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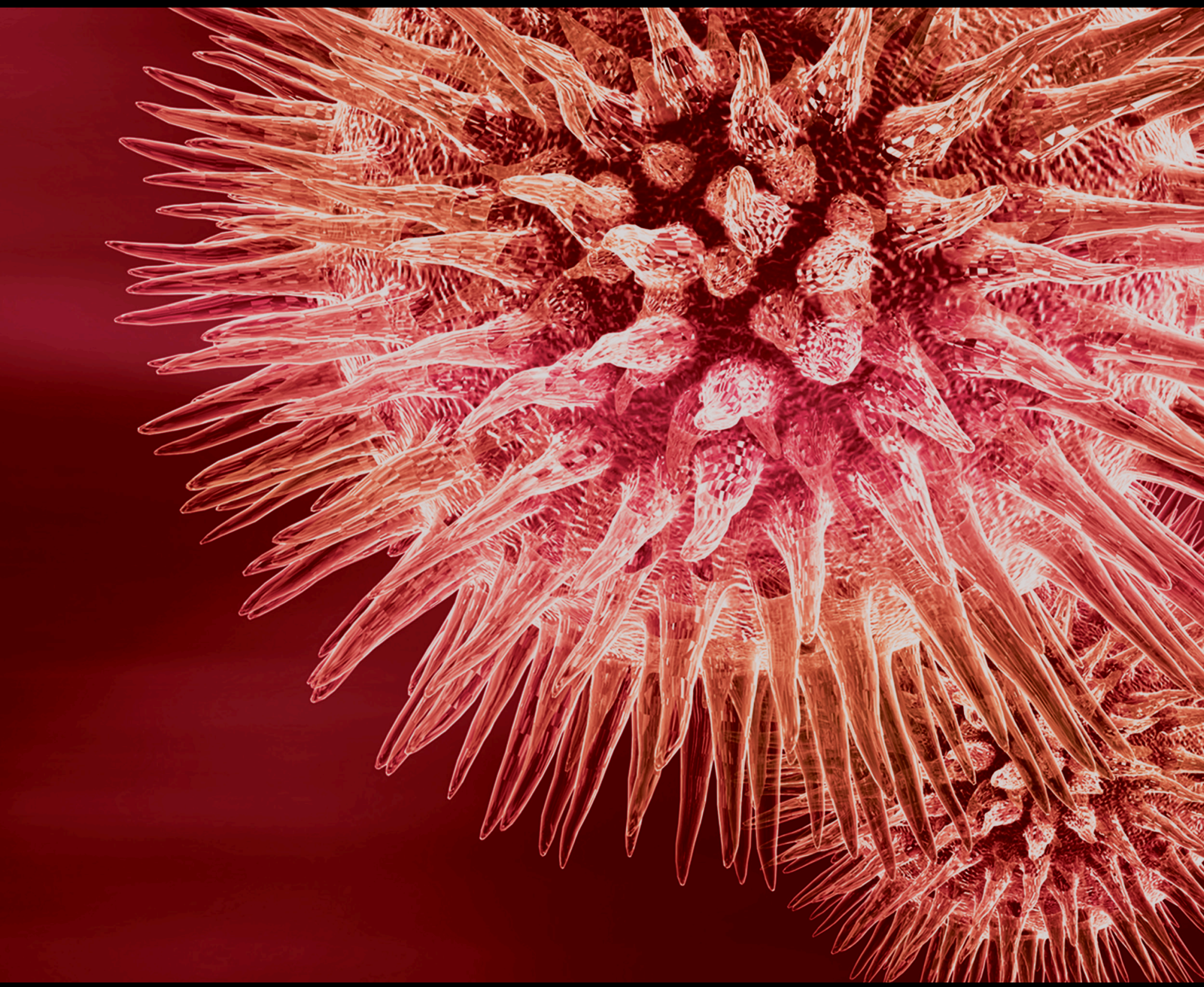
A Novel Role for Extracellular Vesicles in Cytopathology and New Therapeutic Strategies

Lead Guest Editor: Shi-Cong Tao
Guest Editors: Shang-Chun Guo and Johnny Collett



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Editorial Board

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Editorial

A Novel Role for Extracellular Vesicles in Cytopathology and New Therapeutic Strategies

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Extracellular vesicles (EVs) are a newly discovered way by which cells communicate with their neighbouring cells. EVs are lipid nanovesicles that enclose proteins (membrane proteins and internal proteins) and nucleic acids (miRNA, mRNA, lncRNA, and circRNA). EVs include exosomes having a diameter of 30–100 nm (called "small EVs" (sEVs), according to the "Minimal Information for Studies of Extracellular Vesicles 2018," or "MISEV2018," guidelines) and microvesicles having a diameter of 100–1,000 nm (called "large/medium EVs" (l/mEVs), according to the MISEV2018 guidelines). These sEVs and l/mEVs are generated and released from most cell types and, hence, are found in most biological fluids. In particular, sEVs have increasingly attracted attention for their role in physiology and pathology, as well as their possible use as diagnostic and therapeutic tools.

This special issue compiles a series of 5 original contributions and 6 comprehensive reviews that, while not being a complete representation of the field, are an important collection of multifaceted works; we have the pleasure of sharing this knowledge with our readers. These articles cover the relevant aspects of physiology, pathogenesis, diagnoses, and therapies that involve EVs.

X. Wu et al. reported a study that evaluated whether the miRNAs enclosed in circulating sEVs have the predictive values in patients at risk of developing acute respiratory distress syndrome (ARDS) from severe community-acquired pneumonia (SCAP). They found a subset of sEVs carrying miRNAs, including miR-146a, miR-27a, miR-126, and miR-155, in ARDS group samples that exhibited significantly elevated levels than those in non-ARDS group samples. The combined expression of miR-126, miR-27a, miR-146a, and miR-155 predicted ARDS with an area under the curve of 0.909 (95% CI 0.815–1). Only miR-126 had the potential to predict 28-day mortality (OR = 1.002, $P = 0.024$) with its median value classifier. The authors suggest that altered levels of circulating sEVs carrying miRNAs may be a useful biological confirmation of ARDS in patients with SCAP.

C. Dong et al. described the potential roles of circulating sEVs carrying miRNAs during mesenchymal stem cell (MSC) senescence, which is crucial in the development and progression of systemic lupus erythematosus (SLE). The authors found that sEVs derived from the SLE serum increased the proportions of SA-β-gal positive cells, disorganized cytoskeleton structures, and reduced growth rates in MSCs. In SLE patients, miR-146a declined significantly in the level of serum sEVs, compared with healthy controls. This reduction in miR-146a levels could be involved in a novel mechanism of MSC senescence in SLE patients through the targeting of TNF receptor associated factor 6 (TRAF6)/nuclear factor kappa-B (NF-κB) signalling.

J. Wang et al. used high-throughput sequencing to explore the miRNA expression profiles of sEVs in the peripheral blood of kidney recipients having delayed graft function (DGF). They identified 52 known and 5 conserved sEVs containing miRNAs specifically expressed in recipients having DGF. Three coexpressed miRNAs, hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p, were found to be significantly upregulated in kidney recipients with

DGF. Moreover, hsa-miR-151a-5p was positively correlated with the first-week serum creatinine (CR), blood urea nitrogen (BUN), and uric acid (UA) levels in the kidney recipients, after transplantation. In addition, they also analysed functions and signalling pathways of the 3 upregulated miRNA target genes to uncover a putative mechanism of how these miRNA-carrying sEVs functioned during DGF.

J. Ding et al. investigated whether sEVs originated from bone marrow-derived mesenchymal stem cells (BMSCs) preconditioned by deferoxamine (called DFO-sEVs), and explored the mechanisms underlying their superior proangiogenic properties during wound repair. They reported that, in cell-free therapies, DFO-sEVs activate the phosphoinositide 3-kinase (PI3K)/AKT signalling pathway via miR-126-mediated phosphatase and tensin homolog (PTEN) downregulation to enhance healing and angiogenesis in diabetic wounds.

K. Saito et al. are the first to report that second-generation antihistamines, including cetirizine, fexofenadine, azelastine, and terfenadine, exert suppressive effects on Kv1.3-channels in lymphocytes. The efficacy of these drugs may be related to their immunomodulatory mechanisms that reduce inflammatory cytokine synthesis. As a matter of fact, EVs play an important role in inflammatory cells. In a recent study, lymphocytes were found to release EVs which may contribute to type 1 diabetes development. Meanwhile, ion regulatory channels were found to be closely related to EVs. Thus, their findings may suggest a new breakthrough point in extracellular vesicles and new tools for studying them.

R. Dong et al. reviewed the current understanding of sEVs derived from mesenchymal stem cells (MSCs) related to peripheral nerve repair and provide insights on the development of a cell-free MSC therapeutic strategy for peripheral nerve injury (PNI).

J. Lu et al. reviewed the possible relationships between risk factors for bone nonunion and EVs and then discussed the roles of EVs in bone metabolism and regeneration. More and more literature points out that EVs are likely to be the way cells (including osteoclasts, osteoblasts, osteocytes, endothelial cells, and MSCs) communicate with each other in the process of bone healing via the exchange of bioactive substances (such as proteins and nucleic acids). Beyond that, EVs can be used to deliver functional RNAs and mediate cell-to-cell communication, suggesting that EVs may repair bone defects by regulating cells and cytokines involved in bone metabolism.

W. Zhao et al. reviewed the roles of emerging sEV-carried long noncoding RNAs (lncRNAs) in cancer. In addition, the biogenesis of sEVs, the functions of lncRNAs, and the mechanisms of lncRNAs in sEV-mediated cell-cell communication were also summarized.

W. Tian et al. reviewed the latest literature to summarize the action mechanisms and experimental studies of sEVs in cancer metastasis. Studies have shown that sEVs play a vital role in cancer metastasis in forming the premetastatic niche, influencing tumor cells and microenvironment, and determining specific organotropic metastasis. They also raised open questions about the influence of sEVs on metastasis: "(a) Are exosomes still playing a role during tumor latency or after primary tumor resection? (b) What causes the difference of exosomal biomarker levels in serum and plasma? (c) What are the mechanisms governing the specific exosomal cargo targeting between tumor and recipient cells which contribute to inconsistent expression of exosomal inclusions in blood and tissue?"

Z. Zhang et al. summarized recent findings on the function of noncoding RNAs (ncRNAs) and then studied their interaction with premetastatic niche formation, which highlighted the potential of using EVs carrying ncRNAs for cancer diagnosis and therapeutic strategies against it.

S. Li et al. reviewed the role of sEVs in hepatitis B virus (HBV) infection and discussed the following: (1) direct participation of sEVs in HBV replication; (2) modulation of immune responses by sEVs during HBV infections; (3) sEVs carrying RNAs and proteins, which could be novel biomarkers for the diagnosis of HBV infections; and (4) sEVs designed as vaccines against HBV.

Conflicts of Interest

The editors declare that they have no conflicts of interest.

Shi-Cong Tao
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Johnny Collett

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Review Article

MSC-Derived Exosomes-Based Therapy for Peripheral Nerve Injury: A Novel Therapeutic Strategy

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Guest Editor: Shi-Cong Tao

Although significant advances have been made in synthetic nerve conduits and surgical techniques, complete regeneration following peripheral nerve injury (PNI) remains far from optimized. The repair of PNI is a highly heterogeneous process involving changes in Schwann cell phenotypes, the activation of macrophages, and the reconstruction of the vascular network. At present, the efficacy of MSC-based therapeutic strategies for PNI can be attributed to paracrine secretion. Exosomes, as a product of paracrine secretion, are considered to be an important regulatory mediator. Furthermore, accumulating evidence has demonstrated that exosomes from mesenchymal stem cells (MSCs) can shuttle bioactive components (proteins, lipids, mRNA, miRNA, lncRNA, circRNA, and DNA) that participate in almost all of the abovementioned processes. Thus, MSC exosomes may represent a novel therapeutic tool for PNI. In this review, we discuss the current understanding of MSC exosomes related to peripheral nerve repair and provide insights for developing a cell-free MSC therapeutic strategy for PNI.

1. Introduction

Peripheral nerve injury (PNI) is a common neurological disease that seriously threatens human health [1]. In general, it occurs in 2.8%-5% of polytrauma patients, may result in sensory and motor dysfunction, and may even cause permanent disability [2, 3]. Axonal outgrowth following PNI is orchestrated by many factors, such as the transformation of the phenotype of Schwann cells (SCs), the infiltration of immune cells, and neurovascular regeneration [4–6]. Regenerating axons grow only 1 mm per day, which makes the regeneration of peripheral nerves a challenge [7, 8]. Furthermore, the prolonged denervation of proximal nerves may increase the likelihood of irreversible atrophy of the innervated organs [9, 10]. Additionally, the loss of nerve tissue associated with PNI also poses additional problems for nerve regeneration [11]. Although autologous nerve graft is the gold standard technique, the use of nerve grafts is limited due to the shortage of donor sources and the possible loss of neurological function in the donor site [12, 13]. Over the past decade, various studies have focused on developing new methods to promote axonal regeneration without sacrificing other healthy functioning nerves [14, 15]. Although some synthetic nerve conduits permeated with an abundance of SCs or stem cells have been demonstrated to be beneficial, the outcomes of PNI remain far from ideal [16–18].

Mesenchymal stem cell (MSC)-based therapy is thought to be a promising strategy for PNI [19–21]. Previous studies have shown that transplanted MSCs can differentiate into SCs (an important type of glial cell in the peripheral nerve system that mediates axonal regeneration) in vivo, providing the support and nutrition needed for axonal growth [22, 23]. The mechanism by which MSCs facilitate nerve repair has not yet been clarified. Previous studies have shown that MSCs can adopt a Schwann cell phenotype in vitro after they are cocultured with peripheral nerve extracts from the distal segment of a damaged sciatic nerve [24, 25]. However, Sowa et al. found that transplanted MSCs significantly promote axonal outgrowth, the formation of myelin, and the recovery of denervated muscle atrophy; however, transplanted MSCs do not differentiate into Schwann cells [26]. This indicates that the therapeutic effect of transplanted MSCs is more likely

to be attributed to the indirect regeneration of endogenous SCs through a cellular paracrine mechanism than through transdifferentiation.

Exosomes are membrane nanovesicles that are found in almost all biological fluids, such as blood, urine, breast milk, ascites, and saliva [27, 28]. As important mediators of paracrine mechanisms, they have begun to be noticed. Recent studies have indicated that exosomes are associated with pathological and physiological conditions, such as neurodegenerative diseases [29–31], tumors [32–35], and tissue fibrosis [36–41]. Exosomes contain cellular signaling molecules (proteins, lipids, mRNA, miRNA, lncRNA, circRNA, and DNA) that mediate intercellular communication by horizontally transferring these types of cargo [42, 43]. Emerging evidence has shown that exosomes derived from MSCs exert beneficial effects on a variety of disease models, including models of myocardial infarction [44–47], hepatic fibrosis [48–50], and even cancer [51–56]. Significantly, MSC-derived exosomes have the potential to be used for therapeutic strategies for peripheral nerve regeneration. Thus, this review summarizes current studies on MSC exosomes-based therapeutic strategies and discusses the future implications of MSC exosomes as an agent for treating peripheral nerve injury.

2. Concise Review: Current Knowledge Regarding the Use of MSCs for Tissue Repair

MSCs are a population of undifferentiated adult stem cells that possess self-renewal capability, low immunogenicity, and multilineage differentiation potential [57]. As a preclinical agent, MSCs have achieved exciting outcomes in the field of tissue regeneration since they were first described in 1968. Autologous or allogenic MSC transplantation can remarkably improve clinical outcomes via mediating the inflammatory response, modulating cell apoptosis, and promoting cell proliferation [58]. As of now, many studies on MSC-based therapy are ongoing or have been completed, and it is generally considered to be effective and safe.

The clinical testing of and research on MSCs for extensively repairing tissue injury is predicated on the hypothesis that transplanted MSCs can transdifferentiate into appropriate cell types and migrate to target tissues to replace injured cells after transplantation. An increasing number of studies, however, have shown that the number of these cells engrafted within target tissues is extremely low (<1% of cells) [59]. Most transplanted MSCs are found to be trapped in the liver and spleen. Whether engrafted MSCs differentiate into the desired cells in vivo is also controversial. In addition, there are some inherent disadvantages of MSC-based therapy, including the instability of the cellular phenotype, high cost, issues of cellular origin and transport before transplantation, microinfarction caused by MSCs lodged in the pulmonary microvasculature, and ethical issues [60–64]. Therefore, a novel cell-free therapy for PNI with a similar efficacy as that of MSCs needs to be further developed. Notably, MSC-conditioned medium containing MSC secretomes can replicate the therapeutic effects of MSCs [65, 66]. For decades, many investigators have worked to find active secretome of MSCs to explain the mechanism of therapy. Although these active factors are considered promising, there is no single factor that fully explains the efficacy of MSCs. In 2010, Lai et al. first demonstrated that exosomes derived from MSCs can alleviate myocardial ischemia/reperfusion injury [67]. Subsequent studies have also demonstrated that the therapeutic effects of MSCs are closely associated with exosomes secreted by MSCs. MSC exosomes seem to exert comparable effects as those of their parental cells. These novel findings provide new insight into the repair of injured tissue by MSCs and intercellular communication between MSCs and recipient cells and offer a new approach for the development of biological agents for tissue repair. Therefore, MSC exosomes may become a novel therapeutic target.

3. Biogenesis and Biocharacteristic of Exosomes

EVs are membrane-contained vesicles secreted by almost all cell types, such as immunocytes, MSCs, tumor cells, Schwann cells, and epithelial cells, into the extracellular space [68]. It has been demonstrated that EVs exhibit diverse intercellular communication biofunctions, such as antigen presentation, tumor progression and metastasis, and tissue regeneration regulation [69–71]. Exosomes, an important subclass of EVs, have gained much attention in biomedical research.

According to the origin and biogenesis of EVs, EVs can be divided into two main types, namely, exosomes and microvesicles (MVs) [72]. Exosomes are formed by inward budding inside intracellular endosomes, which leads to the formation of multivesicular bodies (MVBs) that then fuse with the PM and are released outside the internal vesicles, while other EVs, such as MVs, are budding vesicles that are shed directly from the PM [68]. Furthermore, exosomes can be distinguished from other EVs by their size. Exosomes are nanosized membrane vesicles that are 40-150 nm in diameter, whereas MVs range from approximately 100 to 1000 nm. Obviously, exosomes are narrower than MVs in the diameter. The characteristics of exosomes and MVs are summarized in Table 1. Upon the observation of exosomes by transmission electron microscopy, their morphology was described as "cup-shaped" [73]. Exosomes are endocytic membrane-derived vesicles with a bilayer lipid structure that consists of cholesterol, ceramide, phospholipids, and glycerophospholipids with long and saturated fatty-acyl chains [74, 75]. The bilayer membrane provides a protected and controlled internal microenvironment for the contents, ensuring that the cargo is not degraded and that it can be moved over long distances [76]. In addition, due to the low number of membrane-bound proteins, such as major histocompatibility complex molecules, in exosomes, they are less immunogenic than their parental cells. This feature can allow them to overcome immune rejection and avoid being cleared by immunocytes [77, 78].

The secretion of exosomes involves a complicated endocytic pathway. At the beginning of the process, endosomes invaginate from a special plasma membrane region into the

Table 1: The characteristics of exosomes and microvesicles.

	Exosomes	Microvesicles
Size	40-150 nm	100-1000 nm
Size range	narrow	broad
Density	1.18-1.21 g/ml	1.16 g/ml
Origin	The invagination of plasma membrane (PM) forms endosomes; endosomes buds inward to form intraluminal vesicles	The outward shedding of PM
Main formation mechanisms	ESCRT-dependent and independent mechanism	Calcium-dependent mechanism
Maker	CD 9, CD 63, CD 91, TSG 101, ALIX	Integrins, selectins, tissue factors
Content	mRNA, miRNA, lncRNA, DNA, protein, lipid, MHC	mRNA, miRNA, protein

ESCRT: endosomal sorting complex required for transport.

cytoplasm. As endosomes mature, the endosomal membrane constantly buds inward to form intraluminal vesicles (IVs). At this stage, the endosomes are called multivesicular bodies (MVBs). After the MVBs fuse with the cell membrane, the MVBs are released into the extracellular space. The released vesicles are called exosomes. At the same time, some MVBs are transferred to lysosomes for degradation [79]. Interestingly, in the process of IV and MVB biogenesis, there are two distinct molecular mechanisms, namely, endosomal sorting complex required for transport (ESCRT)-dependent and ESCRT-independent mechanisms [80, 81]. These are thought to be complex molecular mechanisms that sort and encapsulate specific molecules into IVs. When exosomes are released into the extracellular environment, they can adhere to the surface of recipient cells, interact with lipid ligand receptors, and then be internalized by endocytic uptake or fusion with the cell membrane [79] (see Figure 1).

Compared with other EVs, exosomes also possess their own expression profiles. Exosomes contain abundant proteins associated with membrane transport and fusion, including annexins, ESCRT and Rab GTPases, actin, and β-tubulin. Exosomes are also enriched with specific protein markers, such as tetraspanins (CD9, CD63, CD81, CD82), heat shock proteins (HSP60, HSP70, HSP90), tumor susceptibility gene 101 (TSG101), and ALG-2-interacting protein X (ALIX) [82]. Thus, enzyme-linked immunosorbent assay (ELISA), flow cytometry, and Western blotting are frequently used to detect exosomal biomarkers by analyzing specific proteins [83, 84]. It has been reported that exosomes carry mRNA, ribosomal RNA (rRNA), circRNAs DNA, long noncoding RNA (lncRNA), and some cytokines [85]. Although exosomal miRNAs only account for a small fraction of loaded molecules, many studies have indicated that exosomes are thought to be an important mediator responsible for regulating the phenotype and physiological state of recipient cells by horizontally transferring miRNAs [86]. Mechanically, miRNAs can bind to targeted mRNAs, resulting in mRNA decay or translation dampening and ultimately the regulation of expression [87]. Although their cytoskeleton and cellular metabolism are the same, their cargo and composition are different and depend on the cellular origin and different physiological and pathological states of cells [88]. Obviously, exosomes can also encapsulate loaded molecules based on different functional requirements and external stimuli. In other words, the contents carried by exosomes can be shuttled to regulate cell biological behavior.

4. MSC Exosomes Mediate Axonal Outgrowth

It is well known that endogenous SCs contribute mainly to axonal development, maturation, and regeneration. SCs play a central role in maintaining the homeostasis of the peripheral nervous system and promoting regeneration following injury [89]. Recent studies have indicated that exosomes derived from SCs may be a critical mediator of communication with axons [90]. In other words, a number of protein and genetic components are transferred from SCs to axons though exosomes, thereby promoting axonal regeneration. A study from 2013 showed that exosomes from SCs can be specifically internalized by axons. It was subsequently observed that exosomes from SCs significantly increase axonal regeneration in vitro and enhance regeneration after sciatic nerve injury in vivo [91]. Mechanically, exosomes shift growth cone morphology to a proregenerative phenotype and decrease the activity of the GTPase RhoA, which is involved in growth cone collapse and axon retraction [91, 92]. The above studies suggest that exosomes from glial cells may be a promising therapeutic strategy for facilitating axonal outgrowth. However, harvesting exosomes from SCs requires sacrificing normal neural tissue [93]. More importantly, SCs are difficult to culture in vitro since they are terminal cells. The above shortcoming is the main challenge for SC-based therapeutic strategies for repairing nerve injury. Therefore, subsequent research is needed to find an alternative cell type with efficiency similar to that of SCs.

Fortunately, previous studies have demonstrated that MSCs, similar to SCs, exert a beneficial effect by promoting peripheral nerve regeneration. Many studies have

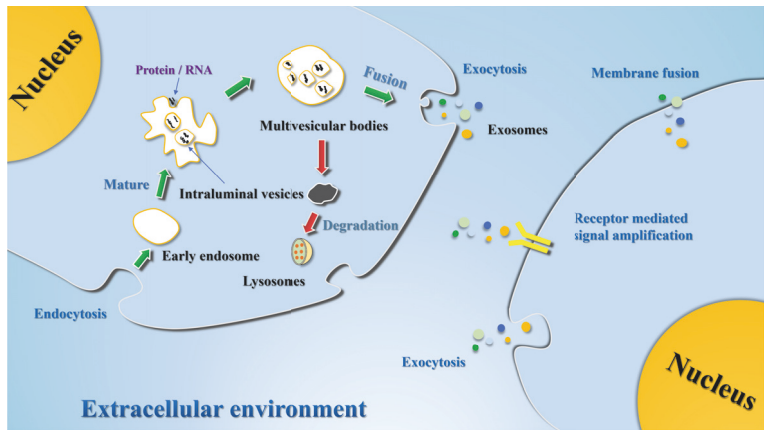

Figure 1: Biogenesis and uptake of exosomes. Endosomes are formed by an invagination in the plasma membrane of cells. As endosomes mature, the endosomal membrane constantly buds inward to form multivesicular bodies (MVBs). Exosomes are released through the fusion of multivesicular bodies with the plasma membrane. Once an exosome is released, receptor cells uptake the exosome through endocytosis, membrane fusion, or receptor-mediated internalization.

indicated that MSC-derived exosomes, as cellular paracrine products, may play a major role in recovery after PNI. In 2016, Lopez-Verrilli et al. reported that MSC exosomes from different sources, including menstrual MSCs and bone marrow mesenchymal stem cells (BMMSCs), can facilitate the neurite outgrowth of dorsal root ganglia neurons and cortical neurons [94]. Another study demonstrated that neuroprotection conferred by MSC exosomes (the source of adipose-derived stem cells) is attributed to the activation of the PI3K/AKT signaling pathway [95]. Furthermore, a recent study reported that gingiva-derived mesenchymal stem cell (GMSC)-derived EVs can induce the upregulation of SC dedifferentiation/repair phenotype-related genes by activating the c-JUN-governed repair phenotype of SCs, thereby facilitating peripheral nerve regeneration [96]. Overall, increasing evidence has demonstrated that MSC exosomes have the potential to be a promising mediator of PNI regeneration.

4.1. MSC Exosomes Mediate the Intercellular Transport of miRNAs to Induce Axonal Outgrowth. Studies have indicated that the miRNA expression profile of SCs and neuron cell bodies following PNI is abnormal, which suggests that local genetic effects largely mediate regeneration after peripheral nerve injury [97, 98]. Moreover, these specifically expressed miRNAs are involved in the proliferation and phenotypic switch of SCs, neurogenesis, axonal outgrowth, and macrophage migration. For example, the overexpression of specific miRNAs (miR-23a, miR-200, miR-133b, miR-17-92 cluster) can promote neurogenesis and myelination [99–102]. Previous studies have reported that miRNAs are transferred from neuronal cell bodies to axons, while recent studies have demonstrated that miRNAs called exosomal shuttle RNAs can be transferred between cells though exosomes [103, 104]. This discovery suggests that exosomes can be packaged with beneficial miRNAs to shuttle these genetic components, thereby mediating neural plasticity and axonal outgrowth.

Emerging studies have shown that MSC exosomes can mediate cell-cell communication by transferring miRNAs to recipient neurons to promote axonal growth. Mead et al. found that BMMSC-derived exosomes can significantly promote the survival of retinal ganglion cells and axonal regeneration. The neuroprotective effect is attenuated by the knockdown of Argonaute 2, a key miRNA effector molecule, confirming that the effect is attributed to the delivery of exosomal miRNAs [105]. Xin et al. demonstrated that, compared with native MSC exosomes, tailored exosomes enriched with the miR-17-92 cluster can enhance neurological recovery following stroke by activating the PI3K/protein kinase B/mechanistic target of rapamycin/glycogen synthase kinase 3β signaling pathway [106]. In addition, tailored exosomes enriched with the miR-17-92 cluster can also activate the PTEN/mTOR signaling pathway and enhance axonal growth by delivering miRNA cargo in vitro [107]. Recently, a study showed that MSC exosomes enriched with miR-133b further stimulate the release of exosomes from astrocytes, indirectly enhancing neural plasticity and neurite outgrowth [108]. MSC exosomes may target neural cells in the neurogenic niche by transmitting exosomal miRNAs to contribute to neurite outgrowth. This may prove to be a meaningful finding for the treatment of PNI.

4.2. MSC Exosomes Shuttle Neurotrophic Factors to Promote Axonal Regeneration. In addition to transmitting genetic components, MSC exosomes contain numerous neurotrophic factors that also play an important role in axonal regeneration. To some extent, the components of MSC exosomes depend on the culture and physiological conditions of the original cells, which influence the biological function of MSC exosomes. A study indicated that exosomes from PEDF-modified ADSCs can activate autophagy and suppress neuronal apoptosis, thereby ameliorating cerebral I/R injury [109]. This study suggests that modifying MSCs to increase the level of bioactive molecular cargo may be a promising approach for accelerating recovery from PNI. Recently, Bucan et al. noted that MSC exosomes can be internalized by SCs in vitro, significantly increasing the proliferation of SCs. In

vivo, MSC exosomes are selectively internalized by axons to promote axonal regeneration [110]. Although this study did not indicate the specific mechanism of peripheral nerve regeneration, it was the first to demonstrate that MSC exosomes are enriched with multiple neurotrophic factors, such as glial cell-derived neurotrophic factor (GDNF), fibroblast growth factor-1 (FGF-1), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), and nerve growth factor (NGF). The beneficial effects exerted by MSC exosomes are closely related to shuttling these neurotrophic factors. Thus, the presence of bioactive molecules related to nerve repair in MSC exosomes provides the possibility of regulating recovery from denervation muscle atrophy, neural survival, and axonal outgrowth.

5. MSC Exosomes Modulate Neuroinflammation in Peripheral Nerve Regeneration

Previous studies have shown that axonal regeneration after PNI is not completely mediated by SCs but rather by macrophages. Although the cellular immune response to nerve injury and infection is relatively conserved in the PNS, a majority of immune cells, such as phagocytic neutrophils and macrophages, migrate to the damaged site within hours or days after peripheral nerve injury [111]. Neuroinflammation plays a crucial role in recovery from PNI. Once a nerve is damaged, myelinating Schwann cells that undergo dedifferentiation are primarily activated in the distal portion of the nerve. Dedifferentiated Schwann cells begin to clear injured myelin and cell debris in a process known as Wallerian degeneration (WD). The release of chemokines and proinflammatory cytokines by dedifferentiated Schwann cells leads to the promotion of the neuroinflammatory response [112, 113]. Neuroinflammatory events lead to the recruitment of circulating macrophages and other peripheral immune cells to the injury site. Circulating macrophages and resident macrophages further facilitate the clearance of myelin and axonal debris, which is necessary for the regeneration of axons because molecules from degenerated axons exert an inhibitory effect on axonal growth, in later stages of WD [114]. Macrophages and other phagocytes accumulate in the region of the injured neuronal cell body in addition to the distal stump. These cells trigger a conditioning lesion response, which is the process by which neurons promote regeneration [115]. However, neuroinflammation exerts a double-edged sword effect. Although neuroinflammation has several beneficial effects in the process of recovery from PNI, it is crucial to nerve regeneration for this inflammatory response to be shut down. Excessive inflammatory responses not only hinder nerve regeneration but also are closely related to neuropathic pain. Hence, promoting a microenvironment that allows a suitable level of inflammation and regeneration might be a crucial target for PNI.

5.1. Immunomodulatory Characteristics of MSC Exosomes. It is well known that MSCs possess immunomodulatory properties though secreting various soluble immunomodulatory mediators, including transforming growth factor (TGF-β), interferon-γ (IFN-γ), indoleamine 2,3-dioxygenase (IDO), prostaglandin E_2 (PGE_2), and heme oxygenase-1, interleukin-10 (IL-10) [116]. However, studies have shown that defective IFN-γ receptors in MSCs and IDO inhibitors induce the loss of IFN-γ and IDO, which does not completely compromise the immune activity of MSCs [117–119]. Hence, the immunomodulatory activities of MSCs cannot be sufficiently explained by the release of the above-mentioned single soluble mediators but is mediated synergistically by multiple complex factors. Among various paracrine factors, MSC-derived exosomes are considered to be major immunomodulatory mediators that contain >200 immunomodulatory proteins [120]. Indeed, many studies have shown that MSC exosomes exert positive immunomodulatory effects in models of different pathologic conditions. In 2014, Zhang and his colleague noted that the treatment of THP-1 monocytes with MSC exosomes can induce high levels of anti-inflammatory IL10 and TGF-β1 in vitro and also attenuate the expression of proinflammatory IL1B, IL6, TNFA, and IL12P40. Additionally, they observed that MSC exosomes induce regulatory T cells (Tregs), which are recognized as a specialized subset of $CD4^+$ T cells that play a role in the establishment and maintenance of immune tolerance, to enhance the survival of allogenic skin grafts [121]. Recently, a study reported that MSC exosomes mediate multifaceted cellular processes, including migration, proliferation, matrix synthesis, macrophage infiltration, and cytokine production, to promote tissues repair. The investigator attributed the positive effect to the presence of CD73, which induces the phosphorylation of both AKT and ERK, thereby activating AKT/ERK signaling, in MSC exosomes [122]. In brief, these data suggest that the diverse factors expressed by MSC exosomes have an immunomodulatory effect rather than an immunosuppressive effect, which makes MSC exosomes a promising candidate for immunomodulation after PNI.

5.2. MSC Exosomes Mediate the Phenotypic Transformation of Macrophages to Promote Axonal Regeneration. Recent studies have indicated that MSC exosomes can regulate the plasticity of macrophages to facilitate polarization into anti-inflammatory phenotypes, thereby reducing the production of proinflammatory cytokines.

Compared with unconditioned MSCs, LPS-preconditioned MSCs not only enhance the paracrine effect to increase the production of exosomes but also increase the accumulation of miRNAs associated with immunomodulation in MSC exosomes. LPS-preconditioned MSC-derived exosomes (LPS pre-Exo) can upregulate anti-inflammatory cytokines by promoting M2 macrophage activation. Mechanically, LPS pre-Exo mediate the TLR 4/NF-κB/STAT-3/AKT regulatory signaling pathway, which regulates macrophage plasticity by shuttling let-7b to alleviate inflammation and maintaining intercellular homeostasis [123]. In addition, MSC exosomes also have the potential to improve brain plasticity by resolving neuroinflammation. Microglial, which are brain-resident immune cells that play a key role in homeostasis under normal conditions, are equivalent to macrophages in the central nervous system (CNS). Recently, it was confirmed that MSC

exosomes can reverse neuronal damage by transmitting exosomal miR-30d-5p to inhibit the autophagy of microglia, ultimately promoting the polarization of microglia to an anti-inflammatory phenotype [124]. This study not only explained the mechanisms by which MSC exosomes mediate neuroinflammation to promote neurogenesis but also suggested that MSC exosomes may be a promising vehicle for gene delivery to attenuate neuroinflammation.

The neuroinflammation triggered by PNI is complex, and the entire reaction is precisely orchestrated and involves multiple immune cells and regulating factors [125]. For decades, the polarization of macrophages post-PNI has been controversial. Ydens et al. observed that only macrophages of the M2 phenotype are present following nerve axotomy, while another investigator reported that macrophages acquire the M1 phenotype post-PNI [126]. This difference in observation may be attributed to differences in time points, the injury model, and macrophage markers used. Furthermore, due to the presence of various stimuli in vivo, the exposure of macrophages to such environments may result in a complex mixed phenotype population. Collectively, although there are few reports that MSC exosomes mediate neuroinflammation to promote axonal outgrowth, this may be a research target for the future.

6. MSC Exosomes Mediate Vascular Regeneration in Peripheral Nerve Regeneration

The reconstruction of the vascular network provides a regenerative microenvironment for the facilitation of axonal growth during peripheral nerve repair [127]. Therefore, maintaining vascular integrity following PNI may be another target for the treatment of peripheral nerve regeneration. Recently, MSC-derived exosomes have attracted attention as paracrine promoters of angiogenesis and are thought to be valuable therapeutic tools for vascular remodeling. Exosomes from induced pluripotent stem cell-derived mesenchymal stem cells promote local angiogenesis by activating the PI3K/AKT signaling pathway in endothelial cells [128]. Additionally, Gong et al. showed that proangiogenic miRNAs can be transferred within endothelial cells though MSCs generated exosomes to improve vascular plasticity [129].

Interestingly, some studies have shown that MSC-derived exosomes can induce angiogenesis, thereby decreasing neurologic deficits. In 2013, Xin et al. demonstrated that the intravenous administration of MSC-generated exosomes can improve functional recovery and enhance neurite remodeling, neurogenesis, and angiogenesis [130]. Similar effects were confirmed in another study in which MSC-generated exosomes were shown to promote endogenous angiogenesis and neurogenesis and reduce inflammation in rats [131]. In summary, the above studies indicate that exosomes derived from MSCs may be mediators of communication with vascular endothelial cells to improve the plasticity of blood vessels after nerve injury. MSC exosomes may provide a new opportunity for PNI repair by facilitating neurovascular regeneration. However, there have been few reports that MSC exosomes enhance angiogenesis and promote peripheral nerve regeneration. Therefore, future studies are warranted to more carefully determine whether MSC exosomes can promote neurovascular regeneration in the process of peripheral nerve repair.

7. MSC Exosomes: Advantages and Challenges for Recovery from PNI

Recent studies have demonstrated that exosomes from MSCs play an important role in nerve regeneration following injury (see Table 2). In the future, MSC exosome-based therapy could be considered a potential alterative therapy for PNI. More attention should be paid to the safety, challenges, and risks of MSC exosomes as an emerging therapeutic agent, as these factors could provide a reasonable basis for the application of MSC exosomes for PNI repair. Therefore, a reasonable explanation of the advantages and challenges of MSC exosomes is required.

It is widely recognized that MSC-based therapy has some inherent risks, including tumor formation, immune rejection, microcirculatory obstruction, and arrhythmia. Unlike MSCs, MSC exosomes are a type of nonliving cell vesicle, and thus their use could radically eliminate the risk of MSC transplantation [132]. Exosomes possess lower immunogenicity because they contain fewer membrane-bound proteins compared with their parental cells [133]. Since MSC exosomes have nanoscale structures and because of the composition of their membranes and the adhesive proteins embedded within them, they can cross biological barriers [91–93]. Furthermore, the bilayer lipid structure of their membrane promotes the stability and bioavailability of their cargo. These features may make them promising natural nanocarriers for transporting drugs, genetic materials, proteins, and lipids and regulating the biological functions of target cells. Moreover, MSC exosomes are also easier to store and can be stored without potentially toxic cryopreservatives in long term at -20°C, greatly reducing the potential for toxic side effects of chemical agents [134]. Unlike those of stem cells, the vitality and potency of MSC exosomes need not be monitored and maintained during manufacturing and storage.

Despite these advantages of the use of MSC exosomes for treating PNI, the challenges cannot be ignored. Currently, one of the major challenges is that there is no standardized technique for the isolation, quantification, and purification of MSC exosomes. In addition, there is no guidance, supervision, or ethical safety assessment. It is necessary to determine the effective route of administration for PNI repair. It is worth exploring whether MSC exosomes are effective when administered systemically, such as through intravenous, subcuticular, or intramuscular routes, which are used for traditional drug therapy. In the last decade, most studies have tended to focus on the use of MSCs combined with artificial neural conduits to generate nerve grafts that mimic autologous nerves for repairing PNI. This approach also gives us great insight into the use of neural conduits combined with MSC exosomes to promote peripheral nerve regeneration. However, there have been few studies that have reported that the implantation of a graft conduit bathed with

Table 2: The beneficial effect of MSCs exosomes for PNI.

Cell source	Exosomal cargo	Effect	The activation of signaling pathway	Reference
hAMSCs	/	Against neuron damage induced by glutamate	the PI3K/Akt signaling pathway	[94]
Gingiva-derived MSCs	/	Promote peripheral nerve regeneration	c-JUN pathway governed repair phenotype of Schwann cells	[95]
rBMMSCs	miR-17-92 cluster	Increase neural plasticity and functional recovery and promote Axonal Growth of Cortical Neurons	the PI3K/protein kinase B/mechanistic target of rapamycin /glycogen synthase kinase 3β, or the PTEN/mTOR signaling pathway	[105, 106]
	miR-133b	Improve neural plasticity and functional recovery	/	[107]
rADSCs	PEDF	Ameliorate cerebral I/R injury	Autophagy and apoptotic pathway	[108]
	Multiple factors	Increase neurite outgrowth in vitro and enhance regeneration	/	
Umbilical cord MSCs	let-7b	Alleviate inflammatory reaction by promoting M2 macrophage activation	The signal axis by TLR 4 / NF-κB / STAT-3 / AKT	[122]
rADSCs	miR-30d-5p	Prevent cerebral injury by mediating microglial polarization to M1	Autophagy pathway	[123]
rBMMSCs	/	Enhance neurite remodeling, endogenous angiogenesis, and neurogenesis and reduce inflammation	/	[129, 130]

"/": not mentioned in the original article; ADSCs: adipose derived mesenchymal stem cells; BMMSCs: bone marrow derived stem cells.

MSC exosomes promotes the repair of PNI. In addition, although many studies have observed that MSC exosomes can accelerate peripheral nerve regeneration after injury, the molecular mechanisms underlying nerve regeneration have not yet been elucidated. Hence, much work is needed to overcome the abovementioned risks and challenges to allow the translation of MSC exosomes as clinical agents for PNI in the near future.

8. Conclusion and Perspectives for the Future

Presently, MSC exosomes are widely recognized as the main regulators of the paracrine mechanism that mediates tissue regeneration [77]. These nanoparticles exert beneficial therapeutic effects similar to those exerted by MSCs and may be potentially powerful tools for cell-free based therapy because they have many advantages over MSCs. Of note, the discovery that MSC exosomes can be used to treat peripheral nerve injury is encouraging. In the peripheral nerve microenvironment, MSC exosomes play an important role in mediating intercellular communication. They can transmit a number of genetic materials, neurotrophic factors and proteins to axons, restoring the homeostasis of the microenvironment, regulating axonal regrowth, and thereby promoting recovery from PNI. These effects are important for adopting MSC exosomes for clinical applications. The therapeutic effect of exosomes reinforces the paradigm that the promotion of tissue regeneration by MSCs is mediated by a paracrine mechanism and provides a new perspective for the development of cell-free therapies [135]. The use of MSC exosomes can eliminate the issues caused by stem cell transplantation. In the future, MSC exosome-based therapy may represent an important field for PNI repair. Although much experimental evidence suggests that the use of MSC exosomes for the treatment of peripheral nerve injury is effective and safe, this field needs to be further researched, and much work is needed to fully determine the potential of MSC exosomes for clinical application.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Serum Exosomal MicroRNAs Predict Acute Respiratory Distress Syndrome Events in Patients with Severe Community-Acquired Pneumonia

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Background. Severe community-acquired pneumonia (SCAP) requiring intensive care unit (ICU) treatment commonly causes acute respiratory distress syndrome (ARDS) with high mortality. This study was aimed at evaluating whether microRNAs (miRNAs) in circulating exosomes have the predictive values for patients at risk of developing ARDS due to SCAP. *Methods*. ARDS/ALI-relevant miRNAs were obtained by literature search. Exosomes in serum were isolated by ultracentrifugation method and identified by Transmission Electron Microscopy. Then the miR profiling in the exosomes using real-time PCR was analyzed in SCAP patients with or without ARDS. Moreover, multivariate Cox proportional regression analysis was performed to estimate the odds ratio of miRNA for the occurrence of ARDS and prognosis. The receiver operating characteristics (ROC) curves were calculated to discriminate ARDS cases. Finally, the Kaplan-Meier curve using log-rank method was performed to test the equality for survival distributions with different miRNA classifiers. *Results*. A total of 53 SCAP patients were finally recruited. Ten miRNAs were picked out. Further, a subset of exosomal miRNAs, including the miR-146a, miR-27a, miR-126, and miR-155 in ARDS group exhibited significantly elevated levels than those in non-ARDS group. The combined expression of miR-126, miR-27a, miR-146a, and miR-155 predicted ARDS with an area under the curve of 0.909 (95 % CI 0.815 –1). Only miR-126 was selected to have potential to predict the 28-day mortality (OR=1.002, P=0.024) with its median value classifier. *Conclusions*. The altered levels of circulating exosomal microRNAs may be useful biologic confirmation of ARDS in patients with SCAP.

1. Introduction

Acute respiratory distress syndrome (ARDS) is a life-threatening disease with a current mortality of about 40% [1]. Severe community-acquired pneumonia (SCAP) is the most frequent cause of ARDS. Despite modifications on clinical definition [2], ARDS patients remain non-specific, not uniformly diagnosis with heterogeneous etiologies or complexity of pathologies, thus responding differently to therapeutic interventions [3]. Therefore, identification of potential biomarkers that facilitate diagnosis of ARDS could improve clinical care and could be used to enroll a more homogeneous group of patients into clinical practice with effective therapies.

It is unlikely that a single biomarker will have adequate specificity for the clinical confirmation of ARDS in patients who have developed pneumonia. MicroRNAs (miRNAs) play crucial roles in the posttranscriptional regulation of gene expression in pathogenesis of lung diseases and infections [4]. Circulating exosomes are membrane vesicles shed by a variety of blood or endothelial cells, and have been shown to be important mediators of intercellular communication and microenvironment, holding the potential to miRNAs delivery [5]. In the presence of devastating stimuli, the compositions of exosomes including miRNAs, could be robustly altered, thus accumulating studies center round the potentials of exosomes to serve as promising biomarkers [6, 7]. Currently, the content of circulating exosomes in patients with or

without ARDS remains elusive. As miRNAs have to be used to construct prognostic classifier for early prediction of disease outcomes [8], it is not yet clear if the biological significance of exosomal miRNAs is superior to a combination of abnormal biomarkers. Although the definition of ARDS is based on clinical criteria, no studies have tested the possible value of a panel of exosomal miRNAs to assist in the diagnosis of ARDS in patients with SCAP.

Therefore, our purpose is to confirm whether the exosomal miRNAs could be used as biomarkers for discriminating the patients at risk of developing ARDS due to SCAP. Herein, we tentatively filtrated a group of ARDS/ALI-relevant miRNAs through several literatures. Then, these profiles of miRNAs carried by exosomes in serum were examined. We also evaluated the association of aberrant exosomal miRNAs expressions with the clinical pathological properties and prognosis, which is probably helpful for understanding of pathogenesis and accurate diagnosis in SCAP patients.

2. Methods

2.1. Subject Recruitment and Sample Acquisition. This prospective, observational study was conducted in the intensive care unit (ICU) of Zhongshan hospital. The consecutive patients suspected of CAP were enrolled from November 2016 to May 2018. CAP was defined as the presence of a new infiltrate on a chest radiograph and at least one of the following signs and symptoms: cough, sputum production, dyspnea, core body temperature > 38.0°C, auscultatory findings of abnormal breath sounds, and rales according to the American Thoracic Society (ATS) criteria, and its severity was evaluated by pneumonia severity index (PSI). Exclusion criteria included pregnancy, terminal illness (malignant cancer of any type, end-stage liver, or renal disease), severe immunosuppression, and refusal of informed consent. Additional exclusion criteria were, prior hospitalization within 15 days before admission, pneumonia developing during hospitalization, and delayed ICU admission for more than 48 hours. The study was approved by the Ethics Committee of the Fudan University (Approval number B2017-061R) and conducted with informed consent forms obtained from all participants.

Demographic information from severe CAP patients was collected including baseline clinical variables, blood PO_2/FiO_2 ratio, laboratory data as well as outcome. The daily clinical variables of interest were recorded to calculate the Acute Physiology and Chronic Health Evaluation II (APACHE II) scores and sequential organ failure assessment (SOFA) scores [9]. During the patient's hospital stay, we evaluated the presence of ARDS that defined by the Berlin definition [2]. Co-morbidities during hospitalization, style of invasive ventilator parameters and hospital stay days were documented for each patient. Also, a follow-up at 28-day mortality was performed to evaluate the prognosis. After enrollment of patients, data was blinded to avoid potential bias.

Blood samples from consenting subjects were collected within 24 hours of admission to the ICU. All biomarkers were assayed in duplicate in thawed serum at enrollment using commercially available ELISAs (IL-6, IL-10, vWF and ANG2, R&D Systems).

Biomarker concentrations were measured on a Bio-Plex System 100 (Bio-Rad, Hercules, CA, USA). Additionally, serum was separated via centrifugation at 1,500 g for 15 min at 4°C, followed by a second centrifugation at 16,000 g for 10 min at 4°C to eliminate residual cells debris. In order to prevent exosomes degradation and loss, samples should be handled within 30 minutes after blood withdrawal.

2.2. Candidate miRNA Selection. Using the combination of keywords and MeSH terms for "ALI" and "microRNA", we searched PubMed for articles that describe associations between the miRNAs and ARDS. To focus on the miRNAs with a high likelihood of relevance, we considered only miRNAs with at least two published references to be potential candidates for investigation. These cross-referenced with miRNA expression data were performed on serum-derived exosomes.

2.3. Bioinformatic Analysis. The databases TargetScan and miRanda were performed to identify experimentally validated and predicted gene targets of differentially expressed exosomal microRNAs.

Subsequently, these genes were input into DAVID Database (http://david.abcc.ncifcrf.gov/) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) for Gene Ontology (GO) or pathway enrichment analysis. The enrichment of related genes in three major GO classifications, that is, cell components (CC), biological processes (BP), and molecular functions (MF). KEGG pathway analysis was carried out to analyze the canonical pathways of relevance to ALI/ARDS. A network graph for these exosomal miRNAs was generated via the cytoscape software, directly showing the association of these genes with miRNAs.

2.4. Serum Exosomes Isolation from Serum and Identification. The size and morphology feature of exosomes were examined by Transmission Electron Microscopy (TEM). Firstly, prepared serum was ultra-centrifuged at 100,000 x g for 120 minutes. Following ultracentrifugation, the supernatant was aspirated and the exosome pellets were re-suspended in 50 ul PBS. For visualization of samples using TEM, exosome suspensions were prepared on formvar-coated copper-palladium grids and negatively stained with uranyl acetate. Image acquisition was conducted using a JEOL 1200EX TEMSCAN electron microscope at Fudan University. The remaining pellets containing the exosome fractions were diluted in sterile PBS for cell treatment or stored up to 6 months for PCR analysis at -80°C. For exosomal total RNA, the pellet fractions were washed twice with PBS and lysed with Trizol. Total RNA was quantified by the NanoDrop ND-2000 (Thermo Scientific) and the RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies).

2.5. Quantitative Validation of miRNAs by Real-Time PCR. Total exosomal RNAs extracted from 250 μl serum were polyadenylated and reverse-transcribed with a poly (T) adapter into cDNAs. The reverse transcription reaction

was carried out with PrimeScript™ II reverse transcriptase (Takara, Japan) at 42°C for 60 min, and 75°C for 15 min in a total volume of 10 μL. To measure the candidated miRNA levels, fold changes were tested by quantitative PCR amplification with SYBR PrimeScript miRNA RT-qPCR Kit (Takara, Japan). All reactions were run in triplicate. Specificity of the RT-qPCR product was confirmed with melting curve analysis. Ct values were determined using default threshold setting. miRNAs with a Ct value of more than 40 and a detection rate of less than 75% in any group were excluded from further analyses. Moreover, miR-39 was used as an endogenous control. Relative fold change of miR expression normalized to miR-39 was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Statistical Analysis. Results for normally distributed continuous variables are presented as mean ± standard deviation and compared between groups by Student's *t* tests (paired groups) or Mann–Whitney U test (unpaired groups). Results for categorical variables are presented as sample rate (constituent ratio) and were compared Chi-squared test. Binary logistic regression analysis was carried out to determine the independent predictors of the occurrence of ARDS or the 28-day mortality. The discriminatory power of each scoring index was measured by receiver operating characteristic (ROC) curves. The areas under the ROC curve (AUC) was calculated to give an estimate of the overall accuracy of each scoring index in predicting different patient outcomes (occurrence of ARDS and 28-day mortality). An area of 0.50 implies that the scoring index is no better than chance, whereas an area of 1 implies perfect accuracy. Spearman correlation analysis was applied to determine the correlation between miRNAs, vWF, ang-2, IL-6, IL-10 and SOFA or APACHE II score. The Kaplan–Meier (KM) curve was generated to evaluate the association between the levels of miR-126/146a and survival rate with log-rank test. All tests were two-sided and the statistical significance was assumed at a value of $P<0.05$.

3. Results

3.1. Characteristics of Enrolled Subjects. A total of 68 patients suggestive of severe CAP were admitted during the study enrollment period. Of the 59 patients who signed consent forms, 6 were excluded because of inadequate laboratory data. Ultimately, 53 were included in the study. The general demographic data, co-morbidities, and associated laboratory findings of enrolled subjects were detailed in Table 1. There were no significant differences between ARDS and non-ARDS groups with respect to sex, age and medical history. In contrast, oxygenation index (PO_2/FiO_2) in patients experiencing ARDS was lower than non-ARDS patients. Subjects who experienced SCAP had no significant differences in their rates of mechanical ventilation, as well as hospital lengths of stay. 28-day mortality rate increased in parallel with a rise in PSI, which tended to be higher in patients with ARDS. However, regarding to the APACHE II score, SOFA score, CRP, PCT, co-morbidities, and noninvasive ventilation parameters PEEP, no statistical differences were observed between them ($P > 0.05$).

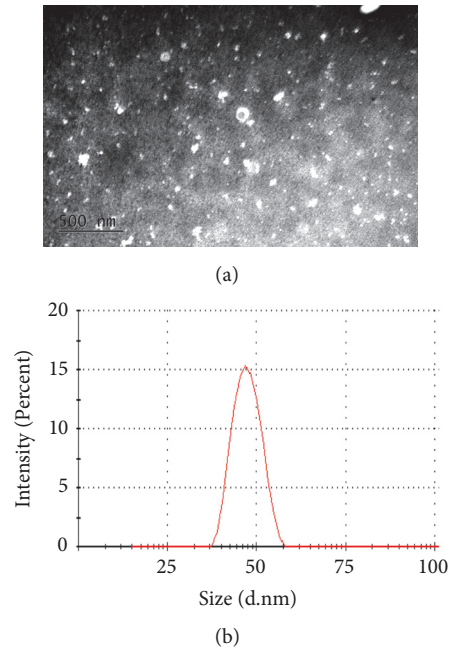

Figure 1: Isolation and characterization of serum exosomes with the standard ultracentrifuge method. (a) A representative electron microscopic image of exosomes showed the characteristic spherical shape and the size (25–75 nm). Scale bar=100 nm. (b) Analysis of the size distribution of exosomes isolated from individual serum.

3.2. The Altered Levels of Exosomal MicroRNAs in Patients with Severe Community-Acquired Pneumonia. Exosomes were purified from serum of SCAP patients using ultracentrifuge method and examined by electron microscopy. The electron micrographs of these revealed rounded structures with a size of approximately 25–75 nm (Figure 1). Meanwhile, these results confirmed the efficacy of our protocol for exosomes isolation.

We identified 27 miRNAs associated with ARDS/ALI, and excluded those not measured or in very low levels. The remaining 10 miRNA were picked out and further analyzed, including miR-15a, miR-16, miR-26, miR-21, miR-27a, miR-181b, miR-126, miR-146a, miR-155, and miR-223. To comprehend the functions and potential mechanisms of exosomal miRNAs involved in ARDS pathogenesis, as a first approach, we identified these experimentally validated and predicted gene targets of miRNAs using TargetScan and miRanda databases. Further, the generated network via the cytoscape software was visualized as Figure 2. In addition, we put these genes input into the David Database for GO enrichment analysis, revealing 27 GO terms with statistical significance ($P <0.05$), shown as Figure 3. KEGG pathway showed seven potential pathways that miRNAs and their related genes got involved in ARDS.

Based on the presence or absence of ARDS, the mean expression levels of the candidate miRNA in serum-derived exosomes was determined by RT-PCR and displayed in

Table 1: Baseline characteristics and outcome parameters in SCAP patients with and without ARDS.

Variables	ARDS (n=24)	Non-ARDS (n=29)	*P* value
Age (yrs)	59.63 ± 15.86	62.47 ± 12.10	0.078
Sex (% men)	18 (76%)	19 (67%)	0.209
APACHE II score	16.08 ± 9.41	12.83 ± 9.01	0.205
SOFA score	5.67 ± 3.55	4.45 ± 3.34	0.208
PSI	97.79 ± 28.38	95.31 ± 21.39	0.031
PaO_2/FIO_2 ratio	170.26 ± 105.49	226.47 ± 88.86	0.04
Co-morbidities			
(i) Pulmonary embolism, n (%)	2 (8%)	4 (14%)	0.541
(ii) Hypertension, n (%)	10 (42%)	10 (34%)	0.6
(iii) Diabetes mellitus, n (%)	6 (25%)	6 (21%)	0.715
(iv) COPD, n (%)	3 (13%)	9 (31%)	0.113
(v) Cardiovascular/cerebrovascular diseases, n (%)	6 (25%)	6 (21%)	0.715
Complication			
(i) Sepsis	7 (29%)	7 (24%)	0.686
(ii) AKI	3 (13%)	4 (14%)	0.893
Platelet count ($\times 10^9$/L)	181.5 ± 77.61	197.9 ± 108.9	0.539
28-day mortality	12 (50%)	10 (34%)	0.262
Microbiological species			
(i) Streptococcus pneumoniae	6 (25%)	6 (21%)	0.715
(ii) Haemophilus influenzae	1 (4%)	2 (7%)	0.676
(iii) Legionella pneumophila	0 (0%)	1 (3%)	0.368
(iv) Mycoplasma pneumoniae	1 (4%)	4 (14%)	0.241
(v) Other	9 (39%)	6 (21%)	0.151
(vi) unknown	7 (29%)	5 (17%)	0.311
Invasive mechanical ventilation, n (%)	16 (67%)	13 (45%)	0.116
PEEP (cmH_2O)	7.04 ± 4.73	4.86 ± 0.833	0.092
Hospital stay, days	20.17 ± 8.25	16.41 ± 8.42	0.109
Laboratory parameters			
(i) CRP (mg/dl)	90.27 ± 72.15	75.3 ± 58.43	0.408
(ii) Procalcitonin	1.75 ± 4.29	1.55 ± 3.76	0.858
(iii) Ang2 (ng/ml)	7.55 ± 1.67	5.08 ± 0.65	0.149
(iv) IL-6 (pg/ml)	62.04 ± 13.39	13.61 ± 2.01	0.002
(v) IL-10 (pg/ml)	10.66 ± 1.04	26.1 ± 7.53	0.07
(vi) vWF (% of control)	449.64 ± 118.45	266.64 ± 18.24	0.001

Data were presented as n (%), mean ± standard deviation. P value <0.05 was considered as statistically significant.
APACHE II, Acute Physiology and Chronic Health Evaluation II score; CRP, C-reactive protein; PSI, pneumonia severity index; SOFA score, Sequential Organ Failure Assessment score; CRP, C-reactive protein; PSI, pneumonia severity index; PEEP, positive end-expiratory pressure; Ang-2, angiopoietin 2; vWF, von Willebrand factor.

Figure 4. The levels of serum exosomal miR-146a, miR-27a, miR-126, and miR-155 were significantly higher in patients with ARDS than in those without (P = 0.004, P = 0.005, P = 0.008, P = 0.014, respectively). Meanwhile, the levels of miR-223 and miR-181b were significantly lower in patients with ARDS than in those without (P = 0.001 and P = 0.038). As PSI, PO_2/FiO_2 and some miRNAs levels were significant different between ARDS and non-ARDS groups, a Pearson linear correlation matrix analysis was further performed. Upregulation of miR-126 was correlated with the severity of ALI as reflected by PSI (r= 0.397, P=0.036). The inverse association between pronounced miR-126 levels and PO_2/FiO_2 was, however, not so significantly evident (r= -0.033, P=0.814). Additionally, miR-16 was inversely related to the PO_2/FiO_2 (r= -0.369, P=0.007). We found significant positive correlation between miR-146a and APACHE II score (r= 0.323, P=0.018), while no associations existed between other miRNA levels and APACHE II score. Furthermore, our study shows that SOFA score did not relate to any exosomal miRNAs. Patients with higher SOFA scores, regardless of ARDS occurrence, did not have a significant trend toward higher mortality rate at enrollment. An overview graph of the above significant linear correlations is given in Figure 5.

With regard to the biomarkers in various aspects of the pathophysiology of ARDS, biomarker panel that includes

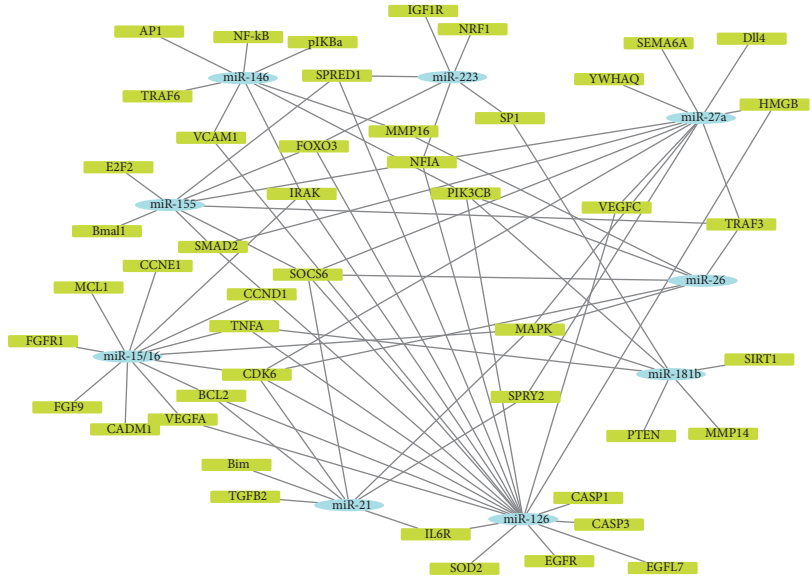

Figure 2: TargetScan and miRanda databases identify several experimentally validated or predicted gene targets of these selected miRNAs in the context of ALI. The interactions between miRNAs (center) and its ambient genes construct the generated network via the cytoscape software.

biomarkers of pulmonary endothelial injury or inflammation may be the most useful for discriminating the patients with ARDS. It is hypothesized that circulating angiopoietin-2 and vWF were generated by the lung endothelium, and associated with pulmonary severity of ALI. To further study clinical significance, we evaluated whether IL-6, Ang-2, IL-10 and VWF levels correlated with the development and clinical outcomes of ARDS. As expected, higher vWF concentrations were significantly found in patients classified in occurrence of ARDS group. Decreased IL-10 levels in parallel with a rise in IL-6 were also assessed among the two patient categories. In all, a Spearman analysis for the correlation coefficients of differentially expressed exosomal miRNAs with IL-6, Ang-2, IL-10, and vWF levels is demonstrated in Table 2.

3.3. The Predictive Value of Differentially Expressed Exosomal miRNAs for ARDS Events. All factors affecting the occurrence of ARDS in SCAP patients were analyzed in multivariate Cox proportional logistic regression. The area under the curve (AUC) values for individual miRNAs ranged between 0.592 and 0.779 as presented in Table 3. Exosomal miR-126 was the most predictive single miRNA for distinguishing SCAP patients with or without ARDS with an AUC of 0.779 [95 % confidence interval (CI) 0.652-0.906]. In a logistic regression model, after adjusting for other confounding factors such as age, sex, co-morbidities, and associated laboratory, miR-126 were independent predictors in SCAP patients for the risks of ARDS (OR = 1.013, P < 0.001). As a predictor variable, miR-27a was also independently associated with the presence of ARDS (OR = 1.01, P = 0.001). Indeed, multivariate logistic regression analysis with occurrence of ARDS as an outcome showed that elevation of circulating exosomal miR-146a predicted ARDS (regression coefficient=1.071, P=0.014). A high correlation coefficient was observed in miR-155 in the development of ARDS (OR = 1.44, P = 0.015). The final multiple marker model included the combination of miR-126, -27a, -146a and -155. The receiver operating characteristics (ROC) curves for miR-126 and miR-146a and the multiple marker model were calculated to differentiate ARDS cases from non-ARDS controls as shown in Figure 6. Taken together, the AUC for the multiple marker model was larger than any individual marker [AUC = 0.909 (95% CI 0.815 – 1)]. The ROC curves for ARDS events showed that a combination of miR-126, -27a, -146a, and -155, in contrast to miRNAs alone, were of predictive value.

3.4. Changes in Exosomal MicroRNAs Reflect ARDS Progression. The final miRNA classifier was obtained by both most differential expression and backwards elimination. In a second set of analyses with 28-day mortality, exosomal miRNA with a potential prognostic role is miR-126, whose expression was obviously higher in non-survivors than those who survived (OR=1.002, P=0.024). To further evaluate the predictive accuracy, we also divided the patients into two groups according to median value. Expression of miR-126 classifier larger than median was assigned as high expression, lower than median was assigned as low expression. The Kaplan–Meier survival curves and log-rank test for 28-day mortality were illustrated in Figure 7. There is a clear tendency that SCAP patients with higher concentration of miR-126 had increased mortality. MiR-146a was also shown to have prognostic utility, but did not reach statistical significance (OR=0.71, P=0.064). However, using the miR-146a classifier, the Kaplan– Meier curve showed statistical difference on 28-day survival rate between the above cutoff group and the below cutoff group, when miR-146a levels were stratified by the cutoff 4.

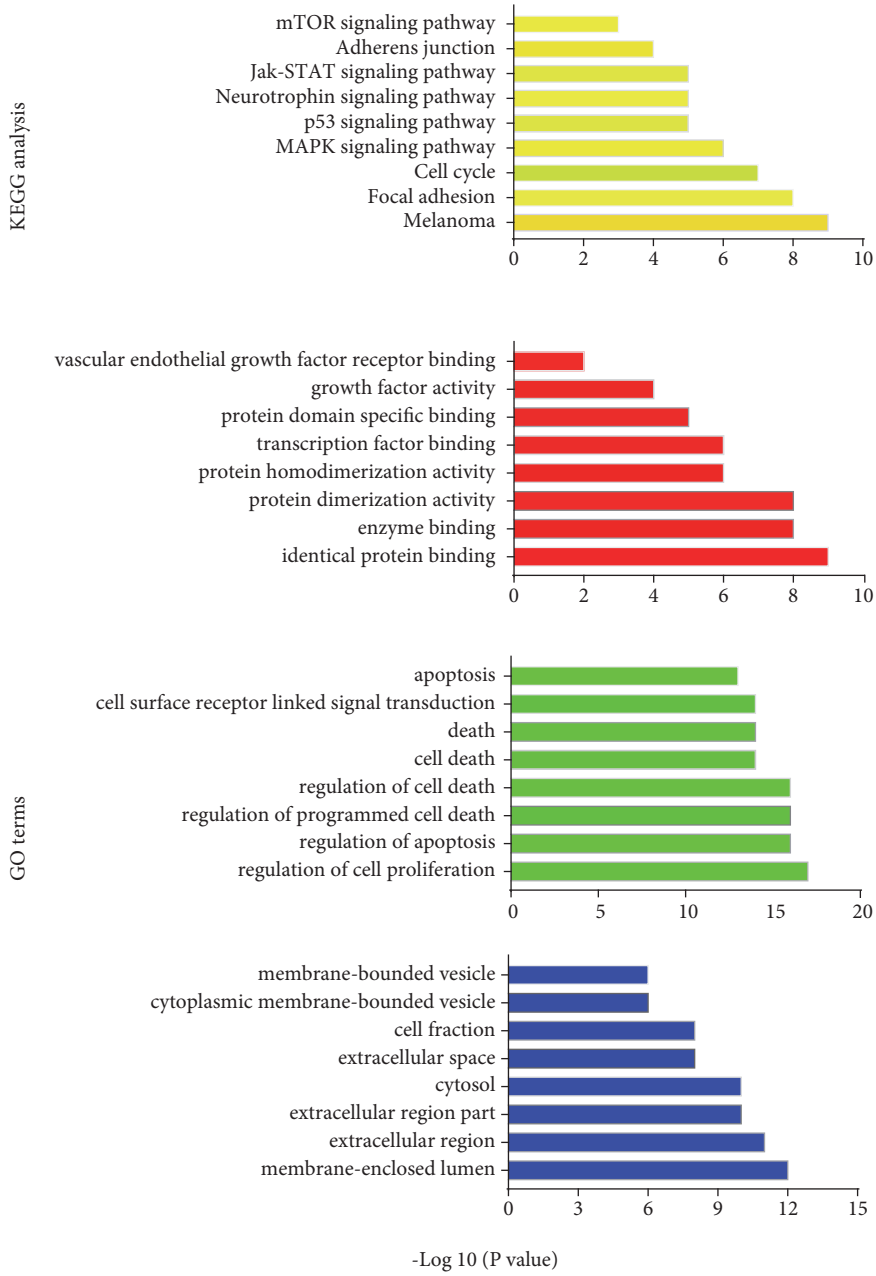

Figure 3: Gene ontology (GO) and KEGG pathway analysis of ARDS-related genes are performed on DAVID database. The function enrichment of related genes in three major GO classifications, that is, cell components (blue), biological processes (green), and molecular functions (red).

Of note, APACHE II score alone was not predictive for 28-day mortality in SCAP patients in receiver operating characteristic curves (minimum area under the curve=0.415, P=0.295). In addition, AUCs of miR-126, miR-155, miR-27a, miR-146a in combination with APACHE II scores were 0.698, 0.59, and 0.664, 0.682 for predicting 28-day mortality, respectively. Thus, these AUCs were lower than those of miRNAs alone (all $P < 0.05$).

4. Discussion

The introduction of exosome served as vehicles of miRNAs, has shed light on novel biomarker discovery. The major finding of the present study demonstrated that levels of some exosomal miRNAs were markedly different in patients with SCAP experiencing ARDS or not. In contrast, conventional indicators such as APACHE II, CRP and PCT showed no

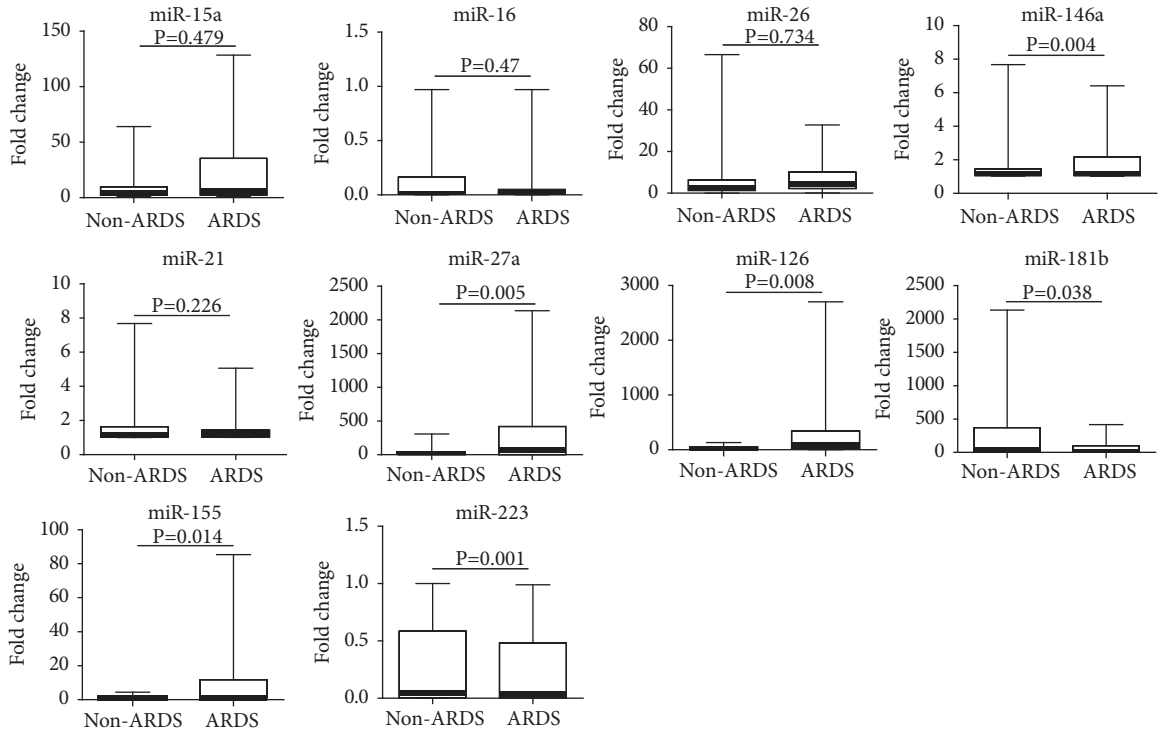

Figure 4: Serum exosomal microRNAs from SCAP patients with or without ARDS were measured by real-time quantitative PCR. The values of the relative gene expression for target microRNA were normalized to miR-39 and calculated using the $2^{-\Delta\Delta CT}$ method. Box plots are displayed where the horizontal bar represents the median, the box represents the IQR and the whiskers represent the maximum and minimum values. Comparisons made by Mann–Whitney U test. The values are expressed as the mean ± SEM. miRNA: microRNA, IQR: interquartile range.

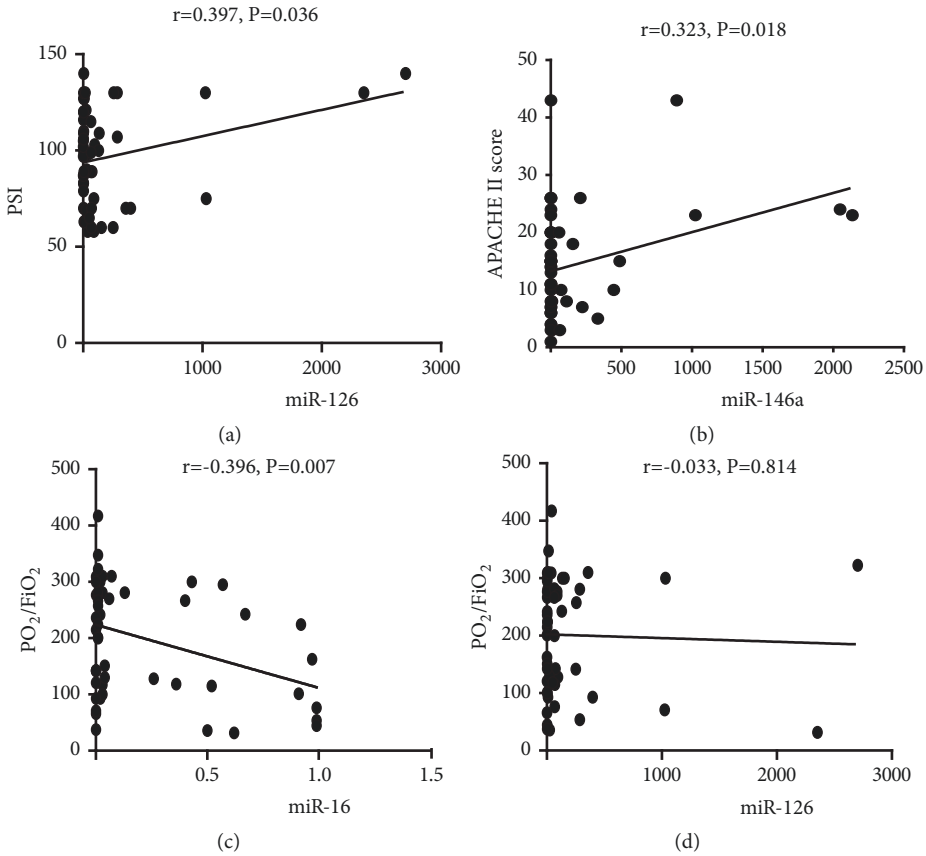

Figure 5: Correlation between miRNAs and the severity of SCAP.

Table 2: Spearman analysis for the correlation of differentially expressed exosomal miRNAs with IL-6, Ang-2, IL-10, and vWF levels in SCAP patients.

Variables	vWF	IL-6	IL-10	Ang-2
miR-126				
(i) coefficient	0.836	0.245	-0.309	-0.063
(ii) *P* value	0.001	0.077	0.024	0.656
miR-27a				
(i) coefficient	0.144	0.636	-0.119	0.447
(ii) *P* value	0.302	0.001	0.397	0.001
miR-181b				
(i) coefficient	-0.27	-0.109	0.333	0.226
(ii) *P* value	0.051	0.435	0.015	0.103
miR-223				
(i) coefficient	0.552	0.288	-0.403	-0.034
(ii) *P* value	0.001	0.036	0.003	0.81
miR-146				
(i) coefficient	0.237	0.644	0.001	0.359
(ii) *P* value	0.088	0.001	0.993	0.008
miR-155				
(i) coefficient	0.16	0.335	0.081	-0.122
(ii) *P* value	0.252	0.014	0.565	0.383
SOFA score				
(i) coefficient	0.261	0.19	-0.199	-0.046
(ii) *P* value	0.059	0.173	0.154	0.741
APACHE II score				
(i) coefficient	0.092	0.098	-0.037	-0.019
(ii) *P* value	0.513	0.487	0.795	0.89

P value <0.05 was considered as statistically significant.
Ang-2, angiopoietin 2; VWF, von Willebrand factor; APACHE II, Acute Physiology and Chronic Health Evaluation II score; SOFA score, Sequential Organ Failure Assessment score.

Table 3: Area under the curve (AUC) for individual miRNAs and for a multivariable logistic regression model.

Markers	AUC	95% CI	*P* value
miR-126	0.779	0.652-0.906	0.001
miR-27a	0.604	0.497-0.831	0.042
miR-146a	0.691	0.512-0.87	0.017
miR-155	0.592	0.429-0.755	0.253
miR-126+miR-27a	0.855	0.741-0.969	0.001
miR-126+miR-27a+miR-146a	0.876	0.761-0.991	0.001
miR-126+miR-27a+miR-146a+miR-155	0.909	0.815-1.00	0.001

miRNA: microRNA; CI: confidence interval.
P value <0.05 was considered as statistically significant.

statistical difference. Modifications in expressive patterns of exosomal miRNAs could regulate the endothelial function, inflammatory response and tissue repair mechanisms, all of which contribute to endothelial activation and barrier injury in the setting of ALI/ARDS. In addition, four abnormal exosomal miRNAs in serum provided excellent discrimination for the diagnosis of ARDS as assessed by ROC curve analysis. Specific exosomal miRNAs also represented the potentially suitable biomarkers of SCAP progression. In particular, exosomal miR-126 is an independent predictor of ARDS events and 28-day mortality, reflecting the severity of SCAP. Therefore, exosomes offer innovative possibilities in detecting and monitoring the development of ARDS evoked by SCAP.

MicroRNA regulation of cellular processes is a complicated issue as each specific miRNA can have a multitude of mRNA targets. Our goal was to investigate possible cellular mechanisms influencing occurrence of ARDS in acute inflammatory response and endothelial injury associated with SCAP. Four of the pathways appeared to be particularly relevant to ARDS including LPS-stimulated MAP kinase signaling, mTOR and Jak-STAT signaling, and cell cycle

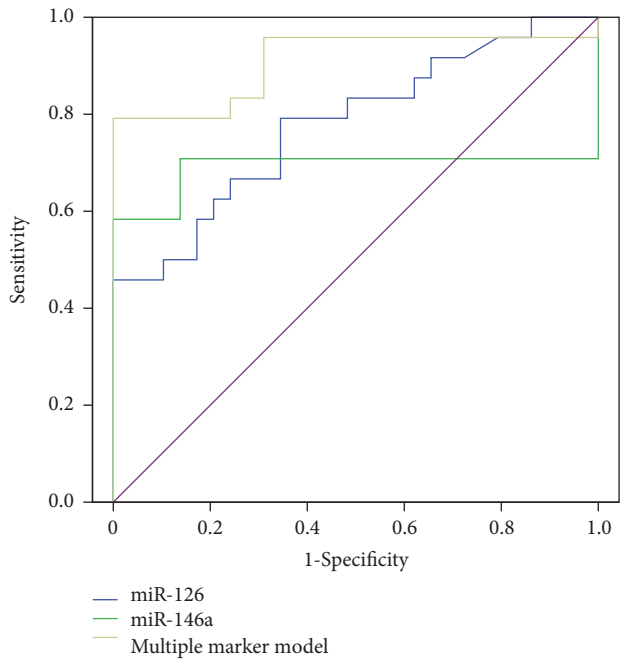

Figure 6: Receiver operating characteristics (ROC) curve analysis of miR-126 and miR-146a and the combination of multiple marker model for predicting the ARDS events in SCAP patients. Three ROC analyses are shown. Predicted probability of ARDS was computed from a multivariable logistic regression model that included the top four miRNA biomarkers.

signaling. The role of miRNAs in pulmonary disease are mostly based in animal models, thus the degree of translation into human medicine is unclear. Although few miRNAs have been detected in the pathogenesis of pneumonia, Abd-El-Fattah et al. demonstrated that miR-155 and miR-21 were markedly increased in patients with pneumonia [10]. Functional miRNAs are capsulated in exosomes and delivered to recipient cells, subsequently regulating gene expression by binding to targeted mRNAs to inhibit their translations. In our separate analysis, the decreased levels of exosomal miR-181b could be explained by the evidence that, it inhibits nuclear factor-κ -gene binding (NF-κB)-mediated endothelial cell activation and vascular inflammation in response to the injury [11]. Wang et al. reported that miR-27a was up-regulated and promoted inflammatory response in sepsis, which was concordant with the elevated expression of exosomal miR-27a in our study [12]. It has been established that alterations in certain miRNAs during ARDS might participate in the modulation of the inflammatory process and tissue repair, as these changes are concomitant to the levels of the inflammatory mediators as well as the recruitment of immune cells in the lung [13, 14]. For example, miRNAs such as miR-146a and miR-155 play crucial roles in regulating the immune response by modulating components of NF-κB signaling [15]. Li et al. demonstrated that miR-15a, miR-16 and miR-223 were found to target the IKK-α expression, and decreased expression of these miRNAs led to an increase in IKK-α in human monocytes [16]. Moreover, ectopic expression of miR-146a could affect IL-6 secretion in primary human fibroblast. Goodwin et al. reported that the down-regulation of exosomal miR-15a and miR-27a may help to reliably identify ICU patients who develop septic shock [17]. These findings were only in good accordance with Poon et al. who have also shown that increased plasma exosomal miR-223 expression during cardiac surgery might play homeostatic roles in downregulating inflammatory responses through intercellular communication [18]. In light of the recent evidence, it may be speculated that, the suppressive role of miR-15a/16 on inflammatory genes might be overruled by the miR-223 leading to an enhanced production of anti-inflammatory factors in ARDS group.

Endothelial dysfunction is a key characteristic of ARDS, giving rise to increasing vascular permeability and then pulmonary edema induced respiratory failure [19]. Considering that miR-126 regulates vascular integrity and angiogenesis by enhancing the actions of VEGF [20], previous study revealed that miR-126 contained in endothelial cell-derived MPs promoted vascular regeneration [21, 22]. Additionally, a significant loss of miR-126 defining the impaired regenerative capacity was reduced in diabetic patients [23]. However, a single endothelial stress can cause microparticles shedding without any cell damage or apoptosis, displaying vector functions for the intercellular exchange of biological information [24]. It can be postulated that miR-126 is largely induced by inflammatory response, thus explaining the elevated miR-126 levels in presence of ARDS. As an endothelial cell-restricted miRNA, miR-126 has been shown to be abnormally expressed in septic patients and correlated with a decreased survival, supporting its possible use as targetable biomarker for early detection and prognosis of ARDS. In regard to miR-126, exosomes represent its major serum compartment, whereas other miRNAs are predominantly found to be transported in the vesicle-free form [25]. Furthermore, exosomes can protect the bioactive substances from repeated freezing and thawing, enzymatic digestion (RNase), degradation, and other adverse conditions. In particular, exosomal miRNAs are stable in the circulation [26].

Our data indicated that the prognostic value of might be different in the case. Exosomes are actively released from cells with different phenotypes including lymphocytes, neutrophils, platelets, endothelial cells, or endothelial progenitor cells in a very selective manner [27], and must be transferred to s recipient cells to repress their target mRNAs, thus exerting different functions that correlated with the clinical course of critical diseases [28]. Nevertheless, these characteristics create a challenge to determining the exact cellular source of circulating miRNA. The complexity of pneumonia underlines the increasing recognition as a systemic disease with repercussions on other organs, not limited to the lung [29]. Regardless of the numerous exosome resources, we found that a combination of four miRNA markers in serum (miR-27a, miR-126, miR-146a, and miR-155) alongside clinical scores had improved prognostic values. In this context, our present study suggested that multifunctional exosomes containing both immune cell- and tissue-specific miRNAs with combined diagnostic values showed a great promise in comparison with other biomarkers.

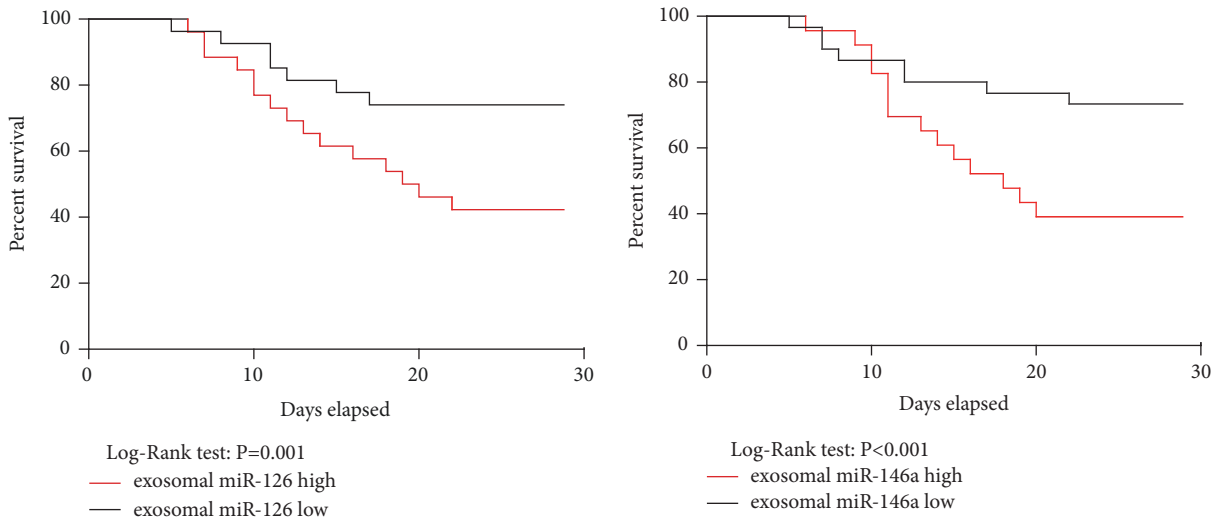

Figure 7: Kaplan–Meier survival analysis and log-rank test were used to evaluate the predictive values of miR-126 and miR-146a grouped by their expression levels.

Several limitations still existed in the present study. Only a selected number of miRs, based on previously published data, were analyzed. SCAP patients were mainly enrolled from the critical care unit of the pulmonary department, and suffered from multiple diseases. Accordingly, the high mortality rate in this small cohort may not be representative of general population of CAP patients. Although classifier containing exosomal miRNAs discovered in this study offered a potentially valuable advantage, novel exosomal miRNA signatures are incompletely recognized and warranted to better predict the prognosis or 28-day mortality in the clinical practice.

To date, no single biomarker has sufficient discriminating power to clearly indicate prognosis in the personalized assessment of the patients [30]. Despite the definition of ARDS based on clinical criteria, altered levels of plasma biomarkers may assist in the precise diagnosis with possible ARDS, as well as potentially selecting patients into clinical practice for the optimization and modulation of intensive care.

Data Availability

The data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Xu Wu and Chengzhi Wu contributed equally to this work. Xu Wu and Wenyu Gu designed the study and wrote the paper; Chengzhi Wu assisted with the statistical analysis; Haiying Ji performed the exosome experiments; Lei Zhu revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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Supplementary Materials

The raw experimental data of clinical parameters, serum exosomal microRNAs, and cytokines levels are provided as Supplementary Material 1. *(Supplementary Materials)*

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Review Article

Exosomes in the Repair of Bone Defects: Next-Generation Therapeutic Tools for the Treatment of Nonunion

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Nonunion with bone defects, a common complication after long bone fracture, is a major challenge for orthopaedic surgeons worldwide because of the high incidence rate and difficulties in achieving successful treatment. Bone defects are the main complications of nonunion. The conventional biological treatments for nonunion with bone defects involve the use of autologous bone grafts or bone graft substitutes and cell-based therapy. Traditional nonunion treatments have always been associated with safety issues and various other complications. Bone grafts have limited autologous cancellous bone and there is a risk of infection. Additionally, problems with bone graft substitutes, including rejection and stimulation of bone formation, have been noted, and the health of the stem cell niche is a major consideration in cell-based therapy. In recent years, researchers have found that exosomes can be used to deliver functional RNA and mediate cell-to-cell communication, suggesting that exosomes may repair bone defects by regulating cells and cytokines involved in bone metabolism. In this review, we highlight the possible relationships between risk factors for nonunion and exosomes. Additionally, we discuss the roles of exosomes in bone metabolism and bone regeneration.

1. Introduction

Traumatic and life-threatening fractures of the long bones have increased dramatically as the demand for motor transport continues to increase in developing countries [1]. Nonunion is the most common complication after long bone fracture, and the rate of nonunion is estimated to be between 5% and 10% [2, 3]. In addition, the higher incidence rates of obesity and musculoskeletal diseases and reduced rates of bone regeneration [4] have increased the occurrence of nonunion [5]. Nonunion is not limited to developing countries but is experienced worldwide. According to the Food and Drug Administration, nonunion is considered if the fracture is not healed after a minimum of 9 months and there are no obvious progressive signs of healing for 3 consecutive months [6]. Patients with bone nonunion often require two or more surgeries, resulting in severe psychological and economic pressure [7, 8] and seriously affecting quality of life [5, 9]. Therefore, an in-depth understanding of the fracture healing process and related mechanisms and the provision of appropriate interventions to accelerate bone regeneration are essential to avoid adverse consequences. Thus, given the burden of nonunion on patients and society, new treatments for nonunion intervention are urgently needed.

Extracellular vesicles are a class of heterogeneous membrane vesicles including three major subpopulations (exosomes, microvesicles, and apoptotic bodies), which has a great diversity of biophysical properties and functions. The International Society for Extracellular Vesicles updated their guidelines in 2018 (Table 1) [10]. Exosomes contain lipids, nucleic acids, proteins, and signalling molecules (Figure 1) [11]. Exosomes were first discovered and described as exfoliated membrane vesicles in 1981 [12]. Firstly, endosomes are produced by plasma membrane internalization of donor cells, and then the proteins and RNAs (including lncRNAs, circRNAs, mRNAs, and miRNAs) are selectively crated into the multivesicular bodies, via endosomal sorting complex required for transport- (ESCRT-) dependent or ESCRT-independent mechanisms. Subsequently, the multivesicular bodies are either fused to the lysosome for degradation

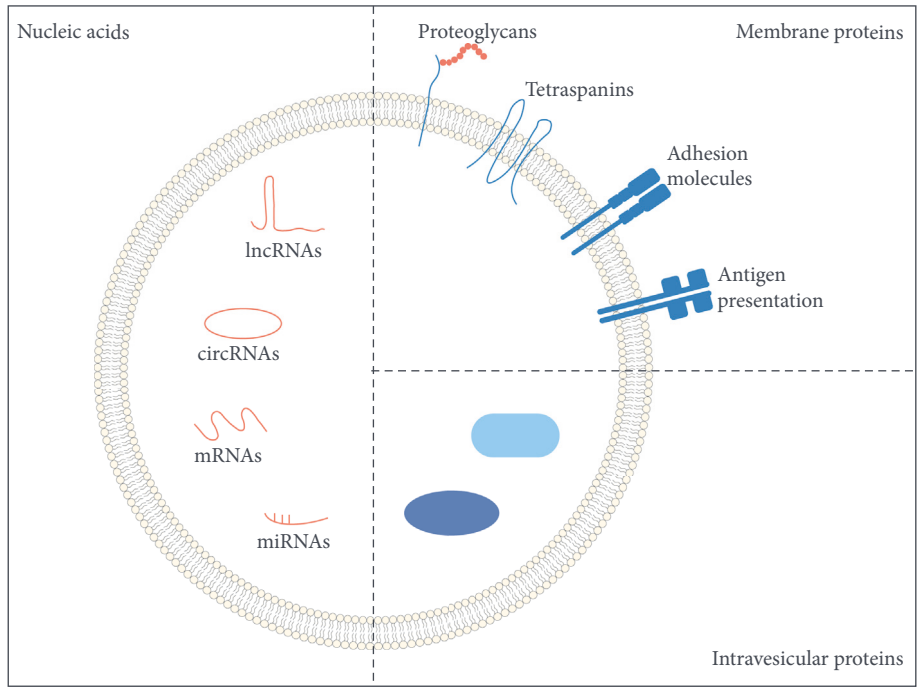

Figure 1: The constitute of exosomes.

Table 1: Types of extracellular vesicles.

Vesicles	Size (nm)	Origin
Exosomes	50 - 100	Endosomes
Microvesicles	100 – 1,000	Plasma membrane
Apoptotic bodies	1,000 – 5,000	Plasma membrane

or released into the extracellular space by fusion with the plasma membrane to produce exosomes. Microvesicles are produced directly on the plasma membrane {Tao, 2018 #264}. Today, exosomes are defined as cellular organelles that are released by various tissues and cells and can be internalized by receptor cells through endocytosis [13]. Exosomes are widely distributed and can be separated from almost all types of biological fluids, particularly semen [14], breast milk [15], saliva [16], synovial fluid [17], and urine [18]. Recent studies have demonstrated that exosomes possess the ability to stimulate the regeneration and repair of tissue and organs [19], including the heart [20], skin [21], and liver [22]. In the past two decades, exosomes have been shown to have various applications as naturally derived nanoparticles. Endocytosis of exosomes can facilitate the absorption of proteins, mRNAs, and microRNAs, thereby affecting target cells [23]. Moreover, these functional RNAs enable intercellular signal communication through exosomes [24].

In this review, we discuss the applications of exosome treatment in nonunion with bone defects, highlighting the physiological mechanisms of fracture, risk factors that may cause nonunion, classifications of nonunion, and treatment intervention using exosomes.

2. Fracture Healing

Bone can heal without scarring and return to its original state, unlike other tissues [25], through the concerted activities of thousands of genes, cytokines, growth factors, chemokines, and other molecules [26]. The newly healed bone is similar in structure and mechanical properties to the original bone [27–29]. Fracture healing involves an initial anabolic stage in which tissue volume increases in relation to newly generated and differentiated stem cells, which form bone and vascular tissue (Figure 2) [30]. First, the injured bone forms a hematoma, which is a fibrin clot caused by periosteal blood vessel haemorrhage beneath the periosteum and the medullary canal [31] as a result of coagulation [32]. Although the mechanical properties are poor, the fibrin clot provides the first step for fracture connection [33]. Simultaneously, macrophages, degranulating platelets, and other inflammatory cells [34] reach the fracture site to clean up the wound [35]. Gradually, endothelial cells and fibroblasts infiltrate, forming new capillaries and collagen matrix, which results in the formation of granulation tissue [32, 36] to fill the fracture gap [37]. Initial granulation tissue is then gradually replaced by fibrous tissue, to form a soft callus [38]. Close to the hypoxic fracture line, as chondrocytes become hypertrophic and begin to undergo apoptosis, the soft callus exhibits endochondral ossification [26]. In the periphery of the new cartilage tissue toward the fracture location, periosteum swelling and bone formation occur through intramembranous ossification [39]. Additionally, following exposure to vascular endothelial growth factor secreted from endothelial cells, the surrounding matrix is digested by chondrocytes and then infiltrated by blood vessels and osteoblasts [40]. The next phase involves the formation of

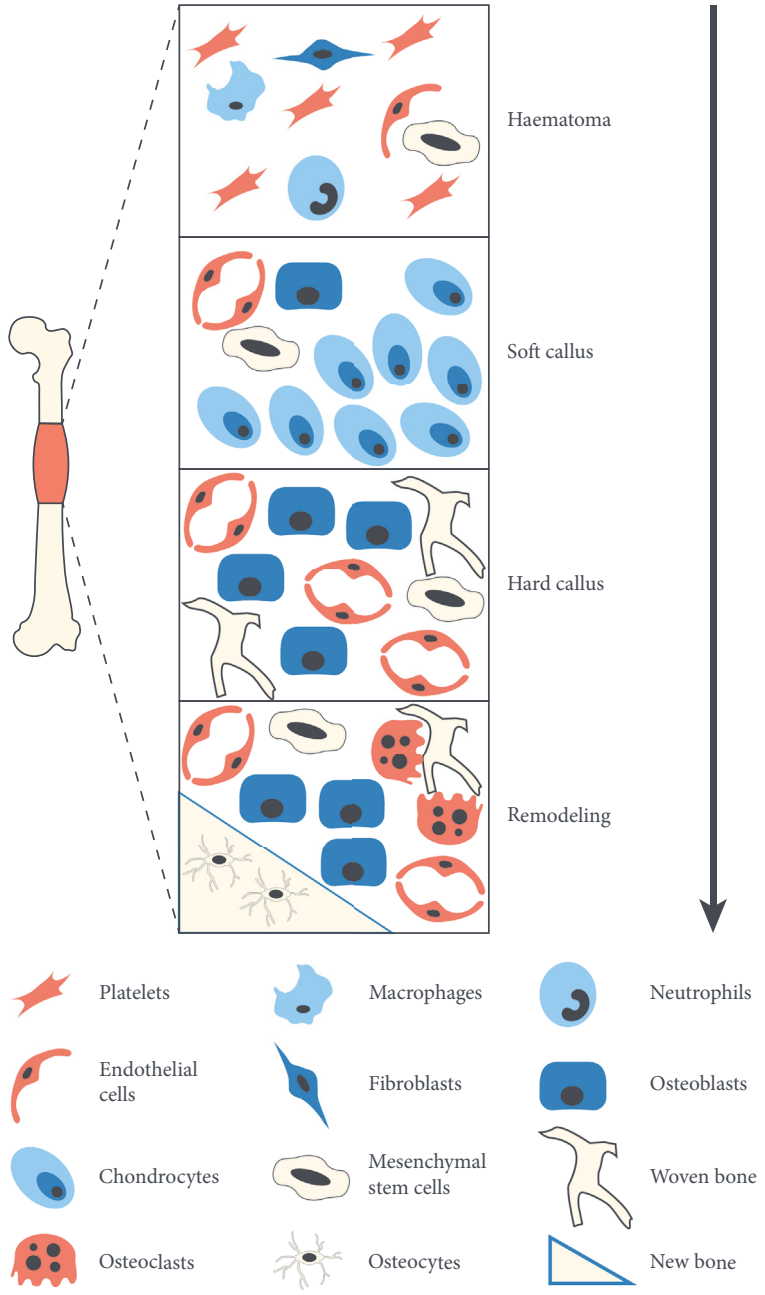

Figure 2: Cells involved in the process of bone healing.

the primary bone [34], which is characterized by replacement of mineralized bone and high levels of osteoblast activity [41], concurrent with cartilage tissue development.

2.1. Indirect (Secondary) Fracture Healing. Indirect healing with callus formation is an orderly process of bone recombination [42]. In general, using fixation with steel plates and screws, the fracture ends are better restored and compressively fixed. Micromotion and weight-bearing enhance indirect fracture healing. However, delayed union and nonunion can also appear when there is too much motion [43].

2.2. Direct Fracture Healing. Direct fracture healing can occur by lamellar bone remodelling when the fracture end is very stable with no gap formation. However, when natural healing of the fracture does not occur, this type of healing usually requires open reduction and internal fixation [42].

3. Contribution of Risk factors to Fracture Nonunion

The relationships between various risk factors affecting fracture healing and nonunion have been confirmed in

recent studies [44]. Risk factors identified as contributing to the development of fracture nonunion include patient-dependent factors, such as age [45] (age-related changes can affect many biological processes during fracture healing), sex [46] (high oestrogen levels in postmenopausal women play important roles in promoting bone formation), nutritional state [47, 48] (during the bone regeneration process, there is an increase in metabolic requirements), diabetes [49] (diabetes is a metabolic disorder that interferes with bone formation and damages fracture healing), osteoporosis [50] (osteoporosis impairs bone regeneration and the ability to restore biomechanical properties), alcohol abuse [51] (alcohol reduces bone repair, repaired tissue stiffness, and ash density), smoking [52] (nicotine inhibits osteoblast proliferation and is a vasoconstrictor, leading to tissue ischemia and hypoxia), and nonsteroidal anti-inflammatories (NSAIDs) [53] (long-term, high-dose treatment with NSAIDs reduces osteoblast numbers and inhibits the formation of prostaglandin). Patient-independent factors include fracture gap [54] (the fracture process is poor when the fracture gap is greater than 2 cm), fracture site [55] (compared with bone fracture of the diaphysis, the incidence of healing defects in the metaphyseal fracture is lower and the healing time is shorter), type of fracture [55, 56] (compared with stable and simple fractures, the more ruptured and unstable the fracture ends, the higher the risk of debris ischemia and necrosis), and infection [57, 58] (infection reduces the strength of the callus and creates conditions for sequestrum and osteolysis). All strategies that help shorten healing time and restore work and activity faster not only improve patient outcomes but also help reduce the financial burden of fracture and nonunion.

4. Classification of Nonunion

The most common classification of nonunions is the Weber and Cech classification [59]. Nonunions include hypertrophic nonunion and atrophic nonunion. Hypertrophic nonunion, also called mechanical nonunion, involves a large number of nonbridged calluses containing cartilage and is characterized by excessive bone formation and poor mechanical fixation. Atrophic nonunion, also called biological nonunion, is characterized by minimal callus or cartilage owing to lack of blood supply or cells. In atrophic nonunion, the fracture end may be hardened or osteoporotic [60].

5. Treatment Intervention

Long bone nonunion is often accompanied with bone defects. For management of nonunion, the main strategies include removal of necrotic bone and tissue, filling most of the bone defect, promoting the recruitment of osteoblasts, increasing the concentrations of osteoinductive substances, and providing a stable mechanical environment [61]. The distribution of blood vessels at the site of nonunion has also been shown to be an important factor in fracture healing [62]. In this section, we summarize various types of reconstruction treatments to achieve bone healing and maintain limb length.

5.1. Surgical Intervention. In order to regenerate hard and soft tissue defects, mechanical stability can be promoted through surgical intervention [61].

Nail dynamization: After treatment of long bone fractures with intramedullary nails, dynamization of intramedullary nails may help increase axial compression and micromotion to stimulate healing [63, 64]. This method may cause axial shortening of the femur via dynamization [65].

Exchange nailing with augmentation plating: Exchange nailing provides biological effects by increasing subperiosteal blood circulation and stimulating osteogenesis [66], growth factor activation [67, 68], and inflammatory responses [69]. As the length of the medullary stenosis and diameter of the intramedullary nail increase, the effective contact area between the intramedullary nail and the medullary cavity is significantly increased, thereby enhancing mechanical stability [70, 71].

Augmentation plating: Rotational instability is also a risk factor in diaphyseal long bone nonunion, which can be solved by augmentation plating [72, 73].

External fixation: External fixation to treat nonunion provides tension and support at the long bone nonunion sites for bone binding [74, 75]. The procedure can improve early weight bearing while increasing the stability of bone healing [76]. The disadvantages of this procedure are that it takes a long time to heal and that the wound is at risk of infection [77].

However, surgical intervention can only overcome factors affecting instability and is not sufficient to cure nonunion. Treatment of nonunion involves not just providing a stable mechanical environment for nonunion but also increasing osteogenic activity to address biological nonunion.

5.2. Autograft and Other Bone Graft Substitutes. Autologous bone grafts are the "gold standard" for the treatment of nonunion because of complete histocompatibility and strong osteoconduction, osteoinduction, and osteogenic activities [78]. However, autograft bone grafts may result in increased blood loss, pain, and possible infection at the donor site [79]. Although allogeneic bone has no osteogenic potential, it can be used as a scaffold to provide osteoconductive material [80]. Demineralized bone matrix is composed of collagen, noncollagenous proteins, bone morphogenic proteins, and growth factors [81], conferring the bone with osteoinductive and some oeteoconductive properties [82]. Ceramics, such as calcium phosphate [83], tricalcium phosphate [84], hydroxyapatite [85], and calcium sulphate [86], have been widely used as osteoinductive carriers and transplant substitutes. Among them, calcium phosphate is chemically similar to human bone minerals and also have bioconductive [87]. Le Nihouannen D [88] proved that microporous biphasic calcium phosphate containing hydroxyapatite and beta-tricalcium phosphate implants into sheep muscle can promote bone formation. Le Nihouannen D et al. [89] also used calcium phosphate ceramics as scaffold combined with fibrin glue based composites to verify osteoinduction (new bone formation) and osteoconduction (bone healing capacity). However, allogeneic bone and bone graft substitutes

may be associated with infection [90] and graft-versus-host disease [91]. Moreover, bone graft substitutes have no cellular components, and their effects are not as good as autologous bone.

5.3. Cell Therapy. Cell-based therapies, which apply a stem cell self-sufficient biological environment and heterogeneity to restore and improve tissue function, have been extensively investigated. Friendstein et al. found that, after hematopoietic necrosis, osteoblast-like bone marrow cells formed new bone *in vitro*, and mesenchymal stem cells (MSCs) were first isolated in this context [92]. Bruder et al. demonstrated for the first time that MSCs isolated from human bone marrow could regenerate normal bone in critical tibial defects of immunocompromised rats [93]. Additionally, MSCs were found to be ideal cells for bone tissue regeneration, not only because of their therapeutic potential and ability to self-renew but also because of their availability from many different tissues [94–96].

Bone marrow-derived stem cells (BMSCs): BMSCs are the most abundant cells in the bone marrow. BMSCs play roles in regulating hematopoietic stem cells and progenitor cells through different signalling pathways, as demonstrated in various studies [97]. When treating nonunion, Connolly et al. reported that 18 of 20 patients with ununited tibial fractures were successfully cured by autologous marrow injection into the nonunion site [98]. Additionally, Quarto et al. [99] and Marcacci et al. [100] have achieved healing results with bone graft substitutes loaded with BMSCs. Similar treatments such as BMSCs and biphasic calcium phosphate biomaterials have been transplanted in nonunion to achieve effective fracture healing and bone growth in clinical trials [101]. This work was supported by European Union's Seventh Framework Programme (FP7/FP7-HEALTH-2009).

Induced pluripotent stem cells (iPSCs): Human iPSCs are similar to embryonic stem cells in multilineage differentiation potential and proliferation ability [102]. Teramura et al. [103] demonstrated that mouse iPSCs could be induced into MSC-like cells and then differentiated into osteoblasts. Although the study of iPSCs is still relatively new, iPSCs may have promising applications in the healing of bone defects.

Other stem cells also have shown excellent osteogenesis capacity. For example, endothelial progenitor cells can form ectopic vascular bone for the treatment of critical size bone defects [104]. In animal studies hydroxyapatite-tricalcium phosphate containing allogeneic MCSs were effective in enhancing the repair of critical-sized defect in the canine femur [105, 106]. Although the treatment outcomes of these novel methods are promising, the molecular mechanisms of MSC repair *in vivo* are still unclear. Increasing evidence suggests that the therapeutic effects of MSCs are related to exosome-mediated paracrine induction [107, 108].

5.4. Cell-Free Therapy. Many studies have supported the roles of exosomes in intercellular communication through paracrine signalling in various tissue repair processes and diseases (Figure 3) [109]. These natural mechanisms can be applied as intercellular signalling pathways to stimulate bone regeneration [109]. Therefore, cell-free therapies that increase the formation of osteoblasts and the interactions between cells may have potential applications in the treatment of nonunion.

As an important component of exosomes, microRNAs have attracted much attention in the study of exosome function owing to their important regulatory roles. Li et al. reported that osteoclast-derived exosomal *miR-214-3p* inhibits osteogenic activity and reduces bone formation; additionally, inhibition of *miR-214-3p* in osteoclasts may have applications in the treatment of nonunion [110]. Qin and colleagues found that muscle-secreting myostatin inhibits osteoblastic differentiation by blocking osteocyte-derived exosomal *miR-218*, suggesting the presence of a potential communication mechanism between muscle and bone [111]. Weilner et al. reported that *miR-31* from vascular endothelial cell-derived exosomes may be a biomarker and potential therapeutic target for osteoporosis [112].

Proteins in exosomes in cell-to-cell communication also play important roles. Ge et al. reported that highly expressed proteins in MC3T3 cell-derived exosomes are rich in osteogenic-related pathways [113]. Moreover, Huynh et al. reported that receptor activator of nuclear factor-κB ligand (RANK), which is highly enriched in exosomes derived from osteoclasts, is a paracrine regulator of osteoclastogenesis [114].

Many recent studies have evaluated the application of stem cell-derived exosomes for bone repair. Qin et al. demonstrated that BMSC-derived exosomes regulate osteoblast expression by *miR-196a in vitro* and improve bone regeneration ability in a Sprague-Dawley rat model of calvarial defects [115]. Additionally, angiogenesis has been shown to be an essential factor in bone regeneration. Ashoo et al. demonstrated that BMSC-derived exosomes are capable of increasing endothelial cell viability *in vitro* and stimulating angiogenesis *in vivo* [116]. Lu et al. also reported that adipose stem cell-derived exosomes promote the proliferation and differentiation of human primary osteoblastic cells [117]. Exosomes released from adipose stem cells (ASCs) have also been shown to promote ASCs-induced angiogenesis [118].

Proteins and RNAs contained in osteoblasts-derived exosomes played an important role in intercellular communication within bone tissue. Ge et al. demonstrated that osteoblast-derived exosomes activate eukaryotic factor 2 to promote osteoblastic differentiation *in vitro* [113]. Weilner et al. demonstrated that galectin-3 levels in osteoblasts-derived exosomes were positively correlated with osteogenesis potential [119]. A proteomic study of exosomes derived from MC3T3 cells (mouse osteoblasts) revealed some osteogenesis-related pathways, including integrin signalling, mammalian target of rapamycin (mTOR) signalling, and eukaryotic initiation factor 2 (EIF2) signalling [113]. Moreover, Cui et al. reported that mineralizing osteoblast-derived exosomes promoted bone marrow stromal cell differentiation into osteoblasts by activating Wnt signalling [120].

Exosomes have been widely reported in the field of regeneration. Qi et al. found that exosomes secreted by MSCs

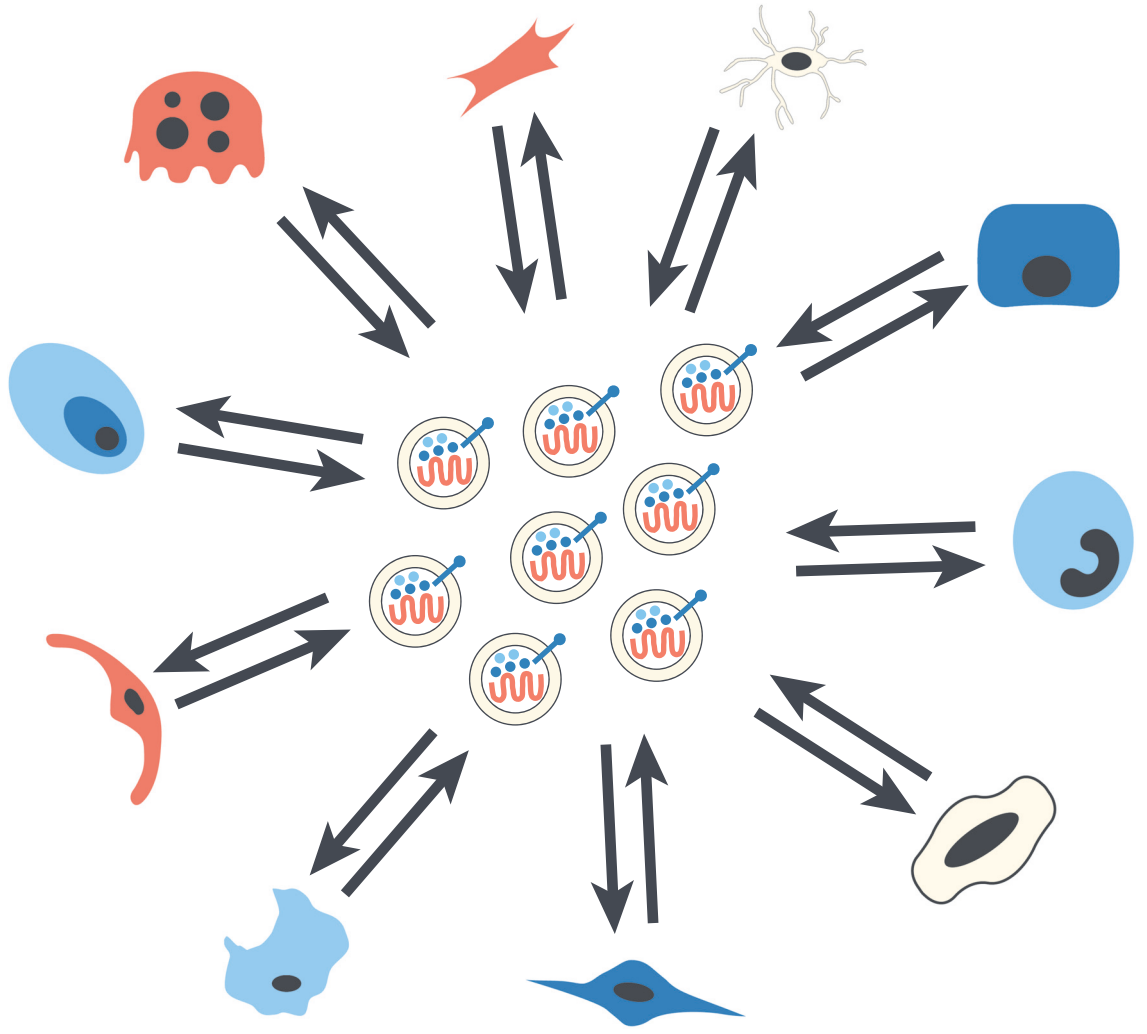

Figure 3: Exosomes are likely to be the way cells communicate with each other in the process of bone healing.

derived from human induced pluripotent stem cells (hiPSC-MSC-Exos) could significantly promote osteogenesis and angiogenesis in rats with osteoporosis [121]. Liu et al. found that, in a model of steroid-induced osteonecrosis in rats, hiPSC-MSC-Exos could prevent femoral head necrosis by activating PI3K/Akt signaling pathways in endothelial cells to promote angiogenesis [122]. In another study, compared with exosomes secreted by synovial membrane MSCs (SMMSC-Exos), exosomes secreted by induced pluripotent stem cell-derived MSCs (iMSC-Exos) had superior therapeutic effect on osteoarthritis (OA) due to the ability to promote chondrocyte migration and proliferation [123]. Du et al. reported that hiPSC-MSC-Exos could alleviate hepatic ischemia-reperfusion (I/R) injury and promote cell proliferation in a rat model of hepatic I/R injury [124].

6. Discussion and Conclusion

Nonunion has major implications for patients and families and can also affect society. Surgical intervention can only solve mechanical nonunion, and interventions for biological bone nonhealing often involve autologous bone or bone graft substitutes, stem cell therapy, and other methods. Nonviable vesicles, such as exosomes, are associated with a lower risk of complications than cell-based treatment. Importantly, exosomes can be stored at −20°C for 6 months without loss of efficacy [125]. Thus, it is particularly important to grasp the relationship between exosome and nonunion healing mechanisms and to solve the problem of nonunion.

Osteoblasts, osteoclasts, proangiogenic factors, and blood platelets play important roles in fracture healing. As described above, osteoblasts release exosomes in a positive feedback loop to promote bone growth [113]. Inder and colleagues reported that prostate cancer cell-derived exosomes can attenuate osteoclast formation and stimulate osteoblast proliferation [126]. In addition to osteoclast-derived exosomes, which can affect osteoclast differentiation, Raimondi et al. reported that multiple myeloma-derived exosomes increase C-X-C motif chemokine receptor 4 expression and activation, thus promoting osteoclast maturation [127]. Additionally, Solberg et al. showed that lysosomal membrane protein 1-positive exosomes contain

RANK ligand, osteoprotegerin, and tartrate-resistant acid phosphatase isolated from rat osteoblasts and osteocytes [128]. Osteoclast formation can be stimulated by RANKL-rich osteoblast-derived exosomes, as shown by Deng et al. [129]. In promoting angiogenesis, exosomes can stimulate endothelial cell migration and angiogenesis through exosomal *miR-129* and *miR-136* [116]. Torreggiani et al. reported that BMSCs treated with platelet-derived exosomes containing proteins and noncoding RNAs showed a significant increase in osteogenesis [130]. Understanding the processes and secreted components of cells involved in fracture healing may guide the development of new treatments for nonunion.

There are some factors that can explain the nonunion of fractures, and treatment of nonunion with these factors can be achieved through the use of exosomes. Xu et al. found that *miR-31a-5p* in rat bone marrow stromal cell-derived exosomes prevents age-related bone loss and reduces osteoclast activity in rats. Thus, they proposed that *miR-31a-5p* may be an age-related potential therapeutic target [131]. Notably, women have a higher probability of developing arthritis than men, and women who are postmenopausal are at increased risk [132]; Kolhe et al. noted that differences in miRNA expression were greater in women with osteoarthritis than in men with osteoarthritis [133], suggesting that some differences in miRNA contents may be related to sex. Additionally, Saha et al. analysed plasma-derived exosomes from alcoholic individuals and reported that alcohol increases exosome production in monocytes; they also found that exosomes containing *miR-27a* released from monocytes promoted naïve monocyte differentiation into M2 macrophages [134]. Goerzl et al. demonstrated that aspirin significantly reduces the levels of plasma platelet-derived exosomes without changing the total number of exosomes [135].

Exosomes have great potential for applications in the treatment of nonunion with bone defects and can be used to adjust the immune microenvironment and promote vascularization, proliferation, differentiation, and mineralization of osteoblasts. Exosomes are eliminated from the blood stream in short time and aggregate in the liver [136, 137]. Thus, future studies are needed to further assess the application and efficiency of exosome-based targeted drug delivery.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Jian Lu is the first author and planned and wrote the manuscript. Qi-Yang Wang helped with the writing. Jia-Gen Sheng provided advice and suggestions. All authors reviewed the manuscript.

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Research Article

Circulating Exosomes Derived-miR-146a from Systemic Lupus Erythematosus Patients Regulates Senescence of Mesenchymal Stem Cells

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The senescence of mesenchymal stem cells (MSCs) plays a crucial role in the development and progression of systemic lupus erythematosus (SLE). Exosomes, small spherical bilayer proteolipid vesicles, contribute to the communication between various cells and their microenvironment by transferring information via their cargo, including the proteins, lipids, and RNAs. While exosomal miRNAs participate in various biological activities, correlations of circulating exosomes with senescent signs of BM-MSCs remain unclear. In our study, we aimed at exploring the roles of circulating exosomal miRNAs in the senescence of MSCs. We found that exosomes derived from SLE serum could increase the proportions of SA-β-gal positive cells, disorganize cytoskeletons, and reduce growth rates. Moreover, the expression of miR-146a declined significantly in serum exosomes of SLE patients compared with healthy controls. miR-146a could be internalized into MSCs via exosomes and participate in MSCs senescence through targeting TRAF6/NF-κB signaling. These results clarified the novel mechanism of MSCs senescence in SLE patients.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic and severe autoimmune disease that affects multiple organs with poor quality of life and substantial mortality [1]. With better knowledge of pathogenesis of SLE, glucocorticoid and immunosuppressive agents have significantly enhanced survival of SLE patients. However, some SLE patients show poor outcome and frequent relapses, which presents a challenge to rheumatologists [2]. Recently, mesenchymal stem cell transplantation (MSCT) has been regarded as a safe and effective therapeutic approach. While allogenic MSCT conferred obvious therapeutic effects on SLE by previous studies, autologous MSCT was ineffective [3–5]. Our previous studies demonstrated that SLE BM-MSCs exhibited senescent characteristics and played a vital role in SLE. Reversing the senescent phenotype of BM-MSCs could improve therapeutic effects of autologous MSCT in SLE [6, 7]. Therefore, exploring the possible senescence mechanisms of BM-MSCs is of great importance. We previously reported that lipopolysaccharide (LPS) could accelerate senescence of dental pulp stem cells (DPSCs) by NF-κB-p53/p21 signaling pathway [8]. The stimulation of IFN-γ could promote cellular senescence in normal BM-MSCs by activating Jak-Stat signaling [9]. Similarly, serum from SLE patients obviously promoted umbilical cord (UC) derived-MSCs senescence [10]. These data suggested that inflammation in microenvironment might participate in the senescence of MSCs, and we are required to further study and investigate the exact mechanism.

Table 1: Details of 10 SLE patients.

Patient	Age	Disease duration	Current treated	SLEDAI
1	21	1 year	Pred 15mg/day	10
			HCQ 0.2g/day	
2	18	9 months	Pred 15mg/day	14
			HCQ 0.2g/day	
			LEF 0.2g/day	
3	24	2 years	Pred 15mg/day	9
			HCQ 0.2g/day	
4	28	1 year	Pred 15mg/day	12
			HCQ 0.2g/day	
5	24	2 years	Pred 10mg/day	14
			HCQ 0.2g/day	
			CTX 0.4g/2 weeks	
6	32	3 years	Pred 15mg/day	12
			HCQ 0.2g/day	
7	43	6 years	Pred 10mg/day	9
			HCQ 0.2g/day	
8	21	4 days	none	13
9	26	1 month	Pred 12.5mg/day	14
			HCQ 0.2g/day	
10	32	1 year	Pred 20mg/day	21
			HCQ 0.2g/day	
			CTX 0.4g/2 weeks	

Pred: prednisolone, HCQ: hydroxychloroquine.
LEF: leflunomide, CTX: cyclophosphamide.

Exosomes, abound in biological fluids, including serum, contribute to the communication between various cells and their microenvironment through transferring information via their cargo, including proteins, DNAs, and RNAs [11]. Increasing evidence suggests that exosomal pathway may be an efficient way to regulate cellular apoptosis, angiogenesis, and inflammatory response in target cells [12]. Previous study showed that exosomes derived from cancer cells in multiple myeloma patients could regulate the proliferation of MSCs via modulating miR-146a and miR-21 [13]. MicroRNA (miRNA), a small noncoding RNA molecule, induces translation repression of the target mRNA and influences cellular metabolism, proliferation, and apoptosis [14]. In the study, we devote ourselves to investigating the effects and potential mechanisms of serum exosomes derived-miRNAs on BM-MSC senescence in SLE.

2. Materials and Methods

2.1. SLE Patients. The diagnosis of all SLE patients was dependent on the criteria determined by the 2009 American College of Rheumatology (ACR) (Table 1). Systemic lupus erythematosus diseases activity index (SLEDAI) was used to evaluate SLE disease activity according to clinical manifestation and laboratory examination. 10 healthy female donors (27.2±7.8 years) and 10 female SLE patients (26.9±7.3 years) were enrolled in this study. Serum samples and BM-MSCs were extracted from the participants. All participators gave written consent to the study, which was approved by the Ethics Committee of Affiliated Hospital of Nantong University (Nantong, China).

2.2. Isolation and Cell Culture of BM-MSCs. BM-MSCs, derived from heparinized BM of healthy donors and SLE patients, were isolated by using Ficoll PREMIUM (1.073g/ml). The BM-MSC layer was collected, washed by phosphate-buffered saline (PBS) three times, then plated in T25/75 cell culture flask, and cultured at 37°C, in 5% CO2 incubator. After 5 days, the medium was replaced every 3 days until the cells reach 80% confluence. The culture medium was Low-glucose Dulbecco Modified Eagle Medium (L-DMEM) with 10% heat inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, USA), 10% normal serum or SLE serum. When BM-MSCs became nearly 80% confluent, the adherent cells were digested with 0.25 % trypsin–EDTA (Gibco, USA) and replated at a density of 1×10^6 cells per T25 flask.

2.3. Extraction of Exosomes from Serum. Serum samples were centrifuged to get rid of cellular debris. The cell-free serum supernatant (200ul) was transferred to a fresh tube and each sample was blended with 1/5 volume (40ul) of Total Exosome Isolation from serum reagent (Invitrogen, USA). The samples were centrifuged at 10,000g at room temperature for 10 min after being incubated at 4°C for 30 min. The supernatant was aspirated and discarded, and the exosome bullet was resuspended in PBS buffer (200ul). It was stored at 4°C for

short term (less than 7 days) or 20°C for longer term. The concentration of exosomes was measured by BCA protein assay kit (Beyotime, Shanghai, China).

2.4. Characterization of Exosomes. After being fixed with 2.5% glutaraldehyde, isolated exosome bullets were centrifuged at 100 000g to remove the glutaraldehyde. The bullets were negatively stained by 3% aqueous phosphotungstic acid and fixed on copper mesh Formvar grids. The morphology of exosomes samples was observed by the JEOL Transmission Electron Microscope (JEM-1230; JEOL, Tokyo, Japan). The size of exosomes was detected by nanoparticle tracking analysis (NTA), using ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and corresponding software ZetaView 8.04.02.

2.5. Exosomes Uptake Assay. Cellular uptake assay of serum exosomes was measured using the PKH-67 labeling kit (Sigma, USA). Exosomes bullets were resuspended in 300ul Diluent C, and then exosomes suspension was incubated with 4 μl PKH-67 dye diluted in 300ul Diluent C at room temperature. After 5 minutes, the suspension was mixed with in 3ml FBS and centrifuged at 100,000g to pellet the PKH-67-labeled exosomes. After incubation with exosomes solution for 3h, BM-MSCs were fixed in 4% paraformaldehyde for 30 minutes at room temperature. The cell nuclei were dyed with DAPI (Sangon Biotech, Shanghai). BM-MSCs were detected by a fluorescence microscope (Leica, Germany).

2.6. Western Blot Assay. Equal parts of proteins were separated by SDS polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% bovine serum albumin and then incubated with primary antibodies against p-p65, p65, p-IκBα (Cell Signaling Technology, USA), p53, CD63, p27, CD9 (Sangon Biotech, Shanghai) at 4°C overnight. After being washed 3 times, samples were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, USA) for 2 h at room temperature. The blots were visualized by using an enhanced chemiluminescence kit (Millipore Corporation, USA).

2.7. SA-β-Gal Assay. The senescence-associated β-galactosidase (SA-β-gal) activity of BM-MSCs was measured by a kit from the Chemical Company (Beyotime, China). Following the manufacturer's instructions, MSCs were cultured into the six-well culture plates at a density of 5×10^4 cells per well. Then cells were washed with PBS and fixed with fixation solution. After incubation with SA-β-gal staining solution at 37°C without CO_2 overnight, BM-MSCs were washed with PBS and analyzed by using the microscope.

2.8. Immunofluorescent Staining. After being fixed with 4% paraformaldehyde for 40-60 minutes, BM-MSCs were washed with PBS containing 0.1% Triton X-100(PBST) and blocked for 30 minutes in PBST supplemented with 10% FBS. BM-MSCs were incubated with the primary antibodies (1:100) in the same solution at 4°C overnight. The primary antibodies were p65 and IκBα (Cell Signaling Technology, USA). Then, BM-MSCs were washed and incubated in secondary antibodies at room temperature for 2 h. The cell nuclei were stained with DAPI (Sangon Biotech, Shanghai). The cells were visualized using a Leica fluorescence microscope (Germany).

2.9. Immunofluorescence Assay of the Skeleton of MSCs. BM-MSCs were washed with PBS and fixed with 4% paraformaldehyde for 40-60 minutes. After permeabilization and block, BM-MSCs were incubated with fluorescein isothiocyanate-conjugated phalloidin (Thermo Fisher, Waltham, USA). The cell nuclei were stained with DAPI (Sangon Biotech, Shanghai). The stained cells were visualized with a Zeiss Confocal Laser Scanning Microscope (Oberkochen, Germany).

2.10. Quantitative Reverse-Transcription PCR. miR-146a is a type of miRNA associated with immunity, participating in cell differentiation, cell proliferation, cell immune response, and release of inflammatory mediators [15]. Exosomal miRNA was extracted using the Total Exosome RNA Kit (Ambion) and MirVana RNA isolation kit (Ambion) following the manufacturer's instructions. U6 was used as the internal reference for qualification of the cellular miRNA. The PCR primers were purchased from Biomics Biotechnologies (Nantong, China).

2.11. Transient Transfection with miR-146a Mimics/Inhibitors. miR-146a-5p mimics or inhibitors and their corresponding negative controls were bought from Biomics Biotechnologies (Nantong, China) and transfected at a final concentration of 50 nM for mimics and 100 nM for inhibitor in BM-MSCs in accordance with the manufacturer's instructions.

2.12. Statistical Analysis. All data are expressed as the mean ± standard deviation (SD). All statistical analysis was performed by the SPSS 21.0 software, and significant differences between these data were determined by using Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. SLE Serum Stimulation Promoted Senescence of MSCs. Previously, our studies demonstrated that BM-MSCs in SLE patients were senescent [9]. To further explore the senescent biological behaviours of BM-MSCs stimulated by SLE serum, we detected SA-β-gal staining, the F-actin distribution, and the expression levels of cell cycle-related proteins (p16, p27, and p53) in BM-MSCs. We found that the ratio of SA-β-gal positive BM-MSCs was upregulated significantly in the group treated with SLE serum, compared with ones treated with normal serum (Figures 1(a) and 1(b)). The F-actin distribution was disordered in MSCs treated with SLE serum (Figure 1(c)). Additionally, the expressions of p16, p27, and p53 proteins were increased in SLE serum-handled BM-MSCs by western blotting (Figures 1(d) and 1(e)). It suggested that SLE serum could enhance the senescence signs of MSCs

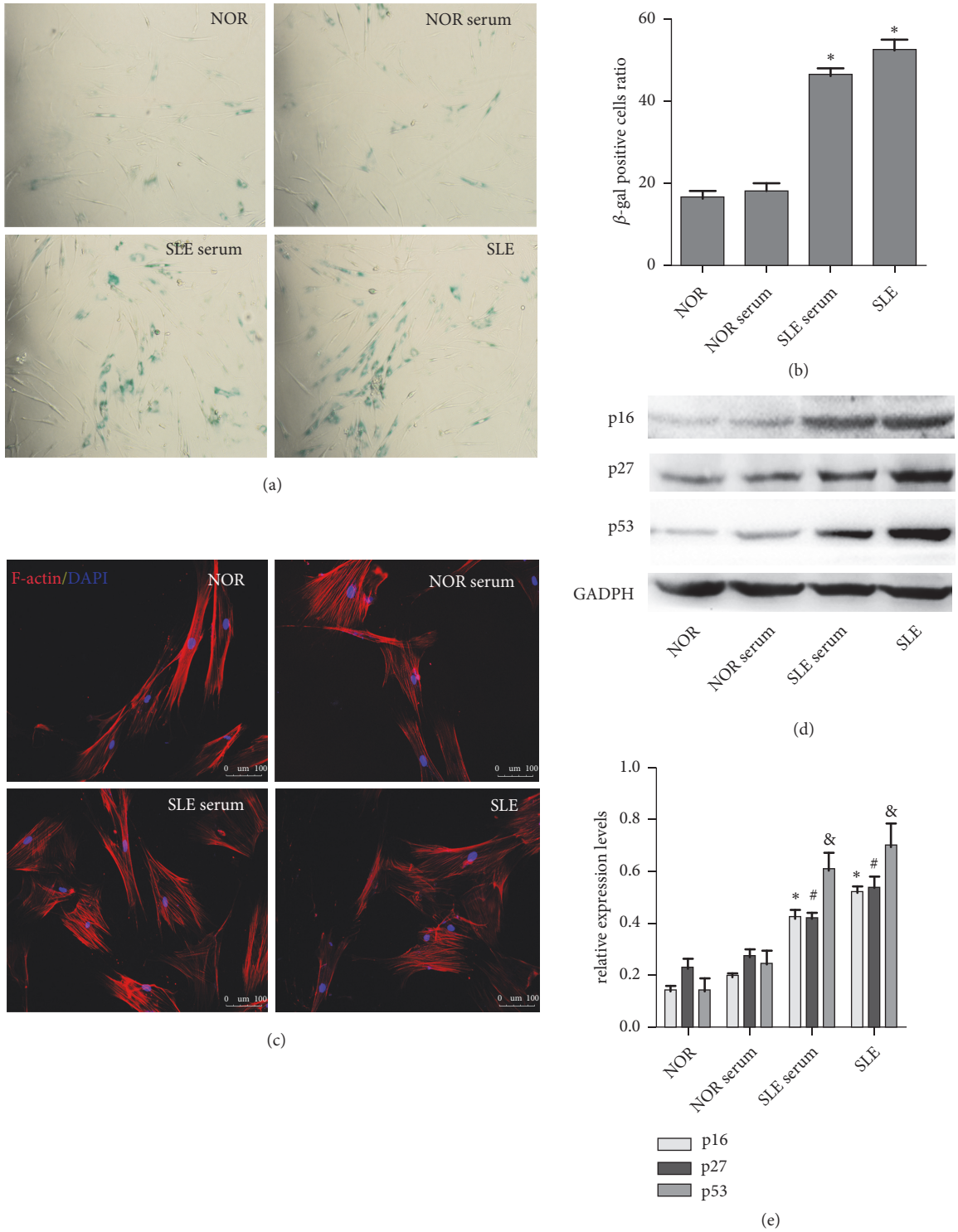

Figure 1: *SLE serum stimulation promoted senescence of MSCs.* (a, b) Cells were cultured in serum from normal persons and SLE patients. BM-MSCs were fixed and stained with SA-β-gal. The number of SA-β-gal-positive cells was increased in the serum-treated normal BM-MSCs in comparison with the normal group. (c) Immunofluorescence showed that the normal distribution of F-actin in the BM-MSCs from normal persons was disordered after being stimulated with SLE serum. (d, e) Western blotting analysis was performed to detect the protein expressions of p16, p27, and p53 ($^{*}P < 0.05$ compared with the normal group, $^{\#}P < 0.05$ compared with the normal group, $^{\&}P < 0.05$ compared with the normal group) (NOR: normal MSCs group, NOR serum: normal MSCs treated with normal human serum, SLE serum: normal MSCs treated with serum from SLE patients, SLE: SLE MSCs group) (SLE MSCs: positive control).

in vitro, which might play a significantly vital role in MSCs senescence.

3.2. Serum Exosomes from SLE Patients Enhanced the Senescence of MSCs by Activating NF-κB Signaling Pathway. To investigate whether exosomes mediated MSCs senescence, serum-derived exosomes were purified by ExoQuick method, it was observed that average size was 100nm in diameter, and membrane vesicles were observed by transmission electron microscopy (Figure 2(a)). CD63 and CD9, representative markers of exosomes, were detected by western blotting; calnexin (a marker of endoplasmic reticulum) was a negative control for exosomes (Figure 2(b)). Nanoparticle tracking analysis was used to detect the size distribution of exosomes (Figure 2(c)). The results demonstrated that serum-derived exosomes were purified successfully. Then, we cultured normal BM-MSCs with SLE serum exosomes for 24h. As shown in Figure 2(d), the fluorescent results showed that PKH-67-labeled exosomes could be localized in the cytoplasm of BM-MSCs. In accordance with the role of SLE serum in MSCs senescence, the ratio of SA-β-gal positive cells treated with SLE serum exosomes was higher than that of cells treated with normal serum (Figures 2(e) and 2(f)). Following the treatment of SLE serum exosomes, the distribution of F-actin in normal BM-MSCs presented derangement distribution (Figure 2(g)). In addition, the expressions of p16, p27, and p53 were determined in MSCs treated with SLE serum exosomes (Figure 2(h)). To explore the molecular mechanism of serum exosomes in promoting MSCs senescence, we found that SLE serum exosomes induced the degradation of IκBα and phosphorylation of p65 (Figure 2(i)). To further examine the nuclear accumulation of p65 in BM-MSCs, immunofluorescence staining showed that p65 could translocate to the nucleus following exposure to SLE exosomes (Figure 2(j)). These results indicated that SLE serum exosomes promoted senescence of MSCs through NF-κB signaling pathway *in vitro*.

3.3. Serum Exosomal miR-146a Regulated the Senescence of MSCs. Given the previous study that miR-146a levels were obviously downregulated in the PBMCs of SLE patients [16], we assessed the levels of miR-146a in serum exosomes from 10 SLE persons. Our results discovered that compared with healthy controls, the levels of miR-146a declined significantly in SLE exosomes (Figure 3(a)). The levels of miR-146a were decreased in MSCs treated by SLE exosomes (Figure 3(b)). To further discover the correlation between the expression of miR-146a and cellular senescence in SLE patients, NOR MSCs or SLE MSCs were transfected with miR-146a inhibitors or mimics, respectively. The transfection efficiency was viewed in Figure 3(c). Interestingly, we found that administration of miR-146a mimics reversed SLE MSCs senescence, as demonstrated by decreased frequencies of SA-β-gal positive cells (Figures 3(d) and 3(e)). Furthermore, the expressions of p16 and p53 were determined in NOR MSCs or SLE MSCs that were transfected with miR-146a inhibitors or mimics (Figures 3(f) and 3(g)). These western blotting results were consistent with previous SA-β-gal assay results. Taken together, these data demonstrated that miR-146a-containing exosomes might regulate the senescence of MSCs.

3.4. miR-146a Directly Targeted TRAF6 and Inhibited TRAF6-NF-κB Signaling Expression. Previous study showed that miR-146a could negatively regulate immune inflammation by suppressing NF-κB signaling pathway activation [17, 18]. According to the bioinformatic algorithms (TargetScan), TRAF6 was selected for further analysis because of its correlation with the senescence regulation of exosomes (Figure 4(a)). The transfection data showed that overexpression of miR-146a significantly decreased the expression level of TRAF6 in BM-MSCs (Figures 4(b) and 4(c)).

4. Discussion

In this study, we further confirmed that SLE serum stimulation could promote senescence of MSCs. In addition, serum-derived exosomes, as a tool for cell communication, could stimulate the senescence of MSCs by activating NF-κB signaling. Exosomal miR-146a might negatively regulate MSCs senescence by targeting TRAF6/ NF-κB signaling (Figure 5).

Previous studies have revealed that SLE is a stem cell-related disease. Senescent MSCs play an important role in SLE. Our groups further discovered that SLE MSCs exhibited senescent characteristics, the increased SA-β-gal staining, disordered cytoskeletons, and low-growth rates [19]. Various signaling pathways are involved in MSCs senescence, including PI3K/Akt pathways, Wnt/β-catenin signaling, and NF-κB pathway. Of them, NF-κB signaling pathway achieves very prominent positions in senescent MSCs [20]. The NF-κB pathway transcriptionally controls a large amount of target genes that contribute to cellular inflammation and senescence [21]. Our group found that NF-κB/p53/p21 signaling pathway participated in the senescence process of DPSC [8]. Similarly, upregulated NF-κB activity in aged MSCs was also observed by a recent study [22]. In the classical pathway of NF-κB signaling, p65 is localized in the cytoplasm predominantly as a complex with the inhibitory IκB protein in inactivated cells. Once stimulated, p65 is shuttled from the cytoplasm to the nucleus with IκB releasing from the inactive complex followed by ubiquitin-mediated proteasomal degradation of IκB [23]. As we expected, western blotting showed that the expression level of p-p65 was elevated in SLE BM-MSCs. p65 translocating to nucleus was increased in SLE MSCs. Therefore, NF-κB signaling was crucial for senescence of MSCs in SLE patients.

Exosomes can carry a lot of bioactive molecules, such as protein, lipid and mRNA, miRNAs, long noncoding RNAs (lncRNAs), and genomic DNA, then transfer their cargos to neighboring cells, and induce the functional modifications in recipient cells [24, 25]. Our group discovered that exosomes from SLE patients could promote MSCs senescence, displaying increased SA-β-gal staining and disordered cytoskeletons. Recent studies have reported that lung cancer cell-derived exosomes can accelerate MSCs to release proinflammatory cytokines, such as IL-8 and IL-6, by activating NF-κB signaling of MSCs [26]. Previous study has depicted

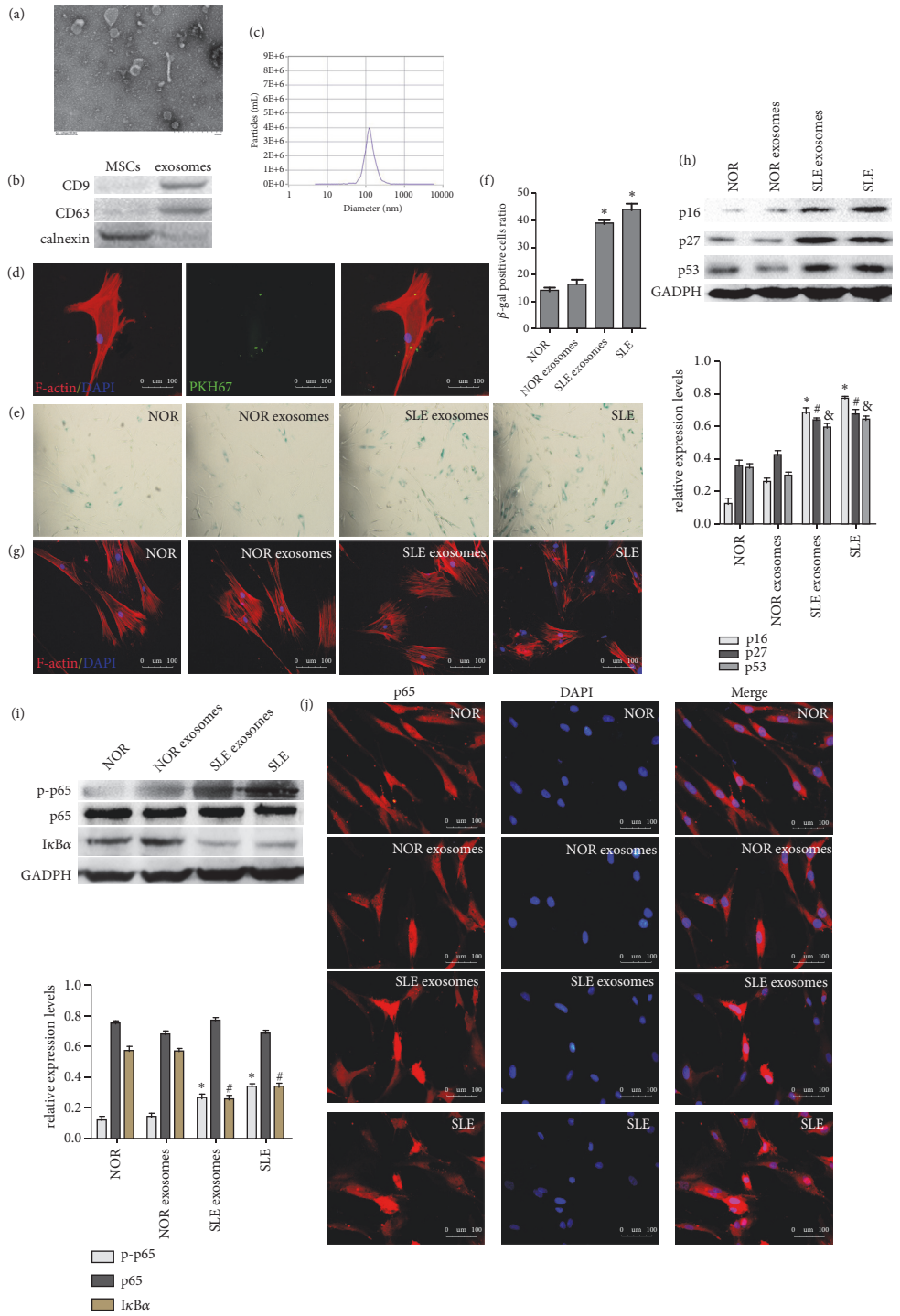

FIGURE 2: *Serum exosomes from SLE patients enhanced the senescence of MSCs by activating NF-κB signaling pathway*. (a) Serum exosomes were observed under a transmission electron microscope. (b) Western blotting analysis of CD9, CD63, and calnexin expression in lysates from purified serum exosomes. (c) Nanoparticle tracking analysis was used to detect the size distribution of exosomes. (d) BM-MSCs were incubated in serum exosomes that were labeled with PKH67 (green). (e, f) The number of SA-β-gal-positive cells was increased in the SLE serum exosomes-treated BM-MSCs in comparison with the normal group. (g) Immunofluorescence showed that distribution of F-actin in the BM-MSCs was disordered after being stimulated with SLE exosomes. (h) The protein expressions of p16, p27, and p53 were increased in MSCs treated with SLE serum exosomes by western blotting analysis. (i) Western blot analysis was performed to examine the protein expressions of p-p65, p65, IκBα. (j) Immunofluorescence staining of p65 in BM-MSCs treated exosomes ($^{*}P < 0.05$ compared with the normal group, $^{\#}P < 0.05$ compared with the normal group, $^{\&}P < 0.05$ compared with the normal group) (NOR: normal MSCs group, NOR exosomes: normal MSCs treated with normal human exosomes, SLE exosomes: normal MSCs treated with exosomes from SLE patients, SLE: SLE MSCs group).

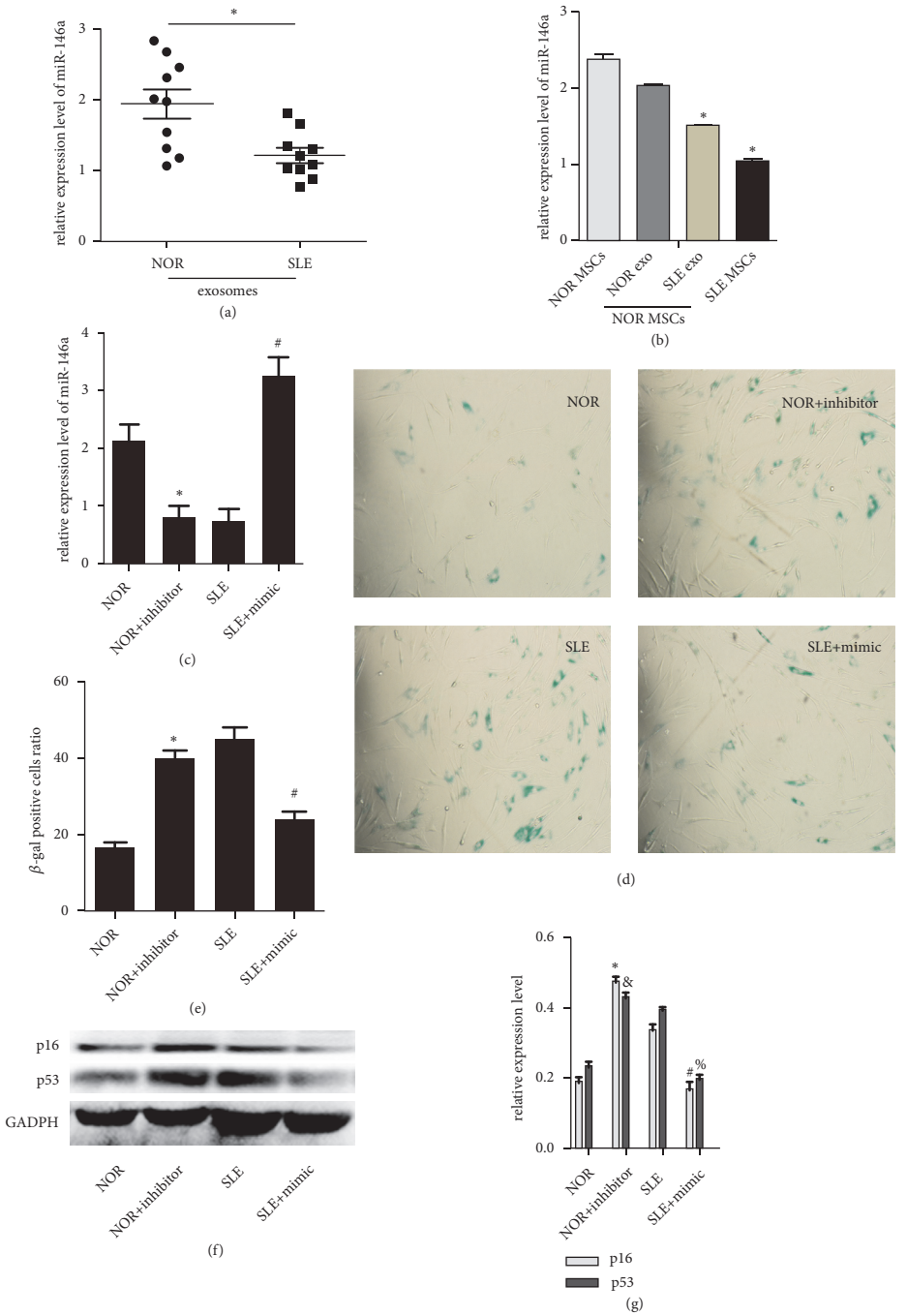

FIGURE 3: *Serum exosomal miR-146a regulated the senescence of MSCs*. (a) QRT-PCR showed the miR-146a expression in serum exosomes of normal persons and SLE patients. (b) QRT-PCR showed the miR-146a expression in MSCs treated with normal exosomes and SLE exosomes. (c) NOR MSCs were transfected with miR-146a inhibitors, and SLE MSCs were transfected with miR-146a mimics. The results showed the transfection efficiency was successful. (d, e) MSCs were fixed and stained for β-gal. The number of SA-β-gal-positive cells was obviously reversed among miR-146a mimics-treated SLE MSCs in comparison with untreated group. (f, g) The protein expressions of p16 and p53 were detected in NOR MSCs transfected with miR-146a inhibitors and SLE MSCs transfected with miR-146a mimics by western blotting analysis ($^{*}P < 0.05$ compared with the normal group,$^{\#}P < 0.05$ compared with the SLE group, $^{\&}P < 0.05$ compared with the normal group) (NOR: normal MSCs group, NOR exo: normal MSCs treated with normal human exosomes, SLE exo: normal MSCs treated with exosomes from SLE patients, SLE: SLE MSCs group).

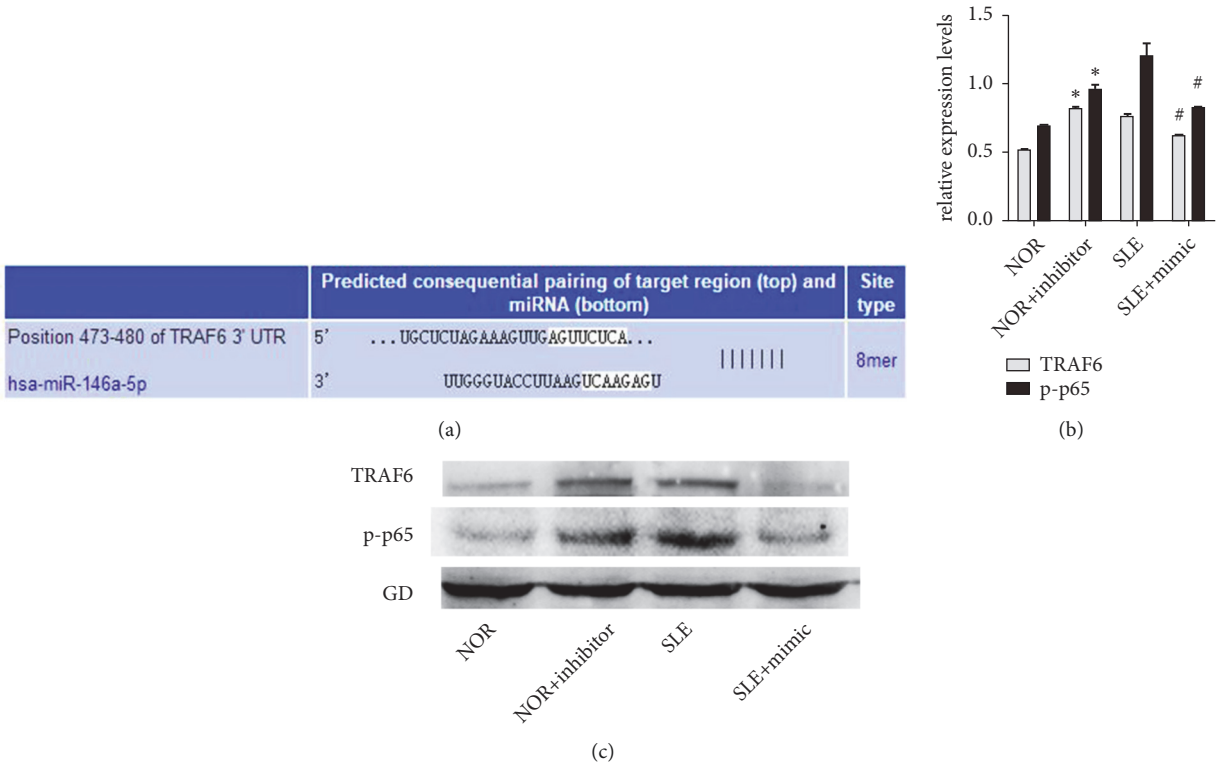

Figure 4: miR-146a directly targeted TRAF6 and inhibited TRAF6-NF-κB signaling expression. (a) TargetScan was used to identify putative seed-matching sites between miR-146a and 3'-UTR of TRAF6. (b, c) NOR MSCs were transfected with miR-146a inhibitors, and SLE MSCs were transfected with miR-146a mimics. After being transfected, each cell was detected for TRAF6 and p-p65 expression levels ($^*P < 0.05$ compared with the normal group, $^\#P < 0.05$ compared with the SLE group).

an important role for NF-κB activity in facilitating senescence of human fibroblasts via the p53/p21 and p16/pRB pathway [27]. In RA patients, serum exosomal miR-548a-3p could restrain the proliferation of pTHP-1 cells by improving the TLR4/NF-κB signaling pathway [28]. To ascertain whether NF-κB signaling pathway was involved in the process of inducing MSCs senescence by serum exosomes, the level of p-p65 was increased in BM-MSCs treated with SLE exosomes. Immunofluorescence staining showed that SLE exosomes treatment was sufficient to increase p65 translocating to nucleus. Hence, we could conclude that serum exosomes from SLE patients enhanced the senescence of MSCs by activating NF-κB signaling pathway.

The previous study provided data that endothelial cells could release exosomes containing miR-214, which could inhibit senescence and promote angiogenesis in target cell [29]. miR-146a is an immune related miRNA, participating in cell differentiation and proliferation, cell immune response, and release of inflammatory mediators [15, 18]. In the majority of many researches, miR-146a acted as a negative regulator of inflammatory signaling pathways. There was evidence showing that chronic, systemic, low-grade inflammation, including IL-1 and TNF-α, contributed to the development of aging [30]. Previous research showed that miR-146a was involved in the senescence of human fibroblasts in IL-1 dependent manner [31]. The level of miR-146a was obviously downregulated in the PBMCs of SLE patients, and it could restrain the expression and secretion of IL-1β, IL-6, IL-8, and TNF-α [32]. It negatively regulated NF-κB signaling pathway and inflammatory reaction activation through targeting TRAF6 [33]. In this study, we observed that serum exosomal miR-146a was decreased in 10 SLE patients in a miRNA level. To further evaluate the role of miR-146a in regulating MSCs senescence, we performed SA-β-gal assay. miR-146a mimics could reverse the SLE MSCs senescence, suggesting that serum exosomal miR-146a regulated MSCs senescence. Furthermore, TRAF6/NF-κB signaling pathway was indicated as the main functional targets of miR-146a in mediating BM-MSCs senescence phenotype.

In summary, this study was the first to report that circulating exosomes derived-miR-146a from SLE patients could promote senescence of MSCs via TRAF6/NF-κB signaling pathway. Targeted-MSCs senescence might improve the transplantation efficacy of BM-MSCs in SLE patients. However, this study has some limitations; we cannot conclude that the effect of serum inducing MSCs senescence is definitively due to exosome uptake, because the remaining portion of the SLE serum might also promote MSCs senescence. The widespread method of exosomes extraction from serum is not perfect, because the remaining supernatant after isolation is the mixture of serum and exosome isolation reagent or diluted serum. In addition, the transfection of miR-146a directly into isolated exosomes needs to be further performed, and the negative effects of exosomal miR-146a mediated MSCs senescence need to be further investigated in the animal model in future study.

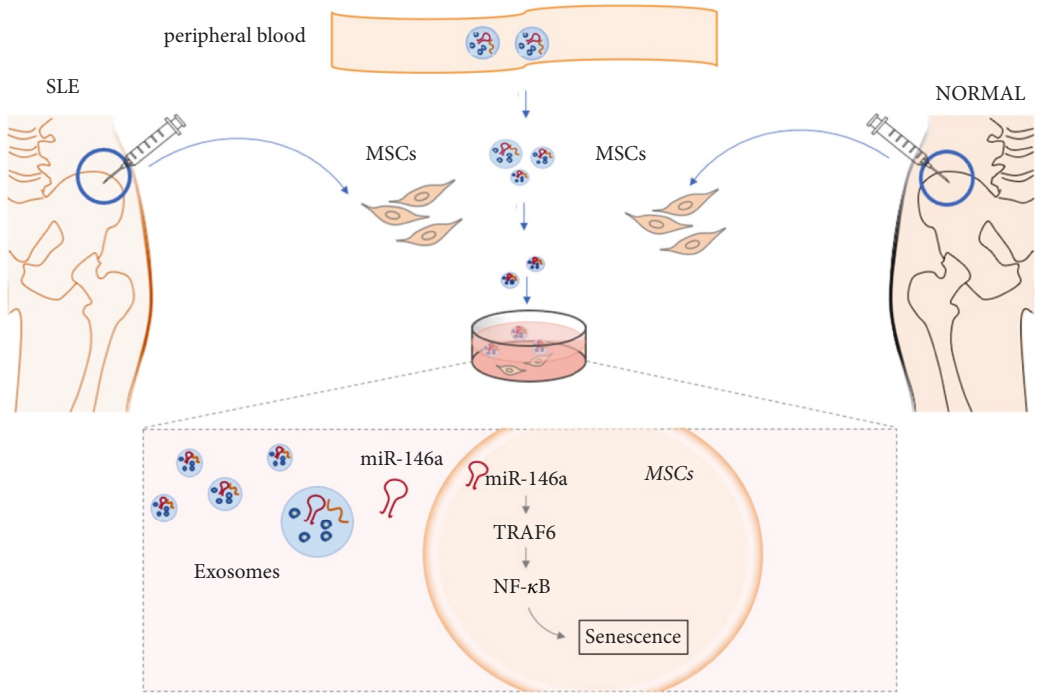

Figure 5: *The schematic mechanism of exosomal miR-146a from SLE patients regulating senescence of MSCs through targeting TRAF6/NF-κB signaling.* BM-MSCs were isolated from bone marrow of healthy donors and SLE patients. Serum-derived exosomes were purified by ExoQuick method and cocultured with BM-MSCs for 24h; then we analyzed the senescent status of BM-MSCs, including SA-β-gal staining, the F-actin distribution, and the expression levels of cell cycle-related proteins. miR-146a levels in SLE exosomes significantly declined in comparison with NOR group. miR-146a might negatively regulate MSCs senescence by suppressing TRAF6/NF-κB signaling pathway activation.

5. Conclusion

Our study demonstrated that circulating exosomes from SLE patients could induce senescence in MSC in vitro and this effect could be partially explained by the loss of miR-146a content.

Data Availability

The data used to support findings of the research are available from the corresponding author upon request.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors' Contributions

Chen Dong, Qiao Zhou, and Ting Fu have equal contribution.

Acknowledgments

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Review Article

Multiple Roles of Exosomal Long Noncoding RNAs in Cancers

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Long noncoding RNAs (lncRNAs) are not transcriptional noise, as previously understood, but are currently considered to be multifunctional. Exosomes are derived from the internal multivesicular compartment and are extracellular vesicles (EVs) with diameters of 30–100 nm. Exosomes play significant roles in the intercellular exchange of information and material. Exosomal lncRNAs may be promising biomarkers for cancer diagnosis and potential targets for cancer therapies, since they are increasingly understood to be involved in tumorigenesis, tumor angiogenesis, and chemoresistance. This review mainly focuses on the roles of emerging exosomal lncRNAs in cancer. In addition, the biogenesis of exosomes, the functions of lncRNAs, and the mechanisms of lncRNAs in exosome-mediated cell-cell communication are also summarized.

1. Introduction

Noncoding RNAs (ncRNAs) account for the majority of transcribed RNA. Long noncoding RNAs (lncRNAs) are ncRNAs that are larger than 200 nucleotides [1, 2]. Rather than being transcriptional noise, lncRNAs regulate biological activities in a variety of ways, including transcriptional regulation, posttranscriptional regulation, translation regulation, and protein cell localization. LncRNAs are also found to play a necessary role in the progression and prognosis of tumors [3, 4]. LncRNAs have important regulatory functions in fundamental pathological and biological processes, which helps to elucidate the use of lncRNAs and their corresponding proteins or peptides for cancer diagnosis and therapy [5]. EVs play an important role in different disease processes, including renal disease [6], osteoarthritis [7], coronary artery disease [8], dermatology [9], and neurodegenerative diseases [10], leukemia [11] and even have immune-modulatory effects on pregnancy and preeclampsia [12]. In addition, EVs are closely related to endothelial damage in sickle-cell disease [13], sinusoidal obstruction syndrome [14], and essential thrombocythemia [15]. The exosome is a kind of vesicle secreted by living cells that has a diameter of 30-100 nm and a bilayer lipid membrane structure. Exosomes are widely present in biological fluids, such as peripheral blood, ascites, urine, saliva, synovial fluid, and cerebrospinal fluid, as well as bronchoalveolar lavage and breast milk [16]. Exosomes can deliver functional molecules, including lipids, proteins, and nucleic acids, to recipient cells. Exosomes participate in intercellular communication and affect various physiological and pathological functions of cells. For example, pancreatic cancer-derived exosomes are involved in the proliferation, progression, and metastasis of pancreatic cancer [17]. However, the mechanisms by which these exosomal elements affect target recipient cells have not been determined to date. Exosomal lncRNAs have been found to participate in the regulation of tumorigenesis, tumor angiogenesis, and drug resistance, which suggests that there are ample opportunities to explore the potential roles of exosomes as biomarkers in cancer therapies. This review summarizes lncRNA functions and exosome biogenesis in exosome-mediated cell-cell communication and specifically focuses on the emerging roles of exosomal lncRNA in cancer. The EVs studied in some articles reviewed have morphological features of exosomes. However, the term EVs was used in these articles since exosomes are a specific subset of vesicles with a distinctive biogenesis.

2. LncRNAs

Currently, increasing evidence suggests that lncRNAs have considerable effects on various molecular mechanisms. Prior studies have indicated that mutations of the noncoding genome are widely involved in common human diseases [18]. Regulatory DNA mutations can widely affect transcription by altering enhancer and promoter activity or chromatin states, which leads to the differential expression of lncRNAs in cancer [19]. Although once considered to be transcriptional noise, lncRNAs exhibit various functions, as illustrated in Figure 1, lncRNAs regulate mRNA selective splicing and stability [20]. Additionally, many lncRNAs regulate gene expression by recruiting chromatin modifiers to special genomic locations, similar to scaffolds [21, 22], or by isolating chromatin modifiers from their regulatory locations, similar to decoys [23]. Moreover, lncRNAs control posttranscriptional regulation by functioning as ceRNAs (competing endogenous RNAs) [24] or miRNA sponges [25]. LncRNAs can also directly interact with important signaling proteins (e.g., phosphorylation) and modulate their functions [26]. Some lncRNAs encode functional micropeptides by small open reading frames (smORFs) [27, 28]. More importantly, Pang Y found several peptides which correspond to nine transcripts annotated as ncRNAs [5]. In addition, two smORFs, which were mainly found in ncRNAs and 5' untranslated regions (UTRs), could bind several ribosomes and participate in translation. Dysregulated lncRNAs have been reported to be involved in regulating the proliferation, metastases, and recurrence of multiple cancers, including lung cancer [29], prostate cancer [30], hepatocellular cancer [31], and ovarian cancer [32].

3. Exosomes

3.1. Exosome Formation. Exosome biogenesis is observed in various cells, including immune cells, mesenchymal stem cells, neurons, epithelial cells, and endothelial cells (ECs). This process is unlike the formation of microvesicles, which are generated via outward budding at the plasma surface [33]. The underlying mechanism of exosome formation includes several steps. First, an endosome forms through the inward budding of the plasma membrane. Then, further inward budding of the limiting membrane inside the endosome leads to the formation of the multivesicular body (MVB) with a diameter of 30-100 nm, peripheral proteins, cytosolic contents, and the transmembrane, which can be merged into the invaginating membrane through the exocytosis pathway and maintained as extracellular vesicles. MVBs rich in cholesterol fuse with the plasma membrane and then release their contents into the extracellular space. Otherwise, MVBs with deficient cholesterol fuse with lysosomes, causing the degradation of vesicular contents [34]. These released vesicles are known as exosomes. MVB packing was thought to be highly conserved. However, MVB packing is now related to the endosomal sorting complexes required for transport (ESCRT) complex proteins [35]. ESCRT-0, -I, and -II are responsible for recognizing and hiding ubiquitinated membrane proteins in endosomal membranes, and ESCRT-III facilitates cutting and inward budding [36]. However, researchers have observed ESCRT-independent MVB packaging pathways [37] (Figure 2).

3.2. Exosomal Molecular Components. Exosomes contain proteins, RNAs, and DNAs [38]. According to the database [ExoCarta (http://www.exocarta.org)], 9769 proteins, 3408 mRNAs, 2838 miRNAs, and 1116 lipids have been identified in exosomes. Extracellular vesicles (EVs) are composed of a lipid bilayer with transmembrane proteins that enclose cytosolic proteins and RNAs [1]. According to the subcellular origin, EVs include microvesicles (100-1000 nm) and exosomes (30-100 nm), which are derived from the internal MVBs [3]. Employing asymmetric flow field-flow fractionation, researchers identified three exosome subgroups: large exosome vesicles (Exo-L, 90-120 nm), small exosome vesicles (Exo-S, 60-80 nm), and "exomeres" (nonmembranous nanoparticles, ~35 nm). Each subpopulation contains a unique component distribution [39]. Metabolic enzymes and hypoxia, microtubule and coagulation proteins, as well as proteins associated with specific pathways, i.e., glycolysis and mTOR signaling, are abundant in exomeres. The proteins contained in Exo-S and Exo-L are involved in endosomal functions, secretion pathways, the mitotic spindle, and IL-2/STAT5 signaling pathways. Additionally, diverse organ distribution patterns have also been observed among those three subpopulations.

3.3. Exosomal Release and Transportation. Intracellular calcium, Rab GTPases, and SNARE proteins are crucial elements in exosome release. However, the precise coordination of events involved in exosome release has not been determined [40–42]. Rab27A, Rab27B, and Rab11 were observed to participate in MVE docking at the plasma membrane and to act as mediators in exosome releases [43, 44]. Another six small GTPases are also associated with secretions (Rab2B, Rab5, Rab7, Rab9A, Rab35, and RAL) [16, 45]. SNARE proteins may participate in the fusion of MVEs with the plasma membrane to release ILVs as exosomes [46]. Ca2+ was observed to be involved in the activation of SNARE complexes in many cell types [47]. However, the precise coordination involved in this event has not been determined.

After being released into the extracellular space, extracellular exosomes can be taken up by the recipient cell membrane, thereby delivering exosomal contents into the cytoplasm. In 2007, Valadi et al. first found that exosomes can function as molecular component cargos after they cocultured HMC-1 human mast cells with exosomes isolated from MC/9 murine mast cells [48]. These researchers found that some RNAs exist in vesicles and can be translated by receptor cells. This exosome-mediated intercellular communication requires several steps: first, exosomes binding to the plasma membrane; second, surface receptor and signaling activation; third, vesicle internalization or fusing with the recipient cells [49]. This binding seems target cell-specific and may be determined by proteins enriched between the exosomal surface and the recipient cell plasma membrane [50]. Several mediators of these interactions are known,

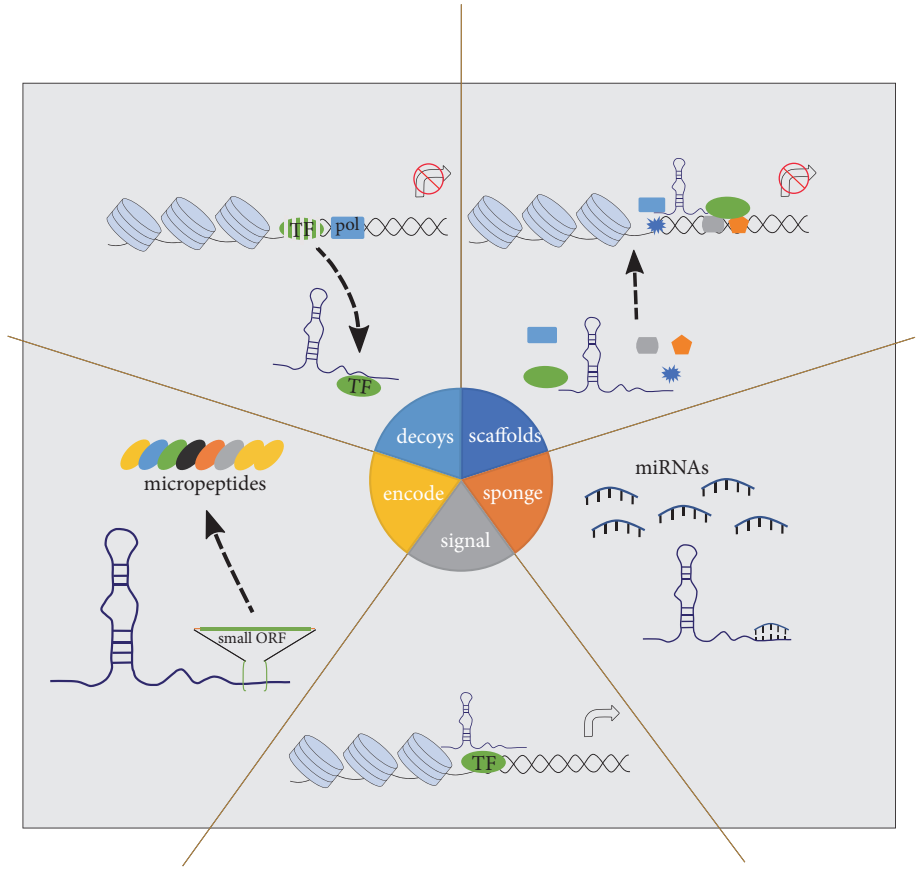

FIGURE 1: *Functions of lncRNA*. Decoys: lncRNAs act as decoys to attract transcription factors and influence protein expression [23]. Scaffolds: LncRNAs regulate gene expression by recruiting chromatin modifiers to special genomic locations acting as miRNA sponges [9, 10]. Sponge: lncRNAs can interact with miRNA, acting as "sponges" [13]. Signal: lncRNAs have a role in signal regulation [26]. Encode: lncRNAs encode functional micropeptides encoded by short open reading frames [27, 28].

including extracellular matrix, tetraspanins [51], heparin sulfate proteoglycans [52], and lectins [53].

Exosomes with different compositions may have different functions. An example of this phenomenon is that the β-amyloid protein present in exosomes derived from neuroblastoma can be specifically internalized by neurons. However, CD-63-enriched exosomes can bind both neurons and glial cells [54]. Additionally, some special structures at the target cell plasma membrane can influence exosome destiny [55]. Once bound to recipient cells, exosomes can be internalized by endocytosis, phagocytosis, or micropinocytosis [56]. After uptake by recipient cells, exosomes fuse with plasma membrane and release their contents or reach MVBs and undergo digestion by lysosomes [57], whereas some exosomes may escape digestion [58].

3.4. Roles of Exosomes in Cancers. Neighboring or distant cells can communicate through the secretion of exosomes. A variety of biological components have been detected in exosomes, such as proteins, mRNAs, and noncoding RNAs [59]. Recent studies have found that tumor-derived EVs participate in promoting antitumor immune responses, helping metastatic dissemination, creating a microenvironment [60], and assisting tumor angiogenesis [61].

4. Exosomal lncRNAs

Exosomes contain various ncRNAs, including lncRNAs. Exosomal lncRNAs can be released from cancer cells and internalized by recipient cells, which induces various effects. RNA sequencing shows that exosomal RNAs reflect the intercellular RNA compositions, which suggests that the RNAs are selectively packed into exosomes [62]. Moreover, it has been found that exosomal secretions of RNAs show discrepancies between cancer cells and normal cells [63]. In addition, researchers have observed that lncRNAs with low expression levels in cells are enriched in secreted exosomes [64]. These findings suggest that tumor cells can secrete specific lncRNA-enriched exosomes and may effectively influence recipient cells, which further affects tumorigenesis. In addition to tumorigenesis, exosomal lncRNAs also influence brain disorders [65] and cardiovascular diseases [66]. Accumulating evidence has shown that lncRNAs can be packed into vesicles and detected, which enables circulating lncRNAs to serve as biomarkers [67, 68].

4.1. LncRNAs Sorted into Exosomes. The exosomal sorting of RNAs has proven to be highly selective and exhibits cell specificity [69]. Additionally, researchers have noticed that

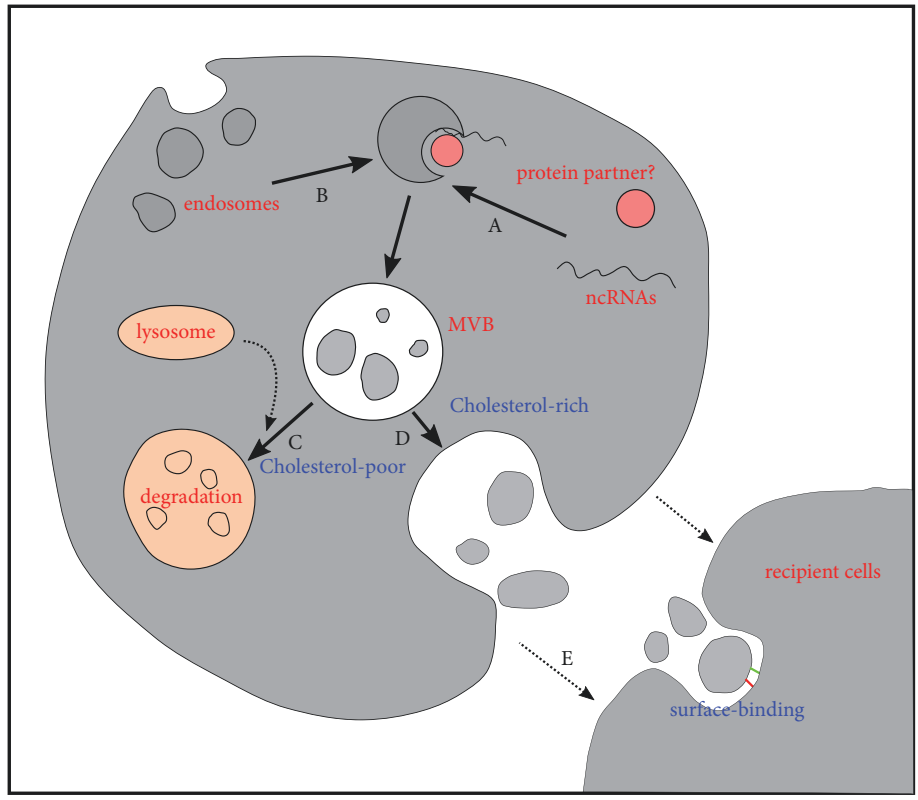

Figure 2: Exosome biogenesis and exosome-mediated delivery of ncRNAs to the recipient cell [37]. A. ncRNAs bind to packing proteins and are selectively secreted. B. Early endosomes are generated from inward budding of the plasma membrane and mature after interacting with Golgi complexes. C. Late endosomes form intraluminal vesicles (ILVs) and incorporate nucleic acids. D. MVB containing ILVs then fuse with the plasma membrane and release exosomes. E. ncRNAs are transferred within exosomes to recipient cells and affect functions.

lncRNA molecules contained in exosomes can reflect the cellular response to stimulation, such as DNA damage. These findings suggest a potential regulatory mechanism of sorting ncRNAs into exosomes. However, the mechanism behind packaging specific biological contents into exosomes is not well-understood at present. Researchers found a specific sequence (GGAG) contained in the exosomal miRNAs, which is identified as the EXOmotif and can be specifically recognized by hnRNPA1 (heterogeneous ribonucleoprotein A1) and hnRNPA2B1, thereby regulating the specific loading of such miRNAs into exosomes [70]. Recently, hnRNPA2B1 has also been found to participate in the sorting of lncRNAs into exosomes by recognizing a specific sequence [71]. Another protein, Y-box–binding protein 1 (YBX1), may also help to sort special RNAs into exosomes via binding to specific structural motifs of RNAs, such as UAAUCCCA and CAGUGAGC of lncRNAs and mRNAs [72].

5. Functions of Exosomal lncRNAs in Cancers

Exosomal lncRNAs can be used as cancer biomarkers and are strongly involved in tumorigenesis, cancer drug resistance, hypoxia signaling, and EMT. These functions of exosomal lncRNAs are listed in Table 1 according to cancer type and are described in the following subsections in detail.

5.1. Cancer Biomarker. The specific lncRNAs contained in cancer cell-derived vesicles may be the measurable and non-invasive clinic biomarkers [73]. Moreover, exosomes prevent proteins and RNAs from being degraded, which renders them intact and functional [74]. In articles published to date, exosomal lncRNAs related to cancer diagnoses and prognoses account for most items.

Serum lncRNAs are commonly used in cancer detection. LncARSR (Ensembl: ENST00000424980) is highly expressed in the plasma of renal cell carcinoma (RCC) patients. In addition, the level of plasma lncRNA-ARSR is decreased after tumor resection and elevated again upon tumor relapse. Correlations between plasma lncRNA-ARSR and progression-free survival (PFS) of RCC patients who underwent sunitinib therapy have also been observed [60]. Exosomal ZFAS1 expression levels are elevated in gastric carcinoma patients and associated with lymphatic metastasis and TNM stage [75]. In addition, with high diagnostic sensitivity and specificity (80.0% and 75.7%), exosomal ZFAS1 is a promising biomarker for gastric cancer diagnosis. Exosomal lncRNAs also exhibit the ability to serve as biomarkers for colorectal

Table 1: Overview of exosomal LncRNAs in cancers.

Cancer type	LncRNA	Source	Function	Related genes	Mechanism	Reference
Hepatocellular Carcinoma	Lnc TUC339	Cell	Tumorigenesis	None	Up or down regulation of TUC339 can effectively influence HCC cell proliferation and metastasis	[83]
	Lnc H19 ©	Cell	Tumorigenesis	None	Exosomes released by CD90+ cancer cells can affect HUVECs by promoting tube formation and cell-cell adhesion	[86]
	Lnc-ROR	Cell	Chemoresistance	None	Lnc-ROR can be selectively enriched in extracellular vesicles by TGFβ1 stimulated HCC/ HepG2 cells	[93]
	Lnc-ROR ©	Cell	Tumor cell ischemia	MiR-145–HIF-1α	Lnc-ROR can modulate intercellular responses to hypoxia via the transfer of extracellular-vesicle.	[99]
	Lnc VLDLR	Cell	Chemoresistance	None	Lnc-VLDLR can be transferred by HCC cell derived EVs and promote chemoresistance in recipient cancer cells	[95]
Lung Cancer	MALAT-1 ©	Serum	Biomarker	None	Serum exosomal MALAT-1 was positively associated with tumor stage and lymphatic metastasis	[69]
Gastric Cancer	Lnc 00152 ©	Serum	Biomarker	None	Serum exosomal lnc 00152 was significantly elevated in gastric cancer patients	[64]
	ZFAS1 ©	Serum/Cell	Biomarker/ tumorigenesis	None	ZFAS1 enriched exosomes can endow recipient cell with proliferation and migration	[75]
	HOTTIP ©	Serum	Biomarker	None	Potential biomarker for GC in diagnosis and prognosis	[101]
Colorectal Cancer	CRNDE-h ©	Serum	Biomarker	None	CRNDE-h specificity discriminates CRC patients from NC and benign disease group with high sensitivity	[77]
	Lnc-PVT1	Cell	Potential biomarker	C-Myc	Lnc-PVT1 shows higher expression in more aggressive colorectal cancer cell line	[89]
	KRTAP5-4, MAGEA3 BCAR4	Serum	Potential biomarker	None	Serum exosomal KRTAP5-4, MAGEA3 and BCAR4 provided the greatest predictive ability for colorectal cancer.	[76]
Prostate Cancer	ELAVL1 and RBMX ©	Cell	RNA binding protein binding	None	N/A	[102]
Cervical Cancer	LncRNA MALAT1, HOTAIR, MEG3 ©	Cervicovaginal lavage	Biomarker	None	RT-PCR in identify different expression lncRNA in cervicovaginal lavage	[79]
	H19	Cell/ Serum	Biomarker	Tumorigenesis	H19 promotes cell proliferation and multicellular tumor spheroid formation	[82]

Table 1: Continued.

Cancer type	LncRNA	Source	Function	Related genes	Mechanism	Reference
Ovarian cancer	Lnc-MEG3	Cell	Drug resistance	MiR-214	Enriched in curcumin treated cell/ mediated cisplatin resistance	[97]
	ENST00000444164, ENST00000437683 ©	Cell	NF-κB phosphorylation	MiR146b-5b/TRAF6/NF-κB/MMP2	Activating the phosphorylation of NF-κB in HUVECs and further affecting tumorogenesis	[87]
Colon Cancer	LncRNA AC007193.8, RUSC1-AS1, TM4SF1-AS1, DLGAP1-AS1, DLGAP1-AS1, SETD5-AS1, DNAJC27-AS1 TTC28-AS1 ©	Cell	None	None	Different lncRNAs enricher in extracellular vesicle subtypes	[103]
Glioma	Lnc-POU3F3 ©	Cell	Endothelial cell angiogenesis	BFGF, VEGFA, bFGFR, and Angio	Lnc-POU3F3 enriched exosomes may induce HBMEC migration, proliferation, and tube formation	[88]
Bladder Cancer	HOTAIR, HOX-AS-2, MALAT1, SOX2, OCT4, Lnc HYMA1, LINC00477, LOC100506688 and OTX2-AS1©	Urine	Biomarker	None	Potentially serving as biomarkers for UBC diagnosis	[80]
	Lnc-UCA1 ©	Cell	Hypoxic resistance/biomarker	HIF-1α, p27, miR-143	Hypoxic derived lnc-UCA1 enriched exosome can elevate tumorigenesis and induce cell EMT transformation	[90]
	Lnc-UCA1 ©	Cell	Drug resistance	HIF-1α, p27, miR-143	Lnc-UCA1 increases the tamoxifen resistance	[96]
laryngeal squamous cell carcinoma	HOTAIR ©	Serum	Biomarker	None	Diagnose combing serum exosomal miR-21 and HOTAIR can have achieve good sensitivity and specificity	[78]
Renal Cancer	LncARSR ©	Serum/Cell	Biomarker/ Drug resistance	HnRNPA2B1, AKT/FOXO axis, miR-34a, miR-449	LncARSR can be specifically packed into exosomes via hnRNPA2B1; LncARSR enriched exosomes can induce sunitinib sensitivity with resistance.	[60]
Cholangiocarcinoma	ENST00000588480.1, ENST00000517758.1 ©	Bile	Biomarker	None	N/A	[71]

© refers to the articles which confirmed the usage of the term exosome. Other articles used the term EVs instead although the EVs studied in these articles have morphological features of exosomes.

adenoma [76, 77], laryngeal squamous cell carcinoma [78], non-small-cell lung cancer [69], and cholangiocarcinoma [71].

In addition to serum, exosomal lncRNAs exacted from other bodily fluids were also found to be plausible biomarkers. Exosomal lncRNA MALAT1, HOTAIR, and MEG3 are differentially expressed in cervical cancer cervicovaginal lavage samples, which suggests that these lncRNAs can be promising biomarkers in detecting cervical cancer [79]. In addition, several lncRNAs (HOTAIR, HOX-AS-2, MALAT1, SOX2, OCT4, HYMA1, LINC00477, LOC100506688, and OTX2-AS1) are enriched in urine exosomes (UEs) from urothelial bladder cancer (UBC) patients [80].

Despite various reports of exosomal lncRNAs functioning as tumor biomarkers, several of these studies did not determine the sensitivity and specificity of the lncRNAs when applied to patients. In addition, many of the studies cannot define the direct relationships of the tested exosomal lncRNAs and cancers. Moreover, methodological differences in EV purification make this approach inadequate in achieving testing reproducibility.

5.2. Tumorigenesis. As mentioned earlier, the expression and function of lncRNAs are associated with various types of cancers [81]. Considering that the roles of lncRNAs in cancer are largely unexplored, research on exosomal lncRNAs is still in its infancy. Most studies investigate the roles of different lncRNAs in tumorigenesis, but they fail to demonstrate that the intercellular transfers of lncRNAs via exosomes play roles in tumorigenesis. For example, Iempridee et al. [82] found that lncRNA-H19 enhances the proliferation and spheroid forming ability of cervical cancer cells and is enriched in cell-derived EVs. Similar experiments performed by Kogure et al. show that lncRNA-TUC339 is most highly expressed in hepatocellular carcinoma cells secreting EVs. Up- or downregulation of TUC339 can effectively influence HCC cell proliferation and metastasis [83]. However, these studies did not find direct evidence to demonstrate that exosomes/lncRNAs can directly affect tumorigenesis.

Lei et al. [75] found that lncRNA-ZFAS1 enriched in exosomes can endow recipient cells (low lncRNA-ZFAS1 expression) with increased proliferation and migration ability, which suggests that ZFAS1 can be delivered by exosomes to promote gastric cancer progression.

Dysregulation of angiogenesis occurs in various pathologies and is one of the hallmarks of cancer [84]. Some studies have illustrated that cancer cell-derived exosomes can affect HUVECs in tube formation, in which exosomal lncRNAs may play a pivotal role. CD90+ hepatic cell carcinoma (HCC) has been described with cancer stem-cell-like (CSC) properties [85]. Conigliaro et al. [86] found that exosomes released by CD90+ cancer cells can affect HUVECs by promoting cell-cell adhesion and tube formation. These researchers further found that lncRNA-H19 is enriched in those exosomes. Another study performed by Wu et al. [87] first showed that exosomes isolated from tumor-associated macrophages (TAMs) can incorporate into HUVECs and block the miR146b-5b/TRAF6/NF-κB/MMP2 pathway, which results in efficient reduction of HUVEC migration. In addition, these researchers used SKOV3-derived exosomes and TAM-derived exosomes to costimulate HUVECs and found that inhibition of migration caused by TAM-derived exosomes is overcome. Two exosomal lncRNAs (ENST00000444164, ENST00000437683) were identified as NF-κB pathway-associated genes. A study conducted by Lang et al. [88] found that exosomes enriched in lncRNA-POU3F3 promote angiogenesis in gliomas. Moreover, exosomal lncRNA-POU3F3 has better function in inducing human brain microvascular endothelial cell (HBMEC) migration, proliferation, tube formation, and elevated angio-related gene expression. These results suggest that lncRNAs carried by exosomes can partly influence angiogenesis and further affect tumorigenesis.

5.3. Hypoxia Signaling and EMT. Hypoxia in cancer pathology is considered to be a significant element. Tumor cells frequently utilize hypoxia signaling to maintain the proliferative response in normoxia and escape growth arrest in hypoxia [89]. Takahashi et al. first revealed that lncRNA-ROR is a hypoxia-responsive lncRNA and can promote the survival of cancer cells under ischemic conditions. More importantly, these researchers found that lncRNA-ROR can modulate intercellular responses to hypoxia via the transfer of extracellular vesicles. In addition, hypoxia signaling often stimulates a cellular epithelial-mesenchymal transition (EMT) process, which is a critical regulator of metastasis. Several exosomal lncRNAs have been shown to affect EMT signaling in cancer cells. Xue et al. [90] found that UMUC2 has a positive effect on cell proliferation, migration, and invasion when incubated with hypoxic 5637 cell-derived exosomes. Moreover, compared to the normoxic cell-derived exosomes, lncRNA-UCA1 is enriched in hypoxic cell-derived exosomes. These hypoxia-derived lncRNA-UCA1-enriched exosomes can elevate tumorigenesis, both *in vivo* and *in vitro*, and induce cell EMT transformation. Transforming growth factor (TGF)-β can promote epithelial-mesenchymal transition (EMT) and further induce invasion and metastasis in pancreatic cancer [91].

5.4. Drug Resistance. LncRNAs can be transported by exosomes and endow the recipient cells with acquired drug resistance. Some studies have demonstrated that lncRNAs have potential functions in delivering drug resistance in recipient cells. TGF-1 has been shown to be involved in obtaining chemoresistance in various human cancers [92]. The groups of Takahashi found that lncRNA-ROR and lncRNA-VLDLR can be selectively enriched in EVs by TGFβ1-stimulated HCC [93]. HCC-derived exosomes can endow HepG2 cells with increased lncRNA-ROR expression and high chemoresistance. Additionally, these researchers found that lncRNA-ROR knockdown can reverse TGFβ-induced chemoresistance in cancer stem-cell-like CD133+ cells [94]. Another study performed by this team also revealed that lncRNA-VLDLR increases in cells and their EVs under chemotherapeutic stress [95]. These researchers found that lncRNA-VLDLR can be transferred by HCC cell-derived EVs and can promote chemoresistance in recipient cancer cells. Xu et al. [96] found that lncRNA-UCA1 shows

high expression in both tamoxifen-resistant LCC2 cells and their derived exosomes. LCC2-derived exosomes facilitate the breast cell line MCF-7 with an increased ability to resist tamoxifen. Moreover, knocking down UCA1 in exo/LCC reverses this phenomenon.

The above studies have proven that exosomal lncRNAs may function in drug resistance; however, they fail to reveal the underlying mechanism of acquired drug resistance related to exosomal lncRNAs. Other articles may better explain the roles of exosomal RNAs in drug resistance. Zhang et al. [97] demonstrated that curcumin-treated cell-derived EVs can reduce the ability of A2780cp cells to induce chemoresistance. LncRNA-MEG3 showed the greatest upregulation in exosomes after curcumin treatment. MEG3 overexpression after curcumin treatment can clearly inhibit miR-214 expression in cells and EVs. These researchers proved that MEG3 can strengthen EV-mediated transfer of miR-214, thereby downregulating drug resistance in recipient cells. These researchers found direct evidence proving that lncRNA-ARSR can be secreted from sunitinib-resistant cells to sensitive cells and induce sunitinib resistance. Intracellular lncRNA-ARSR elevation is directly due to exosome fusion, rather than an increase in intracellular synthesis. LncRNA-ARSR elevation caused by exosomal delivery functions as competing endogenous RNA for miR-449 and miR-34 to facilitate AXL and c-MET expression, which further affects sunitinib resistance.

6. Conclusion

In general, exosomes are secreted in almost all types of cells. Exosomes can selectively carry various elements and function as cell-to-cell carriers. LncRNAs secreted by exosomes also play an essential role in cancers. Liquid biopsy through exosomal lncRNAs provides a novel method for diagnosing cancer. Additionally, extracellular lncRNAs packed by exosomes help us evaluate the prognoses and therapeutic effects of the cancers. Moreover, exosomal lncRNAs have been determined to participate in inducing drug resistance in recipient cells, which provides a potential method of cancer therapy. Despite significant progress made in recent years, more work is needed to achieve a better understanding of exosomal lncRNAs in the function and regulation of tumorigenesis.

7. Perspective

LncRNAs have shown their utility in the diagnosis and prognosis of some cancers. Unlike commonly used cell-free DNAs (cfDNAs), which originate from dying cells, exosomal nucleic acids (exoNAs), which are derived from living cells, can better reflect the underlying cancer biology [98]. Recently, researchers have presented a novel EGFR T790M assay based on exosomal cfDNAs and RNAs/DNAs from plasma and achieved 92% sensitivity and 89% specificity [99]. However, the use of lncRNAs as biomarkers for cancer diagnosis and prognosis remains limited. First, different methods of isolation, mainly ultracentrifugation-based isolation and exosome precipitation techniques, were used in the aforementioned studies. The methodological differences in exosome isolation and lncRNA extraction make the experimental results difficult to compare. Second, only a small number of lncRNAs have already been investigated, and many of them have been functionally characterized. The construction of an extravascular lncRNA database has greater potential for the study of exosomes.

Moreover, as the natural transporter of functional small RNAs and proteins, exosomes have been suggested to have potential applications in the drug delivery field. It has been demonstrated that specific lncRNAs enriched in exosomes can change the phenotypes of neighboring cells [100]. Moreover, lncRNAs delivered by exosomes can induce drug resistance and angiogenesis in recipient cells. In the field of other exosomal RNAs, researchers have found that MSC-derived exosomes inhibit breast cancer growth by downregulating vascular endothelial growth factor (VEGF) and transferring miR-16 in mice [101]. Additionally, in the field of lncRNAs, intercellular transfer of lncRNA-ARSR through exosomes can significantly dampen the response of RCC xenografts to sunitinib, with increased lncRNA-ARSR expression being observed in tumors. A phase II trial has recently evaluated IFNγ-DC-derived exosomes loaded with MHC I/II confined cancer antigens as maintenance immunotherapy after chemotherapy in advanced patients without tumor progression, and exosomes may be used as anticancer vaccines in the future. However, the modulation of lncRNAs *in vivo* is not easy to achieve; therefore, there have been no lncRNA drugs brought into clinical trials to date.

Conflicts of Interest

The authors have no conflicts of interest.

Authors' Contributions

Wenyuan Zhao and Yuanqi Liu contributed equally to this work and should be considered co-first authors.

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Review Article

Potential Role of Exosomes in Cancer Metastasis

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High cancer mortality is attributed to metastasis to a large extent. However, cancer metastasis remains devoid of dynamic monitoring and early prevention in terms of current advances in diagnostic means and therapeutic modalities. Meanwhile, studies have shown that reciprocal crosstalk among cells via exosomes plays a critical role in maintaining normal physiological state or triggering disease progression, including cancer metastasis. Therefore, in this review, we focus on the latest literature (primarily from 2018) to summarize action mechanisms and experimental studies of exosomes in cancer metastasis and put forward some problems as well as new outlooks of these studies.

1. Introduction

Cancer is responsible for approximately 1 out of every 6 deaths and is the second-leading cause of death (following cardiovascular diseases) worldwide [1]. Meanwhile, metastases as well as their treatment consequences are the leading causes for cancer death [2]. Cancer statistics in 2019 from the American Cancer Society show the following estimates: the largest number of cancer deaths will be attributed to lung, prostate, and colorectal cancer in men. In women, lung, breast, and colorectal cancer will be largest. Moreover, the mortality of lung cancer will account for 25% of cancer deaths in 2019 [3].

Despite advances in cancer therapy, including chemoradiotherapy, immunotherapy, and molecular targeted treatment, there has yet to be satisfactory clinical outcome for patients within cancer metastasis [2, 4]. In addition, most new therapeutic strategies were developed according to their anticancer activity against tumorigenesis and primary growth, rather than their antimetastatic activity. Preclinical evidence and further clinical therapy applications of agents with antimetastatic activity are still lacking [4]. Therefore, it will be very important to develop specifically antimetastatic drug for clinical application. This will require researchers to focus their efforts on the mechanisms of cancer metastasis.

Cancer metastasis refers to the process of primary tumor cells arriving to other sites of the body, proliferating there and finally forming new tumors. It includes four main stages: intravasation (from primary tumor sites to blood vessels), extravasation (from blood circulation to future metastasis sites), tumor latency, and formation of micrometastasis and macrometastasis. The process of metastasis is modulated by epithelial-mesenchymal transition (EMT) and the reverse (MET), extracellular matrix (ECM) remodeling, activity of immune system, characteristics alteration of tumor cells, reprogramming of microenvironment cells (fibroblasts, macrophages, endothelial cells, etc.), and recruitment of bone marrow-derived cells (BMDC), such as mesenchymal stem cells (MSC) [5, 6]. In addition, the organ specificity of metastasis has gradually been unveiled by the "seed" and "soil" theory of Paget and studies of Isaiah Fidler [5]. Another intriguing finding is that organs targeted for metastasis can be altered to become suitable for tumor colonization before the arrival of cancer cells, that is, by formation of a premetastatic niche [6, 7].

Further studies have shown that exosomes play a vital role in cancer metastasis, namely, contributing in forming the premetastatic niche, influencing tumor cells and microenvironment, and determining specific organotropic metastasis [2, 4, 7]. Exosomes are formed by the inward budding of early endosomes to produce multivesicular endosomes and their fusion with cell plasma membranes [8]. They belong to the so-called extracellular vesicles (EVs) which generally include three types: apoptotic bodies, cellular microparticles

/ microvesicles / ectosomes, and exosomes [9]. Comparisons among the three types are shown in Table S1 of Supplementary Material [8–15]. Exosomes can transfer nucleic acids, proteins, and lipids from parent cells to recipient cells in three ways including surface receptor binding, membrane fusion with target cells, or vesicle internalization, then influencing the cell functional state [8].

Therefore, in this review, we will discuss the study of the influence of exosomes in cancer metastasis, which may provide new horizon for monitoring cancer progression, finding new therapeutic targets and realizing early intervention on metastasis.

2. Exosomes in Cancer Metastasis

Exosomes, serving as a cell complement, function mainly via monitoring the specific organotropism of primary tumor cells, and altering the microenvironment of targeted organs and primary tumor organs. They influence the function of tumor cells, and they change the efficacy of chemotherapy, thereby possibly functioning as dynamic monitoring biomarkers and therapeutic targets for cancer metastasis.

2.1. Role of Exosomes in Organ-Specific Targeting. The pioneering study from group of Prof. Layden [16] has demonstrated that exosomal integrins (ITGs) play an important role in organ-specific metastasis and colonization of tumor cells in distant sites. Their main ideas include the following. (i) tumor-derived exosomal ITGs determine the metastatic sites of the primary tumor cells; namely, exosomal ITG$\alpha_6\beta_4$ and -$\alpha_6\beta_1$ are associated with lung metastasis, while ITG$\alpha_v\beta_5$ is associated with liver metastasis, and ITGβ_3 is associated with brain metastasis. (ii) These ITGs mediate the interaction of exosomes and specific resident cells of the targeted organ, namely, lung-tropic tumor-derived exosomes and lung fibroblasts and epithelial cells, liver-tropic tumor-derived exosomes and liver Kupffer cells, brain-tropic tumor-derived exosomes, and brain endothelial cells. (iii) The above interactions depend on exosomal ITGs selectively adhering to the ECM associated with specific resident cells, including laminin of lung microenvironments and fibronectin of liver microenvironments, respectively. (vi) Exosomal ITGs regulate the function of targeted cells by activating proto-oncogene tyrosine-protein kinase Src (Src) and increasing the expression of S100 (a family of genes whose symbols use the S100 prefix) gene to promote migration and inflammation. (v) Exosomal ITG content is positively associated with cancer progression. Another report from the above group has shown that pancreatic ductal adenocarcinomas (PDAC) cells-derived exosomes play a part in determining liver-tropic metastasis. These exosomes transfer migration inhibitory factor (MIF) to Kupffer cells. Thus Kupffer cells secret more transforming growth factor beta (TGF-β) and promote the production of fibronectin by hepatic stellate cells. Subsequently, the accumulation of fibronectin is advantageous in recruiting bone marrow-derived macrophages and forming the premetastatic niche [17]. Moreover, exosomal ITG$\alpha_2\beta$ is also correlated with brain-tropic metastasis, while exosomal ITG$\alpha_4\beta_1$ and -$\alpha_v\beta_3$ promote the metastasis to bone, and exosomal ITGα_4 is related to lymph node (LN) metastasis [18]. Figure 1 summarizes the above content.

2.2. Influence of Exosomes in Altering the Tumor Microenvironment. Tumor cells-derived and microenvironment cells-derived exosomes modify the microenvironment of the primary tumor and make targeted organ suitable for tumor progression (Table 1).

2.2.1. Tumor Cells-Derived Exosomes. The tumor cells-derived exosomes transfer some crucial miRNAs, lncRNAs, and proteins to the cancer microenvironment cells, mainly containing epithelial cells, macrophages, endothelial cells, and fibroblasts. This contributes to inflammatory cell infiltration, angiogenesis, obtainment of tumor-associated cell phenotypes, and tumor innervation.

The binding of RNA to toll-like receptor (TLR) of epithelial cells or macrophages can induce tumor microenvironment inflammatory phenotypes. Liu et al. [19] have shown that exosomal small nuclear RNAs (snRNAs) of Lewis lung carcinoma (LLC) or B16/F10 melanoma cells activate TLR3 of alveolar epithelial cells and then promote chemokine release which recruits neutrophils to the lung microenvironment. Furthermore, these exosomal RNAs promote the metastasis progression by influencing the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) and mitogen-activated protein kinase (MAPK) pathways. In addition, it is reported that colorectal cancer (CRC) cells-derived exosomal miR-21 activates TLR7 in cytoplasm of liver macrophages. This activation results in proinflammatory phenotype transformation of macrophages with increasing expression of interleukin (IL)-6, S100 calcium-binding protein A (S100A), and matrix metalloproteinases (MMPs). Meanwhile, by a positive feedback, the above upregulated IL-6 can stimulate the expression of miR-21 mediated by signal transducer and activator of transcription 3 (STAT3) [20, 21].

The crosstalk between cancer cells and endothelial cells facilitates angiogenesis. Epithelial ovarian cancer (EOC) cells-derived exosomes enhance proangiogenic properties of human umbilical vein endothelial cells (HUVECs) via metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) trafficking which may stimulate the expression of vascular endothelial growth factor (VEGF)-A, VEGF-D, epithelial-derived neutrophil-activating protein 78 (ENA-78), placental growth factor (PlGF), IL-8, angiogenin, basic fibroblast growth factor (bFGF), and leptin in HUVECs [22]. In addition, exosomal miR-25-3p from CRC cells can be internalized by HUVECs, which gives rise to decreasing expression of Krüppel-like factor 2 (KLF2) and KLF4 with the respective functions of inhibiting angiogenesis and maintaining the integrity of endothelial barrier [23]. Pessolano et al. have studied the role of exosomal annexin A1 (ANXA1) in pancreatic cancer via the MIA PaCa-2 model and knock-out technology of clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9). They have indicated that ANXA1 can elevate exosomes production. Moreover, exosomal ANXA1 can promote migration, invasion, and EMT of pancreatic cancer cells, as well as angiogenesis by

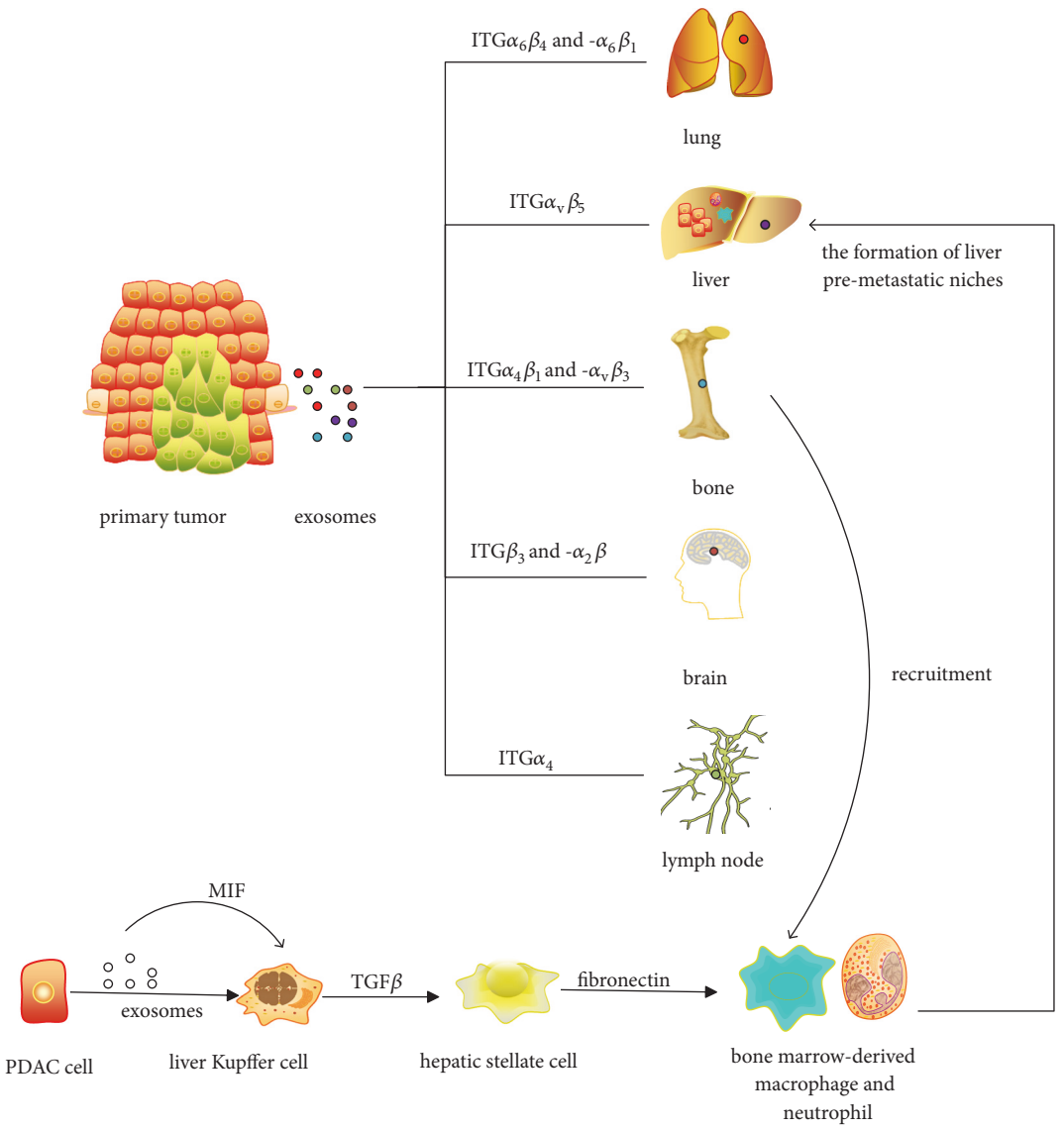


Figure 1: Role of exosomes in organ-specific targeting. Pancreatic ductal adenocarcinoma, PDAC.

interaction with HUVECs [24]. Tumor released-exosomal miR-221-3p promotes lymphangiogenesis and LN metastasis in cervical squamous cell carcinoma (CSCC) by its transmission to human lymphatic endothelial cells (HLECs), which results in the activation of miR-221-3p-vasohibin-1- (VASH1-) extracellular signal-regulated kinase (ERK)/serine/threonine-protein kinase Akt (AKT) signal axis [25].

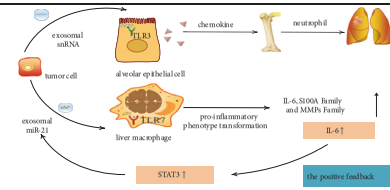
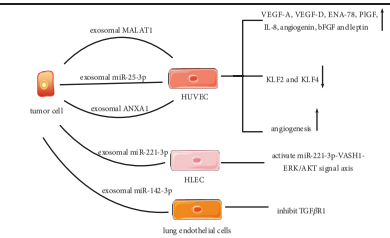
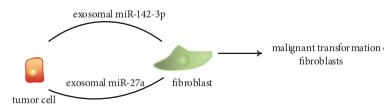
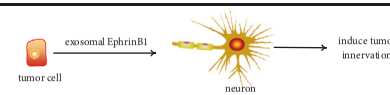
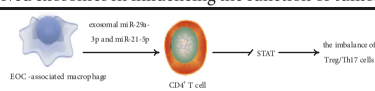
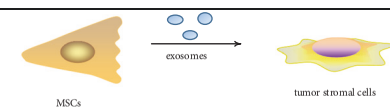
Exosomes communicating with fibroblasts also trigger reprogramming of recipient cells into cancer-associated phenotypes. These exosomes released from lung adenocarcinoma cells (LAC) transfer miR-142-3p to lung endothelial cells and fibroblasts, which promotes angiogenesis mediated by inhibiting TGFβR1 in endothelial cells and induces fibroblasts tumor-associated phenotypes but may be irrelevant to TGFβ signaling pathway [26]. Wang et al. have demonstrated that exosomal miR-27a from gastric cancer cells are also relevant to malignant transformation of fibroblasts [27].

Exosomes can also increase the nerve distribution of the microenvironment to elevate the malignant degree of tumor cells. Head and neck squamous cell carcinomas (HNSCC) released-exosomal EphrinB1 can induce tumor innervation in the PC12 neuronal model in vitro and the murine model in vivo, and patients with increased tumor innervation are prone to suffer from cancer metastasis [28].

2.2.2. Tumor Associated Microenvironment Cells-Derived Exosomes. Meanwhile, surrounding stromal cells-derived exosomes are also involved in preparing microenvironment amenable for tumor colonization.

EOC-associated macrophages transfer miR-29a-3p and miR-21-5p to $CD4^+$T cells via exosomes, which synergistically inhibits the activity of STAT3 and causes the imbalance of regulatory T cells (Treg)/helper T cell 17 (Th17). This contributes to form an immune-suppressive microenvironment [29].

Table 1: Influence of exosomes in altering the tumor microenvironment.

Donor cells	Recipient cells	Mechanisms of action	Effects	Ref.
The role of tumor cells-derived exosomes in influencing the function of tumor microenvironment cells				
LLC or B16/F10 melanoma cells CRC cells	Alveolar epithelial cells Liver macrophages		Promote ECM remodeling, the formation of inflammatory tumor microenvironment and pre-metastatic niche	[19] [20, 21]
EOC cells CRC cells Pancreatic cancer cells CSCC cells LAC cells	Umbilical vein endothelial cells (HUVECs) Lymphatic endothelial cells (HLECs) Lung endothelial cells		Contribute to angiogenesis	[22] [23] [24] [25] [26]
LAC cells Gastric cancer cells	Fibroblasts Fibroblasts		Promote the cancer-associated phenotype transformation of fibroblasts	[26] [27]
HNSCC cells	neuronal models		Increase the nerve distribution of tumor microenvironment	[28]
The role of tumor microenvironment cells-derived exosomes in influencing the function of tumor microenvironment cells				
EOC-associated macrophages	$CD4^+$ T cells		Form an immune-suppressive microenvironment	[29]
MSCs	tumor stromal cells		Affect angiogenesis, immune response, migration and invasion of tumor	[30, 31]

Note: Lewis lung carcinoma, LLC; Colorectal cancer, CRC; Epithelial ovarian cancer, EOC; Cervical squamous cell carcinoma, CSCC; Lung adenocarcinoma, LAC; Head and neck squamous cell carcinoma, HNSCC; Mesenchymal stem cell, MSC; Human umbilical vein endothelial cell, HUVEC; Human lymphatic endothelial cell, HLEC; ↑, Upregulated or activated; ↓, Downregulated or inhibited; ⟶, Inhibited.

Table 2: Involvement of exosomes in influencing the function of tumor cells.

Cancer type	Donor cells	Recipient cells	Study molecule	Signal axis	Effect	Ref.
The role of tumor cells-derived exosomes in influencing tumor cells						
Melanoma	Tumor cells	Tumor cells	RAB27A		Migration and invasion↑	[32]
Lung cancer	Tumor cells	Tumor cells	lnc-MMP2-2	lnc-MMP2-2⟶MMP2↑	Migration and invasion↑	[33]
CRC	Hypoxic tumor cells	Normoxic tumor cells	HIF1A	HIF1A⟶Wnt4-activated β-catenin signaling pathway↑	Migration and invasion↑	[34]
PDAC	Tumor cells	Tumor cells	miR-222	miR-222⟶p27↓	Proliferation, invasion and migration↑	[35]
Breast cancer	Tumor cells	Tumor cells	CAV1		Migration and invasion↑	[36]
Breast cancer	Exosomes from plasma of healthy donor(the exception of study mode)	Tumor cells	surface proteins	surface proteins⟶FAK signaling pathway↑	Adhesive ability and migration↑	[37]
The role of microenvironment cells-derived exosomes in influencing tumor cells						
CRC	Tumor associated M2 macrophages	Tumor cells	miR-21-5p and miR-155-5p	miR-21-5p and miR-155-5p ⟶ BRG1↓	Migration and invasion↑	[38]
OSCC	CAFs	Tumor cells	miR-34a-5p	miR-34a-5p⟶AXL↓⟶AKT/GSK-3β/β-catenin signaling pathway↑	Proliferation, EMT and metastasis↑	[39]
HCC	CSCs	Tumor cells	exosomal molecules	exosomal molecules⟶Bax and p53↓, Bcl2↑; VEGF↑; P13K, ERK and MMP9↑, TIMP1↓; TGFβ1↑	Tumor progression↑	[40]
	BM-MSCs		exosomal molecules	contrary to the above expression changes	Tumor progression↓	

Note: Colorectal cancer, CRC, Pancreatic ductal adenocarcinoma, PDAC; Oral squamous cell carcinoma, OSCC; Cancer-associated fibroblast, CAF; Hepatocellular carcinoma, HCC; Cancer stem cell, CSC; Bone marrow-mesenchymal stem cell, BM-MSC; ↑, Upregulated or activated; ↓, Downregulated or inhibited.

MSCs play dual roles-stimulative or inhibitory in tumor progression by the interaction of MSC-derived exosomes and tumor microenvironment cells, which affects angiogenesis, immune response, migration, and invasion of tumors [30, 31].

2.3. Involvement of Exosomes in Influencing the Functions of Tumor Cells. Tumor cells- and microenvironment cells-derived exosomes commonly act on changing the proliferation activity, migration, invasion, and further distant metastasis of tumor cells (Table 2).

2.3.1. Tumor Cells-Derived Exosomes. Tumor cells-released exosomes affect activities of tumor cells via autocrine and paracrine processes.

Ras-related protein Rab-27A (RAB27A) is upregulated in melanomas compared with normal skin or nevi and is related to the advanced stage of melanomas for patients. Exosomes enriched with RAB27A can rescue the invasion phenotype of the melanoma cells after the knockdown of RAB27A, which reveals that exosomes promote melanoma metastasis by changing the ability of invasion and motility of surrounding melanoma cells [32]. Exosomal lnc-matrix metalloproteinase 2-2 (lnc-MMP2-2) mediated by TGF-β upregulates the expression of MMP2 in lung cancer cells by its enhancer activity, which leads to increasing migration and invasion of tumor cells via the increasing vascular permeability [33]. Hypoxic CRC cells-derived exosomes promote the migration and invasion of normoxic CRC cells via protein Wnt-4- (Wnt4-) activated β-catenin signaling pathway, and the function depends on the hypoxia-inducible factor 1-alpha (HIF1A) expression of hypoxic cells. Upregulated HIF1A increases Wnt4 expression in hypoxic CRC cells and their released exosomes [34]. In PDAC, exosomal miR-222 transmission to cancer cells is functional to promote proliferation, invasion, and migration through two ways: (i) decreasing cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) (p27) expression levels directly; (ii) activating AKT by inhibition of serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform (PPP2R2A), which increases p27 phosphorylation and cytoplasmic p27 expression coupled with reduced nucleus expression [35]. Breast cancer cells-derived exosomal caveolin-1 (CAV1) can facilitate migration and invasion of cells with knockout of CAV1 in vitro. CAV1 is positively associated with cancer stages, which may suggest that exosomal CAV1 transferred to recipient cells promotes cancer metastasis in vivo [36].

In addition, there is a distinct model for studying exosomes function. When most studies focus on tumor-derived exosomes, Shtam et al. pay attention to exosomes from plasma of healthy donor. They have found that these exosomes can increase adhesive ability of breast cancer cells in vitro and migratory activities in Zebrafish model, which is dependent on the interaction of exosomal surface proteins and breast cancer cells, and the activation of focal adhesion kinase (FAK) signaling pathway [37].

2.3.2. Tumor Associated Microenvironment Cells-Derived Exosomes. When tumor cells-derived exosomes modify diverse tumor associated microenvironment cells, in turn, these cells release exosomes acting on the functions of tumor cells.

For CRC metastasis, exosomes derived from tumor associated M2 macrophage transfer miR-21-5p and miR-155-5p to CRC cells, which results in downregulated expression of transcription activator BRG1 (BRG1) and enhanced migration and invasion of cancer cells [38]. In oral squamous cell carcinoma (OSCC), cancer-associated fibroblasts-(CAFs-) secreted exosomes deliver miR-34a-5p to cancer cells. Then miR-34a-5p activates AKT/glycogen synthase kinase-3 beta (GSK-3β)/β-catenin signaling pathway via the inhibition of tyrosine-protein kinase receptor AXL (AXL), which causes increased nuclear location of β-catenin and further upregulated expression of zinc finger transcription factor SNAIL (SNAIL) as well as MMP-2 and MMP-9. This finally plays an essential role in accelerating proliferation, EMT, and metastasis of cancer cells [39]. By the application of diethylnitrosamine- (DEN-) inducing long-term animal models of hepatocellular carcinoma (HCC), Alzahrani et al. have found that hepatic cancer stem cells- (CSCs-) derived exosomes function as protumor factors while bone marrow-mesenchymal stem cells (BM-MSCs) released-exosomes play an inhibitory role in tumor progression. These exosomal molecules influence apoptosis, angiogenesis, metastasis, and invasiveness as well as EMT of tumor cells via altering the expression of targeted molecules. These molecules include apoptosis regulator BAX (Bax), cellular tumor antigen p53 (p53), apoptosis regulator Bcl-2 (Bcl2), VEGF, phosphoinositide 3-kinase (P13K), extracellular signal-regulated kinase (ERK), MMP9, tissue inhibitor of metalloproteinases 1 (TIMP1), and TGFβ1 [40].

2.4. Influence of Exosomes in Changing the Efficacy of Chemotherapy. Exosomes can transfer resistance to chemotherapy via two different ways (Figure 2): (i) the tumor induces chemotherapy resistance and, reversely, (ii) chemotherapy also promotes drug resistance.

A recent study shows that in hypoxic tumor microenvironment of EOC, tumor associated macrophages- (TAMs-) derived exosomes induce chemotherapy resistance of tumor cells via delivering miR-223 and activating miR-223/ phosphatase and tensin homolog- (PTEN-) PI3K/AKT signaling pathway [50]. In turn, chemotherapy may promote cancer metastasis. Keklikoglou et al. have demonstrated that in the breast cancer model, chemotherapy promotes the formation of lung premetastatic niche by increased release of tumor-derived EVs. These chemotherapy-stimulated EVs function as the prometastatic factor by transferring annexin A6 (ANXA6) to lung endothelial cells and then activating NF-KB signaling pathways, which causes C-C motif chemokine 2 (CCL2) upregulation, lymphocyte antigen 6C positive and C-C chemokine receptor type 2 positive ($Ly6C^+$ $CCR2^+$) monocyte accumulation, and tumor cells colonization in lung [51].

2.5. Exosomes as Potential Biomarkers of Cancer Metastasis. Some studies focus on difference analysis based on different molecular components to select exosomal biomarkers, which sets the stage for in-depth mechanism investigation (Table 3).

2.5.1. Exosomal RNAs. Exosomal miR-140-3p, miR-30d-5p, miR-29b-3p, miR-130b-3p, miR-330-5p, and miR-296-3p are associated with the migration ability of hepatocarcinoma cells by the comparison analysis of exosomal miRNAs profile in fast- and slow-migrating groups of patient-derived liver cells (PDLCs). The migration ability is assessed by the wound closure percentage of wound healing assay [41]. Serum exosomal miRNA-21 and lncRNA activated by tumor growth factor-beta (lncRNA-ATB) levels in HCC patients are positively related to tumor progression [42]. miR-9 and miR-155 levels are higher in metastatic breast cancer-derived exosomes and the two miRNAs downregulate the expression of PTEN and dual specificity protein phosphatase 14 (DUSP14) in recipient cells [43]. In castration-resistant prostate cancer (CRPC), the high level of plasma exosomal miR-1290 and miR-375 is connected with poor prognosis of patients [44]. Moreover, the study of Cannistraci et al. has indicated that the expression of exosomal tyrosine-protein kinase Met (Met)/miR-130b axis in serum is related to the risk that patients with prostate cancer become resistant to castration therapy and suffer from metastasis [45]. In serum and urine of urothelial carcinoma of the bladder (UCB) patients, exosomal protein arginine N-methyltransferase 5 circular RNA (circPRMT5) levels are upregulated and associated with metastasis. The binding of circPRMT5 to miR-30c inhibits the function of miR-30c. Therefore circPRMT5 boosts EMT of UCB cells via increasing expression of SNAIL1 and reducing expression of E-cadherin, the downstream target of SNAIL1 [46].

2.5.2. Exosomal Proteins. Wang et al. have shown that the level of CD82 antigen (CD82) in exosomes is negatively correlated with that in tissue for breast cancer patients, and the content of serum exosomal CD82 is higher in cancer group than that in the benign group and healthy control group. CD82 expression in serum exosomes is also positively correlated with cancer clinical stage. Therefore, there may be a redistribution of CD82 from tissue to serum exosomes, which reflects tumorigenesis and progression of breast cancer [47]. Ohshima K et al. have indicated that exosomal epidermal growth factor receptor pathway substrate 8 (Eps8) protein content is higher in metastatic cells-derived exosomes by the comparative proteome analysis of exosomes, which are purified from human pancreatic cancer cell lines with distinct stages [48]. For CRC patients with lung metastasis, studies have revealed that C-X-C chemokine receptor type 7 (CXCR7) and C-X-C motif chemokine ligand

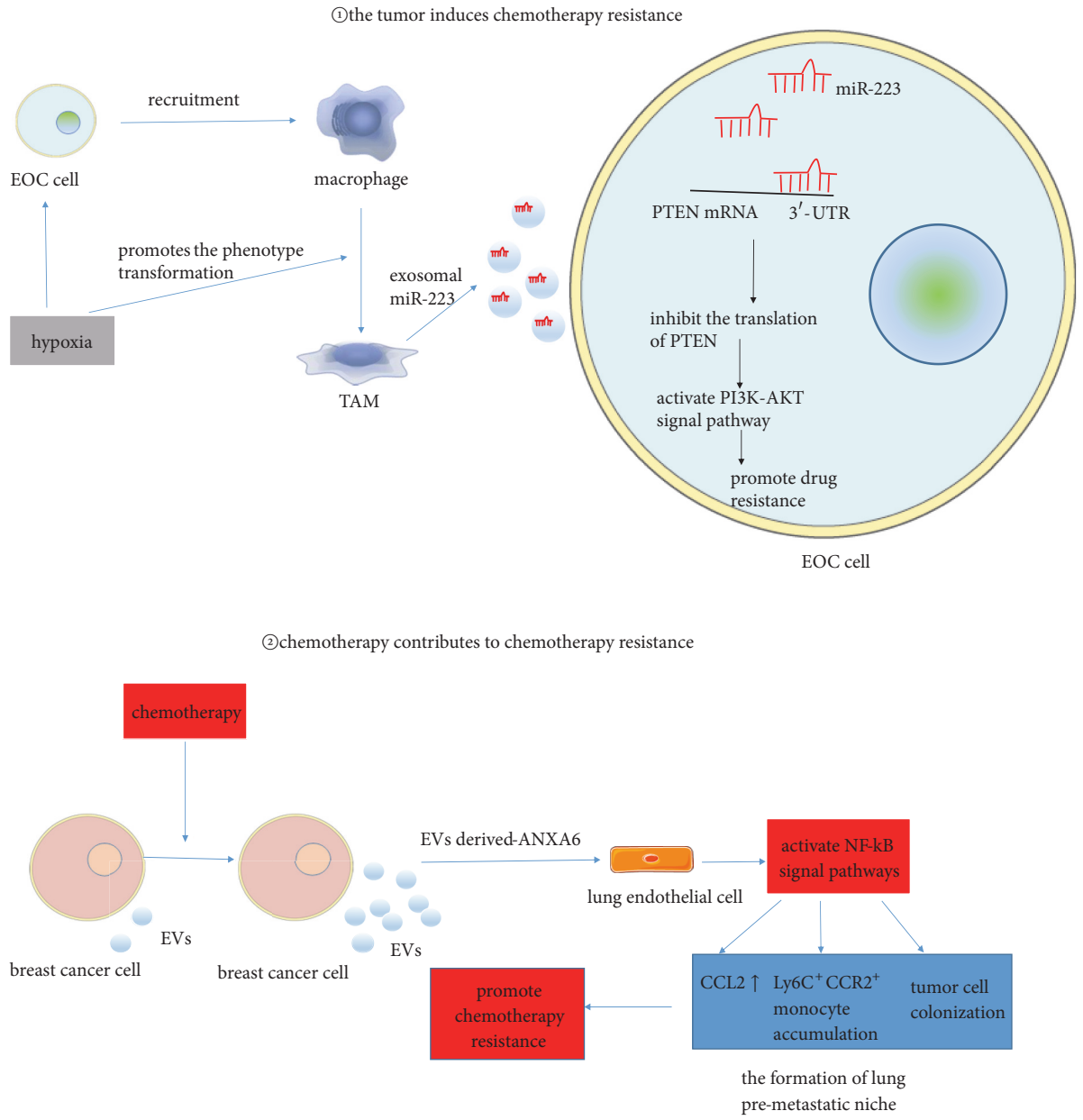


Figure 2: Influence of exosomes in changing the efficacy of chemotherapy. Tumor associated macrophage, TAM; epithelial ovarian cancer, EOC; ↑, upregulated.

12 (CXCL12) expression is significantly higher in metastatic site than in primary lesion, and CXCL12 expression is higher in nontumor lung tissue of patients with CRC than in control lung tissue with benign lesion. In addition, after injection of exosomes isolated from CRC cell line (CT26) into BALB/c female mice, CXCL12 expression is increased in lung tissue before cancer metastasis. Based on the above finding, the authors have stated that CRC cells-derived exosomes elevate CXCL12 expression levels in lung before metastasis [49].

The multidirectional communications of tumor cells and tumor associated microenvironment cells via the trafficking of exosomes facilitate the enhancement of malignant phenotypes of tumor cells, promote the formation of premetastatic niche, and finally exhibit clinically detectable metastasis.

In view of the important involvement of exosomes in cancer metastasis, more in-depth studies of exosomes are expected to shed more light on its biogenesis, release, and relevant functions. However, these exosome results may be questionable, due to the lack of standard isolation and characterization methods. Another disturbing factor is the fact that other EV types are likely interfering with the analysis of exosomes [9]. Indeed, the used methods currently based on size, protein composition, and morphology are not sufficient to completely separate one type of EVs from the others [8]. As is shown in Table S1, the overlap of size range occurs among the three main types of EVs. Moreover, the size range is slightly inconsistent in the literature possibly due to the various cell origin and different isolation methods among

Table 3: Potential exosomal biomarkers of cancer metastasis.

	Potential biomarkers	Comparison analysis	Ref.
Exosomal RNAs	miR-140-3p, miR-30d-5p, miR-29b-3p, miR-130b-3p, miR-330-5p, miR-296-3p	Exosomes derived from fast- and slow-migrating groups of PDLCs	[41]
	miRNA-21 and lncRNA-ATB	Serum exosomes isolated from patients with different HCC stages	[42]
	miR-9 and miR-155	Exosomes derived from breast cells with different metastatic ability	[43]
	miR-1290 and miR-375	Plasma exosomes derived from CRPC patients with different prognosis	[44]
	miR-130b and Met	Serum exosomes isolated from prostate cancer patients and healthy donors	[45]
	circPRMT5	Serum and urine exosomes from normal people and patients with UCB	[46]
Exosomal proteins	CD82	Exosomes derived from tissue, serum, and plasma in breast cancer patients	[47]
	Eps8	Exosomes purified from human pancreatic cancer cell lines with distinct stages	[48]
	CXCR7 and CXCL12	Exosomes isolated from tissues of primary tumor, lung metastasis, and benign lung disease in CRC patients	[49]

Note: Patient-derived liver cell, PDLC; Hepatocellular carcinoma, HCC; Castration-resistant prostate cancer, CRPC; Urothelial carcinoma of the bladder, UCB; Colorectal cancer, CRC.

laboratories. Therefore, more standard and specific isolation and characterization methods are required for exosomes, in order to be suitable for clinical application. We refer the readers to a recent review including methodological classification, detection principle, and new technological methods for analyzing EVs [52].

Moreover, microvesicles as one of the EV types also gave rise to much attention in the cancer field. The prostate cancer cells-derived large oncosomes (a new class of shedded vesicles) are endocytosed by fibroblasts, which activates Myc proto-oncogene protein (MYC) of recipient cells via active AKT1, giving these fibroblasts a protumor phenotype [53]. Bertolini et al. have demonstrated that glioma stem cells-derived large oncosomes deliver homeobox genes and V-ATPase subunit to tumor cells and nontumor cells, which facilitates their malignant transformation [54, 55]. Therefore, the intricate identities and functions of the different EVs warrant further investigation.

3. Open Questions about the Influence of Exosomes on Metastasis

(a) Are Exosomes Still Playing a Role during Tumor Latency or after Primary Tumor Resection? During tumor latency, there are both quiescent single cells and micrometastasis. Duration of the dormant state differs in different cancers [5]. It has been well documented that metastasis sometimes still occurs after primary tumor resection.

A further question arises as to what stimulates these dormant cells into active states and promotes metastasis without a primary tumor. The contributor may be partially remaining exosomes derived from these seemingly stationary tumor cells in predetermined metastasis sites.

To demonstrate this hypothesis, it might be necessary to monitor exosomes alteration in blood of patients without detectable metastasis and then conduct long-term tracking of exosomal biomarkers for patients after tumor resection.

(b) What Causes the Difference of Exosomal Biomarker Levels in Serum and Plasma? Exosomal CD82 content in serum is different from that in plasma. Serum exosomal CD82 content in the malignant group is higher than that in the benign group and in the healthy group. However, the content difference between the above groups for plasma exosomes has no statistical significance; therefore serum exosome CD82 is proposed as the biomarker for breast cancer [47].

The study reminds us that detection of exosomal biomarkers in blood is dependent on selection of an appropriate specimen. Serum or plasma may give differing diagnostic test values. We need to further investigate the origin of these observed differences for a better prognosis monitoring.

(c) What Are the Mechanisms Governing the Specific Exosomal Cargo Targeting between Tumor- and Recipient Cells Which Contribute to Inconsistent Expression of Exosomal Inclusions in Blood and Tissue? The levels of miR-486-5p are down-regulated in CRC tissue while upregulated in plasma of patients [56]. Therefore, we can postulate that redistribution of miR-485-5p from tissues to exosomes gives rise to partial expression difference between tissue and blood. Low levels of miR-486-5p in tumor cells might consequently influence cell function.

Under the above speculation, exosomes are putative molecular transporters modifying their levels both in tumor cells and in recipient cells. They further alter the state of the two kinds of cells, being either beneficial or obstructive for

tumor progression. Deciphering this important question is only in its infancy.

4. Conclusion

It can be expected that more specific therapeutic targets for cancer metastasis will be developed following these studies. Some research has already demonstrated that tumor cells are inhibited by reducing the production of some exosomes, by interfering with their encapsulated content before or after its packaging, as well as by modifying exosomes as drug carriers [57, 58].

Abbreviations

List 1 (Abbreviations of Cancer Cells, Microenvironment Cells, Cell Components, Organs, and Biological Processes)

BMDC: Bone marrow-derived cell
BM-MSC: Bone marrow-mesenchymal stem cell
CAF: Cancer-associated fibroblast
CRC: Colorectal cancer
CRPC: Castration-resistant prostate cancer
CSC: Cancer stem cell
CSCC: Cervical squamous cell carcinoma
ECM: Extracellular matrix
EMT: Epithelial-mesenchymal transition
EOC: Epithelial ovarian cancer
EV: Extracellular vesicle
HCC: Hepatocellular carcinoma
HLEC: Human lymphatic endothelial cell
HNSCC: Head and neck squamous cell carcinoma
HUVEC: Human umbilical vein endothelial cell
LAC: Lung adenocarcinoma cell
LLC: Lewis lung carcinoma
LN: Lymph node
MET: Mesenchymal-epithelial transition
MSC: Mesenchymal stem cell
OSCC: Oral squamous cell carcinoma
PDAC: Pancreatic ductal adenocarcinoma
PDLC: Patient-derived liver cell
TAM: Tumor associated macrophage
Th17: Helper T cell 17
Treg: Regulatory T cell

List 2 (Abbreviations of Different Molecular Components)

AKT: Serine/threonine-protein kinase Akt
ANXA1: Annexin A1
ANXA6: Annexin A6
AXL: Tyrosine-protein kinase receptor AXL
Bax: Apoptosis regulator BAX
Bcl2: Apoptosis regulator Bcl-2
bFGF: Basic fibroblast growth factor
BRG1: Transcription activator BRG1
CAV1: Caveolin-1
CCL2: C-C motif chemokine ligand 2
$CCR2^+$: C-C chemokine receptor type 2 positive
CD82: CD82 antigen
CircPRMT5: Protein arginine N-methyltransferase 5 circular RNA
CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9
CXCL12: C-X-C motif chemokine ligand 12
CXCR7: C-X-C chemokine receptor type 7
DEN: Diethylnitrosamine
DUSP14: Dual specificity protein phosphatase 14
ENA-78: Epithelial-derived neutrophil-activating protein 78
Eps8: Epidermal growth factor receptor pathway substrate 8
ERK: Extracellular signal-regulated kinase
FAK: Focal adhesion kinase
GSK-3β: Glycogen synthase kinase-3 beta
HIF1A: Hypoxia-inducible factor 1-alpha
IL: Interleukin
ITG: Integrin
KLF: Krüppel-like factor
lnc-MMP2-2: Lnc-matrix metalloproteinase 2-2
lncRNA-ATB: LncRNA-activated by tumor growth factor-beta
$Ly6C^+$: Lymphocyte antigen 6C positive
MALAT1: Metastasis-associated lung adenocarcinoma transcript 1
MAPK: Mitogen-activated protein kinase
Met: Tyrosine-protein kinase Met or Hepatocyte growth factor receptor
MIF: Migration inhibitory factor
MMP: Matrix metalloproteinase
MMP2: Matrix metalloproteinase 2
MYC: Myc proto-oncogene protein
NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells
p27: Cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$)
p53: Cellular tumor antigen p53
PI3K: Phosphoinositide 3-kinase
PIGF: Placental growth factor
PPP2R2A: Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform
PTEN: Phosphatase and tensin homolog
RAB27A: Ras-related protein Rab-27A
S100: A family of genes whose symbols use the S100 prefix; the "S100" symbol prefix is derived from the fact that these proteins are soluble in 100% ammonium sulfate at neutral pH
S100A: S100 calcium-binding protein A
SNAIL: Zinc finger transcription factor SNAIL
snRNA: Small nuclear RNA
Src: Proto-oncogene tyrosine-protein kinase Src
STAT3: Signal transducer and activator of transcription 3
TGFβ: Transforming growth factor beta

TIMP1: Tissue inhibitor of metalloproteinases
TLR: Toll-like receptor
VASH1: Vasohibin-1
VEGF: Vascular endothelial growth factor
Wnt4: Protein Wnt-4.

Additional Points

MIA PaCa-2. The cell line was established by A. Yunis et al. in 1975 from tumor tissue of the pancreas obtained from a 65-year-old Caucasian male. The information is obtained via ATCC website (https://www.atcc.org/). *Tumor Associated M2 Macrophage*. Macrophages generally consist of the two types: M1- and M2 macrophages. Studies have shown that M2 macrophages are more likely to promote tumor progression.

Conflicts of Interest

The authors declare no conflicts of interest

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Supplementary Materials

Table S1: Difference among the three main types of EVs. *(Supplementary Materials)*

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Research Article

Expression Profiling of Exosomal miRNAs Derived from the Peripheral Blood of Kidney Recipients with DGF Using High-Throughput Sequencing

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Delayed graft function (DGF) is one of the major obstacles for graft survival for kidney recipients. It is profound to reduce the incidence of DGF for maintaining long-term graft survival. However, the molecular regulation of DGF is still not adequately explained and the biomarkers for DGF are limited. Exosomes are cell-derived membrane vesicles, contents of which are stable and could be delivered into recipient cells to exert their biological functions. Consequently, exosome-derived proteomic and RNA signature profiles are often used to account for the molecular regulation of diseases or reflect the conditional state of their tissue as biomarkers. Few researches have been done to demonstrate the function of exosomes associated with DGF. In this study, high-throughput sequencing was used to explore the miRNA expression profiling of exosomes in the peripheral blood of kidney recipients with DGF. We identified 52 known and 5 conserved exosomal miRNAs specifically expressed in recipients with DGF. Three coexpressed miRNAs, hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p, were observed to be significantly upregulated in kidney recipients with DGF. Moreover, hsa-miR-151a-5p was positively correlated with the first-week serum CR, BUN, and UA levels of the kidney recipients after transplantation. Furthermore, we also analyzed functions and signaling pathways of the three upregulated miRNAs target genes to uncover putative mechanism of how these exosomal miRNAs functioned in DGF. Overall, these findings identified biomarker candidates for DGF and provided new insights into the important role of the exosomal miRNAs regulation in DGF.

1. Introduction

Delayed graft function (DGF) defined as the dialysis requirement in the first week after transplantation is a manifestation of acute renal failure [1]. DGF occurs in as many as 2%-50% of the immediate post-kidney-transplant cases and is a major obstacle for graft survival [2]. A meta-analysis of 34 studies from 1988 through 2007 demonstrated a 49% incidence of acute rejection for patients with DGF compared to 35% incidence for non-DGF patients [1]. In addition, DGF was associated with a 41% and 53% increase in allograft dysfunction and death for patients with DGF, respectively [1, 3]. Thus, it is profound to reduce the incidence of DGF for maintaining long-term graft survival. DGF is commonly considered as a consequence of kidney tubular damage due to ischemia and reperfusion injury (IRI). Moreover, recent studies suggest that the generation of cytotoxic mediators and activation of innate and adaptive immune response could be also correlated to DGF [4]. However, the molecular regulation of DGF is still not adequately explained and the biomarkers for DGF are limited.

Exosomes are cell-derived membrane vesicles (40-100 nm of diameter) present in fluids such as blood, urine, amniotic fluid, breast milk, platelets, synovial fluid, bronchoalveolar lavage fluid, and malignant ascites [5–7]. Exosomes are reported to play a key role in many processes such as cellular activities regulation, intercellular communication, and waste management [6, 8]. Various cell products including protein, DNA, mRNA, and miRNA could be carried by exosomes

[9–11]. The contents of exosomes are stable and could be delivered into recipient cells to exert their biological functions; consequently, exosome-derived proteomic and RNA signature profiles are often used to account for the molecular regulation of diseases or reflect the conditional state of their tissue as biomarkers [12–14]. Evidence is accumulating that exosomal contents are also involved in the rejection of transplantation. $CD4^+$ $CD25^+$ regulatory T cells-derived exosomes could prolong kidney allograft survival in a rat model [15]. Upregulation of exosomal miR-142-3p was also observed during cardiac allograft rejection and it could augment vascular permeability through downregulating the expression of endothelial RAB11FIP2 [16]. In addition, two exosomal proteins (TSPAN1 and HPX) were observed to be significantly higher in patients with acute T cell mediated rejection than in patients without rejection, while exosomal mRNAs transcripts (gp130, CCL4, TNFα, CAV1, DARC, and SH2D1B) were helpful to distinguish antibody-mediated rejection patients from cellular rejection recipients [17, 18]. However, few researches have been done to demonstrate the function of exosomes associated with DGF occurring in kidney transplantation.

Therefore, high-throughput sequencing was used to explore the miRNA expression profiling of exosomes in the peripheral blood of kidney recipients with or without DGF and elucidate the regulation of miRNAs in the process of DGF. In this study, we identified a series of known, conserved, and novel exosomal miRNAs in the case of DGF and found that hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p were significantly upregulated in kidney recipients with DGF. Moreover, hsa-miR-33a-5p_R-1 and hsa-miR-151a-5p were positively correlated with the first-week serum CR and BUN levels of the kidney recipients after transplantation, indicating that these two exosomal miRNAs had the potential to be used as biomarkers of DFG in kidney recipients. Furthermore, we also analyzed functions and signaling pathways of the three upregulated miRNAs target genes to uncover putative mechanism of how these exosomal miRNAs function in DGF occurring in kidney transplantation.

2. Materials and Methods

2.1. Patients and Samples. A total of 9 patients who received donation after cardiac death (DCD) kidney grafts in Provincial People's Hospital of Henan Province were involved in this study. The recipients were divided into two groups: the DGF group and the control group based on whether they needed more than one dialysis within the first week after transplantation or not [19, 20]. Their first-week clinical data after kidney transplantation were collected from Provincial People's Hospital of Henan Province. Clinical data from 5 patients without kidney transplantation were also analyzed as normal controls.

2.2. Exosome Isolation. One week after kidney transplantation, exosomes were isolated and purified from the peripheral blood of recipients using the exoEasy Maxi Kit (QIAGEN). Firstly, the blood samples were centrifuged for 10 min at 3000 rpm and 4°C to remove cells and large debris. The upper plasma phase was carefully collected and filtered with a 0.22 μm filter to remove additional cellular fragments. Then larger protein complexes abundant in blood could be removed during the binding and washing steps as follows: (1) mix the filtered sample with XBP buffer (1:1) at room temperature (RT), add the mix onto the exoEasy spin column, centrifuge at 500 × g for 1 min, and discard the flow-through and place the column back into the same collection tube; (2) add 10 mL XWP buffer, centrifuge at 5000 × g for 5 min, and discard the flow-through together with the collection tube. Finally, transfer the spin column to a fresh collection tube, add 400 μl XE buffer to the membrane and incubate for 1 min, and centrifuge at 5000 × g for 5 min to collect exosomes in the eluate.

2.3. Nanoparticle Tracking Analysis and Transmission Electron Microscopy. The concentration and size distribution of the isolated exosomes were detected by ZetaView (Particle Metrix, Germany) and analyzed using the in-build nanoparticle tracking analysis (NTA) software ZetaView 8.02.31. A 20 μL aliquot that contained about 4-5 × 10^6 particles was fixed with 1% glutaraldehyde and loaded onto a carbon-coated grid for 2 min at room temperature. The grid was negatively stained with 2% aqueous phosphotungstic acid for 1 min and then imaged under a transmission electron microscope (JEM-1400, Japan).

2.4. Western Blot. Exosomal lysates were prepared using RIPA buffer with protease inhibitor mixture (Bi Yuntian, China), and protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Scientific, USA). About 20 μg of exosomal lysates from each sample in both DGF and control groups was loaded onto SDS-polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (0.45 μm pore size, Millipore, USA). The blots were blocked in 5% non-fat dry milk for 1 h at room temperature. After blocking, the membranes were incubated overnight at 4°C with primary rabbit anti-CD63 antibody (1:1000; ab68418, Abcam, USA), rabbit anti-CD9 antibody (1:1000; ab92726, Abcam, USA), or rabbit anti-β-actin antibody (1:1000; ab8227, Abcam, USA). After being washed by PBST, the membranes were incubated with HRP conjugated anti-rabbit IgG (1:20,000; Thermo Scientific, USA) for 1 h at 37°C. Subsequently, blots were developed using Lumi-Light Western Blotting Substrate (Roche, Switzerland), imaged, and quantified using ChemiDoc MP imaging system (Bio-Rad, USA).

2.5. RNA Extraction. Total RNAs from exosomes were isolated using the mirVana miRNA Isolation Kit (Ambion, USA) according to the manufacturer's protocol. The RNA quality and concentration were assessed with the OD ratio 260/280 using a Nanodrop 1000 (Thermo Scientific, USA).

2.6. miRNA Libraries Construction and Sequencing. TruSeq MiRNA Sample Prep Kit (Illumina, USA) was used to construct miRNA sequencing libraries according to the manufacturer's protocol. The 3′- and 5′-adapter were ligated

Table 1: Basic information of the kidney recipients.

Patient ID	Height (cm)	Weight (kg)	Age	Gender	Type of transplantation	Induction therapy	Maintenance immunosuppressive drugs
DGF 1	178	78	52	Male	DCD	Thymoglobuline	Tac+MMF+Pred
DGF 2	170	72	48	Male	DCD	Thymoglobuline	Tac+MMF+Pred
DGF 3	171	62	51	Male	DCD	Thymoglobuline	Tac+MMF+Pred
DGF 4	163	50	24	Male	DCD	Thymoglobuline	Tac+MMF+Pred
Ctrl 1	169	67.5	38	Male	DCD	Thymoglobuline	Tac+MMF+Pred
Ctrl 2	180	71	32	Male	DCD	Thymoglobuline	Tac+MMF+Pred
Ctrl 3	160	42	40	Female	DCD	Thymoglobuline	Tac+MMF+Pred
Ctrl 4	168	55	40	Male	DCD	Thymoglobuline	Tac+MMF+Pred
Ctrl 5	155	48	29	Female	DCD	Thymoglobuline	Tac+MMF+Pred
Ntr 1	173	83	51	Male	-	-	-
Ntr 2	172	72	44	Male	-	-	-
Ntr 3	160	69	37	Female	-	-	-
Ntr 4	166	64	32	Male	-	-	-
Ntr 5	173	70	26	Male	-	-	-

DGF: delayed graft function; Ctrl: control; Ntr: normal control (patients without kidney transplantation); DCD: donation after cardiac death; Tac: tacrolimus; MMF: mycophenolate mofetil; Pred: prednisone.

to approximately 1 μg of total RNA from each sample using T4 RNA ligase (Epicentre, USA) at 28°C for 1 h, respectively. The adapter-ligated miRNAs were dissolved in RNase free water and used as templates for RT-PCR to create and amplify the cDNA constructs. Then the PCR-amplified cDNA products from the 6% TBE PAGE gels were extracted and then purified. After the completed libraries were evaluated with a quantitative real-time PCR to ensure acceptable quality and confirm that adapters were correctly added, the high-throughput sequencing of the cDNA was done on the HiSeq 2500 (Illumina, USA). Image analysis and base calling were performed with the ACGT101-miR software (LC Sciences, USA).

2.7. miRNA Target Prediction, Functional Annotation, and Pathway Enrichment Analysis. Two algorithms, miRanda and TargetScan, were used to predict the target genes of exosomal miRNAs which were differentially expressed between DGF and control groups [21, 22]. Only the miRNA targets identified by both miRanda and TargetScan algorithms composed the final predicted targets. Subsequently, the gene ontology terms (http://www.geneontology.org/) and KEGG pathway terms (http://www.genome.jp/kegg) enriched in predicted target genes were determined to explore the function and related pathway of the targets [23].

2.8. Ethics Statement. Written informed consent was obtained from all patients. This study was approved by the Protection of Human Subjects Committee, Provincial People's Hospital of Henan Province.

2.9. Statistical Analysis of Data. Statistical assessment was performed using SPSS 19.0 for Windows. Firstly, we conducted the Shapiro-Wilk normality test to assess whether continuous data have been drawn from a normally distributed population. Since all continuous data were normally distributed, they were assessed by one-way ANOVA or t-test. Categorical data were evaluated by nonparametric test. Pearson correlation was used for correlation analysis miRNA expressions and the first-week serum CR or BUN levels from the kidney recipients. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Basic Information and Clinical Data of Kidney Recipients with DGF. Nine patients who received donation after cardiac death (DCD) kidney grafts were involved in this study. Immunosuppressive treatment with mycophenolate mofetil, prednisone and tacrolimus, and induction therapy with thymoglobuline were given to the recipients after transplantation for preventing acute rejection. The basic information of these kidney recipients was shown in Table 1. One week after transplantation, the blood and urine samples from the recipients were collected and then their serum creatinine (CR), blood urea nitrogen (BUN), uric acid (UA), hemoglobin (HGB), albumin (ALB), platelet (PLT), and urine protein (Pro) levels were detected. We divided the recipients into two groups: those with delayed graft function (DGF, n = 4) and those with immediate graft function, considered as the control group (n = 5), and found that the CR levels, BUN levels, and UA levels of the recipients in DGF group were significantly higher than those of the recipients in control group, respectively (CR: $P = 0.0002$; BUN: $P < 0.0001$; UA: $P = 0.0120$; Table 2). Clinical data from patients without kidney transplantation (n = 5; basic information shown in Table 1) were also analyzed as normal controls. When compared with patients without kidney transplantation, recipients in DGF group exhibited significantly higher levels of CR and BUN (CR: $P = 0.0002$; BUN: $P < 0.0001$; Table 2), whereas clinical data of kidney recipients in control group and patients without kidney transplantation were similar (Table 2).

3.2. Characterization of Exosomes in Peripheral Blood of Recipients after Kidney Transplantation. Based on the unique

Table 2: First-week clinical data of the kidney recipients after transplantation.

Variable	DGF	Ctrl	Ntr	*P1*	*P2*	*P3*
CR (μmol/L)	565.30 ± 224.00	74.40 ± 14.67	71.60 ± 14.55	0.0002	0.0002	0.9992
BUN (mmol/L)	33.70 ± 8.47	10.18 ± 2.16	6.16 ± 1.14	<0.0001	<0.0001	0.3914
UA (μmol/L)	496.00 ± 129.40	233.00 ± 118.30	332.80 ± 85.66	0.0120	0.1167	0.3640
HGB (g/L)	91.50 ± 13.80	109.80 ± 22.53	130.20 ± 25.34	0.4459	0.0535	0.3336
ALB (g/L)	36.15 ± 1.97	38.96 ± 4.50	43.30 ± 4.46	0.5576	0.0506	0.2364
PLT ($\times 10^9$/L)	157.80 ± 58.59	122.00 ± 53.37	202.60 ± 43.35	0.5718	0.4253	0.0734
Pro, -/+/++/+++	1/0/1/2	3/0/1/1	5/0/0/0	0.7890[a]	0.0735[a]	0.6924[a]

Data were expressed as mean antibody concentration ± SD. *P1*, *P2*, and *P3* values referred to the comparison of the patients between DGF group and Ctrl group, DGF group and Ntr group, and DGF group and Ntr group, respectively. [a]Nonparametric test. DGF: delayed graft function; Ctrl: control; Ntr: normal control (patients without kidney transplantation); CR: serum creatinine; BUN: blood urea nitrogen; UA: uric acid; HGB: hemoglobin; ALB: albumin; PLT: platelet; Pro: urine protein.

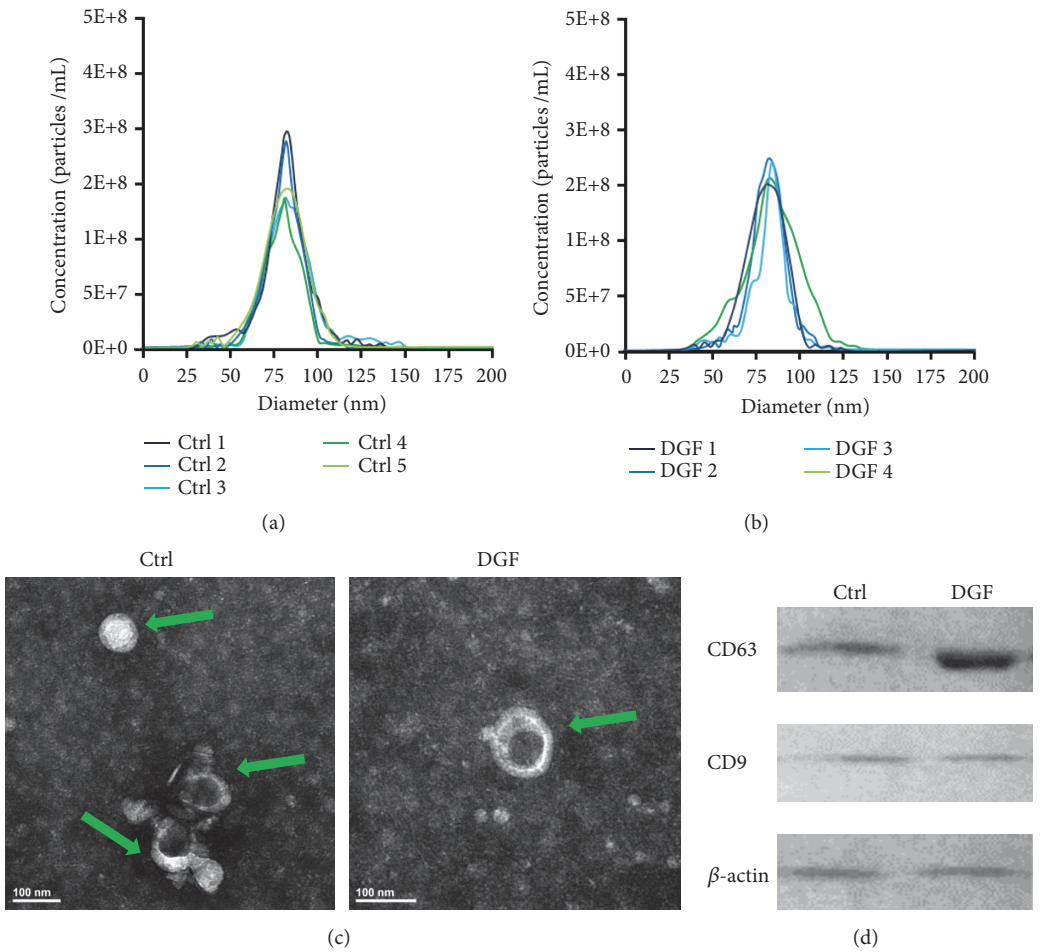

Figure 1: *Identification of exosomes through nanoparticle tracking analysis, transmission electron microscopy, and Western blot.* (a) Concentration and size of exosomes in control (a) and DGF (b) groups were analyzed by the nanoparticle tracking analysis. (c) Exosomes isolated from the peripheral blood of recipients one week after kidney transplantation have a saucer-like shape that was limited by a lipid bilayer. Green arrowheads point to exosomes. Scale bar = 100 nm. (d) Exosomes are positive for CD63 and CD9, as determined through Western blot analysis. β-Actin was used as a positive control.

size and density, exosomes were isolated from the peripheral blood of recipients in both DGF and control groups one week after kidney transplantation using the exoEasy Maxi Kit (QIAGEN). Then the exosomes were identified through nanoparticle tracking analysis, transmission electron microscopy, and Western blot. The results showed that small vesicles with diameters ranging from approximately 60 to 90 nm and a lipid bilayer could be observed under the transmission electron microscopy (Figures 1(a), 1(b), and 1(c)). Moreover, Western blot analysis confirmed presence

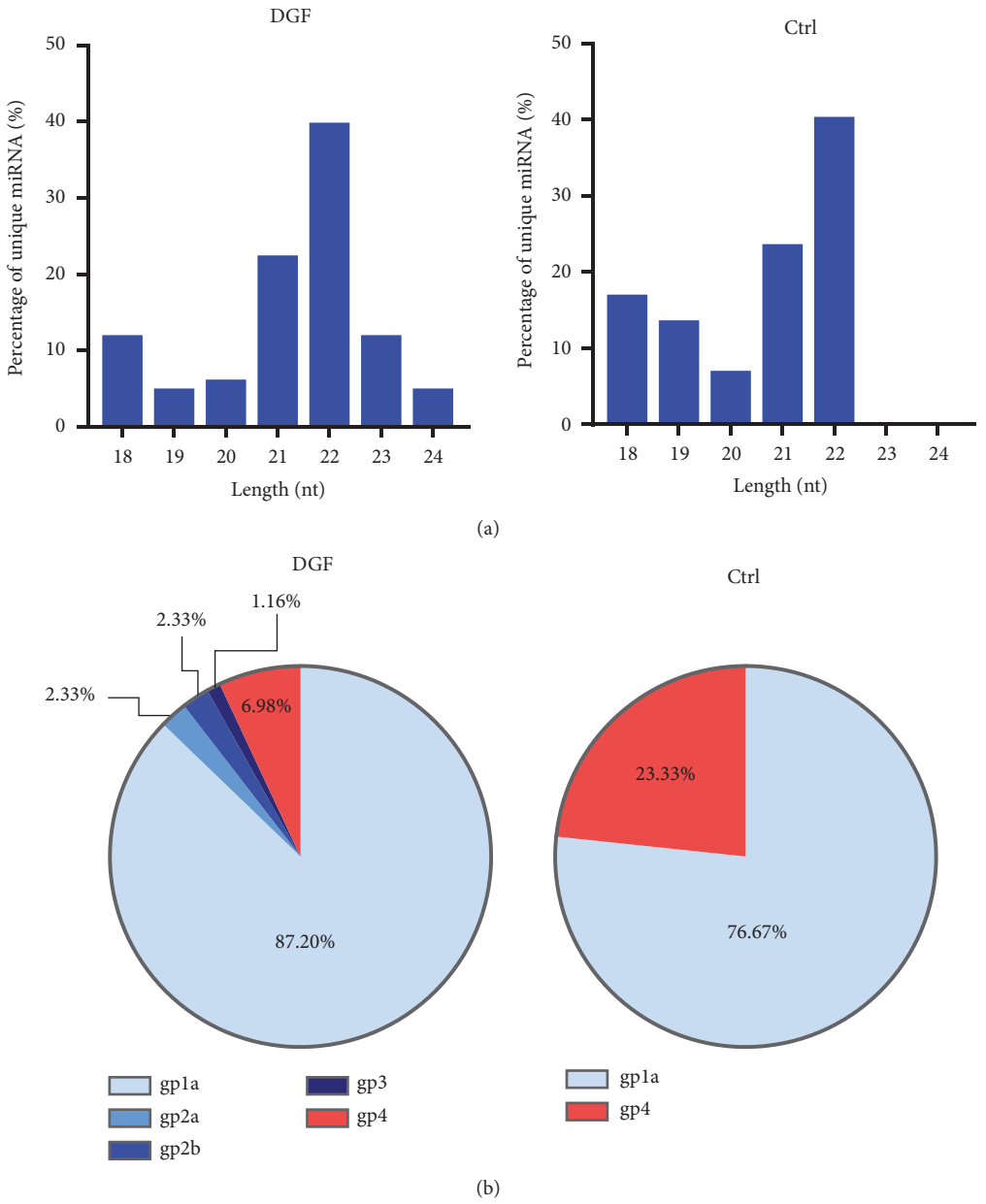

Figure 2: *Length and category distribution of sequenced exosomal miRNAs in the peripheral blood of kidney recipients.* (a) Category distribution of sequenced exosomal miRNAs. Reads with length 18–26 nt were chosen for mapping. Length distribution of sequenced miRNAs is shown. The nucleotide (nt) lengths of cloned miRNAs are shown on x-axis; the number of total reads by deep sequencing is shown on y-axis. (b) Category distribution of sequenced exosomal miRNAs. Gp1a: reads were mapped to miRNAs/pre-miRNAs of specific species in miRBase and the pre-miRNAs were further mapped to genome and EST. gp2a: reads were mapped to miRNAs/pre-miRNAs of selected species in miRBase and the mapped pre-miRNAs were not further mapped to genome, but the reads (and of course the miRNAs of the pre-miRNAs) were mapped to genome. The extended genome sequences from the genome loci may form hairpins. gp2b: reads were mapped to miRNAs/pre-miRNAs of selected species in miRBase and the mapped pre-miRNAs were not further mapped to genome, but the reads (and of course the miRNAs of the pre-miRNAs) were mapped to genome. The extended genome sequences from the genome loci may not form hairpins. gp3: reads were mapped to miRNAs/pre-miRNAs of selected species in miRBase and the mapped pre-miRNAs were not further mapped to genome, and the reads were not mapped to genome either. But the reads were mapped to the miRNAs (Matures). gp4: reads were not mapped to pre-miRNAs of selected species in miRBase. But the reads were mapped to genome and the extended genome sequences from genome may form hairpins.

of the known exosomal membrane proteins, CD63 and CD9 (Figure 1(d)). These results demonstrated that the small vesicles we isolated were exosomes.

3.3. Identification of Exosomal miRNAs. A cDNA library of miRNAs was constructed from the exosomes in the peripheral blood of kidney recipients one week after the

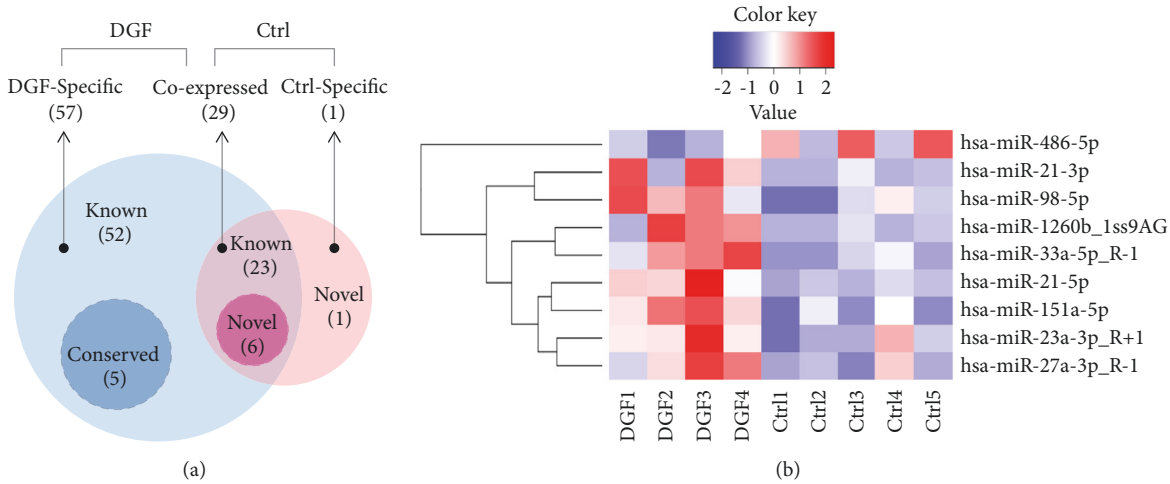

Figure 3: *Analysis of differentially expressed exosomal miRNAs in DGF and control groups.* (a) Venn diagram of differentially expressed exosomal miRNAs in DGF (blue) and control (red) groups. (b) Heat map of the differentially expressed exosomal miRNAs in DGF and control groups. The color of each pattern represents the fold change as log 2, from high (red) to low (blue).

transplantation. After removing low-quality reads, contaminants, and adaptors, we obtained 87 unique miRNAs of 18 and 26 bases in length through Solexa sequencing. To guarantee the accuracy of sequence data, only the sequences that were observed in all samples from DGF or control group were selected as miRNA molecules (Table 3). The length distribution of unique exosomal miRNAs (18–24 nt) varied in the DGF and control groups. The lengths of miRNAs in the DGF group ranged from 18 to 24 nt (Figure 2(a), left panel), while the length of the miRNAs in the control group ranged from 18 to 22 nt (Figure 2(b), right panel). The most abundant size class in both groups was 22 nt, followed by 21 nt and then by 18 nt (Figure 2). To further identify the unique miRNA we obtained by high-throughput sequencing, all the unique miRNA clean reads were compared with the known human miRNAs in miRBase 18.0 (University of Manchester, Manchester, UK). Among the 87 unique miRNAs we identified, 86 and 30 miRNAs could be detected in the DGF and control groups, respectively. The known (gp1a), conserved (gp2a, gp2b, and gp3), and novel (gp4) miRNAs accounted for, respectively, 87.2%, 5.82%, and 6.98% of the total miRNAs in the DGF group (Figure 2(b), left panel), while known and novel miRNAs separately made up 76.67% and 23.33% of the total miRNAs in the control group (Figure 2(b), right panel). In conclusion, these results demonstrated that the exosomal miRNA profiling in the DGF and control group is significantly different.

3.4. Analysis of Differentially Expressed Exosomal miRNAs in DGF and Control Groups. We summarized the coexpressed and specific exosomal miRNA sequences between the DGF and control group. Among the exosomal miRNAs we identified, 29 miRNAs were shared in both the DGF and control groups, while 57 miRNAs containing 52 known miRNAs and 5 conserved miRNAs and 1 novel miRNA were specifically expressed in the DGF and control groups, respectively (Figure 3(a)). Moreover, we analyzed the expressions of the 29 coexpressed miRNAs in the DGF and control group (Figure 3(a)). The coexpressed miRNAs were composed of 23 known miRNAs and 6 novel miRNAs (Figure 3(a)). Among the known coexpressed exosomal miRNAs, 3 miRNAs, hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p, were detected at noticeably higher levels in the DGF group than that in the control group (P = 0.007, 0.024 and 0.027, resp.; fold change > 2; Figure 3(b)). The detailed information of the differentially expressed exosomal miRNAs was expressed in Table 4. These results suggested that the exosomal miRNAs, hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p, had the potential to be the biomarkers for DGF.

3.5. Correlation Analysis of Differentially Expressed Exosomal miRNAs with Clinical Parameters. Since the CR, BUN, or UA levels of the kidney recipients in the DGF and control group were significantly different, the correlation analysis of the hsa-miR-33a-5p_R-1, hsa-miR-98-5p, or hsa-miR-151a-5p miRNA expressions with these clinical parameters was conducted to further assess the potential of the three exosomal miRNAs to be the diagnostic indicators of DGF. Noticeably, the expression of hsa-miR-151a-5p was positively correlated with CR (r = 0.8131, P = 0.0077; Figure 4(a)), BUN (r = 0.8150, P = 0.0074; Figure 4(b)), and UA (r = 0.6958, P = 0.0374; Figure 4(c)) levels, while the expressions of hsa-miR-33a-5p_R-1 only showed significant positive correlation with the CR (r = 0.9404, P = 0.0002; Figure 4(a)) and BUN (r = 0.7991, P = 0.0098; Figure 4(b)) levels. No significant correlation existed between the hsa-miR-98-5p expression and the CR, BUN, and UA levels (Figures 4(a), 4(b), and 4(c)). These results demonstrated that hsa-miR-151a-5p was not only a biomarker candidate for DGF but could reflect the CR, BUN, and UA levels.

3.6. Functional Analysis of Differentially Expressed miRNA Target Genes. The target genes of the three differentially expressed exosomal miRNAs, hsa-miR-33a-5p_R-1,

Table 3: Exosomal miRNAs identified in the peripheral blood of kidney recipients.

miRNA name	Length	Sequence	Group	Expression level	Common/specific
hsa-let-7a-5p	22	TGAGGTAGTAGGTTGTATAGTT	gp1a	high	DGF specific
hsa-let-7b-5p	22	TGAGGTAGTAGGTTGTGTGGTT	gp1a	high	common
hsa-let-7d-5p	22	AGAGGTAGTAGGTTGCATAGTT	gp1a	high	DGF specific
hsa-let-7f-5p	22	TGAGGTAGTAGATTGTATAGTT	gp1a	high	DGF specific
hsa-let-7g-5p	22	TGAGGTAGTAGTTTGTACAGTT	gp1a	high	DGF specific
hsa-let-7i-5p_R-1	21	TGAGGTAGTAGTTTGTGCTGT	gp1a	high	common
hsa-miR-100-5p_R-1	21	AACCCGTAGATCCGAACTTGT	gp1a	middle	DGF specific
hsa-miR-101-3p_R+1	22	TACAGTACTGTGATAACTGAAG	gp1a	high	common
hsa-miR-103a-3p_R-2	21	AGCAGCATTGTACAGGGCTAT	gp1a	high	common
hsa-miR-106a-5p_1ss1AC	23	CAAAGTGCTTACAGTGCAGGTAG	gp1a	high	DGF specific
hsa-miR-106b-5p_R-1	20	TAAAGTGCTGACAGTGCAGA	gp1a	high	common
hsa-miR-107_R-2	21	AGCAGCATTGTACAGGGCTAT	gp1a	high	common
hsa-miR-10b-5p_R-1	22	TACCCTGTAGAACCGAATTTGT	gp1a	middle	DGF specific
hsa-miR-125b-5p	22	TCCCTGAGACCCTAACTTGTGA	gp1a	high	DGF specific
hsa-miR-126-3p	22	TCGTACCGTGAGTAATAATGCG	gp1a	high	common
hsa-miR-126-5p	21	CATTATTACTTTTGGTACGCG	gp1a	high	DGF specific
hsa-miR-128-3p	21	TCACAGTGAACCGGTCTCTTT	gp1a	middle	DGF specific
hsa-miR-142-3p_L-1	22	GTAGTGTTTCCTACTTTATGGA	gp1a	high	DGF specific
hsa-miR-142-5p_L+2R-3	20	CCCATAAAGTAGAAAGCACT	gp1a	high	DGF specific
hsa-miR-143-3p_R+1	22	TGAGATGAAGCACTGTAGCTCT	gp1a	high	DGF specific
hsa-miR-144-3p_R-1	19	TACAGTATAGATGATGTAC	gp1a	high	common
hsa-miR-145-5p	23	GTCCAGTTTTCCCAGGAATCCCT	gp1a	middle	DGF specific
hsa-miR-146a-5p	22	TGAGAACTGAATTCCATGGGTT	gp1a	middle	DGF specific
hsa-miR-148a-3p	22	TCAGTGCACTACAGAACTTTGT	gp1a	high	DGF specific
hsa-miR-148b-3p	22	TCAGTGCATCACAGAACTTTGT	gp1a	middle	DGF specific
hsa-miR-150-5p	22	TCTCCCAACCCTTGTACCAGTG	gp1a	middle	DGF specific
hsa-miR-151a-5p	21	TCGAGGAGCTCACAGTCTAGT	gp1a	middle	DGF specific
hsa-miR-152-3p	21	TCAGTGCATGACAGAACTTGG	gp1a	middle	DGF specific
hsa-miR-15a-5p_R-1	21	TAGCAGCACATAATGGTTTGT	gp1a	high	common
hsa-miR-15b-5p_R-3	19	TAGCAGCACATCATGGTTT	gp1a	high	common
hsa-miR-16-5p	22	TAGCAGCACGTAAATATTGGCG	gp1a	high	common
hsa-miR-17-5p	23	CAAAGTGCTTACAGTGCAGGTAG	gp1a	high	DGF specific
hsa-miR-181a-5p	23	AACATTCAACGCTGTCGGTGAGT	gp1a	middle	DGF specific
hsa-miR-185-5p	22	TGGAGAGAAAGGCAGTTCCTGA	gp1a	middle	DGF specific
hsa-miR-191-5p	23	CAACGGAATCCCAAAAGCAGCTG	gp1a	high	DGF specific
hsa-miR-199a-3p	22	ACAGTAGTCTGCACATTGGTTA	gp1a	middle	DGF specific
hsa-miR-19b-3p_R-2	21	TGTGCAAATCCATGCAAAACT	gp1a	middle	DGF specific
hsa-miR-20a-5p	23	TAAAGTGCTTATAGTGCAGGTAG	gp1a	high	DGF specific
hsa-miR-21-5p	22	TAGCTTATCAGACTGATGTTGA	gp1a	high	common
hsa-miR-221-3p_R-1	22	AGCTACATTGTCTGCTGGGTTT	gp1a	high	DGF specific
hsa-miR-223-3p_R+1	23	TGTCAGTTTGTCAAATACCCCAA	gp1a	high	DGF specific
hsa-miR-22-3p	22	AAGCTGCCAGTTGAAGAACTGT	gp1a	high	common
hsa-miR-22-5p_R-1	21	AGTTCTTCAGTGGCAAGCTTT	gp1a	middle	DGF specific
hsa-miR-23a-3p_R+1	22	ATCACATTGCCAGGGATTTCCA	gp1a	high	DGF specific
hsa-miR-23b-3p_R-5	18	ATCACATTGCCAGGGATT	gp1a	middle	DGF specific
hsa-miR-24-3p	22	TGGCTCAGTTCAGCAGGAACAG	gp1a	middle	DGF specific
hsa-miR-25-3p	22	CATTGCACTTGTCTCGGTCTGA	gp1a	high	common
hsa-miR-26a-5p	22	TTCAAGTAATCCAGGATAGGCT	gp1a	high	common
hsa-miR-26b-5p_R+1	22	TTCAAGTAATTCAGGATAGGTT	gp1a	high	common

Table 3: Continued.

miRNA name	Length	Sequence	Group	Expression level	Common/specific
hsa-miR-27a-3p_R-1	20	TTCACAGTGGCTAAGTTCCG	gp1a	high	common
hsa-miR-27b-3p	21	TTCACAGTGGCTAAGTTCTGC	gp1a	high	DGF specific
hsa-miR-29a-3p_R-1_1ss10CT	21	TAGCACCATTTGAAATCGGTT	gp1a	high	common
hsa-miR-29c-3p_R-1	21	TAGCACCATTTGAAATCGGTT	gp1a	high	common
hsa-miR-30a-5p_R+2	24	TGTAAACATCCTCGACTGGAAGCT	gp1a	high	DGF specific
hsa-miR-30b-5p	22	TGTAAACATCCTACACTCAGCT	gp1a	high	DGF specific
hsa-miR-30c-5p_R+1	24	TGTAAACATCCTACACTCTCAGCT	gp1a	high	DGF specific
hsa-miR-30d-5p_R+2	24	TGTAAACATCCCCGACTGGAAGCT	gp1a	high	DGF specific
hsa-miR-30e-5p_R+2	24	TGTAAACATCCTTGACTGGAAGCT	gp1a	high	DGF specific
hsa-miR-320a-3p	22	AAAAGCTGGGTTGAGAGGGCGA	gp1a	high	common
hsa-miR-339-5p_R-4	19	TCCCTGTCCTCCAGGAGCT	gp1a	middle	DGF specific
hsa-miR-33a-5p_R-1	20	GTGCATTGTAGTTGCATTGC	gp1a	middle	DGF specific
hsa-miR-363-3p_R-1	21	AATTGCACGGTATCCATCTGT	gp1a	middle	DGF specific
hsa-miR-378a-3p_R-1	21	ACTGGACTTGGAGTCAGAAGG	gp1a	high	DGF specific
hsa-miR-423-3p	23	AGCTCGGTCTGAGGCCCCTCAGT	gp1a	middle	DGF specific
hsa-miR-423-5p	23	TGAGGGGCAGAGAGCGAGACTTT	gp1a	middle	DGF specific
hsa-miR-425-5p_R-1	22	AATGACACGATCACTCCCGTTG	gp1a	middle	DGF specific
hsa-miR-451a_R-1	21	AAACCGTTACCATTACTGAGT	gp1a	high	common
hsa-miR-484_R-1	21	TCAGGCTCAGTCCCCTCCCGA	gp1a	middle	DGF specific
hsa-miR-486-5p	22	TCCTGTACTGAGCTGCCCCGAG	gp1a	high	common
hsa-miR-652-3p_R-1	20	AATGGCGCCACTAGGGTTGT	gp1a	middle	DGF specific
hsa-miR-92a-3p	22	TATTGCACTTGTCCCGGCCTGT	gp1a	high	common
hsa-miR-93-5p	23	CAAAGTGCTGTTCGTGCAGGTAG	gp1a	high	DGF specific
hsa-miR-98-5p	22	TGAGGTAGTAAGTTGTATTGTT	gp1a	middle	DGF specific
hsa-miR-99a-5p_R-1	21	AACCCGTAGATCCGATCTTGT	gp1a	high	DGF specific
hsa-miR-99b-5p	22	CACCCGTAGAACCGACCTTGCG	gp1a	middle	DGF specific
eca-miR-451_L+1R-1_1ss20TA	22	CAAACCGTTACCATTACTGAGT	gp2a	middle	DGF specific
efu-miR-9226_L-4_1ss22GA	18	GTCCCTGTTCGGGCGCCA	gp2a	middle	DGF specific
mmu-miR-3968_L-3_1ss14AT	18	ATCCCACTCCTGACACCA	gp2b	middle	DGF specific
cgr-miR-1260	18	ATCCCACCGCTGCCACCA	gp2b	middle	DGF specific
bta-miR-2478_L-2	18	ATCCCACTTCTGACACCA	gp3	middle	DGF specific
PC-3p-1061_715	18	AGTCCACGATCCCCGTGG	gp4	high	common
PC-3p-2112_237	19	CCCACCACGTTCCCCGTGG	gp4	middle	common
PC-3p-4600_77	19	TTCCACCACGTTCCCGGTG	gp4	middle	ctrl specific
PC-3p-4883_70	18	CACGTTCCCGGGCCGTGG	gp4	middle	common
PC-3p-857_1046	18	ATTCCAACGTTCCCGTGG	gp4	high	common
PC-5p-1596_363	18	GTTCCCGTGGTCCCGTGA	gp4	high	common
PC-5p-3627_109	18	CTCCACCACTTCCCGTGG	gp4	middle	common

gp1a: reads were mapped to miRNAs/pre-miRNAs of specific species in miRBase and the pre-miRNAs were further mapped to genome and EST. gp2a: reads were mapped to miRNAs/pre-miRNAs of selected species in miRBase and the mapped pre-miRNAs were not further mapped to genome, but the reads (and of course the miRNAs of the pre-miRNAs) were mapped to genome. The extended genome sequences from the genome loci may form hairpins. gp2b: reads were mapped to miRNAs/pre-miRNAs of selected species in miRBase and the mapped pre-miRNAs were not further mapped to genome, but the reads (and of course the miRNAs of the pre-miRNAs) were mapped to genome. The extended genome sequences from the genome loci may not form hairpins. gp3: reads were mapped to miRNAs/pre-miRNAs of selected species in miRBase and the mapped pre-miRNAs were not further mapped to genome, and the reads were not mapped to genome either. But the reads were mapped to the miRNAs (Matures). gp4: reads were not mapped to pre-miRNAs of selected species in miRBase. But the reads were mapped to genome and the extended genome sequences from genome may form hairpins.

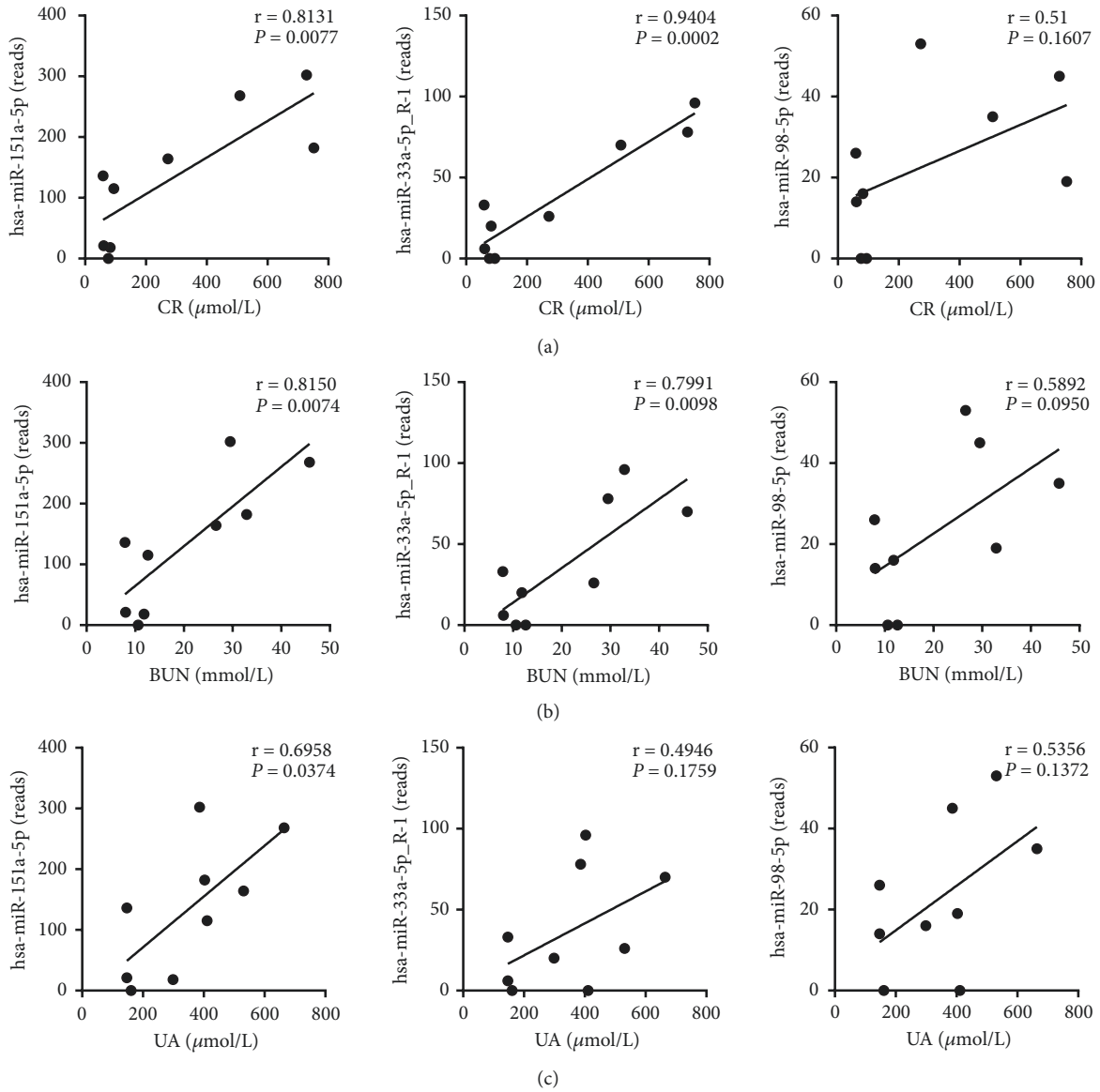

Figure 4: *Correlation analysis of differentially expressed exosomal miRNAs with clinical parameters*. Pearson correlation was performed to examine the relationship between hsa-miR-151a-5p, hsa-miR-33a-5p_R-1, and hsa-miR-98-5p and the first-week serum (a) CR, (b) BUN, or (c) UA levels in the kidney recipients after transplantation. Pearson correlation coefficient (r) and *P* value are plotted in each case.

Table 4: The differentially expressed miRNAs between DGF and Ctrl ($P < 0.05$).

miR name	miR sequence	Genome ID	Pre-miRNA sequence	Fold change	log 2 (fold change)	*P* value
hsa-miR-33a-5p_R-1	GTGCATTGTAGTTGC ATTGC	chr22	ggcagctgtgGTGCATTGTAGTTG CATTGCatgttctggtggtacccatgCA ATGTTTCCACAGTGCATCAca gaggcctg	3.954	1.983	0.007
hsa-miR-98-5p	TGAGGTAGTAAGTT GTATTGTT	chrX	aggattctgctcatgccagggTGAGGTA GTAAGTTGTATTGTTgtggggtag ggatattaggccccaattagaagataactatac aacttactactttccctggtgtgtggcatattca	5.777	2.530	0.024
hsa-miR-151a-5p	TCGAGGAGCTCACA GTCTAGT	chr8	ggggcaaagatgactaaaacacttttcctgccc TCGAGGAGCTCACAGTCTAG TatgtctcatcccctaCTAGACTGAA GCTCCTTGAGGacagggatggtcata ctcacctc	3.375	1.755	0.027

DGF: delayed graft function; Ctrl: control; Fold change: fold change (DGF(mean)/Ctrl(mean)); *P* value: DGF versus Ctrl, *t*-test.

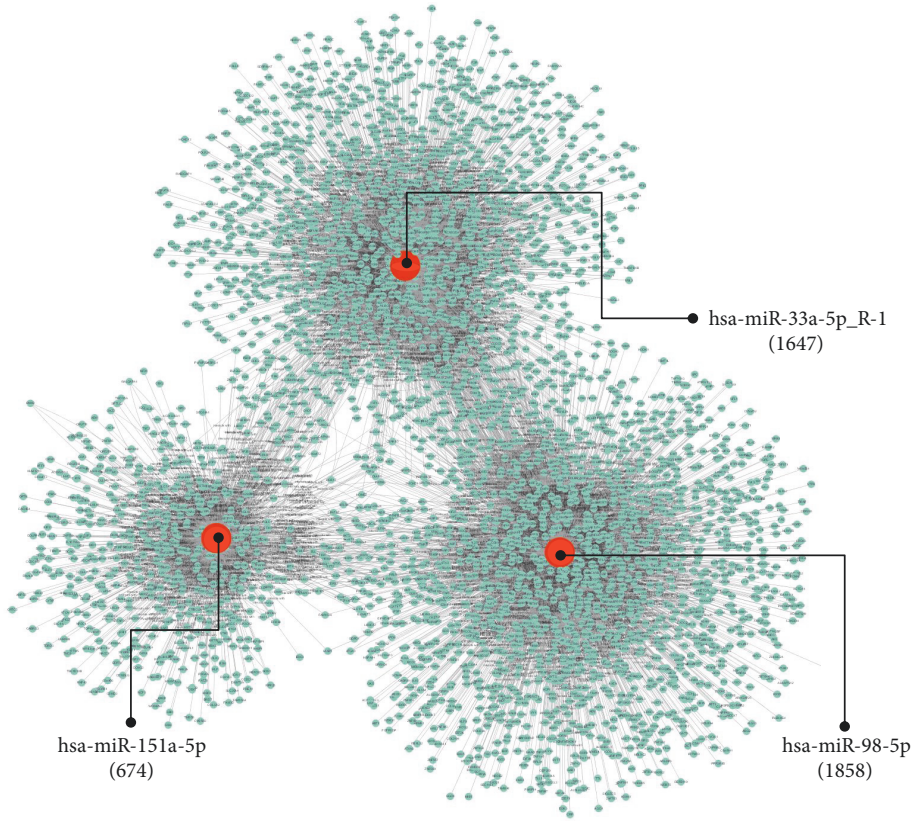

Figure 5: *The regulatory network between miRNAs and target genes associated with DGF*. The red and green roundness represented the miRNAs and genes, respectively. The target gene number was expressed in parentheses.

hsa-miR-98-5p, or hsa-miR-151a-5p, were predicted using TargetScan and miRanda software. 1647, 1858, and 647 target genes of hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p were identified, respectively (Figure 5). To further highlight the functional features of exosomal miRNAs, the putative target genes were subsequently annotated in GO terms. As shown in Figure 6, the target genes were mainly enriched in "protein binding" (1850 genes, P = 9.807E-15), "cytoplasm" (1,307 genes, P = 3.997E-14), "nucleus" (1,275 genes, P = 1.027E-07), "cytosol" (971 genes, P = 1.158E-05), "metal ion binding" (825 genes, P = 4.330E-15), "nucleoplasm" (665 genes, P = 2.626E-08), "regulation of transcription, DNA-templated" (573 genes, P = 3.456E-10), "transcription, DNA-templated" (532 genes, P = 1.681E-09), and so forth (Figure 6). KEGG enrichment analysis demonstrated that target genes were significantly enriched in 52 signaling pathways. Among these signaling pathways, "MAPK signaling pathway" (82 genes, P = 1.158E-05), "Proteoglycans in cancer" (62 genes, P = 1.158E-05), "Axon guidance" (58 genes, P = 1.158E-05), "cGMP - PKG signaling pathway" (51 genes, P = 1.158E-05), and "Oxytocin signaling pathway" (48 genes, P = 1.158E-05) were the five most significantly enriched signaling pathways (Figure 7).

4. Discussion

Since few researches have been done to demonstrate the role of exosomal miRNAs associated with the process of DGF in kidney transplantation, in this study, we utilized high-throughput sequencing to explore the miRNA expression profiling and function of exosomes in the peripheral blood of kidney recipients with or without DGF. The lengths of the miRNAs we identified in the DGF group ranged from 18 to 24 nt, while the length of the miRNAs in the control group ranged from 18 to 22 nt. Though the length distributions of unique miRNAs varied in the DGF and control groups, they exhibited the canonical size range distribution common to mammalian miRNAs [24]. Through comparing the miRNA sequences with the known miRNAs in the miRBase, we identified 87 exosomal miRNAs in the peripheral blood of kidney recipients with DGF after transplantation.

Among the 87 exosomal miRNAs, 57 were specific and were expressed specially in the recipients with DGF, while 29 exosomal miRNAs were coexpressed in the DGF and control groups. Three coexpressed exosomal miRNAs, hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p, were further found to be significantly upregulated in the peripheral blood of kidney recipients with DGF, indicating that these exosomal miRNAs could be biomarker candidates for DGF. Mean values of CR, BUN, and UA were observed to be significantly increased in the liver recipients with (acute kidney injury) AKI at postoperative days 1, 3, and 7 [25]. In our study, the CR, BUN, and UA levels of the recipients in DGF group were also significantly higher than those of the recipients in control group, respectively. It was remarkable that the hsa-miR-151a-5p expression showed significant positive correlation

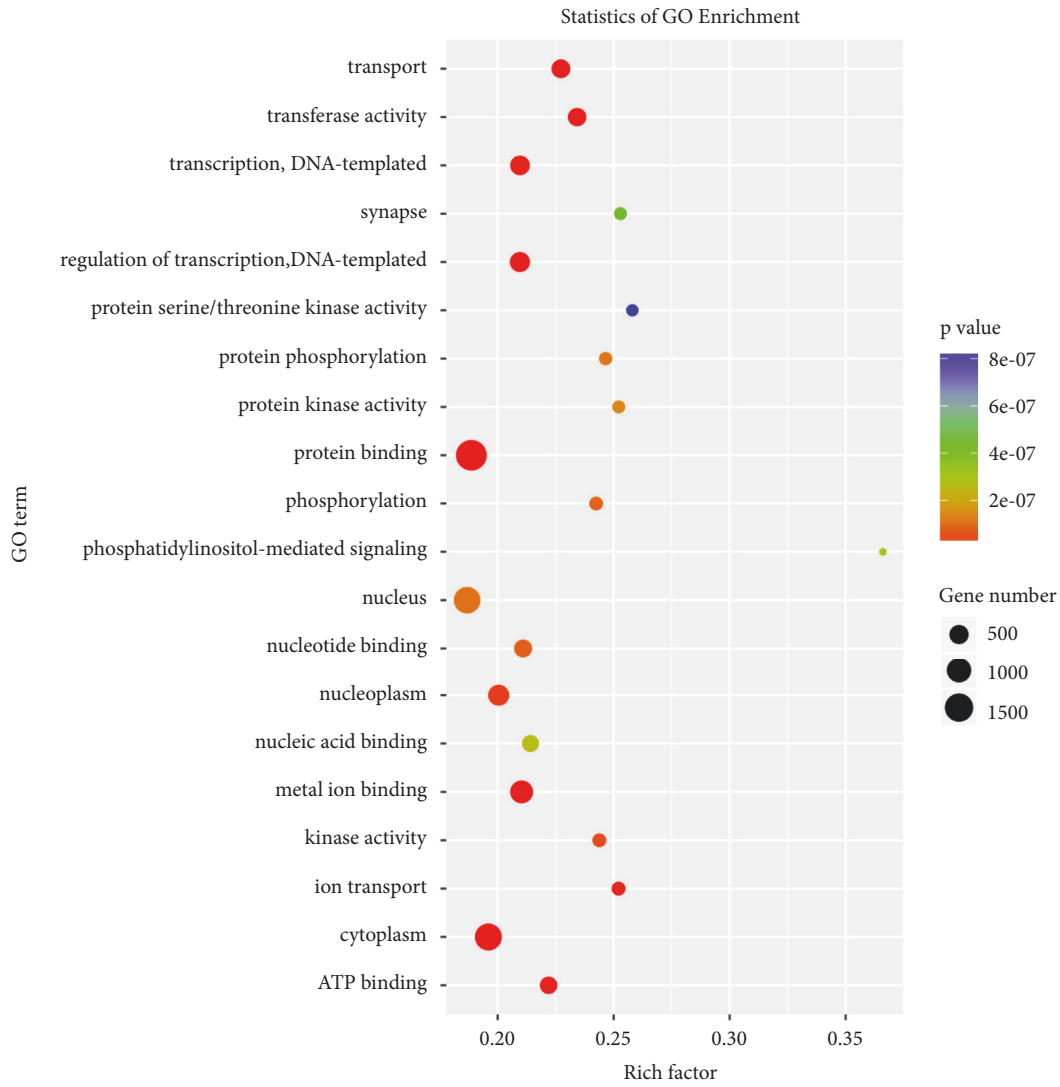

Figure 6: *Go analysis of target genes of the predicted differentially expressed miRNAs*. Advanced bubble chart shows enrichment of differentially expressed genes in GO terms. y-axis represented pathway, and x-axis represented rich factor, which meant the ratio of the amount of differentially expressed genes enriched in the pathway and the amount of all genes annotated in this pathway. Size and color of the bubble represented the amount of differentially expressed genes enriched in the pathway and enrichment significance, respectively.

with CR, BUN, and UA levels of the patients as well. Thus, compared with two other exosomal miRNAs, hsa-miR-151a-5p seemed to have more clinical value, for it could not only be a biomarker candidate for DGF but also reflect CR, BUN, and UA more accurately.

Few evidences in previous research were provided to demonstrate the relationship between kidney transplantation and hsa-miR-33a-5p_R-1, hsa-miR-98-5p, or hsa-miR-151a-5p. In this study, GO and KEGG pathway enrichment analyses were utilized to reveal biological processes and functions of differentially expressed exosomal miRNA target genes relevant for DGF. Our results indicated that the differentially expressed genes were mainly involved in MAPK signaling pathway and biological processes including DNA-templated transcription. MAPK signaling pathway was shown to play a pivotal role in chronic allograft dysfunction of kidney transplant recipients by regulating kidney interstitial fibrosis. Renal fibrosis was ameliorated by interfering the MAPK signaling pathway in an animal model of unilateral ureteral obstruction [26–28]. P38 MAPK signaling pathway could be observed to be activated in human kidney (HK-2) cells stimulated by IL-33 and kidney recipients with chronic allograft dysfunction [29]. Recent studies also uncovered that MAPK signaling pathway was associated with cerebral, cardiac, and retinal ischemia reperfusion injury [30–32]. DGF is primarily a consequence of ischemia and reperfusion (IR) injury resulting in postischemic acute tubular necrosis [2]. Therefore, probably, the differentially expressed exosomal miRNAs, hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p, might moderate the process of DGF by regulating the MAPK signaling pathway associated with ischemia reperfusion injury.

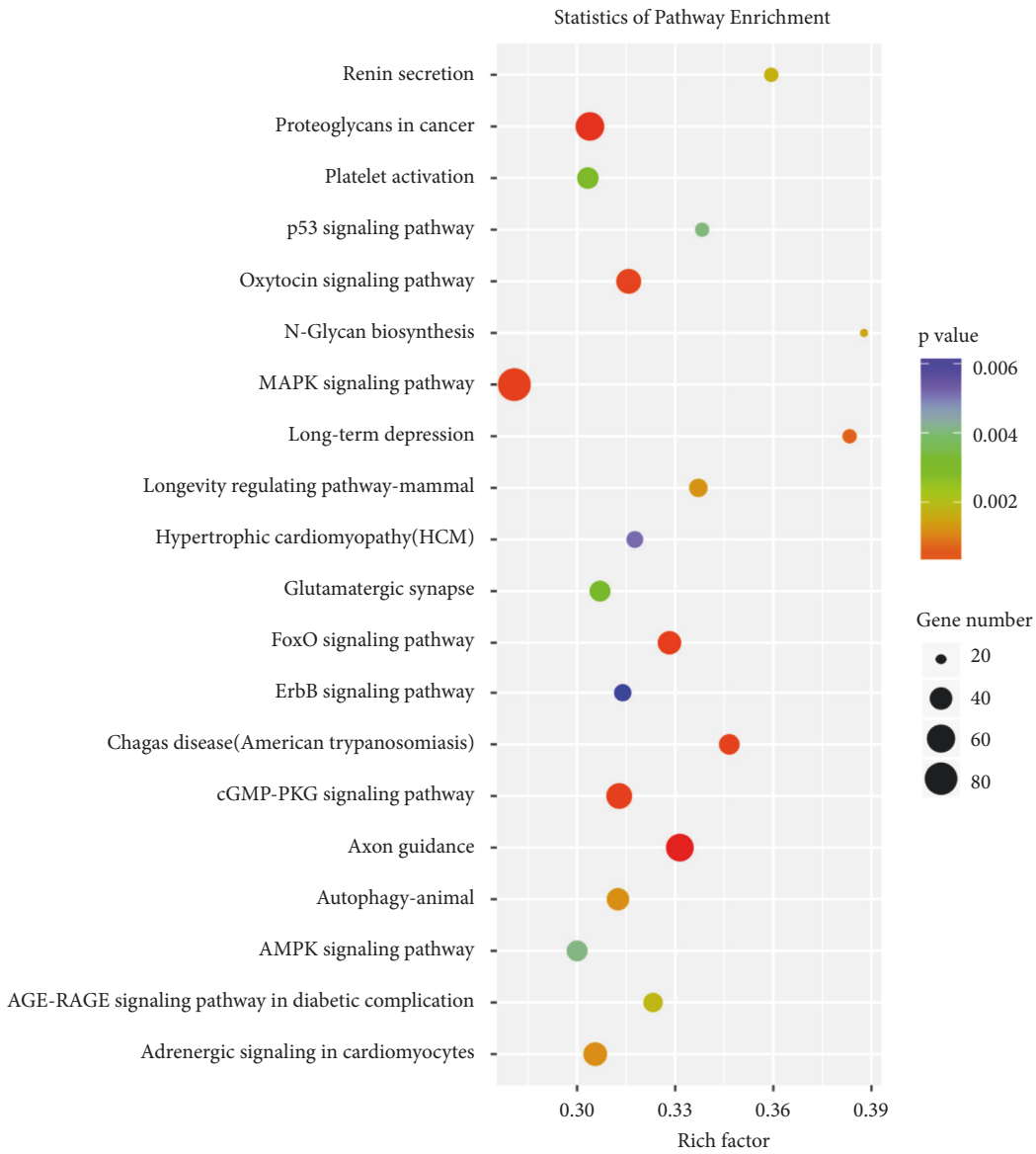

Figure 7: *KEGG pathways significantly enriched in the predicted target genes of differentially expressed miRNAs*. Advanced bubble chart shows enrichment of differentially expressed genes in signaling pathways. y-axis represented pathway, and x-axis represented rich factor, which meant the ratio of the amount of differentially expressed genes enriched in the pathway and the amount of all genes annotated in this pathway. Size and color of the bubble represented the amount of differentially expressed genes enriched in the pathway and enrichment significance, respectively.

Noticeably, two DGF-specific exosomal miRNAs that we identified, hsa-miR-20a-5p and hsa-miR-17-5p, were reported also to be significantly upregulated in the biopsy specimens of DGF kidneys with acute tubular necrosis [33]. DGF is regarded as a consequence of ischemia and reperfusion injury (IR) resulting in postischemic acute tubular necrosis [2]. Essentially, these results were consistent with ours and further confirmed the possibility of exosomal hsa-miR-20a-5p and hsa-miR-17-5p in the peripheral blood of the kidney recipients to be the biomarkers of DGF. hsa-miR-17-5p was also found to be involved in liver allograft rejection by regulating PD-L1 expression [34], suggesting that the regulation of PD-L1 may be a possible mechanism for hsa-miR-17-5p to function in DGF. However, the studies about the role of hsa-miR-20a-5p in kidney transplantation were very limited; most researches about hsa-miR-20a-5p are associated with cancer [35, 36]. Though significant upregulation of exosomal miRNAs hsa-miR-21-5p and hsa-miR-106b-5p could be detected in DGF kidney in the previous study [33], our results demonstrated that the expressions of these two exosomal miRNAs were similar in the peripheral blood of the kidney recipients with or without DGF.

Overall, in this study, we identified the expression profiling of exosomal miRNAs in the peripheral blood of kidney recipients with DGF using Solexa high-throughput sequencing and provided new insights into the important

role of the exosome-derived miRNA regulation in DGF. The miRNA candidates, hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p, which were upregulated in the process of DGF, have the potential to be used as biomarkers to predict graft function in the kidney recipients and the inhibitors of these miRNAs might offer a novel treatment option in transplantation.

Data Availability

The clinical data of the patients used to support the findings of this study are included within the article. The original sequencing data of the microRNA used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Junpeng Wang and Xin Li contributed equally to this work.

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Review Article

Noncoding RNAs Act as Tumor-Derived Molecular Components in Inducing Premetastatic Niche Formation

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Cancer metastasis has been demonstrated as it is the culmination of a cascade of priming steps. Increasing evidence has shown that tumor-derived molecular components (TDMCs) are known as extra cellular vesicle and nonvesicle factors and serve as versatile intercellular communication vehicles which can mediate signaling in the tumor microenvironment while creating the premetastatic niche. Noncoding RNAs (ncRNAs) as one of the TDMCs have been proved in participating in the formation of the premetastatic niche. Understanding the premetastatic niche formation mechanisms through TDMCs, especially ncRNAs may open a new avenue for cancer metastasis therapeutic strategies. In this review, recent findings regarding ncRNAs function were summarized, and then the interaction with the premetastatic niche formation was studied, which highlight the potential of using ncRNAs for cancer diagnosis and therapeutic effect.

1. Introduction

Increasing tumor patients pass away eventually owing to tumor metastatic progression every year, even though significant advancements have been achieved in cancer therapies [1]. Therefore, it emphasizes the important goal of cancer research efforts to identify the pivotal molecular and cellular components in every step of tumor metastasis to develop new strategy for prevention and control of tumor metastasis. About 100 years ago, "seed and soil" hypothesis was originally proposed by Stephen Paget [2] which indicated that metastatic tumor cell "seed" chose the fertile "soil" to colonization. Under numerous efforts of research groups, it is now widely accepted that primary tumors prior modulate the local microenvironment of distant organs in preparation for tumor cell colonization even before their arrival. The microenvironment is termed the premetastatic niche with a series changes including immunosuppression, inflammation, lymphangiogenesis, organotropism, angiogenesis, and vascular permeability [3], which is crucial for the development of metastasis and has attracted more and more attention in the last several years [4].

TDMCs, one of the primary tumor-secreted molecules can be divided into nonvesicle tumor-derived secreted factors (TDSFs) and tumor-derived secreted extracellular vesicles (EVs) and carry diverse molecular components, such as proteins, lipids, RNA, and DNA molecules [5]. They have the functions of recruiting BMDCs, making the immune cells heterogeneity, altering the vasculature, and reprogramming the stromal cells and metabolic progress [6]. Studies have shown that ncRNAs are involved in regulating tumor transcription, invasion, and metastasis, etc., and may control cancer-related genes by acting on proteins [7].The expression of ncRNAs can be used to reflect the origin, grade, and other pathological constants of tumors and provide a method for the diagnosis and prognosis. In recent years, ncRNAs, as a new intercellular communication mechanism of TDMCs, have been established as an important part of premetastatic niche induction [8]. These new advances can indicate new direction to guide future research and better illustrate the

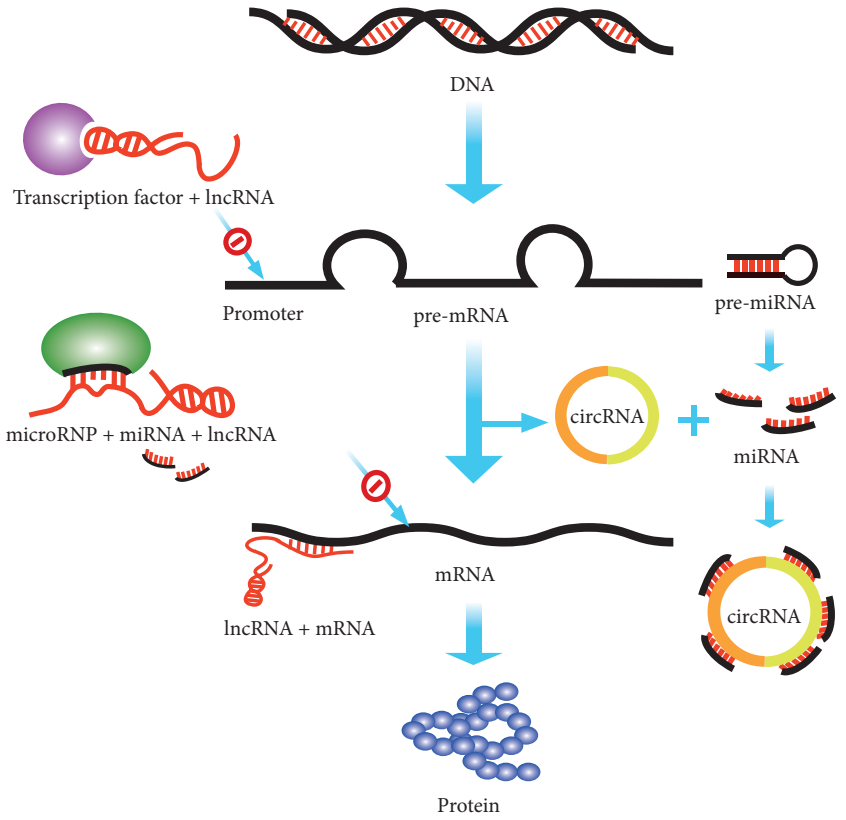

Figure 1: The functions of ncRNAs. The molecular functions of lncRNAs can be summarized into decoy and miRNAs sponge and affect the stability of mRNAs by combining with mRNAs. And circRNAs competitively bind miRNAs as ceRNA.

potential mechanisms of tumor metastasis to design more effective diagnosis and treatment strategies to fight cancer. Here we focus on the most recent findings on the ncRNAs and their underlying induced mechanisms of the premetastatic niche formation.

2. NcRNAs as a Sort of TDMCs

In human genome sequence, only 2% of capacity is mRNA which can encode protein [9]. ncRNAs, known as non-messenger refer to the RNAs without protein encoding function due to the lack of an open reading frame, and they are often transcribed from the complementary strand of protein encoding genes [10]. According to the length, ncRNAs are divided into long ncRNAs (lncRNAs) and short ncRNAs (< 200nt), and short ncRNAs include microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs). Based on the position, ncRNAs can be divided into nonvesicle and extracellular vesicle sort. With the development of extensively parallel sequencing technology and high-resolution microarrays, ncRNAs especially lncRNAs, miRNAs, and circRNAs have caught extended attentions. Massive lines of evidence suggested that ncRNAs are not only involved in life activities but also closely related to tumor cell differentiation, proliferation, migration, invasion, and infiltration [8, 11].

When ncRNAs function as one category of TDMCs, they mainly exist in the EVs and the minority of them are free [12]. EVs carry a distinctive repertoire of lncRNAs and short ncRNAs [13, 14]. What is more, packaging RNA into the EVs is also a selective progress [15]. EVs can transfer special sorts of RNAs from donor to recipient cells under pathological and physiological conditions [16] (Figure 1).

3. NcRNAs: A Key to the Formation of the Premetastatic Niche

Previous research has found primary tumor-derived vesicles contribute to the formation of the premetastatic niche [17]. Exosomes secreted by different cell types contain different RNAs and proteins that germinate from the primary tumor cells and enter the bloodstream, which may help the cancer change the metastatic microenvironment and invade new organs [18].

3.1. MiRNAs. Increasing angiogenesis and vascular permeability are essential for tumor development and metastasis, and tumor-derived EV miRNAs promote this progress by affecting endothelia cells [19, 20]. In the premetastasis niche, metastatic breast cancer cells secret EV-encapsulated miR-105 to destroy the vascular barriers by downregulating cellular endothelial tight junction protein zonula occludens 1 (ZO-1), which induces vascular leakiness in distant organs to promote metastasis [21]. Brain metastatic cancer cells-derived EVs of breast cancer contain miR-181c, which modulate the actin

dynamics by the downregulation of phosphoinositide dependent kinase 1 (PDPK1) in brain endothelial cells to trigger blood brain barrier (BBB) destruction and facilitate cancer cells migration [22]. Distant lung fibroblast derived miR-30s stabilize pulmonary vessels in premetastatic lungs, the low expression of miR-30s could directly target the skp2 and promote pulmonary vascular destabilization via activating of matrix metalloproteinase 9 (MMP9) [23]. MiR-9, packaged in the SK23 melanoma cell-derived microvesicles (MVs) and delivered to the endothelial cells directly target the negative regulator suppressor of cytokine signaling 5 (SOCS5) and activating JAK-STAT signaling pathway to promote endothelial cell migration and tumor angiogenesis [24]. Exosomal miR-92a, derived from the leukemia cell line K562, has also shown the same function [19]. Exosomal miR-135b from chronic hypoxia multiple myeloma cells directly suppressed its target factor-inhibiting hypoxia inducible factor 1 (FIH-1) in endothelial cells and enhances the endothelial tube formation under hypoxia via the HIF-FIH signaling pathway [20]. Similarly, hypoxia can stimulate various miRNAs contain miR-210 overexpression in exosomes, so that promoting tumor angiogenesis [25].

The formation of premetastatic niche not only involves the angiogenesis and vascular permeability, but also the metabolic reprogramming and stromal reprogramming to facilitate tumor metastasis. Breast cancer can secret miR-122 enriched vesicles, which have been proved associated with breast cancer metastasis [26]. It can be taken up by the recipient premetastatic niche stromal cells such as astrocytes and lung fibroblasts to downregulate the glucose metabolism by decreasing the glycolytic enzyme pyruvate kinase (PKM) and glucose transporter 1 (GLUT1) [27]. Due to the availability of nutrient for the upcoming tumor cells, this new mechanism may be more important at an early stage prior to cancer-induced angiogenesis. What is more, EVs, rich in miR-494 and miR-542-3p, accelerate the premetastatic niche formation by targeting cadherin-17 and overexpressing the MMP2 and MMP3 in premetastatic lung stromal cells [28]. Transfection with a large amount of exosomal miRNAs (miR-100-5p, miR-21-5p, and miR-139-5p) in normal prostate fibroblasts increased the level of MMP2, MMP9, and MMP13 which relate to ECM and receptor activator of NF-κB ligand expression [29]. Meanwhile, prostate cancer stem cells- (CSCs-) derived exosomes are different from bulk tumor derived in miRNA content which may modify the premetastatic niche by fibroblast migration and contribute to invasion [29]. In addition, exosomal miRNAs (miR-16, miR-21, and miR-29a) from tumor can bind to Toll-like receptors (TLR) in surrounding immune cells and trigger a TLR-mediated prometastatic inflammatory response [30]. Recently, a research shows that lung epithelial cells are critical for lung metastatic niche formation by tumor exosomal ncRNAs via TLR3 [31].

Therefore, most of the cancer-derived miRNAs, which packaged into vesicles as a class of small ncRNAs, to a certain extent play a crucial role in regulating numerous components and signaling pathway of premetastatic environment to facilitate metastasis. There is still spacious room for development in the function of various kinds of miRNAs in premetastatic niche formation.

3.2. LncRNAs. LncRNAs, such as CCAT2, DREH, HOTAIR, H19, lincROR, MALAT, and BCAR4, have relevant data and clinical significance in vivo and in vitro metastasis [32]. However, due to the less knowledge in the field, lncRNAs have been rarely studied in premetastatic niches.

With the increased studies of lncRNAs, they found that the imbalance of lncRNAs is involved in the regulation of tumor progression [33]. They can also release exosomes, change the cell physiology of remote nontumor cells, and enable the early survival of disseminated tumor cells in the premetastatic niche [34]. In CD90+ hepatocellular carcinoma cells, H19 is transferred and internalized by endothelial cells, and promotes angiogenesis and intercellular adhesion by upregulating the production and release of VEGF [35, 36]. Integrin β1/α5/JNK/c-JUN signaling pathway participates in higher matrix stiffness, which is induced LOXL2 increasing in liver cancer cells. LOXL2 promotes the production of fibrin, the expression of MMP9 and CXCL12, and the recruitment of BMDCs to help the formation of premetastatic niche [37]. In PDAC, exosome derived protein MIF can induce the formation of hepatic premetastatic niche and enhance the expression of liver metastatic. LncRNA Sox2ot regulate Sox2 expression to promote epithelial-mesenchymal transformation (EMT) and stem cell-like properties by competitively binding the miR-200 family [38]. Thereby, it promotes the invasion and metastasis of PDAC. The researchers from the University of Wisconsin School of medicine and public health hypothesized that anaplastic thyroid carcinoma (ATC) cancer stem-like cells (CSC) secrete lncRNAs transferred by exosome. It is important that linc-ROR induces EMT and establishes premetastatic niche [39] (Table 1).

3.3. CircRNAs. CircRNAs were once thought to be RNA splicing errors. However, they have now been shown to exist in many eukaryotic transcripts [40]. CircRNAs are mainly reported as miRNA sponges that affect downstream miRNAs target genes, regulate selective splicing, and affect host gene transcription [41]. Among circRNAs studies, miR-7 is the most studied miRNAs, which regulates multiple functions in the process of cancer, such as cell development, proliferation, and apoptosis [42].

The presence of circRNAs in exosomes was confirmed in cancer cell lines of hepatocellular carcinoma, colon cancer, lung cancer, gastric cancer, breast cancer, and cervical cancer. But circRNAs have not been reported in premetastatic niches.

3.4. Cross Talking. MiRNAs, LncRNAs, and circRNAs all have mutual regulation relationships, forming a cross talking network. NcRNAs and mRNAs can form a well-regulated interaction network [43]. It is well known that miRNAs play a role by targeting the 3 UTR of mRNAs [44]. LncRNAs-mRNAs interaction is like that of miRNAs-mRNAs. LncRNAs can bind to multiple mRNAs, and one mRNA can be targeted by multiple lncRNAs [45].

Table 1: Recently reported ncRNAs-mediated adaptations in a premetastatic niche.

ncRNAs species		Cancer type	Biological function	Mechanism	Refs
miRNA	miR-9	melanoma	promote migration and angiogenesis	target to SOCS5 and activating JAK-STAT signaling pathway	[24]
	miR-16, miR-21, miR-29a	lung cancer	promote tumor growth and metastasis	bind with TLR, trigger TLR-mediated pro-metastatic inflammatory response	[30]
	miR-92a	leukemia	regulate endothelial gene expression	Downregulate target gene integrin a5, play a similar role in endogenous miRNAs in HUVECs	[19]
	miR-100-5p, miR-21-5p, miR-139-5p	prostate cancer	modify pre-metastatic niche	increase MMP2, 9, 13, RANKL and fibroblast migration	[29]
	miR-105	breast cancer	promote metastasis	destroy the vascular barriers by downregulating ZO-1	[21]
	miR-122	breast cancer	induce angiogenesis	downregulate the glucose metabolism by decreasing PKM and GLUT1	[26]
	miR-135b	multiple myeloma	enhance endothelial tube formation under hypoxia	suppress FIH-1 in endothelial cells via the HIF-FIH signaling pathway	[20]
	miR-181c	brain-metastatic breast cancer	facilitate migration	downregulate PDPK1 in brain endothelial cells to trigger BBB destruction	[22]
	miR-210	leukemia	affect angiogenesis	exosomes containing a high expression level of miR-210 downregulate EFNA3	[25]
	miR-494, miR-542-3p	pancreatic adenocarcinoma	accelerate the pre-metastatic niche formation	target cdh-17 and over express MMP2 and MMP3	[28]
lncRNA	H19	hepatocellular carcinoma	promote angiogenesis and intercellular adhesion	up-regulate the VEGF, promote MMP9, CXCL12, and the recruitment of BMDCs	[35–37]
	LncRNA Sox2ot	Pancreatic duct adenocarcinoma	promote the invasion and metastasis	regulate Sox2 expression by binding the miR-200 family competitively	[38]
	lncRNA MALAT1, linc-ROR	anaplastic thyroid carcinoma	establish pre-metastatic niche	induce EMT	[39]

MD et al. summarized four ways of interaction between lncRNAs and miRNAs. The first is that LncRNAs are precursor molecules of miRNAs, for example, the precursor of H19 encoding miR-675 [46]. The second is that miRNAs reduce the stability of lncRNAs. Yoon et al. reported that RNA binding protein HuR recruits let-7/Ago2 to degrade lncRNA-p21 [47]. Thirdly, LncRNAs compete with miRNAs. For instance, the antisense transcript of CDR1 is almost completely complementary to miR-671 which can guide the degradation of the antisense transcript of CDR1 by AGO2-dependent methods [48]. Finally, as a sponge of miRNAs, LncRNA X chromosome inactivated specific transcript (XlST), inhibited the expression of miR-499a and promoted the expression of B-cell lymphoma-2 (Bel2) [49]. LncRNAs competitively bind to miRNAs as competitive endogenous RNA (ceRNA) [50].

CircRNAs have a sponge-isolating effect on miRNAs [51]. Cirs-7 (CDR1as) was found to isolate tumor suppressor miR-7 which inhibits EGF receptors in oncogenes such as IRS-1, IRS-2, and Raf1 in glioblastoma [52]. The ciRs-7/miR-7/miR-671 axis confirmed the significance of circRNAs entrapment on miRNA in cancer (Figure 2).

4. Clinical Utility of ncRNAs

4.1. TDMCs: A Source of Novel and Specific Biomarkers of Cancer. Early detection of reliable cancer biomarkers is critical to diagnosis. Ideally, biomarkers should be targeted to specific tumor types and detected noninvasive techniques prior to transfer. Tumor-derived exosomes are involved in the interstitial interaction between tumor cells and surrounding tissues and promote the development of malignant tumors.

Circulating miRNAs have great potential as a biomarkers. They bind to exosomes/microvesicles or protein complexes and are chemically stable to RNase activity and reproducible

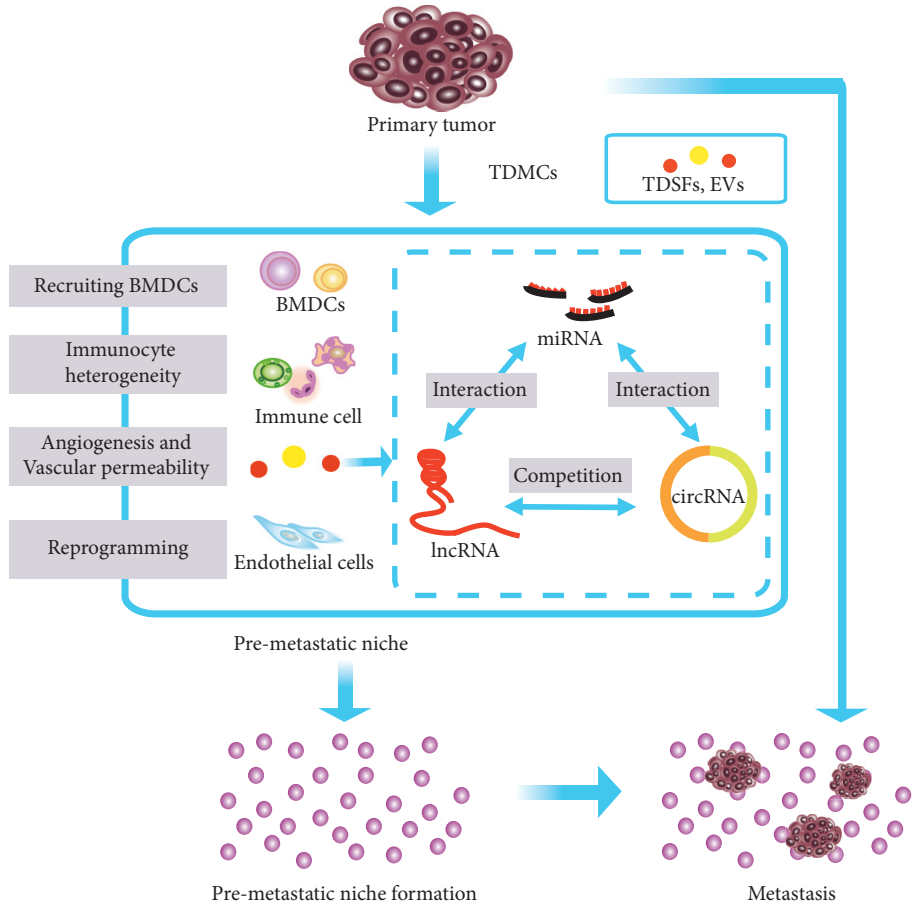

Figure 2: Mechanism of premetastatic niche formation. Primary tumors prior modulate the premetastatic niche in preparation for tumor cell colonization even before their arrival. TDMCs have the functions of recruiting BMDCs, making the immune heterogeneity, altering the vasculature, and reprogramming. They create premetastatic niche as the signal transduction mediators. NcRNAs, one of TDMCs, are involved in the formation of premetastatic niche. MiRNAs, lncRNAs, and circRNAs are building a well-regulated interaction network by directly regulating with each other.

in the blood. Rodriguez M et al. confirmed that miR-196a-5p and miR-501-3p were downregulated in prostate cancer samples by rt-qPCR analysis in an independent cohort study, suggesting that specific miRNAs in urinary exosomes may serve as a noninvasive biomarker for the diagnosis of prostate cancer [53]. A higher level of circulating miR-122 was detected in early breast cancer metastasis progression [54], and a lower level of miR-125b in serum circulating exosomes of advanced melanoma could be used as a predictive marker [55]. In addition, these EVs can be used for the detection of tumor metastasis. The exosomes of patients with metastatic sporadic melanoma were compared with familial melanoma or the unaffected control group, and higher expression levels of miR-17, miR-19a, miR-21, miR-126, and miR-149 were found [56]. Exosome miR-21 is associated with esophageal cancer recurrence and distant metastasis [57]. Fleming et al. demonstrated that evaluating miR-15b, miR-30d, miR-150, and miR-425 was more likely to predict melanoma recurrence than TNM staging [58].

In addition to miRNAs, lncRNAs associated with tumor-derived exosomes are also attractive as potential biomarkers. LncRNA PCA3 (DD3) is an approved urinary biomarker and prebiopsy diagnosis of prostate cancer [59]. Elevated lncRNA ZFAS1 in serum exosomes of GC patients may represent a better biomarker for GC diagnosis [60]. In 2015, the plasma study of liver cancer patients found that lncRNA XLOC_014172 and LOC149086 are potential biomarkers for liver cancer metastasis [61]. Furthermore, the release of the regulation of high specific expression of lncRNAs in tumor tissues can be used as targets for the treatment of cancer [62].

CircRNAs, which are stably expressed in exosomes, are promising candidates for cancer biomarkers. Many circRNAs exhibit tissue-specific and developmental stage-specific patterns. CircRNAs are also described as biomarkers of aging in Drosophila [63]. In gastric cancer, the abundance of Hsa_circ_002059 was low. It was also lower in cancer tissues and adjacent tissues [64]. Chen et al. found that circPVT1 was upregulated in gastric cancer. It was an independent prognostic indicator for overall survival and disease-free survival of gastric cancer patients. This finding suggests that circPVT1 is a new prognostic marker for gastric cancer [65].

NcRNAs in circulating EVs may be a new and specific source of cancer biomarkers. Currently, the use of multiple circulating ncRNAs as reference genes may be more applicable.

4.2. Targeting ncRNAs in the Premetastatic Niche for Cancer Therapeutics

4.2.1. The Therapeutic Potential of ncRNAs. NcRNAs have been proved to play an important role in tumorigenesis, typing, metastasis, drug resistance, and therapeutic effect [66]. Hence, many ncRNAs can be targeted for tumor therapy. What needs to be further investigated is how to consume EV or block the uptake of EV in cancer patients [67]. Functional studies have confirmed that miRNAs dysfunction is the cause of many cancers. MiRNA mimics and miRNA-targeting molecules (antimiRs) show promise in preclinical development [68]. MiRNAs have also been used in studies to sensitize tumor cells to chemotherapy. MiR-375 cotransports cisplatin nanoparticles as a promising treatment for liver cancer [69]. The fact that most lncRNAs are carcinogenic and upregulated during cancer suggests the possibility of therapeutic intervention. The specificity of lncRNAs has been used to selectively kill tumor cells, providing a pathway for targeted therapy. In addition, gene therapy can be tried to deliver beneficial tumor suppressor lncRNAs [70]. As for circRNAs, inhibiting the relevant f-circRNAs may be a new therapeutic approach, especially in the fight against cancer drug resistance [71].

4.2.2. Therapeutic Target Groups of Specific ncRNAs. Before precise tumor treatment, it is very necessary to study the best miRNA candidates or miRNA targets for each disease. Recently, miR-CLIP seq technology can identify miRNA-mRNA association and sequencing [72]. The miR-CLIP capture technique can also identify the interaction between miRNAs and lncRNAs [73]. For example, the sponge effect of lncRNA H19 on mir106a upregulated the corresponding mRNA level [74]. In addition, it will be necessary to further understand the interaction between lncRNAs and circRNAs.

4.2.3. Small Interfering RNA (siRNA) Therapy. To date, miRNAs and small interference RNAs (siRNA) play an important role through RNAi mechanism and are widely used in gene therapy, especially in cancer therapy. The efficiency of siRNA depends on a number of factors, such as targeted sequences, end modification, and whole-body delivery methods [75]. One of the most commonly used methods to inhibit the upregulation of carcinogenic lncRNAs is to deliver siRNA to target cells. Furthermore, lncRNAs expression was inhibited by the use of antisense oligonucleotide (ASO) or small molecule inhibitors [76]. Among them, some are undergoing Phase III clinical trials in prostate cancer, so far; however, most are still in the period of basic research [77].

4.2.4. NcRNAs Replacement Therapy. One is the use of antimiRs, common inhibitors AntigomiRs, locked nucleic acid (LNA), etc., to block the expression of miRNAs targeting mRNA in tumor and premetastatic niche [78]. MiR-15a and miR-16-1 provide indications for miRNA-based treatment, and strong regulation of clonal amplification of CLL plays a role at the level of mature leukemia [79]. AntimiRs targeting miR-122 have reached phase II clinical trials for hepatitis [80]. MiR-10b antagomirs inhibit the metastasis of breast cancer in mice by silencing endogenous miR-10b, which is well tolerated in animals and is a promising candidate for the development of new antimetastatic drugs [81].

The second is the use of miRNAs mimics, which are a new therapeutic approach. In order to protect miRNAs from degradation by serum nucleases, miRNAs mimics were specifically delivered to tumor interested miRNAs by using the nanoparticle delivery system [82, 83]. For example, MiR-125b was directly injected into subcutaneous tumors using a nonviral vector composed of cationic polymer polyethylamine (PEI), targeting VE-cadherin to induce nonfunctional angiogenesis and inhibit tumor growth [84]. MiR-34, an analogue of tumor suppressor, has reached stage I clinical trials for the treatment of cancer [85]. Other ncRNAs have not been explored for their functional regulation.

AntimiRs drugs combined with chemotherapy or targeted therapeutic drugs (such as small molecules or antibodies) are used to reduce miRNAs, potential checkpoint inhibitors, and to reduce adverse reactions. Therefore, further studies are needed to resolve some controversial issues to achieve the clinical application of exosomes in the microenvironment of premetastatic niche.

5. Conclusions and Perspective

From traditional therapy to molecular targeted therapy, great progress has been made in the treatment of primary tumors. However, for the treatment of the formation of premetastatic niche, how to identify and kill the premetastatic niche at an early stage, and how to avoid the toxic and off-target effects of ncRNAs drugs targeted premetastatic niche need to be solved urgently.

As one of TDMCs, ncRNAs has been confirmed to be involved in the formation of premetastatic niche. By understanding the formation mechanism of premetastatic niche through TDMCs, especially ncRNAs, the hypothesis of premetastatic niche is further studied, which may provide new strategies for us to develop new targeted antitumor drugs, thus greatly improving the therapeutic effect and prognosis of tumors. In this regard, siRNA therapy and ncRNAs replacement therapy through exosomes are promising strategies with initial results, while the physiological and pathological effects of exosomes such as lncRNAs and circRNAs in the premetastatic niche still need to be further studied. Moreover, the latest miR-CLIP seq technology and miR-CLIP capture technology recognize the association between miRNAs and mRNAs, miRNAs and lncRNAs, respectively. The cross talking of miRNAs with circRNAs, lncRNAs, and circRNAs is for further study. In the future, identify the interactions between ncRNAs before performing precise tumor therapy, study the optimal ncRNA candidates or ncRNA targets for each disease, and target inhibitors for their

interaction, which may provide the next generation of precise personalized drugs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Exosomes Modulate the Viral Replication and Host Immune Responses in HBV Infection

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Although current diagnosis and treatment of hepatitis B virus (HBV) infection can maintain viral suppression, new therapies need to be invented to sustain off-treatment virologic suppression and reduce side effects. Exosomes act as intercellular communicators to facilitate direct transfer of proteins, lipids, and nucleic acids between cells *in vitro* and *in vivo*. Pioneering work has demonstrated that exosomal cargos changed markedly during HBV infection. An improved understanding of the functions of exosomes during HBV infection could lead to a powerful new strategy for preventing and treating HBV. In this review, we point out the role of exosomes in HBV infection: (1) exosomes could directly participate in HBV replication; (2) exosomes modulate immune response during HBV infections; (3) exosomal RNAs and proteins might be selected as novel biomarkers for the diagnosis of HBV infections; and (4) exosomes can also be designed as vaccines.

1. Introduction

Since Blumberg et al. discovered the hepatitis B virus (HBV) in 1965 [1], much has been elucidated regarding its genome, sequence, epidemiology, and hepatocarcinogenesis. HBV belongs to hepadnaviridae and mainly infects hepatocytes [2]. Despite much has been yielded in the effort to discover effective treatments for HBV, the interferon α (IFN-α) and Nucleotide Analogs (Lamivudine, Telbivudine, Entecavir, Adefovir, and Tenofovir) have been proved as the effective treatment for HBV chronic infection [3, 4], HBV infection continues to be a significant public health problem worldwide. Approximately, more than 2 billion people were infected by HBV and the infection appeared more frequently in the Asia-Pacific region. Though the exact pathogenesis of HBV is not yet completely understood, the current thought is that the virus replication is not directly cytotoxic to hepatocytes, but rather, it is the immune response that mediates the damage of infected liver cells [5, 6].

Exosomes are multiform, 30-150 nm diameter cup-shaped vesicles which are secreted by almost all types of cells. Exosomes can be found in most body fluids (like serum, plasma, lymph, saliva, urine, tears, sweat, semen, cerebrospinal fluid, and breast milk), as well as cell culture supernatants [7]. Exosomes have variable components which can reflect the status of host cells, and many researches found that exosomes can encapsulate lipids and multiple types of proteins including membrane fusion-related proteins, proteins involved in vesicle formation, integral membrane proteins, components of the major histocompatibility complex (MHC) classes I and II, proteins related to the cytoskeleton and cell metabolism, and cell surface proteins involved in oncogenesis [8, 9]. Additionally, nucleic acids including mRNA, miRNA, long non-coding RNAs (lncRNAs), and DNA have also been detected inside exosomes [10, 11]. In addition, the exosomal membrane helps to protect these cargos from enzymatic degradation, and exosomes have other attractive features, such as low immunogenicity, high biocompatibility, and the ability to overcome biological barriers [12]. So recent studies highlight the importance of exosomes in intercellular communication by transmitting biological signals between cells to regulate a diverse range of physiological and pathological processes. For example, neuronal exosome release is triggered by Ca^{2+} entry through

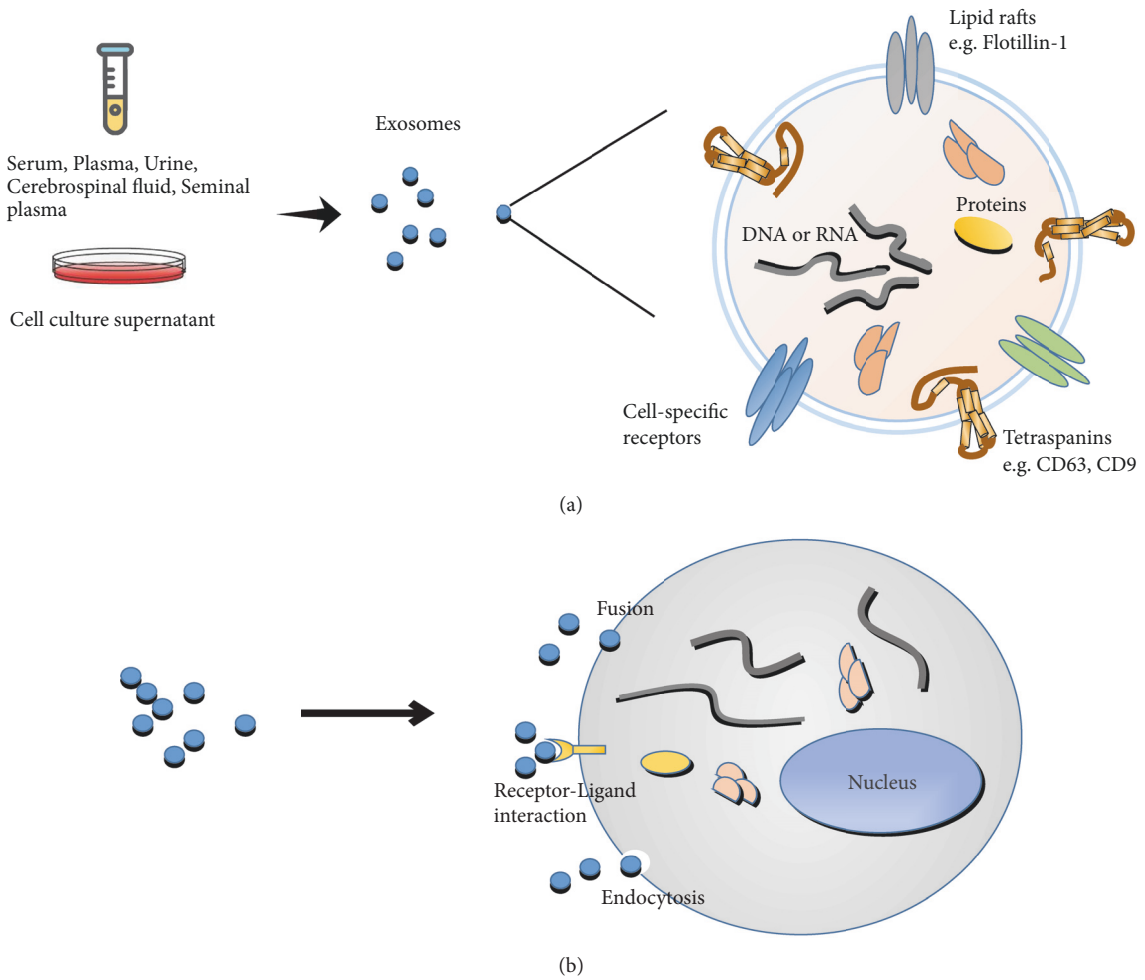

Figure 1: The characteristics of exosomes. (a) Exosomes are nanovesicles originated from membranes, the diameter from 30 nm to150 nm. In normal and pathological conditions, cells release exosomes to extracellular matrix and can be detected in many types of body fluids (serum, plasma, lymph, cerebrospinal fluid, saliva, urine, tears, sweat, etc.) and cell culture supernatants. (b) Exosomes play the role of information transactors among cells through three ways: (1) fusion directly with the target cell membrane; (2) the exosomal ligands binding to receptors of target cell; (3) the soluble components of exosomal proteins active endocytosis of target cells. Then, exosomes transfer bioactive nucleic acids, proteins, and lipids to target cells.

N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors at glutamatergic synapses, suggesting that exosome release may be part of normal synaptic physiology [13]. These exosomes can play the important role of cell communication on both neighboring and distal cells in the central nervous system (CNS). Besides, exosomes have been tightly linked to pathological processes to transport tumorigenesis factors such as CD97 which can activate of MAPK signaling pathway to promote gastric cancer (GC) cell proliferation and invasion [14], as well as the spread of autoantigens such as GAD65 and IA-2 which may accelerate diabetes onset *in vivo* by stimulating the autoimmune response [15]. Because exosomes with low immunogenicity, so *in vivo*, dendritic cells derived exosomes probably need a danger signal to be presented by an "exosome-presenting cell" for inducing an immunogenic response; without a danger signal, dendritic cells derived exosomes could mediate tolerance [12]. And studies found that during viral infections, exosomal cargos changed profoundly, and many researches indicated that exosomes isolated from virus-infected cells contain pathogen agents such as HCV RNA [16] and HIV-1 [17] which can, respectively, promote HCV and HIV infections [18, 19]. Meanwhile, some specific contents in exosomes can also play pivotal anti-infection roles through directly inhibiting viral replication and/or inducing antiviral immune responses [20]. Via exosomes transmitting and delivering, these functional components enter into recipient cells to process intracellular communication and various biological activities (Figure 1). In this review, we summarize the role and probable mechanisms of exosomes participating in cellular crosstalk during HBV infection.

2. Exosomes Participate in HBV Replication

Many enveloped viruses are reported to employ exosomes related proteins to form enveloped virions. HBV envelope

proteins colocalize with multivesicular bodies (MVB, including exosomes) related proteins AIP1/ALIX and VPS4B, and either of these proteins being negative mutant can block the production and/or release of enveloped HBV virions [21]. Some viruses (like HCV and HIV) can hijack host exosomes; they can assimilate viral constituents into exosomes. And these exosomes distribute from infected cells transport various vital components to neighboring cells which help in regulating cellular responses and producing infections [22]. This has led to the proposal of the "Trojan exosome hypothesis," and HBV and exosomes can have similarities in their biogenesis (Table 1). HBV RNA and viral DNA were detected in $CD81^+$ exosomes from HepG2 cells with pHBV [23]. And exosomes derived from CHB patients serum also contained HBV DNA (rcDNA and cccDNA) and RNA (HBx and HBs/p), as well as HBsAg. And these exosomes can transmit HBV to uninfected hepatoma cells. Furthermore, exosomes derived from CHB patients suppress the cytotoxicity of NK cells, the expression of degranulation molecule CD107a and activating receptor NKp44, and the production of IFN-γ and the tumor necrosis factor (TNF)-α. These data suggest that exosomes derived from CHB patients can transmit HBV infection as free virus and depress the function of NK-cell dysfunction [24]. Several proteins encoded by the HBV genome, including large S, core and P proteins, were found inside exosomes isolated from an HBV-inducible cell line HepAD38. Besides, the differences of exosomal protein contents secreted by the HepAD38 cell line with or without HBV replication were evaluated by label-free proteomic analysis; 1412 protein groups were identified and bioinformatic analysis revealed that 32.98% exosomal proteins were plasma membrane-associated proteins as well as some proteins participating in the regulation of cytokine production or mediating signaling pathways. These data may provide insights into the potential function of exosomes in HBV-host interaction and immunomodulatory effect during HBV infection [25]. Since the first report of EBV can encode miRNAs (such as ebv-miR-BHRF1-1 and ebv-miR-BART1), it has been demonstrated that many viruses, like HIV and HCV, can encode specific viral miRNAs which play important roles in diverse cellular processes, including interactions between virus and host [26]. An HBV-encoded miRNA (called HBV-miR-3) was identified by deep sequencing, the HBV-miR-3 expression was detected in patients with HBV infection, and the expression level was significantly higher in the sera of patients with HBV infection in the acute phase than in those in convalescence. Meanwhile, HBV-miR-3 was released into the circulation by exosomes isolated from HepG2.2.15 (a human liver hepatocellular carcinoma cell line which contains complete HBV genome and is capable of stable HBV expression and replication in culture). The exosomal HBV-miR-3 can target a unique site of HBV transcript to reduce the level of HBV pregenomic RNA (pgRNA) and HBV core protein (HBc) protein and finally inhibit the HBV replication and protein expression [27]. These data indicate that HBV-encoded miRNAs may control self-replication by targeting viral transcripts, and the process may contribute to HBV persistent infection in patients. HBV x (HBx) protein is required for viral infection and replication, and it is closely associated with the development of hepatocellular carcinoma (HCC). Moreover, both HBx mRNA and protein exist in exosomes isolated from the HBx-expressing hepatoma cells and mass spectrometry (MS) studies suggest that the entrapped HBx mRNA could be translated into viral protein in hepatic stellate cells. And HBx could increase the production of exosomes through inducing the activity of nSMnase2 (the key enzyme involved in exosomes biogenesis) [28]. These data presented establish that HBx can modulate the biogenesis of host exosomes and alter neighboring liver cells. During HBV infection, HBx selectively decreases intracellular APOBEC3G (A3G) protein level and its effects on intracellular A3G level by affecting its export through exosome production and secretion. A3G belongs to apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) family which has been generally considered as a restricting factor of HBV infection [29, 30]. These data might provide a new insight into the mechanism of HBx-mediated activation of HBV which might involve regulating host restriction factor A3G. In addition, proteomic analysis data demonstrate the content of exosomal proteins change markedly isolated from Huh-7 cells (originated from human liver cells) infected with HBx, HBV, and HBV(HBx-), respectively, and these exosomal proteins display little overlap between each group. Furthermore, compared with HBV negative controls, exosomal proteins isolated from HBV-infected patients showed specific changes. These exosomes contained higher levels of HCC related proteins, like heat shock protein 90B1 (HSP90B1) and valosin containing protein (VCP) [31]. Even these results highly illustrated that exosomes contribute to HBV spread and can modulate host biological activities, but whether exported components contained in exosomes have any effects on anti-HBV defense of recipient cells still needs future investigations, and the interaction between HBV induced exosomes and host is also worth pursuing.

3. Exosomes Modulate Immune Response during HBV Infection

Exosomes can elicit immune response as well as serve to transfer pathogens to their reservoirs in order to support latency. Therefore, altered exosomal components could determine the fate of viral infection and disease progression [32]. $CD81^+$ exosomes released from hepatocytes with pHBV induced NKG2D ligand expression in macrophages through MyD88, TICAM-1, and MAVS-dependent pathways; these data suggest the importance of exosomes for macrophage function. Moreover, exosomal miR-21 and miR-29a, as well as other immunosuppressive miRNAs levels, were markedly increased by HBV infected HepG2-NTCP cells. miR-21 downregulates IL-12p35 mRNA expression and miR-29a is known to suppress IL-12p40 mRNA expression. So these exosomal miRNAs may inhibit NK cells activity through downregulating IL-12 expression [23]. These observations indicate that exosomes play a crucial role in the innate immune response against HBV. And exosome would mediate viral escape from the host innate immune response through downregulating IL-12. When NK cells from health donors

Table 1: HBV elements packaged within exosomes and their effects.

HBV element		Source	Effect	Ref.
HBV DNA	rcDNA cccDNA	CHB patients serum	Transmit HBV infection	[23, 24]
HBV RNA	HBx HBs/p	HBx-expressing hepatoma cells; CHB patients serum	Transmit HBV infection	[23, 24, 28]
HBV surface protein	HBsAg	CHB patients serum	Transmit HBV infection	[24]
HBV-encoded proteins	Large S Core protein P protein	HepAD38 cell line with HBV replication	Transmit HBV infection	[25]
	HBx protein	HBx-expressing hepatoma cells	Support viral spread and pathogenesis; export of intracellular A3G via exosome	[28, 29]
HBV-encoded miRNA	HBV-miR-3	HBV infected patients sera; HepG2.2.15 cells	Regulate HBV replication	[27]

co-cultured with exosomes derived from chronic HBV infection patients sera, the HBV rcDNA and HBV RNA can be transmitted into NK cells. And these transmitted HBV components can reduce CD107a (a cytotoxicity mediator of NK cells) to lower cytotoxicity of NK cells, as well as the proliferation of NK cells [24]. This means HBV-induced exosomes can influence the function and survival of NK cells. Therefore, the intricate relationships among various viral components and host factors could determine whether viral clearance or persistence occurs. Besides, exosomes can deliver proteasome subunit proteins to monocytes. HepAD38, the HBV-inducible cell line, secreted exosomes contain higher proteasomal activity proteins. Transmitting via exosomes these specific proteins can suppress IL-6 expression in monocytes [25]. These data demonstrated that HBV-induced exosomes might influence the production of proinflammatory molecules in the recipient monocytes. In addition, exosomes isolated from HepAD38 cells with HBV replication can strongly upregulate programmed-death ligand 1 (PD-L1) expression in monocytes compared with exosomes secreted from HepAD38 cells without HBV replication [33]. Programmed-death protein 1 (PD-1) expressed on T cells binding with PD-L1 induces the T cell exhaustion and protects target tissues from immune-mediated damage. So HBV-induced exosomes might promote HBV infection by suppression of T cells. Type I interferons (IFNs), mainly interferon-α (IFN-α) and IFN-β, serve as an important role in controlling viral replication during the initial stages of infection [34]. IFN-α can induce the transfer of resistance to HBV from nonpermissive liver nonparenchymal cells (LNPCs) to permissive hepatocytes via exosomes. Some specific antiviral activity molecules can be sorted into exosomes from IFN-α-treated LNPC. These antiviral molecules can be transferred through internalizing exosomes to hepatocytes and can attenuate HBV replication in hepatocytes [35]. Also, viral antigen expression and DNA quantification results indicate that exosomes isolated from IFN-α treated macrophages can efficiently transfer IFN-α-induced anti-HBV activity to HepG2.2.15 cells [36, 37]. IFNs function as natural antiviral mechanisms and have various therapeutic applications. After binding to the interferon receptor complex (IFNAR1-IFNAR2), IFN-α and IFN-β can be signalled through a kinase of the Jak family to the signal transduction and activator of transcription (STAT) pathway to the transcription of interferon-stimulated genes (ISGs: ISG15, ISG56, MxA, etc.), which are under the control of interferon-stimulated response elements (ISREs) [38]. The expression of ISGs establishes an antiviral state in host cells during viral infections. A previous study demonstrated that human liver endothelial cells could release ISG-enriched exosomes, which can inhibit HCV replication in liver parenchymal hepatocytes [39]. Later, exosomes from human brain microvascular endothelial cells were identified which contain antiviral factors including several key ISGs, such as ISG15, ISG56, and Mx-2 [40]. These data and findings have introduced and expanded the idea that exosomes enriched with ISGs may be vital in sensing and controlling viral infection. Whether HBV infection can induce ISGs enrichment in exosomes remains to be determined. As mentioned, exosomes and HBV are mutually influenced and mutually stimulated, and the function of exosomes during HBV infection is like a double-edged sword (Figure 2).

4. Exosomes as Markers for HBV Diagnosis

As described above, exosomes are involved in multiple steps during HBV infection; quantitative and qualitative analyses of the differences in the composition of exosomes in health and infected patients have been extensively reported. These differences, together with easy isolation and relatively stable for storage, make exosomes become excellent biomarker reservoirs as well as potential applications for diagnosis [41]. Recent studies identified some specific exosomal cargos as biomarkers for different liver diseases [42]. The level of HBV DNA and serum alanine aminotransferase (ALT) has been commonly used in estimating liver disease and as the important criterion for defining which patients need therapy. However, relying on serum ALT levels as a prerequisite to choosing treatment candidacy has limitations [43]. For example, 18 exosomal miRNAs (like hsa-miR-221-3p, hsa-miR-25-3p) were upregulated and 6 exosomal miRNAs (like hsa-miR-372-3p, hsa-miR-10a-5p) were downregulated in persistently normal ALT (PNALT) patients with the liver tissue inflammation. So these exosomal miRNAs are more sensitive than ALT to assess liver inflammation in the CHB patients with PNALT [21]. In addition, the levels of hnRNPH1 mRNA and miR-21 in serum exosomes isolated from HCC patients were remarkably higher than in CHB group. Receiver operating characteristic (ROC) curve analyses showed that exosomal hnRNPH1 mRNA level can discriminate HCC from CHB. The detection of serum exosomal miR-21 is also more sensitive than in serum, so hnRNPH1 mRNA and miR-21 in exosomes may serve as sensitive and specific biomarkers to diagnose HCC and distinguish CHB [44, 45]. The interest in using miRNAs within circulating exosomes as noninvasive biomarkers has increased rapidly; because of the exosomal lipid bilayer membrane, miRNAs are protected from degradation and keep stable in the body fluids. Despite the great benefits of exosomal miRNAs in diagnosis, there are several issues that still need to be addressed. Firstly, the selection of suitable reference genes as normalization factors is necessary to accurately compare exosomal miRNA transcripts. In particular, U6 (CCG-1) or miR-181a (RG-5d) had lower sensitivity for the comparability of miR-21 expression between CHB patients and HCC patients. The combination of miR-221, let-7a, miR-191, miR-26a, and miR-181a (RG-5d) was the optimal reference gene set, for the comprehensive investigation into the progression of CHB to HCC [46]. These findings highlight the importance of validating reference genes before quantifying target miRNAs. Secondly, biological body fluids are the rich source of exosomes with different origins, which make it difficult to isolate HBV-induced exosomes. The specificity of exosomal miRNAs still needs to be validated. For example, exosomal miR-21 is also highly expressed in glioblastomas pancreatic cancer, colorectal cancer, breast cancer, and colon cancer [47–49]. Therefore, large-scale studies of HBV and HCC patients would determine the value of differentially expressed

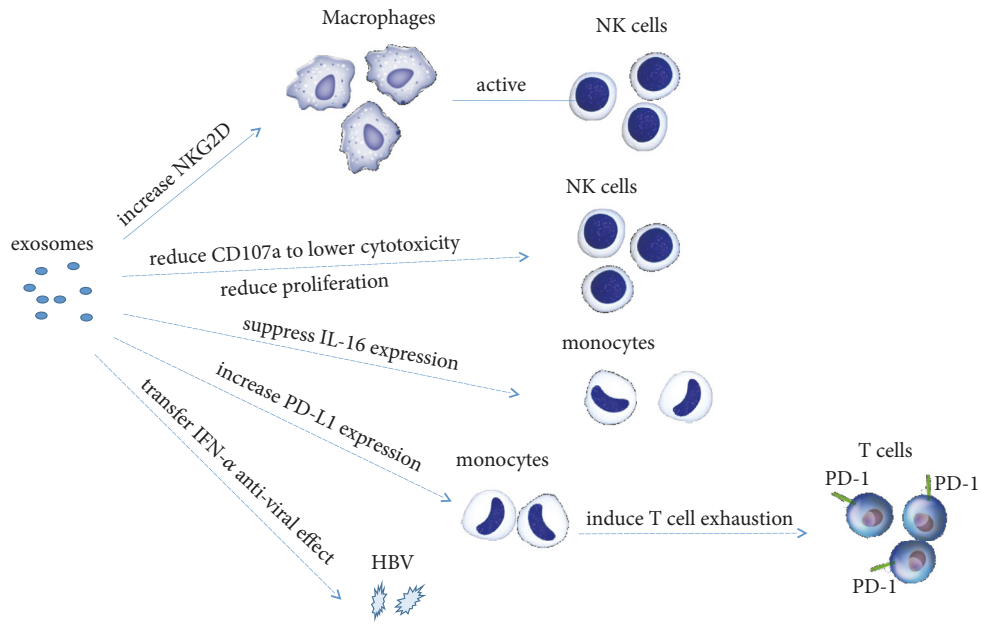

Figure 2: Exosomes modulate immune response during HBV infection. On the one hand, exosomes can play anti-HBV infection roles by increasing macrophages and NK cells function and delivering antiviral molecules among cells. On the other hand, HBV induced exosomes can promote HBV infections by inhibiting immune responses directly or indirectly and influencing cytokine-mediated signaling pathways and cytokine production.

exosomal miRNAs as potential biomarkers for distinguishing HBV patients from HCC. CHB patients on long-term treatment with nucleoside or nucleotide analogs are at risk of selecting drug-resistant HBV mutation strains. In two cases of HBV-infected patients whose treatment with entecavir (ETV) and tenofovir (TDF) is ineffective, rtS78T mutation was found in the reverse transcriptase (RT) gene of the HBV genome. The mutation causes a premature stop codon at sC69 and thereby deletes almost the entire small HBV surface protein in viral particles and exosomes. These changes facilitate replication and resistance to ETV and TDF treatment [50]. Further studies are needed to predict drug resistance according to specific changes in exosomal contents. With the deep study of these correlations between exosomal components and diseases, exosomes gradually become potential diagnostic and prognostic tools.

5. Exosomes as New Therapies for HBV Infection

As the carriers of functional RNAs and proteins, exosomes have attracted much attention as novel targets to develop new drugs. As potential vehicles, exosomes can also deliver some therapeutic agents, and the therapeutical molecules can be loaded into exosomes using either passive or active means. It was found that exosomes isolated from lipopolysaccharide endotoxin (LPS)-stimulated human monocytic cell line (THP-1) can induce a proinflammatory profile in healthy mice through the induction of cytokines such as tumor necrosis factor alpha (TNF-α), chemokine (C-C motif) ligand 5 (CCL5), and interleukin 1 beta (IL-1β). Moreover, when these exosomes are used as adjuvants for hepatitis B recombinant antigen (HBsAg), the cellular immune response is induced in mice and triggered an immunomodulatory effect on the cellular immune response by increased IFN-γ concentration and hastened the appearance of IgG antibody production [51]. These results showed that the unmodified exosomes can trigger immunostimulatory effect which could make them attractive coadjuvants. Engineered exosomes can be loaded with specific molecular and delivery *in vivo* for treating diseases. Based on the fusion between exosome-anchoring protein Nef mutant (Nefmut) and HBV core protein, elicited exosomes contain huge amounts of HBV core protein. When translated in animals, cytotoxic T lymphocyte (CTL) immunization against HBV can be induced by the engineered exosomes. The activated HBV-specific CTLs reconstitution can be of significant therapeutic effect, so these engineered exosomes open a new way for vaccine candidates against HBV [52]. As further investigations on exosomes, identification and delivery of specific antiviral molecules or therapeutic agents through exosomes will be the potential therapeutic strategy for HBV treatment and control, and many additional tests will be necessary to apply them in therapy.

6. Conclusion

Exosomal vesicles can transmit signals between pathogens and the host cells regarding various aspects of the host defense. In this review, we focus on the exosome functions in the relationship with HBV infection and anti-infection. On the one hand, the exosomes secreted by HBV infected cells are

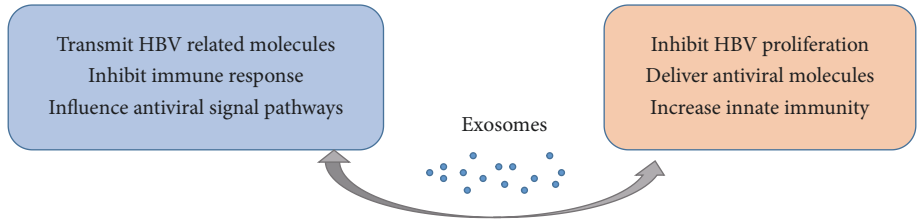

Figure 3: The dual effects of exosomes in HBV transmission and antiviral response. HBV induced exosomes containing viral proteins and RNAs can promote infection in three ways: (1) causing further infection by transmitting viral-related molecules; (2) inhibiting immune responses directly or indirectly; (3) influencing cytokine production and cytokine-mediated signaling pathways, while exosomes can play anti-infective roles by (1) inhibiting pathogen proliferation and infection directly; (2) delivering antiviral molecules among cells; and (3) increasing monocyte-macrophages and NK cells function. There must be a balance between infection and anti-infection processes, and exosomes as crucial messengers might modulate this balance in different ways as discussed in the present review.

responsible for the transmission of viral information, and the exosomal cargos may be involved in viral transmission and pathogenicity. On the other hand, exosomes can transport antiviral agents and induce antiviral response (Figure 3). Through modulating immune response, exosomes may play promoting or limiting role in the process of HBV infection, so they can be used to develop preventive and therapeutic vaccines. In addition, because exosomes originated from all cell types, the levels of nucleic acid and proteins encapsulated in exosomes will change under pathological conditions, so exosomes in body fluids can be used as noninvasive markers which have potential application prospects in early diagnosis of HCC from CHB, prediction of drug resistance, and so on.

As the understanding of exosomes functions in the process of HBV infection is still in the early phase, identification of exosomal proviral and antiviral components for cell-cell communications during HBV infection needs to be further clarified. Further functional analyses of exosomal cargoes may be important in understanding the mechanisms of HBV infection and identifying sensitive therapeutic targets.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding this work.

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Research Article

Exosomes Derived from Human Bone Marrow Mesenchymal Stem Cells Stimulated by Deferoxamine Accelerate Cutaneous Wound Healing by Promoting Angiogenesis

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The exosomes are derived from mesenchymal stem cells (MSCs) and may be potentially used as an alternative for cell therapy, for treating diabetic wounds, and aid in angiogenesis. This study, aimed to investigate whether exosomes originated from bone marrow-derived MSCs (BMSCs) preconditioned by deferoxamine (DFO-Exos) exhibited superior proangiogenic property in wound repair and to explore the underlying mechanisms involved. Human umbilical vein endothelial cells (HUVECs) were used for assays involving cell proliferation, scratch wound healing, and tube formation. To test the effects in vivo, streptozotocin-induced diabetic rats were established. Two weeks after the procedure, histological analysis was used to measure wound-healing effects, and the neovascularization was evaluated as well. Our findings demonstrated that DFO-Exos activate the PI3K/AKT signaling pathway via miR-126 mediated PTEN downregulation to stimulate angiogenesis in vitro. This contributed to enhanced wound healing and angiogenesis in streptozotocin-induced diabetic rats in vivo. Our results suggest that, in cell-free therapies, exosomes derived from DFO preconditioned stem cells manifest increased proangiogenic ability.

1. Introduction

Diabetes mellitus has not only seen an increased incidence but the complications associated with this condition have also increased in severity [1]. A ubiquitous and incapacitating impediment in diabetes is decreased diabetic wound healing. This is a medical stumbling block of today that is relevant to patients who either have feet ulcers or are recuperating from complex surgeries that have a possibility of impeding regular activity, chronic ischemic skin lesions, and possible limb amputation of limbs [2]. Wound healing thus depends on an important process of angiogenesis that is involved in delivering nutrition and oxygen to sites of wounds allowing for the proliferation of fibroblasts, synthesis of collagen, and reepithelialization [3]. Healing of wounds, however, gets extended in diabetic patients due to weakened angiogenesis [4]. Diverse biological agents, such as different platelet concentrates, have determined their potential to improve the angiogenesis both in vivo and in vitro [5]. Platelet gel, a recently developed platelet concentrates for topical use, is widely used in different areas of regenerative medicine, such as bone osteoradionecrosis, severe oral mucositis, and wound healing [6, 7]. In order to hasten the process of healing diabetic wounds, the use of new approaches that supplement the local angiogenesis looks promising.

In order to hasten the process of healing diabetic wounds, the use of new approaches that supplement the local angiogenesis looks promising. Mesenchymal stem cells that are multipotent have several potentials for differentiation and immunomodulation [8]. Cell-based therapy appears to be an optimistic therapy for alleviating a worsened diabetic wound. But this direct therapeutic approach still holds risk factors such as the development of tumors and immune responses that are not anticipated [9, 10]. Thus, there is an urgent need to devise new approaches of using stem/progenitor cells that will not have any likely harmful effects because of their direct use and will most effectively realize their therapeutic potential. Several studies have pointed out at a key class of

paracrine mediators that function between MSC and target cells: exosomes, which are small particles that are membrane-associated and have an endosomal origin with sizes in the range of 30-150 nm. These mediators bring miRNAs, mRNAs, and proteins to recipient cells and thus have an important part in intercellular communication [11]. Exosomes can be used effectively for direct treatment because they have regenerative attributes like stem cells and may surpass several unwanted effects associated with treatments involving stem cell transplantation. When administered as injections locally, exosomes cause angiogenesis and can promote proliferation and migration of skin cells and closure of wound in animal models with diabetic or burn wound [12, 13], suggesting the exosomes, as a promising therapeutic approach for wound healing.

Stem cells live in niches, which are complex microenvironments, play important roles in directing the function, division, and differentiation of stem cells [14]. Nevertheless, the important approach for in vitro and in vivo enhancement of MSC functions includes preconditioning of MSC culture conditions. The conditions that have shown improvement through tissue engineering and regenerative medicine include cytokines, hypoxia, trophic factors, physical factors, and chemical and pharmacological agents. The hypoxic preconditioning could not only increase the angiogenesis and neuroprotection but also improve proliferation and migration of MSCs, improving the efficiency of the transplantation of MSCs compared to that observed in MSCs under normal culture [15]. Various studies have determined that exosomes derived from mesenchymal stem cells preconditioned by hypoxia own better properties compared with the common MSC-exosome [16, 17]. Preconditioning with other chemical and biological factors could mimic the effect of the hypoxia, such as valproic acid (VPA), CoCl2, deferoxamine (DFO), and dimethyloxaloyl glycine. Various studies have proved that compared with the hypoxia, hypoxia mimetic agent could also induce related hypoxic genes, improving the stemness of MSCs for angiogenesis in studies and clinical tests in a more practically [18–20]. However, hypoxia mimic preconditioned MSCs have not still been used in any research to collect exosomes for being used in therapy. Several hypoxia-response genes have been shown to be induced by an agent, Deferoxamine, that is classical hypoxic-mimetic and mimics the oxygen deprivation effects [21–23].

In this study, we endeavored to study whether exosomes released from BMSCs stimulated by DFO could improve the angiogenic activity in vivo and in vitro and enhance the healing of the impaired diabetic wound.

2. Materials and Methods

2.1. Cell Culture. Human bone marrow mesenchymal stem cells (BMSCs; ScienCell) were cultured in MEM (Minimum Essential Medium) Alpha Medium (Corning) and 10% fetal bovine serum (FBS, Gibco, NY, USA). BMSCs between passages 4 and 8 were used for the subsequent experiments. Human umbilical vein endothelial cells (HUVECs; ScienCell) were cultured in Endothelial Cell Medium (ECM; ScienCell) containing 5% FBS (ScienCell) and 1% endothelial cell growth supplement (ScienCell). For DFO stimulation, DFO was dissolved in distilled water, which was then diluted in culture medium before use. BMSCs at 80% confluency were incubated for 48 hours in complete medium with exosomes-free FBS and supplemented with 50 μM DFO (Sigma-Aldrich), 100 μM DFO, 200 μM DFO, 400 μM DFO or distilled water as a negative control.

2.2. Cell Viability Assay. Cell viability was determined using the cell counting kit-8 (CCK8) assay. The BMSCs were plated at 5000 cells/well into 96-well plates and treated with medium containing 0, 50, 100, 200, and 400 μM of DFO. After 48 hours of incubation, 10 μL CCK8 reagent (Dojindo, Japan) was added to the culture medium and the absorbance of each well was observed at 450 nm by a microplate reader (Bio-Rad 680, Hercules, USA).

2.3. Exosomes Isolation. After the harvest of BMSCs conditioned medium, the cells were removed by centrifuging for 10 min at 500 g. Next, the vesicles of apoptosis along with debris were removed by centrifugation of the supernatant for 20 min at 12,000 g and filtering using a filter 0.22 μm pore size. This was followed by ultracentrifugation for 70 min at 110,000 g (Beckman Optima™ XPN, 45Ti) to collect the exosomes in the form of a pellet, which was further resuspended in phosphate-buffered saline (PBS) for further purification by ultracentrifugation for 70 min at 110,000g to remove the contaminating protein. Finally, exosomes were stored at -80°C after being resuspended in PBS. The protein content of exosomes was determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

2.4. Exosomes Characterization and Internalization. qNano platform (iZON® Science, UK) was used to analyze the distribution and absolute size of the exosomes. The morphological examination of isolated exosomes was done through transmission electron microscopy (TEM; HT7700, Hitachi, Japan). The expression of CD9, CD63, TSG101, and GM130 (the cis-Golgi matrix protein, a negative exosomal marker) in exosomes was evaluated by western blot analysis, and the following primary antibodies were used: CD9, CD 63, TSG101 (1:1000; rabbit IgG, Proteintech and GM130 (1:500; rabbit IgG, Abcam). A green fluorescent dye (DIO; Life Technologies) was utilized to label the exosomes in accordance with the instructions of the manufacturer. Ultracentrifugation at 110,000 g at 4°C for 70 min was performed to remove excess dye. HUVECs were incubated with DIO-labeled exosomes at 50 μg/mL concentration for 8 hours, followed by using of 4,6-diamidino-2-phenylindole (DAPI; Southern Biotech, Birmingham, AL, USA) to stain nuclei and then analyzed by a fluorescent microscope.

2.5. Transfection of miRNA Inhibitor. MiR-126 inhibitor and a negative control inhibitor (RiboBio, Guangzhou, China)) at 100 nmol/L were used to transfect BMSCs at 80% confluence with Lipofectamine 2000 in Opti-MEM (Invitrogen) in accordance with the instructions of the manufacturer. After

transfection for 5 hours, the cells were cultured in complete medium with or without DFO for 24 h. The supernatant of the culture was used to isolate exosomes.

2.6. Real-Time PCR. TRIzol reagent was used for isolation of total RNA from cells while exosomal miRNAs were isolated by using Exosome RNA Purification Kit (Qiagen). For miRNA analysis, the reverse transcription reactions of miRNAs were performed using 4 × Reverse Transcription Master Mix kit (EZBioscience). The qRT-PCR analyses of miRNAs were carried out with FastStart Universal SYBR Green Master (Roche). U6 small nuclear RNA was used to normalize the results.

2.7. Western Blot Analysis. Cells were harvested using RIPA lysis buffer, quantified by using the BCA protein assay kit. Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to polyvinylidene fluoride (PVDF) membranes. Blocking of membranes was done using 5% nonfat milk for 60 minutes and incubated at 4°C overnight with antibodies against the following proteins: PTEN, p-AKT, AKT (all 1:1000; Abcam), and actin (1:10,000; Thermo Fisher Scientific). Membranes were then incubated with HRP-conjugated anti-rabbit (1:1000) or anti-mouse (1:4000) secondary antibodies (Cell Signaling Technology).

2.8. The Assay for Cell Proliferation. Into 96-well plates, HUVECs at 2×10^3 cells/well were seeded. This was followed by the addition of the Exos or DFO-Exos at 50 μg/mL concentration or an equal volume of PBS to the culture medium for 48 h. The proliferation of cells was determined using CCK8 kit. A microplate reader was used to determine the optical density (OD) at 450 nm.

2.9. Scratch Wound-Healing Assay. In 6-well plates, HUVECs at 2×10^5 cells/well were seeded. On reaching confluence, sterile tip of a micropipette was used to make scratch wounds across each well. Each well was washed two times with PBS, and basal medium was added containing Exos or DFO-Exos at 50 μg/mL final concentration. Images for 3 fields of view per scratch were taken at 0h, 24h. The residual fractional wound area was determined and the migratory effect was evaluated using *ImageJ* software.

2.10. Transwell Assay. The upper chamber of a 24-well transwell plate (Corning, Corning, NY) was seeded with 5×10^4 cells. 600 μL of medium supplemented with 50 μg/mL Exos, DFO-Exos, or an equal volume of PBS was added to the lower chamber. After 12 hours of incubation, 0.5% crystal violet was used to stain cells for 5 minutes. A cotton swab was used to remove the cells from the up surface of the membranes after washing thrice with PBS. The number of migrated cells was quantified under an optical microscope (Leica).

2.11. Tube Formation Assay. Matrigel from BD Biosciences was used for tube formation assay. In matrigel-coated 24-well plate, 2×10^4 HUVECs per well were seeded and cultured with 50 μg/mL Exos, DFO-Exos, or an equal volume of PBS for 16 h at 37°C. After 16 h incubation, tube formation was determined under optical microscope (Leica). The total length of the network structures was measured using *ImageJ* software.

2.12. Animal Model to Study Skin Wound Healing on Diabetic Rats. 250-300 g male Sprague Dawley (SD) rats were used in our study. The Animal Research Committee of the Sixth People's Hospital at the Shanghai Jiao Tong University approved all the procedures. 60 mg/kg of STZ (Sigma-Aldrich, USA) given as one dose intraperitoneally was used to induce diabetes mellitus. Rats with more than 300 mg/dL blood glucose level were chosen for the next stage of the study. Prior to creating wounds in the skin, the diabetic rats were observed for a total of two weeks.

Pentobarbital of 50 mg/kg concentration was injected intraperitoneally to anesthetize STZ-induced diabetic rats. Two excisional wounds of diameter 20 mm were made on the upper back of the rats after shaving, and then they were assigned randomly to three treatment groups. Rats among these groups were administered subcutaneously around the wounds at four injection sites either with Exos (100 μg Exos in 100 μL PBS), DFO-Exos (100 μg DFO-Exos in 100 μL PBS), or 100 μL PBS. The decrease in wound-size was determined: wound-size reduction (%) = (A0 – At)/A0 × 100, where A0 is the area of the wound initially, and At is the area of the wound at week 1 or 2 following wounding.

2.13. Microfil Perfusion and Microcomputed Tomography (Micro-CT). On the 14th day after wounding, rats in three groups were perfused with Microfil (Microfil MV-122, Flow Tech, USA) after anesthetization. For a proper polymerization of the contrast agent, rats were kept at 4°C overnight. The new blood vessels in the wound sites were detected using micro-CT (Skyscan 1176; Kontich, Belgium) for scanning at 9 μm resolution. The original datasets were analyzed by CTAN (Skyscan, Bruker). The threshold is used to distinguish blood vessels from soft tissue by binarization, and the number of blood vessels was quantified.

2.14. Histological Analysis. On day 14 after wounding, the skin specimens were fixed in 10 % formalin, dehydrated with a series of graded-alcohol, and embedded in paraffin. After being cut into 4-μm-thick sections, Hematoxylin and eosin (H&E) and Masson's trichrome staining were performed to evaluate extent of wound healing.

2.15. Immunohistochemical and Immunofluorescent Analyses. For immunohistochemical analysis, the sections were rehydrated, blocked, and incubated with primary anti-CD31 antibody at 4°C overnight. After incubation with secondary at room temperature, the stained sections were visualized using the DAB substrate and finally counter-stained using hematoxylin.

The formation of blood vessels in granulation tissues was determined at day 14 after wounding by immunofluorescent

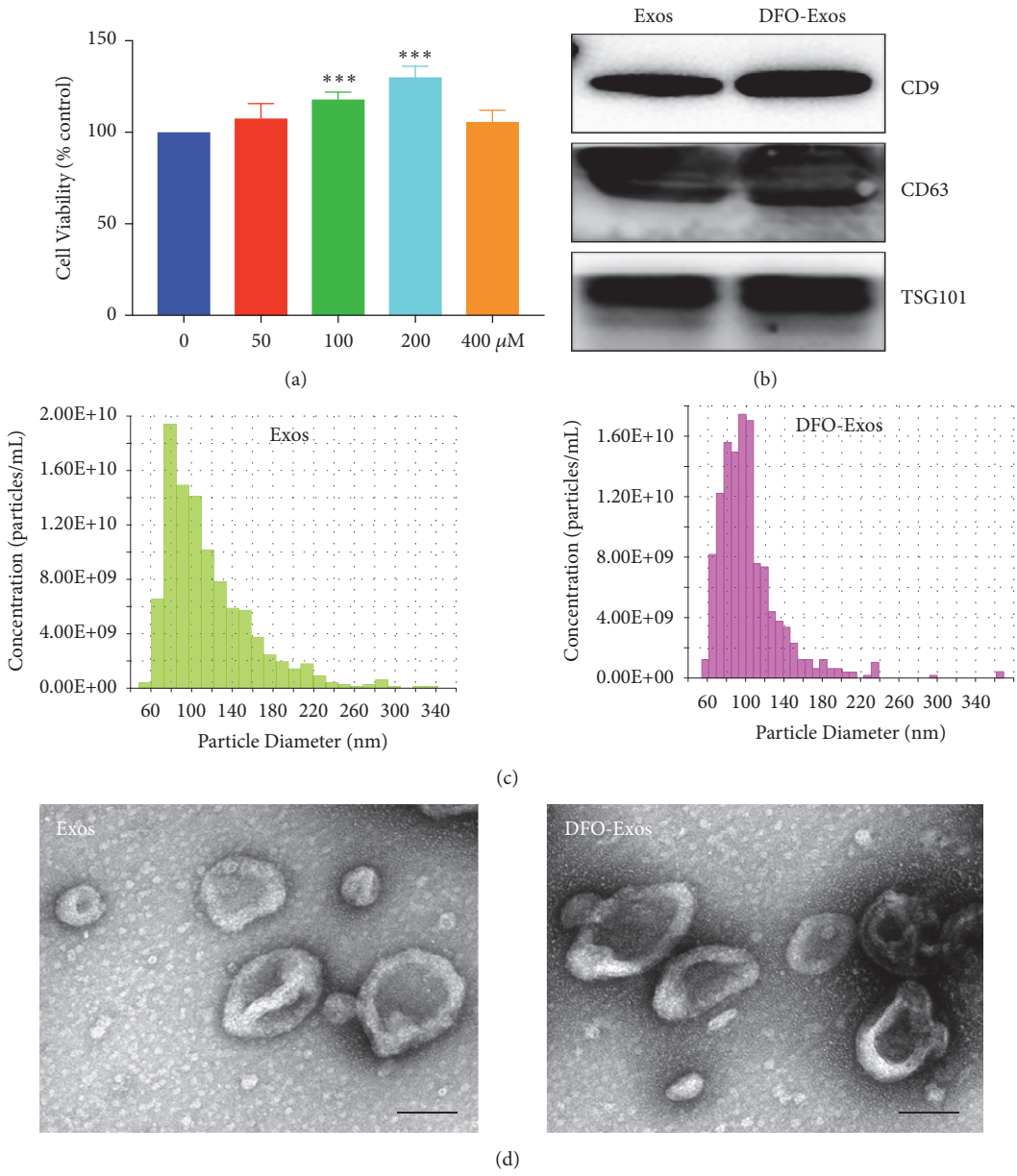


Figure 1: Characterization of exosomes. (a) Viability assay of BMSCs treated with various concentrations of DFO for 48 hours. DFO-treated BMSCs at 100 and 200 μM concentration showed significantly more viability when compared to control group. *** $P < 0.001$ versus control. (b) Western blotting showed the presence of exosomal markers including CD9, CD63, and TSG101 in Exos and DFO-Exos. (c) qNano analysis results of Exos and DFO-Exos. (d) TEM photomicrographs of Exos and DFO-Exos. Scale bar = 50 nm.

staining against CD31 and alpha-smooth muscle actin (α-SMA). After incubation with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies, DAPI was used to stain the nuclei.

2.16. Statistical Analysis. Means ± SEM was used to express data. Student's t-test was used for comparing single comparisons. For multiple comparisons, one-way analysis of variance (ANOVA) was performed. A value of $P < 0.05$ was used as a benchmark for statistical significance.

3. Results

3.1. Isolation and Characterization of Exosomes. To analyze the optimal working concentration of DFO without impact cell viability in our study, BMSCs were incubated with DFO at concentrations of 0, 50, 100, 200, and 400 μM of DFO. After 48 hours of incubation, the viability of cells was examined by CCK8 assay. The result showed that DFO-treated BMSCs at 100 and 200 μM concentration showed significantly more viability when compared to control group (Figure 1(a)). So, 48 hours treatment with 200 μM of DFO was used to collect DFO-Exos for all experiments in this study.

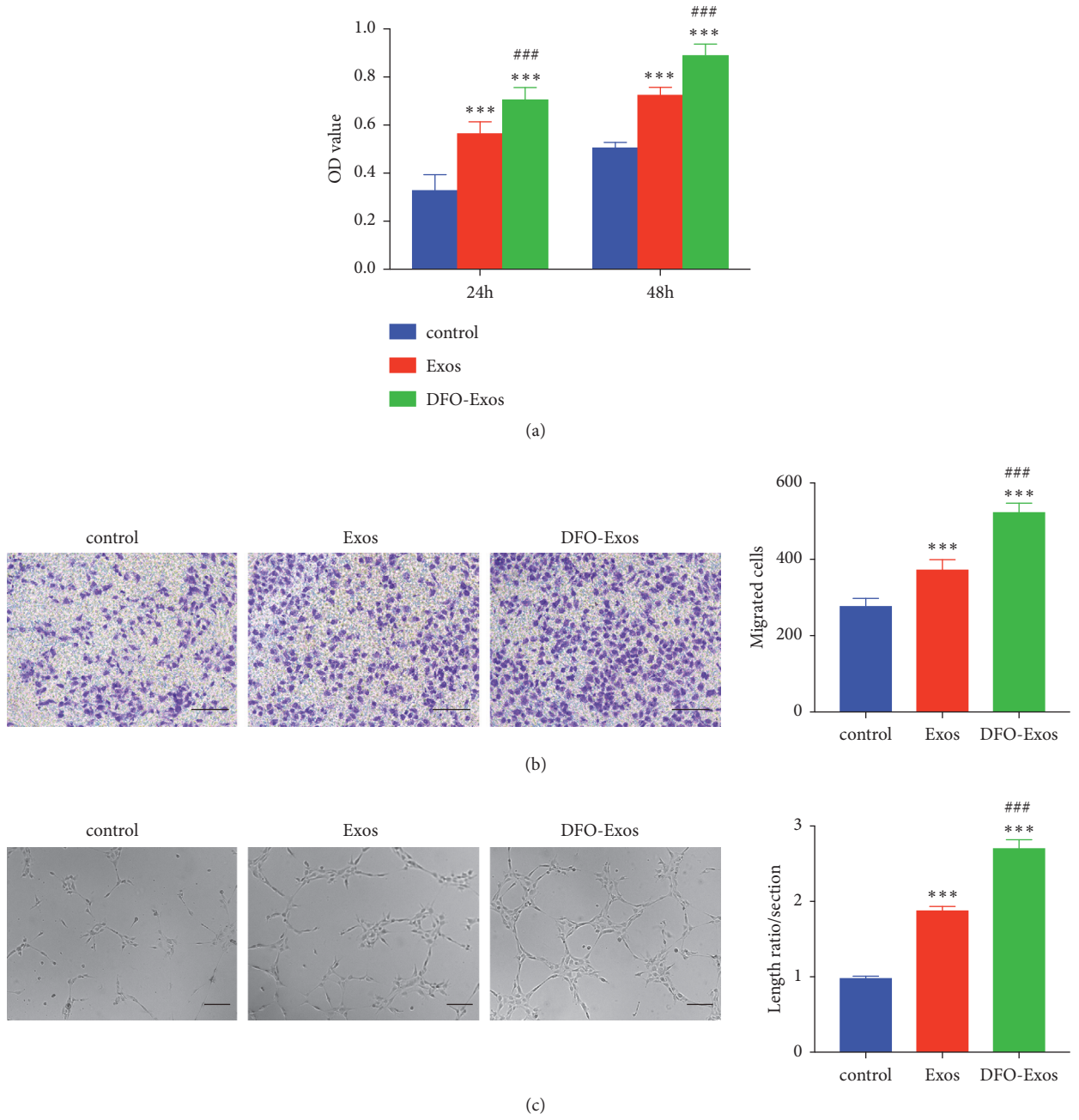


FIGURE 2: The proliferation and proangiogenic effects of Exos and DFO-Exos on HUVECs. (a) Proliferation of HUVECs treated with Exos or DFO-Exos at 50 μg/mL concentration or an equal volume of PBS. *** $P < 0.001$ versus control, $^{###}$ $P < 0.001$ versus Exos group. (b) Left panel, representative images of transwell assay, scale bar = 200 μm; right panel, quantitative analysis of the assay. *** $P < 0.001$ versus control, $^{###}$ $P < 0.001$ versus Exos group. (c) Left panel, representative images of tube formation assay from three groups, scale bar = 200 μm; right panel, quantitative analysis of the assay. *** $P < 0.001$ versus control, $^{###}$ $P < 0.001$ versus Exos group.

As presumed, the presence of exosomal marker proteins CD9, CD63, and TSG101 was detected in both Exos and DFO-Exos (Figure 1(b)). qNano analysis was performed to identify Exos and DFO-Exo, finding that the size distribution of most exosomes in two groups was in the range of 50-150 nm (Figure 1(c)). Using TEM analysis, these vesicles with characteristic cup-shaped morphology were observed (Figure 1(d)).

3.2. Exosomes Promoted Angiogenesis In Vitro. To determine the activities of exosomes released from BMSCs and DFO-treated BMSCs, we performed an endothelial cell proliferation assay. Compared to the control group, Exos and DFO-Exos enhanced the proliferation of endothelial cell significantly at 24 and 48 hours (Figure 2(a)). And, DFO-Exos exhibited stronger proproliferative effect when compared with Exos group (Figure 2(a)). Transwell assay

(Figure 2(b)) and scratch wound-healing assay (Supplementary Figure 1) were used to evaluate the promigratory effects, showing that Exos and DFO-Exos remarkably upregulated the motility of HUVECs while DFO-Exos possessed much stronger promigratory effect that Exos did. Tube formation assay was performed to find that a greater number of cord-like structures were formed on Matrigel in DFO-Exos group, in comparison to the Exos group and control (Figure 2(c)). These results revealed that BMSCs treated with DFO enhance the proangiogenic capacity of exosomes.

3.3. Accelerated Wound Healing in Diabetic Rats after Exosomes Treatment. To determine the effects of Exos and DFO-Exos on diabetic wound healing, diabetic rats were established by injection of STZ and full-thickness cutaneous wounds were created on dorsal skin regions, followed by subcutaneous administration of Exos, DFO-Exos or an equal volume of PBS. On days 7 and 14 after wounding, wound closure was accelerated in animals that received Exos treatment in comparison to control PBS, and a higher rate of wound closure was observed in rats treated with DFO-Exos compared with that treated with Exos (Figures 3(a) and 3(b)). H&E staining was carried out to evaluate the extent of wound healing, finding that much longer newly formed epidermis and dermis were observed in the wounds treated with Exos, as compared with wounds in the control group at day 14 after operation (Figure 3(c)). Quantitative analysis confirmed that Exos-treated wounds had a lower level of scar formation than the Exos group (Figure 3(d)). In addition, the further significantly promoted effect on wound healing was observed in DFO-Exos group (Figures 3(c) and 3(d)). Masson's trichrome staining showed the largest amounts of wavy collagen fibers in DFO-Exos-treated-wounds compared to other two groups (Figure 3(e)).

3.4. Promoted Angiogenesis in the Wound Sites after Exosomes Treatment. On the 14th day after operation, Microfil perfusion was done to evaluate the blood vessels in the wound sites. The reconstructed 3D images after micro-CT scanning showed a significant increased density of blood vessels after administration of Exos and DFO-Exos, when compared to that in the control group. Additionally, the DFO-Exos-treated wound sites exhibited a greater degree of formation of blood vessels (Figure 4(a)). Immunohistochemical staining against CD31 and immunofluorescence double-staining for CD31 and α-SMA were done to identify the blood vessels in wound sites that received various treatment. According to the results, the number of overall (Figure 4(b)) as well as mature blood vessels (Figure 4(c)) in the wound sites that received DFO-Exos and Exos treatment was enhanced remarkably compared to that in the control group, and a larger number of these two parameters were identified in the DFO-Exos group, when compared to that in Exos group (Figures 4(c) and 4(d)). These results revealed that DFO pretreatment enhances BMSCs-derived exosomes mediated angiogenesis in wound healing.

3.5. Exosomal Transferred-miR-126 from BMSCs Is Promoted to Activate PI3K/AKT Pathway after DFO Treatment. Quantitative real-time PCR (qRT-PCR) was utilized to examine the levels of key proangiogenic miRNAs, such as miR-214, miR-21, miR-126, miR-125b, miR-27b, and miR-19b in exosomes. Among these detected miRNAs, miR-126 was found to display marked upregulation after DFO treatment (Figure 5(a)). Next, we determined whether Exos or DFO-Exos could be internalized into HUVECs, cause internalization is an essential condition for transfer of miRNAs via exosomes. Exos and DFO-Exos were labeled by the green fluorescent dye (DIO) and then were incubated with HUVECs for 8 hours. Fluorescence microscopy confirmed that DIO-labeled Exos and DIO-labeled DFO-Exos had been transferred to perinuclear region of HUVECs (Figure 5(b)). To verify the transfer of miR-126, HUVECs were treated with Exos and DFO-Exos. Then the cells from different groups were harvested for PCR analysis. As shown in Figure 5(c), miR-126 levels in HUVECs were significantly increased after the cells were incubated with Exos and DFO-Exos for 3 hours respectively, and the level of miR-126 was higher significantly in DFO-Exos-treated HUVECs. Studies have shown that miR-126 can target PTEN in cells to induce the activation of PI3K/AKT pathway. Analysis by western blot in HUVECs after different treatments showed much more reduction of phosphatase and tensin homolog (PTEN) and an increase in the amount of phosphorylation of AKT (p-AKT) in cells subjected to DFO-Exos treatment, when compared to that in Exos and control groups (Figure 5(d)). These results suggested that exosomes released from BMSCs by DFO stimulation directly by more miR-126 to endothelial cell by activating PI3K/AKT pathway.

Then, BMSCs were transfected with miR-126 inhibitor or negative control inhibitor before DFO stimulation, as shown in Figure 6(a), and the miR-126 level was significantly inhibited in DFO-treated BMSCs-derived exosomes (126I-DFO-Exos). To explore the effects of exosomes derived from miR-126 knockdown DFO-BMSCs, western blot analysis showed that PTEN was upregulated and p-AKT was downregulated in 126I-DFO-Exos group (Figure 6(b)). The transwell assay showed that the number of migrated cells in the group treated with miR-126 knockdown DFO-Exos was significantly decreased (Figure 6(c)), as well as the length of tubes and branch numbers in the 126I-DFO-Exos group determined by tube formation assay (Figure 6(d)). These results suggested that miR-126 plays the vital role in the enhanced proangiogenic effect of exosomes from DFO-stimulated BMSCs.

4. Discussion

In this study, we investigated exosomes derived from MSCs preconditioned by DFO which exhibit superior proangiogenic properties in vitro and in vivo. The results in this study show that, in presence of DFO-Exos, the levels of miR-126, a proangiogenic component, were enhanced remarkably in the HUVECs. Furthermore, in the diabetic skin wound animal model, the proangiogenic effects of exosomes were increasingly mediated by miR-126.

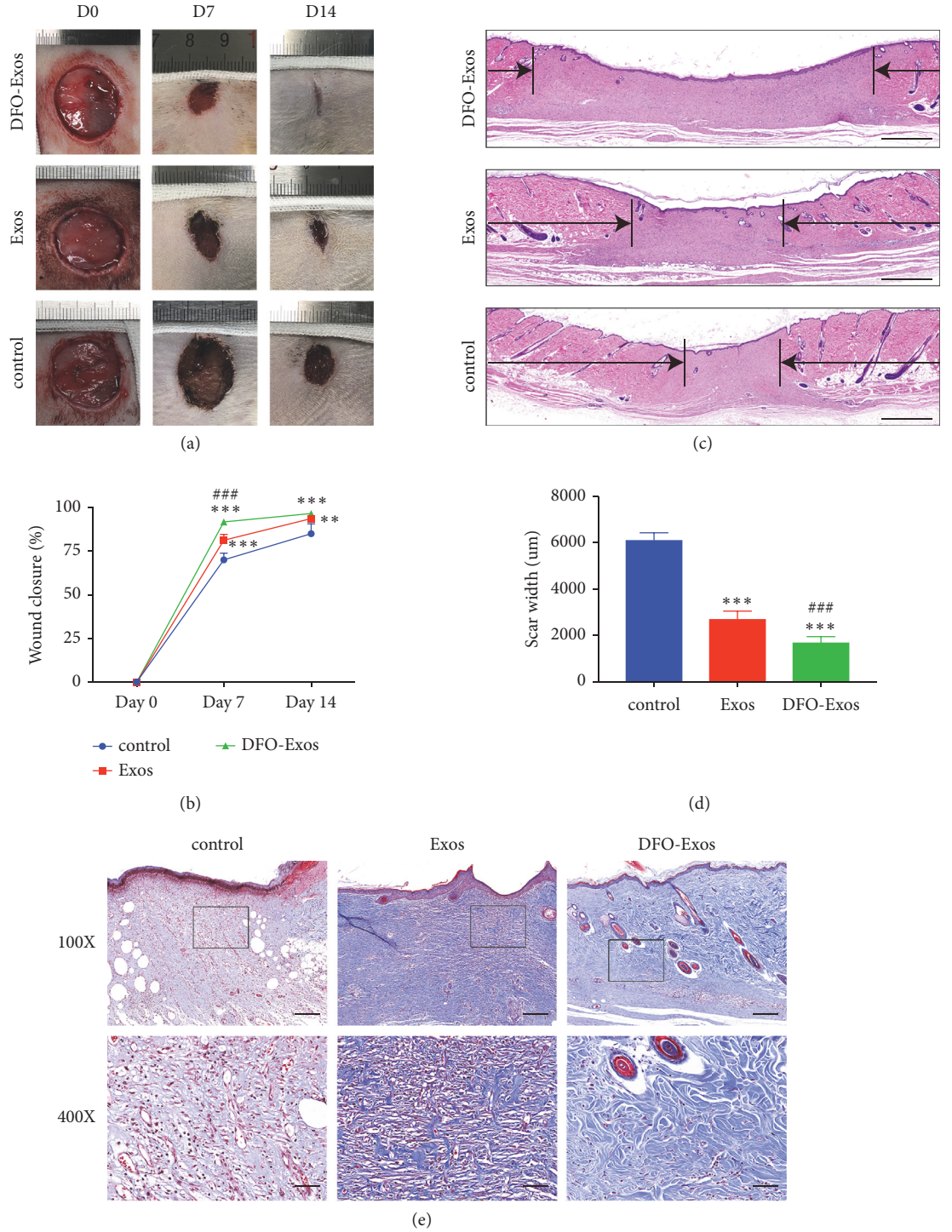


FIGURE 3: Cutaneous wound healing in diabetic rats is accelerated after Exos and DFO-Exos transplantation. (a) Gross view of wounds treated with Exos, DFO-Exos or PBS on days 0, 7, and 14 after wounding. (b) The wound-closure rates on specific days of three groups. *** $P < 0.001$; ** $P < 0.01$ versus control, ### $P < 0.001$ versus Exos group. (c) H&E staining of wound sections from three groups at 14 days after wounding. The black arrows point out the scar edges. Scale bar = 1000 μm. (d) Quantitative analysis of the scar widths. *** $P < 0.001$ versus control, ### $P < 0.001$ versus Exos group. (e) Masson's trichrome staining for the evaluation of collagen maturity. Scale bar = 100 μm (top) or 50 μm (bottom).

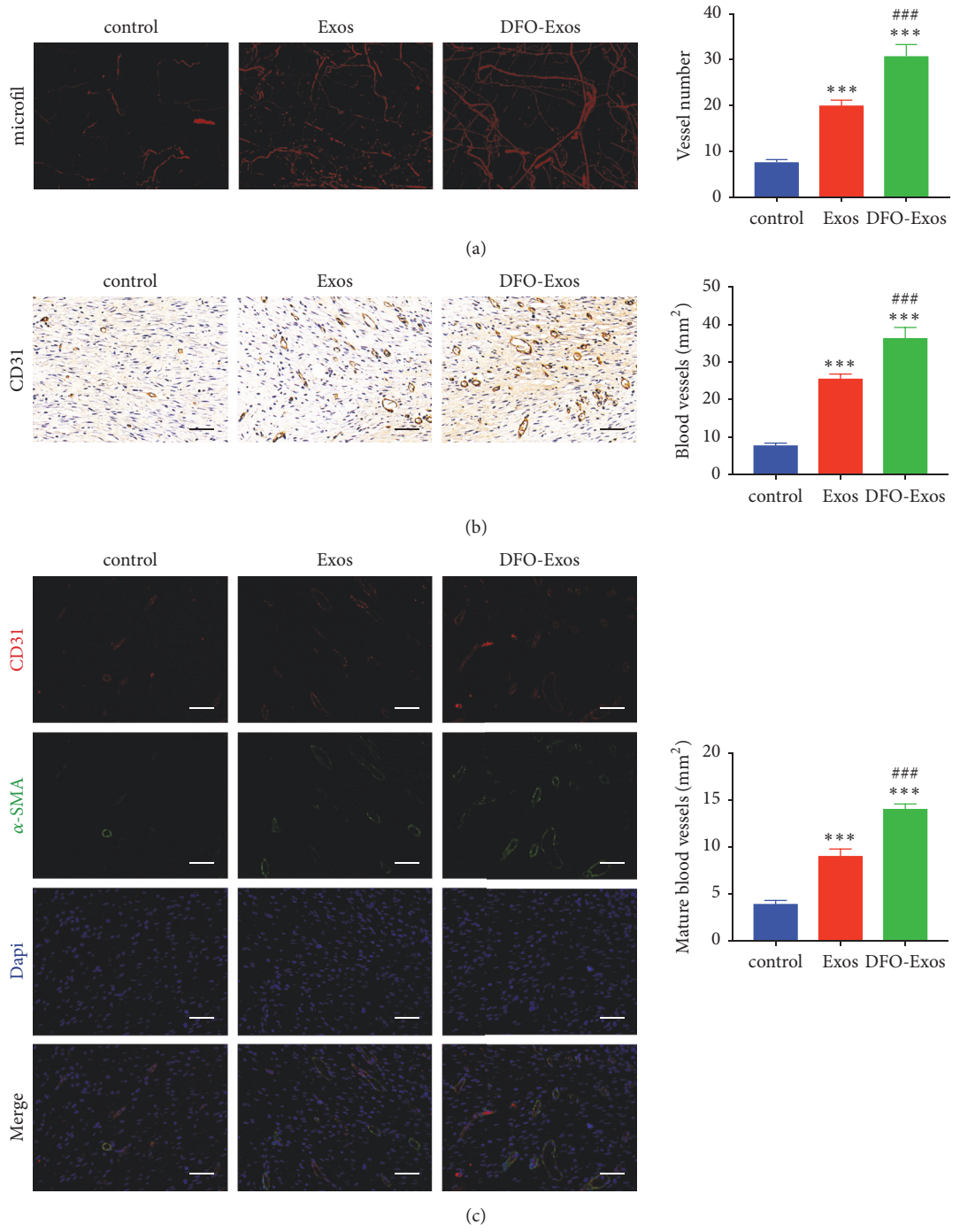


FIGURE 4: Promoted angiogenesis in the wound sites after exosomes treatment. (a) Left panel, Microfil perfusion for determination of blood vessel density in the wound sites on day 14 after wounding; right panel, quantitative analysis of blood vessel density in three groups. *** $P < 0.001$ versus control, ### $P < 0.001$ versus Exos group. (b) Left panel, immunohistochemical staining against CD31 of wound sections on day 14 after wounding. Scale bar = 50 μm; right panel, quantitative determination of the number of total blood vessels. *** $P < 0.001$ versus control, ### $P < 0.001$ versus Exos group. (c) Left panel, immunofluorescent staining against CD31 and α-SMA of wound sections from three groups on day 14 after wounding. Scale bar = 50 μm; right panel, quantitative determination of the number of mature blood vessels. *** $P < 0.001$ versus control, ### $P < 0.001$ versus Exos group.

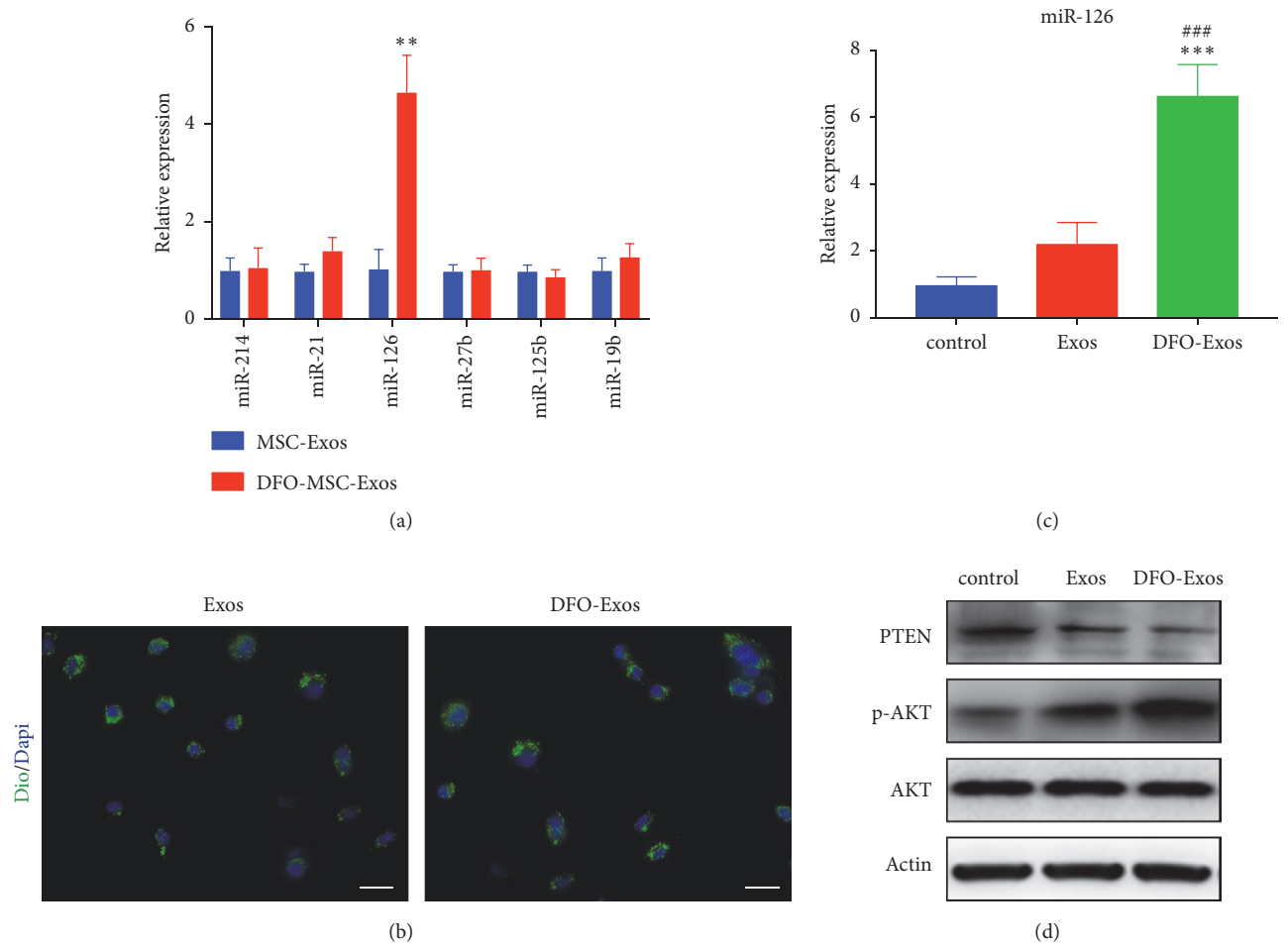
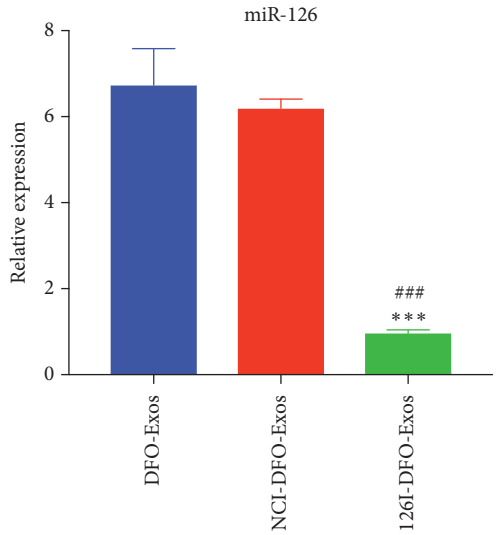


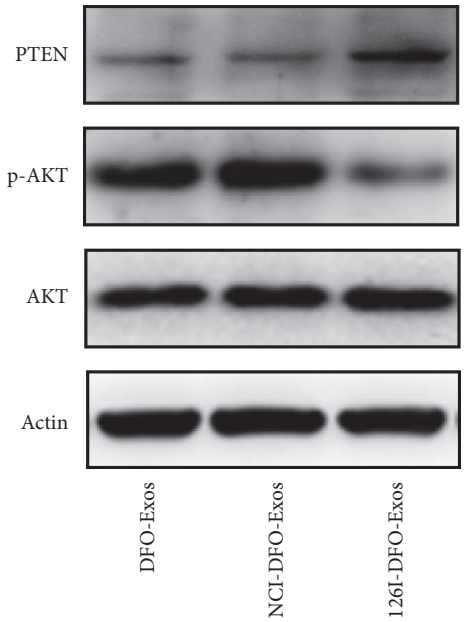
Figure 5: DFO-Exos deliver more miR-126 into the endothelial cells. (a) The levels of angiogenic miRNAs among Exos and DFO-Exos were determined by qRT-PCR. ** $P < 0.01$ versus Exos group. (b) Fluorescent microscopy showed the internalization of DIO-labeled Exos of DFO-Exos by HUVECS. Exosomes could be seen in the perinuclear region of HUVECs. Scale bar = 20 μm. (c) The level of miR-126 in HUVECs after DFO-Exos, Exos, or PBS treatment was evaluated. *** $P < 0.001$ versus control, ### $P < 0.001$ versus Exos group. (d) Western blotting analysis of PTEN, p-AKT, and AKT expression in HUVECs among three groups.

Preconditioning using hypoxia causes an increase in angiogenesis induced by MSCs as well as alterations of mRNA and proteins in exosomes; furthermore, interactions between these exosomes and endothelial cells boosted the angiogenic potential of the latter, according to several studies [16, 24, 25]. This current work involved an isolation of exosomes from BMSCs that were exposed to a pretreatment of DFO that displayed greater proangiogenic properties in vitro. A direct communication between the exosomes and their target cells in order to deliver particular miRNAs and proteins prototypical of the origin of the exosomes is thought to be the mechanism of angiogenesis mediated by exosomes [17]. The levels of miR-296, miR-21, and miR-126 that are vital microRNAs involved in angiogenesis were evaluated in our study. A surprising observation was an elevation of significance in levels of miR-126 in DFO-Exos. The levels of this miRNA were increased in the HUVECs after the treatment by the DFO-Exos. A vital role has been implicated for miR-126 in development of vascular tissues [26]. Concurrently, exosomes released by CD34b peripheral blood mononuclear cells also contain miR-126, which regulates angiogenesis after being transferred to endothelial cells [27]. This is the first study that reports an upregulation of expression of miR-126 in exosomes derived from MSCs by stimulation by DFO and exhibits higher effects of proangiogenesis in vitro and in vivo.

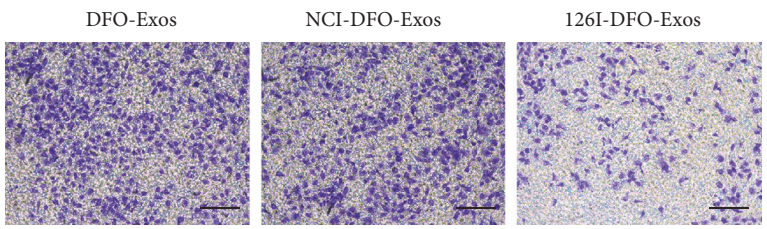
In addition, it was also observed that the quantity of an established target of the miRNA: PTEN was lowered in endothelial cells when DFO-Exos were administered while the levels rose when miR-126 was inhibited. This is suggestive of a regulation of expression of the target in endothelial cells by microRNA uptake. The proangiogenic pathway is inhibited in endothelial cells by PTEN through PI3K/AKT signaling to suppress growth factor signaling [26]. Along the same lines, the HUVECs cultured with DFO-Exos showed elevated levels of phosphorylation of AKT while the level was lower in HUVECs that were cultured with exosomes that had inhibited miR-126. These results are indicative of proangiogenic functions of exosomes from MSCs that are released by DFO stimulation.

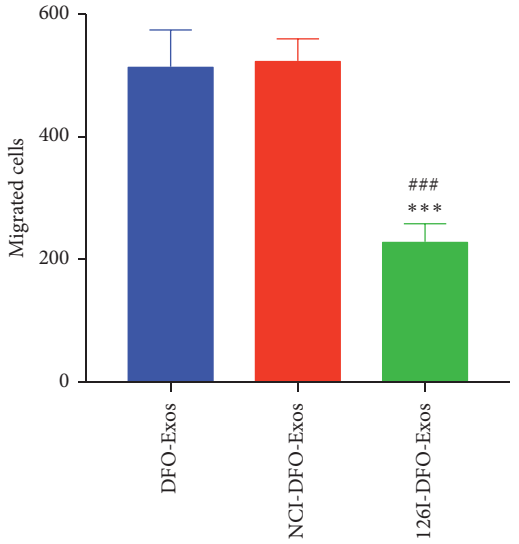


(a)



(b)





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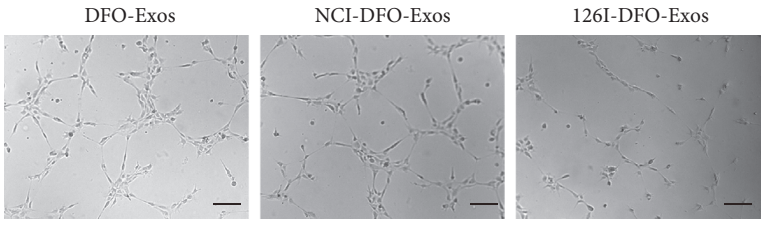
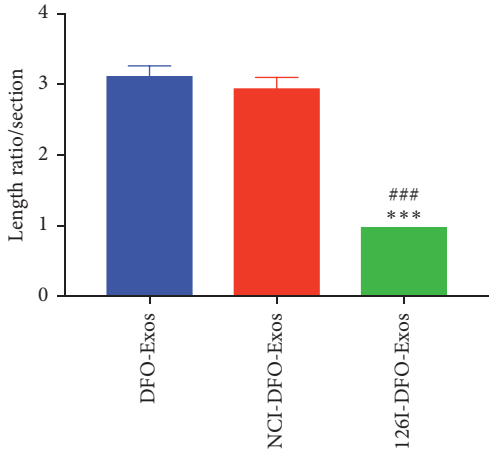
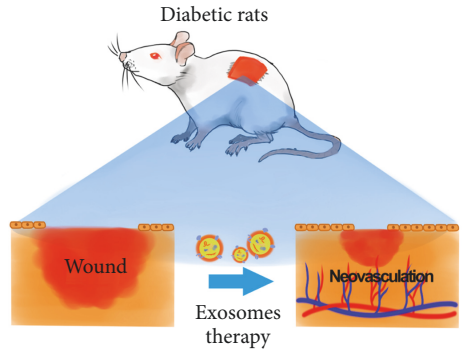




(d)

Figure 6: Continued.



(e)

Figure 6: Effects of DFO-Exos with miR-126 knockdown on angiogenesis of endothelial cells. Levels of miR-126 in exosomes from BMSCs treated with DFO + PBS (DFO-Exos), DFO + negative inhibitor control (NCI-DFO-Exos), or DFO + miR-126 inhibitor (126I-DFO-Exos) were analyzed by qRT-PCR. *** $P < 0.001$ versus DFO-Exos, ### $P < 0.001$ versus NCI-DFO-Exos group. (b) Western blotting analysis of PTEN, p-AKT, and AKT expression in HUVECs among three groups. (c) Left panel, representative images of transwell assay, scale bar = 200 μm; right panel, quantitative analysis of the assay. *** $P < 0.001$ versus DFO-Exos, ### $P < 0.001$ versus NCI-DFO-Exos group. (d) Left panel, representative images of tube formation assay from three groups, scale bar = 200 μm; right panel, quantitative analysis of the assay. *** $P < 0.001$ versus DFO-Exos, ### $P < 0.001$ versus NCI-DFO-Exos group. (e) Schematic diagram depicts the enhanced proangiogenic effects of DFO-Exos on wound healing. Followed by subcutaneous administration of DFO-Exos, Exos, or PBS, higher rate of wound closure was observed in rats treated with DFO-Exos due to the improvement of angiogenesis.

In conclusion, our study showed that MSC derived exosomes stimulated by DFO in low doses promote neovascularization via the PI3K/AKT pathway and enhance skin wound recovery in the diabetic skin wound animal model. Thus, DFO may cause exosomes to be triggered in order to show augmented proangiogenic potential in cell-free therapeutic applications.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare the absence of any conflicts of interest with regard to this paper.

Authors' Contributions

Jianing Ding, Xin Wang, and Bi Chen contributed equally to this work.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1. The promigratory effects of Exos and DFO-Exos on HUVECs. Left panel, scratch wound-healing assay of HUVECs treated with Exos or DFO-Exos at 50 μg/mL concentration or an equal volume of PBS, scale bar = 200 μm; right panel, quantitative analysis of the assay. *** $P < 0.001$ versus control, ### $P < 0.001$ versus Exos group. *(Supplementary Materials)*

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Research Article

Second-Generation Histamine H1 Receptor Antagonists Suppress Delayed Rectifier K^+-Channel Currents in Murine Thymocytes

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Background/Aims. Voltage-dependent potassium channels (Kv1.3) are predominantly expressed in lymphocyte plasma membranes. These channels are critical for the activation and proliferation of lymphocytes. Since second-generation antihistamines are lipophilic and exert immunomodulatory effects, they are thought to affect the lymphocyte Kv1.3-channel currents. *Methods*. Using the patch-clamp whole-cell recording technique in murine thymocytes, we tested the effects of second-generation antihistamines, such as cetirizine, fexofenadine, azelastine, and terfenadine, on the channel currents and the membrane capacitance. *Results*. These drugs suppressed the peak and the pulse-end currents of the channels, although the effects of azelastine and terfenadine on the peak currents were more marked than those of cetirizine and fexofenadine. Both azelastine and terfenadine significantly lowered the membrane capacitance. Since these drugs did not affect the process of endocytosis in lymphocytes, they were thought to have interacted directly with the plasma membranes. *Conclusions*. Our study revealed for the first time that second-generation antihistamines, including cetirizine, fexofenadine, azelastine, and terfenadine, exert suppressive effects on lymphocyte Kv1.3-channels. The efficacy of these drugs may be related to their immunomodulatory mechanisms that reduce the synthesis of inflammatory cytokine.

1. Introduction

Among antiallergic drugs, second-generation histamine H1 receptor antagonists, such as fexofenadine, cetirizine, terfenadine, and azelastine, are widely used in the treatment of allergic disorders, such as allergic conjunctivitis, chronic rhinitis, urticaria, and asthma [1–3]. They differ from first-generation antihistamines in their higher selectivity for peripheral H1 receptors but lower affinity for H1 receptors in the central nervous system [3]. In addition to their antiallergic properties, second-generation antihistamines exert immunomodulatory properties by actually suppressing the proinflammatory cytokine production from T-lymphocytes [4–8]. Patch-clamp studies revealed that delayed rectifier K^+-channels (Kv1.3) are predominantly expressed in lymphocyte plasma membranes [9] and that these channels are critical for the initiation of the immune reaction [10–12]. Recently, using murine thymocytes, we revealed drugs, including calcium channel blockers, macrolide antibiotics, and statins, suppressed Kv1.3-currents, and exerted immunomodulatory properties [13–16]. Based on our results, in suppressing the channel currents, these lipophilic drugs appear to generate microscopic changes in the membrane surface structure and thus collapsed the channels conformationally. Among second-generation antihistamines that are more lipophilic than the first-generation ones [17, 18], azelastine and terfenadine have relatively higher lipophilicity [19–21]. Therefore, they were more likely to directly disrupt the thymocyte membranes and thereby suppress the Kv1.3-channel currents. To reveal this, using the patch-clamp whole-cell recording

technique in T-lymphocytes (murine thymocytes), we compared the effects of second-generation antihistamines, such as cetirizine, fexofenadine, azelastine, and terfenadine, on the channel currents and membrane capacitance. Here, we show for the first time that these drugs inhibit lymphocyte Kv1.3-channels. We additionally show that the efficacy of azelastine and terfenadine occurred through interactions of the drugs with cellular membranes, which was monitored electrophysiologically by the decreased membrane capacitance.

2. Materials and Methods

2.1. Cell Sources and Preparation. Four- to 5-week old male *ddy* mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were anaesthetized deeply using isoflurane. They were sacrificed by dislocating the cervical spine. The Animal Care and Use Committee of Tohoku University Graduate School of Medicine approved our protocol for the use of animals. As we previously described, [13, 15, 16, 22, 23], we separated thymocytes from mouse thymus and resuspended them in external solution containing 145 mM NaCl, 4.0 mM KCl, 1.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 5.0 mM Hepes, and 0.01% bovine serum albumin, adjusted with pH 7.2 by titrating NaOH. We kept the isolated cells at room temperature (22-24°C) to use in 4 hours.

2.2. Electrical Setup and Patch-Clamp Recordings. Using an EPC-9 patch-clamp amplifier system (HEKA Electronics, Lambrecht, Germany), standard whole-cell patch-clamp recordings were conducted [13, 15, 16, 22, 23]. The patch pipette resistance was maintained 4-6 MΩ when filled with internal (patch pipette) solution containing (in mM): KCl, 145; $MgCl_2$, 1.0; EGTA, 10; Hepes, 5.0 (pH 7.2 adjusted with KOH). After we formed a giga-seal, suction was applied briefly to the pipette to rupture the patch membrane. We maintained the series resistance of the whole-cell recordings below 10 MΩ during the experiments. We normalized peak and pulse-end currents by the membrane capacitance, which were expressed as the current densities (pA/pF). All experiments were carried out at room temperature.

2.3. Drug Delivery. We purchased cetirizine dihydrochloride and azelastine hydrochloride, from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and fexofenadine hydrochloride from LKT Laboratories, Inc. (St. Paul, Min., USA). We separately dissolved these drugs in the external solutions to make the final concentration of 100 μM. Terfenadine from Tocris Bioscience (Minneapolis, MN, USA) was dissolved at 10 μM. We delivered one of the drugs to the cells by the standing hydrostatic pressure of 3 cmH_2O from a nearby pipette, as described previously [13, 15, 16, 22, 23]. Then, we recorded whole-cell membrane currents before and after 30 s exposure to the drugs. To eliminate the possibility that the observed effects were derived from the procedure alone, the external solution was simply applied to the cells and the absence of any significant changes in the channel currents was confirmed.

2.4. Membrane Capacitance Measurements. We used the Lock-in amplifier within the EPC-9 Pulse program and employed a sine plus DC protocol [13, 15, 16, 23]. Thus, we monitored the thymocytes' membrane capacitance. We superimposed an 800-Hz sinusoidal command voltage by holding the membrane potential with -80 mV. We continuously recorded the membrane capacitance (Cm), membrane conductance (Gm) and series conductance (Gs), before and after exposing to the drugs for 30 s during the whole-cell patch-clamp recording. Mathematically, we calculate a whole-cell Cm from a parallel-plate capacitor formula: $Cm = \varepsilon A/d$, where ε indicates the dielectric modulus of the plasma membrane; A indicates the membrane surface area; and d indicates the membrane thickness [24]. Under a physiological condition where ε and d are almost constant, the changes in Cm are attributable to the alteration in the membrane surface area (A) [25].

2.5. Electron Microscopy. We fixed the thymocytes, incubated in the external solutions containing no drug, 100 μM azelastine, or 10 μM terfenadine for 10 min, with 4% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 2 hours at room temperature. After trimming, the specimen was cut into small pieces, postfixed in 1% osmium tetroxide for 1 hour at 4°C, rinsed in PBS, dehydrated in a graded series of alcohol and propylene oxide, and finally embedded in epoxy resin. We prepared ultrathin (80 nm) sections on an ultramicrotome (Ultracut R, Leica, Heerbrugg, Switzerland) with a diamond knife. Then we stained with uranyl acetate and lead citrate and viewed using an electron microscope (JEM-1200, JOEL, Tokyo, Japan).

2.6. Statistical Analyses. We used an EPC-9 amplifier and PulseFit software (HEKA Electronics, Lambrecht, Germany), IGOR Pro 6.2 (WaveMetrics, Lake Oswego, Oreg., USA), and Microsoft Excel 2013 (Microsoft Corporation, Redmond, Wash., USA). Then we analyzed the data and expressed them as means ± SEM. We employed two-way ANOVA and Student's or Dunnett's t test, assessing whether they were statistically significant. We considered a p-value < 0.05 being significant.

3. Results

3.1. Effects of Cetirizine, Fexofenadine, Azelastine, and Terfenadine on Kv1.3-Channel Currents in Murine Thymocytes. To examine the effects of the second-generation antihistamines on Kv1.3- channel currents, we applied external solutions including either 100 μM cetirizine, 100 μM fexofenadine, 100 μM azelastine, or 10 μM terfenadine, to the thymocytes and monitored the changes in the whole-cell currents (Figures 1(a) and 1(b)). When orally administered in humans, the serum concentrations of these drugs are usually below 1 μM under a physiological condition [26–29]. Nevertheless, in some *in vitro* studies, isolated cells, such as cardiomyocytes, needed much higher concentrations of these drugs, including 10 μM terfenadine and as high as 100 μM cetirizine, 100 μM fexofenadine, and 100 μM azelastine, to effectively elicit

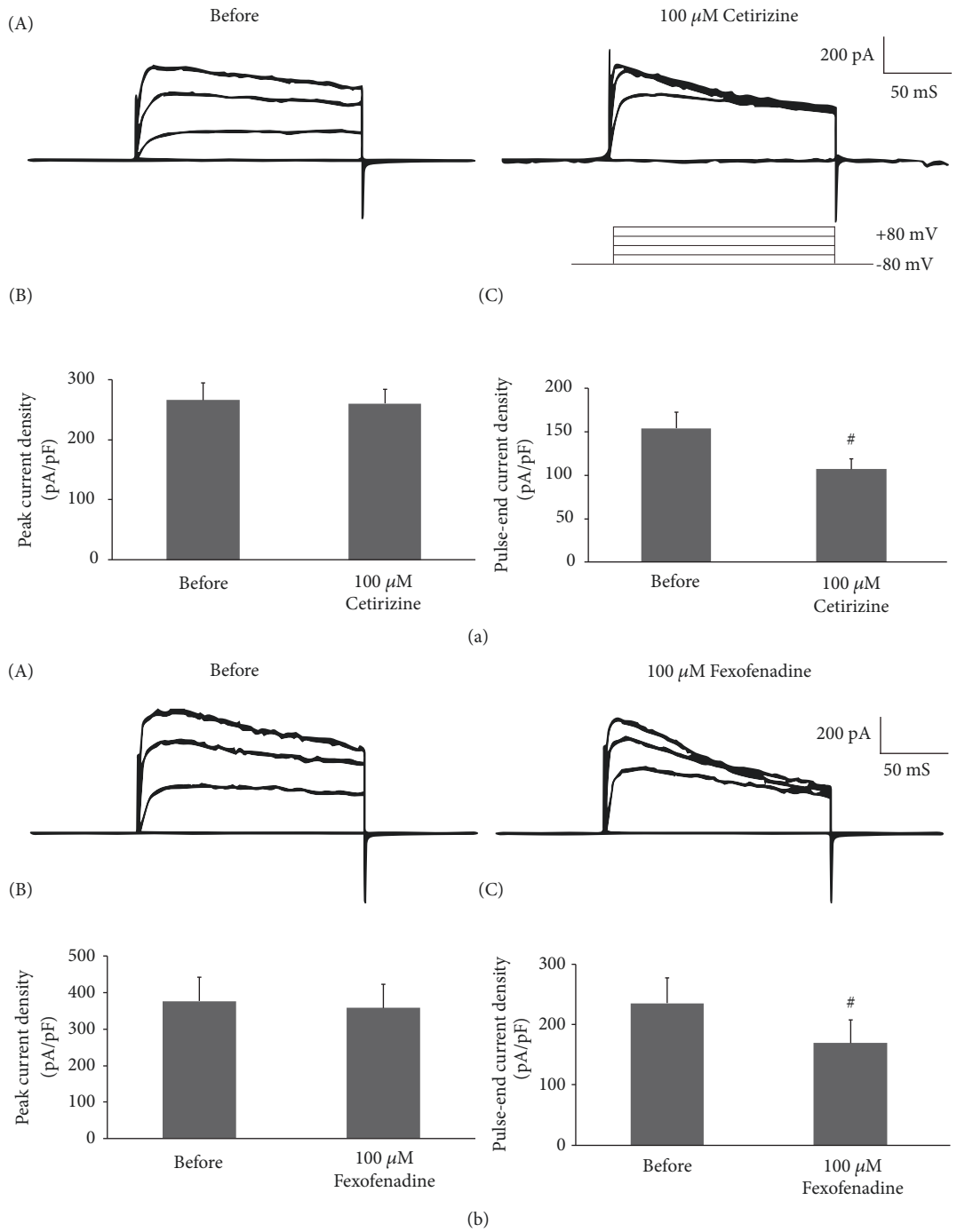

Figure 1: *Effects of cetirizine and fexofenadine on Kv1.3 channel currents in murine thymocytes*. The effects of 100 μM cetirizine (a) and 100 μM fexofenadine (b). (A) Typical whole-cell current traces at different voltage-steps recorded before and after the application of either drug. The currents were elicited by voltage-steps from the holding potential of -80 mV to -40, 0, 40, and 80 mV, as depicted in the voltage protocol. Each pulse was applied for a 200-ms duration between 10-second intervals. (B) Peak current densities (peak currents normalized by the membrane capacitance) obtained from the records in (A) at the voltage-step of 80 mV. (C) Pulse-end current densities (pulse-end currents normalized by the membrane capacitance) obtained from the records in (A) at the voltage-step of 80 mV. $^{\#}$ $p < 0.05$ *vs.* before the drug application. Values are means ± SEM (cetirizine, $n = 16$; fexofenadine, $n = 13$). Differences were analyzed by ANOVA followed by Dunnett's or Student's t test.

their inhibitory properties on cardiac K^+- or Ca^{2+}-channel currents [30–33]. In those studies, we dissolved the drugs in the liquid cultured media in advance or persistently injected into the chamber. In our study, however, because the drugs were applied by a puff application method, the exposure of drugs to the cells could be partial or insufficient. To overcome such issues, we applied the drugs to the isolated thymocytes at higher concentrations than those in physiological condition [26–29], such as 100 μM cetirizine, 100 μM fexofenadine, 100 μM azelastine, or 10 μM terfenadine, which were prepared into the patch-pipettes.

Step-like alterations of the membrane potential, from -80 mV (holding potential) to the various levels of depolarizing potential, generated membrane currents in the thymocytes (Figure 1). These currents represented voltage-dependent activation and inactivation patterns that are identical to Kv1.3 [22]. In our previous study, we actually demonstrated in murine thymocytes that margatoxin, a selective inhibitor of the channel, totally inhibited the channel currents [22]. Cetirizine and fexofenadine did not significantly affect the peak Kv1.3-channel currents in thymocytes at the highest voltage-step of + 80 mV (cetirizine: from 266 ± 28.5 to 260 ± 23.7 pA/pF, $n = 16$, Figure 1(a)(B); fexofenadine: from 363 ± 62.3 to 357 ± 62.7 pA/pF, $n = 13$, Figure 1(b)(B)). These drugs rather tended to enhance the peak currents at the lower voltage-steps (Figures 1(a)(A) and 1(b)(A)). However, both cetirizine and fexofenadine significantly lowered the pulse-end currents as demonstrated by the significant decrease in the amplitudes (cetirizine: from 154 ± 18.3 to 107 ± 11.7 pA/pF, $n = 16$, $p < 0.05$, Figure 1(a)(C); fexofenadine: from 228 ± 39.4 to 163 ± 35.6 pA/pF, $n = 13$, $p < 0.05$, Figure 1(b)(C)). Azelastine and terfenadine inhibited the Kv1.3 channel currents in the thymocytes (Figures 2(a)(A) and 2(b)(A)). However, compared to cetirizine or fexofenadine (Figure 1), the peak currents were more dramatically inhibited (azelastine: from 137 ± 24.4 to 67.0 ± 18.4 pA/pF, $n = 12$, $p < 0.05$, Figure 2(a)(B); terfenadine: from 189 ± 39.7 to 117 ± 31.2 pA/pF, $n = 9$, $p < 0.05$, Figure 2(b)(B)) and the pulse-end currents were almost completely suppressed (azelastine: from 101 ± 16.2 to 14.9 ± 4.4 pA/pF, $n = 12$, $p < 0.05$, Figure 2(a)(C); terfenadine: from 114 ± 30 to 34.0 ± 14.7 pA/pF, $n = 9$, $p < 0.05$, Figure 2(b)(C)).

3.2. Effects of Cetirizine, Fexofenadine, Azelastine, and Terfenadine on the Inactivation of Kv1.3-Channel Currents. "Inactivation" is the closing tendency of voltage-dependent ion channels responding to prolonged voltage stimuli after "activation". Recent advances in molecular biology regarding voltage-dependent *Shaker* family K^+-channels revealed the presence of two types of inactivation pattern: N-type and C-type [34]. Although the mechanisms are not fully understood, "N-type inactivation" is referred to as the "fast" inactivation, while "C-type inactivation" is as the "slow" inactivation. According to the existing biophysical and mutational evidence [35, 36], N-type inactivation, conferred by a "ball and chain mechanism" involves a blockade of the intracellular mouth of the pore by the partial binding of the extreme N-terminal residues. In contrast, C-type inactivation involves an alteration of residues in the conserved core domain, which leads to the closing of the external mouth.

In the present study, to make the degree of the current inactivation influenced by the second-generation antihistamines more evident, the ratio of the pulse-end currents, which were considered as a "steady-state" currents (*Iss*), to the peak currents (*Ip*) was additionally calculated (Figure 3). Cetirizine and fexofenadine significantly lowered the *Iss/Ip* ratio (Figures 3(a) and 3(b)), indicating that the drugs facilitated the process of inactivation. In contrast, azelastine and terfenadine did not significantly affected the Iss/Ip ratio (Figures 3(c) and 3(d)), suggesting that the drugs inactivated the currents more slowly than cetirizine and fexofenadine did.

3.3. Effects of Cetirizine, Fexofenadine, Azelastine, and Terfenadine on Whole-Cell Membrane Capacitance in Murine Thymocytes. From our results, cetirizine and fexofenadine facilitated the inactivation of the currents faster than that before the application of drugs (Figures 3(a) and 3(b)). According to kinetic studies [34], the results indicated the "N-type inactivation" current patterns, suggesting that these drugs plugged into the open-pores of the channel, inhibiting the currents. In contrast, both azelastine and terfenadine inactivated the currents more slowly than cetirizine and fexofenadine did (Figures 3(c) and 3(d)). These indicated the "C-type inactivation" patterns according to the kinetic studies [34] and suggested that the drugs conformationally collapsed the inactivation gates (selectivity filters) of the pore-forming domains within the potassium channels [22]. Regarding such pharmacological efficacy, azelastine and terfenadine are likely to change the structure of lymphocyte plasma membranes [16]. Using thymocytes, we precisely detected microscopic changes of the cellular membrane surface by measuring the whole-cell membrane capacitance (Cm) [16, 23]. Thus, we employed this electrophysiological technique in the present study to detect the structural changes induced by the drugs within the thymocyte plasma membranes (Figure 4). Table 1 summarizes the numerical changes in the parameters. When the external solution was simply applied to thymocytes, it did not cause any significant changes in the Cm or other parameters, including the membrane conductance (Gm) and series conductance (Gs) (Table 1). This confirmed the technical precision of our procedure with a puff application of the reagents by a constant hydrostatic pressure using a nearby pipette. The inclusion of 100 μM cetirizine or 100 μM fexofenadine in the patch-pipettes did not significantly alter Cm and other parameters (Figures 4(a) and 4(b); Table 1). However, inclusion of either 100 μM azelastine or 10 μM terfenadine in the patch-pipettes caused significant decreases in the Cm immediately after the drugs were applied (Figures 4(c) and 4(d); Table 1). From these results, azelastine and terfenadine were thought to actually cause the microscopic changes of the structure within the thymocyte membranes. Since the cessation of these drugs during the observation period did not reverse the decreases in the Cm (Figures 4(c) and 4(d); Table 1), the drugs were thought to induce persistent changes in the thymocyte membrane structures.

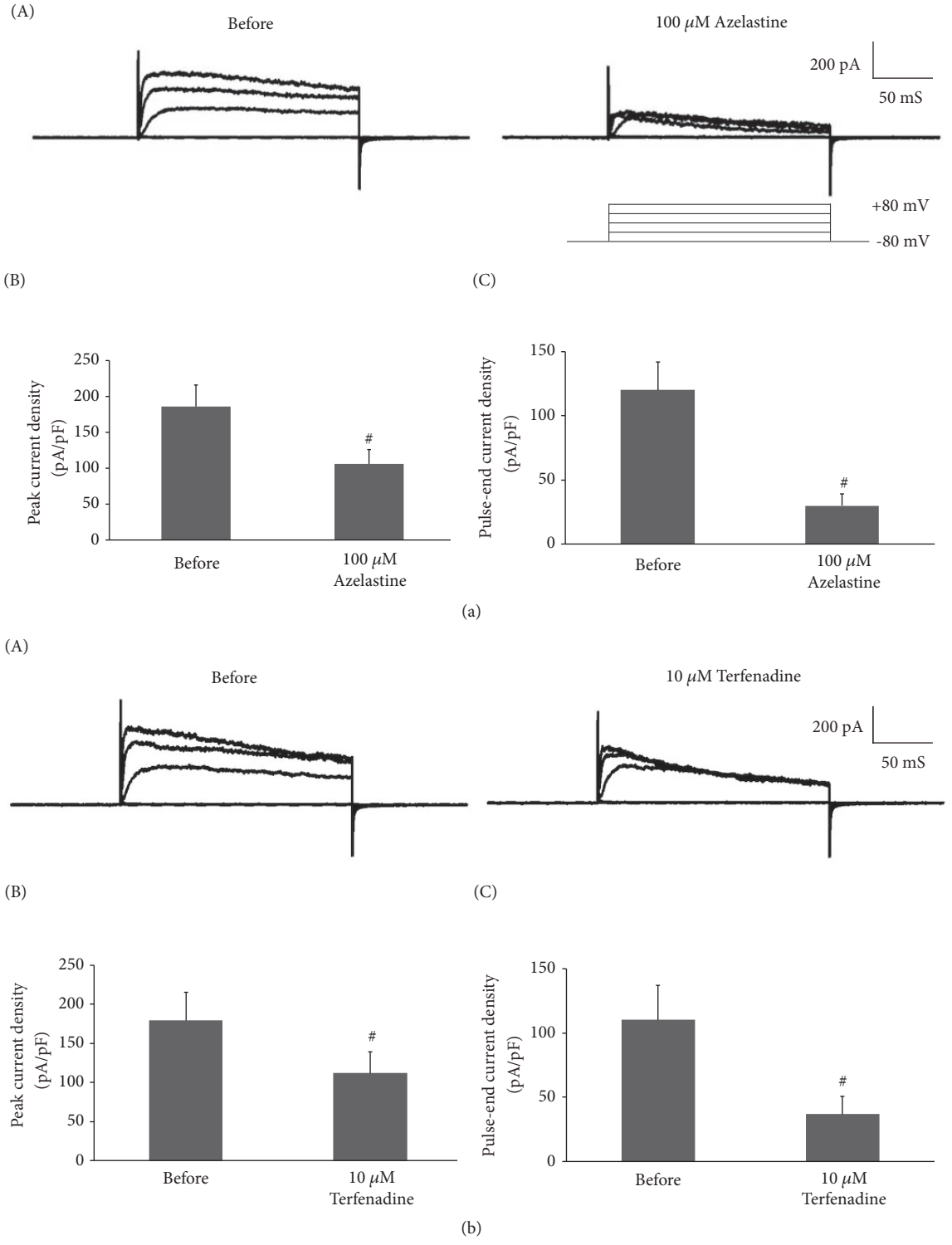

Figure 2: *Effects of azelastine and terfenadine on Kv1.3 channel currents in murine thymocytes*. The effects of 100 μM azelastine (a) and 10 μM terfenadine (b). (A) Typical whole-cell current traces at different voltage-steps recorded before and after the application of either drug. The currents were elicited by voltage-steps from the holding potential of -80 mV to -40, 0, 40, and 80 mV, as depicted in the voltage protocol. Each pulse was applied for a 200-ms duration between 10-second intervals. (B) Peak current densities (peak currents normalized by the membrane capacitance) obtained from the records in (A) at the voltage-step of 80 mV. (C) Pulse-end current densities (pulse-end currents normalized by the membrane capacitance) obtained from the records in (A) at the voltage-step of 80 mV. $^{\#}$ $p < 0.05$ *vs.* before the drug application. Values are means ± SEM (azelastine, $n = 12$; terfenadine, $n = 9$). Differences were analyzed by ANOVA followed by Dunnett's or Student's t test.

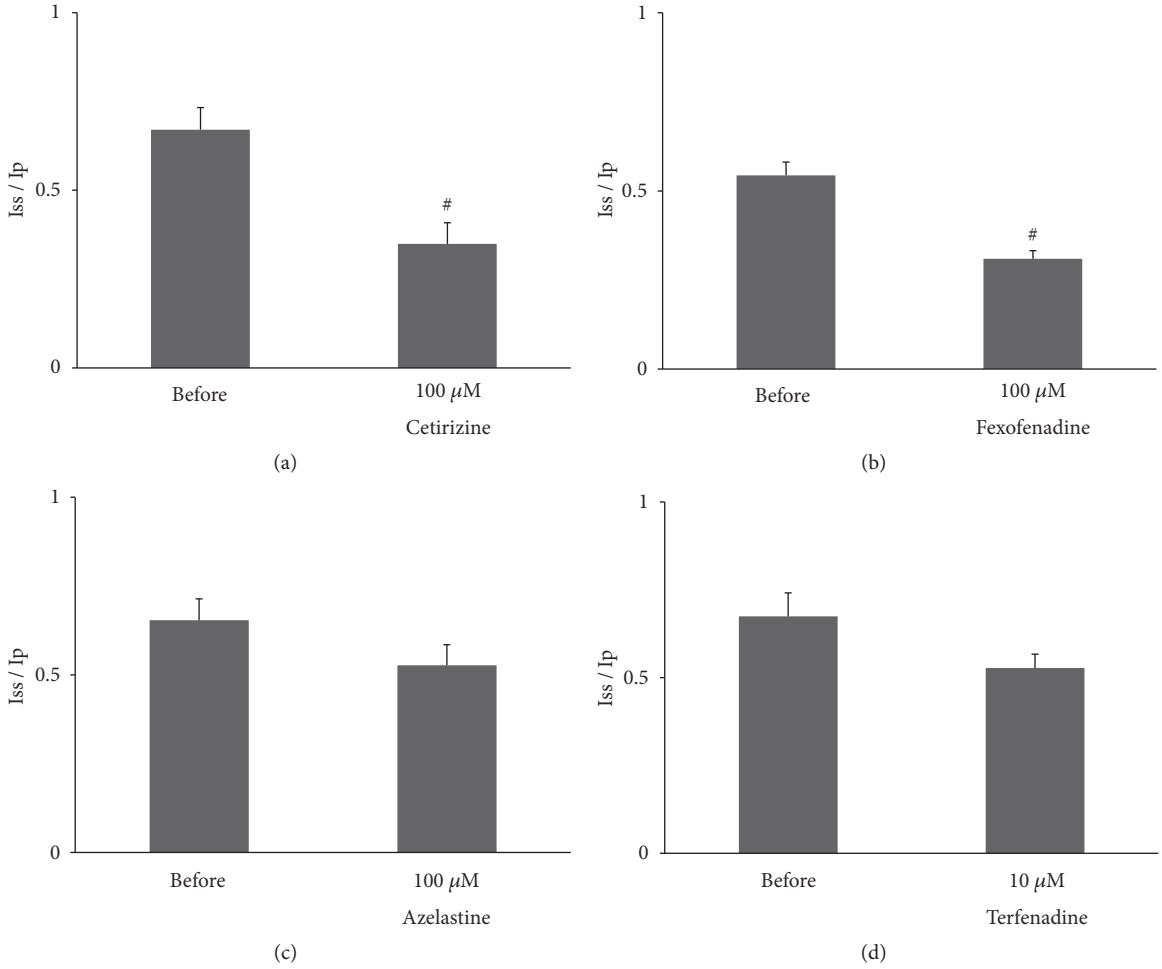

Figure 3: *Effects of second-generation antihistamines on the degree of inactivation of Kv1.3 channel currents*. The effects of 100 μM cetirizine (a), 100 μM fexofenadine (b), 100 μM azelastine (c), and 10 μM terfenadine (d) on the ratio of the pulse-end currents, which were considered as a "steady-state" currents (*Iss*), to the peak currents (*Ip*) obtained from the current traces at the voltage-step of 80 mV. $^{\#}$ $p < 0.05$ *vs.* before the drug application. Values are means ± SEM (cetirizine, $n = 5$; fexofenadine, $n = 5$; azelastine, $n = 5$; terfenadine, $n = 4$). Differences were analyzed by ANOVA followed by Dunnett's or Student's t test.

3.4. Effects of Azelastine and Terfenadine on the Size of Thymocytes and Endocytosis. In T-lymphocytes, membrane trafficking as a result of endocytosis is an important process that regulates the surface expression of membrane proteins, such as T cell receptors [37]. For their recycling, endocytosis constitutively occurs in T-lymphocytes to modulate their immune response [38, 39]. In previous patch-clamp studies, the process of endocytosis in lymphocytes was well monitored by the continuous decrease in the Cm [40, 41]. In our results, since both azelastine and terfenadine significantly decreased the Cm in thymocytes (Figure 4; Table 1), we examined their effects on endocytosis by electron microscopy (Figure 5). At lower magnification, these drugs did not apparently affect the total size of the thymocytes (Figures 5(a)(A), 5(b)(A), 5(c)(A), and 5(d)). At higher magnification of the thymocytes incubated in the external solution alone (Figure 5(a)(B)), there were a few small vesicles in the cytoplasm (arrows) and indentations on the membrane surface (arrow heads), indicating the ongoing process of endocytosis. In thymocytes incubated in azelastine or terfenadine-containing solutions (Figures 5(b)(B) and 5(c)(B)), similar small vesicles were observed in the cytoplasm (arrows). Statistically, there were no significant differences in the total numbers of small vesicles and membrane indentations between thymocytes incubated in the external solution alone and those incubated in the drug-containing solutions (Figure 5(e)). These findings suggested that neither azelastine nor terfenadine affected the process of endocytosis in thymocytes.

4. Discussion

In recent studies, second-generation antihistamines were demonstrated to exert immunomodulatory effects by functionally suppressing human leukocytes when they produce proinflammatory cytokines [4–8]. In our study, we demonstrated for the first time that these drugs, which generally antagonize the histamine H1 receptors, additionally suppress the thymocyte Kv1.3-channel currents. By using specific

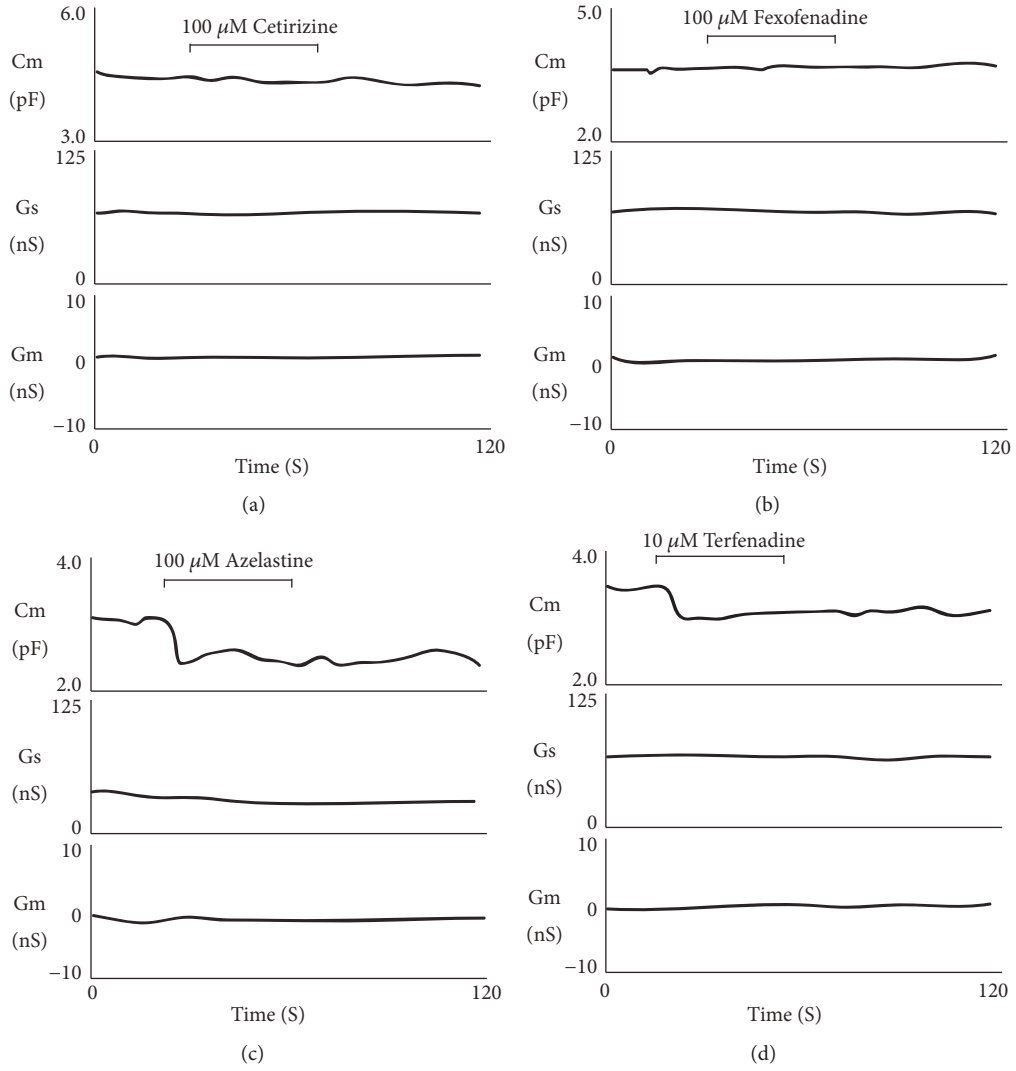

Figure 4: *Second-generation antihistamines-induced changes in thymocyte membrane capacitance, series, and membrane conductance*. After establishing the whole-cell configuration, external solutions containing 100 μM cetirizine (a), 100 μM fexofenadine (b), 100 μM azelastine (c), or 10 μM terfenadine (d) were delivered for 30 sec to single thymocytes. Membrane capacitance, series, and membrane conductance were monitored for at least 2 min. Cm, membrane capacitance; Gs, series conductance; Gm, membrane conductance.

inhibitors of the channel, patch-clamp studies revealed that the Kv1.3-channels are critical for promoting calcium influx and trigger the proliferation and activation of lymphocytes [42–44]. Regarding the molecular mechanisms that are involved, the increased concentration of the intracellular calcium activates the phosphatase calcineurin, which subsequently dephosphorylates the nuclear factor of activated T cells (NFAT), leading to its accumulation in the nucleus and binding to the promoter region of the interleukin 2 (IL-2)-encoding gene [12]. In previous *in vitro* studies, the Kv1.3-channel inhibition by highly selective channel blockers, including ShK-Dap^{22} and margatoxin, was well correlated with the decreased IL-2 production from lymphocytes [9, 45]. Therefore, from our results, the immunosuppressive properties of the second-generation antihistamines are thought to be due to their inhibitory effects on the Kv1.3-channel currents. Our hypothesis is that the second-generation antihistamines' suppression on this delayed rectifier K^+-channel currents may be involved in the control of cytokines secretion by lymphocytes in inflammatory diseases treated with these drugs in the clinical practice. However, more studies are necessary to calculate that the concentrations of antihistamines used in our study were similar to those obtained in the plasma serum by the use of these same antihistamines on the therapy clinical practice.

From our results, in contrast to cetirizine and fexofenadine, which failed to affect the peak amplitude of the Kv1.3-channel currents (Figures 1(a) and 1(b)), azelastine and terfenadine significantly inhibited the currents (Figures 2(a) and 2(b)). In our previous study, the amplitude of peak currents was deeply related to the "activation" of the Kv1.3-channel currents, although kinetic studies are necessary to confirm this [22]. In this context, at the lower voltage-steps, cetirizine and fexofenadine may have stimulated the

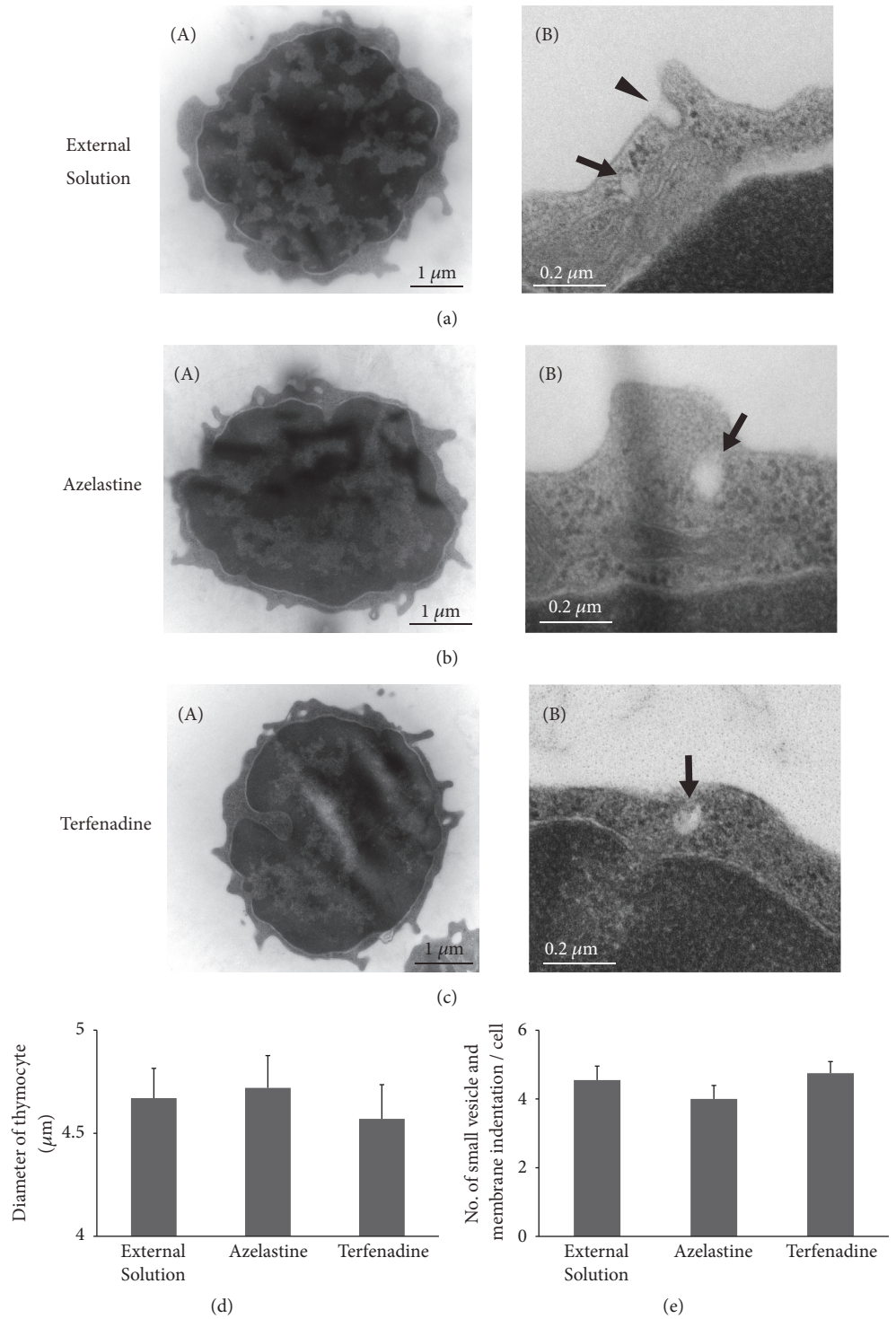

Figure 5: *Electron microscopic images of thymocytes after incubation in azelastine or terfenadine*. Thin-section electron micrographs of thymocyte membrane surface after incubating the cells in the external solutions containing no drug (a), 100 μM azelastine (b) or 10 μM terfenadine (c). Low-power (A) and high-power (B) views. (d) Diameters of thymocytes incubated in each solution were measured and averaged. (e) Endocytosis was quantified in thymocytes incubated in each solution by counting the numbers of small vesicles in the cytoplasm (arrows) and indentations (arrow heads) on the membrane surface per cells. Values are means ± SEM ($n = 20$). Differences were analyzed by ANOVA followed by Dunnett's or Student's t test.

Table 1: Summary of changes in membrane capacitance after application of cetirizine, fexofenadine, azelastine, and terfenadine.

Agents	n	Cm before drug application (pF)	Cm after drug application (pF)	ΔCm (fF)
External Solution	6	3.48 ± 0.30	3.41 ± 0.29	70.0 ± 29.1
100 μM Cetirizine	5	2.66 ± 0.28	2.61 ± 0.27	86.0 ± 11.2
100 μM Fexofenadine	6	3.41 ± 0.43	3.36 ± 0.44	65.0 ± 14.8
100 μM Azelastine	6	4.34 ± 0.73	3.63 ± 0.54$^{\sharp}$	706 ± 228
10 μM Terfenadine	5	2.93 ± 0.42	2.67 ± 0.39$^{\sharp}$	267 ± 74.4

Values are means ± SEM. Cm = membrane capacitance.
$^{\sharp}p < 0.05$ *vs*. Cm before drug application.

opening of the activation gates of the channels, as we previously demonstrated with chloroquine [22]. In contrast, our results suggested that azelastine and terfenadine were more likely to cause membrane depolarization in thymocytes than cetirizine or fexofenadine. The membrane depolarization undermines the Ca^{2+} flux into the cytoplasm, causing a marked decrease in cellular immunity. This may contribute to the stronger immunosuppressive potency of azelastine and terfenadine than the other second-generation antihistamines, as actually revealed by the decreased production of inflammatory cytokines [6–8].

To reveal the mechanisms of drug-induced prolongation of QT intervals on electrocardiograms, previous studies examined the effects of second-generation antihistamines on various ion channels expressed in cardiomyocytes [18]. In patch-clamp studies using isolated cardiomyocytes or transfected cultured cells, second-generation antihistamines, including cetirizine, fexofenadine, azelastine, and terfenadine, all suppressed the currents of delayed rectifier K^+-channels, such as Kv1.5 or Kv11.1, which is codified by the human ether-a-go-go related gene (*hERG*) [31, 33, 46, 47]. In these studies, azelastine and terfenadine more potently inhibited the Kv11.1 than cetirizine and fexofenadine did. Kv11.1 is responsible for the cardiac repolarizing K^+ currents and both azelastine and terfenadine actually increased the action potential duration of cardiomyocytes [33, 48]. Therefore, the blockade of this channel was considered to be the primary mechanism by which azelastine and terfenadine cause QT interval prolongation.

Mathematically, we calculate a whole-cell Cm from a parallel-plate capacitor formula: $Cm = \varepsilon A/d$, where ε indicates the dielectric modulus of the plasma membrane; A indicates the membrane surface area; and d indicates the membrane thickness [24]. Under a physiological condition where ε and d are almost constant, the changes in Cm are attributable to the alteration in the membrane surface area (A) [25]. Therefore, we frequently measured the Cm to monitor the process of exocytosis in secretory cells or endocytosis in phagocytic cells, in which the total membrane surface area is gradually increased or decreased [40, 41, 49–52]. In the present study, however, despite the decrease in the Cm (Figures 4(c) and 4(d)), neither azelastine nor terfenadine apparently affected the size of the thymocytes (Figures 5(a)(A), 5(b)(A), 5(c)(A), and 5(d)), nor did they affect the process of endocytosis in the cells (Figures 5(a)(B), 5(b)(B), 5(c)(B), and 5(e)), indicating that "A" remained constant in the formula after the drug application. In such a condition, the increase in d was likely to be primarily responsible for the decreased Cm [12]. Generally, first-generation antihistamines are more lipophilic than second-generation antihistamines [17]. Among second-generation antihistamines, since azelastine and terfenadine are relatively more lipophilic than the others [19–21], they were thought to distribute more freely into the lipid bilayers of the plasma membranes. Thus, the decrease in the Cm was considered to be mainly attributable to the increased membrane thickness (d) caused by the drug-membrane interactions [53]. Then, from inside the membranes, the drugs may directly intrude into the composite domains of the channels, constricting or conformationally collapsing the selectivity filters of the pore-forming domains within the channel [34]. Consequently, azelastine and terfenadine were thought to induce "C-type inactivation" of the Kv1.3-channel currents (Figures 2 and 3).

According to our previous animal studies, Kv1.3-channels were overexpressed in lymphocytes and were pathologically responsible for their *in situ* proliferation within kidneys and the deterioration of renal fibrosis [54]. We further revealed that benidipine, one of the dihydropyridine calcium channel blockers (CCBs), actually improved the progression of renal fibrosis [54] by strongly inhibiting the lymphocyte Kv1.3-channels [13]. Recently, targeting the channels, we suggested novel pharmacological approaches in the treatment of "chronic inflammatory diseases", including chronic kidney disease (CKD), chronic obstructive pulmonary disease (COPD), and inflammatory bowel disease (IBD) [55–57]. From our results, similar to CCBs, statins and macrolide antibiotics [13–16], second-generation antihistamines, such as cetirizine, fexofenadine, azelastine, and terfenadine, also efficiently suppressed the Kv1.3-channel currents in thymocytes (Figures 1 and 2). Therefore, these drugs may be additively used as potent inhibitors of "chronic inflammatory diseases".

5. Conclusion

In conclusion, this study revealed for the first time that second-generation antihistamines, including cetirizine, fexofenadine, azelastine, and terfenadine, inhibit the Kv1.3-channel currents in lymphocytes. Of note, azelastine and terfenadine significantly lowered the membrane capacitance. Since these drugs did not affect the process of endocytosis in lymphocytes, they were thought to have interacted directly

with the plasma membranes. Such efficacy of these drugs may be related to their immunomodulatory mechanisms by which they reduce the inflammatory cytokine synthesis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Kazutomo Saito and Nozomu Abe contributed equally to this article.

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