Chapter

The Role of Extracellular Vesicles in the Progression of ALS and Their Potential as Biomarkers and Therapeutic Agents with Which to Combat the Disease

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that impairs motor neuron function, leading to severe muscular atrophy. The non-cell autonomous and heterogeneous nature of the disease has so far hindered attempts to define ALS etiology, leaving the disease incurable and without effective treatments. Recent studies have focused on the pathologic role of intercellular communication between nerve cells to further our understanding of ALS pathophysiology. In this chapter, we summarize recent works investigating the role of extracellular vesicles (EVs) as a means of cellular crosstalk for ALS disease propagation, diagnosis, and treatment. There is growing evidence that EVs secreted by the majority of mammalian cells serve as effective biomolecule carriers to modulate recipient cell behavior. This underscores the need to understand the EV-mediated interplay that occurs within irreversibly degenerating nervous tissue in ALS patients. Additionally, we highlight current gaps in EV-ALS research, especially in terms of the pathologic role and responsibilities of specific EV cargos in diseased cells, specificity issues associated with the use of EVs in ALS diagnosis, and the efficacy of EV-mediated treatments for the restoration of diseased neuromuscular tissue. Finally, we provide suggestions for future EV-ALS research to better understand, diagnose, and cure this inveterate disease.

Keywords: ALS, extracellular vesicle, exosome, propagation, biomarker, therapeutic agent

1. Introduction

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is a heterogeneous neurodegenerative disease that primarily impairs both upper and lower motor neurons [1, 2]. 5000 people in the United States are diagnosed with ALS each year, mostly between the ages of 40 and 70. The irreversibly progressive nature of ALS leads to the death of most patients within 2–5 years of diagnosis,

typically due to respiratory failure [1, 3]. In most patients, the cause of ALS is unknown. Only 10% of patients have a familial history of ALS, caused by specific mutations in their genomes, while 90% are exhibit sporadic ALS due to unknown causal factors [1, 4, 5]. The lack of understanding concerning ALS disease mechanisms has led to the development of very few FDA approved drugs. To date, those drugs that have progressed to the US market merely slow down disease progression by a few months and do little to restore patient's neuromuscular function [6]. As progressive degeneration and the non-cell autonomous nature of ALS are known to be major reasons for this stalemate, the aim of much current ALS research is focused on understanding the diverse interplay between neurons and non-neuronal components in neuromuscular tissue. Among the non-neuronal components, glial cells and their crosstalk with neurons have been major targets of study regarding symptomatic development and progression in ALS [7–10]. Accordingly, research into a myriad of proteins and RNAs known to be transmissible between neurons and glial cells has been gaining interest in recent years. It is already well-established that such agents can act as molecular 'messengers' to alter the behavior of nearby cells in ALS, but the detailed mechanisms that underpin specific cargo selection, initiation of transport, and subsequent activation of the internalized biomolecules are not fully understood.

Extracellular vesicles (EVs) are lipid bi-layered particles, less than 2 μm in size, secreted by the majority of mammalian cells, including nerve cells, to mediate diverse paracrine signaling pathways [11–13]. These vesicles are typically classified into three categories: microvesicles, apoptotic bodies, and exosomes [14]. Microvesicles and apoptotic bodies are normally 0.1–2 μm in size and bud directly from the plasma membrane. Exosomes, on the other hand, are smaller (50–150 nm) and are generated within cytoplasmic multivesicular bodies (MVBs) before being secreted to the extracellular space through subsequent fusion of MVBs with the plasma membrane [11, 15].

In many neurodegenerative disorders, EVs derived from nerve cells have been proposed to be responsible for spreading neurotoxins to normal cells, accelerating disease progression [16–20]. In the diseased cell, misregulated proteins serve as 'templates' for subsequent protein oligomerization, generating insoluble toxic aggregates. Such aggregates are then either degraded by lysosomes using a 'self-clearance' mechanism, or incorporated into MVBs and/or the plasma membrane facilitating subsequent release into the extracellular space [21]. EV-loaded biomolecules can then be transmitted to recipient cells primarily by endocytosis but also by endosomal fusion of the EV membrane with cell's plasma membrane [22]. For example, exosomes facilitate the intercellular delivery of amyloid beta (Aβ) peptides in Alzheimer's disease, leading to plaque formation in the recipient cells [19, 20]. A similar trend of EV-mediated or free protein spreading in a 'prion-like' manner is observed in Parkinson's disease (PD). Specifically, studies with mice have demonstrated that grafted cells containing aggregated α-synuclein can transfer these protein aggregates to healthy brain tissue [23].

As the contents of EVs reflect the physiological status of the original cell, recent studies have begun to evaluate the possibility of using these structures as biomarkers with which to gauge the onset and progression of a diverse range of neurodegenerative diseases. Issues remain with identifying tissue specificity and cell type of origin for pathogenic EVs found in body fluids. However, convenient sampling and improved understanding of internal cargo molecules' function has led to EV's being seen as one of the strongest candidate classes for next generation prognosis/diagnosis screening. As with other degenerative diseases of the central nervous system (CNS), much recent work on ALS has focused on investigating the pathological role of EVs in diseased neuromuscular tissue, as well as evaluating their

applicability in the early diagnosis and further treatment of the disease. In this review, we will highlight recent research studying the diverse roles of EVs in ALS progression, diagnosis, and treatment. In addition, we will discuss possible solutions for unsolved problems in this area and suggest future directions for ALS-EV research to further our understanding of ALS pathology and help develop advanced diagnosis and treatment methods using EVs.

2. EVs as dysregulated biomolecule carriers for disease propagation in ALS

ALS tissues commonly contain cells supporting dysregulated protein aggregates in their cytoplasm and these structures often exert detrimental effects on cell viability and function. SOD1 (superoxide dismutase 1) and TDP-43 (transactive DNA binding protein 43 kDa), encoded by the *SOD1* and *TARDBP* genes respectively, are the most well-studied proteins susceptible to ALS-associated aggregation. Aggregation of these proteins, in some cases due to destabilizing mutations, are known to be actively involved in motor neuron degeneration in both familial and sporadic ALS [1, 24, 25]. The SOD1 enzyme resides in the cytoplasm of normal cells to regulate oxidative stress by converting free superoxide radicals into molecular oxygen [2]. There are more than 180 mutations reported in the *SOD1* gene, and oligomerization of the encoded proteins has been shown to cause increased intracellular oxidative stress and anomalous metal binding [2]. However, the pathologic pathway connecting *SOD1* mutation, protein dysregulation, and subsequent neurodegeneration have yet to be defined in a comprehensive manner [2, 26]. TDP-43 is a highly conserved nuclear RNA and DNA binding protein, known to be involved in transcriptomic regulation, primarily by RNA splicing but also by effects on mRNA transport and stability, effects on microRNA production, and participating in DNA repair [27–31]. Approximately 97% of ALS patients have abnormally aggregated TDP-43 in their neurons, even without direct mutation of the *TARDBP* gene, leading to ALS proteinopathic characteristics in their pathology [6, 32].

Strikingly, recent ALS studies have demonstrated that abnormally transformed proteins, such as SOD1 and TDP-43, do not merely impair the cells of origin, but migrate to neighboring cells by means of extracellular exosome release, resulting in a spread of their cytotoxic effect to recipient cells [33, 34]. Exosome shuttling has been shown to be a preferred cellular mechanism for removing intracellular toxic molecules to the extracellular space [35]. The exosomal loading of cytotoxic protein aggregates is beneficial to host cells since it minimizes the physiological damage caused by these structures. Such phenomena could be promoted by the host cell recognizing an increase in intracellular protein aggregation or impaired lysosomal autophagy. Interestingly, normal neurons cultured with TDP-43 aggregate-loaded exosomes induce cytoplasmic redistribution of endogenous TDP-43, leading to an exacerbation of the disease phenotype in mice [36]. Moreover, proteins packaged in extracellular vesicles are preferentially taken up by recipient cells and exhibit a greater detrimental physiological effect compared to free protein release, highlighting a crucial role for EV and plasma membrane tethered proteins in regulating protein internalization and functional activation [37]. These studies suggest an important interaction between exogenous TDP-43 transported via extracellular vesicles and endogenous TDP-43 expressed by the recipient cell. Such interactions constitute a pre-requisite for 'prion-like' seeding followed by cytoplasmic protein redistribution and offer a potential mechanism for the rapid propagation of disease phenotypes throughout the motor neuron pool. Indeed, when insoluble TDP-43 aggregates taken from ALS brain were introduced to neuron-like SH-SY5Y cells

endogenously expressing normal TDP-43, the treatment induced significant aggregation of TDP-43 in recipient cells in a self-templating manner [38]. Similarly, SOD1 aggregates transferred via exosomes have been shown to work as self-templating 'seeds' in recipient cells, leading to the propagation of a misfolded protein that persists in culture over multiple passages and population doublings [34].

Although recent studies have observed intercellular protein transmission via EVs, their specific roles in neuromuscular pathophysiology are poorly understood. In particular, how the transfer of abnormal proteins induces subsequent neuronal damage and a breakdown in their electrophysiological function has yet to be elucidated. Furthermore, by taking into account that ALS exhibits non-neuron autonomous characteristics, it is essential to obtain a more comprehensive understanding of whether and how non-neuronal nerve cells can damage neuromuscular function via the exosomal transfer pathway. **Table 1** provides an overview of the studies performed so far relating to the role of EVs in propagating ALS pathologies to neighboring cells and these studies are discussed in more detail throughout this section.

Among non-neuronal cell types, astrocytes have gained significant interest as carriers of detrimental protein aggregates in ALS tissues. Secretome analysis of astrocytic exosomes from SOD1 (G93A) mutant mice were reported to contain SOD1 aggregates, and the exosomal release of these structures accounted for a larger proportion of SOD1 transport than free SOD1 release [4, 14]. In addition, proteomic analysis has revealed that proteins involved in vesicle trafficking are downregulated in mutant astrocytes, indicating a possible impairment of protein disposal in ALS astrocytes. Moreover, wild-type mice transplanted with astrocyte progenitors expressing mutant SOD1 exhibit motor neuron impairment, raising the reasonable postulation that diseased astrocytes utilize extracellular vesicle-mediated paracrine communication to deliver pathogenic protein aggregates to motor neurons [4, 14]. Another quantitative proteomic analysis of EVs derived from ALS nervous tissue showed a relative absence of the microglial marker (CD11b) but positive expression for the astrocyte marker (GLAST) and the synaptic marker (SNAP25), indicating that astrocytes and neurons may constitute the major cell types involved in EV-mediated communication in the CNS [7]. However, as the main function of microglia in the CNS is immune response regulation under pro-inflammatory conditions, microglia might also actively secrete EVs within an immune-active environment to modulate the immune response of other nerve cells. Since ALS is reported to have autoimmune disease characteristics as well [39, 40], a pathogenic role for EVs secreted from diseased microglia in ALS tissue could be another important subject to explore.

Do skeletal muscle cells also secrete EVs for neuronal uptake? The breakdown of neuromuscular junctions (NMJs; the synapses connecting lower motor neurons and skeletal muscle fibers) is a critical early indicator of pathological onset in ALS. However, it remains unclear whether NMJ breakdown occurs due to general ill health of the motor neuron or some aberrant signaling between these neurons and their afferent synaptic contacts. If the latter is true, a reasonable hypothesis would be that ALS progression is affected by paracrine communication between skeletal muscle and motor neurons. To this point, an interesting study in 2017 described the involvement of skeletal muscle in EV-based crosstalk in neuromuscular tissues. In ALS muscle biopsies, noticeable accumulation of MVBs containing exosome-like vesicles were measured, with significant increases in vesicular protein concentrations reported when compared with controls. In addition to the denser protein accumulation reported in the EVs, ALS skeletal muscle-derived EVs were shown to exclusively damage neuronal viability *in vitro* [4, 14].

Role of EV	Study method	Source of EV	Significance	Unknowns	Reference
Dysregulated protein and RNA carrier	*In vitro* study with astrocytes and mouse motor neurons	Directly differentiated astrocytes from fALS (C9ORF72) patient-derived fibroblast	Micro-RNAs in ALS astrocyte-derived extracellular vesicle (EV) caused neuronal network degeneration and growth cone impairment	The role of C9ORF72 protein for dysregulated miRNA encapsulation in EV	Varcianna et al. [41]
	In vitro/ex vivo study for whole tissue vesicle isolation	Brain- and spinal cord tissue from NTg and SOD1 mutant mice	Brain-derived astrocytes and neurons, but not microglia, were the main EV source in CNS	Detailed cargo modification pattern of EVs in the diseased cells	Silverman et al. [7]
	Clinical research with sporadic ALS patients' body fluid for EV analysis	Venous blood of sALS patients	Microvesicles of ALS patients were enriched with potentially pathological protein (SOD1, TDP-43, FUS), while exosomes did not show any protein changes	Contradictory result with other (*in vitro*) studies that suggested huge loading of dysregulated proteins in exosomes of ALS mutant cells	Sproviero et al. [59]
	In vitro study with ALS mouse muscle-derived exosomes treated to primary/iPSC-derived motor neurons	Skeletal muscle cells from SOD1 mutant mouse	EVs derived from ALS myotubes encapsulated H2-AX (neurotoxin) which suggests possible dissemination of various neurotoxic molecules from diseased skeletal muscle to normal neurons	Is H2-AX a major neurotoxin in human ALS skeletal muscle as well?	Gall et al. [60]
	In vitro study using Neuro2a and primary neurons to study the role of exosomes in ALS proteinopathy	Primary neurons and Neuro2a cells from ALS mouse brain	Exosome secretion was beneficial in neuronal clearance of pathological TDP-43, but also it might be responsible for the propagation of the toxic TDP-43 aggregates to the other cells	Should we inhibit exosome secretion to prevent aggregated TDP-43 propagation or promote the secretion for TDP-43 clearance in neurons?	Iguchi et al. [36]
	In vitro co-culture of NSC-34 with cortical	Mutant DPR (dipeptide repeat proteins)	DPRs can be transmitted to neurons and glial cells with/	Difference between propagated DPRs and other	Westergard et al. [61]

Role of EV	Study method	Source of EV	Significance	Unknowns	Reference
	neurons and astrocytes	transfected NSC-34 cells	without exosome involvement	dysregulated proteins (TDP-43, SOD1) in terms of their neurodegeneration effect?	
	In vitro study using ALS patient-derived exosomes and human glioma cell line (U251)	CSF from 18 sALS patients	ALS-CSF incubation with U251 cells increased mis-located TDP-43 in the glioma cells and induced their apoptotic behavior and macro autophagy process	Connection between propagated TDP-43 and autophagy mechanism in recipient cells	Zhou et al. [62]
	In vitro study using primary neurons and TDP-43 transfected HEK cells	ALS post-mortem lysate, primary cortical mouse neurons and HEK cells	TDP-43 oligomers were present in EVs and showed that microvesicular TDP-43 exerts higher toxicity than free TDP-43	EV encapsulating pathway of neurotoxic TDP-43 oligomers	Feiler et al. [37]
	In vitro study with neuroblastoma cells with brain tissue obtained from ALS/FTD patients	Neuroblastoma cells expressing TDP-43 and SOD1	Misfolded wild-type proteins could traverse cell-to-cell as a self-templating 'seed' either as free protein aggregates or loaded on the surface of exosomes	Which specific receptors control the uptake of misfolded TDP-43/ SOD1 presented on exosomes?	Nonaka et al. [38]
	In vitro study analyzing neurotoxic effect and amount of SOD1 protein encapsulated in astrocytic exosomes	Primary astrocytes from mouse expressing human mutant SOD1	Mutant SOD1 astrocytes released increased number of exosomes, which were toxic for motor neurons	Protein factors which involved in mutant SOD1 astrocytic exosome	Basso et al. [26]

Table 1.
Overview of the recent findings regarding roles for extracellular vesicles in ALS disease propagation.

Although dysregulated protein transfer is of major interest in ALS-EV research, the role of transmissible miRNAs in facilitating ALS progression should not be overlooked. EVs obtained from astrocytes derived from ALS patient post-mortem tissue (*C9ORF72* mutation) have been shown to display a neurotoxic phenotype, inducing mouse motor neuron degeneration, though fewer EVs were secreted from diseased astrocytes than normal controls [41]. Dysregulated miR-494-3p was identified as a main component of the diseased EVs. This astrocyte-specific miRNA is

known to negatively regulate semaphorin-3A expression, which is highly involved in axonal growth and maintenance [41]. Similarly, exosomes secreted by mouse-derived motor neuron-like cells (NSC-34) transfected with a human SOD1 mutant variant were enriched with miR-124. This miRNA was in turn transmissible to the microglia, where it induced impairment of their phagocytic ability and an increase in pro-inflammatory gene expression [42]. These results indicate the presence of a multi-directional intercellular communication network in ALS that is mediated by miRNAs encapsulated in EVs. Researchers have only begun to scratch the surface of EV-mediated miRNA transfer in ALS and this area requires more comprehensive study to fully disentangle the pathologic milieu that exists among the different types of nerve cells in ALS nervous tissue.

Research investigating EV-mediated ALS pathophysiology is in an incipient stage, and impressive results in previous studies are still raising a number of important questions that must be addressed. For example, it is unclear whether the beneficial effect of elimination of intracellular toxic proteins outweighs the deleterious effects of uptake of those proteins by neighboring cells. Understanding this point will be critical when designing therapeutic methods to inhibit the propagation process. Furthermore, to promote extracellular disposal of toxic protein aggregates while inhibiting their uptake by neighboring cells, we need to fully understand the interactions between surface proteins on detrimental EVs and those on the plasma membranes of motor neurons. Alternatively, efforts could be focused on enhancing lysosomal degradation process over EV-mediated protein disposal in diseased cells, but it is unclear whether the majority of detrimental contents in their multivesicular bodies can alternatively be delivered to autophagosomes for degradation. Another gap in EV-ALS research is a lack of studies focused on analyzing motor neuron-derived EVs to understand the phenotypic effect of transmitting neuron-originated proteins to other neurons and/or non-neuronal cells within ALS tissues. As motor neurons are the main target in ALS pathogenesis, pathogenic proteins are likely to be enriched in motor neuron-derived EVs and could potentially directly damage glia and/or NMJ structures, or even directly affect skeletal muscle contractility. We believe that answering these questions, in addition to better characterizing non-neuronal EV cargos, should constitute the principle focuses of future studies aimed at improving our understanding of EV-mediated ALS progression. The results of such work would doubtlessly provide invaluable insights into ALS pathophysiology and help identify suitable targets for future therapeutic development.

3. Can EVs serve as reliable biomarkers for ALS?

There is no reliable biomarker established for ALS, neither for confirming disease onset nor for characterizing disease progression [43]. The lack of a reliable biomarker for ALS makes the correct prognosis challenging, and even limits diagnosis to relatively late stages, after patients recognize their neuromuscular symptoms. As the typical life expectancy for ALS patients is approximately 2–5 years after disease onset, early and accurate diagnosis is crucial not only for developing early-stage applicable therapies, but for improving quality of life for patients during their follow-up period. A new detection method should be robust and convenient for clinical settings, and, critically, have sufficient detection sensitivity, specificity, and reproducibility to ensure confidence in the result. Meeting all these requirements simultaneously is an extremely challenging goal, given the extremely heterogeneous nature of the disease. Recent studies are evaluating ALS patient-derived EVs as potential biomarkers of ALS for prognosis, early diagnosis, and patient stratification. RNAs collected from patient's blood or cerebrospinal fluid (CSF;

often used as a surrogate for nervous tissue sampling) are the main target of analyses so far and advanced RNA-sequencing techniques are being employed to analyze deregulated RNAs exclusively in ALS patient-derived EVs. Recently, Otake et al. reported a new methodology using highly sensitive exoRNA-sequencing for comprehensive analysis of exosomal mRNAs in patient CSF, to identify abnormally expressed mRNAs in exosome samples from ALS patients [43]. The technique identified 543 mRNAs exhibiting statistically different expression patterns compared to normal samples. In particular, this analysis revealed that the gene *CUEDC2* was only detected in ALS patient-derived exosomes. As the gene is known to regulate the ubiquitin-proteasome pathway as part of the inflammatory response, its abnormal expression is postulated to cause potential neuroinflammation in ALS, making *CUEDC2* mRNA a strong biomarker candidate [43]. Follow-up studies are necessary to demonstrate a specific causal relationship between exosomal *CUEDC2* mRNA presence (rather than free intracellular *CUEDC2* expression) and ALS development. Furthermore, work demonstrating an omnipresence of the same RNA mis-regulation in EVs from other ALS patients is also required. However, the described study highlights how exosomal mRNAs could be attractive biomarker candidates, given their stability within body fluids, as well as the convenience of sample collection and ease of subsequent data analysis. Efforts to date to characterize EV cargos as ALS biomarkers, including those discussed in detail above and below, are summarized in **Table 2**.

As non-coding RNAs are also reported to be involved in ALS onset and progression, miRNAs loaded in EVs represent another candidate biomarker class for ALS diagnosis. Study of free miRNA for ALS detection has been previously reported. Microarray analyses were performed on ALS mutant mouse-derived cells and patient serum, and the results identified the expression of 10–13 dysregulated miRNAs in diseased samples [44, 45]. In addition to free-miRNA analyses, Saucier et al. tried identifying ALS-associated miRNA signatures in EVs to discriminate blood between healthy and ALS individuals. Extensive exosomal RNA analysis using high-throughput sequencing coupled with droplet digital PCR enabled the identification of a group of dysregulated miRNAs, including miR-183-5p, miR-9-5p, miR-338-3p and miR-1246, which were all remarkably downregulated in ALS patient-derived exosomes [45].

EV-based biomarker studies in ALS have so far given promising results and strong motivation for follow-up studies to further specify molecular candidates with higher disease relevancy. However, limited information on the exosomal RNAs, in terms of the specific signaling pathways they regulate, is currently available. This lack of understanding makes it difficult to determine the relevance of each when attempting to define a diagnostic EV-RNA signature for ALS. Also, as significant difficulty lies in identifying the cellular origin of EVs collected from body fluids, their practical application in diagnostics could be challenging, especially if the same miRNAs secreted from different cells of origin reflect different states of the disease. Furthermore, the RNA profile in different subtypes of EVs, such as exosomes versus microvesicles, that exist in patient's body fluids has not yet been investigated. As such, more comprehensive RNA analysis, using entire EV populations, might give discordant results to those reported from exosomal RNA analysis. Additionally, to date there has not been any analysis of when specific RNAs arise during ALS disease progression and whether certain expression patterns are indicative of certain stages of the pathology. Such an understanding is crucial in determining whether expression of certain RNAs can be employed effectively as diagnostic tools or as methods to chart disease progression. Finally, as hundreds of exosomal RNAs in ALS patient body fluids have been found to exhibit significant differences in their expression levels relative to normal controls, clinically

Role of EV	Study method	Source of EV	Significance	Unknowns	Reference
Biomarker	Sequencing exosomal mRNAs in CSF	CSF of ALS patients and normal donors	A new methodology for comprehensive analysis of exosomal mRNAs in human CSF using newly developed exoRNA-seq, which showed potential applicability to identify specific ALS biomarkers	Specificity level of mRNAs detected in ALS exosome using the technique	Otake et al. [43]
	Clinical study comparing the expression of miR-27a-3p in serum-derived exosomes	Serum of ALS patients and healthy subjects	The expression of miR-27a-3p in patients with ALS was significantly downregulated than that in healthy human serum exosomes	Specific role of miR-27-3p in the expression of disease phenotypes in ALS	Xu et al. [63]
	Clinical analysis of miRNA profile in 16 ALS patients	Serum of ALS patients and healthy controls	Distinct miRNA expression profile was observed in ALS patient's serum-derived exosomes compared to healthy controls	Are ALS-associated miRNAs actually involved in post-transcriptional regulation of neurons?	Saucier et al. [45]
	In vitro study with NSC-34 and N9 microglia to discover ALS specific miRNAs	Motor neuron like NSC-34 cells with mSOD1 expression	Increased level of miR-124 in circulating exosomes of NSC-34 may be used as potential biomarker of motor neuron degeneration in ALS	miR-124 expression in other health state? (false positive issue)	Pinto et al. [42]
Therapeutic agent	*In vitro* study using adipose-derived stem cells and human SOD1 overexpressing mouse NSCs	Adipose-derived stem cells	Adipose-derived stem cell exosomes showed paracrine effect to SVG neurons to alleviate their disease phenotypes and mitochondrial dysfunction in ALS	Major exosomal cargos that exert each of therapeutic effects on recipient neurons	Lee et al. [48]
	In vitro study assessing efficacy of stromal cell-derived exosomes on NSC-34 cells expressing hSOD1 mutants	Murine adipose-derived stromal cells	The ASC-derived exosomes were able to protect motor neuron-like NSC-34 (SOD1 mutant) from oxidative stress and increase their viability	Where does the beneficial effect of ASC-exosomes come from?	Bonafede et al. [50]
	Proteomic profiling of exosomes derived from mASC	Murine adipose-	Proteomic analysis revealed mASC-derived exosomes	Exosomal effect on actual restoration of	Bonafede et al. [47]

Role of EV	Study method	Source of EV	Significance	Unknowns	Reference
	with *in vitro* assay of exosome treatment on NSC-34 cells	derived stem cells	contain proteins for cell adhesion and negatively regulate cell apoptosis	neuronal function	

Table 2.
Summary of the applications of extracellular vesicles in ALS diagnosis and treatment.

applicable diagnosis will be difficult until we better understand the physiological outcomes of those misregulated RNAs in neuromuscular tissue maintenance and function. Future research elucidating the expression patterns of EV cargos across diverse ALS populations at different disease stages, as well as studies of the signaling pathways regulated by EV-RNAs in ALS tissue, are therefore necessary before the value of these molecules in ALS diagnostic medicine can be fully evaluated.

4. EVs for therapeutic agents in ALS treatment

EVs hold distinct advantages for function as stable biomolecule carriers. Their lipid bilayers, decorated with transmembrane proteins, protect cargo molecules from enzymatic degradation in the extracellular space as well as making them immune-tolerant. Functional transmembrane proteins also promote EV internalization into recipient cells, which naturally occurs through active fusion of EV membranes with cell's plasma membranes. This in turn facilitates release of EV molecular cargo to recipient cell cytoplasms or induces endosome uptake for functional activation [23, 46].

Accordingly, an attractive hypothesis is that therapeutic molecules loaded in vesicles could be delivered to target cells to subsequently modulate phenotypes in neurodegenerative disease. In such a model, cargo molecules could encompass proteins and mRNAs, or non-coding RNAs for post-transcriptional protein regulation, or even specific compounds for sustained activation with avoidance of enzymatic attack. Research efforts to test this hypothesis are summarized in **Table 2** and discussed in detail below. As very few drug candidates with a proven efficacy for treating ALS exist, research on 'drug-loaded EVs' has not yet begun in earnest. However, adipose-derived stromal cells and stem cells have shown a capacity to generate exosomes capable of conferring a therapeutic effect on defective neurons. Exosomes from murine adipose-derived stromal cells alleviated oxidative stress and reduced hydrogen peroxide-induced apoptosis in NSC-34 cells overexpressing a human SOD1 mutant variant [47]. Safe availability and a capacity to migrate to damaged tissues for their reparative processes make stromal cells good candidates for EV sources. Although the study demonstrated that stromal cell-derived EVs can reverse SOD-1 induced cell death, such applications have not yet been specifically targeted to restore electrophysiological phenotypes in ALS neurons. Consequentially, the applicability of stromal cell-derived EVs may be limited if the specific cargo molecule responsible for restoring motor neuron function is not identified.

Retaining the remarkable therapeutic potential of stem cells, while avoiding issues with tumorigenicity, insufficient therapeutic specificity, and substantial cell loss during treatment, stem cell-derived EVs offer an exciting alternative to direct stem cell therapy. Indeed, EVs obtained from undifferentiated stem cells have shown a capacity to alleviate disease phenotypes in ALS mutant neurons. Specifically, exosomes from adipose-derived stem cells reduced SOD1 aggregation and

rescued normal mitochondrial protein expression in mouse neurons [48]. Another adipose-derived stem cell study performed proteomic analysis of EVs and found 189 exosomal proteins, mainly involved in regulating cell adhesion and negative regulation of apoptosis. Stress response proteins, such as SOD1, were also included in these exosomes and were found to replace the enzymatic function of mutant SOD1 in NSC-34 cells. Additionally, the study reported the presence of ribonuclease RNase 4 in the examined exosomes. This could represent a potential neuroprotective molecule applicable in ALS, as RNase 4 has been reported to have angiogenic, neuroprotective properties [49, 50].

The field of EV-mediated therapeutics for ALS treatment is still in its infancy. However, since glial cells and neural stem cells are responsible for regulating neuron differentiation, protection, and synaptic function [13, 51–54], EVs derived from those cells could constitute attractive subcomponents for therapeutic agent development. As mentioned above, EVs possess distinct advantages for therapeutic development, including sustained cargo delivery and avoidance of physiological degradation. Naturally derived EVs, as well as engineered EVs loaded with optimal therapeutic materials, therefore represent a powerful candidate for the future of ALS treatment. However, the following issues should be addressed to facilitate the practical application of EVs in treating this disease. First, since EVs secreted from one type of cell usually do not target a specific cell type for cargo delivery, higher delivery specificity to eliminate potential off-target effects is essential. EV membrane engineering to attach proteins with exclusive affinity to target neuron cell surfaces could be an attractive approach to consider [55–58], if reliable surface markers for diseased cells can be defined. Second, a lack of information regarding the physiological function of specific cargo molecules in glia or stem cell-derived EV limits their applicability in ALS treatment. As disease phenotypes in ALS are extremely heterogeneous, investigating the role of each EV-loadable molecule with more categorized efficacy studies beyond cell viability is also highly required. Production scale of EVs is a critical issue for the actual application of this technology in ALS clinics. Although EV collection technology continues to advance to minimize EV loss and reduce collection time and cost, most EV experiments are still done in small-scale benchtop studies. This is not a huge issue during these early stages of research and development, but will cause a critical problem for scale up and

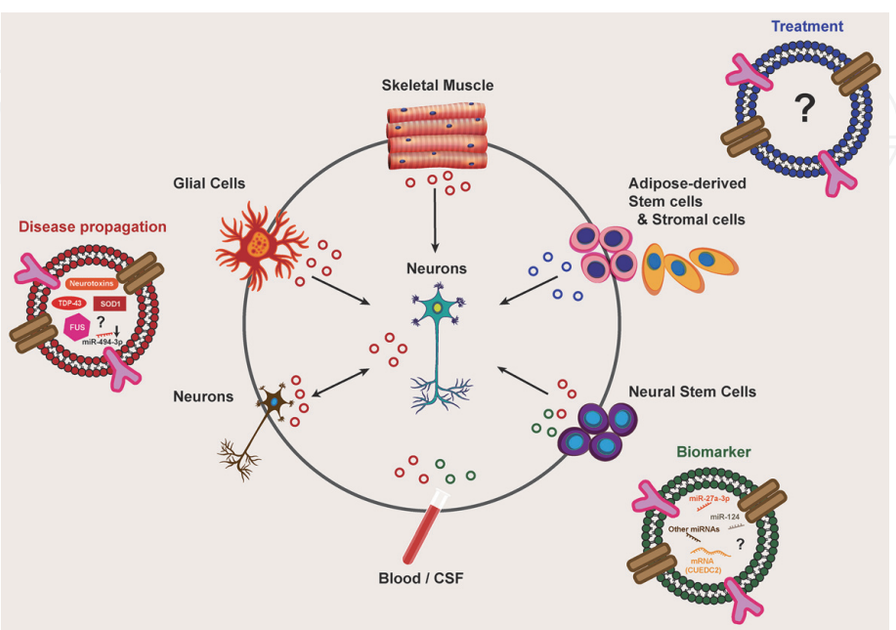

Figure 1.
Schematic summary of the major role EVs play in ALS propagation, diagnosis, and treatment.

administration as we move closer to clinical trials and subsequent distribution. Lastly, cargo molecule purity is another significant issue. Even with a single cell source, EV cargo composition is likely to be different based on culture conditions, and may fluctuate due to other unknown factors; especially if cell-derived EVs is advanced to large-scale production (**Figure 1**).

5. Conclusions

Current EV research in relation to ALS is weighted toward investigating the pathogenic role of intercellularly transmissible vesicles, with a particular focus on dysregulated protein propagation. This is likely due to the fact that SOD1 and TDP-43 mutant cells are already known to produce neurotoxic protein aggregates, which makes the hypothesis that their dissemination via EVs contributes to the nature of irreversible degeneration of neuromuscular tissue in ALS a straightforward one. Several studies have demonstrated that EVs collected from ALS patient's body fluids and from mutant non-neuronal cells can be internalized by healthy cells. Their capacity to induce neurodegenerative behavior in these cells supports the notion that these structures contribute to the rapid disease progression characteristic of ALS. However, the effect of collected EV components in specific ALS disease phenotypes is currently quite ambiguous, especially for correlating neuromuscular tissue level abnormalities with defined EV component expression. To address this, EV-mediated interplay, occurring at the diseased NMJ may be a good potential target for future investigations. EVs secreted from skeletal muscle and motor neurons are known to contribute significantly to the development of normal synaptic formation at the NMJ and better understanding how these signaling processes are disrupted in ALS could significantly improve our understanding of early disease etiology. Work in these early stages of ALS and EVs has also highlighted the potential for using EVs as either a biomarker for ALS diagnostics or even a potential therapeutic agent. Although evidence of a causal relationship between misregulated EV-RNAs and functional impairments in ALS neurons is currently sparse, notable differences in ALS EV-RNA expression patterns detected by next generation sequencing does support their potential as future diagnostic tool. EV's exogenous nature and pre-established cellular internalization mechanism also provides substantial motivation to continue research that applies these vesicles in therapy development, either as a molecule carrier or as a naturally-derived drug in and of itself. Further studies addressing the non-specificity of EV delivery and issues with production scale will raise their status as a potent therapeutic means to combat ALS in the future.

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