Review Article

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Apoptotic cell-derived micro/nanosized extracellular vesicles in tissue regeneration

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Abstract: Extracellular vesicles (EVs), products released by cells in multiple biological activities, are currently widely accepted as functional particles and intercellular communicators. From the orthodox perspective, EVs derived from apoptotic cells (apoEVs) are responsible for cell debris clearance, while recent studies have demonstrated that apoEVs participate in tissue regeneration. However, the underlying mechanisms and particular functions in tissue regeneration promotion of apoEVs remain ambiguous. Some molecules active during apoptosis also function in tissue regeneration triggered by apoptosis, such as caspases. ApoEVs are generated in the process of apoptosis, carrying cell contents to manifest biological effects and possess biomarkers to target phagocytes. The regenerative effect

of apoEVs might be due to their abilities to facilitate cell proliferation and regulate inflammation. Such regenerative effect has been observed in various tissues, including skin, bone, cardiovascular system, and kidneys. Engineered apoEVs are produced to amplify the biological benefits of apoEVs, rendering them optional for drug delivery. Meanwhile, challenges exist in thorough mechanistic exploration and standardization of production. In this review, we discussed the link between apoptosis and regeneration, current comprehension of the origination and investigation strategies of apoEVs, and mechanisms in tissue regeneration of apoEVs and their applications. Challenges and prospects are also addressed here.

Keywords: extracellular vesicles, apoptosis, tissue regeneration, biomaterials, engineered nanoparticles

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Abbreviations

AiP	apoptosis-induced compensatory proliferation
ApoEVs	extracellular vesicles derived from apoptotic cells
BMM	bone marrow macrophages
DLS	dynamic light scattering
EVs	extracellular vesicles
FACS	Fluorescence activated cell sorting
HUVEC	human umbilical vein endothelial cells
MSCs	mesenchymal stem cells
MSNs	mesoporous silica nanoparticles
PtdSer	phospholipid phosphatidylserine
ROS	oxygen species
SEM	scanning electron microscope
TEM	transmission electron microscope
UV	ultraviolet

1 Introduction

Extracellular vesicles (EVs) are micro/nanosized vesicles containing small particles with lipid membranes secreted

by cells in multiple biological activities. EVs serve as information communicators of cells [1,2], which were formerly considered cell debris. According to biogenesis and size, EVs can be roughly classified as exosomes (endosome origin, 30–100 nm in diameter), microvesicles (plasma origin, 50–1,000 nm), and apoptotic bodies (apoptotic cell origin, 1–5 μ m) [3,4]. EVs of various sizes from apoptotic cells have been studied over the last several decades and they are not just confined to apoptotic bodies. As a result, apoptotic vesicles are used to refer to all types of EVs derived from apoptotic cells (apoEVs) [5–7].

Apoptosis is a highly ordained general physiological process that occurs every second throughout the body, resulting in the death of billions of cells every day [8,9]. Different from necrosis, apoptosis is unlikely to cause a traumatic defect or aberrant tissue shrinkage. As a result, apoptosis is well-known to play a role in tissue homeostasis and life cycle regulation, although there are still many unsolved mysteries in the field [10]. In recent years, tissue renewal, such as epithelium, skin, and liver renewal, has been widely described as one of the major biological effects mediated by apoptosis [11–13]. ApoEVs are products in the apoptotic process. Traditionally, the uptake of apoEVs by phagocytes was thought to be the end of apoptosis, which biochemically signals the death of cells, but new studies suggest that their fates include transfer and reuse [14–17]. Apoptosis-like regeneration properties have also been demonstrated in the application of apoEVs, which come in a variety of sizes and are produced through different apoptotic pathways. Recent studies indicate that apoEVs might promote regeneration in tissue maintenance, including skin, bone, cardiovascular system, and kidneys, by functioning bioactive effects through recruitment of targeted cells and delivery of biomolecules [18–22]. To better utilize and amplify the therapeutic properties of apoEVs, engineered methods implicating targeting, releasing, and retention have been investigated [23,24]. Figure 1 demonstrates the production, isolation, functions, and applications of apoEVs. The culture medium of apoptotic cells was collected and centrifuged to obtain apoEVs. By recruiting targeting cells and delivering biomolecules, apoEVs promote cell proliferation and regulate inflammation, thus being applied in tissue regeneration. ApoEVs are modified to function in broader fields as well. Recent studies have discussed the functions and applications of apoEVs in immune regulation, inflammation mediation, mesenchymal stem cell (MSC) transplantation, and tumor progression [3,25–28]. Nonetheless, a few documents have comprehensively discussed the regenerative effect of apoEVs and analyzed the underlying mechanisms. In this review, the mechanisms of apoEVs in tissue regeneration are

addressed here, along with their applications in both medicine and engineering. We hope this work will provide an indicative view of the mechanisms in tissue regeneration of apoEVs and inspiration for novel application of apoEVs in the future.

2 Apoptosis and regeneration

2.1 Apoptosis with caspases

Since the term “apoptosis” was proposed for the first time by John Foxton Ross Kerr and his colleagues at the University of Queensland [29], it has been proven to participate in various physiological processes, including development, aging, immune homeostasis, and tissue integrity maintenance [3,16,30]. Apoptosis, the most prominent pattern of programmed cell death, is characterized by several morphological changes, involving cell shrinkage, cytoplasmic and nuclear condensation, membrane blebbing, and the subsequent formation of apoptotic bodies.

Apoptosis, a conserved physiological process, can typically be triggered by the intrinsic pathway and the extrinsic pathway. A general summary is addressed here to clarify the processes of apoptosis. Caspases are fundamental participants in both pathways of apoptosis. The intrinsic pathway of apoptosis is closely associated with mitochondrial regulation, in which stimuli (for example, DNA damage) generate biochemical changes inside the cells. Stimuli elicit the dissipation of mitochondrial transmembrane potential, thus forming mitochondrial permeability transition pore on the outer membrane and leaking proapoptotic mitochondrial contents. Cytochrome *c* is then released into the cytoplasm to induce oligomerization of apoptotic peptide activating factor 1, which recruits procaspase-9 and forms apoptosome, the activator of caspase-9. Subsequently, activated caspase-9 in turn cleaves response caspases, activating caspase cascade reactions [16,31,32]. The extrinsic pathway of apoptosis initiates with the activation of death receptors. Upon contact with extracellular ligands, mainly TNF and Fas, the death receptors on the cell membrane transfer death signals toward the cytoplasm. The binding of ligands and receptors initiates the recruitment of Fas-associated death domain protein, causing autocleavage of procaspase-8. The activated caspase-8 then cleaves procaspase-3 to yield activated caspase-3 responsible for executing the downstream degradation process [16,30,32].

Figure 1: The production, isolation, functions and applications of apoptotic cell-derived extracellular vesicles (apoEVs). The culture medium of apoptotic cells was collected and centrifuged to obtain apoEVs. By recruiting targeting cells and delivering biomolecules, apoEVs promote cell proliferation and regulate inflammation, thus being applied in tissue regeneration. ApoEVs are modified to function in broader fields as well.

2.2 Regeneration with apoptosis

The mechanism of apoptosis is much more sophisticated than the brief sketch above, concerning a series of interactions and cascade signaling pathways, in which caspases have a well-defined role. However, increasing evidence implies the function of caspases in tissue repair and regeneration [33]. Dead cells are replaced by compensatory proliferated cells to maintain tissue homeostasis. Apoptotic cells deliver mitogenic signals to trigger the compensatory proliferation of neighboring cells, which is so-called “apoptosis-induced compensatory proliferation (AiP)” [11,34].

Caspases, the initiator and effector of apoptosis, are the major activators of AiP [11]. In *Drosophila*, cell death was blocked by the effector caspase inhibitor *p53* even with high expression of the initiator caspase Dronc, rendering Dronc functioning apart from apoptosis [35,36]. Actually, Dronc activated AiP via the JNK signaling pathway to express Wingless and Decapentaplegic mitogens, triggering compensatory proliferation of surrounding cells [34,37]. In Dronc-induced AiP, extracellular reactive oxygen species (eROS) produced by NADPH oxidase Duox activated macrophage-like hemocytes, which in turn amplified the JNK signal by TNF ortholog Eiger, thus promoting epithelial cell growth [11,12]. The effector caspases DrICE and Dcp-1 induced AiP in the eye tissue via Hedgehog signaling [36,38].

In some other species, such as *Xenopus tadpole* and *Hydra*, ROS are pivotal compartments for AiP. Profuse ROS at the wound activated Wnt/ β -catenin signaling and downstream *fgf20*, initiating active cell proliferation in tail regeneration of *Xenopus tadpole* [39]. In *Hydra*, ROS produced immediately after injury elicited MAPK signaling [11] and Wnt3 [40] in head regeneration.

In mammals, regeneration due to AiP is also linked to caspases. In mice, the lack of caspase-3 or caspase-7 leads to deficiency in the skin and liver regeneration [13]. The cleaved caspase-3 and caspase-7 initiate calcium-independent phospholipase A2 to upregulate the expression of arachidonic acid, which further mediates the production of prostaglandin E2, facilitating tissue repair and stem cell proliferation [13]. The activation of caspase-3 enlarged YAP-dependent organs, while inhibition of caspase-3 attenuated cell proliferation and diminished the sebaceous gland [41]. Some mediate evidence also proves the function of caspases in regeneration. The self-renewal property of embryonic stem cells was remarkably inhibited when lacking caspase-3 [42]. Deficient caspase-3 in mice resulted in the impaired differentiation of bone marrow stem cells and decreased bone density by the activation of the TGF- β /Smad2 signaling pathway [43,44], which was ameliorated by secretion from apoptotic MSCs [44]. In conclusion, participants in apoptosis, such as caspases and ROS, function in tissue renewal, coupling cell death, and regeneration.

3 Apoptotic extracellular vesicles (apoEVs)

Extracellular vesicles derived from apoptotic cells (apoEVs), the products of the programmed cell clearance process, are intercellular signal transporters harboring biomolecules from dying cells, which were previously recognized as cell debris [8,25]. Traditionally, apoEVs are indicated to be apoptotic bodies with a diameter of 1–5 μm [8,29,32]. Some smaller EVs are also generated in apoptosis, described as apoptotic microvesicles (apoMVs, 50–1,000 nm) [4,7,28,44] and apoptotic exosomes (apoExos) (<150 nm) [6,45], because their size resembles microvesicles and exosomes released by viable cells [46]. Since the conventional identification of subtypes of apoEVs is currently ambiguous [25], apoEVs could be a general description of micro- to nanoscale vesicles [47]. Here, we demonstrated several representative morphologies of apoEVs (see Figure 2).

3.1 Biogenesis and characteristics

Nowadays, the biogenesis of apoEVs is described as three sequential well-coordinated steps with corresponding

morphological changes: plasma membrane blebbing, thin membrane protrusions formation, and fragmentation [8,25]. In the formation of membrane protrusions, different protrusions might be generated, including microtubule spike, apoptopodia, and beaded apoptopodia. However, for some specific cells, not every step is necessary for the biogenesis of apoEVs (Figure 3). Apoptotic membrane blebbing, the earliest morphological change in apoptosis, is driven by cytoskeleton collapse and increased hydrostatic pressure [32]. Caspases and various protein kinases regulate this mechanism, preventing apoptosis when they are inhibited [31]. Rho-associated protein kinase 1, a target of active caspase-3, induces phosphorylation of the myosin light chain (MLC), causing actomyosin contraction [32]. MLC kinase facilitates the assignment of degraded chromatin into blebs [48]. Serine/threonine LIM domain kinase 1 inhibits the actin-binding protein cofilin to promote actin polymerization [49]. This membrane blebbing is known as fundamental for membrane protrusions, but some cells undergo different membrane deformation in this process, indicating diverse mechanisms in addition to blebbing solely in apoEVs formation (Figure 3). Apoptotic A431 epithelial cells formed a rigid membrane protrusion, the microtubule spike, in the process of apoptosis without blebbing [50]. Human Jurkat T cells generated apoptopodia

Figure 2: Several representative images of apoEVs. (a) Scanning electron microscopy (SEM) image of MSC-derived apoEVs. Scale bar, 1 μm [18]. (b) Transmission electron microscopy (TEM) image of MSC-derived apoEVs. Scale bar, 125 nm [68]. (c) Live differential interference contrast (DIC) microscopy image showing generation of apoEVs from THP-1 cells undergo UV-induced apoptosis. Scale bar, 5 or 10 μm as indicated [5].

Figure 3: Morphological changes of apoptotic cells in the biogenesis of apoEVs. It could be described as three steps with corresponding morphological changes: plasma membrane blebbing, membrane protrusions formation, and cell fragmentation. In step 2, different protrusions might be generated, including microtubule spike, apoptopodia, and beaded apoptopodia. Dotted arrows mean that apoEVs can be generated by some specific cells without step 1 or step 2.

following blebbing, which is regulated by the caspase-activated four-pass transmembrane pannexin 1 channel, a negative regulator of apoptosis [51]. Recently, a novel “beads-on-a-string” type of membrane protrusion in apoptosis was described in apoptotic human THP-1 cells and monocytes. This beaded apoptopodia forms abundant apoEVs (approximately 10–20) with diameters of 1–4 μm due to the formation of protrusions on the “string” with a length eight times that of the dying cell, rendering it much more efficient than the previous two patterns of membrane protrusion [52]. After the formation of blebs and/or membrane protrusions, fragmentation is responsible for the drop of single apoEV (Figure 3). While the specific mechanism or molecules mediating this process are currently unclear now, shear force in circulation or intercellular physical force of cell disassembly might be involved [8].

By the end of apoptosis, “find-me” and “eat-me” signals are indispensable for the attraction and clearance of phagocytes [53], avoiding cell content leakage and secondary necrosis [32]. The “find-me” signal is a chemo-attractive gradient generated by the released soluble molecules in apoptosis, such as ATP, UTP [54], and CX3CL1/fractalkine [55], which was also found to be associated with apoEVs [25,56]. Some receptor proteins in the membrane of phagocytes, the G protein-coupled receptor G2A, for example, induce the recognition of “find-me”

signals, even though the precise pathway of this process and quantified attractive gradient remain to be elucidated [57]. The most universally studied “eat-me” signal is the exposure of phospholipid phosphatidylserine (PtdSer) [55], anchoring on the surface of apoEVs. In the membrane of healthy cells, PtdSer is strictly confined to the inner side of the bilayer. While undergoing apoptosis, PtdSer flips from the inner of the lipid bilayer of the plasma membrane to the outer layer during blebbing, which is induced by caspase-activated scramblases, a set of phospholipid translocases [57]. The appearance of PtdSer on the outer layer in both apoptotic cells and apoEVs transmits the dying signal, inducing recognition and clearance of phagocytes. In addition, ICAM-3, a confirmed mediator of apoptotic cell clearance, was also present on the surface of apoEVs to attract macrophages [58]. Notably, apoEVs seem to recruit phagocytes selectively, which might be due to content diversity caused by different parental cells and specific physiological and pathological processes. Adipocyte-derived apoEVs recruited macrophages rather than neutrophils [59]. However, neutrophils’ infiltration was observed in mice, in which larger apoEVs (1–3 μm in diameter) attracted neutrophils, whereas smaller ones (<1 μm) could not. Inflammatory chemokine IL-1 appeared in larger apoEVs but not in smaller ones to induce sterile neutrophil inflammation, which might

originate from the nuclear IL-1 α precursor that translocated into the nucleus during apoptosis [60]. The equilibrium between apoEVs and phagocytes is indispensable for homeostasis. Secondary necrosis might occur when massive apoptosis and subsequent excessive apoEVs containing inflammatory factors overwhelm the clearance ability of phagocytes or when the function or quantity of phagocytes is impaired in diseases.

In most studies, apoEVs were described as bioactive carriers transporting functional molecules [3,16,31], including DNA debris [61], RNA [21,62–64], and proteins [65,66]; yet, cargo differences among each group of apoEVs exist widely. The proteomes of healthy and cirrhotic human biliary epithelial cells showed a huge divergence in the shotgun proteomics of apoEVs [65]. Furthermore, exosome-like apoEVs carried more active 20S proteasome core than that in apoBDs, which is responsible for their immunogenic activity [45]. More interestingly, DNA and RNA could not be packed into apoEVs of HL-60 cells simultaneously. Over 90% of apoEVs containing RNA were free of DNA, and *vice versa* [62]. Additionally, nuclear material was absent in beaded apoptopodia [52], suggesting that some bioactive molecules may enter apoEVs with tropism. Different groups of apoEVs possess diverse properties and functions due to their distinct biogenesis processes and unequal contents that result from different parental cells and their previous physiological and pathological changes.

3.2 Production and identification

Technically, it is necessary for researchers to study apoEVs in a relatively uniform condition to acquire convincing information, whereas cells in a general culture condition are less likely to undergo apoptosis under control. Thus, some stimuli of apoptosis in specific physiological or pathological conditions have been applied in studies to produce apoEVs. Here, we reviewed production and isolation methods in recent years studying apoEVs, along with their documented times, donor cells, and experimental models (Table 1). The most universally used stimulus of apoEVs is staurosporine [18,19,23,64,67,68], a potent inhibitor of protein kinase C, which induces apoptosis by activating caspase-3 and suppressing the AKT/MAPK pathway [69,70]. Serum starvation might affect the cell cycle and promote apoptosis [71]. Exposure to gamma rays or ultraviolet radiation injured the stability of nucleic acids [67,72,73], which also appeared when applying some chemotherapeutic drugs, such as cisplatin [67]. For some specific cells, the corresponding stimulus was applied.

Alendronate was used to produce apoEVs from osteoclasts due to endoplasmic reticulum stress in osteoclast precursors caused by alendronate [74].

For isolation of apoEVs, apoMVs, a subtype of apoEVs resembling microvesicles in size [4], were obtained in a centrifuge at 16,000–50,000g. ApoBDs and apoExos were isolated in a centrifuge at 1,000–5,000g and 100,000–200,000g, relatively. The size and morphology were observed and determined by dynamic light scattering (DLS) analysis, scanning electron microscope (SEM), transmission electron microscope (TEM), or confocal microscope. Fluorescence-activated cell sorting (FACS) was used to quantify and purify apoEVs. Several molecules were detected to identify apoEVs. The most widely accepted biomarker is Annexin V, a calcium-binding protein of the annexin superfamily, which can interact with the “eat-me” signal PtdSer. Thrombospondin and complement protein C3b, the products of membrane changes during apoptosis, are also well-accepted biomarkers. Cleaved caspase-3 and C1q were also applied in some cases. Nuclear granularity and hypochromicity were detected by propidium iodide but their quantities were not equal and were even absent in some apoEVs [5,15,18–21,23,44,63,64,67,68,72,73,75].

However, it is noteworthy that the current classification is based on size while ignoring their biogenesis in cells and the contents of each group. Besides, apoEVs derived from different cells might differ in size, and apoEVs would collapse over time *in vitro* [5]. Actually, the current studies are less likely to identify and analyze the subtype properties of apoEVs utilized in experiments, rendering disturbances from different groups inevitable. It is pivotal to further analyze the contents and properties and uncover the key proportion of each group of apoEVs, which contributes to standardizing protocols for isolation and identification, thus obtaining rigorous scientific results with diminished confounding.

4 Functions of apoEVs in tissue regeneration

ApoEVs regulate cell function by transferring various and abundant cargo, which involves many bioactive factors participating in tissue repair and regeneration. ApoEVs derived from different parental cells might differ in content and affect cell activities *via* diverse pathways. In tissue regeneration, the beneficial effects of apoEVs have been studied and discussed. Figure 4 demonstrates the main mechanisms of apoEVs in tissue regeneration, including promoting the proliferation of neighboring cells by

Table 1: Production and isolation strategies of apoEVs in recent years

Ref	Origin of apoEVs	Inducer of apoptosis	Models	Isolation strategy
Hristov <i>et al.</i> [118]	HUVEC	Serum starvation	<i>In vitro</i> : epithelial progenitor cell	16,000g*
Zernecke <i>et al.</i> [21]	HUVEC; human atherosclerotic plaque material, mouse whole blood	Serum starvation	<i>In vitro</i> : HUVEC	16,000g for supernatant; FACS for tissue and blood
Zhu <i>et al.</i> [63]	BMM	Lipopolysaccharide	<i>In vivo</i> : rat	2,000g
Shen <i>et al.</i> [72]	T cell, neutrophil	UV exposure	<i>In vitro</i> : A549 epithelial cell	100,000g
Liu <i>et al.</i> [44]	MSC	Staurosporine	<i>In vitro</i> : Th cell	1 and 5 µm filters followed by 2,000g
Brock <i>et al.</i> [75]	Epithelial stem cell	Metronidazole	<i>In vitro</i> : HUVEC	—
Hardy <i>et al.</i> [64]	HUVEC	Serum starvation	<i>In vivo</i> : zebrafish	50,000g for apoBDs and 200,000g for apoExos
Chen <i>et al.</i> [73]	Primary murine thymocytes, Jurkat cell	Gamma ray or UV exposure, serum starvation	<i>In vitro</i> : macrophage	180,000g
CoralGarcía-Pastor <i>et al.</i> [67]	HK-2 cell	Cisplatin, UV exposure	<i>In vivo</i> : mice	5,000g
Ma <i>et al.</i> [20]	BMM, osteoclast	Alendronate	<i>In vitro</i> : MC3T3-E1 cell	1 and 5 µm filters followed by 3,500g
Tyukavin <i>et al.</i> [90]	Cardiomyocyte, fibroblast	Serum starvation	<i>In vivo</i> : rat	16,500g
Liu <i>et al.</i> [15]	MSC	Staurosporine	<i>In vitro</i> : HUVEC	16,000g
Ma <i>et al.</i> [19]	Osteoclast	Staurosporine	<i>In vivo</i> : rat	3,000g
Dou <i>et al.</i> [23]	T cell, HUVEC	Staurosporine	<i>In vitro</i> : endothelial cell	1,000g
Liu <i>et al.</i> [18]	MSC	Staurosporine	<i>In vitro</i> : macrophage	16,000g
Zheng <i>et al.</i> [68]	MSC	Staurosporine	<i>In vivo</i> : mice	16,000g
			<i>In vitro</i> : macrophage	
			<i>In vivo</i> : mice	

*Centrifuge speed; HUVEC: human umbilical vein endothelial cells; FACS: fluorescence activated cell sorting; BMM: bone marrow macrophages; UV: ultraviolet; MSC: mesenchymal stem cells.

compensatory proliferation signals and remote cells by transferring therapeutic molecules and suppressing inflammation by regulating macrophage polarization (Figure 4).

4.1 Promoting cell proliferation

Tissue integrity is maintained in the homeostasis of dynamic cell death and compensatory proliferation. Apoptosis has been implied to activate compensatory proliferation signaling, and hence, dying cells mediate compensatory proliferation in adjacent cells [11,13,30,34,76,77], which is also called AiP [11,34]. ApoEVs, the product of dying cells, seem to inherit such a compensatory proliferation function. ApoEVs produced by different apoptotic stimuli were reported to trigger neighboring cell proliferation in both transformed and primary cells upon contact *via* compensatory proliferation signaling [78], which is related to CrkI, a type of protein responsible for cell proliferation and cytokinesis [79,80] (Figure 4). Epithelial stem cell-released apoEVs also triggered cell proliferation of standby healthy stem cells in such a compensatory proliferation way, maintaining regeneration of the epithelium [75]. In addition to the near approach, apoEVs were also implicated in promoting cell proliferation in remote sites. ApoEVs from macrophages

delivering miR-221/222 induced lung epithelial cell growth by suppressing the expression of cyclin-dependent kinase inhibitor 1B, a tumor suppressor controlling cell cycle at G1 [63] (Figure 4). Circulating apoEVs from MSCs transferring ubiquitin ligase RNF146 and miR-328-3p recovered the self-renewal and differentiation properties of distant impaired MSCs [44] (Figure 4). Intriguingly, but predictably, the Wnt/ β -catenin signaling pathway participated closely in cell growth and renewal promoted by apoEVs [44,75,78]. Focusing on miRNAs and proteins modulating the Wnt pathway in apoEVs could be a possible way to elucidate the mechanisms by which apoEVs promote tissue regeneration.

4.2 Regulating inflammation

Inappropriate inflammation impedes repair and regeneration progress in multiple diseases [81–84]. ApoEVs are capable of attenuating inflammation by modulating the cell activities of inflammatory cells, thereby smoothing the tissue regeneration process. Suppression of inflammatory cytokines, including MCP-1 and IL-6, caused by upregulated TSG-6 was observed after treatment with apoptotic MSCs [85] (Figure 4). TSG-6 has been reported to reduce proinflammatory signals from macrophages [86] and

Figure 4: The main mechanisms of apoEVs in tissue regeneration, including promoting the proliferation of neighboring cells by compensatory proliferation signals and remote cells by transferring therapeutic molecules, and suppressing inflammation by regulating macrophage polarization.

Figure 5: Therapeutic effects of apoEVs in regeneration of the skin, bone, cardiovascular system, and kidneys.

inhibit the activation of macrophages [87]. Furthermore, apoEVs have been implicated in modulating macrophage polarization both *in vivo* and *in vitro*. In mouse models, apoEVs from MSCs promoted polarization of anti-inflammatory M2 macrophages, whereby they increased the anti-inflammatory cytokines IL-10 and TGF- β and enhanced the migration and proliferation of fibroblasts [18] (Figure 4). Biomimic apoEVs were taken up more effectively by activated M1 macrophages and led to more intensive M2 macrophage polarization, ameliorating inflammation by upregulating anti-inflammatory cytokines [88]. In addition, apoEVs derived from activated T cells were likely to possess trophic properties to inflammatory sites similar to that of their parental cells, meanwhile, promoting M2 macrophage polarization [23]. In short, apoEVs help to recover a healthy microenvironment of repair and regeneration by ameliorating excessive local inflammation.

Besides all the above, many other intricate mechanisms contribute to the regeneration function of apoEVs. ApoEVs from MSCs promoted the proliferation of endothelial cells [15] and endothelial progenitor cells [89], thus inducing angiogenesis. ApoEVs derived from different parental cells manifested specific functions in regeneration resembling their parental cells, which was observed in preosteoclasts and mature osteoclasts [19,20], cardiomyocytes [90,91], and proximal tubular cells [67]. From this perspective, stem cells might be a promising origin of apoEVs due to their intrinsic regenerative properties. However, it is difficult to draw a definite conclusion from these

scattered studies since there is a little convincing result indicating the direct correlation between the regenerative property of apoEVs and specific mechanisms.

5 Therapeutic effects of apoEVs in regeneration

In recent years, researchers have probed and utilized the regeneration ability of apoEVs in numerous tissue regeneration processes. Here, we summarize and discuss the promotion of repair and regeneration of the skin, bone, heart, and kidneys by apoEVs (Figure 5).

5.1 ApoEVs in skin regeneration

ApoEVs produced by MSCs facilitated wound healing as MSCs [18,85,92]. ApoEVs induced M2 macrophage polarization, the anti-inflammatory subtype of macrophages, and sequentially promoted the viability of fibroblasts, accelerating cutaneous wound repair [18] (Figure 5). Furthermore, apoEV mimics carrying miR-21/curcumin boosted M2 polarization [23], indicating that the membrane of apoEVs and these therapeutic factors cooperated in skin regeneration. Taking advantage of the macrophage targeting property, apoEVs delivering antibiotic

vancomycin relieved intracellular methicillin-resistant *Staphylococcus aureus* infection of macrophages [24]. Remaining dormant in macrophages, intracellular *Staphylococcus aureus* escape from the immune system and antibiotics, causing persistent cutaneous infection, which results in impaired wound healing and skin regeneration [93]. Moreover, after the apoptotic induction of TNF- α , apoEVs enveloping more IL-1RA were released by MSCs, promoting gingival and skin regeneration through the Fas/Fap-1/Cav-1 axis [92]. Reduced hypertrophic scar formation in the process of skin regeneration was also observed after treatment with TSG-6 secreted by apoptotic human MSCs [85]. TSG-6, a protective factor in the inflammatory response, has been reported to be one of the dominant mediators in the regenerative ability of MSCs [94].

5.2 ApoEVs in bone regeneration

ApoEVs produced by osteoclasts also manifested similar biological effects as parental cells [19,20]. Preosteoclast-derived apoEVs promoted angiogenesis, while mature osteoclast-apoEVs promoted osteogenesis, similar to perspective parental cells both in bioinformatics analysis and *in vivo* [19]. Mechanistically, mature osteoclast-derived apoEVs induced the differentiation of osteoblasts that mediate osteogenesis by activating receptor activators of NF- κ B ligand reverse signaling [20,95], coupling bone resorption to formation (Figure 5). Notably, MSCs released apoEVs rescued the osteopenia phenotype in both apoptosis-deficient mice and ovariectomized mice by restoring stem cell properties of impaired MSCs (Figure 5). The uptake in the circulation of ubiquitin ligase RNF146 and miR-328-3p carried by apoEVs activated the Wnt/ β -catenin pathway [44], a conservative signaling pathway crucial in the development and tissue regeneration [96,97]. These results demonstrate the indispensable role of apoEVs in regulating bone remodeling and maintaining bone homeostasis.

5.3 ApoEVs in the cardiovascular system

ApoEVs derived from different donor cells provide varied biological benefits for cardiovascular system protection and regeneration. Endothelial apoEVs deliver miR-126 ameliorated atherosclerosis and plaque *in vivo* by reducing macrophage infiltration and apoptotic cells. Endothelial apoEVs upregulated the expression of chemokine CXCL12

and mobilized Sca-1⁺ progenitor cells, thus inhibiting CXCR4-dependent atheroprogession [21]. Similarly, an apoEV biomimetic liposome enhanced the stability of atherosclerotic plaques, which means a lower possibility of plaque rupture and subsequent thrombosis by improving macrophage-induced inflammation to protect the cardiovascular system [88] (Figure 5). In another aspect, MSC-derived apoEVs enhanced angiogenesis and cardiac function recovery in myocardial infarction mice, which was due to recipient endothelial cells activating autophagy [15]. Moreover, apoEVs derived from cardiomyocytes regulated the differentiation of cardiac stem cells to cardiomyocyte precursors and improved the contractility of the myocardium [90,91], while apoEVs released by fibroblast produced no such effect [90] (Figure 5).

5.4 ApoEVs in renal regeneration

In renal regeneration, a novel type of apoEVs containing Crkl has been described, inducing mitosis and proliferation of nearly 100% analyzed recipient cells including renal parietal epithelial cells [78]. Podocyte-derived apoEVs containing Crkl might promote regeneration of injured tubular epithelial cells [22] (Figure 5). Surprisingly, apoEVs released by the first apoptosis in proximal tubular cells (PTCs) caused by cisplatin mediated the secondary apoptosis of recipient PTCs, while apoEVs produced by this secondary apoptosis stimulated tubular cell proliferation [67]. These controversial effects of apoEVs derived from different apoptosis processes might be explained by the propagation of toxic effects of cisplatin or apoptotic stimulus of Fas ligand that was probably expressed on apoEVs [98]. Nevertheless, specific comparison experiments concerning apoEVs induced by cisplatin and other stimuli, and the interaction and homeostasis between apoptosis and regeneration of PTCs need to be addressed further. In other words, apoEVs from diverse origins may have distinct therapeutic effects or play different roles in different biological processes.

6 Engineered apoEVs

EVs have been reported to possess tropism targeting a specific tissue or cell [99–103], known as the “homing effect” [101–103]. Moreover, EVs show natural advantages as drug delivery systems over synthetic nanoparticles, with the ability to cross biological barriers efficiently

and interact with the plasma membrane *via* ligand/receptor responses, while possessing low immunogenicity and toxicity [100,104–107]. These intrinsic properties of apoEVs qualify them as optimal bioactive materials to be modified for tissue regeneration, causing more precise tissue targeting, enhanced inflammation modulation, and longer drug retention. Thus, apart from the direct utilization of natural apoEVs, modified apoEVs or biomimetic apoEVs are also a concern.

The unique identifying signal of apoEVs for phagocytes has piqued the interest of researchers in harnessing their targeting ability. ApoEV biomimicking liposomes were constructed by connecting PtdSer onto the surface of liposomes [88], inspired by the “eat-me” signal from the exposed PtdSer of apoEVs. The PtdSer-modified liposomes effectively escaped from clearance by the reticuloendothelial system owing to PEGylation [88,108]. Compared with liposomes, the modified liposomes that targeted M1 macrophages were engulfed more effectively as expected. As a sign of suppressed inflammation, inhibited M1 polarization and motivated M2 polarization were observed from increased anti-inflammatory cytokines such as IL-4 and IL-10 and decreased proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α [88] (Table 2). In addition, apoEVs derived from some kinds of cells might inherit the particular targeting property of their parental cells [109], such as leukocytes. Taking this into consideration, apoEVs released by T cells were manipulated to target inflammatory sites and then macrophages [23]. In this research, natural membranes of T cell-derived apoEVs without cargos were obtained and then encapsulated mesoporous silica nanoparticles (MSNs) that were preloaded with anti-inflammatory molecules. These chimeric apoEVs showed comparable CD44 and Mac-1 protein expression to T cells, which were responsible for recognition between inflamed endothelium and leukocytes [110,111], thus displaying inflammation targeting capacity both *in vitro* and in colitis models (Table 2). Promoted macrophage-specific phagocytosis and M2 polarization was observed as well [23]. Similarly, cancer cell-derived apoEVs were reconstructed for targeting the same type of cancer cells as well as targeting macrophages [24].

Combined with their targeting property dictated by their membrane markers and cargo content [105], the natural lipid membrane potentialized apoEVs as drug delivers. Co-bathing in sonication is one of the common strategies to load therapeutic molecules into apoEVs [23] (Table 2). To boost the efficiency and stability of drug loading, therapeutic molecules could be preloaded into MSNs, an outstanding nanoplatform as a drug carrier [112,113], by electrostatic interactions [23] or undergoing a freeze–thaw process bathing with apoEVs [24]. By a

Table 2: Modification of engineered apoEVs in recent years

Methods of modification	Forms of modification	Purposes of modification	Functional parts	Results of modification
Connecting PtdSer onto the surface of liposomes	Liposomes with “eat-me” signal of apoEVs	Targeting property for macrophages	PtdSer on the surface	M2 macrophage polarization; inflammation amelioration
Co-bathing in sonication of the membrane of apoEVs and MSNs	Chimeric T-cell apoEVs with MSNs loading anti-inflammatory molecules	Targeting property for inflammatory sites	CD44 and Mac-1	Inflammation amelioration
Freezing and thawing apoEVs with vancomycin	Reconstructed apoEVs carrying vancomycin	Delivering drugs into cells	Lipid membrane of apoEVs; vancomycin	Antibacterial effect inside macrophages
Transient transfection of miRNA/siRNA into donor cells	apoEVs containing miRNA transfected	Loading and delivering miRNA into apoEVs	Lipid membrane of apoEVs; therapeutic miRNAs	Proved possibility of loading miRNA into apoEVs
Embedding apoEVs into PF-127 hydrogel	Combination of apoEVs and bioactive scaffolds	Longer drug retention	Sustained release of apoEVs in PF-127 hydrogel	Improved skin regeneration

PtdSer: phosphatidylserine; MSN: mesoporous silica nanoparticles; miRNA: micro-RNA; siRNA: silencing RNA.

mechanically extruding technique, apoEVs were processed into a relatively uniform size of 80–150 nm and thereby obtained an enhanced encapsulation efficiency of vancomycin [24] (Table 2). Otherwise, genetic manipulation can also be considered. Transient transfection into donor cells of therapeutic miRNA could also be transferred into apoEVs successfully [68], providing another cargo loading approach (Table 2).

Another drug delivery strategy is loading apoEVs into materials or scaffolds with biocompatibility, not concerning the targeting property or membrane superiority, but aiming to achieve longer drug retention and controlled release. When embedded into PF-127 hydrogel, apoEVs showed the fastest skin regeneration [18] (Table 2). Although a concentration–time curve is lacking in this research, a compelling retention time and promoted regeneration ability of other EVs combined with biomaterials have been provided [114–116]. In general, the combination of apoEVs and biomaterials is an attractive method with the potential for sustainable therapy.

7 Challenges, prospects, and conclusions

ApoEVs, the products, and meanwhile the information disseminators, seem to inherit the therapeutic role of apoptosis in tissue regeneration, changing death to another beginning of life. Different biogenesis pathways of apoEVs seem to be linked to cell types. To obtain information on the reason why different biogenesis patterns of apoEVs occur, investigating the functions of cells undergoing diverse apoptotic ways and respective apoEVs might be a possible direction. Moreover, morphological diversities in different biogenesis patterns of apoEVs might contribute to the clearance of apoptotic cells. For example, suppressed blebbing in Jurkat T cells caused inhibited uptake by macrophages in cell disassembly [117]. The interaction between microtubule spikes of A431 epithelial cells and phagocytes was reduced when the formation of microtubule spikes was inhibited [50]. Although a conclusive explanation for such diverse patterns still needs to be clarified, diversity among the contents of apoEVs originating from different sources exists. For example, apoEVs undergoing “beads-on-string” apoptosis contain no nuclear material, unlike other groups of apoEVs [52]. In addition, research has seldom compared the underlying discrepancies in different production protocols or differences among subtypes of apoEVs. In limited literature that analyzed the content diversity of apoEVs,

different attractive abilities for neutrophils were demonstrated in various groups of apoEVs. The group containing inflammatory chemokines caused secondary inflammation, which we will not anticipate to occur in tissue regeneration. This means that it is not that more apoEVs there are, the better the regenerative effect, but the appropriate quantity and prompt processing of phagocytes, along with the optimal group of apoEVs, or the specific contents of apoEVs; in another word, matter more. Figuring out these puzzles is fundamental to probe into the particular portion of apoEVs functioning directly or mediately as key therapeutics in different diseases, thus further formulating instructive protocols to study or manufacture in the future, aiming at specific pathological situations.

In conclusion, apoptosis, the silent closure of one cell, and the initiation of other cells play pivotal roles in tissue integrity maintenance. ApoEVs may promote tissue regeneration, including the tissue of skin, bone, cardiovascular system, and kidneys, by activating targeted recipient cells or transferring biomolecules (DNA, RNA, proteins) to facilitate proliferation and regulate inflammation, both locally and remotely. Nevertheless, the reason why apoEVs could be generated in different patterns *in vivo*, and the connection between different biogenesis patterns and the properties of the respective apoEVs remain unclear. Controlling the distribution and release of biomolecules or drugs carried by apoEVs is also important for therapeutic usage, for which manipulation by engineered means and combination with biomaterials can be optional. There is still a long way to utilize the regenerative and therapeutic functions of apoEVs, and many comprehensive research studies need to be finished.

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