pathway, or microRNAs (miRNAs) such as miR-let7a, miR-21 and miR-222 to activate the pathway by targeting its different components. EVs can also affect the crosstalk between the PI3K/AKT/mTOR signalling pathway and other pathways, such as the Wnt/$\beta$-catenin pathway or the NF-$\kappa$B pathway. *EVs* extracellular vesicles

oxygen and nutrients to the wound site and facilitates the removal of waste products and inflammatory cells [140]. The VEGF signalling pathway is activated by the binding of VEGF ligands to VEGFRs on the surface of endothelial cells [141]. This binding triggers a cascade of downstream signalling pathways that mediate endothelial cell proliferation, migration, survival and permeability, as well as the expression of genes that promote angiogenesis [140]. The VEGF signalling pathway interacts with other pathways involved in wound healing, such as the MAPK and PI3K-AKT pathways, which are located downstream of VEGF signalling and modulate its effects [142]. EVs derived from different sources can modulate the VEGF signalling pathway in wound healing by delivering various cytokines, miRNAs and proteins that are involved in angiogenesis [143] (Figure 4). For instance, EPC-EVs can deliver VEGF-A and VEGFR-2, which activate the VEGF/PI3K/AKT/eNOS pathway and enhance the healing of soft tissue wounds by stimulating angiogenesis and collagen synthesis in fibroblasts and keratinocytes [125, 144, 145]. HUCMSC-EVs can deliver VEGF-A, IL-15 and EGF, which can activate the PI3K/AKT and mTOR pathways and regulate the expression of IGF-1 in DETCs that is beneficial to wound healing in diabetic mice [108, 146]. MSC-EVs can deliver miR-126-3p and miR-210-3p, which can target SPRED1 and PTP1B and increase AKT phosphorylation in keratinocytes and fibroblasts, thereby enhancing their proliferation and migration [147].

**Modulation of TGF-$\beta$ pathway: a major promoter of inflammation and collagen deposition** The TGF-$\beta$ signalling pathway is a complex and multifunctional pathway that regulates various cellular processes, such as proliferation, differentiation, migration, apoptosis and ECM synthesis [148]. Among the TGF-$\beta$ family, TGF-$\beta$1 plays a dominant role in skin wound healing and mainly mediates pathological healing, such as chronic inflammation and excessive scarring [149]. The TGF-$\beta$ signalling pathway can be activated by various stimuli, including cytokines, growth factors and

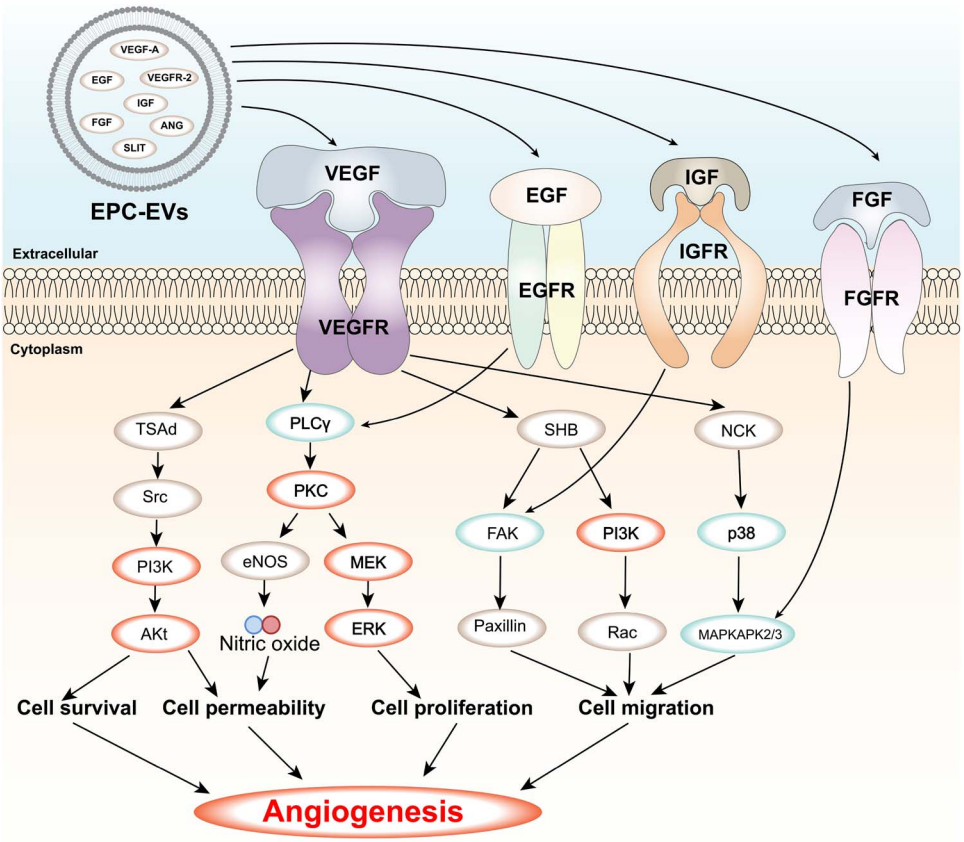



**Figure 4.** VEGF signalling pathway and its regulation by EVs contents. VEGF is a key factor that stimulates the formation of new blood vessels from existing ones or from the embryonic circulatory system. VEGF binds to its receptors (VEGFRs) on the surface of endothelial cells and activates various downstream pathways, such as Ras/MAPK, FAK/paxillin, PI3K/AKT and PLC$\gamma$/PKC, that regulate cell proliferation, migration, survival, permeability and angiogenesis. EVs are small membrane vesicles that are released by various cell types and contain bioactive molecules, such as proteins and RNAs. EVs can modulate the VEGF signalling pathway by delivering their contents to target cells or by interacting with VEGFRs on the cell surface. Some of the EVs contents that have been shown to affect the VEGF signalling pathway are SLIT, IGF, ANG, EGF, FGF, VEGFR-2 and VEGF-A. The figure shows the main components and effects of the VEGF signalling pathway and how EVs regulate this pathway through their contents. *FGF* Fibroblast growth factor, *FGFR* FGF receptor, *IGF* insulin-like growth factor, *IGFR* IGF receptor, *EGF* epidermal growth factor, *EGFR* EGF receptor, *MAPKAPK2/3* mitogen-activated protein kinase-activated protein kinase 2/3, *MEK* MAPK/ERK kinase, *ERK* extracellular signal-regulated kinase, *Ra*, Ras-related C3 botulinum toxin substrate, *PI3K* phosphatidylinositol 3-kinase, *p38* p38 MAPK, *NCK* non-catalytic region of tyrosine kinase adaptor protein, *FAK* focal adhesion kinase, *SHB* Src homology 2 domain-containing adapter protein B, *eNOS* endothelial nitric oxide synthase, *PKC* protein kinase C, *PLC$\gamma$* phospholipase C gamma, *AKT* protein kinase B, *Src* proto-oncogene tyrosine-protein kinase Src, *TSAd* T cell-specific adapter protein, *VEGF* vascular endothelial growth factor, *VEGFR* VEGF receptor, *EVs* extracellular vesicles

mechanical stress, and can interact with other pathways involved in wound healing, such as the JAK–STAT, MAPK/ERK, PI3K/AKT/mTOR and Wnt/$\beta$-catenin pathways [150]. The function of the TGF-$\beta$ pathway is context-dependent and can be either beneficial or detrimental for wound healing depending on the stage, type and location of the wound [151]. EVs derived from different sources can modulate the TGF-$\beta$ signalling pathway in wound healing by delivering various cytokines, miRNAs and proteins that are involved in inflammation and ECM remodelling [152] (Figure 5). For instance, HUCMSC-EVs can deliver miR-21, miR-125b, miR-23a and miR-145, which inhibit the TGF-$\beta$/SMAD2 pathway and reduce the expression of collagen and $\alpha$-SMA in fibroblasts and myofibroblasts [153]. This action can prevent excessive scar formation and fibrosis in the late stage of wound healing. MSC-EVs can deliver proteins that can regulate MMP levels and the TGF-$\beta$ signalling pathway, such as tissue inhibitor of metalloproteinases (TIMP)1 and TIMP2 [154]. This sequence of events modulates the balance between ECM synthesis and degradation and regulates $\alpha$-SMA and collagen deposition in the wound site [155]. MSC-EV treatment can also aid in the contraction of newly formed scar tissue by altering the ratio of type III to type I collagen and MMP3 to TIMP1 [156, 157].

**Modulation of JAK–STAT pathway: a key mediator of cytokine responses and macrophage polarization.** The JAK–STAT signalling pathway is a key pathway that mediates cellular responses to various cytokines and growth factors, such as interleukins, interferons and colony-stimulating factors. The JAK–STAT pathway can regulate various aspects of wound healing, such as inflammation, angiogenesis, cell proliferation and migration, and ECM synthesis [158, 159]. The activation of the JAK–STAT pathway depends on the binding of cytokines or growth factors to their specific receptors, which leads to the phosphorylation of

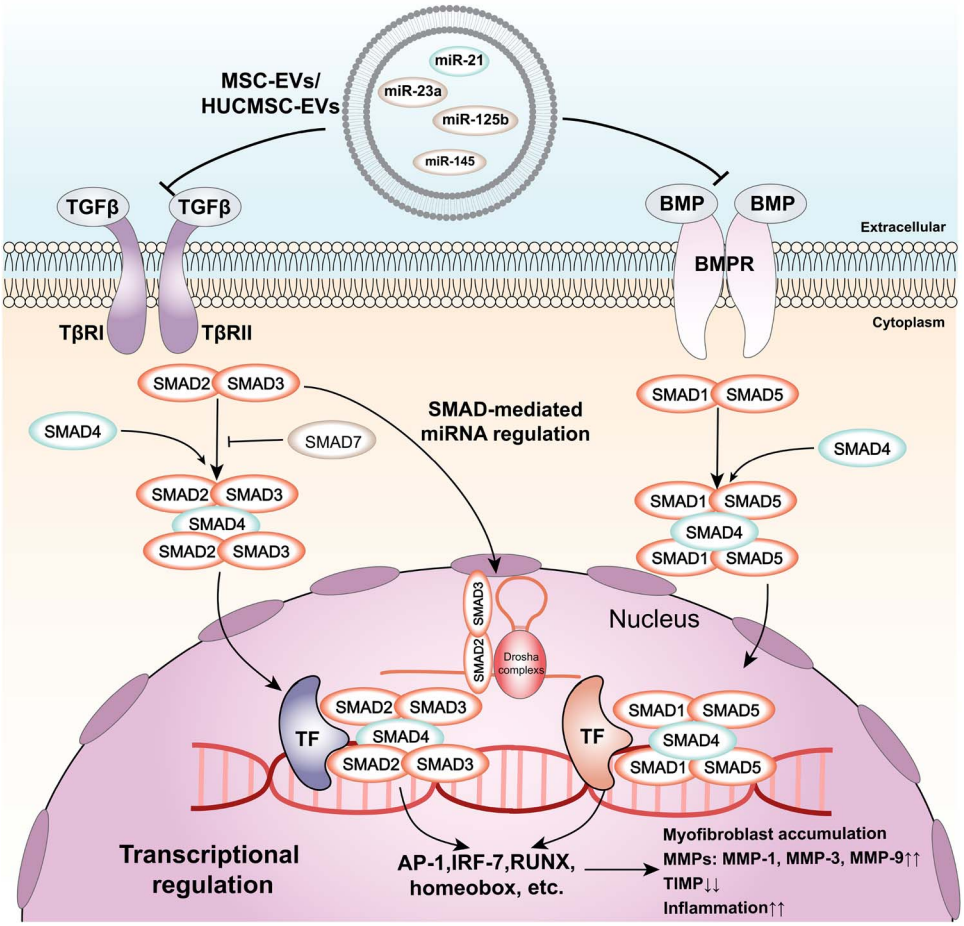



**Figure 5.** TGF-$\beta$ signalling pathway and its regulation by EVs. The figure shows the major components and regulatory effects of TGF-$\beta$ signalling and how cellular EVs regulate TGF-$\beta$ signalling through inclusions. TGF-$\beta$ and BMP bind to their respective receptors (T$\beta$RII and BMPR) and activate SMAD proteins (SMAD2/3 for TGF$\beta$ and SMAD1/5 for BMP). SMAD4 forms complexes with SMAD2/3 or SMAD1/5 and translocates to the nucleus, where it interacts with transcription factors (TF) to regulate gene expression. SMAD-mediated miRNA regulation involves Drosha complexes and SMAD2/3. The final effect of TGF$\beta$ signalling is myofibroblast accumulation, MMPs and TIMP expression, and an inflammatory condition. Exosome contents, such as miR-145, miR-125b, miR-23a and miR-21, can modulate TGF$\beta$ signalling by targeting different components of the pathway. *T$\beta$RII* TGF-$\beta$ receptor type II, *TGF-$\beta$* transforming growth factor beta, *BMPR* bone morphogenetic protein receptor, *BMP* bone morphogenetic protein, *TF* transcription factor, *AP-1* activator protein 1, *IRF-7* interferon regulatory factor 7, *RUNX* runt-related transcription factor, *MMPs* matrix metalloproteinases, *TIMP* tissue inhibitor of metalloproteinases, *miR* microRNA, *EVs* extracellular vesicles

JAKs and STATs. The phosphorylated STATs then dimerize and translocate to the nucleus, where they regulate the transcription of target genes [160, 161]. EVs derived from different sources can modulate the JAK–STAT signalling pathway in wound healing by delivering various cytokines, miRNAs and proteins that are involved in inflammation and angiogenesis [162] (Figure 6). For example, EVs derived from fibrocytes can deliver anti-inflammatory miRNAs (miR-124a, miR-125b, miR-126 and miR-let7b) and HSP90a, which can promote the phosphorylation of STAT3 and the polarization of macrophages to the M2 type [163, 164]. This can reduce the production of proinflammatory cytokines such as TNF-$\alpha$, IL-1$\beta$ and IL-6 and enhance the secretion of anti-inflammatory cytokines such as IL-10 and TGF-$\beta$ [165, 166]. EVs derived from MSCs can deliver miR-21, which can regulate the JAK–STAT pathway and promote the activation of Ras protein [167]. This can enhance the PI3K/AKT pathway and stimulate angiogenesis and collagen synthesis in the wound site.

**Optimal EV therapy for various wound types** Different types of wounds have different requirements and objectives for optimal healing. Therefore, the choice of EV sources and contents should be tailored to the specific characteristics and challenges of each wound type [168]. Some examples of how exosome sources and contents can be matched to wound types are as follows (Table S1, see online supplementary material).

(1) For wounds that suffer from poor blood supply and oxygenation, such as diabetic ulcers, pressure ulcers and venous ulcers, EPC-EVs or MSC-EVs might be beneficial [169]. These EVs can contain VEGF-A and VEGFR-2, which are key molecules for stimulating the formation and maturation of new blood vessels in the wound area [170]. By enhancing angiogenesis, these EVs can improve the delivery of

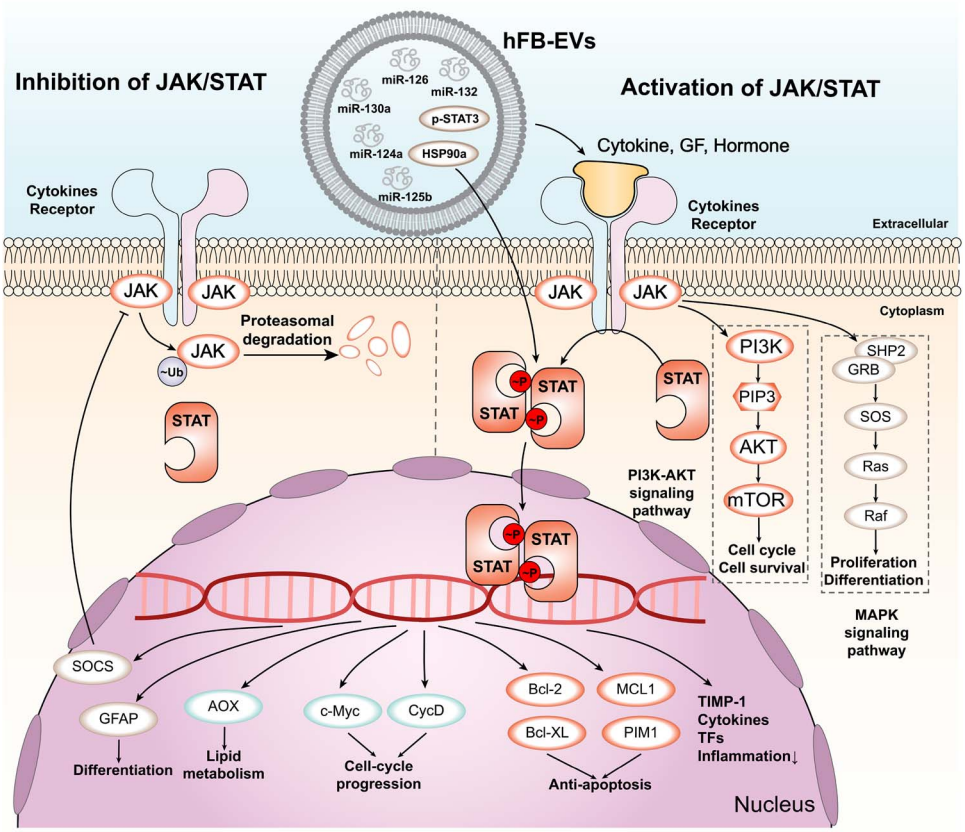



**Figure 6.** JAK/STAT signalling pathway and its regulation by EVs. The main components of the JAK/STAT signalling pathway are shown. Cytokines, growth factors (GF) and hormones bind to their receptors on the cell membrane, activating JAK kinases that phosphorylate (p) STAT proteins. p-STAT proteins form dimers and translocate to the nucleus, where they regulate the transcription of target genes. Ubiquitylated JAK proteins are degraded by proteasomes. The downstream target genes of p-STAT proteins include those involved in anti-apoptosis, cell-cycle progression, lipid metabolism and differentiation. Some examples of these genes are PIM1, MCL1, Bcl-XL, Bcl-2, CycD, c-Myc, AOX, GFAP, SOCS, TIMP-1, cytokines and transcription factors (TFs). The JAK/STAT signalling pathway interacts with other signalling pathways, such as the PI3K-AKT and MAPK pathways. The PI3K-AKT pathway regulates cell cycle and survival through AKT, PIP3, PI3K and mTOR. The MAPK pathway regulates proliferation and differentiation through Raf, Ras, SOS, SHP2, GRB2 and ERK. EVs are extracellular vesicles that contain various molecules, such as microRNAs (miRNAs), proteins and lipids. EVs can modulate the JAK/STAT signalling pathway by delivering or removing some of these molecules. For example, EVs can carry miR-124a, miR-130a, miR-125b, HSP90a, p-STAT3, miR-132 and miR-126 to target cells and affect their gene expression and signalling activity. *EVs* extracellular vesicles, *STAT3* signal transducer and activator of transcription 3, *TIMP-1* tissue inhibitor of metalloproteinases, *MAPK* mitogen-activated protein kinase, *ERK* extracellular signal-regulated kinase

oxygen and nutrients to the wound site and facilitate wound healing [168, 171].

(2) For wounds that are prone to excessive inflammation and scar formation, such as burns, surgical wounds and lacerations, HUCMSC-EVs or MSC-EVs might be helpful. These exosomes can contain anti-inflammatory miRNAs, such as miR-181c, or proteins, such as interleukin-10 (IL-10), which modulate the inflammatory response and reduce the production of proinflammatory cytokines, such as TNF-$\alpha$, IL-1$\beta$ and IL-6 [108, 172, 173]. By reducing inflammation, these exosomes can prevent tissue damage and infection and promote wound resolution. Moreover, these exosomes can also inhibit the expression of collagen and the activation of the TGF-$\beta$ pathway, which are involved in scar formation. By inhibiting scar formation, these exosomes preserve the function and appearance of the skin [174–176].

(3) For wounds that need to accelerate cell growth and movement, such as abrasions, contusions and punctures, human amniotic epithelial cell derived EVs or MSC-EVs might be effective. These exosomes can contain growth factors, such as EGF, or proteins that activate the PI3K/AKT/mTOR pathway, such as Wnt4 [171, 172, 177]. These molecules can stimulate the proliferation and migration of various cell types involved in wound healing, such as keratinocytes, fibroblasts and endothelial cells. By enhancing cell proliferation and migration, these exosomes promote re-epithelialization, granulation tissue formation and wound closure [174, 178, 179].

## Engineered EVs

**Enhancing the therapeutic potential of EVs through engineering** EVs have shown great potential for promoting wound healing and skin regeneration by enhancing angiogenesis, inflammation, keratinization, ECM remodelling and scar reduction. However, traditional EVs derived from natural sources have some limitations and shortcomings that can

affect their therapeutic effect, such as low yield, impurity, lack of targeting and low drug delivery rate. These limitations can reduce the efficiency, specificity and safety of exosome therapy for wound healing [168, 180, 181]. To overcome these limitations, engineered EVs have been developed by modifying or enhancing the properties of natural EVs [182]. Engineered EVs can be derived from different cell sources, such as stem cells, and loaded with various molecules, such as growth factors, cytokines or drugs, to target specific pathways or processes in wound healing [183]. They can also be modified on their surface or membrane to improve their stability, biocompatibility, homing ability and delivery efficiency. Engineered EVs have shown promising results in preclinical and clinical studies for wound healing and skin regeneration [184] (Table S2, see online supplementary material). For example, they can trigger wound healing and skin regeneration in ischaemic wounds by delivering TGF-$\beta$ and VEGF signals to the wound site [185]. They can also modulate the immune response and enhance the antimicrobial activity of macrophages in infected wounds by delivering interferon-gamma and nitric oxide [186, 187]. Engineered EVs are a potential solution to the limitations of traditional EVs for wound healing. They can offer more control over the biotherapeutic performance and delivery by manipulating their cargo or surface markers.

**Characteristics, production methods and applications of engineered EVs** Engineered EVs are EVs that are modified to carry specific therapeutic agents or molecules. They can be used to deliver drugs or RNA molecules to target cells and tissues and to attenuate the oncogenic activity of cancer cells [188]. Engineered EVs can be obtained by modification of natural membrane vesicles, such as by electroporation, transfection or genetic engineering [189] (Figure 7). Electroporation is a technique that uses electric pulses to create pores in the membrane of EVs, allowing the entry of drugs or RNA molecules [190]. Transfection is a technique that uses liposomes or other agents to introduce DNA or RNA molecules into EVs [191]. Genetic engineering is a technique that uses viral vectors or the CRISPR–Cas9 system to modify the genes of EV-producing cells, resulting in altered expression or secretion of EV proteins or RNAs [192]. Engineered EVs have been explored for various clinical applications, such as cancer therapy, immunotherapy, gene therapy, tissue regeneration and diagnosis. For example, Codiak Biosciences has developed engineered EVs that carry a tumour antigen and a stimulatory molecule to activate T cells against cancer cells [193]. Engineered EVs can deliver therapeutic RNAs to target cells and tissues affected by rare diseases resulting from genetic mutations [194]. These engineered EVs can also carry anti-inflammatory and pro-regenerative factors to modulate the inflammatory and regenerative responses in cardiac and muscular disorders [195]. Engineered EVs have several advantages over conventional EVs or synthetic nanoparticles, such as biocompatibility, low immunogenicity, high stability, natural targeting ability and easy modification. Therefore, engineered EVs have promising prospects for biomedical applications [196]. However, there are also some challenges and limitations that need to be addressed, such as scalability, standardization, quality control, safety evaluation, regulatory approval and cost-effectiveness [197]. More research and development are needed to overcome these hurdles and to translate engineered EVs from the laboratory to the clinic.

**Cost-effectiveness and feasibility of engineered EV therapy** Engineered EVs are modified or produced artificially to enhance their therapeutic or diagnostic potential. They can be classified into different types based on their origin (autologous or allogeneic), source (stem cell or nonstem cell), specificity (tissue-specific or nonspecific) and method (natural or artificial) [198, 199]. The current production and cost status of engineered EVs may vary depending on these factors, as well as the quality and quantity requirements for different applications [200]. Generally, engineered EVs may have higher yield and lower cost than traditional EVs if they are produced by artificial methods that do not require donor cells or complex isolation procedures, such as nanovesicles or exosome-mimetics [201]. However, they may have lower yields and higher costs than traditional EVs if they are produced by natural methods that require donor cells and multiple isolation and modification steps, such as hybrid EVs [199]. Moreover, engineered EVs may have lower yields and higher costs than traditional EVs if they are modified to introduce specific functions or targets, which can increase the complexity and difficulty of engineering [202]. Some methods that expand production and effect the cost of engineered EVs may include the following. (1) Developing standardized and scalable protocols for isolation, purification, characterization and modification of EVs from different sources and methods [203]. (2) Optimizing the culture conditions, engineering strategies and quality control measures for producing high-quality and high-purity EVs with desired properties and functions [204]. (3) Exploring novel biomaterials, nanotechnologies and bioengineering approaches for generating artificial or hybrid EVs with enhanced stability, specificity and efficacy [205]. (4) Reducing the production costs by using renewable or low-cost resources, such as plant-derived or microbial-derived EVs, or by recycling or reusing the waste materials from exosome production [206]. (5) Increasing the production efficiency by using automated or integrated systems, such as microfluidic devices or bioreactors, for continuous or large-scale production of EVs [204].

Engineered EVs have shown great promise in various biomedical applications, such as drug delivery, gene therapy, tissue regeneration, immunotherapy, diagnosis and biomarker discovery. They have several advantages over other nanocarriers or cell therapies, such as low toxicity, high biocompatibility, natural targeting ability, cargo loading capacity, editable surface structure and immune evasion [207, 208]. However, engineered EVs also face some challenges and limitations, such as lack of clear definition and classification, heterogeneity and variability of exosome

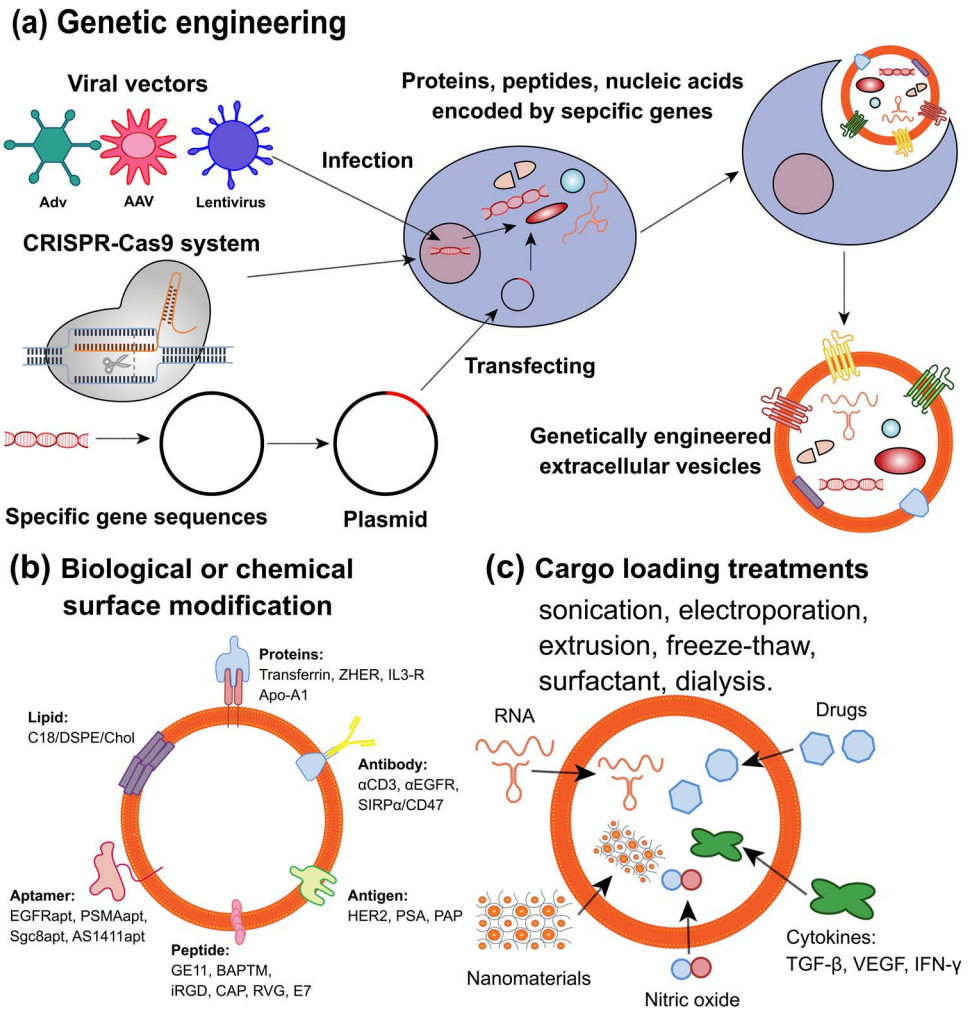



**Figure 7.** Different methods of EV engineering and their applications. EVs are small membrane-bound vesicles that can be engineered to modify their surface molecules, cargo contents or targeting specificity. Exosome engineering can be achieved by three main approaches: (**a**) genetic engineering, (**b**) surface modification, and (**c**) cargo loading. (a) Genetic engineering involves manipulating the genetic information of exosome-secreting cells using viral vectors, CRISPR-Cas9 system or plasmid transfection of specific gene fragments. This can result in the synthesis of specific proteins, peptides or nucleic acids that can be incorporated into EVs. For instance, EVs can be engineered to express fluorescent proteins, therapeutic genes or immunostimulatory molecules. (b) Surface modification involves attaching biological or chemical molecules to the surface of EVs using covalent or non-covalent bonds. This can alter the biophysical properties, stability or targeting ability of EVs. For example, EVs can be modified with proteins, antibodies, lipids, aptamers, peptides or antigens that can enhance their binding affinity, specificity or immunogenicity to target cells or tissues. (c) Cargo loading involves introducing exogenous molecules into the lumen of EVs using physical or chemical treatments. This can increase the functional diversity and efficacy of EVs. For instance, EVs can be loaded with RNA, nanomaterials, drugs, cytokines or gaseous molecules that can modulate gene expression, imaging contrast, drug delivery, inflammation or vasodilation in target cells or tissues, *EGF* epidermal growth factor, *EVs* extracellular vesicles, *FGF* fibroblast growth factor, *VEGF* vascular endothelial growth factor, *TGF-β* transforming growth factor-beta, *IFN-γ* interferon-gamma

characteristics and functions, potential immunogenicity and transmission of pathogens or malignancies, ethical and regulatory issues, and technical difficulties in engineering and manufacturing [197, 209]. Therefore, more research is needed to address these challenges and limitations, as well as to explore new applications and mechanisms of action of engineered EVs [197, 210]. Furthermore, more clinical trials are needed to evaluate the safety and efficacy of engineered EVs in human patients with various diseases. In summary, engineered EVs have a bright future in biomedicine as a novel and versatile delivery platform with multiple benefits [200].

## EV therapy: a promising strategy

In recent years, research has found that EVs have good regulatory potential and play an important role in a variety of biological processes [211]. EVs can not only be used for chronic wound healing but also show great potential in many therapeutic areas, including cosmetic dermatology, cardiovascular dysfunction, cancer and neurodegenerative diseases [21, 75, 212]. These complex vesicles have received much attention in the field of biomarker research and are now seen as an alternative strategy for stem cell-based regenerative therapy, which is similar in treatment mechanism to stem cell therapy but with better efficacy, lower side effects, and is

easier to artificially regulate and modify [213]. In addition to the direct use of EVs for wound treatment, EV-mediated drug delivery is also a good therapeutic approach with low toxicity, low immunogenicity and high engineering ability, and is expected to be used as cell-free therapy for a variety of diseases. Compared with the disadvantages of stem cell therapy, such as low cell survival rate, high immunogenicity and tumourigenicity, EVs as a cell-free therapy have significant advantages [214].

However, the current research on EVs for wound treatment focuses more on the research of EVs extracted from certain stem/progenitor cells applied to the wound site and whether they promote healing. How to achieve artificial modification of EVs, i.e. to obtain engineered EVs, is a more promising field. For instance, therapeutic drugs such as small molecules or nucleic acid drugs can be incorporated into EVs and then delivered to specific types of cells or tissues for targeted drug delivery. Targeted delivery increases the local concentration of the therapeutic agent and minimizes side effects [215]. EVs can be engineered through genetic or chemical methods to achieve targeted drug delivery [216]. There are many directions for engineering modification of EVs, which can target both the membrane components of EVs and the contents of EVs, including: (1) artificial incorporation of cargos [217]; (2) modification or regulation of surface lipids or proteins; and (3) changes in the content and composition of RNA and proteins [207]. The functions of EV membrane components are numerous and important. The surface proteins of EVs can mediate their targeting and adhesion to specific cells or tissues, which gives them an advantage over liposomes, which have lower targeting and adhesion abilities. [84]. The membrane component can avoid activating the immune clearance of the body, is well tolerated and will not induce toxicity [218]. The targeting of EVs to wound sites is also related to their membrane surface receptors. Engineered modification of the membrane components of EVs is designed to increase the local concentration of EVs at the lesion site, thereby reducing toxicity and side effects and maximizing therapeutic efficacy [139]. The engineering of the contents is more complex and promising, for instance, regulating the sorting mechanism of EV cargo, changing the type and volume of the contents, and achieving specific transport of therapeutic agents into EVs.

However, the current research is limited to characterization. Although surface engineering has been widely used for targeted drug delivery, how it affects EVs, their cell stability entry pathway and tissue distribution *in vivo* remain to be elucidated. Characterization of the sorting of EV contents is insufficient, which increases the difficulty for artificial regulation. More studies are needed to explore the sorting mechanism of the exosome cargo, i.e. how EVs selectively obtain intracellular components.

## Conclusions

In this review article, we have summarized and discussed how EVs derived from different sources can promote wound healing and skin regeneration by modulating various signalling pathways and their key proteins. We focused on the Wnt/$\beta$-catenin, PI3K/AKT/mTOR, VEGF, TGF-$\beta$ and JAK–STAT signalling pathways, which are involved in various aspects of wound healing, such as inflammation, angiogenesis, cell proliferation and migration, extracellular matrix remodelling and scar formation. We have also highlighted the recent advances in engineering and modifying EVs to optimize their biotherapeutic performance and delivery for wound healing applications. We have discussed the definition, source, production method and clinical application of engineered EVs, as well as the prospects and challenges of this emerging field. We believe that this review article provides a comprehensive and updated overview of the current state of knowledge on how EVs promote wound healing and skin regeneration and that it can inspire further research and innovation in this field.

## Abbreviations

ALIX: Programmed cell death 6 interacting protein; ECM: Extracellular matrix; EGF: Epidermal growth factor; eNOS: Endothelial nitric oxide synthase; EPCs: Endothelial progenitor cells; ERK: Extracellular signal-regulated kinase; EVs: extracellular vesicles; FGF fibroblast growth factor; HUCMSC-EVs: Human umbilical cord mesenchymal stem cells derived extracellular vesicles; IL-1$\beta$: Interleukin-1$\beta$; ILVs: Intraluminal vesicles; MAPK: Mitogen-activated protein kinase; miRNAs: MicroRNAs; MMPs matrix metalloenzymes; MSCs: Mesenchymal stem cells; mTOR: Mammalian target of rapamycin; MVBs: Multivesicular bodies; NF-$\kappa$B: Nuclear factor-$\kappa$B; PI3K: Phosphoinositide 3-kinase; SMA: $\alpha$-Smooth muscle actin; TGF: Transforming growth factor; TIMP-1: Tissue inhibitor of metalloproteinases; TNF-$\alpha$: Tumor necrosis factor alpha; TLR: Toll-like receptor; TSG101: Tumor susceptibility gene 101; VEGF: Vascular endothelial growth factor; VEGFRs: Vascular endothelial growth factor receptors. CAGR: compound annual growth rate; COX-2: cyclooxygenase-2; PCNA: proliferating cell nuclear antigen; YAP: yes-associated protein; LATS: large tumor suppressor kinase; STAT3: signal transducer and activator of transcription 3; SPRED1: sprouty-related EVH1 domain-containing protein 1; PTP1B: protein tyrosine phosphatase 1B; TEAD: TEA domain transcription factor; PTEN: phosphatase and tensin homolog.

## Supplementary data

Supplementary data is available at *Burns & Trauma* Journal online.

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## Authors' contributions

BY and Y-QS conceived and designed the main ideas of this article. BY performed the literature review, wrote the first draft of the

manuscript and revised it according to feedback. YML, YBH and NXZ provided feedback and revisions to improve the manuscript. Y-QS supervised the writing process and reviewed the manuscript several times to determine the final version. All authors read and approved the manuscript for publication.

## Conflict of interests

The authors declare that they have no competing interests or connections, direct or indirect, that might raise the question of bias in the work reported or the conclusions, implications or opinions stated in this review. The authors have no pertinent commercial or other sources of funding for the individual author(s) or for the associated department(s) or organization(s), personal relationships, or direct academic competition related to this review. The authors have followed the guidelines of the journal for writing a conflict of interest statement and have obtained the relevant information from all co-authors.

## Data availability

The data that support the findings of this review are available from the corresponding author upon reasonable request. The data include the web page context, the search query, the search results, and the conflict of interest statement. The data are stored in a secure database that complies with the journal's data sharing policy.

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