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Review

Engineered Extracellular Vesicles: A potential treatment for regeneration

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SUMMARY

Extracellular vesicles (EVs) play a critical role in various physiological and pathological processes. EVs have gained recognition in regenerative medicine due to their biocompatibility and low immunogenicity. However, the practical application of EVs faces challenges such as limited targeting ability, low yield, and inadequate therapeutic effects. To overcome these limitations, engineered EVs have emerged. This review aims to comprehensively analyze the engineering methods utilized for modifying donor cells and EVs, with a focus on comparing the therapeutic potential between engineered and natural EVs. Additionally, it aims to investigate the specific cell effects that play a crucial role in promoting repair and regeneration, while also exploring the underlying mechanisms involved in the field of regenerative medicine.

INTRODUCTION

Extracellular vesicles (EVs) are membrane-bound vesicles released by cells that plays a pivotal role in intercellular communication by transporting important signaling molecules from parental cells to recipient cells.[1,2] EVs can be classified into four subclasses because of their different diameters and modes of occurrence: exosomes, apoptotic bodies, microvesicles and oncosomes.[1] In this review, we mainly focus on exosomes, which are generated through the endosomal pathway. The formation of exosomes begins with the invagination of the cytoplasmic membrane to form endosomes. Subsequently, the endosomal membrane further buds inward, leading to the formation of multivesicular bodies (MVBs). During this process, cytoplasmic components and transmembrane proteins are incorporated into the vesicles.[2] When MVBs fuse with the cell membrane, the vesicles inside the MVBs are released as exosomes into the extracellular environment through internal budding (Figure 1).

EVs are derived from various types of cells, and their cargo mainly includes proteins, nucleotides, lipids and metabolites. The differences in EV composition have different biological effects due to the active components inherited from their source cells. These components give EVs the characteristics of homologous targeting, immune escape and physiological regulation,[3] which also provides inspiration for our engineering strategies.[4–6] EVs can enter recipient cells through direct fusion, receptor targeting, or endocytosis (Figure 1) and activate related signaling pathways to exert their effects. Compared with other nanomedicines, such as liposomal nanoparticles, EVs exhibit several distinct advantages. First, EVs possess enhanced biocompatibility due to their natural origin as vesicles released by cells.[7] This feature reduces the risk of immune reactions and cellular toxicity, making EVs suitable for therapeutic applications. Second, EVs demonstrate superior cycle stability, which enables them to withstand the harsh extracellular environment and enhances their efficacy in delivering therapeutic payloads. Moreover, EVs have inherent abilities to capture pathogens,[8] allowing them to neutralize pathogens and making them potential candidates for treating infectious diseases. One of the most significant advantages of EVs is their ability to transport cargo across biological barriers. The presence of specific membrane proteins and receptors on EVs enables targeted delivery to specific cell types or tissues, thereby reducing off-target effects and enhancing therapeutic efficacy. However, the direct use of EVs faces challenges, such as insufficient targeting, efficacy, and dosage. To overcome these limitations, various engineering strategies are being used to optimize EVs for therapeutic purposes. These strategies include surface modification of EVs to enhance targeting capabilities, the loading of specific therapeutic agents, and genetic engineering to enhance therapeutic efficacy.

As a promising next-generation nanoplatform, engineering EVs allows them to carry specific types of cargo to cells. In this review, we first summarize the applications of engineered EVs in various repair and regeneration processes in recent years (Table 1). Next, we review the available strategies for engineered EVs in regenerative medicine and divide them into two categories: engineered EVs produced by donor cells and directly engineered EVs. In addition, we explore the potential mechanism of the biological effects at the cellular level. Finally, we discuss the advantages and limitations of engineering in preclinical applications and summarize the key opportunities and challenges currently faced by engineered EVs.

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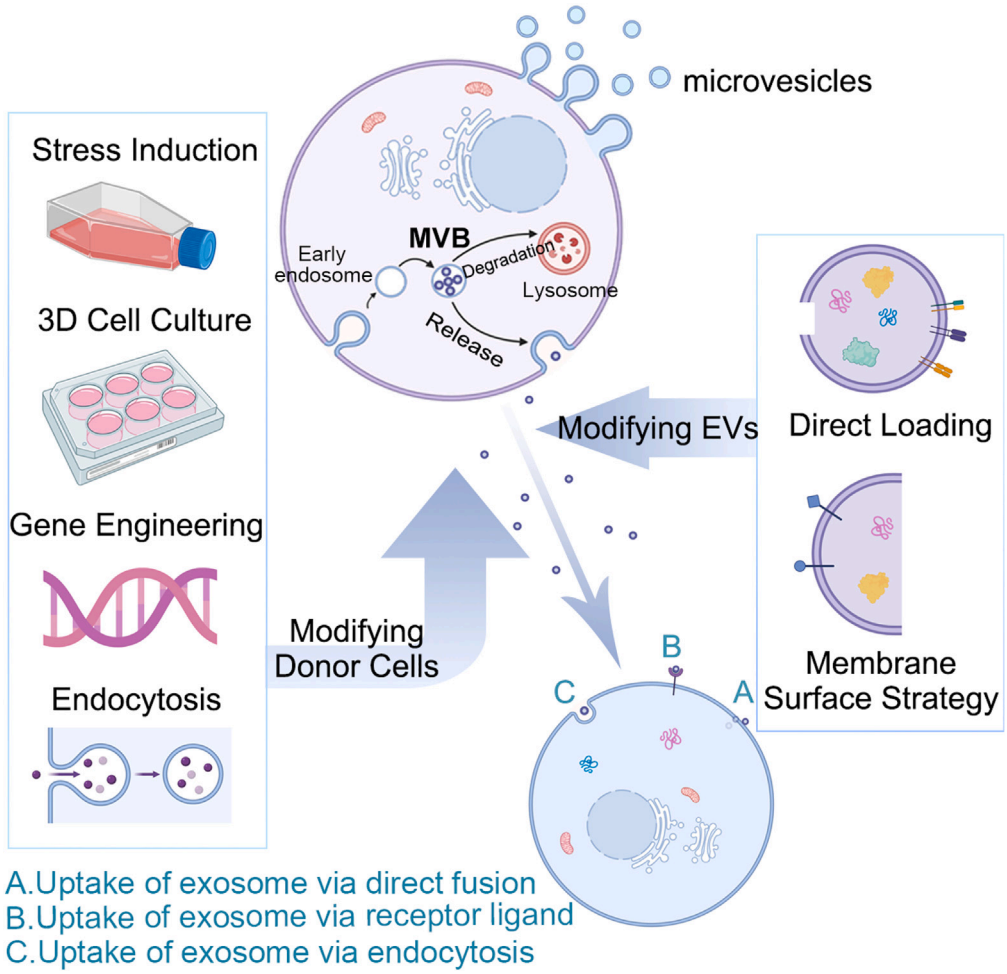

Figure 1. Engineering strategy of EVs: Divided into modifying donor cells (left) and modifying EVs (right)

Treatments of donor cells include stress induction、3D cell culture、gene engineering and endocytosis. Treatments of EVs include direct loading and membrane surface strategy. Exosomes are released into the extracellular environment by budding within MVBs. Recipient cells receive exosome mainly through three ways: A. uptake of exosome via direct fusion B. uptake of exosome via receptor ligand interaction C. uptake of exosome via endocytosis.

STRATEGIES FOR EXTRACELLULAR VESICLE ENGINEERING

Due to a lack of natural EVs or the limitations of inefficient cell targeting, on-demand delivery and treatment feedback, many methods have been developed to improve the performance of EVs.[9,10] EV engineering methods are mainly divided into two categories. One method can regulate the generated EVs by modifying the donor cells,[11] and the other can directly modify EVs to obtain optimized performance.[12] Modifying donor cells indirectly produces engineered EVs by changing the cell culture environment (such as stress induction or 3D cell culture) or changing the cell contents (such as genetic engineering or endocytosis). The direct engineering method can be divided into direct loading and membrane surface strategies according to the specific operation mode.

Modifying donor cells

Stress induction

In response to stress induction (ischemia, hypoxia, inflammation, and so forth) and treatment with exogenous compounds, donor cells can secrete EVs with different properties.[13] The yield of donor cell-derived EVs increases in response to low pH treatment.[14] Additionally, Gong's study[15] demonstrated that low pH reprogramming of EVs significantly enhanced cell targeting specificity. This effect may be attributed to alterations in the proportion of lipid components on the surface of EVs induced by low pH culture conditions, and the distinct composition of lipids could explain the observed differences in the uptake properties of EVs. Compared to cells cultured under normal oxygen conditions, a range of components are encapsulated in EVs and transferred to recipient cells to exert protective effects,[16] such as promoting wound healing[17] and angiogenesis,[18] regulating the extracellular matrix (ECM),[19] and inhibiting inflammation.[20,21] Furthermore, the oxygen-glucose deprivation (OGD) environment changes the expression profile of donor cells.[22] Accumulating evidence suggests that EVs generated under

Table 1. Application of engineered extracellular vesicles in injury

Cell source	Tissue	Specific components	Modification methods	Biological effects	publication date	Reference
USCs	cartilage	miR-140–5p	gene engineering	Overexpression of miR-140-5p and alleviation of OA progression by down-regulating VEGFA.	2022	Liu et al.[51]
ADSCs	cartilage	curcumin	incubation	Reduce oxidative stress and protect chondrocytes.	2022	Xu et al.[142]
BMSCs	cartilage	miR-216a-5p	hypoxia	Cartilage repair in osteoarthritis via delivery of miR-216a-5p.	2021	[143]
BMSCs	cartilage	circRNA_0001236	gene engineering	Up-regulate circRNA _0001236 and inhibit cartilage degradation through miR-3677-3p/Sox9	2021	Mao et al.[144]
M1 macrophage	bone	miR-21a-5p	gene engineering	The osteogenic differentiation of MC3T3-E1 was accelerated by directly targeting GATA2 with miR-21a-5p.	2023	Luo et al.[145]
M2 macrophages	bone	H_2S	incubation	H2S induced the polarization of M2 macrophages and modified the protein profile of exosomes, with a significant enrichment of the moesin protein.	2023	Zhou et al.[146]
BMSCs	bone	miR-26a	gene engineering	Promoting bone regeneration by suppressing genes associated with osteoclast differentiation instead of causing harm to osteoclasts.	2023	Kuang et al.[147]
U937	bone	Cathelicidin/LL-37	gene engineering	Engineered exosomes possess enhanced antimicrobial and angiogenic activities, and more effectively promote proliferation and migration of skin cells.	2022	Su et al.[148]
BMSCs	bone	RGD	incubation	Synergistic effects of EVs and RGD enhance osteogenic differentiation and mineralization of BMSCs *in vitro*.	2022	Ma et al.[108]
BMSCs	bone	miR-935	gene engineering	Targeting STAT1 by delivery of miR-935 promotes osteoblast proliferation and differentiation.	2021	Zhang et al.[109]
BMSCs	muscle	IL6ST	incubation	Selective inhibition of IL6 signaling pathway, thereby inhibiting STAT3 phosphorylation.	2021	Conceição et al.[149]
PMSCs	muscle	collagen-binding peptide SILY	click chemistry	Enhanced adhesion of EVs to collagen surface.	2022	Hao et al.[150]
C2C12	muscle	miR-29b	gene engineering	Prevent muscle atrophy induced by dexamethasone, angiotensin II and tumor necrosis factor alpha.	2022	Chen et al.[151]
BMSCs	muscle	TGF-β1	incubation	Targeting FABP3 by delivery of miR-29a.	2022	Xu et al.[152]
Fibroblasts	nerve	TFAP2C	COS Incubation	COS induce fibroblasts to produce TFAP2C-enriched exosomes, which are then transferred into axons to promote axon regeneration via miR-132-5p/CAMKK1.	2023	Zhao et al.[153]
BMSCs	nerve	SIRPα	gene engineering	Accelerating hematoma clearance and improving secondary white matter injury following intracerebral hemorrhage.	2023	Gao et al.[154]
UCMSCs	nerve	miR-146a-5p	gene engineering	Promoting neurological recovery in rats with acute spinal cord injury by targeting neurotoxic astrocytes.	2022	Lai et al.[155]
HEK293	nerve	Tom40	gene engineering	Protect cells reduce oxidative stress.	2022	Sayeed et al.[52]
ADSCs	nerve	miR-25	Oxygen-glucose deprivation	Promoting differentiation, maturation and migration of oligodendrocyte precursor cells.	2021	Zhai et al.[53]
VSC4.1	nerve	miR-126-3p	Oxygen-glucose deprivation	The up-regulated miR-126-3p may have a protective effect by regulating PI3K/Akt and NF-κB signaling pathways mediated by PIK3R2.	2021	Wang et al.[156]

(*Continued on next page*)

Table 1. *Continued*

Cell source	Tissue	Specific components	Modification methods	Biological effects	publication date	Reference
ADSCs	vascular	circ-Snhg11	hypoxia	Circ-Snhg11 regulates the migration, proliferation, and vascular regeneration potential of vascular endothelial cells through the miR-144-3p/NFE2L2/HIF1α pathway.	2023	Hu et al.[157]
ADSCs	vascular	FPG3	electrostatic and hydrophobic interaction	Enhancing the biological function of extracellular vesicles in cell migration and angiogenesis	2023	Ma et al.[158]
BMSCs	vascular	islet-1	gene engineering	Increased retention of extracellular vesicles in endothelial cells and enhanced resistance to apoptosis, proliferation and angiogenesis.	2022	Hu et al.[54]
DPSCs	vascular	LOXL2	hypoxia	Up - regulated LOXL2 may be involved in angiogenesis mediated by engineered extracellular vesicles.	2022	Li et al.[17]
UCMSCs	liver	HSTP1	gene engineering	Precise treatment of hepatic stellate cells in complex liver tissues.	2022	Lin et al.[159]
A549	lung	ACE2	gene engineering	Surface protein receptors mediate host defense by binding and inhibiting pore-forming toxins secreted by bacterial pathogens.	2022	Ching et al.[160]
PSCs	lung	siRNA	electroporation	Specific targeting of lung tissue and inhibition of SARS-CoV-2 pseudovirus infection *in vivo*.	2022	Fu et al.[161]
plasma	colon	siRNA	gene engineering	Rapidly alleviates intestinal inflammation by inhibiting pro-inflammatory responses in colon macrophages and T cells.	2022	Zhou e al.[162]
ADSCs	corneal	miRNA 24-3p	gene engineering	Accelerate corneal epithelial defect healing and epithelial maturation	2023	Sun et al.[163]

ACE2, angiotensin-converting enzyme 2; ADSCs, adipose-derived mesenchymal stem cells; Akt, protein kinase B; A549, adenocarcinomic human alveolar basal epithelial cells; BMSCs, bone marrow mesenchymal stem cells; C2C12, mouse myoblast cell line; COS, chitosan oligosaccharides; FPG3, fluorinated peptide dendrimers; HIF1α, hypoxia inducible factor-1α; HSTP1, HSTP1 peptide; IL6ST, interleukin 6 cytokine family signal transducer; LOXL2, lysyl oxidase homolog 2; NFE2L2: nuclear factor, erythroid 2 like 2; NF-κB, nuclear Factor kappa B; OA, osteoarthritis; PI3K, phosphatidylinositol 3-kinase; PIK3R2, phosphoinositide-3-kinase regulatory subunit 2; PSCs, pluripotent stem cells; RGD, Arg-Gly-Asp; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SIRPα, signal regulatory protein α; STAT1, signal transducer and activator of transcription 1; TFAP2C, transcription factor AP-2 gamma; Tom40, mitochondrial membrane protein; UCMSCs, umbilical cord mesenchymal stem; USCs, urine-derived stem cells; U937, human macrophage cell line; VSC4.1, spinal cord anterior horn motor neuron tumor cells.

OGD conditions contribute to neuroprotection and facilitate neuronal functional recovery in a cerebral ischemia model by modulating autophagy.[22–24] Electrical stimulation therapy also exerts significant therapeutic effects on neurological damage. After being subjected to electrical stimulation, Schwann cells increase their secretion of EVs and upregulate the expression of neurotrophic factors.[25] This effect is attributed to the ability of electrical stimulation to induce dorsal root ganglion cells to secrete glutamate, which binds to glutamate receptors on oligodendrocytes and promotes calcium influx.[26] Studies have shown that the increase in intracellular calcium levels in various cell types triggers the fusion of lysosomes and cell membranes, thereby inducing exocytosis and increasing the release of EVs.[27] Additionally, employing 3D culture systems, incorporating growth factors, reducing passage number, and co-culturing with certain cell types are all potential approaches that can enhance cellular state and result in the secretion of EVs with distinct regenerative capabilities.[28,29]

Mesenchymal stem cells (MSCs) are often used as donor cells due to the large number of EVs secreted and the absence of any toxicity or immunogenicity *in vivo* or *in vitro*.[1] Inflammatory stimuli such as TNF-α, IFN-γ, LPS, and thrombin[30,31] enhance the therapeutic potential of MSCs. *In vitro*, TNF-α-pretreated MSC-EVs inhibited proinflammatory M1 markers (such as IL-1β and iNOS) and increased reparative M2 markers (such as Arg1 and CD206), and enhanced immunomodulatory properties. *In vivo* experiments showed more bone regeneration than in the control group.[32] TNF-α stimulation not only increased the number of EVs but also enhanced the expression of CD73 on the surface of EVs.[33,34] Zhang showed that the rapid proliferation and migration of cells during repair were attributed to the adenosine activation of AKT and ERK signal transduction mediated by CD73 on the surface of EVs,[35] but the precise molecular mechanism mediated by exogenous CD73 has not been verified. After LPS pretreatment, the expression of glutathione reductase (GSR) and superoxide dismutase (SOD1) in EVs secreted by MSCs was upregulated. These proteins can consume ROS and reduce oxidative damage. In addition, engineered EVs promote the polarization of macrophages to the M2 phenotype through ROS/ERK signaling and inhibit the inflammatory response.[36]

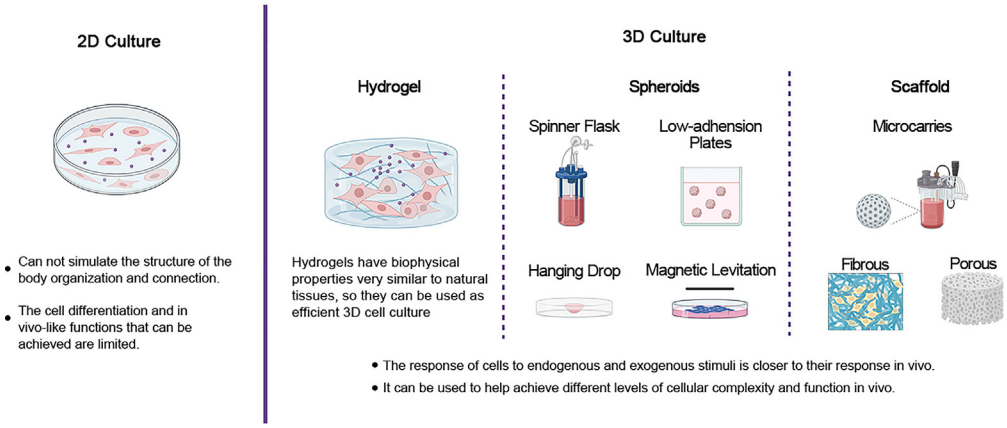

Figure 2. Characteristics of 2D culture and 3D culture: In 2D culture, cells grow on the plastic surface of the culture bottle or dish (left)
3D culture is mainly divided into hydrogel, cell aggregation and attachment scaffold culture (right).

Various studies have shown that pretreatment methods can enhance the regenerative potential of EVs, the efficacy of these strategies is contingent upon the specific exogenous compounds used and their concentrations, as well as the way these factors interact with donor cells. To improve the effect of engineering, we often need higher concentrations of compounds and longer exposure times. However, exceeding a certain limit may damage cell viability or even change the phenotype of the donor cells. Therefore, exploring more suitable compound concentrations and exposure times to maximize the effect of engineering may be a future research direction.

3D cell culture

The cellular microenvironment is regulated by many factors, and the ECM surrounding the cell provides necessary biochemical and mechanical signals.[37] Some scholars have noted that the traditional 2D *in vitro* culture system cannot faithfully imitate the characteristics of cells in the physiological state.[28] 2D culture affects not only cell movement but also gene expression and the secretion of paracrine factors.[38] Compared with 2D culture, 3D culture allows cells to grow in a physiological topology, and the most prominent manifestation is a significant increase in EV secretion[39] (Figure 2). Evidence shows higher yields of MSC-derived EVs cultured from 3D bioreactors, and these cells secrete more interleukins and cytokines than MSCs cultured in 2D.[40,41] Some scholars hypothesize that the differential expression of genes in spherical culture is partly due to the neutral hypoxic environment of the sphere. Hypoxia-related genes such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), stromal cell-derived factor 1 (SDF-1) and Transforming growth factor β (TGF-β) are overexpressed in the spheres.[42,43] There is evidence that EVs produced in a 3D culture system promote the transformation of macrophages from a proinflammatory phenotype to an anti-inflammatory phenotype in the periodontal tissue of mice with periodontitis, and the mechanism may be related to miR-1246 in EVs.[44] Studies have shown that EVs in a 3D culture system restore the Th17 cell/Treg balance through the miR-1246/Nfat5 axis, providing a basis for the EV treatment of periodontitis and inflammatory bowel disease.[45]

Further research shows that imaging, analysis, and optimization of 3D culture systems have become key challenges.[40] Moreover, various 3D culture methods are needed to determine the optimal 3D culture conditions for different cell lines.[46] Overall, existing studies have shown that dynamic 3D culture systems have great potential in increasing the yield and bioactivity of the secretome (Table 2). 3D culture is expected to become a popular trend in cell culture.

Genetic engineering

EV cargo enter and reprogram target cells, and these methods are increasingly popular EV engineering strategies.[47] During EV engineering, the target peptide or ligand[48] is first fused with the transmembrane protein expressed on the EV surface. Subsequently, the plasmid encoding the fusion protein is transfected into donor cells, and the donor cells secrete engineered EVs with targeted ligands on their surface.[48] Among them, LAMP-2B is the most widely used exosome surface protein.[2,11,48] The N-terminus of LAMP-2B is expressed on the surface of EVs and can be attached to a targeting sequence. LAMP-2B can also be genetically fused with targeting proteins or antibody fragments to result in targeting proteins on exosomes.[49] However, the targeting portion introduced into the EV membrane may affect the normal function of the membrane protein. In addition, engineered EVs may require improved isolation and purification methods compared to unmodified EVs.

In addition, lentivirus transfection technology is often used to make donor cells obtain specific gene sequences.[11,50] Lentiviral vectors are gene therapy vectors based on HIV-1 and have been widely used for EV cargo loading.[51–54] Compared to the passive introduction of exogenous DNA plasmids through transfection,[55] lentiviruses can actively integrate exogenous DNA into host cells, and the transfection efficiency is high.[56,57] For some cells that are difficult to transfect or even unable to be transfected by conventional methods, the virus can also achieve efficient transient expression of the target gene and achieve efficient and stable transduction of cells and animals.[11]

Table 2. 3D culture

MSCs source	3days Formation method	EVs efficiency	Reference
ADSCs	Cellhesion VP	With the increase of EVs production, the expression levels of OCT4, NANOG and SSEA4 were significantly increased, and the wound healing ability was improved.	Kim et al.[164]
BMSCs	spheroids	Increased EVs production and decreased expression of F-actin may provide a favorable environment for the synthesis and secretion of EVs.	Kim et al.[165]
AMSCs	spheroids	With increased EVs production, hAMSC cells remain viable and pluripotent, promoting angiogenesis and immunosuppression.	Miceli et al.[43]
UMSCs	hollow-fiber bioreactor	A 7.5-fold increase in EVs production and activation of transforming growth factor β1 and Smad2/3 signaling promote cartilage regeneration.	Yan et al.[166]
DPSCs	ultra-low-attachment culture dish	Increased EVs production restores Th17/Treg balance through the miR-1246/Nfat5 axis	Zhang et al.[45]
BMSCs	Vertical-Wheel bioreactor	Increased EVs production, significantly enhanced axon growth, elongation and complexity	Jalilian et al.[39]
BMSCs	3D aggregates	The expression of neuroprotective and anti-apoptotic miRNAs increased significantly with the increase of EVs production.	Yuan et al.[167]

Cellhesion VP, an insoluble fiber composed of chitin-based polysaccharides; ADSCs, adipose-derived stem cells; AMSCs, amniotic mesenchymal stem cells; NANOG, nanog homeobox; OCT4, octamer-binding transcription factor 4; SSEA4, stage specific embryonic antigen 4; UMSCs, umbilical cord mesenchymal stem cells.

Endocytosis

Endocytosis refers to the process in which extracellular substances enter cells through membrane invagination and internalization. This is an important way for cells to obtain macromolecules and granular substances from the extracellular space.[58] Donor cells can internalize a variety of exogenous particles and produce a relatively high yield of engineered EVs containing nanoparticles.[59] Engineered EVs are internalized by cancer cells after coincubation to achieve effective cancer cell targeting and killing.[59,60] Studies by Kim[61] and Zhang[62] showed that MSCs and neutrophils that were pretreated with superparamagnetic iron oxide nanoparticles (SPIONs) produced magnetic EVs. SPIONs can move the drug to the designated area in the body in response to an external magnetic field with good targeting. However, the interaction between SPIONs and the magnetic field is complex. The parameters should be continuously adjusted according to the position of SPIONs in different organs and the exposure time of the magnetic field.[63] In addition, the magnetic field intensity generated by a single magnet decreases exponentially with the distance from the magnet, and magnetic targeting of SPIONs is typically limited to the surface area. The use of SPIONs is a challenge that requires a trade-off between immunotoxicity, magnetic responsiveness and increased deep tissue permeability.

Directly modifying extracellular vesicles

Direct loading

Direct EV loading methods generally include mixed incubation, electroporation, ultrasound, saponin treatment, freeze–thaw cycles and extrusion.[1,9,64] The advantage of electroporation is that not only can conventional small molecule drugs be loaded, but macromolecular DNA and nanoparticles can also be included.[65] This technique omits the inconvenience of designing and constructing miscellaneous vectors by directly inserting cargo into EVs via nanoscale channels.[9] However, the process of electroporation can also affect the zeta potential and colloidal stability of EVs and lead to RNA aggregation, resulting in low drug loading.[66] This method exhibits great potential for loading small RNAs, such as miRNAs and siRNAs, but remains technically challenging for loading lncRNAs, mRNAs, or other large nucleic acid drugs.[67] Compared with room temperature incubation and electroporation, mild ultrasound has the greatest loading capacity for the anticancer drug paclitaxel.[68] However, these EVs did not show encouraging therapeutic effects *in vivo*, which may be due to the mechanical shear force generated by the ultrasonic probe, which destroys the integrity of the phospholipid bilayer of the EVs, potentially allowing the loaded cargo to spread out when the membrane deforms.[69] The direct loading of EVs has some limitations, such as low loading efficiency,[70] EV aggregation and destroying EV membrane integrity, which affects EV activity *in vivo*.

While natural vesicles possess some inherent targeting functions, they are mostly limited to passive targeting, and their capabilities for on-demand drug release and treatment feedback are restricted.[12] However, in recent years, extensive research has been carried out on external engineering strategies.

Membrane surface strategies

The EV membrane or cell membrane surface strategy is an important engineering method that gives EVs specific functional ligands or high loading efficiency.[71] Direct EV membrane modification mainly involves the functionalization of EVs by covalent or noncovalent methods.[72] Typically, covalent methods such as biological coupling, aldehyde amine condensation and click chemistry are used to bind the targeting portion to the EV membrane through covalent bonds, while noncovalent methods are used to bind the targeting portion to the EV membrane through hydrophobic insertion, fusion, receptor–ligand binding and multivalent electrostatic interactions.[73] Conjugation reactions covalently and stably modify EV surface proteins,[74,75] and lipids or amphiphilic molecules can be inserted into the lipid bilayer of EVs, allowing their hydrophilic portions to be displayed externally.

The emergence of click chemistry, which is rooted in oligosaccharide engineering, has revolutionized the approach to modifying EV membranes. This innovative technique has resulted in a wide array of click reactions that can be used to effectively modify the surface of EVs.[76–79] For example, the amine groups on the surface of EVs can be easily modified with alkynyl groups,[12,78] and alkyne-labeled exosomes can be coupled with azide-containing reagents through a copper-catalyzed azide-alkynyl cycloaddition (CuAAC) click reaction. These clickable EVs can bind to specific targeting ligands on the cell surface under pathological conditions and achieve efficient and specific drug delivery through click chemistry.[80–82] Furthermore, biorthogonal engineering has garnered widespread attention as a one-step biological conjugation technique that replies on site-specific copper-free click chemistry.[77,80,83,84] This approach offers a rapid, biocompatible, and specific reaction within biological systems. Based on bioorthogonal reactions, biomacromolecules can be specifically labeled, allowing real-time observation of their dynamic behavior and functional changes within living cells.[77,80] This approach also aids in determining the suitability of EVs as site-specific delivery vehicles for therapeutic purposes.[84,85] Moreover, chemical strategies rely on the biological conjugation of targeted ligands to surface proteins, but the complexity of EV surface can lead to the inactivation of surface proteins or the aggregation of EVs.[73]

Research on noncovalent EV modification strategies is increasing.[86] The lipid bilayer membrane of EVs can spontaneously fuse with other types of membrane structures.[87] As a common drug carrier, liposomes can be mixed with EVs. Mixed nanovesicles typically have the beneficial characteristics of both EVs and liposomes.[88] Sun designed a fibroblast-derived EV and clodronate-loaded liposome mixed drug delivery system. This mixed vesicle preferentially accumulated in the fibrotic lung and had significantly increased penetration within fibrotic pulmonary tissue through targeted delivery, further enhancing the inhibitory effect of clodronate on fibrosis.[89] Apart from drugs, large nucleic acids, including CRISPR/Cas9 expression vectors, can be encapsulated in mixed EVs, providing a new strategy for gene manipulation. While there is a possibility that lipid self-assembly could increase exosome toxicity,[64] it cannot be denied that mixed nanovesicles are an efficient approach to engineering EVs.[90] By avoiding mechanical methods such as electroporation and extrusion, this strategy prevents vesicle damage and enhances assembly efficiency.

In order to track EVs *in vivo*, various strategies have been developed to effectively label EVs. Molecular imaging techniques, such as fluorescence imaging, bioluminescence imaging, radioactive isotopes, and magnetic resonance imaging, can non-invasively monitor the absorption, distribution, metabolism, and excretion of EVs, providing valuable data for regenerative therapies.

EV membranes typically have negative potentials.[91] Scholars have fixed EVs on the surface of cationic materials through electrostatic interactions to improve the targeting of engineered EVs.[66] Recent studies have shown that protein coronas can spontaneously form around EVs in plasma in some cases.[92] Previous studies have regarded proteins in EV preparations as pollutants, but an increasing number of studies have shown that EVs can associate with specific molecules in the blood.[93] This leads us to hypothesize that the formation of this corona is common in biofluids and that the mechanisms may also involve protein aggregation and electrostatic interactions. Furthermore, this finding suggests that we should further explore the interaction and mechanism between EV membranes and matrix components. The formation of coronas provides a new perspective for engineering EVs.

The shortcomings of traditional drug delivery systems lie in their inability to precisely deliver drugs to specific disease sites, resulting in poor treatment outcomes. Additionally, synthetic nanocarriers cannot match EVs in terms of biological distribution and bioavailability.[80,94] In contrast, EVs, which are natural drug carriers, have the potential to become primary drug delivery vehicles because they can avoid the negative effects of systemic drug delivery. Although EVs generally have the ability to cross the blood–brain barrier, after systemic administration, EVs tend to accumulate in the liver[49] and then distribute to the spleen, gastrointestinal tract, and lungs.[94,95] Apart from intravenous injection, other routes of administration, such as local injection (intramuscular, subcutaneous, intraperitoneal), inhalation,[96] and implantation,[97] can better facilitate the accumulation of EVs around the injection site. Furthermore, direct targeting of EVs can avoid complex cell engineering procedures and reduce the relative dose, thereby enhancing the effectiveness of intravenous EV delivery.[49,85]

The incorporation of targeted molecules into EVs not only enhances their binding affinity to target cells but also reduces the uptake of therapeutic drugs by nontarget cells during delivery. One effective approach is to modify EVs to allow them to evade macrophage-mediated clearance. A relatively novel method to prolong circulation time and reduce the clearance of nanomedicines by the mononuclear phagocyte system (MPS) is achieved by depleting macrophages using EVs modified with cationized mannan. This cationized dextran modification enhances the targeting of EVs to macrophages, leading to MPS saturation and minimizing the clearance of subsequently administered nanomedicines. By incorporating or expressing different molecules, such as CD24, CD3, CD44, and CD47, on the surface of EVs, it is possible to prolong their circulation time, reduce the frequency and dose of administration, minimize off-target interactions, and enhance the ability of EVs to reach the required concentration levels for desired biological effects in target tissues/organs. These targeting molecules are inspired by mechanisms used by tumor cells to evade the immune system. These engineering strategies provide opportunities for research and the application of EVs as potential nanocarriers for drug delivery (Figure 3).

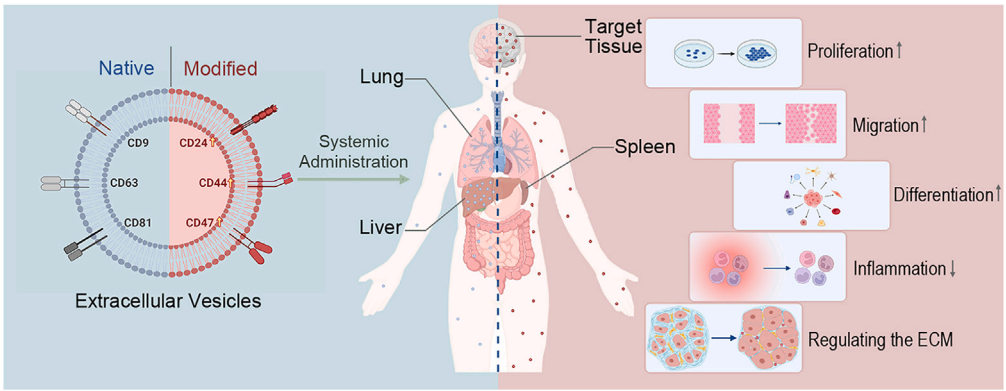

Figure 3. The mechanism of modified EVs avoiding phagocytosis and promoting repair and regeneration: After being administered systemically, EVs tend to accumulate in the liver and subsequently disperse to organs such as the spleen and lungs

To enhance the bioavailability and extend the circulation duration of EVs, various molecules such as CD24, CD44, and CD47 can be integrated onto the EVs' surface (left). Modified EVs have become potential regenerative therapies of great concern by promoting the proliferation, migration, and differentiation of receptor cells, reducing inflammation levels, and regulating the surrounding extracellular matrix to promote regeneration (right).

MECHANISMS BY WHICH EXTRACELLULAR VESICLES PROMOTE REPAIR AND REGENERATION

Promoting cell proliferation and migration and regulating differentiation

In previous studies, a variety of cell-derived EVs were associated with promoting proliferation and differentiation, including the proliferation of damaged tissue cells and the proliferation,[9] migration,[98] and tube formation of endothelial cells.[19,99,100] Engineered EVs secreted by hypoxic preconditioned cells have been shown to promote the proliferation of a variety of cell types.[101,102] Jagged1 (a Notch ligand) is upregulated in hypoxia-pretreated MSC-derived EVs. Coculture of these EVs with hematopoietic stem cells (HSCs) can enhance the self-renewal, proliferation and clonal formation of HSCs.[103] In a wound healing model, hypoxic preconditioning enhanced EV-mediated paracrine functions of UCMSCs, promoted the proliferation and migration of endothelial cells by upregulating miR-125b targeting of tumor protein p53 inducible nuclear protein 1 (TP53INP1), inhibited apoptosis, and obtained better results than EVs obtained from normal oxygen culture conditions.[104] In a study of fracture healing, hypoxic preconditioning enhanced the production of miR-126 in EVs by activating HIF-1α. MiR-126 activates the Ras/Erk pathway by inhibiting the expression of SPRED1, thereby causing proliferation, migration and angiogenesis in human umbilical vein endothelial cells (HUVECs) *in vitro*. *In vivo* experiments showed that hypoxic exosome administration promoted fracture healing through exosomal miR-126 transfer.[101]

Gene overexpression or silencing can also magnify repair. Genetic engineering can explicitly overexpress or silence the target gene, but this is limited to regeneration projects in which some specific mechanisms are known.[11,47,58,105] The level of miR-21 was increased in in EVs released by ADSCs overexpressing miR-21. In addition, *in vitro* experiments revealed that the expression levels of HIF-1α, SDF-1, VEGF, Collagen I, Collagen-III, *p*-Akt and *p*-ERK1/2 were upregulated in miR-21-overexpressing engineered EVs.[47,58] Experiments showed that the enrichment of miR-21 significantly enhanced the proliferation and migration of HUVECs and induced angiogenesis through Akt and ERK activation and the upregulation of HIF-1α and SDF-1 expression.[47] Adipsin-overexpressing EVs can be taken up by cardiac microvascular endothelial cells (CMECs), thereby increasing CMEC proliferation, inhibiting CMEC apoptosis, mitigating cell migration defects and accelerating wound healing.[105] These results pave the way for EV treatment, demonstrating that engineered EVs can mediate distant communication between different cells through the differential expression of RNA, thereby reducing cell damage, promoting cell proliferation and migration, and improving wound healing.

Cell differentiation in tissue regeneration is closely related to culture conditions.[98] The 3D culture system can activate the mechanical transducer and transcription factor YAP, which may be one of the mechanisms by which 3D-EVs stimulate osteogenesis.[106] Compared with that in traditional 2D culture, the expression of Runt-related transcription factor 2 (Runx2), osteopontin, collagen type I alpha 1 and alkaline phosphatase was significantly increased after treatment with EVs secreted by 3D culture, and the osteogenic ability of BMSCs was enhanced.[107] The use of EVs to deliver osteogenic-related peptides[108] and coding RNAs[53,109,110] to promote cell differentiation and regeneration is also a useful strategy.

Reducing inflammatory injury

Inhibiting the release of inflammatory cytokines is the most direct way to reduce inflammation. Gupta[111] used genetic engineering to add the cytokine binding domains of TNFR1 and IL-6ST to the surface of EVs as bait for TNF-α and IL-6, respectively, which significantly increased the survival rate of mice by inhibiting the level of inflammatory cytokines. In addition to binding to inflammatory factors, EVs engineered with specific proteins on the surface can bind to autoantibodies, cytokines or bacterial toxins to protect against invasion. In response to bacterial DNA, human and mouse cells can secrete EVs carrying ADAM10.[112] ADAM10 binds to pore-forming toxins as bait to protect the body from bacterial toxins. Scientists have suggested that circulating miR-21-5p levels may be associated with inflammation and the cell senescence

factors known as inflammatory miRNAs, and inhibiting this expression can promote tissue regeneration.[113] Recent studies have reported that miR-21-5p overexpression in EVs promotes the fibrotic process of tendon-to-osseointegration.[114] Additionally, some studies have shown that miR-21-5p in EVs promotes injury repair by enhancing autophagy to promote cell proliferation[115] and inhibit osteoclast differentiation.[116] MiR-21-5p does not play a negative role. We have reason to believe that miR-21-5p may be a double-edged sword for signal regulation. Its function may vary depending on the cell state and the specific molecular network involved.

Inflammation is the body's defense response to stimuli such as injury and infection, and immune regulation plays an important role in each stage of inflammation.[32] Macrophages have three major phenotypes: the nonpolarized state M0, the activated inflammatory M1 phenotype (induced by bacterial LPS, IFN-γ, or TNF-α) and the activated anti-inflammatory M2 phenotype (induced by IL-4 and IL-13).[30] As immune-related factors, M1 macrophages exhibit increased secretion of proinflammatory mediators, while M2-like macrophages have an anti-inflammatory phenotype.[33] A variety of MSC-derived EVs can change macrophage polarization from proinflammatory (M1) to anti-inflammatory (M2) *in vitro* and *in vivo*, and some pretreatment methods can amplify this effect.[21] In many diseases, macrophages cannot convert to the M2 phenotype. A continuous M1 proinflammatory state can hinder the healing process.[117,118] However, M2 macrophages are not the only cell type that is beneficial to repair. On the one hand, the role of M1 macrophages in antibacterial activity and promoting the secretion of angiogenic factors in the early stage of inflammation is often underestimated.[34] On the other hand, excessive accumulation of M2 macrophages at the site of injury can become unfavorable for tissue repair.[31] In fact, so-called M1 and M2 macrophage markers can be simultaneously expressed by a single cell, indicating the diversity of polarization states.[36] To make the comparisons of studies more transparent, some scholars suggest adding a description of the stimulation parameters to the naming of macrophages, such as M(LPS) rather than M1.[119] Understanding the behavior of macrophages is critical to deciphering the etiology of a disease. We need to reconsider the definition and standardization of macrophage phenotypes.

Regulating the extracellular matrix

Inflammation and remodeling are the results of profibrotic factor activation.[120] Tissue fibrosis is an irreversible lesion. Fibrotic tissues lose their normal function and can be life-threatening in severe cases.[121] Hepatic fibrosis is a common pathological process in various chronic liver diseases.[2,67,122,123] Hepatic stellate cells play an important role in liver injury. After being activated, these cells become myofibroblasts, which are the main effector cells of liver fibrosis and respond to liver injury by producing excessive amounts of ECM.[123] Compared with that in normal 2D culture, miRNA array data showed that miR-6766-3p expression was upregulated in EVs secreted by 3D cultured stem cells. Engineered EVs deliver miR-6766-3p to activated human hepatic stellate cells and inactivate SMAD signaling by inhibiting TGF-β receptor (TGFβRII) expression to inhibit cell proliferation and promote fibrosis, thereby ameliorating liver injury.[122] Relaxin (RLN) is an antifibrotic peptide hormone. Studies have shown that there is a correlation between the reversal of activated hepatic stellate cells *in vivo* and the endogenous peptide hormone RLN.[2] In a mouse model of CCl4-induced liver fibrosis, RLN-pretreated macrophage-derived EVs showed higher levels of miR-30a-5p than natural macrophage-derived EVs, delaying the progression of liver fibrosis by reducing serum alanine aminotransferase and aspartate aminotransferase and reducing the expression of fibrosis-related genes.[123]

Glial cell line-derived neurotrophic factor (GDNF) is a tissue morphogen that affects stem cells and can reduce renal fibrosis.[124] In a model of unilateral ureteral obstruction (UUO), GDNF-overexpressing EVs showed increased SIRT1 expression and eNOS activation, which seems to be a mechanism to reduce peritubular capillary loss and prevent renal fibrosis.[50] A recent study showed that engineered mouse satellite cell-derived EVs with Lamp2b fused with rabies virus glycoprotein peptide (RVG) had increased renal targeting efficacy. In the UUO model, these modified EVs delivered miR-29 and had higher antifibrotic effects (reducing renal alpha-smooth muscle actin, collagen protein, wave protein, and fibronectin levels) than unmodified EVs.[125] Electroporation can facilitate the introduction of various cargoes into cells, including genes,[8] proteins,[10] viruses and other macromolecules. After being loaded with miR-29b mimics via electroporation, EVs can be effectively internalized by cardiac fibroblasts. In a mouse model of myocardial infarction, EVs loaded with miR-29b mimics were shown to upregulate the expression of miR-29b and downregulate the expression of fibrosis-associated proteins, thereby inhibiting myocardial fibrosis and improving cardiac function.[126]

Since fibrosis is a highly dynamic process, the use of this type of treatment is important for treating fibrosis.[127] Although there is still a gap between the identification of antifibrotic targets and translating research into effective treatments, there is increasing evidence that EVs, which are emerging antifibrotic agents, can reduce or reverse tissue fibrosis, providing a new perspective for effective antifibrotic treatment in the future.

INFORMED CONSENT STATEMENT

This article has obtained written informed consent from all authors. All co-authors agree to add Yanhong Zhao as a corresponding author. Written consent, named "Written Consent," has been uploaded as a JPG file.

DISCUSSION

In recent years, there has been a significant surge in research on EVs, and the study of exosomes has gradually transitioned from basic science to clinical applications.[128] There have been some clinical applications of engineered EVs documented on ClinicalTrials.gov. A clinical study reported beneficial effects of EVs overexpressing CD24, which underwent a biosafety review and targeted patients with moderate-to-severe

COVID-19 infections. Furthermore, several clinical projects involving the targeted delivery of exosomes and their use as mRNA drug delivery vehicles for gene therapy are expected to be completed in the coming years.

Despite these advancements, there remain several challenges that must be addressed for the successful clinical application of EVs.[129] During clinical translation, there is a close connection between the production process and the quality of the final product. The quality of the product, including yield, composition, and biological activity, can be significantly influenced by factors such as upstream and downstream processing choices. The culture conditions in the upstream process, including cell passaging, cell density, and EV harvesting frequency, are all crucial factors. Currently, there is no uniform standard for engineering methods, such as hypoxia induction, exposure time and other parameters that affect the experimental results.[101,130] Furthermore, the tolerance of different cell types to hypoxia varies.[17] Hypoxia may lead to the secretion of differently engineered EVs by cells, and we cannot determine whether there is a correlation between cell death and EV release levels.

Although urine,[131] **milk,**[132] and plant-derived EVs[133] have relatively high yields, their targeting ability and cargo efficiency are relatively poor, posing challenges for their use as clinical therapeutic drugs. Bioreactors can be used to increase the yield of EVs, and 3D culture allows for affinity interactions between cells and provides more diffusion space. In a dynamic 3D microcarrier-based system, MSCs expanded more than 13 times in 5–6 days.[134] By using a scaffold-perfusion bioreactor system, the yield of EVs increased by more than 100-fold compared to that in traditional 2D flask culture.[135] Cell culture bioreactors with industrial-grade volumes are already available, and airlift bioreactors and stirred tanks are the most commonly used types.[134] However, one drawback of traditional bioreactors is that due to enhanced cell metabolism, the culture medium needs to be frequently replaced, which increases the risk of contamination during the opening process.

Technical difficulties in the isolation and characterization of pure populations of specific subtypes are a key limitation to the precise characterization of EVs.[1,136] Currently, commonly used methods for isolating EVs include differential ultracentrifugation (UC), tangential flow filtration (TFF), density gradient centrifugation, size exclusion chromatography (SEC), polymer-based precipitation, and immunomagnetic bead capture. Each method has its own advantages and disadvantages. Among them, differential ultracentrifugation is considered the gold standard for EV isolation.[137] This large-scale production technique typically only allows for separation based on EV size, but the product may contain small EVs and non-EVs.[1,138,139] Therefore, unless their multivesicular body origin has been determined, these factors are collectively referred to as EVs.[138] To achieve large-scale and efficient recovery of EVs without compromising purity, multiple methods are needed. The International Society for Extracellular Vesicles (ISEV) proposed the Minimal Information for Studies of Extracellular Vesicles (MISEV) in 2014 to define EVs and their functions. In 2018, an updated version was released to promote the establishment of unified standards for the isolation, identification, quantification, and quality control of EVs.[137]

Overall, the field of engineered EVs regenerative medicine shows great potential. To enhance treatment efficacy, it is imperative to delve deeper into the specific mechanism and develop engineered EVs with increased targeting precision and precise cargo loading. Some studies have not significantly reduced the accumulation of EVs in clearance systems such as the liver,[140] even if they are specifically targeted. Attempts to fully understand the effective targeting of EVs, the required therapeutic dose,[141] the effect on targeted tissues, and long-term safety depend on further studies. With the rapid accumulation of preclinical data,[128] research on EVs should focus on improving production and purification techniques and developing more standardized guidelines. In summary, standardized EV production, separation, and characterization methods will improve the effectiveness of engineered EVs in regenerative medicine.

LIMITATION

While this review aims to provide a comprehensive overview of EVs, it is important to acknowledge its limitations. The field of EVs research is rapidly evolving, and new discoveries are made frequently. Therefore, this review may not include all the latest findings. Additionally, due to the vast number of studies on EVs, we have focused on specific aspects related to exosomes, excluding other EVs such as apoptotic bodies. The heterogeneity of EV populations, isolation methods, and characterization techniques pose challenges in comparing and interpreting results across studies. Limited space prevents extensive discussions. Although EVs show great potential in biomedical applications, further research is needed to fully understand their mechanisms and clinical implications. Future studies should address these limitations and fill the gaps in our knowledge.

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AUTHOR CONTRIBUTIONS

W.C. and YH.Z. were responsible for conceptualizing the article. YR.S. and C.X. were involved in the initial drafting of the article. YQ.S. and Y.L. contributed to the design and preparation of the figures. W.C. and YM.Z. conducted a critical review and revision of the article. Q.Y. played a role in acquiring funding for the project. All authors have read and approved the final version of the article for publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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