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Functionalized extracellular vesicles of mesenchymal stem cells for regenerative medicine

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Abstract

Stem cell-derived extracellular vesicles (EVs) have emerged as a safe and potent alternative to regenerative medicine in recent decades. Furthermore, the adjustment of EV functions has been recently enabled by certain stem cell preconditioning methods, providing an exceptional opportunity to enhance the therapeutic potential or confer additional functions of stem cell-derived EVs. In this review, we discuss the recent progress of functionalized EVs, based on stem cell preconditioning, for treating various organ systems, such as the musculoskeletal system, nervous system, integumentary system, cardiovascular system, renal system, and respiratory system. Additionally, we summarize the expected outcomes of preconditioning methods for stem cells and their EVs. With recent progress, we suggest considerations and future directions for developing personalized medicine based on preconditioned stem cell-derived EVs.

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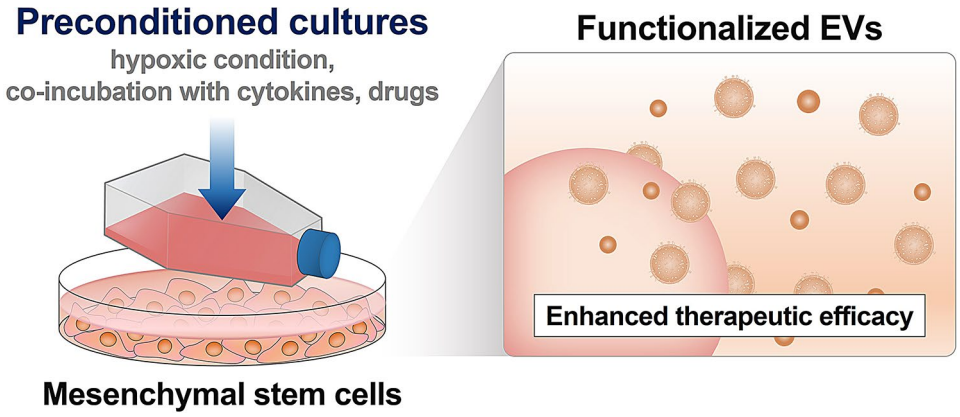
Graphical abstract



Keywords Extracellular vesicle, Exosome, Preconditioning, Nanomedicine, Regenerative medicine

Introduction

Regenerative therapy refers to treatments for dealing with not only the alleviation of tissue damage but the support of the innate regenerative capacity, thereby normalizing malfunctioning tissues [1]. Typically, the innate regenerative process is divided into three distinct phases. In the initial stage of the regenerative process, known as the inflammation phase, pro-inflammatory cytokines, including IL-1, IL-6, and tumor necrosis factor-α (TNF-α), are released to facilitate the clearance of external pathogens and cell debris [2]. Thereafter, the proliferation phase fills the damaged tissues with collagen and proliferated cells, by releasing anti-inflammatory cytokines, such as IL-4, IL-10, and tumor growth factor-β (TGF-β) [2, 3]. Finally, the remodeling phase resolves the scar by adjusting the collagen density of granulation tissues and replacing them with appropriate cells for the recovery of tissue function [4, 5]. Thus, various ways to modulate inflammatory reactions, cell proliferation, and collagen production can be applicable to facilitate tissue regeneration [5]. Furthermore, the tissue regeneration must be elaborately orchestrated step by step to recover the tissue function successfully. To date, various drugs have been discovered as regenerative medicine, such as antibiotics (e.g., tetracycline [6]) to prevent infection, and anti-inflammatory drugs (e.g., corticosteroids [7]) to reduce tissue damage. However, there is still no fundamental treatment that not only regulates inflammatory reactions but facilitates cell proliferation.

Of numerous trials, stem cell therapy has been considered the most successful alternative in regenerative medicine. Stem cells are self-renewing and undifferentiated cell types that differentiate into specialized cells within one particular lineage [8]. For instance, mesenchymal stem cells (MSCs) can differentiate into bone, cartilage, adipose tissue, and connective stromal cells. These functions are essential to the reconstruction of tissues as well as the recovery of functions by facilitating cell migration, angiogenesis, epithelialization, and granulation tissue formation [9]. For this reason, stem cell therapy has emerged as a promising alternative to conventional regenerative therapy and has been extensively studied in academic and clinical fields. However, stem cell therapy has several obstacles as a cell-based therapy in practical applications so far. Firstly, allogenic MSCs can provoke an immune response in the recipient due to allogeneic antigens derived from the donor [10]. Secondly, the proliferation rate and differentiation capacity of the cells are reduced by increasing the number of passages under the prolonged culture condition [11, 12]. These results cause decreases in cell viability and engraftment rate. Lastly, the functions of MSCs vary depending on biological factors such as the health status of the donor, tissue sources, and culture condition [12]. As this heterogeneity causes poor reproducibility of stem cell functions and the range of differentiable cell lineages, it has remained a significant challenge of MSCs for clinical application in regenerative medicine. Thus, recent studies have focused on discovering and exploiting bioactive components of stem cells, rather than injecting living stem cells directly.

Mounting evidence suggests that the paracrine signaling of stem cells heavily relies on extracellular vesicles (EVs), a group of membranous vesicles that are released from the cells [13]. In general, EVs are classified as exosomes, ectosomes, and apoptotic bodies, each of which distributes to maintain tissue homeostasis in different ways. Among EVs, exosomes are the most significant type of EVs in cell-to-cell communications. As shown in Fig. 1, exosomes are typically spherical and range in diameter from 30 to 150 nm [14]. Notably, these vesicles resemble

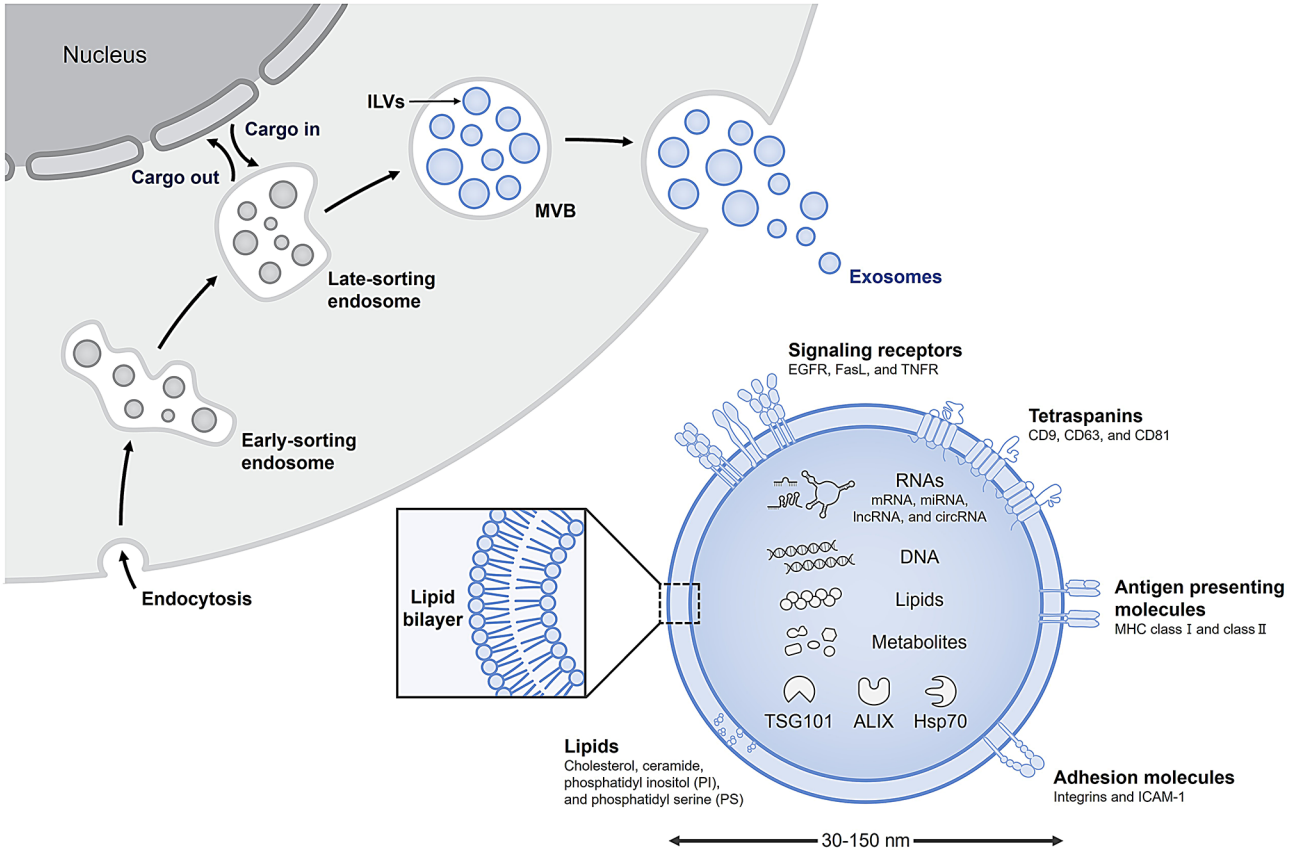


Fig. 1 Characteristics of exosomes: the intracellular machinery of biogenesis and secretory pathway, physicochemical characteristics, and contents

the biological characteristics of donor cells by carrying abundant RNA (mRNA, miRNA, lncRNA, and circRNA), DNA, lipids, and metabolites. Physiologically, the biogenesis of exosomes is featured to double the invagination of plasma membranes [15, 16]. The primary invagination of the plasma membranes is caused by endocytosis involving the internalization of plasma membrane proteins and extracellular milieu [17]. Gradually, bud formation into the cytoplasm leads to early-sorting endosomes (ESEs), forming the contents of the ESEs by exchanging cargoes through interaction with the endoplasmic reticulum and trans-Golgi network [18]. At this stage, the exosomes are enriched with a variety of RNAs and proteins expressed by the cell, thereby undergoing maturation into signaling mediators that can effectively regulate other cells, even in small numbers. After ESEs mature into late-sorting endosomes (LSEs), LSEs cause the multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) to form secondary invagination. Consequently, certain MVBs undergo fusion with lysosomes, resulting in the degradation of their internal contents [19]. On the other hand, other MVBs fuse with the luminal side of cells through cytoskeletal and microtubule networks, releasing ILVs into the extracellular space as exosomes reflecting the biological characteristics of donor cells. Therefore, exosomes can tone the recipient cell to be likely the donor cell and contribute to cell-to-cell communication in a broad range of biological reactions [20]. With these regard, EVs, isolated from various cells, have been extensively studied as potential regenerative medicine in recent years. In particular, stem cell-derived EVs have been demonstrated to influence tissue regeneration by upregulating the production of anti-inflammatory cytokines and suppressing the inflammatory phenotypes of immune cells [21–23]. Furthermore, stem cell-derived EVs have superior storage longevity, and lower immunogenicity compared to living stem cells as non-living materials, and they are barely influenced by low cell viability, tumorigenesis, and vascular embolism, featured in current stem cell therapies [24, 25]. Taken to these benefits, stem cell-derived EVs have emerged as a safer and more prominent alternative to stem cell therapy in the recent decade [26].

Moreover, one significant advantage of EVs is the adjustability of the composition, since various stimuli can perturb the genetic expression of origin cells and cargoes transferred into EVs (Fig. 2a) [27]. Therefore, there has been an enormous effort to strengthen the innate regenerative potential of stem cell-derived EVs or offer additional therapeutic functions by preconditioning MSCs

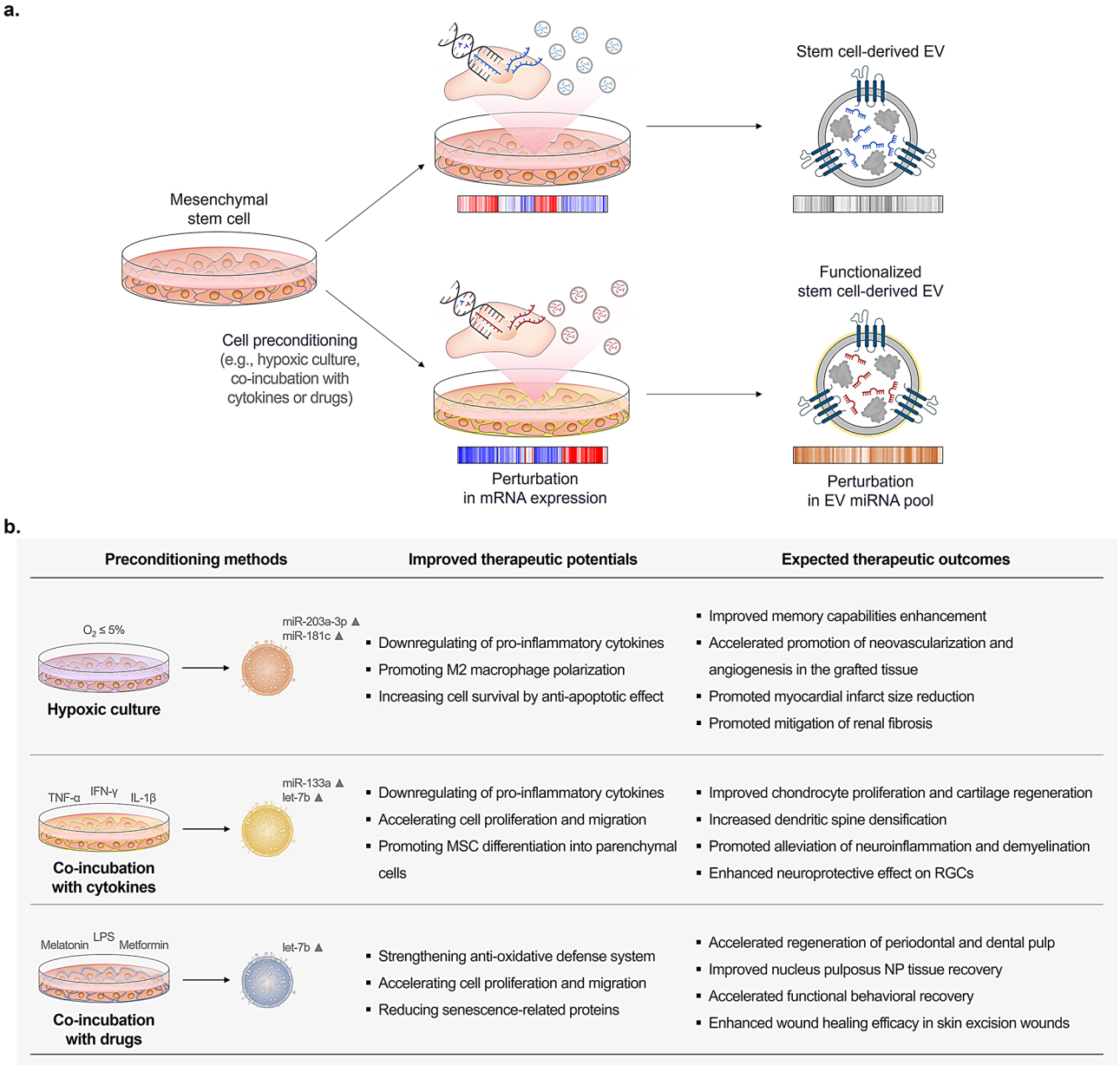


Fig. 2 Schematic of stem cell preconditioning for the functionalization of EVs. **(a)** Perturbation of cell preconditioning in MSCs and their EVs. **(b)** Representative preconditioning methods for functionalizing MSC-derived EVs and anticipated therapeutic outcomes

(Fig. 2b). For instance, changes in the culture condition (e.g., hypoxia, serum deprivation, and 3D culture) can stimulate gene expressions of stem cells, leading to the upregulation of genes (e.g., HIF-1α, Bcl-2, and Bax) that promote cell survival [28]. Additionally, recent studies have indicated that EVs derived from hypoxic-cultured stem cells exhibit enhanced anti-apoptosis and cell proliferation compared to EVs from non-preconditioned stem cells [29–31]. In the meantime, co-incubation of stem cells with certain chemicals (e.g., pro-inflammatory cytokines, lipopolysaccharides (LPS), and nitric oxide) can direct the immunomodulatory function of stem cells toward anti-inflammatory [28]. Particularly, co-incubation of inflammatory cytokines can significantly improve the therapeutic potential of EVs in promoting cell proliferation and repairing cellular damage [32–34].

Taken together, some of the trials achieved significant remarks in enhancing the regenerative effect of stem cell-derived EVs, and these advances make stem cell-derived EVs prominent candidates for treating various diseases (e.g., kidney injury, acute lung injury (ALI), and cartilage injury [35–37]). Nonetheless, the majority of studies attempting to functionalize EVs by preconditioning stem cells have remained in the early stages. They have thus far

only been able to achieve fragmentary modulation of the active components rather than precisely control the content of EVs under the identification of miRNA pools in EVs. In addition, inconsistent preconditioning methods are interfering with the practical application of functionalized EVs, and the standardization of the preconditioning method has remained a conundrum. Therefore, there is an urgent need for a comprehensive review discussing the recent progress of functionalized EVs and suggesting their future directions.

In this review, we thoroughly discuss the preconditioning of MSCs to functionalize EVs with recent progress of pretreatment of MSCs to functionalize EVs for the treatment of various organ systems, including the musculoskeletal, nervous, integumentary, cardiovascular, renal, and respiratory systems (Table 1; Fig. 3). The contents of this paper cover the biochemical influence in the cellular level of each preconditioning method and the therapeutic outcomes of their EVs in terms of the expression of miRNA on anti-inflammatory, proliferation, and migration. Additionally, current limitations and future directions for this strategy will be suggested.

Functionalized extracellular vesicles for treating various organs

Musculoskeletal system

The musculoskeletal system is the organ system that is responsible for supporting organ systems and moving the body. Throughout a whole life, the musculoskeletal system is subjected to continuous abrasion, causing innumerable cycles of micro-damage and subsequent repair. As the musculoskeletal system ages, it eventually reaches a point where it can no longer repair itself, leading to the loss of structural integrity as well as functionality. Furthermore, aging in the musculoskeletal system causes a decrease in bone density and muscle fiber and progressive loss of intervertebral discs, thereby the development of inflammatory diseases such as lumbar disc herniation, scoliosis, osteoporosis, rheumatoid arthritis (RA), and osteoarthritis (OA) [38]. In particular, the social expenses for treating age-related diseases and their complications have gradually increased by escalating the aging population [39]. Traditionally, pharmacotherapies, including nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and muscle relaxants, are frequently used to treat musculoskeletal diseases, however, these treatments only provide temporary pain relief and slow down the progression of disease [40]. Thus, there have been numerous endeavors to develop the treatment, offering the fundamental restoration of the tissue and function as well.

Among alternative approaches, EVs have emerged as potent candidates for the regeneration of damaged tissues by facilitating the proliferation of cells and alleviating inflammatory reactions [41]. For example, Zhang et al. demonstrated that intra-articular injection of bone marrow MSCs (BMSCs)-derived EVs can not only release anti-inflammatory cytokine by promoting the transformation of synovial macrophage polarization from M1 to M2 but reduce damage of articular cartilage by inhibiting the release of inflammatory cytokine [42]. Thus, BMSCs-derived EVs can relieve progressing OA.

To date, there have been several trials to enhance the anti-inflammatory and regenerative effects of EVs by preconditioning the cells. Representatively, Zhang et al. reported that hypoxia (5% O_2)-preconditioning of BMSCs could regulate the expression of miRNA in EVs toward anti-inflammatory, proliferation, and migration [30]. They found that the hypoxic preconditioning of BMSCs can differentially upregulate hsa-miR-181c-5p, hsa-miR-18a-3p, hsa-miR-376a-5p, and hsa-miR-337-5p than that of EVs from normoxically cultured BMSCs. Interestingly, these miRNAs are highly associated with the inhibition of mitogen-activated protein kinase 1 (MAPK1), signal transducer and activator of transcription 3 (STAT3), which trigger inflammatory responses in OA. Consequently, in vivo studies showed that EVs from hypoxia-pretreated BMSCs exhibited superior therapeutic effects in inhibiting chondrocyte migration and apoptosis, which may be attributed to the differential upregulation of miR-181c-5p and miR-18-3p. Similarly, Shen et al. reported hypoxic (3% O_2)-preconditioned BMSCs-derived EVs, upregulated miR-205-5p, promote therapeutic effects of H-EXOs about articular chondrocytes (ACs) proliferation, migration, anabolism, and anti-inflammatory by activating phosphatase and tensin homolog (PTEN)/RAC-alpha serine/threonine-protein kinase (AKT) pathway [37]. Additionally, Tian et al. studied the influence of hypoxia (1% O_2) preconditioning of dental pulp stem cells (DPSCs) on EVs in the production rate and differential upregulation of miR-210-3p. They demonstrated that hypoxia preconditioning of DPSCs can upregulate exosomal miR-210-3p, allowing the capables of EVs to inhibit macrophage M1 polarization and osteoclastogenesis [31].

Cytokines that mediate the immune system can induce M1/M2 macrophage polarization and cell proliferation by mimicking inflammation or anti-inflammatory reactions in cells, regulating the miRNA or proteins that allow cells to respond to internal situations. Nakao et al. attempted to precondition gingival tissue-derived MSCs (GMSCs) by TNF-α (100 ng/mL) for 48 h [33]. The upregulated CD73 of preconditioned GMSCs-derived EVs promotes recipient macrophages toward M2 polarization by activating AKT and ERK. Thus, it alleviates inflammation and prevents periodontitis leading to bone loss in the periodontal tissue. MiR-1260b in EV inducing the Wnt5a-mediated receptor activator of nuclear

Table 1 Regenerative natural EVs functionalized by preconditioning strategies to better treat various diseases

Preconditioning	Target disease	Dose	Source of EVs	EV component changes	Outcomes	Ref.
Musculoskeletal system						
Hypoxia	Osteoarthritis	5% O_2	(Mouse) BMSCs	12 miRNAs ▲ 17 miRNAs▼ (miR-181c-5p, miR-18a-3p, miR-376a-5p, and miR-337-5)	miR-181c-5p ▼ → NF-κB signaling pathway ▼ → TNF-α, IL-1β ▼ miR-18a-3p/JAK-STAT, miR-181c-5p/MAPK pathway ▲ → OA repression	[30]
	Cartilage injury	3% O_2	BMSCs	72 miRNAs ▲ (miR-205–5p, miR-196b-5p, miR-361–3p, miR-181c-3p, and miR-203a-3p) 111 miRNAs ▼	miR-205-5p ▲ → PTEN ▼→ PI3K-AKT signaling pathway ▲ → proliferation, migration, anabolism and anti-apoptosis of ACs ▲ → cartilage regeneration	[37]
	Inflammatory osteolysis	1% O_2	DPSCs	45 miRNAs ▲ (miR-1249, miR-7578, miR-203a-3p, miR-187-3p, miR-221-5p, and miR-210-3p) 64 miRNAs ▼	miR-210-3p ▲ → inhibiting NF-κB1 expression → M1 (IL-1β, IL-6, TNF-α, iNOS) ▼, M2 (IL-10, Arg1) ▲ → osteoclastogenesis (Acp5, CTSK, c-FOS, DC-STAMP, Atp6v0d2) ▼	[31]
Cytokine	Periodontitis	TNF-α 100 ng/mL	GMSCs	655 miRNAs differential expression miR-1260b ▲	CD73 ▲ → M2 macrophage ▲ miR-1260b ▲ → inactivating Wnt5a-mediated RANKL pathway → osteoclastogenesis ▼	[33]
	Inflammatory osteolysis	TNF-α 1 ng/mL	ADSCs	Wnt-3a protein ▲	Wnt-3a protein ▲ → proliferation, migration, and osteogenic differentiation of HOB ▲	[43]
	Osteoarthritis	TGF-β1 10 ng/mL	MSCs	miR-135b ▲	miR-135b ▲ → Sp1 ▼ → chondrocyte proliferation, cartilage repair ▲	[32]
Drug	Osteoarthritis	LPS 100 ng/mL	SMSCs	18 miRNAs (let-7b) ▲ 64 miRNAs ▼	let-7b → ADAMTS5 ▼ → aggrecan, COL2A1 ▲ → cartilage destruction in the mouse model of OA ▼	[44]
	Periodontal disease	LPS 0.25 μg/mL	DFCs	-	Periostin, OPN, OCN, and Runx2 ▲ COL I, CEMP-1, fibronectin, and periostin ▲ → periodontal regeneration	[45]
	Dental pulp disease	LPS 1 μg/mL	DPSCs	-	VEGF, neurofascin, ALP, and DSPP of BMSC ▲ TGF-β ▲→ M2 polarization ▲ → dental pulp regeneration	[46]
	Interver-tebral disc degeneration	Metformin 1 mM	BMSCs	70 differentially expressed proteins (ITIH4)	AMPK ▲ → TNF-α, IL-1β, p16, p21 ▼ → NP tissue recovery in a rat model of IDD	[47]
Nervous system						
Hypoxia	Traumatic spinal cord injury	1% O_2	(Mouse) BMSCs	Expression of miR-216a-5p ▲	miR-216a-5p ▲ → the activity of TLR4 ▼ → TNF-α, IL-1β, and IL-6 ▼, TGF-β, IL-4, and IL-10 ▲ → M2 polarization of microglia/macrophages ▲ → lesion area ▼	[55]
	Alzheimer's disease	95% N_2, 5% CO_2 for 12 h.	(Mouse) BMSCs	-	The activation of STAT3 and NF-κB ▼ → pro-inflammatory cytokines (TNF-α and IL-1β) ▼, anti-inflammatory cytokines (IL-4 and IL-10) ▲ The level of miR-21 in the brains of AD mice ▲ → memory capabilities of AD mice ▲	[56]

Table 1 (continued)

Preconditioning	Target disease	Dose	Source of EVs	EV component changes	Outcomes	Ref.
Cytokine	Alzheimer's disease	TNF-α 20 ng/mL, IFN-γ 25 ng/mL	BMSCs	-	COX-2, IDO, and PGE2 ▲ → anti-inflammatory effects (IL-6 and IL-1β ▼, IL-10 ▲) → M2 polarization Iba-1 expression ▼ → microglia activation in 3xTg AD mice ▼, dendritic spine density in 3xTg AD mice ▲	[57]
	Cerebral stroke	IFN-γ 20 ng/mL	(Human) NSCs	5 miRNAs (hsa-miR-206, hsa-miR-133a-3p, hsa-miR-4677-5p, hsa-miR-205-5p, and hsa-miR-3656) ▲ 2 miRNAs (hsa-miR-34c-3p and hsa-miR-3151-5p) ▼	Cell viability, proliferation, and migration of hNSC ▲ miRNAs (hsa-miR-206, hsa-miR-133a-3p, and hsa-miR-3656) ▲ → the important role of cell survival Infarct volume ▼, microglia activation ▼ in a mouse model of stroke → neurological functional recovery	[58]
	Multiple sclerosis	IFN-γ 10 ng/mL	UC-MSCs	Non-coding RNA ▲ ~ 48 IDO in IFN-γ-Exo MIC-1, Gal-1, HSP70, and LTBP ▲	Iba-1 ▼ → T cell (Th1 and Th17) ▼, Treg ▲ → neuroinflammation, demyelination in mouse model ▼	[34]
Drug	Spinal cord injury	Melatonin 1 μmol/L	BMSCs	875 proteins ▲ (USP29) 347 proteins ▼	NRF2 stabilization by USP29 → iNOS, TNF-α, and IL-1β ▼, ArgI, CD206, and YM1/2 ▲ → M2-like microglia/macrophage polarization, mitochondrial function ▲ → functional behavioral recovery ▲	[59]
Respiratory system						
Hypoxia	Acute lung injury	Hypoxic conditions for 72 h.	(Rat) BMSCs	A level of lncRNA XIST ▲	Inflammatory factors (IL-2, TNF-α, and TGF-β) ▼ Bcl-2 ▲, Bax, cleaved caspase-9 ▼ → apoptosis ▼ → cell viability ▲ Expression of lncRNA XIST in EVs ▲ → miR-455-3p ▼ → protein of claudin-4 in vivo ▲ → protective effect against ALIv	[36]
Drug	Sepsis-induced lung injury	EPA 10 μM	ADSCs	-	RvD1, PGE2, IL-10, TGF-β ▲ → lung and distal organ damage in CLP mice ▼ → clinical score and survival rate ▲	[67]
Integumentary system						
Hypoxia	Human adipose tissue graft	5% O_2	ADSCs	VEGF, EGF, FGF, VEGF-R2, VEGF-R3, MCP-2, and MCP-4 ▲	EGF, FGF, VEGF, VEGF-R, ANG-1, and Tie-2 ▲ → expression of CD31 around the graft ▲ → neovascularization, angiogenesis in the grafted tissue ▲	[75]
Drug	Chronic cutaneous wound	LPS 100 ng/mL	UC-MSCs	let-7b, miR-1180, miR-183, miR-550b, and miR-133a ▲	let-7b ▲ → TLR4, p-P65 ▼, p-STAT3, p-AKT ▲ → M2 macrophage ▲, M1 macrophage ▼ → inflammatory and diabetic cutaneous wounds ▼	[76]
	Cutaneous wound	Thrombin (40 U)	UCB-MSCs	Angiogenin, angiopoietin-1, HGF, and VEGF ▲	AKT, ERK1/2 phosphorylation → HUVECs proliferation, migration, and proangiogenic effects ▲, hDF proliferation ▲ → wound healing efficacy in rats subjected to skin excision wounds ▲	[77]
Cardiovascular system						

Table 1 (continued)

Preconditioning	Target disease	Dose	Source of EVs	EV component changes	Outcomes	Ref.
Hypoxia	Myocardial infarction	1% O_2	BMSCs	12 miRNAs ▲ (mmu-miR-5112, mmu-miR-711, mmu-miR-125b-5p, mmu-miR-92a, mmu-miR-7025, and miR-7045)	miR-125b-5p ▲ → p53, BAK1 ▼ → injured H9C2 cardiomyoblasts apoptosis ▼→ infarct size ▼	[84]
	Myocardial ischemia-reperfusion injury	100% N_2 (5 cycles of anoxia in 60 min. with intermittent reoxygenation in 30 min.)	(Mouse) ADSCs	41 miRNAs ▲(miR-224-5p)	miR-224-5p ▲ → Bcl-2/Bax, GATA4 ▲, TXNIP, cleaved caspase-1 ▼ → anti-apoptosis effect on cardiomyocytes	[85]
Drug	Myocardial infarction	LPS 100 ng/mL	BMSCs	-	NF-κB signaling pathway ▼ → pro-inflammatory cytokines ▼ AKT1 P ▲, AKT2 P ▼ → M1 (IL-6, TNF-α, IL-1β, and CD-11b) ▼, M2 (IL-10, CD206, and Arg1) ▲ → post-infarction inflammation and myocardial injury restoration	[86]
Renal system						
Hypoxia	Renal fibrosis after ischemia-reperfusion injury	1% O_2, 94% N_2, and 5% CO_2 for 48 h.	P-MSCs	-	BUN, vimentin, collagen I, and α-SMA ▼ → interstitial fibrosis, tubular atrophy ▼ CPT1A, CPT2, and ACOX2 ▲ → recovery of mitochondrial homeostasis → renal fibrosis ▼	[92]
Drug	Renal ischemia-reperfusion injury	Melatonin 5 μM	BMSCs	-	Oxidative stress (MDA, HIF-1α, and NOX2) ▼, antioxidant status (SOD, CAT, GPX, and HO1) ▲ Caspase-3 activity, Bax and PARP1 ▼, Bcl-2 ▲ → apoptosis ▼ MPO activity, ICAM1, IL-1β, and NF-κB▼, IL-10 ▲ → inflammation ▼ Basic FGF, HGF, and SOX9 ▲ → regeneration ▲ VEGF ▲ → angiogenesis ▲ BUN, creatinine ▼ → kidney function ▲ Ischemic and pro-inflammatory lesions ▼	[93]
Other organs						
Cytokine	Injured retina	TNF-α 10 ng/mL	BMSCs	-	PEDF, VEGF-A, and PDGF-AA ▲ → RGCs neuroprotective effects	[98]

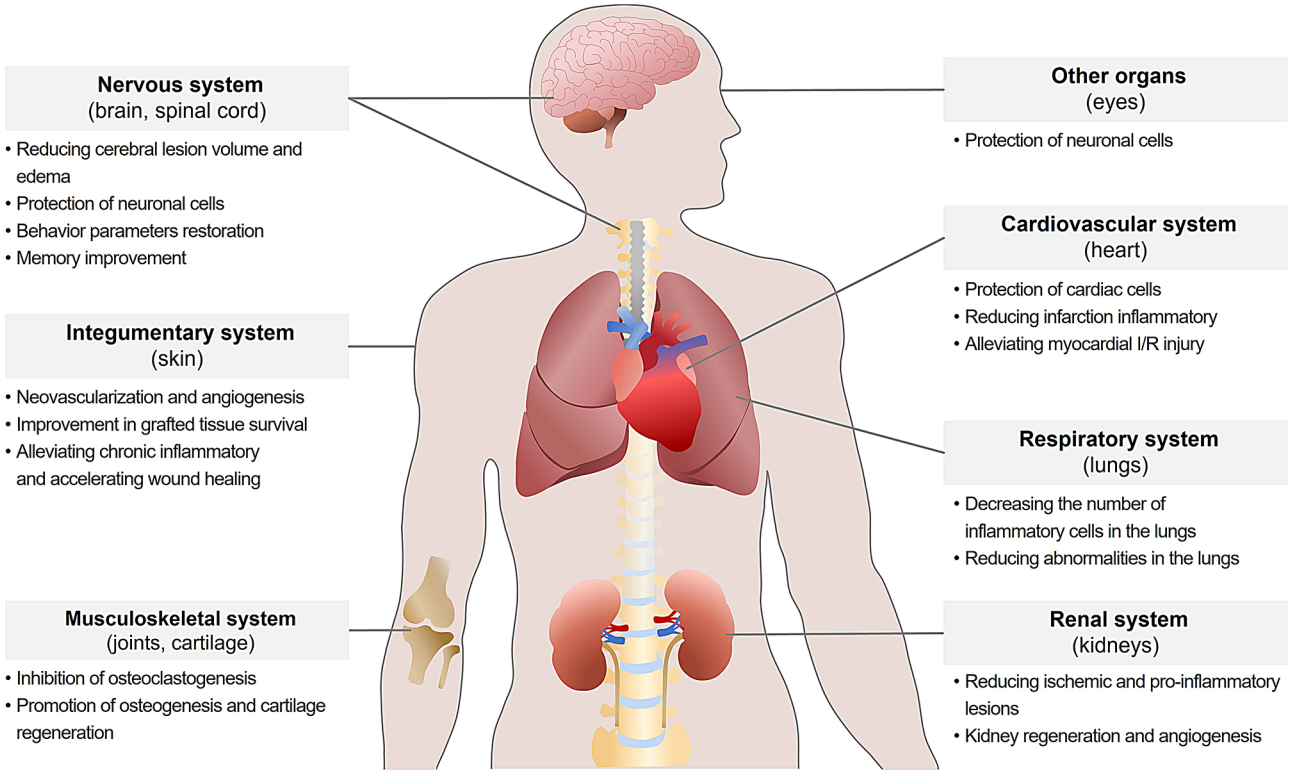


Fig. 3 Therapeutic applications of functionalized EVs for treating various organs. Created by reconstructing the researches in Table 1 based on their therapeutic effects

factor kappa-B ligand (RANKL) pathway suppresses osteoclastogenesis as well. As the same cytokine, Lu et al. preconditioned adipose tissue-derived MSCs (ADSCs), using TNF-α (1 ng/mL) for 3 days [43]. Preconditioned ADSC-derived EVs contained abundant Wnt-3a protein activating Wnt signaling and improved proliferation and osteogenic differentiation of human primary osteoblastic cells (HOB). Wang et al. regulated the paracrine activity of EVs by treating TGF-β1 (10 ng/mL) to MSCs [32]. TGF-β1 primed MSCs-derived EVs encouraged chondrocyte proliferation by down-regulating specificity protein 1 (SP1) expression via miR-135b. Similarly, their in vivo demonstrated cartilage protection effect of TGF-β1 primed MSCs-derived EVs in OA-induced rat.

Drug preconditioning advances the functional properties of cell-derived EVs by inducing resistance against extracellular environments. Duan et al. studied that lipopolysaccharide (LPS, 100 ng/mL)-preconditioned synovial MSCs (SMSCs)-derived EVs improved chondrocyte proliferation and migration, inhibited apoptosis. Moreover, they found that EVs from preconditioned MSCs could suppress ECM degradation by upregulating exosomal let-7b, which blocks the degradation of aggrecan and collagen type II alpha 1 (COL2A1) [44]. Likewise, the analysis revealed that LPS-preconditioned SMSCs-secreted EVs successfully alleviate the course of cartilage degradation in vivo. Shi et al. reported that LPS (0.25 μg/mL)-preconditioned dental follicle cells (DFCs)-derived EVs not only relieve periodontitis due to gene expression of COL I, cementum protein 1 (CMEP-1), fibronectin, and periostin but more secreted from DFCs after LPS preconditioning [45]. Through osteogenic-related genes such as osteopontin (OPN), osteocalcin (OCN), and runt-related transcription factor 2 (Runx2), it stimulates the production of collagen, osteogenic and cementation formation, and adhesion. Moreover, in vivo, activating the OPG/RANK/RANKL signaling pathway inhibits bone resorption of periodontal tissue. Chen et al. harnessed LPS (1 μg/mL)-preconditioned human dental pulp stem cells (hDPSCs)-derived EVs to demonstrate dental pulp regeneration by regulating BMSCs proliferation, migration, angiogenesis, and differentiation [46]. Moreover, expression of vascular endothelial growth factor (VEGF), neurofascin, alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP), and TGF-β was significantly elevated compared to EVs derived from hDPSCs without the preconditioning. Alternatively, Liao et al. harnessed EVs secreted from BMSCs which are preconditioned with metformin [47]. Metformin known as an FDA-approved oral anti-hyperglycemic agent commonly used in diabetes mellitus, is a novel therapeutic way of intervertebral disc degeneration (IDD). Interestingly, metformin broadly influences the secretion of EVs from BMSCs by altering the genetic expression of BMSCs. For

example, metformin significantly upregulates microtubule-associated protein 1 A/1B-light chain 3-II (LC3-II) and phosphorylation of synaptosome-associated protein 29 (SNAP29) promoting the secretion of EVs quantitatively. Furthermore, metformin tones the EV protein profiles to upregulate adenosine monophosphate-activated protein kinase (AMPK) pathway-related proteins, such as inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), which plays a significant role in anti-aging, through the AMPK pathway. Accordingly, EVs from metformin-preconditioned BMSCs were capable of downregulating senescence-associated secretory phenotype (SASP) (e.g., TNF-α and IL-1β) and senescence markers (e.g., p16 and p21) in nucleus pulposus (NP). Furthermore, the in vivo therapeutic evaluation result shows that EVs from metformin-preconditioned BMSCs more effectively retarded the progression of IDD stages of the rat than that from non-treated BMSCs.

Nervous system

The nervous system, which spans most of our body, is divided into the central and peripheral nervous systems. It governs our motor abilities and sensations, and damage to the nervous system can lead to severe consequences that impede body functions. In particular, nerve injuries such as traumatic brain injury (TBI), spinal cord injury (SCI), and stroke frequently accompany various complications, which are highly associated with mortality [48]. Typically, nerves have been known to be time-limited in recovery, therefore, these are less resilient than other types of cells. In particular, the fibrotic environment due to the recovery of damaged tissues restricts the delivery of neurotransmitters and induces neurolysis, rendering damaged nerves difficult to recover functionally. Therefore, conventional treatment strategies have only been able to achieve short-term recovery, and no pharmacological treatment has been found to fully restore nervous functions [49]. Although there have been remarkable advances, including stem cell transplantation, for treating neurological diseases, the development of technology to elaborately regulate inflammatory response and tissue regeneration for functional recovery of nerves has remained a challenge so far [50].

Meanwhile, recent studies have revealed that EVs from MSCs are more likely to be associated with clinical mechanisms of MSCs-mediated nerve regeneration than the conversion of MSCs. Moreover, MSCs-derived EVs exhibit exceptional potential to treat neurological diseases due to the crossing the biological barriers through transcytosis. For example, Lu et al. reported the therapeutic potential of BMSCs-derived EVs in treating SCI by crossing the blood-spinal cord barrier (BSCB) [51]. Similarly, facilitated crossing of the blood-brain barrier (BBB) of MSCs-derived EVs and thereby exceptional benefit in treating neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS), have been extensively studied in recent [52–54]. Furthermore, recent findings in MSCs-derived EVs for treating neurological diseases EVs have shown that preconditioning of MSCs can provide an additional therapeutic function or improve the therapeutic efficacy of EVs.

In particular, the hypoxia preconditioning of MSCs provides an exceptional opportunity to enhance the regenerative effect of EVs. For example, Liu et al. applied hypoxia (1% O_2)-preconditioned BMSCs-derived EVs to recover the abnormal function of the spinal cord following SCI [55]. Notably, their results indicate that the hypoxia preconditioning of BMSCs adjusted EV contents to be more favorable to suppress inflammation not only quantitatively but also qualitatively. For example, hypoxia-preconditioned BMSCs-derived EVs show upregulated miR-216a-5p that polarized microglia phenotype from M1 to M2 by suppressing TRL4 than that released EVs from normoxic conditions. In addition, Cui et al. demonstrated that EVs released from hypoxia (95% N_2, 5% CO_2)-preconditioned MSCs have the potential to prevent AD through miR-21 by regulating inflammatory responses [56]. The in vivo study shows that EVs derived from hypoxia-preconditioned MSCs not only significantly improve cognition and memory impairment of mice by lowering plaque and amyloid-β (Aβ) accumulation but regulate inflammatory cytokines by reducing activation of astrocytes and microglia. Similarly, Losurdo et al. introduced the ameliorative effect on AD by EVs, derived from BMSCs that are incubated in the culture medium supplemented with TNF-α (20 ng/mL) and interferon γ (IFN-γ) (25 ng/mL) [57]. Noteworthy, cytokines supplementation of BMSCs improved the efficacy of EVs by upregulating cyclooxygenase 2 (COX2) and indoleamine 2, 3-dioxygenase (IDO), hence, constitutively lowering the secretion of pro-inflammatory cytokines in microglia. Moreover, intranasal (IN) injection of the EVs significantly weakens the microglia activation and elevates dendritic spine density. Recently, Zhang et al. suggested EVs-based cerebral stroke therapy by mimicking the paracrine signaling between the host immune system and grafted stem cells via EVs [58]. As the signal based on the IFN-γ/interferon gamma receptor 1 (Ifngr1) complex pathway, they functionalized EVs by preconditioned human neural stem cells (hNSCs) with IFN-γ (20 ng/mL). As a result, the treatment of EVs released from IFN-γ-preconditioned hNSCs improved stem cell survival and reduced cell apoptosis suggesting the protective abilities of neuronal cells, both in vivo and in vitro, compared to EVs from hNSCs without preconditioning. Specifically, miRNAs in EV, including hsa-miR-206, hsa-miR-133a-3p, and hsa-miR-3656, were found

exclusively in IFN-γ-preconditioned hNSCs-discharged EVs, indicating their critical role in cell survival. Similarly, Riazifar et al. discovered the potential of IFN-γ (10 ng/mL)-preconditioned human umbilical cord MSCs (UC-MSCs)-derived EVs to recover autoimmune and neurodegenerative disorders [34]. The EVs significantly induced a decreasing number of macrophages, microglia, and pro-inflammatory T cells occupying the spinal cord by reducing pro-inflammatory cytokines. Besides, the fundamental approach to the central nervous system autoimmune inflammation is indicated by inducing increased Tregs significantly via anti-inflammatory RNAs in EV. Increases anti-inflammatory RNAs in EV and Treg numbers suggest that the EVs can fundamentally approach the treatment of central nervous system autoimmune inflammation.

Melatonin (MT) is a well-known anti-inflammatory agent by reprograming the polarization of macrophages from M1 to M2 [59]. Recently, Liu et al. reported the advantages of MT-preconditioning of BMSCs in potentiating the anti-inflammatory effects of EVs. They identified that the administration of MT-preconditioned BMSCs-derived EVs, particularly enriched with ubiquitin-specific protease 29 (USP29) compared to normalized EVs, inhibited the degradation of nuclear factor-like 2 (NRF2), regulating M2 polarization. Consequently, it ascertained assisting roles in functional behavioral improvements and axonal regeneration after SCI in mice.

Respiratory system

The respiratory system is an organ system that circulates the air from the body to the pulmonary alveoli, located at the ends of the bronchi [60]. In this process, alveoli not only act as a gas-blood barrier but participate in innate immune responses that prevent pathogen invasion as well [61]. Structurally, pulmonary alveoli feature extremely thin linings to expand the surface area of capillary for efficient gas exchange. Since this thin lining confers the fragility to the adhesion of each layer occurring the blockage of gas exchange, ALI and thereby immune responses often cause severe interruptions in oxygen supply, such as acute respiratory distress syndrome (ARDS) [62]. Among the causes of ALI, sepsis, which features pathological characteristics such as loss of alveolar endothelial and epithelial cells, alveolar-capillary injury, increased numbers of neutrophils, and release of inflammatory cytokines, has been tried to treat with various traditional approaches, including fluid management, nutritional support, lung protective ventilation, statins, exogenous surfactant, and glucocorticoid [63]. However, the complete restoration of pulmonary structures and functions has remained a conundrum, due to the delicate and elaborate structure of pulmonary alveoli. As a result, ALI is still considered a life-threatening disease, resulting in substantial morbidity and an in-hospital mortality rates up to 38–46% [64]. In particular, most patients have been suffering from post-treatment symptoms from remaining irreversible damage to pulmonary structures.

Therefore, stem cell-based therapies have been highlighted as prominent treatments for treating ALI in recent decades, since they provide exceptional opportunities for alleviating pulmonary damage and restoring pulmonary functions by reorganizing pulmonary structures [65]. However, concerns regarding teratoma formation and immune-related adverse effects have hindered the use of MSCs despite a significant benefit in improving survival rates of ALI patients. With similar functions and fewer side effects, MSCs-derived EVs have been attracted as a promising alternative to MSCs in recent years. For instance, Shen et al. reported that miR-125b-5p in ADSCs-derived EVs ameliorated inflammation and inhibited ferroptosis in pulmonary microvascular endothelial cells from sepsis-induced acute lung injury [66]. Therefore, EVs that fundamentally alleviate protective effects along with functional improvements on the lungs are in the spotlight as alternative therapeutics for ALI.

Recent trials have revealed that certain preconditioning methods could improve the therapeutic potential of MSC-derived EVs in treating ALI. As an example, Ren et al. attempted to enhance the cytoprotective effect of BMSCs-derived EVs in lung injury by applying hypoxic condition to BMSCs for 72 h [36]. EVs from hypoxia-preconditioned BMSCs (Hypo-Exos) more efficiently debilitated immune cells in bronchoalveolar lavage fluid and diminished their release of inflammatory cytokines such as IL-2, TNF-α, and TGF-β. Also, treating Hypo-Exos more efficiently proliferates lung epithelial cells by increasing anti-apoptotic factors and suppressing apoptotic factors than that of EVs from BMSCs under normoxic conditions. Their results indicate that the treatment of hypoxia preconditioning to BMSCs significantly upregulates EV contents of long non-coding RNA (lncRNA) X-inactive specific transcript (XIST). Furthermore, the upregulated lncRNA XIST confers the potential to attenuate lung injuries via the miR-455-3p/claudin-4 axis. It might be attributed to the sponging of miR-455-3p by lncRNA XIST allows the upregulation of claudin-4, crucial for reorganizing cell-to-cell connections. On the other hand, the preconditioning of eicosapentaenoic acid (EPA) to MSCs is proposed to be a novel method for enhancing the anti-inflammatory potential of MSC-derived EVs. Since EPA broadly alters the expression of inflammation-related genes to be anti-inflammatory, it commonly plays a key role in adjusting the activity of various inflammatory cells in inflammatory and recovery responses. In recent, Silva et al. found EVs, isolated from EPA (10 μM)-treated ADSCs, were intravenously injected into cecal ligation and puncture (CLP)-induced

mice, and it reduced alveolar collapse, interstitial edema, alveolar septal inflammation, and collagen fiber content in the lung [67]. Furthermore, it not only effectively decreased total leukocytes, monocytes, and neutrophils in the blood, but reduced morphological abnormalities in distal organs such as the liver, kidney, heart, spleen, and small bowel, thereby significantly increasing the survival rate of CLP-induced mice.

Integumentary system

The integumentary system, the largest organ in the outermost layer, includes the skin, hairs, nails, and glands. The skin, the major part of the integumentary system, is composed of multiple layers and serves as the frontline defence of the human body against external intruders. Given that the skin is constantly exposed to microbial infections and physical damage, the dermal microenvironment is toned to recruit immune cells and accompany inflammatory responses for inhibiting invasion of external pathogens, thereafter performing a self-healing process involving hemostasis, inflammation, cell proliferation, and tissue remodeling [68]. However, excessive damage to the skin often occurs due to the delay of the self-healing process, thereby interrupting the reconstruction of the extracellular matrix and proliferation of surrounding cells, leading to the formation of chronic wounds [69]. To prevent the aforementioned, wound dressings, iodine-containing compounds, and antibiotics are commonly used to inhibit microbial infection, however, the way to accelerate early wound healing has remained a puzzle so far [70]. Despite the intensive study and long-standing utilization of epidermal skin grafts using skin substitutes these often result in flap necrosis and complications, leading to deterioration of the surgical region [71]. Consequently, there is a need for not only the regulation of inflammatory responses in the dermal microenvironment, but also the facilitation of the regeneration of damaged tissues. In this regard, MSC-based treatments have considered the representative successful strategies for skin wound healing among the available alternatives, due to their capables for immune regulation and tissue regeneration [72]. However, disadvantages of cell-based therapy, such as the formation of teratomas, low viability of cells, and host immune rejections, hindered their clinical applications.

Recently, MSCs-derived EVs have been highlighted as a promising candidate for complementary treatment of skin wound healing since it has been reported to show immune regulation and cell proliferation. Furthermore, recent studies have revealed that preconditioned MSCs-derived EVs can facilitate extracellular matrix production and angiogenesis, which are crucial to the restoration of damaged tissue [73, 74].

Han et al. attempted to evaluate the survival of human umbilical vein endothelial cells (HUVECs) and grafted adipose tissue through EVs released from human ADSCs, maintaining oxygen concentration at 5% [75] Han et al. suggested the potential application of EVs from hypoxia-preconditioned (5% O_2) ADSCs for improving the survival of adipose tissue engraftment. In the proliferation test using human umbilical vein endothelial cells (HUVECs), the survival of HUVECs is remarkably increased by treating EVs from hypoxia-preconditioned ADSCs. Furthermore, the elevated expression of these proteins in the peri-implant tissue has been shown to alleviate neoangiogenesis in a nude mice model. It might be attributed to the differential increase of exosomal content of proteins relevant to tissue regenerations, such as VEGF, epidermal growth factor (EGF), fibroblast growth factor (FGF), their receptor (VEGF-R2, VEGF-R3), and monocyte chemoattractant protein (MCP-2, MCP-4) by preconditioning hypoxia to ADSCs.

In a study by Ti et al., the paracrine effects of UC-MSCs were enhanced by LPS (100 ng/mL) to regulate macrophage polarization and accelerate neoangiogenesis in the context of chronic cutaneous wound healing in mice [76]. It is noteworthy that LPS-treated UC-MSCs-derived EVs containing let-7b played a significant role in the regulation of macrophage polarization via toll-like receptor 4 (TLR4)/nuclear factor kappa B (NF-κB)/STAT3/AKT signaling pathway. In other ways, Sung et al. evaluated the proangiogenic activity and cutaneous wound healing by treating EVs released from thrombin (40 U)-preconditioned human umbilical cord blood-derived MSCs (hUCB-MSC) compared with H_2O_2 (50 μM), LPS (1 μg/mL), or hypoxia (10% O_2) preconditioning to hUCB-MSC [77]. In vitro, EVs derived from thrombin-treated hUCB-MSC demonstrated a remarkable capacity to promote the proliferation of HUVEC and human dermal fibroblast, as well as the formation of capillary-like structures. Furthermore, the EVs exhibited enhanced efficacy in promoting wound healing in rats subjected to a skin excision model when compared to EVs from hUCB-MSC without preconditioning, irrespective of the number of EVs based on the number of MSCs (5×10^5) or EV protein concentration (20 μg/10 μL).

Cardiovascular system

The heart consecutively circulates blood, nutrients, and oxygen to all organs through a process of repetitive contractions and relaxations. Given that the myocardial muscle is responsible for generating the force that drives blood circulation, it also requires sufficient oxygen and nutrients to function optimally. Therefore, disorders in the myocardial muscle can have a detrimental effect on life, even in the short term.

Decreases or obstructions in blood flow to the myocardial muscle, caused by coronary artery disease, can result in a condition known as myocardial infarction, which leads to the necrosis of myocardial muscle tissue. Severe cases can result in a myocardial infarction (MI), the leading cause of mortality worldwide [78]. The etiology of myocardial infarction is multifactorial, but the most prevalent underlying causes are atherosclerosis, thrombosis, coronary artery spasm, obesity, and diabetes [79]. In particular, atherosclerosis, an age-related disease characterized by endothelial inflammation and the formation of plaque that restricts blood flow, is highly associated with an increased risk of MI. In the majority of cases, MI has no specific symptoms in the early stages. However, once plaque formation obstructs narrow coronary arteries, it significantly progresses MI and leads to the deterioration of heart function by forming blood clots and subsequently blocking a myocardial vessel, requiring emergency treatment. Even worse, patients who have experienced an MI have a high relapse rate due to the complexity of regeneration in damaged cardiac tissues, and most patients demonstrate a gradual exacerbation of their condition. Conventional treatments for MI include analgesics (e.g., morphine and meperidine), anticoagulants (e.g., aspirin), thrombolytics (e.g., tissue plasminogen activator), fibrinolytic enzymes (e.g., streptokinase and urokinase), antihypertensive drugs (e.g., diuretics, β-blockers angiotensin-converting enzyme inhibitors, and calcium channel blockers), and vasodilatory drugs (e.g., nitroglycerin) have been shown to alleviate pain and prevent additional damage. Despite substantial efforts in the development of MI therapeutics, current approaches exhibit limited effects in the recovery of peri-infarct zones to date [80]. Therefore, MSCs-derived EVs have recently been proposed as a novel therapeutic approach for the treatment of myocardial disorders, given their capability to protect against ischemic shock and regeneration of damaged myocardiocytes [81]. For example, Zou et al. reported that BMSCs-derived EVs, which induce the upregulation of autophagy, prevent myocardial injuries and myocardiocyte apoptosis caused by MI [82]. On the other hand, Ma et al. demonstrated that endothelial progenitor cells-derived EVs have the capacity to stimulate proliferation of endothelial cells against ischemia-reperfusion (I/R) injury by improving the function of mitochondria [83].

Furthermore, recent reports have revealed that preconditioning of MSCs in hypoxic or hypoxia-mimic conditions can improve the therapeutic efficacy of EVs by upregulation of genes related to enhancing cell survival and enduring cellular stress. Zhu et al. observed that hypoxia (1% O_2)-preconditioned EVs derived from BMSCs significantly upregulated the expression of 6 miRNAs compared with normoxia EVs [84]. Among them, the upregulation of miR-125b-5p has been shown to reduce myocardial infarct size and prevent I/R-induced cardiac dysfunction by suppressing p53 and BAK1, which is responsible for modulating cardiomyocyte proliferation and survival physiologically. In other cases, Mao et al. documented that hypoxia (100% N_2) preconditioning tones mouse ADSCs-derived EVs to upregulate 41 miRNAs [85]. Especially, the expression of miR-224-5p in EVs reduced the pyroptosis and apoptosis of cardiomyocytes following myocardial I/R injury (MIRI) by diminishing thioredoxin-interacting protein (TXNIP) and cleaved caspase-1, raising Bcl-2/Bax ratio and inhibiting of GATA4 degradation.

Also, the preconditioning of drugs has been suggested as a potential alternative to the preconditioned MSCs-derived EV therapy for the treatment of myocardial infarction. Xu et al. validated EVs isolated from BMSCs treated with LPS (100 ng/mL) increased M1-related markers (IL-6, TNF-α, IL-1β, CD11b, and iNOS) and decreased M2-related markers (IL-10, CD206, and arginase I (ArgI)) [86]. The EVs subsequently recovered post-infarction inflammation and myocardial injury by lessening the NF-κB signaling pathway and regulating the AKT1/AKT2 pathway.

Renal system

The renal system, comprising the kidney, ureter, and urethra, is responsible for maintaining homeostasis by means of the reorganization of blood components through a process of filtration, secretion, and reabsorption. In particular, the kidney is a central organ that is capable of filtering blood and balancing the ionic concentration and pH level of blood. However, renal injury can result in a decline in kidney function by accumulating surplus metabolism or waste, which can lead to complications. Depending on the duration of the disorder, the stage of kidney damage can be classified as either acute renal failure (ARF), which occurs in a short period of time, or chronic renal failure (CRF), which lasts for more than three months [87]. The etiology of ARF is frequently associated with three causes: (i) sepsis, cardiac dysfunction in the prerenal region, (ii) glomerulonephritis, renal vasculitis in the renal region, (iii) nephrolithiasis, urolithiasis in the postrenal region. In contrast, CRF arises from conditions such as diabetes, hypertension, or glomerulonephritis, and is marked by uremia, metabolic acidosis, and anemia [88]. To date, the treatment for recovering renal functions has remained a puzzle so far, and renal transplantation has considered the only way to recover renal functions, despite its difficulties in finding compatible donors and poor success rate [89]. Thus, the treatment of renal failure is highly dependent on hemodialysis for delaying multiple organ dysfunctions by temporarily treating the renal functions [90]. In the past decade,

the therapeutic potential of EVs in the regeneration of renal functions has been intensively studied, due to their anti-inflammatory and proliferative effects. In acute kidney injury, Huang et al. investigated that MSCs-derived EVs promote the proliferation of renal tubular cells, and angiogenesis in a porcine model [35]. In chronic kidney injury, Nassar et al. reported that intra-arterial and intravenous injections of MSCs-derived EVs normalize the immune system and improve kidney function through anti-inflammation, anti-fibrosis, and anti-apoptosis [91].

Meanwhile, fatty acid oxidation (FAO) is the key dynamics of energy metabolism within active proximal tubular cells (PTCs). In PTCs, FAO is facilitated by carnitine palmitoyltransferase 1 A (CPT1A), carnitine palmitoyltransferase 2 (CPT2), and acyl-coenzyme A oxidase 2 (ACOX2). However, I/R injury-induced mitochondrial dysfunction can cause the depletion of CPT1A and CPT1A-mediated FAO, resulting in metabolic disruption and subsequent renal fibrosis [92]. Recently, Gao et al. proposed that the preconditioning of human placenta-derived MSCs (hP-MSCs) in a hypoxic environment could enhance the functionalization of EVs, thereby inducing the normalization of cellular metabolism by increasing the expression of CPT1A. The application of hypoxic culture conditions (1% O_2, 94% N_2, and 5% CO_2) significantly upregulates the EV gene encoding CPT1A and its relevant enzymes. Interestingly, the treatment of EVs from hypoxia-preconditioned hP-MSCs showed an improved therapeutic effect in a renal I/R injury model when compared with hP-MSCs without preconditioning. The maintenance of CPT1A, along with mitochondrial oxidative phosphorylation proteins (ATP synthase subunit beta (ATPB), succinate dehydrogenase subunit B (SDHB), cytochrome c oxidase IV (COX IV)), was found to be crucial in the process of normalizing FAO and mitochondrial homeostasis.

In several studies, the preconditioning of MSCs with MT has been studied as a facile strategy to develop functionalized EVs for treating I/R injury. For example, Alzahrani reported the therapeutic advantages of MT preconditioning of BMSCs in functionalizing EVs for treating renal ischemia-reperfusion injury (RIRI) [93]. They demonstrated that EVs from MT-preconditioned BMSCs can alleviate not only renal ischemic and pro-inflammatory lesions but renal function in vivo. Furthermore, their mechanistical studies revealed that MT-preconditioned BMSCs-derived EVs can promote tissue regeneration and angiogenesis by reducing oxidative stress and regulating inflammation.

Other organs

In the optical system, retinal ganglion cells (RGCs) are located in the ganglion cell layer of the retina and receive electrical signals, subsequently transmitting them to the brain. RGCs are vulnerable to loss of optical nerve through trauma or degenerative optic neuropathies [94]. Glaucoma, a disease caused by the death of RGCs, is a major cause of severe vision loss. Current treatment strategies include laser therapy, incisional surgery, and pharmacotherapies to reduce intraocular pressure [95]. Although reducing intraocular pressure is the primary therapeutic approach for managing glaucoma, it is inadequate to halt its progression. Recent research has revealed that stem cell therapy, known for its regenerative effects, can decrease intraocular pressure, restore damaged RGCs, and prevent vision loss [96]. To overcome the shortcomings of MSCs remaining in the vitreous body after injection, MSCs-derived EV treatments have been devised and demonstrated to offer outstanding protection and regenerative effects on RGCs through the effective delivery of neurotrophins and upregulated miRNA [97].

The preconditioned MSCs-derived EVs promote abundant contents by inflammatory cytokine compared to traditional EV treatments. For instance, Mead et al. isolated EVs derived from recombinant human TNF-α-primed MSCs after 48 h [98]. TNF-α-primed MSCs-derived EVs promote neuroprotection by increasing pigment epithelium-derived factor (PEDF), VEGF-A, and platelet-derived growth factor (PDGF)-AA in EVs. Consequently, TNF-α preconditioning can upregulate neurotrophic proteins and isolate efficiently EVs without loss.

Anticipated therapeutic outcomes of each preconditioning method

In an attempt to enhance the function of MSCs-derived EVs, a variety of research strategies have been developed that involves the isolation of EVs from culture media by varying the culture conditions of MSCs. Although the impacts of preconditioning methods have not yet been systematically organized, mounting evidence suggests that each preconditioning method indicates a common effect. Based on recent progress in the field, we provide a brief overview of the expected therapeutic outcomes of preconditioning of MSCs on EVs according to each preconditioning method as outlined below.

Hypoxic culture

Typically, the tension of oxygen gas in the adult tissues is lower than the inhaled ambient oxygen tensions (21%), and it is heterogeneous in the microenvironment of each tissue. For example, most tissues have oxygen tensions in a range of 2–9% [99], while certain tissues exhibit a relatively hypoxic condition (bone marrow 1–7% [100], adipose tissue 3% [101], fetal circulation ~ 5% [102]). When oxygen tensions drop below a particular threshold, tissues activate hypoxia-inducible factor-1 (HIF-1) to maintain cellular homeostasis through angiogenesis, vascular

tone, metabolism, and cell survival-related gene expression [29]. Among these, HIF-1α, a subunit of HIF-1, plays a role in tissue responses against hypoxic conditions. In particular, HIF-1α promotes the proliferation of MSCs in the surrounding tissue and sustains the multipotency of MSCs by reducing chromosomal aberrations, markers of DNA damage response, aneuploidy, double-strand breaks (DSBs) and telomere shortening rates brought on by oxidative stress [103, 104]. Furthermore, recent studies revealed that HIF-1α can adjust the function of MSCs-derived EVs toward anti-inflammatory, anti-apoptotic, functional recovery, and wound healing processes by modulating proliferation, differentiation, angiogenesis, and mobility-related molecules. Therefore, there have been continous attempts to functionalize MSC-EVs by preconditioning HIF-1α for the regulation of inflammatory responses.

In respective studies, conducted by Zhang et al., and Cui et al., hypoxia preconditioned BMSCs-derived EVs have showed to result in the downregulation of TNF-α and IL-1β in both OA and AD via the inactivation of the NF-κB signaling pathway, even though the oxygen tension of each studies are different [30, 56] In a similar manner, the treatment approach for inflammatory osteolysis by Tian et al. likewise demonstrated that hypoxia preconditioned DPSCs-derived EVs showed downregulation of IL-1β, TNF-α, IL-6, and iNOS via inhibiting NF-κB expression [31]. Furthermore, Liu et al. demonstrated that hypoxia preconditioned BMSCs-derived miR-216a-5p in EV not only reduced pro-inflammatory cytokines (IL-2, TNF-α, and TGF-β) but shifted microglial polarization from M1 to M2 phenotype, downregulating TLR4 protein level by directly targeting TLR4 3′UTR [55]. With regard to the suppression of cellular apoptosis, Zhu et al. utilized 1% O_2 preconditioned BMSCs-derived EVs in MI to suppress the mRNA and protein levels of pro-apoptotic effectors, including p53 and BAK1. Conversely, Mao et al. assessed the cytoprotective effect of 100% N_2 preconditioned ADSCs-derived EVs in MIRI by means of upregulating Bcl-2 and GATA4 but downregulating TXNIP, Bax and cleaved caspase-1 [84, 85]. In a parallel study, Mao et al. and Ren et al. reported that EVs from hypoxia preconditioned BMSCs enhanced cell viability as a result of regulating both anti-apoptotic (Bcl-2) and apoptotic factors (Bax, and cleaved caspase-9) [36].

Cytokine co-incubation

Cytokines are critical immune system regulators that respond to stimuli by modulating gene expression and thereby suppressing or stimulating immune responses [105]. For example, pro- or anti-inflammatory cytokines (e.g., IFN-γ, TNF-α, and TGF-β) typically trigger Janus kinases (JAK)/signal transducers and activators of transcription (STAT) [106], NF-κB [107], and phosphoinositide 3-kinase (PI3k)/Akt [108], which are involved in cellular behaviors, such as cell proliferation, differentiation, cell survival, and migration. Additionally, these cytokine-mediated genetic perturbations tone to the EV contents and influence adjacent cells by transferring adapted proteins and genes to recipient cells. Therefore, EVs play a key role in these processes, and the upregulation of anti-inflammatory or regenerative factors within cells has the potential to enhance EV functions. Wang et al. used TGF-β1 preconditioned MSCs-derived EVs to demonstrate the proliferation of chondrocyte and cartilage repair in OA as a result of upregulating specific miRNA (miR-135b) in EVs [32]. Also, Zhang et al. proved that EVs secreted from IFN-γ preconditioned hNSCs are superior to hNSCs-derived EVs in enhancing cell viability, proliferation, and migration of hNSCs injected into the ischemic regions of the brain [58]. Furthermore, Lu et al. confirmed the effect of EVs released from TNF-α preconditioned ADSCs promoting not only proliferation and migration but differentiation of HOBs as a novel approach to bone regeneration [43]. Meanwhile, Nakao et al. and Losurdo et al. insisted that pro-inflammatory cytokines preconditioned MSCs-derived EVs induced upregulation of anti-inflammatory cytokines, followed by the prohibition of persistent inflammation and alleviation of injuries through an increase in transformation of M2 polarization [33, 57].

Drug co-incubation

Preconditioning of certain drugs (e.g., LPS, MT, metformin, thrombin, and EPA) has been shown to enhance the therapeutic potency of EVs. Especially, functionalized EVs derived from LPS-preconditioned MSCs have been the focus of recent studies. Researchers have utilized EVs which encapsulated anti-inflammatory molecules via transition to an anti-inflammatory response after the TLR pathway proceeded by treating the LPS in MSCs. Duan et al., Shi et al., and Chen et al. used LPS-treated MSCs-derived EVs in the musculoskeletal system to normalize organs by diminishing tissue destruction and regenerating damaged tissue [44–46]. In common, they showed regenerative factors such as COL2A1, COLI, VEGF, aggrecan, fibronectin, periostin, and neurofascin. In the cardiovascular system, Xu et al. validated that LPS-preconditioned BMSCs-derived EVs reduce post-infarction inflammation and myocardial injury, regulating M1/M2 macrophage polarization in MI as a cell-free therapy substitute for stem cell therapy [86]. Meanwhile, MT, mainly secretion by the pineal gland, has been shown to upregulate M2 macrophage polarization of MSCs by playing the role of antioxidants, including the revitalization of antioxidant enzymes and augmentation of the efficiency of oxidative phosphorylation in mitochondria [109]. Consequently, MT-preconditioned

MSCs-derived EVs are capable of controlling antioxidative stress and M1/M2 macrophage polarization. Liu et al. and Alzahrani et al. assessed M2-like factors using MT-preconditioned BMSCs-derived EVs [59, 93]. As a result, Liu et al. validated functional behavioral recovery after SCI in mice and Alzahrani et al. reported not only decreased ischemic and pro-inflammatory lesions but oxidative stress in RIRI. Concurrently, Liao et al. documented that metformin preconditioned BMSCs-derived EVs decreased senescence markers (e.g., p16 and p21) by upregulating AMPK, unveiling new opportunities in intervertebral disc degeneration [47].

Discussions and future perspectives

Traditionally, stem cell therapy has been considered a major branch of regenerative medicine that offers an indispensable opportunity to address intractable diseases. However, the inherent limitations of stem cells as living entities, including challenges related to mass production and quality control, have historically impeded their clinical translation. In recent decades, studies on EVs have advanced from the finding of their role in paracrine effects of MSCs to functionalization of their regenerative potential by preconditioning. As a non-living material, MSCs-derived EVs are low immunogenic, tumorigenic, and suitable for long-term storage and transportation. For this reason, they have emerged as a safer and more effective alternative to MSCs for regenerative therapy. Additionally, adjusting the EV contents, such as membrane proteins and inner contents provides an exceptional advantage as nanomedicine.

In this review, we have focused on summarizing the reinforced therapeutic effect of MSCs-derived EVs following certain preconditioning (e.g., hypoxia, cytokines, and drug). Coincidentally, it has been found that under certain conditions, EVs isolated from MSCs can have altered EV content or enhanced EV production. Through many trials, it was found that certain preconditioning can enhance the therapeutic potential of EVs for certain diseases [47, 77]. These pre-conditioned EVs can regulate a more specific range of cells, as opposed to the broad cytostatic and immunosuppressive effects of conventional MSC-EVs. For example, Shen et al. showed that BMSC-derived EVs cultured in 3% oxygen applied to cartilage injury readily upregulated miRNAs, especially miRNA-205-5p, compared to normoxic EVs, and these changes could accelerate musculoskeletal and neurological repair, alleviate inflammatory responses more efficiently, and induce a normalized immune response, all of which are irreversible with conventional MSC-EVs [37]. Meanwhile, Riazifar et al. used IFN-γ, a representative anti-inflammatory, to precondition UC-MSCs [34]. The application of IFN-γ preconditioning allows highly accumulated anti-inflammatory-related noncoding RNA such as tRNA, lincRNA, miRNA, and antisense RNA in EVs. Hence, it shows the potential of treatment for autoimmune and neurodegenerative disorders via inhibition of immunocyte infiltration and pro-inflammatory. As such, the functionalization of MSC-EVs through this preconditioning provides a groundbreaking opportunity to modify inanimate materials, MSC-EVs, to target diseases, just as living stem cells actively respond to their environment to exert therapeutic effects. However, several scientific considerations have remained significant hurdles in practical applications of functionalized EVs by preconditioned MSCs.

First, the difficulty of identifying and controlling active ingredients has remained a significant hurdle. EVs contain thousands of types of miRNAs and proteins, which has made it difficult to identify the active component and mode of action in their therapeutic effects. Additionally, the perturbation of preconditioning into MSCs and their EVs is difficult to predict because it acts on a wide range of mRNAs and miRNAs. However, the experimental results so far have been too fragmented to be organized, since most studies have only considered a small fraction of the influences by preconditioned MSCs and their EVs. In particular, the role of EVs other than exosomes, and the effects of preconditioning on them, remains largely unknown. For example, apoptotic bodies contribute to the maintenance of tissue homeostasis in multicellular tissues, while ectosomes contribute to intercellular signalling, albeit to a lesser extent than exosomes. However, their physiological effects are still in the early stage, and the separation of EVs into each subtypes remains a challenge to date. Therefore, the vast majority of studies on functionalization of MSC-EVs have focused on exosomes. Therefore, we expect that in the future, as the basic research on EVs is further developed, the scope of our work can be extended to the functionalization of other EV types through pretreatment.

Second, the consistency and reproducibility of previous research are controversial due to the lack of uniformity in experimental conditions for each study. Furthermore, the absence of a standardized methodology for the preparation of EVs has also given rise to discrepancies in EV concentration and dose.

Third, the reproducibility of MSCs and their EVs has hindered the standardization of EV-based therapeutics, since the functions of MSCs vary depending on biological factors such as the health status of the donor, tissue sources, and culture condition. Finally, the requisite improvements to EV production technology have been demanded for the practical implementation of EV-based therapies. Due to the function and proliferation of MSCs are decreasing by repeated proliferations, the reproducibility and production yield of MSC-derived EVs have been limited. Additionally, the absence of optimized

technologies for the large-scale production and purification, in conjunction with long-term storage, has persisted as a significant impediment to the practical application of EVs. Meanwhile, in the assessment of given these obstacles, the majority of studies in EVs and functionalized EVs for therapeutic applications remain at the proof-of-concept stage rather than progressing to advanced research into clinical trials and commercial applications.

To surmount the current challenges of functionalized EVs in practical applications, the following future perspectives have been suggested. First, it is crucial to identify the relationship between EV components and the proteome and transcriptome of MSCs in order to gain a deeper understanding of the influences of preconditioning on the therapeutic potential of EVs. Probably, a meta-study would be an excellent tool for exploring the hidden pieces of a thorough understanding of relationships between each former study. From an analogous perspective, Liu et al. used the variable nearest neighbor method based on molecular structure to predict chemical-induced genome wide gene expression changes [110]. Likewise, efforts to standardize EV production and analysis methods will provide a foundation for the machine learning method linked to multicenter study. It is thought to be applicable for precisely predicting the specific miRNAs in EVs based on preconditioning. Moreover, the ability to predict the perturbation of cell preconditioning will facilitate the development of personalized medicine based on each patient's disease microenvironment. Therefore, it is imperative to establish precise good manufacturing practice (GMP) regarding the size, specific markers, source, and isolation methodology to enhance reproducibility and purity in the large-scale production of clinical-grade EVs. In addition, for preconditioning with cytokines or drugs, it is necessary to evaluate the existence of the remaining cytokine or drug in the EVs in order to identify the exact active ingredient.

On the other hand, limited delivery routes of EVs have remained a significant hurdle, limiting the clinical applications of EV-based therapeutics. Since EVs have a size in a range of several tenth nanometers, they are unavailable for crossing most biological barriers, which have tight junctions, such as epidermal barrier, intestinal barrier, corneal barrier. Therefore, their administration route has been limited to intravenous or other locally invasive approaches. Accordingly, advanced formulations to facile delivery of EVs by incorporating EVs into microneedles or inhalables may extend the applicability of EVs and improve the patient compliance [111, 112]. Furthermore, the therapeutic efficacy of EVs can be enhanced by introducing additional therapeutic modalities through co-loading adjuvants into the formulation [113]. By addressing these conundrums, we believe that preconditioned MSC-derived EVs have the potential to represent a breakthrough in the treatment of intractable diseases, with the ability to usher in a new era of regenerative medicine.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare no competing interests.

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