

## Review

## Extracellular Vesicle-Associated Proteins in Tissue Repair

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**The administration of (stem) cell-derived extracellular vesicles (EVs) promotes tissue repair through management of different inflammatory, proliferative and remodeling processes in the body. Despite the widely observed biological and therapeutic roles of EVs in wound healing and tissue repair, knowledge on how EVs activate recipient cells and which EV cargo is responsible for the subsequent functional effects is limited. Recent studies hint toward an important role for proteins as functional EV cargo. Here, we provide an overview of how EV-associated proteins promote tissue repair processes and discuss current challenges in evaluating their contribution to EV function and future directions for translating fundamental insights into clinically relevant EV therapies.**

### Extracellular Vesicles: Natural Vehicles of Biological Cargo in Tissue Homeostasis and Repair

Apart from **conventional secretory pathways** (see [Glossary](#)), cells secrete proteins, nucleic acids, and lipids that are delivered to other cells through vesicles generated via the endosomal pathway or upon direct budding of the plasma membrane. Collectively, these vesicles are called **extracellular vesicles (EVs)**. Despite initial thoughts about being nothing more than the garbage disposal system of cells, it is now well established that these EVs also play important roles in **tissue homeostasis** through short- and long-distance intercellular communication. EVs isolated from multiple cell types, tissues, and body fluids promote endogenous **tissue repair** through the modulation of several physiological processes in the body. As a result, EVs bear potential as therapeutics in **regenerative medicine**, and have been demonstrated to be a functional component of the **stem and progenitor cell secretome** [1]. Indeed, EVs have been found to promote different reparative processes in the body. Although many studies have set out to identify the functional cargo and cell activation pathways of EVs in tissue repair, the exact mechanisms by which these EVs exert their effects remain elusive. In this review, we describe why proteins expressed on (or in) EVs might be important contributors to EV function, and address the role of specific EV proteins in tissue repair processes in the body. Challenges in the study of individual EV-associated proteins and the implication for the translation of these insights into EV therapeutics will be discussed.

### EV Biogenesis and Recipient Cell Activation

EVs may be classified based on a set of characteristics including their size, density, process of cellular release, and their protein/lipid composition [2,3]. Three such subgroups are exosomes, microvesicles, and apoptotic bodies ([Box 1](#)). Although distinct in biogenesis, it is hard to capture and assign the heterogeneous population of EVs upon isolation toward different classes as they share overlapping physiochemical characteristics and biological markers [4]. To encompass all different vesicle types, the general term EV is increasingly used, as also in this review. EVs are enriched in distinct proteins and nucleic acids compared with their cells of origin, which suggests that a dedicated packaging mechanism is associated with EV biogenesis and loading. **EV cargo**

### Highlights

EV-associated proteins modulate different stages of endogenous as well as therapeutic tissue repair at multiple cellular levels.

Proteins present on EVs activate intracellular pathways in recipient cells involved in tissue repair through direct receptor binding; through their delivery into the cytosol, where they exert their biological effect; or through the capture, delivery, or conversion of bioactive molecules, in turn leading to recipient cell activation.

Some EV-associated proteins have specific immune-modulatory, reparative, and remodeling effects in individual tissues, while others exert pleiotropic effects in different tissues.

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**Box 1. EV Biogenesis and EV-Mediated Activation of Intracellular Signaling**

EVs can be subdivided in three main categories based on their biogenesis. Exosomes (diameter 30–120 nm) are produced in intracellular multivesicular bodies (MVBs) through invagination of an endosomal membrane, thereby capturing cytosolic components. After fusion of MVBs with the plasma membrane, exosomes are released into the extracellular space. Microvesicles, sometimes also referred to as microparticles or ectosomes, are more heterogeneous in size (diameter 50–1000 nm) and encapsulate cytosolic components via direct cytoplasmic membrane budding and fission. Apoptotic bodies (diameter 50–5000 nm) are released upon activation of apoptotic pathways during cell death. The biogenesis of EV subtypes has been reviewed in more detail elsewhere [104,105]. EVs can be characterized based on the expression of specific proteins associated with vesicle trafficking and biogenesis such as tetraspanins (CD9, CD63, and CD81), endosomal proteins (syntenin-1, Tsg101, and Alix), maturation-related proteins (flotillin and annexin), and heat-shock proteins (HSPs) [3,106]. Also other endosomal or plasma membrane proteins, such as integrins, selectins, and CD40 ligand have been demonstrated to be enriched on EVs.

The interaction of EVs with recipient cells is suggested to be mediated via specific protein and sugar moieties at, and the lipid composition of, both the EV membrane and the plasma membrane of the recipient cells. EV membrane proteins such as proteoglycans, lectins, and integrins, as well as ECM components, mediate the EV–plasma membrane interaction [104,107]. EVs may remain at the plasma membrane and directly initiate intracellular signaling pathways through receptor–ligand interactions, but may also be internalized via clathrin-dependent or clathrin-independent pathways including macropinocytosis, phagocytosis, and caveolae- or lipid raft-mediated endocytosis, leading to EV processing in the canonical endosomal pathway [6,104]. The uptake of EVs into endosomes and subsequent trafficking to lysosomes for degradation leads to the recycling of their contents within the recipient cell. Alternatively, the event of fusion of EVs with the membrane of endosomes or the plasma membrane leads to the delivery of their luminal cargoes into the cytoplasm of the recipient cells, and may lead to the exchange of transmembrane proteins and lipids [6]. EV-mediated recipient cell activation may also occur through the capture or delivery of extracellular molecules by EV-associated transmembrane proteins which prevents or promotes their binding to receptors at the plasma membrane. Also, EV-associated (proteolytic) enzymes can convert or release bioactive molecules which in turn can activate recipient cells.

is determined by the donor cell type as well as different physiological, pathological, and stress conditions of the tissue of origin [5].

EVs can activate recipient cells by directly activating receptors on the plasma membrane, or by intracellular delivery of their cargo (Box 1). However, it remains unresolved if targeting to recipient cells is specific or stochastic. Some EV types have been reported to be preferentially internalized by specific cell types, while others can be taken up by different cell types [6,7]. Upon recipient cell activation, EV cargo can activate a plethora of **intracellular signaling** cascades in recipient cells modulating cellular responses and tissue homeostasis.

**Proteins as Functional EV Cargo**

The physiological and therapeutic roles that EVs display depend on their cargos and their capacity to transfer proteins, nucleic acids, and other molecules into the recipient cells. Although many groups confirmed the presence of **miRNAs** in EVs and demonstrated the ability of these miRNAs to induce biological responses in target cells [8], its low abundance in EVs makes it less likely that miRNAs account for the majority of functional effects observed [9–12].

In tissue repair, the importance of EV surface proteins in recipient cell activation is demonstrated by the observation that proteinase K or trypsin digestion of EV surface proteins leads to a loss in downstream functional effects [13]. Although the removal of EV surface proteins also hampers EV uptake, recipient cell activation is often observed within minutes upon EV exposure which hints toward direct receptor–ligand interactions instead of intracellular delivery of functional cargo that includes miRNAs [14,15]. This is further underpinned by the observation that intracellular pathways can be activated even when only limited numbers of EVs are taken up, postulating that EVs might affect recipient cell function by direct surface contact or direct release of their soluble contents [16,17]. In the following sections, we highlight current evidence that EV function in tissue repair might, to a great extent, be regulated via a protein-based mechanism.

**Glossary**

**Angiogenesis:** process in which new blood vessels are formed out of pre-existing ones, contributing to nutrient and oxygen supply to (newly formed) tissue.

**Apoptosis:** regulated process in response to harmful external or internal stimuli resulting in changes in cell morphology and cell death.

**Canonical hedgehog signaling:** intracellular signaling pathway that orchestrates a plethora of developmental and physiological processes including angiogenesis and vascular remodeling, activated by Shh isoforms and mediated by the Gli family of transcription factors.

**Conventional secretory pathways:** trafficking route of secretory proteins when transported from the endoplasmic reticulum to the Golgi apparatus, and subsequently to the plasma membrane where they are released in the extracellular space.

**Extracellular vesicle (EV):** lipid bilayer-enclosed particle secreted by cells, transporting selective biomolecules including lipids, proteins, and nucleic acids.

**EV cargo:** content of EVs including different lipids, nucleic acids, and proteins dependent on donor cell type and secretory pathway.

**Fibrosis:** process of excessive deposition of extracellular matrix components replacing normal tissue, leading to scar tissue formation.

**Heat shock proteins (HSPs):** family of proteins known for their protein (un)folding function in the endosomal pathway. Various different HSPs are enriched on EVs, thereby contributing to EV-mediated cell activation directly or through their function as chaperone.

**Inflammation:** defense mechanism of the innate immune system in response to harmful stimuli to eliminate injurious agents and to remove damaged tissue components.

**Intracellular signaling:** series of molecular cascades by which a signal is transmitted through the cell, which is part of any communication process that governs basic cell activities. It regulates the ability of cells to perceive and correctly respond to their microenvironment.

**Mesenchymal stem cell (MSC):** an undifferentiated cell present among differentiated cells in tissues or organs.

## EVs in Modulating Tissue Repair: Individual Proteins Highlighted

Tissue injury can result from various stimuli, including infections, autoimmune reactions, toxins, radiation, and trauma. Multiple processes are essential in the subsequent repair of damaged tissues: (1) **apoptosis** inhibition and promotion of cell survival to prevent extensive cell loss, (2) modulation of the immune response to prevent uncontrolled **inflammation**, (3) **angiogenesis** in which endothelial cells migrate and proliferate to form new blood vessels, (4) cell proliferation and differentiation to repopulate lost cell types or to replace injured cells, and/or modulation of **fibrosis** and extracellular matrix (ECM) synthesis and subsequent scar tissue formation. These immune modulatory and tissue remodeling processes are regulated via EVs from several differentiated, mostly stromal, cells in the human body, and may be further stimulated by EVs released from stem and progenitor cells (Figure 1). Investigation into the specific protein composition of EVs responsible for these reparative effects has led to the discovery of specific EV-enriched proteins and EV-induced signaling cascades and mechanisms of cell activation (Table 1). Examples from cancer models are also included as they could provide more insights into EV protein-mediated cell activation. The functional contribution of EV-associated proteins in different reparative processes in the body will be discussed in more detail in the following sections.

### Prevention of Cell Death

In response to internal cellular damage or external (hypoxic) signals during tissue injury, several cell death pathways are initiated, triggering scar formation and tissue remodeling. Preventing cellular apoptosis and increasing cellular survival are essential in the process of tissue repair and maintaining organ homeostasis. Cell apoptosis is a process characterized by mitochondrial leakiness and dysfunction in response to the activation of proapoptotic signaling pathways, which can be prevented by EV-associated proteins in several tissues [18,19] (Figure 1A). Upon myocardial injury, exogenously administered **mesenchymal stem cell** (MSC) and cardiac progenitor cell (CPC) EVs reduce scar size and improve cardiac function through delivery of cardioprotective proteins [20] (Table 1). Also therapeutic overexpression of stromal-derived factor 1 (SDF1) and macrophage migration inhibitory factor (MIF) on these MSC-derived EVs prevents cell apoptosis and scar formation through the activation of intracellular prosurvival pathways [18,21] (Figure 2A).

An important EV-mediated strategy of tissue protection during ischemia/reperfusion injury is alleviation of oxidative stress caused by an imbalance in the generation of reactive oxygen species (ROS), for example, through the delivery of antioxidant enzymes [22]. A family of proteins harboring antioxidative and antiapoptotic properties are **heat shock proteins (HSPs)**. HSP molecules are not secreted via the conventional protein secretion pathway as they do not possess the signal peptides mediating this process [23]. One of the mechanisms of release of HSPs is through EVs, after which they protect cells and tissues against cell death and ischemia/reperfusion injury through direct receptor binding or through their function as chaperone [16,24]. By contrast, HSP70 is displayed in high levels on rodent and human plasma EVs released under hyperglycemic conditions, yet these EVs are unable to prevent apoptosis [25]. The absence of protective effects may be explained by changes in HSP glycation status, leading to protein inactivation. Moreover, HSP70 and HSP90 present on mouse lung carcinoma EVs induce malignant muscle wasting, which can be caused by their immunomodulatory properties and the induction of pro-inflammatory cytokine release [26]. This highlights the importance of the (disease) status of the donor cells and tissues as well as the disease model system to HSP configuration and function.

In protection against tissue damage, vascular endothelial growth factor (VEGF) plays an important role. Both deficiency and excess of VEGF appears to be detrimental to the physiological integrity of tissues, and an imbalance between VEGF and VEGF receptor 1 (VEGFR1) has been reported in many diseases [27]. VEGF-containing EVs, released by human umbilical cord MSCs, attenuate

MSCs can self-renew or differentiate into a specialized cell of multiple lineages.

**microRNA (miRNA):** small non-coding RNA that has a role in post-transcriptional regulation of gene expression through its function in RNA silencing.

**Regenerative medicine:** field of research that aims to repair, replace, or engineer damaged or diseased tissue and organs.

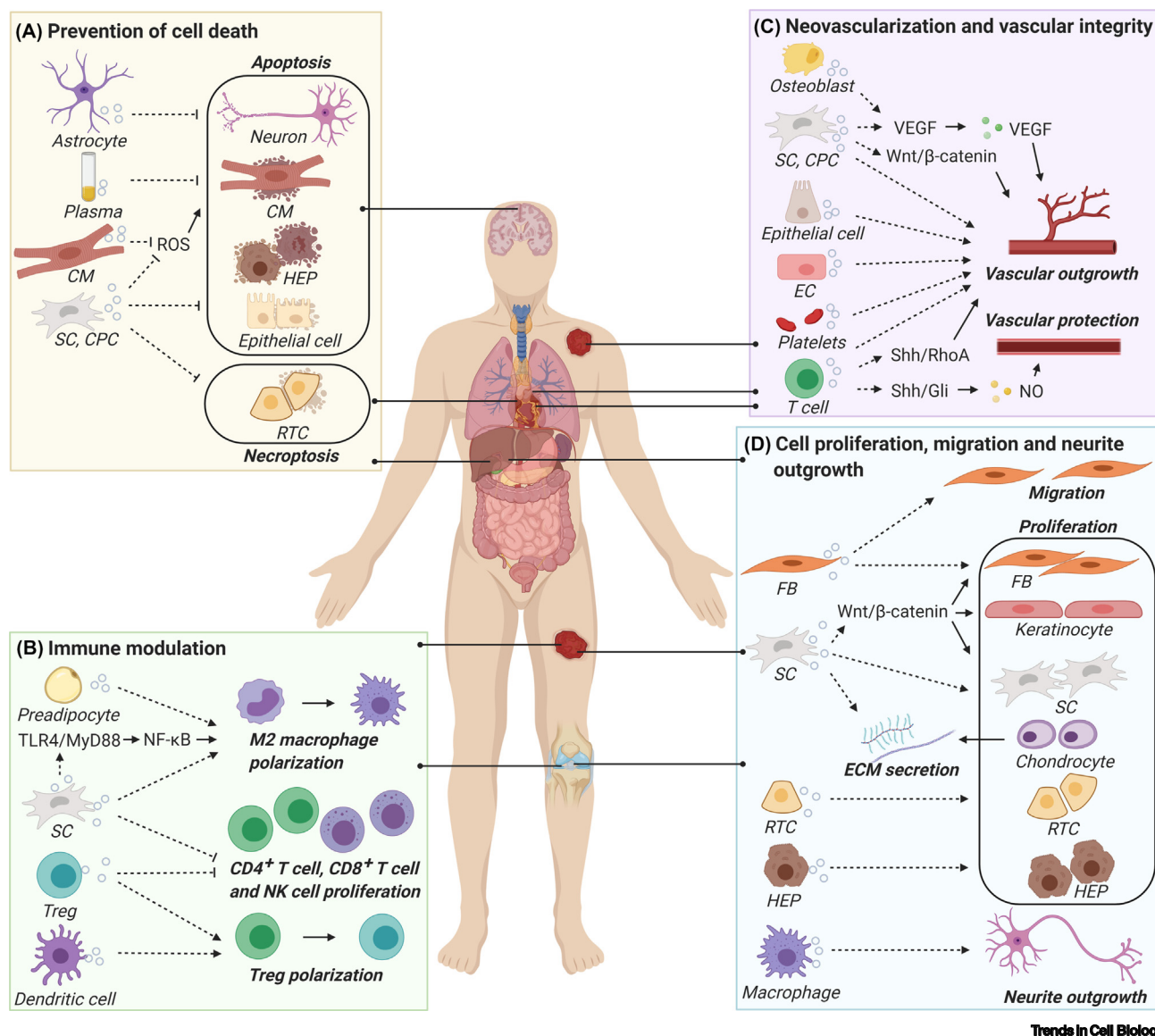
**Sonic hedgehog (Shh):** morphogen controlling vertebrate development, influencing cell fate, and affecting cell growth and survival through activation of intracellular canonical and non-canonical hedgehog signaling. Shh contains cholesterol and palmitate modifications for transport on EVs.

**Stem and progenitor cell secretome:** all proteins and cellular components of a cell secreted into the extracellular space.

**Tissue homeostasis:** balanced regulation of organ function in response to changes, to ensure healthy organ function.

**Tissue repair:** collective term for different processes in the body which are initiated after tissue injury, restoring original structures and function of the damaged tissue.

**Wnt ligands:** family of secreted hydrophobic glycoproteins that activate canonical and non-canonical intracellular Wnt signaling cascades. These pathways regulate cell proliferation, polarity, migration, and fate specification, thereby controlling development and tissue homeostasis.



Trends in Cell Biology

**Figure 1. Tissue Repair Processes Influenced by Extracellular Vesicle (EV)-Associated Proteins.** Proteins present on EVs isolated from multiple different cell types are able to prevent cell death, promote neovascularization and vascular integrity, modulate the immune system at different cellular levels, and induce cell proliferation, migration, and neurite outgrowth and the secretion of extracellular matrix (ECM) components. EV activity is represented by dotted lines. (A) EV proteins prevent apoptosis and necroptosis of several cell types. One postulated mechanism is through EV-mediated reduction of reactive oxygen species (ROS) levels. (B) Proteins present on EVs stimulate T cells and macrophages to undergo polarization toward anti-inflammatory regulatory T cell (Treg) and M2 phenotypes, and inhibit the proliferation of inflammatory CD4<sup>+</sup> T, CD8<sup>+</sup> T, and natural killer (NK) cells. An important mechanism of immune modulation is through the activation of TLR4/myeloid differentiation primary response 88 (MyD88)–NF-κB signaling. (C) Angiogenesis is promoted by the EV-mediated upregulation of intracellular pathways in endothelial cells including vascular endothelial growth factor (VEGF), Wnt/β-catenin, and sonic hedgehog (Shh)/RhoA signaling. Activation of Shh/Gli signaling and subsequent nitric oxide (NO) release lead to vascular protection. (D) EV-associated proteins activate migration and proliferation of multiple cell types. Proliferation of different cell types in the skin can be stimulated by the activation of Wnt/β-catenin signaling. Other EV-associated proteins induce ECM secretion and promote neurite outgrowth. Abbreviations: CM, cardiomyocyte; CPC, cardiac progenitor cell; EC, endothelial cell; FB, fibroblast; HEP, hepatocyte; RTC, renal tubule cell; SC, stem cell.

H<sub>2</sub>O<sub>2</sub>-induced epithelial cell death and lung injury in mice [28], while amniotic fluid stem cell-derived EVs capture excess of VEGF through VEGFR1 on their surface to protect against VEGF-induced glomerular endothelial cell apoptosis [27] (Figure 2A). These examples

Table 1. EV-Associated Proteins Promoting Tissue Repair Processes

Protein	Donor cell	Model	Target cell(s)	EV isolation method	Method of cargo manipulation	Reparative effect	Involved molecular mechanism	Refs
Prevention of cell death								
MIF	hB-MSC	<i>In vitro</i> ; MI rat model	CM	RIBO Exosome Isolation Reagent	Overexpression	Inhibition of apoptosis; reduction of mitochondrial fission and fragmentation; reduction of infarct size and improvement of cardiac function	Decreased ROS production; enhanced AMPK phosphorylation	[18]
MIF <sup>a</sup>	m-pancreatic ductal adenocarcinoma	<i>In vitro</i>	Hepatic stellate cell; Kupffer cell; macrophage	dUC	shRNA	Activation toward fibrotic microenvironment; premetastatic niche formation promoting liver metastasis	Increase of $\alpha$ -SMA, TGF $\beta$ , and FN expression	[97]
GJA-20k / Connexin 43	r-astrocytes	<i>In vitro</i> ; traumatic brain injury rat model	Neuron	dUC	shRNA	Inhibition of apoptosis; recovery of mitochondrial function; improved brain recovery	Reduction of Bcl-2, Bax, cytochrome C, and caspase-3; downregulation of Cx43 phosphorylation	[19]
PAPPA	hCPC	<i>In vitro</i> ; MI rat model	CM	dUC	siRNA	Inhibition of apoptosis; reduction of scar size; improvement of cardiac function	Cleavage of IGFBP4/IGF complexes; stimulation of Akt, ERK1/2, and IGFR phosphorylation; inhibition of caspase-7 activation	[20]
SDF1/CXCL12	huc-MSC	<i>In vitro</i> ; MI mouse model	Primary mouse myocardial cell	dUC	Overexpression	Stimulation of angiogenesis; inhibition of apoptosis; decreasing infarct size and inflammation	Upregulated expression of MMP-2, MMP-9, VEGF; increase in PI3K, Akt, and mTOR phosphorylation; increase in Bcl-2 expression, decrease in Beclin-1 and Bax expression	[21]
MnSOD (SOD2)	huc-MSC	<i>In vitro</i> ; hepatic I/R injury rat model	Hepatocyte	dUC	siRNA	Reduction of respiratory burst of neutrophils and prevention of oxidative stress-induced cell death; reduction of infiltrating neutrophils and alleviating oxidative stress	Decrease in proinflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ); reduction of ROS levels; reduction of caspase-3	[22]

HSP20	m-primary CM	<i>In vitro</i> ; STZ-induced diabetic mouse model	EC; CM	dUC	Overexpression	Stimulation of angiogenesis; inhibition of apoptosis; improved cardiac function	Increase of phosphorylated Akt, survivin, and SOD1 expression in EVs (chaperone function); reduction of ROS levels; increase in SOD1 expression in recipient cell	[24]
HSP70	Human and rat plasma	<i>In vitro</i> ; I/R rat Langendorff model	CM	dUC	Ab	Reduction of apoptosis; decrease in infarct size	Activation of TLR4 and p38MAPK; increase of ERK1/2 and HSP27 phosphorylation	[16]
HSP70	Plasma of Goto Kakizaki rats with type II diabetes; hyperglycemic HUVEC	<i>In vitro</i>	CM	dUC	/	No protective effects		[25]
HSP70, HSP90 <sup>a</sup>	m-lung carcinoma cell	<i>In vitro</i>	Myotubes	ExoQuick (System Biosciences)	Ab; siRNA	Stimulation of catabolic muscle wasting	Activation of TLR4; increase in p38 phosphorylation; elevation of proinflammatory cytokines	[26]
VEGF	huc-MSC	<i>In vitro</i> ; newborn Sprague-Dawley rats exposed to hyperoxia	Rat lung epithelial cell challenged with H <sub>2</sub> O <sub>2</sub>	dUC	siRNA	Attenuation of cell death; attenuation of hyperoxic lung injury; stimulation of alveolarization, angiogenesis, and macrophage activation	Activation of proinflammatory cytokine response	[28]
VEGFR1	h-amniotic fluid SC	<i>In vitro</i> ; glomeruli of mouse with Alport syndrome	Glomerular EC with VEGF overexpression	dUC	shRNA	Prevention of cellular damage; amelioration of proteinuria levels	Trapping of extracellular VEGF; reduction of VEGFR2 phosphorylation; decrease in VEGFR1 expression	[27]
SP1	h-iPSC-MSC	<i>In vitro</i> ; renal I/R injury rat model	Renal tubular cell	ExoQuick (System Biosciences)	CRISPR/Cas9	Inhibition of necroptosis; protection against I/R injury	Transcriptional activation of SK1; formation of S1P	[111]
Immune modulation								
PD-L1	TGF- $\beta$ -stimulated h-lung FB	<i>In vitro</i>	FB; T cell	dUC	Ab (Avelumab); siRNA	Stimulation of FB migration; prevention of T-cell proliferation		[33]

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Table 1. (continued)

Protein	Donor cell	Model	Target cell(s)	EV isolation method	Method of cargo manipulation	Reparative effect	Involved molecular mechanism	Refs
EDA-FN1	h-embryonic MSC	<i>In vitro</i> ; allogeneic skin graft mouse model	Monocyte; CD4 <sup>+</sup> T cell	TFF, SEC	Anti-EDA Ab	Induction of M2 polarization, thereby mediating CD4 <sup>+</sup> T-cell differentiation toward Treg and enhancing the survival of allogeneic skin	TLR4 activation; MyD88-dependent alkaline phosphatase expression; NF- $\kappa$ B translocation	[15]
CCR2	mBM-MSC	I/R renal injury mouse model	Macrophage	dUC	siRNA	Suppression of M1 and reduction of renal damage after I/R injury	CCL2 binding; inhibition of p65 phosphorylation	[34]
pSTAT3	m-adipose-derived SC	<i>In vitro</i> ; diet-induced obesity mouse model	Macrophage	ExoQuick (System Biosciences)	Inhibitor (cryptotanshinone)	Induction of M2 polarization	Increase in STAT3 phosphorylation and Arg-1 expression	[112]
TSG-6	Dog adipose tissue MSC	<i>In vitro</i> ; dextran sulfate sodium-induced colitis mouse model	T cell; macrophage	dUC	siRNA	Alleviation of inflammation in colon; induction of M1 to M2 polarization and Treg differentiation	Decrease in TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$ , IL-6, and iNOS expression; increase in IL-10 expression	[35]
TSG-6	huc-MSC	Neonatal hyperoxia-induced BPD mouse model	Lung, brain	dUC	siRNA; Ab	Reduction of brain and lung cell death; decrease in proinflammatory cytokine release; decrease in alveolar-capillary leakage and disrupted alveolar-capillary barrier	Reduced IL-6, TNF- $\alpha$ , and IL-1 $\beta$ expression	[113]
HSP70	mBM-DC exposed to supernatant of I/R-treated primary hepatocytes	<i>In vitro</i> ; liver I/R mouse model	Naive T cell	dUC	Ab	Induction of Treg differentiation; prevention of Th17 cell differentiation; reduction of I/R liver injury	Induction of PI3K expression, and of Akt and mTOR phosphorylation	[14]
HSP27 <sup>a</sup>	h-differentiated macrophage cell	<i>In vitro</i>	Macrophage	dUC	Addition of recombinant protein	Induction of anti-inflammatory macrophage activation	Activation of TLR4-NF- $\kappa$ B and IL-10 release	[32]
HSP90 $\alpha^a$	Murine melanoma cell	<i>In vitro</i> ; subcutaneous melanoma engraftment mouse model	CD4 <sup>+</sup> T cell; CD8 <sup>+</sup> T cell; Breg	Magnetic bead capture with LC3b Ab	Ab	CD4 <sup>+</sup> T-cell activation and differentiation, leading to Breg activation and inhibition of CD4 <sup>+</sup>	Activation of TLR2-MyD88-NF- $\kappa$ B signaling; induction of Akt, STAT3, and p38 phosphorylation; increase in IL-6, IL-10,	[48]

									and CD8 <sup>+</sup> antitumor response; stimulation of tumor growth			and IL-21 production	
HSP90	Serum of mice with alcoholic liver disease	<i>In vitro</i> ; alcohol naïve mouse model	Macrophage; Kupffer cell	ExoQuick (System Biosciences)	Inhibitor (17-DMAG)	Stimulation of immune cell recruitment to liver; activation of Kupffer cells and induction of macrophage polarization	Induction of TNF- $\alpha$ expression; decrease in IL-10 production	[114]					
HSP72 <sup>a</sup>	m-mammary or colon carcinoma cells	<i>In vitro</i> ; xenograft mammary or colon carcinoma mouse model	MDSC	dUC	Ab; shRNA	Activation leading to suppression of T-cell activation	TLR2-MyD88 activation; induction of Stat3 phosphorylation; IL-6 production	[115]					
HSP70 <sup>a</sup>	h-lung cancer cell	<i>In vitro</i> ; subcutaneous xenograft lung cancer mouse model	MSC	dUC	Ab	Induction of proinflammatory phenotype, leading to macrophage recruitment and stimulation of tumor growth	Elevated secretion of IL-6, IL-8, and MCP-1; activation of p65 phosphorylation and nuclear translocation; increase in IKK $\alpha$ / $\beta$ phosphorylation; activation of TLR2 signaling	[50]					
IDO1	IFN- $\gamma$ -treated h-amniotic fluid SC	<i>In vitro</i> ; allogeneic skin graft mouse model	T cell	dUC	siRNA	Decrease of T-cell proliferation and increase in Treg		[37]					
HLA-G	CD105 <sup>+</sup> renal cancer SC	<i>In vitro</i>	DC	dUC	Ab	Inhibition of DC differentiation and maturity, indirectly regulating T-cell immune response		[38]					
TGF- $\beta$ 1	Canine Wharton's jelly-MS	<i>In vitro</i>	CD4 <sup>+</sup> T cell	dUC	Ab	Inhibition of CD4 <sup>+</sup> T-cell proliferation	TGF $\beta$ -RI and adenosine A2A receptor activation	[13]					
TGF- $\beta$ 1	h-fetal liver MSC	<i>In vitro</i>	NK cell	dUC	Ab	Impairment of proliferation, differentiation, and cytotoxicity	Stimulation of the nuclear translocation of phosphorylated SMAD2/3	[39]					
TGF- $\beta$	h-endometrial MSC	<i>In vitro</i>	PBL; CD4 <sup>+</sup> T cell	dUC	Ab	Suppression of differentiation into effector memory cells		[44]					
TGF- $\beta$	hMSC	<i>In vitro</i>	PBMC; Treg; Th17 cell	dUC	Ab	Inhibition of islet antigen T-cell activation	Decrease of IFN- $\gamma$ , IL-17, IL-10 secretion; increase of TGF- $\beta$ , PGE <sub>2</sub> , IL-10, and IL-6 secretion	[45]					
TGF- $\beta$ 1 <sup>a</sup>	h-mesothelioma cell	<i>In vitro</i>	T cell	dUC, sucrose cushion	Ab	Inhibition of proliferation		[31]					

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Table 1. (continued)

Protein	Donor cell	Model	Target cell(s)	EV isolation method	Method of cargo manipulation	Reparative effect	Involved molecular mechanism	Refs
TGF- $\beta$ <sup>1a</sup>	Irradiation-induced apoptotic mT-cell tumor	Xenograft lymphoma tumor mouse model	DC	dUC	Ab	Inhibition of DC-stimulated CD8 <sup>+</sup> T-cell response and antitumor immunity		[116]
TGF- $\beta$ <sup>a</sup>	h-prostate cancer cell, h-mesothelioma cell	<i>In vitro</i>	FB	dUC, sucrose cushion	Ab	Induction of myofibroblast differentiation	Activation of SMAD3 signaling; increase of $\alpha$ -SMA expression; increase in FGF-2 secretion	[95]
TGF- $\beta$ <sup>a</sup>	h-prostate cancer cell line	<i>In vitro</i>	BM-MSC	dUC, sucrose cushion	Ab	Induction of myofibroblast differentiation	increase of $\alpha$ -SMA expression; induction of VEGF-A, HGF, and MMP-1, -3, -13 secretion	[96]
CD73, CD39 <sup>a</sup>	h-bladder cancer cell	<i>In vitro</i>	T cell	dUC, sucrose cushion	Inhibitor (AMOPC; POM-1)	Inhibition of proliferation and induction of proinflammatory cytokine release	Conversion of ATP to 5'-AMP, and 5'-AMP to adenosine	[36]
CD73	Serum of mice receiving hBM-MSC	<i>In vitro</i> ; human-into-mouse xenogeneic graft-versus-host disease model	CD4 <sup>+</sup> Th1 cell	Total exosome isolation reagent (Invitrogen)	A2A receptor blockade	Reduction of cell engraftment and inflammatory cytokine production	Conversion of 5'-AMP to adenosine	[40]
CD73	huc-MSCs	<i>In vitro</i>	T cell	dUC	Inhibitor (AMPCP)	Suppression of proliferation and induction of immunosuppressive response	Conversion of 5'-AMP to adenosine	[41]
CD73	m-primary CD4 <sup>+</sup> CD25 <sup>+</sup> Foxp3 <sup>+</sup> Treg	<i>In vitro</i>	CD4 <sup>+</sup> T cell	dUC	Inhibitor (AMPCP)	Suppression of proliferation	Conversion of 5'-AMP to adenosine	[43]
Adenosine <sup>a</sup>	h-breast cancer cell	<i>In vitro</i>	CD8 <sup>+</sup> cytotoxic T cell	dUC	Inhibitor (ADA)	Suppression of perforin secretion		[42]
ILK	mBM-EPC (with IL-10 knock out), cardiac TNF- $\alpha$ -treated EC	<i>In vitro</i> ; acute MI mouse model	EC	dUC	siRNA	Inhibition of angiogenesis; reduction of neovascularization and cardiac function; increase in apoptosis and scar size	Activation of NF- $\kappa$ B nuclear translocation and downstream proinflammatory gene expression	[49]
HMGB1 <sup>a</sup>	m-tumor cells	<i>In vitro</i> ; E. G7-OVA-bearing mouse model	B cell	dUC, Percoll gradient	shRNA	Stimulation of B-cell differentiation toward Breg leading to T-cell suppression	Activation of TLR2-MyD88-NF- $\kappa$ B signaling; increase in IL-10 production	[47]

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Table 1. (continued)

Protein	Donor cell	Model	Target cell(s)	EV isolation method	Method of cargo manipulation	Reparative effect	Involved molecular mechanism	Refs
Wnt3a	hBM-MSC	<i>In vitro</i>	Dermal FB; EC	dUC, sucrose cushion	Overexpression	Induction of FB proliferation and migration; stimulation of angiogenesis	Stimulation of LRP6; Wnt/TCF/LEF- $\beta$ -catenin reporter activation	[52]
Wnt4	huc-MSC	<i>In vitro</i> ; second-degree skin burn injury rat model	EC	dUC	shRNA	Stimulation of angiogenesis	$\beta$ -catenin translocation to nucleus; upregulation of PCNA, cyclin D3, and N-cadherin; downregulation of E-cadherin	[51]
TF	h-microvascular EC	<i>In vitro</i> ; mouse Matrigel plug assay; hindlimb ischemia mouse model		dUC	Overexpression	Stimulation of angiogenesis and vascular perfusion	Increase in $\beta$ 1-integrin and Rac1 expression; induction of ERK1/2 and ETS1 phosphorylation; elevated CCL2 production	[65]
uPAR, uPA	TNF- $\alpha$ -stimulated hEC	<i>In vitro</i>	EC	dUC	Ab	Stimulation of angiogenesis	Plasminogen to plasmin conversion	[66]
HSP90 $\alpha/\beta$ <sup>a</sup>	h-breast cancer cell; h-glioma cell	<i>In vitro</i>	Tumor cell	dUC	Ab	Stimulation of migration	Binding of tPA and conversion of plasminogen to plasmin; pro-MMP-2 activation	[67]
Shh	Stimulated hT cell	<i>In vitro</i> ; myocardial I/R injury mouse model	EC	dUC	Inhibitor (cyclopamine)	Enhanced perseverance of endothelial integrity and functionality	PI3K activation; upregulation of eNOS and eNOS and caveolin phosphorylation; induction of NO production; decrease of ROS levels	[54]
Shh	Stimulated hT cell	Hind limb ischemia mouse model	Aorta; skeletal muscle	dUC	Inhibitor (cyclopamine)	Stimulation of flow recovery	Upregulation of VEGF and eNOS expression, and of eNOS and Akt phosphorylation; induction of NO production	[68]
Shh	Stimulated hT cell	Angiotensin II-induced hypertension and endothelial dysfunction mice model	Aorta	dUC	Inhibitor (cyclopamine)	Correction of angiotensin II-induced hypertension and endothelial dysfunction	Increase in NO production; decrease in O <sub>2</sub> <sup>•-</sup> production	[69]
Shh	Stimulated hT cell	<i>In vitro</i>	EC	dUC	Inhibitor (cyclopamine)	Stimulation of angiogenesis	Increase in RhoA and VEGF expression and in FAK phosphorylation	[55]
Shh	hBM CD34 <sup>+</sup>	<i>In vitro</i>	EC; FB (reporter)	dUC, sucrose	Overexpression	Transfer and activation	GlI/ $\beta$ -galactosidase	[70]

	hematopoietic SC	<i>In vitro</i>	HEK293T (reporter)	cushion	Overexpression; Ab	Reporter activation	reporter activation	
Shh	hHEK293T	<i>In vitro</i>	HEK293T (reporter)	dUC, sucrose cushion	Overexpression; Ab	Reporter activation	Gli/β-galactosidase reporter activation	[71]
β1-integrin	Shh overexpressing hHEK293T	<i>In vitro</i>	Mouse embryonic SC	dUC, sucrose cushion	Ab	Induction of motor neuron differentiation	Increase in HNF3β expression	[71]
Shh	Mouse insulin-resistant adipocyte	<i>In vitro</i>	Macrophage	dUC	Ab; overexpression; inhibitor (cyclopamine)	Increase of macrophage adherence; induction of M1 polarization	Ptch receptor binding and Gli signaling activation and PI3K phosphorylation	[72]
Shh <sup>a</sup>	h-squamous carcinoma cell line	<i>In vitro</i>	EC	dUC	RhoA inhibition in target cell	Stimulation of angiogenesis	Induction of Shh/RhoA signaling	[74]
AT1R	Hypotonic or angiotensin II-stimulated HEK293T	<i>In vitro</i> ; angiotensin II induced hypertension mouse model	FB; CM	dUC	Overexpression; AT1R KO animals	Reconstitution of angiotensin II blood pressure response	Increase in ERK1/2 phosphorylation; AT1R incorporation in distal heart- and skeletal muscle and mesenteric vessels	[75]
EGFR <sup>a</sup>	h-squamous cell carcinoma	<i>In vitro</i>	EC	dUC	Ab	Transfer and activation	Induction of Akt and ERK1/2 phosphorylation, and VEGF expression; autocrine VEGFR2 activation	[76]
EGFR <sup>III</sup> <sup>a</sup>	h-glioblastoma cell	<i>In vitro</i>	Glioblastoma cell	dUC	Overexpression	Transfer and stimulation of proliferation	Increase in VEGF production and phosphorylation of ERK1/2 and Akt; activation of ErbB signaling	[117]
EGFR <sup>a</sup>	h-gastric cancer cell	<i>In vitro</i> ; mouse orthotopic tumor model	Liver cell	dUC	siRNA; overexpression	Transfer and activation	Induction of HGF secretion	[118]
DI4 <sup>a</sup>	h-glioma cancer cell	<i>In vitro</i> (3D)	EC	dUC	Overexpression	Inhibition of sprouting; increase in EC migration; and suppression of proliferation	Activation of Notch signaling; transcription of Hey1 and Hes1	[102]
DI4 <sup>a</sup>	h-glioma cancer cell	<i>In vitro</i> (2D); glioma xenograft mouse model		dUC	Overexpression	Stimulation of angiogenesis; transfer to membrane	Inhibition of Notch signaling; inhibition of NICD accumulation and Hey1 and Hey2 transcription	[103]
CD47	hT cell	<i>In vitro</i>	T cell; EC	ExoQuick (System Biosciences)	siRNA	T-cell and EC activation; stimulation of angiogenesis	Increase in VEGFR2 phosphorylation; induction of CD69 expression	[119]
CD47 <sup>a</sup>	m-primary fibroblast	Orthotopic pancreatic cancer mouse model	Circulating AF647+ monocyte	dUC	Ab; knock-out	Decrease in circulating monocytes; suppression of tumor growth	Increase in EV half-life in circulation; interaction with SIRPα	[120]

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Table 1. (continued)

Protein	Donor cell	Model	Target cell(s)	EV isolation method	Method of cargo manipulation	Reparative effect	Involved molecular mechanism	Refs
Proliferation, migration, and neurite outgrowth								
GPRC5B	Canine kidney cysts	<i>In vitro</i>	Kidney cell	dUC	Overexpression; shRNA	Stimulation of outward cyst growth; increasing cell migration	Increase in ERK1/2 phosphorylation	[77]
SK2	m-hepatocytes	<i>In vitro</i> ; hepatectomy mouse model	Hepatocyte	dUC	siRNA; KO mice; inhibitor (FSBA)	Increase of proliferation	SiP generation	[78]
Wnt4	huc-MSC	<i>In vitro</i> ; second-degree skin burn injury rat model	Skin cell	dUC	shRNA	Stimulation of proliferation and migration; improved wound healing; reduction of apoptosis	$\beta$ -catenin translocation to nucleus; upregulation of PCNA, CK19, N-cadherin, cyclin D1, and cyclin D3; increase of Akt and GSK3 $\beta$ phosphorylation	[79]
Wnt11	huc-MSC	<i>In vitro</i> ; second-degree skin burn injury rat model	Skin cell	?	/	Induction of proliferation and migration; improved wound healing	Activation of Wnt/ $\beta$ -catenin signaling	[80]
Wnt8a	Zebrafish apoptotic epithelial SC	Zebra fish epidermal DNA damage model	p63+ basal SC	dUC (Apoptotic bodies)	KO animal (CRISPR/Cas9); overexpression	Increase in proliferation	Activation of Wnt signaling	[82]
Jagged1	h-fetal dermal MSC	<i>In vitro</i> ; full-thickness dermal wound injury mouse model	Adult dermal FB	ExoQuick-TC (System Biosciences)	siRNA	Stimulation of proliferation and migration; improved cutaneous wound healing	Activation of Notch signaling; increased Jagged1, Notch1, and Hes1 expression	[83]
Tspan8/CD151 <sup>a</sup>	m-MCA-induced tumors; serum of Tspan8/CD151 KO mice	<i>In vitro</i> ; MCA xenograft mouse model	EC; BM cell; MCA tumor cell	dUC	KO mice	Stimulation of tumor cell migration and invasion; increase in tumor cell apoptosis resistance; induction of angiogenesis, lymphogenesis, and BM cell maturation	Upregulation of proangiogenic integrins and RTK	[84]
Tspan8/CD151 <sup>a</sup>	r-pancreatic adenocarcinoma cell	<i>In vitro</i> ; pancreatic adenocarcinoma xenograft rat model	Tspan8/CD151-knockdown pancreatic adenocarcinoma cell; stromal cell; hematopoietic cell	dUC	shRNA	Stimulation of migration and invasion; increase in tumor metastasis	Induction of Notch signaling and EMT-related gene expression; upregulation of different cytokine/chemokine and receptor expression; integrin and protease	[85]

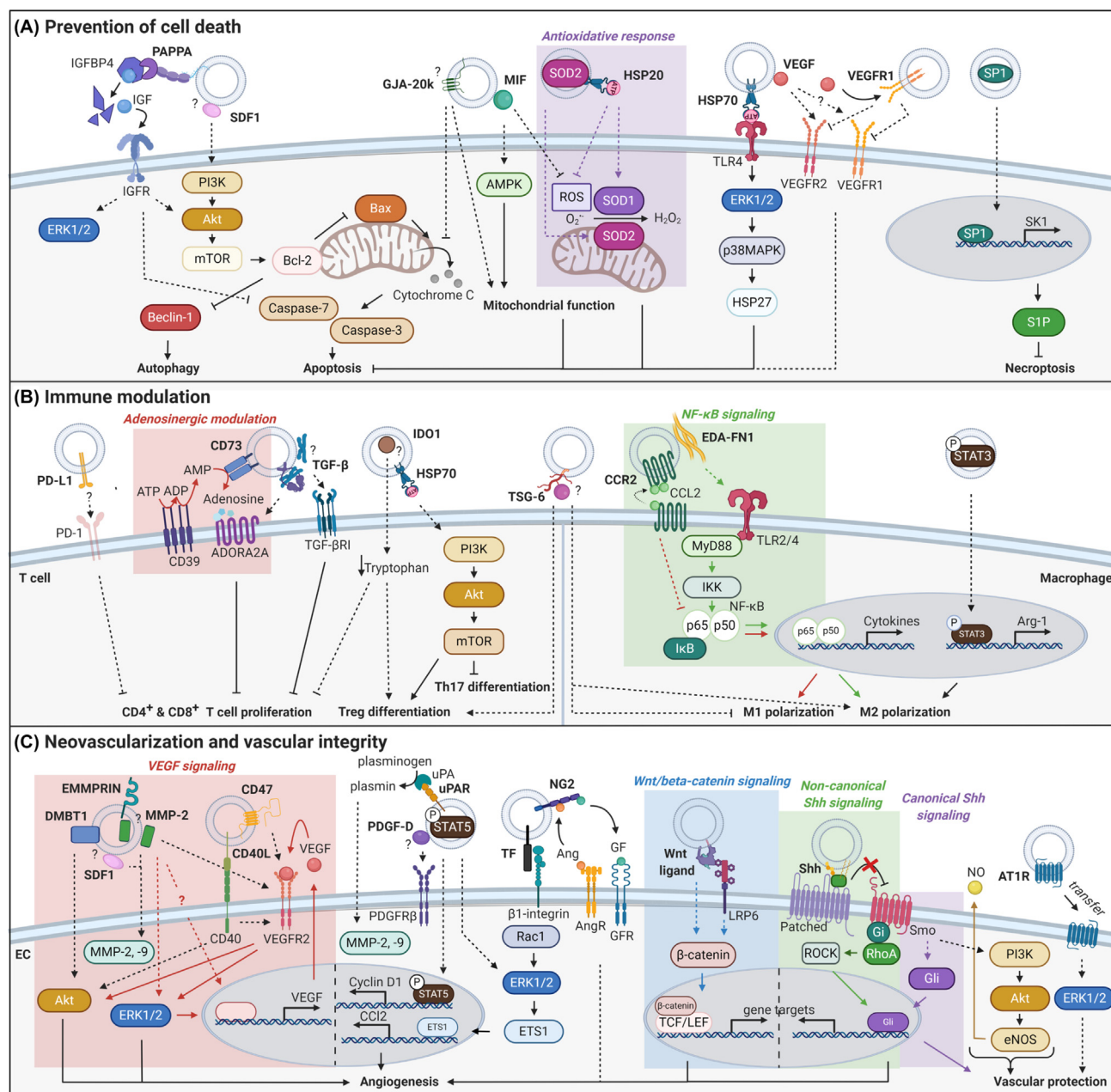
CD73	h-embryonic MSC	<i>In vitro</i> ; osteochondral defect rat model	Chondrocyte	TFF	Inhibitor (AMPCP)	Promotion of chondrocyte migration and proliferation; induction of ECM secretion; increase in M2 macrophage infiltration; decrease in M1 macrophages and inflammatory cytokines; improved cartilage repair	5'-AMP to adenosine conversion; increase in Akt and ERK1/2 phosphorylation	[86]
FN	h-fetal senescent and non-senescent lung FB	<i>In vitro</i>	FB	dUC	Ab	Stimulation of migration and invasion	Interaction with $\alpha 5\beta 1$ integrin; increased FAK and Src phosphorylation	[87]
FN	r-brain microvascular EC	<i>In vitro</i>	Oligodendrocyte precursor cell	ExoQuick (System Biosciences)	Heparitinase	Increase in survival and proliferation	Binding to heparin sulfate proteoglycans	[89]
FN <sup>as</sup>	h-fibrosarcoma cell	<i>In vitro</i>	Rab27a-knockout fibrosarcoma cell	dUC, OptiPrep gradient	FN depletion in culture medium	Stimulation of migration	Interaction with $\alpha 5\beta 1$ integrin	[88]
FN <sup>as</sup>	h-myeloma cell	<i>In vitro</i>	Myeloma cell; EC	dUC	Ab (Hep-II heparin-binding domain of FN)	Enhancement of migration and invasion	Binding to heparin sulfate proteoglycans; increase in p38 and ERK1/2 phosphorylation	[90]
NOX2	mBM-macrophages	<i>In vitro</i> ; sciatic nerve crush in Lysm-cre;Nox2fl/fl mice	Neuron	ExoQuick (System Biosciences)	KO mice	Stimulation of dorsal route ganglion axonal outgrowth; increase in number of regenerating axons	Increase in superoxide production; inactivation of PTEN; increase of Akt phosphorylation	[91]
PTEN	RAR $\beta$ agonist treated m-cortical neuron	<i>In vitro</i> ; cervical avulsion rat model	Astrocyte	Total exosome isolation reagent (Invitrogen)	siRNA	Inhibition of proliferation and stimulation of normal arrangement; reduction of scar formation and increased neurite outgrowth	PTEN transfer	[92]

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Table 1. (continued)

Protein	Donor cell	Model	Target cell(s)	EV isolation method	Method of cargo manipulation	Reparative effect	Involved molecular mechanism	Refs
MET <sup>a</sup>	h-malignant melanoma cell	<i>In vitro</i> : orthotopic xenograft mouse tumor model	BMDc: melanoma cell	dUC	shRNA; inhibitor (crizotinib)	Induction of BMDc recruitment and differentiation toward provascular phenotype; increase in vascular leakiness and tumor cell metastasis	Induction of HGF-induced S6 and ERK1/2 phosphorylation; increase in c-Kit and Tie2 expression	[99]
MET <sup>a</sup>	h-malignant melanoma cell	Orthotopic xenograft mouse tumor model	Melanoma cell	dUC	shRNA	Induction of tumor cell metastasis		[100]
Integrins <sup>a</sup> (α6β1, α6β4, α5β5)	h-breast and pancreatic cancer cell	<i>In vitro</i> : xenograft tumor mouse model	Lung epithelial cell; lung FB; liver Kupffer cell	dUC	shRNA; overexpression	Increase in tumor cell metastasis	Induction of Src phosphorylation and proinflammatory S100 gene expression	[107]

Abbreviations: 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; α-SMA, α-smooth muscle actin; Ab, (neutralizing) antibody; ADA, adenosine deaminase; AMPCP, adenosine 5'-α-β-methyleneidiphosphate; AMPK, 5'-monophosphate-activated protein kinase; ANXA1, endogenous annexin A1; Arg-1, arginase 1; AT1R, angiotensin II receptor type 1; Bax, Bcl-2 associated X; Bcl-2, B-cell lymphoma 2; bFGF, basic fibroblast growth factor; BM, bone marrow; BMDc, bone-marrow-derived progenitor cells; BPD, bronchopulmonary dysplasia; Breg, regulatory B cell; CCL2, chemokine (C-C motif) ligand 2; COR2, C-C motif chemokine receptor-2; CK18, cytokeratin 18; CK19, cytokeratin 19; CK8, cytokeratin 8; CM, cardiomyocyte; CPC, cardiac progenitor cell; DC, dendritic cell; DL4, delta-like 4; DMBT1, deleted in malignant brain tumors 1; dUC, differential ultracentrifugation; EC, endothelial cell; ECM, extracellular matrix; EDA-FN1, alternatively spliced domain A-containing fibronectin 1; EGFR, epidermal growth factor receptor; EGFRvIII, epidermal growth factor receptor variant III; EMMPRIN, extracellular matrix metalloproteinase inducer; EMT, epithelial to mesenchymal transition; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-related kinase 1/2; ETS1, ETS Proto-Oncogene 1; FPR2, formyl peptide receptor 2; FSBA, 5-p-fluorosulfonylbenzoyl-adenosine; GJA-20k, gap junction alpha 1 – 20k; GPRC5B, factor 2; FN, fibronectin; Foxp3, forkhead box P3; FPR1, formyl peptide receptor 1; FPR2, formyl peptide receptor 2; FSP1, formyl peptide receptor 1; FSP2, formyl peptide receptor 2; FSP3, forkhead box P3; FSP4, forkhead box P4; FSP5, forkhead box P5; FSP6, forkhead box P6; FSP7, forkhead box P7; FSP8, forkhead box P8; FSP9, forkhead box P9; FSP10, forkhead box P10; FSP11, forkhead box P11; FSP12, forkhead box P12; FSP13, forkhead box P13; FSP14, forkhead box P14; FSP15, forkhead box P15; FSP16, forkhead box P16; FSP17, forkhead box P17; FSP18, forkhead box P18; FSP19, forkhead box P19; FSP20, forkhead box P20; 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Trends in Cell Biology

**Figure 2. Recipient Cell Activation by Extracellular Vesicle (EV)-Associated Proteins.** Proteins present on EVs activate intracellular signaling cascades through different mechanisms (see Box 1), represented by dotted lines. For some proteins (question marks) the exact localization on EVs is unclear. (A) EV-associated proteins prevent apoptosis through, for example, the upregulation of PI3K–Akt–mTOR signaling and downregulation of proapoptotic signaling cascades, leading to a decrease in the mitochondrial release of cytochrome C and expression of caspases, or by reducing ROS levels by influencing the antioxidative response. (B) EV proteins modulate the immune system at different levels. CD73 converts 5' AMP to adenosine, which binds adenosine A2A receptor (ADORA2A) to suppress T-cell proliferation. Activation of PI3K–Akt–mTOR signaling and reduction in tryptophan induces regulatory T-cell (Treg) differentiation. Activation and inhibition of MyD88–IKK–NF-κB signaling cascade by different EV-associated proteins induce M2 and inhibit M1 macrophage polarization, respectively. (C) Several proteins expressed on EVs directly or indirectly stimulate vascular endothelial growth factor (VEGF) signaling, leading to extracellular VEGF release and binding to endothelial growth factor receptor 2 (VEGFR2), promoting angiogenesis. Other proteins induce transcription of Cyclin D or CCL2. Wnt ligands induce β-catenin translocation to the nucleus and its binding

(Figure legend continued at the bottom of the next page.)

demonstrate the two-edged sword of EV-mediated VEGF signaling in cell death and the capability of EV transmembrane proteins to capture secreted proteins to prevent binding to their receptors on the recipient cell membrane.

### Immune modulation

Inflammation is a mechanism of innate immunity in response to harmful stimuli including damaged cells or irritants. An uncontrolled inflammatory response is associated with diverse acute inflammatory diseases, but is also considered the root cause of most chronic diseases and even cancer. Several proteins such as Fas ligand, programmed death-ligand 1 (PD-L1), and antigen presenting complexes are expressed on EVs released by immune and tumor cells and modulate the immune response in the tumor microenvironment to favor tumor growth, as reviewed elsewhere [29,30]. These studies demonstrate the importance of EVs as protein carrier as some proteins might have stronger immunomodulatory effects in circulation and tumor microenvironments when associated to EVs as compared with their soluble EV-free form [31,32]. In tissue remodeling, PD-L1 is secreted on human lung fibroblast EVs in response to transforming growth factor-beta (TGF- $\beta$ ), thereby inhibiting T-cell proliferation and fibroblast migration [33].

During investigations into the therapeutic properties of stem cell-derived EVs in tissue repair, it has become clear that their therapeutic features are to a large extent due to their immune-modulating activities. Stem cell EVs modulate inflammatory responses by inducing the differentiation of M2 macrophages and the suppression of M1 macrophages [15,34,35], by the suppression of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation and the induction of regulatory T-cell (Treg) polarization [13,14,36,37], by impairing dendritic cell maturation [38], and by inhibiting NK cell proliferation [39] (Figure 1B, Table 1). One of the mechanisms of MSC-EV-mediated immunosuppression is through the expression of CD73 on their surface. CD73 converts AMP to adenosine, leading to suppression of T cells [40,41], a mechanism which is also employed by cancer cells and Tregs [36,42,43] (Figure 2B). TGF- $\beta$  present on stem cell EVs is an interesting immune modulatory protein as it is transported in both latent membrane form and active form [39,44], thereby modulating the immune response at different levels [13,39,44,45] (Table 1).

In innate immunity, the activation of Toll-like receptors (TLRs) initiates MyD88- or TRIF-dependent intracellular signaling pathways, culminating in the activation of the transcription factor nuclear factor-kappaB (NF- $\kappa$ B). After activation and nuclear translocation, NF- $\kappa$ B binds to DNA response elements and induces expression of many genes including cytokines, chemokines, and adhesion molecules [46]. EV proteins are potent inducers of TLR-MyD88-NF- $\kappa$ B signaling leading to anti-

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to TCF/LEF transcription factors, leading to the transcription of proangiogenic gene targets. EV-delivered sonic hedgehog (Shh) activates noncanonical Shh/RhoA signaling, leading to angiogenesis, while canonical Shh/Gli and Akt/eNOS activation induces vascular protection. Abbreviations: AMPK, 5'-monophosphate-activated protein kinase; Ang, angiotensin; AngR, angiotensin receptor; Arg-1, arginase 1; AT1R, angiotensin II receptor type 1; Bax, Bcl-2 associated X; Bcl-2, B-cell lymphoma 2; CCL2, chemokine (C-C motif) ligand 2; CCR2, C-C motif chemokine receptor-2; DMBT1, deleted in malignant brain tumors 1; EDA-FN1, alternatively spliced domain A-containing fibronectin 1; EMMPRIN, extracellular matrix metalloproteinase inducer; eNOS, endothelial nitric oxide-synthase; ERK1/2, extracellular signal-related kinase 1/2; ETS1, ETS proto-oncogene 1; GF, growth factors; GFR, growth factor receptor; Gi, Gi proteins; GJA-20k, gap junction alpha 1-20k; Gli, glioma-associated oncogene; HSP20, heat-shock protein 20; HSP27, heat-shock protein 27; HSP70, heat-shock protein 70; IDO1, indoleamine 2,3- dioxygenase 1; IGF, insulin growth factor; IGFBP4, insulin like growth factor binding protein 4; IGFR, insulin growth factor receptor; I $\kappa$ B, I $\kappa$ B kinase; LEF, lymphoid-enhancer factor; LRP6, low density lipoprotein-related protein 6; MIF, macrophage migration inhibitory factor; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; mTOR, mammalian target of rapamycin; MyD88, myeloid differentiation primary response 88; NF- $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NG2, neural/glia antigen 2; NO, nitric oxide; p38MAPK, p38 mitogen-activated protein kinase; PAPP, pregnancy-associated plasma protein A; PD-1, programmed cell death protein 1; PDGF-D, platelet-derived growth factor D; PDGFR $\beta$ , platelet-derived growth factor receptor-beta; PD-L1, programmed death-ligand 1; PI3K, phosphoinositide 3-kinase; RhoA, Ras homolog family member A; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; S1P, sphingosine-1-phosphate; SDF1, stromal-derived factor 1; SK1, sphingosine kinase 1; Smo, smoothened receptor; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; SP1, specificity protein 1; STAT3, transcription 3; STAT5, transcription 5; TCF, T-cell factor; TF, tissue factor; TGF- $\beta$ , transforming growth factor-beta; TGF- $\beta$ RI, TGF- $\beta$  receptor 1; Th17, T helper cell 17; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; TSG-6, tumor necrosis factor alpha-stimulated gene 6; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; VEGFR1, vascular endothelial growth factor receptor 1.

inflammatory cytokine production and immune suppression [15,47,48], but can also induce pro-inflammatory gene expression [49,50]. Interestingly, proteins inhibiting NF- $\kappa$ B signaling evoke immune suppression, which demonstrates the complex interplay between EV proteins and NF- $\kappa$ B signaling in innate immune activation [34] (Figure 2B). HSP70 modulates the immune response also at different levels: when expressed on mouse bone marrow dendritic cell EVs, HSP70 induces Treg differentiation [14], while when present on human cancer cell EVs it promotes a pro-inflammatory MSC phenotype, leading to an activated immune response [50]. It might be questioned, however, whether both anti-inflammatory and proinflammatory properties are unique to these HSPs, or whether other EV cargo influences these HSP-linked functional changes.

### Neovascularization and Vascular Integrity

Angiogenesis is defined as the formation of new blood vessels from pre-existing ones, and is a fundamental process to restore the delivery of oxygen, nutrients, and growth factors to damaged tissue. Neovascularization during recovery after ischemia/reperfusion injury and cutaneous wound healing can be promoted by the administration of EVs released from different stem and progenitor cells [1,21,51,52]. Also EVs from differentiated cells such as endothelial, epithelial, and immune cells are potent promoters of angiogenesis through protein-mediated activation of intracellular signaling pathways in endothelial cells [53–55] (Figure 1C). An important intracellular mechanism mediating angiogenesis is the initial upregulation of VEGF-A signaling and release in response to vascular damage, which leads to activation of endothelial cells of the surrounding blood vessels through binding to VEGF receptor 2 (VEGFR2). Endothelial VEGF signaling and up-regulation of VEGFR2 expression can be activated by the (over)expression of several proteins on EVs, including deleted in malignant brain tumors 1 (DMBT1) and SDF1 [21,56–59] (Figure 2C). In cancer, VEGF-A can be transported on the exterior of EVs, leading to direct activation of VEGFR2 and its downstream signaling cascades [60,61]. Also other growth factors have been reported to be attached to EVs and upon delivery activate their receptors at the recipient cell membrane [62]. A postulated mechanism of growth factor transport is through binding to transmembrane EV proteins [63], which demonstrates the capability of EVs to serve as a transport vehicle for secreted proteins and thereby contributing to long-distance delivery.

Other angiogenic effects are accompanied with an upregulation of genes related to cell migration and proliferation through EV-mediated delivery of transcription factors [64], **Wnt ligands** [51,52], or through the expression of bioactive enzymes at the exterior of the EV membrane [65] (Figure 2C). EV-associated urokinase-type plasminogen activator receptor (uPAR) binds urokinase-type plasminogen activator (uPA) and thereby converts plasminogen to plasmin, suggested to activate matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9), resulting in the release of proangiogenic factors from the ECM [66], a mechanism also employed by tumor-cell derived EVs to promote tumor cell migration [67].

Vascular protection is regulated by endothelial cell release of vasoactive substances, including nitric oxide (NO), and is important for maintaining blood pressure. **Sonic hedgehog** (Shh), expressed on T-cell EVs, induces vascular protection through **canonical hedgehog signaling** [54,68,69]. Also Shh transport by EVs from other cell types induces such Shh/Gli signaling [70–72]. Shh has a high affinity for membranes through cholesterol and palmitate modifications [73], explaining how EVs can contribute to long-range Shh signaling. Interestingly, noncanonical Shh signaling stimulated by EVs leads to angiogenesis [55,74]. In response to cardiac pressure overload, vascular function can be maintained through EV-mediated transfer and incorporation of angiotensin II receptor type 1 into distal myocytes and mesenteric vessels [75]. Also other receptors can be trafficked into or onto EVs for release in the extracellular space and circulation instead of being recycled in the donor cell, leading to endothelial cell activation [76].

### Cell Proliferation, Migration and Neurite Outgrowth

In several pathological conditions, lost tissue can be replaced through the proliferation and migration of adjacent remaining healthy cells, or by the deposition of ECM components to form scar tissue. In response to stress or injury, healthy cells can secrete EVs harboring bioactive proteins that promote cell proliferation and migration (Figure 1D). Hepatic growth factor (HGF)-treated kidney cysts excrete EVs carrying G-protein-coupled receptor 5B (GPRC5B), which together with HGF induces kidney tubulogenesis [77]. Sphingosine kinase 2 (SK2) on hepatocyte EVs promotes the proliferation of adjacent hepatocytes through the induction of sphingosine-1-phosphate (S1P) expression [78]. During cutaneous wound healing, the proliferation of different cell types in the skin is promoted through the therapeutic delivery of MSC EVs displaying morphogen Wnt [51,79,80]. Wnt ligands comprise a family of secreted signaling molecules that require essential lipid modifications (palmitoylation) for secretion, demonstrating the importance of EV-like carriers for long-distance signaling [81]. Upon delivery, Wnt ligands elicit canonical Wnt- $\beta$ -catenin-LEF/LCF signaling in recipient cells [52,82]. The activation of intracellular Notch signaling also contributes to the proliferation of skin cells leading to epidermal wound repair [83], whereas its activation is also important for epithelial-to-mesenchymal transition [84,85].

In joint regeneration, MSC EVs contribute to chondrocyte proliferation, migration, and ECM secretion through expression of CD73 [86] (Figure 1D). PD-L1, expressed on TGF- $\beta$ -stimulated fibroblast EVs, induces fibroblast migration [33], a process that is also induced by EV-associated fibronectin (FN) [87]. The transport of FN by EVs is an interesting phenomenon because it highlights that apart from carrying transmembrane proteins and intraluminal proteins, EVs have the capability to catch ECM components on their surface, most likely through binding to integrins and heparin sulfate proteoglycan chains on the EV surface. FN favors EV cell adhesion and modulates recipient cell function by, for example, creating a niche favoring cell migration [88–90].

Neurite outgrowth is a regenerative mechanism upon nerve injury and evoked by the inflammatory and oxidative environment. Dorsal root ganglion outgrowth can be induced through EV-mediated release of active NADPH oxidase 2 (NOX2) complexes that activate ROS production. Here, increasing ROS levels have no detrimental role in cell homeostasis, but participate in cellular signaling favoring neurite outgrowth [91]. Also tumor suppressor phosphatase and tensin homolog (PTEN) has a dual role in neuronal regeneration. PTEN is known to be a major negative regulator of neuronal regeneration, but upon transfer by EVs it inhibits astrocyte proliferation and scar formation, creating a permissive environment for neurite outgrowth [92].

### Dual Roles of EV-Associated Proteins in Tissue Repair

As indicated above, several proteins have been discovered to be present on EVs evoking different reparative functions in the body. Interestingly, some generic proteins seem to stimulate only specific processes in a single tissue. For example, EV-associated Wnt ligands have not yet been demonstrated to contribute to the repair of other tissues than the skin, despite their presence on EVs derived from different cell sources [93,94]. By contrast, other EV-associated proteins seem to be capable of stimulating various processes in multiple tissues. For example, CD73 expression on MSC EVs both promotes chondrocyte proliferation and reduces T-cell proliferation [40,41,86], with the latter also being reduced by cancer cell EV-expressed CD73 [36,43]. Some EV-associated proteins induce beneficial effects in one tissue while hampering repair in others [13,39,44,45,95,96]. Shh promotes vascular protection and angiogenesis while associated to T-cell EVs [68], but induces proinflammatory M1 polarization when associated to insulin-resistant adipocyte EVs [72]. This illustrates the complexity of EV-induced tissue repair and raises the question of why specific proteins seemingly exert distinct functions in different tissues. Differences in recipient cell activation could be explained by differences in total EV

composition [18,97] and experimental models used, or due to an interaction of the protein of interest or other EV components with tissue- or cell-specific structures. It is known that overactivation of intracellular signaling cascades is detrimental for tissues, and that inhibition of one signaling cascade may tip the balance to activation of other cascades involved in wound repair [98], thereby making the contribution of a single protein less relevant to the total process of tissue repair. To resolve if EV function is tissue specific or pleiotropic, more in-depth studies should focus on the contribution of specific EV cargo to the repair of multiple different tissues.

### Biological and Technical Challenges in the Study of EV-Associated Proteins

Investigating the contribution of individual proteins to EV function is hampered by several challenges. Technical challenges include constantly evolving EV isolation, characterization, and normalization techniques (Box 2), and the use of different cell culturing methods and donor cell types between different studies, leading to variations in EV yields and characteristics. The resulting EV heterogeneity and differences in EV cargo hamper reproducibility [99,100] and may mask the contribution of a single protein to EV function. Insufficient purification can also result in co-isolation of contaminant (secreted) proteins, while high-shear forces during ultracentrifugation may affect the localization of EV-associated proteins, subsequently leading to differences in activation of downstream signaling cascades in recipient cells [52].

Biological challenges include the study of EV protein localization, its manipulation, and the use of relevant experimental models. The exact EV location of the protein of interest is often not reported, which questions sometimes how this protein can exert its functional effects. Especially for secreted proteins such as platelet-derived growth factor D (PDGF-D) and SDF1, which function through receptor binding at the recipient cell membrane, it might be questioned how they are contained in EVs since they lack transmembrane properties [21,62]. Other proteins such as DMBT1 are present in different isoforms, some secreted and some membrane bound, but which isoform is present on EVs has not yet been reported [56]. In order to demonstrate the exact localization of a specific protein on EVs, isolation techniques including density gradients in combination with single EV characterization techniques and/or proteinase protection assays could be used [16,20,91]. EV cargo manipulation is an additional challenge, since effective knock down and overexpression techniques have the risk of influencing not only the expression of the target protein, but also the expression of other functional proteins [24], or even EV release in general [101].

#### Box 2. EV Isolation and Characterization

Different methods for EV isolation from conditioned media or bodily fluids exist and are based on distinct physical properties of EVs such as density, size, and surface composition. EV isolation and purification involves preclearing (serial centrifugation) and/or filtration steps before EVs can be retrieved. No consensus about the most optimal EV isolation technique has been established, with each isolation technique having its own strengths and limitations [106]. Differential ultracentrifugation (dUC) allows pelleting of EVs from fluids, with the advantage that EVs from different fluids and volumes can be readily obtained. A disadvantage is that dUC might induce EV aggregation and changes in EV structure, and there is a risk for co-isolation of protein aggregates and other soluble factors. Density gradient centrifugation (e.g., iodixanol or sucrose-based gradients) can be used for further EV purification. Size-exclusion chromatography retrieves EVs with better integrity, but is hampered by the fact that EV-containing medium should be concentrated before injection into purification columns, resulting in potential EV loss and increased processing times. More recently developed techniques include immunoaffinity capture using beads conjugated with antibodies directed toward specific EV surface markers, ultrafiltration, field-flow fractionation, and microfluidic approaches [106].

For comparative functional studies, EV isolations should be normalized either by the characteristics of the isolated EVs or by the amount of starting material. EVs can be characterized based on particle numbers, total amount of biomolecules present (e.g., nucleic acids, proteins, or lipids), or the presence or activity of specific EV-associated molecules. Comparing multiple EV characteristics provides an indication of EV purity and presence of EV (sub)types. A combination of characterization and normalization techniques is required for optimal comparative functional studies.

Finally, a major challenge is the extrapolation of EV function from *in vitro* to *in vivo* models. Two-dimensional cell cultures do not reflect the normal 3D habitat of cells and are often stimulated with supraphysiological doses of EVs [36]. In the body, a more constitutive release of EVs is observed and multiple cell types are involved in the process of tissue repair. Also the contribution of endogenous proteins levels present in the tissue should be taken into account. For example, T cells express high levels of CD73, which questions to what extent exogenously supplied EV-associated CD73 can contribute to further inhibition of T-cell function [41]. The influence of different biological models on outcome is demonstrated by glioma cell EV expression of DLL4 and its activation of downstream Notch signaling in 2D and 3D, leading to contradicting effects in endothelial cell motility [102,103]. These examples highlight that EV heterogeneity, but also differences in experimental models, might explain why some EV-associated proteins evoke beneficial effects in certain tissues and not in others.

### Concluding Remarks and Future Perspectives

Based on current knowledge, it is clear that we have just scratched the surface of the complex signaling involved in EV-mediated tissue remodeling and repair. EV-associated proteins promote several processes in the body that contribute to endogenous tissue remodeling and therapeutic tissue repair. So far, several EV-based therapeutics entered early phase clinical trials, in which stem cell EVs have been demonstrated to be safe and to possess anti-inflammatory properties, or to be potent inducers of wound closure in different (inflammatory) diseases (Box 3). In order to get a safe and effective translation of EVs as cell-free therapeutic in organ repair, future research should be directed to investigate if the contribution of a particular protein to EV function is specific to an EV (sub)type, and if reparative functions are tissue specific or pleiotropic (see Outstanding Questions). It will be necessary to perform studies in a comprehensive manner that includes proper molecular, cellular, and functional characterization to address EV function and to test different EV (sub)types and EV-associated proteins in the same experimental

### Outstanding Questions

What EV cargo is responsible for the therapeutic and tissue remodeling function of EVs, and does this depend on EV (sub)type?

What determines the tissue specific- or pleiotropic effects of EV-associated proteins?

How can the true contribution of specific EV cargo to EV function be investigated, taking EV heterogeneity and variability in experimental models into account?

How do we optimally use EVs for regenerative medicine purposes? Which EV donor cell type is most effective to induce tissue repair?

How can the bioavailability and efficacy of EV therapeutics be improved?

### Box 3. Clinical Application and Challenges

Stem- and progenitor cell-derived EVs have been shown to mediate therapeutic effects in various preclinical disease models, which resulted in the initiation of several early phase clinical trials. Ongoing registered studies ([ClinicalTrials.gov](https://clinicaltrials.gov)) mainly evaluate the safety and efficacy of different routes of therapeutic EV application such as delivery to the brain (NCT03384433) and aerosol inhalation (NCT04313647<sup>†</sup>, NCT04276987<sup>‡</sup>, NCT04491240<sup>§</sup>), and investigate their immune modulatory properties (NCT02138331<sup>¶</sup>, NCT04213248<sup>¶</sup>, NCT04356300<sup>¶</sup>, NCT04270006<sup>¶</sup>). So far, it has been demonstrated that MSC EVs are effective in reducing proinflammatory cytokine response in patients with graft-versus-host disease after multiple injections [108], ameliorate chronic renal inflammation and improve overall kidney function in grade III–IV chronic kidney disease patients [109], and contribute to the healing of large macular holes after intravitreal injection (NCT03437759<sup>¶</sup>) [110]. Other ongoing interventional trials evaluate MSC- and plasma-derived EVs for their contribution to wound closure (NCT02565264<sup>¶</sup>, NCT04173650<sup>¶</sup>).

Although encouraging, as safety of therapeutic EV application in regenerative medicine is demonstrated, some aspects need to be considered. Appropriate quality control is required since culture conditions and isolation methods might affect EV content and structure (see Box 2). EV production and isolation methods should be optimized for large-scale EV production and standardized for each donor cell type. Isolated EVs should be quantified and characterized in accurate and relevant potency assays to safeguard reproducibility. Storage strategies not hampering EV function and increasing shelf-life should be explored. Pharmacokinetics, pharmacodynamics, and biodistribution of each specific EV type in the body, and their optimal dose and route of administration for each disease and target tissue should be determined. Other safety and regulatory aspects are discussed the position paper from the International Society of Extracellular Vesicles [1].

EVs are able to cross cell and tissue barriers, making them a promising therapeutic agent for difficult-to-reach tissues. However, as EVs are quickly trapped in the liver, lungs, and spleen after (systemic) administration, clinical application is limited by their bioavailability. Bioavailability and efficacy could be increased by targeting EVs to specific tissues or cell types; by a more local, sustained, or repetitive delivery; or by other biophysical and biochemical methods to modify EV properties. This demonstrates the importance of investigating local delivery methods [110], the use of biomaterials to control spatial and temporal release of EVs, or to modify EV cargo in order to improve therapeutic function.

model. These aspects will be crucial to unravel which identified protein cargo and activated intracellular mechanisms contribute to EV-mediated tissue repair, and how we can ultimately use EVs therapeutically in regenerative medicine.

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### Resources

<sup>i</sup><https://clinicaltrials.gov/ct2/show/NCT03384433>

<sup>ii</sup><https://clinicaltrials.gov/ct2/show/NCT04313647>

<sup>iii</sup><https://clinicaltrials.gov/ct2/show/NCT04276987>

<sup>iv</sup><https://clinicaltrials.gov/ct2/show/NCT04491240>

<sup>v</sup><https://clinicaltrials.gov/ct2/show/NCT02138331>

<sup>vi</sup><https://clinicaltrials.gov/ct2/show/NCT04213248>

<sup>vii</sup><https://clinicaltrials.gov/ct2/show/NCT04356300>

<sup>viii</sup><https://clinicaltrials.gov/ct2/show/NCT04270006>

<sup>ix</sup><https://clinicaltrials.gov/ct2/show/NCT03437759>

<sup>x</sup><https://clinicaltrials.gov/ct2/show/NCT02565264>

<sup>xi</sup><https://clinicaltrials.gov/ct2/show/NCT04173650>

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