EXTRACELLULAR VESICLES AS NEXT GENERATION THERAPEUTICS

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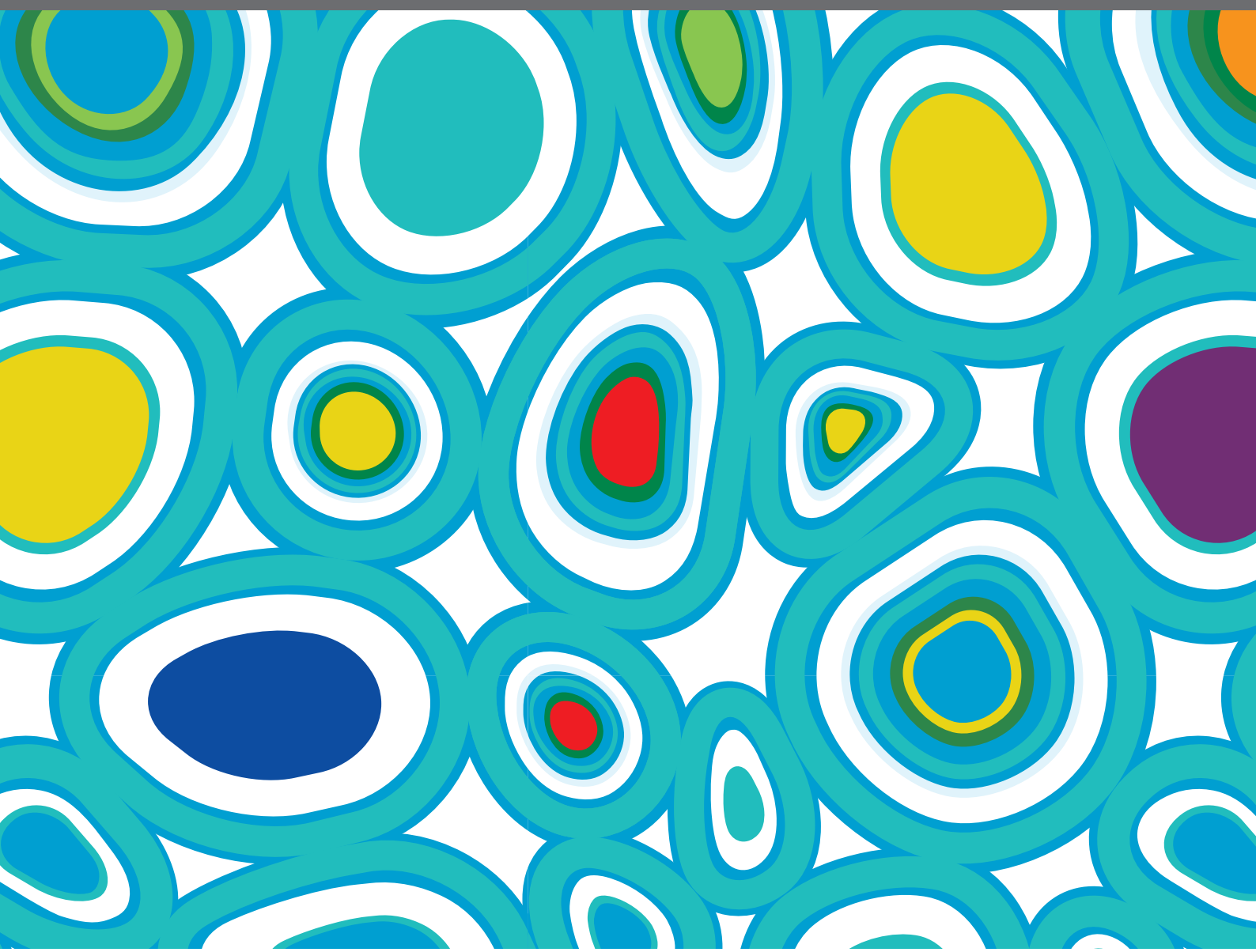

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EXTRACELLULAR VESICLES AS NEXT GENERATION THERAPEUTICS

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EDITORIAL
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Editorial: Extracellular Vesicles as Next Generation Therapeutics

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Keywords: extracellular vesicles, therapy, regeneration, disease treatment, drug delivery, vaccine, bioengineering, nanomedicine

Editorial on the Research Topic

Extracellular Vesicles as Next Generation Therapeutics

Extracellular vesicles (EVs) are nano-sized (30–200 nm) cell membrane-derived circular structures, released by virtually any cell type. They carry variety of bioactive molecules, including proteins, lipids and RNA species, by which they can influence phenotype and properties of other cells. Owing to such properties, EVs are increasingly recognized as important mediators of cell-to-cell communication system, which can be successfully leveraged for therapeutical use in the treatment of human diseases (Wiklander et al., 2019). This Research Topic aimed at presenting recent advances in utilizing EVs as future medicines, regarding different aspects of EVs engineering, manufacturing and testing their therapeutic efficacy.

We have collected four original articles, four reviews and two perspectives, which cover the most important aspects of biomedical applications of EVs and EV-derived bioactive molecules (**Figure 1**). The list of medical indications, which can benefit from EVs usage, is increasingly growing. Some of these areas are considered in this article collection, including heart disease, stroke, pulmonary fibrosis, dermatological conditions, immunomodulation and cancer. However, the information provided here does not fully exhaust the broad possibilities of EV utility as next-generation therapeutics.

A comprehensive review on the recent progress in clinical applications of EVs, bioengineering and the complexity of EV-molecular cargo, is presented by Claridge et al. The authors mark new avenues for future work related to EVs, with the goal to improve EV-based therapeutic strategies. Importantly, current limitations of EV utility are also discussed, such as manufacturing, scalability, molecular characterization and targeted delivery.

Further elaboration on therapeutic potential of exosomes is provided in the review by Shi et al. The authors focused on dermatological conditions, including wound recovery, burning, senescence and scars. They comment on regulatory function of exosomes in skin development and implicate a novel role of exosomes as biomarkers of cutaneous diseases. In turn, Wan et al. discuss the hurdles and perspectives for EV therapy in the treatment of stroke. They outline an important role of microglia in neurogenesis and neuroinflammation, including pathogenesis of stroke. Importantly, EVs from different sources may affect the activity of microglia and ameliorate stroke progression.

Apart from native cargo, bioactive content of EVs can also be modified to enhance their pro-regenerative capacity. This approach was taken by Ibrahim et al. On the basis of their prior work, the authors introduced beta-catenin and gata4 into therapeutically inert fibroblasts, to obtain activated specialized tissue effector EVs (ASTEX) with proven pro-regenerative capacity. ASTEX were able to reduce fibrotic markers in fibroblasts and endothelial cells, *via* the activity of anti-fibrotic miRNAs. Application of ASTEX in bleomycin induced lung damage, exerted beneficial effects in the experimental animals, both short- and long-term, proving their utility as anti-fibrotic agents.

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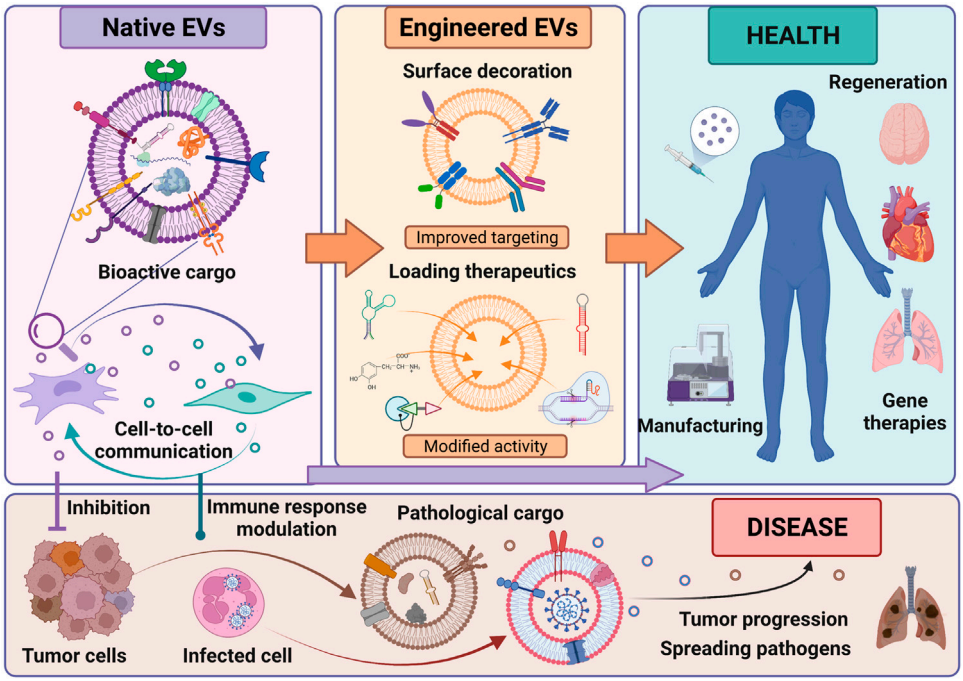

FIGURE 1 | Characteristics, bioengineering and role of extracellular vesicles (EVs) in health and disease. EVs contain bioactive cargo in the form of proteins, lipids and nucleic acids which exert biological effects on target cells and thereby influence their fate. To improve functionality and specificity of EVs, they can be engineered by surface modification and/or loading a specific therapeutic cargo. Native and modified EVs can be used as next-generation cell-free therapeutics to improve health. On the contrary, EVs released in pathological conditions by, e.g. cancer cells or infected cells, participate in disease spreading and progression.

Similarly, pro-regenerative activity another miRNA molecule contained in the EVs, was investigated by Sanchez-Sanchez et. al. They explored cardioprotective properties of hypoxia-stimulated mesenchymal stem cell (MSC)-derived EV-containing miR-4732-3p. Activity of this miRNA was demonstrated to promote angiogenesis and ameliorate fibrosis, both *in vitro* and *in vivo,* in a rat model of myocardial infarction.

EVs were shown not only to directly stimulate tissue-residing cells for regeneration, but also to modulate function of immune cells, which are the major players in tissue injury and resolution. With this respect, Peck et al. showed that introducing a specific molecule exerting immunomodulatory function in neutral fibroblasts, resulted in the production of EVs with enhanced immunomodulatory capacity. Specifically, they selected tryptophan 2,3-dioxygenase (TDO2), a target gene of canonical Wnt-β-catenin pathway. Applying TDO2-enriched EVs in an animal model of acute myocardial infarction, greatly improved heart function.

Another dimension of immunomodulatory activity of EVs was presented by Cochran and Kornbluth, in the context of fighting cancer. EVs isolated from immune cells—natural killer NK3.3 cell line, were shown to contain an array of cytotoxic enzymes, as well as variety of miRNAs associated with anti-tumor activity. In a cell killing assay, such EVs were able to inhibit proliferation and induce apoptosis in hematopoietic and non-hematopoietic tumor cell lines. Importantly, the observed effect was tumor cell specific, since normal cells remained unaffected.

With respect to tumor cells, however, it was shown that they produce EVs which promote tumor cell proliferation and metastasis, leading to tumor progression (**Figure 1**). Such EVs can alter tumor microenvironment, allowing tumor cells to escape recognition and clearance by immune cells (Xu et al., 2018). A summary of recent findings in the field of tumor-related EVs, with emphasis on oral squamous cell carcinoma (OSCC), is presented in a review by Lu et al. OSCC represents 90% of oral cancers and is associated with low survival rate, thus constitutes a serious health problem. One of novel treatment options for OSCC may constitute EV isolated from therapeutically-approved cells, such as MSCs.

Apart from tumor cells, pathological EVs can also be released during inflammation and they are involved in spreading pathogens. In the light of recent SARS-CoV-2 pandemic, which affected millions of individuals worldwide and resulted in the onset of COVID-19 disease, the contribution by Pironti et al., provides a timely discussion on the potential link between virus spreading *via* EV secretory pathway and cardiovascular manifestations of COVID-19. It has been investigated that COVID-19 disturbs the renin-angiotensin system, thereby exacerbating heart function. To improve heart condition, EVs derived from therapeutically beneficial cells, such as MSCs or cardiosphere-derived cells (CDCs), can be applied.

The Research Topic of EV engineering, to enhance their biological activity and improve targeting toward specific cell or tissue type is comprehensively discussed by Nazimek and Bryniarski. It is known that upon delivery to a living organism, EVs are rapidly cleared by phagocytes, which significantly reduces their therapeutic potential. As

presented by Nazimek et al., EVs co-incubation with antigen-specific antibodies and light chains induces their aggregation. This modification extends their stability in body fluids and allows their escape from urinary excretion. Moreover, EV decoration with a specific antibody can greatly enhance tissue targeting. These advancements may boost therapeutic potential of EVs and their applicability to treat human diseases.

Concluding, this Research Topic provides the state-of-the-art of recent discoveries in the field of EVs and highlights the importance of further research needed to fully explore their therapeutic potential. Transferring this knowledge to clinical practice will be a milestone achievement in contemporary medicine.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Mechanistic and Therapeutic Implications of Extracellular Vesicles as a Potential Link Between Covid-19 and Cardiovascular Disease Manifestations

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Extracellular vesicles (EVs), which are cell released double layered membrane particles, have been found in every circulating body fluid, and provide a tool for conveying diverse information between cells, influencing both physiological and pathological conditions. Viruses can hijack the EVs secretory pathway to exit infected cells and use EVs endocytic routes to enter uninfected cells, suggesting that EVs and viruses can share common cell entry and biogenesis mechanisms. SARS-CoV-2 is responsible of the coronavirus disease 2019 (Covid-19), which may be accompanied by severe multi-organ manifestations. EVs may contribute to virus spreading via transfer of virus docking receptors such as CD9 and ACE2. Covid-19 is known to affect the renin angiotensin system (RAS), and could promote secretion of harmful EVs. In this scenario EVs might be linked to cardiovascular manifestations of the Covid-19 disease through unbalance in RAS. In contrast EVs derived from mesenchymal stem cells or cardiosphere derived cells, may promote cardiovascular function due to their beneficial effect on angiogenesis, fibrosis, contractility and immuno-modulation. In this article we assessed the potential impact of EVs in cardiovascular manifestations of Covid-19 and highlight potential strategies to control the extracellular signaling for future therapies.

Keywords: extracellular vescicles, COVID–19, renin—angiotensin—aldosterone system, cardiovascular disease, MSC derived exosomes, cardiosphere-derived cell, exosomes, cardiomyopathy

INTRODUCTION

Cells are continuously secreting extracellular vesicles (EVs) that include large apoptotic bodies (1–5 μm), microvesicles (100–1,000 nm) or small exosomes (30–100 nm), which vary in their abundance, size, and composition. Enclosed by a lipid bilayer, EVs are thus very stable and convey cytoplasmic molecules including proteins, RNAs, lipids, and metabolites (Robbins and Morelli, 2014).

Exosomes originate from processing of early endosomes by endosomal sorting complex to form intraluminal vesicles within larger multivesicular bodies (MVB) that traffic in the cytoplasm (Thery et al., 2002). The fusion of MVB to the plasma membrane allows the release of their intraluminal

vesicles (now called exosomes) into the extracellular microenvironment (Thery et al., 2002). Initially these vesicles were thought to be secreted by cells merely to eliminate obsolete molecules, but the importance of exosomes in conveying cellular information, with potent autocrine and paracrine biological activities, is now well known as they are released by many different cell types and can be found in most bodily fluids (van Niel et al., 2018).

Microvesicles have size overlap and share some surface markers, such as CD9 and CD63 (Crescitelli et al., 2013; Andreu and Yanez-Mo, 2014) making even more challenging the distinction from exosomes, thus they both are often referred as extracellular vesicles. However, microvesicles have a distinctive biogenesis originating from outward budding of plasma membrane (Kalra et al., 2016). The apoptotic bodies represent the largest EVs and are secreted by cells undergoing programmed cell death, by membrane blebbing and bulging, promoting the clearance of apoptotic material. The intrinsic capacity of EVs in conveying biologic material between proximal and distant cells have been used for therapeutic applications in order to facilitate drug delivery, but it is also used by pathogens to facilitate infection spreading and cause multi-organ diseases. This aspect should be taken in consideration in the perspective of engineering EVs with implemented delivery capacity. In this review we use the term EVs to refer to the two categories microvesicles and exosomes.

CAN EVs PROMOTE SARS-COV-2 VIRUS SPREADING?

Many viruses, including coronaviruses, are known to enter the EVs avenue during synthesis and intra-host spreading, in a caveolin-1-dynamin-dependent mechanism (Owczarek et al., 2018; Badierah et al., 2020). Whether the SARS-CoV-2 virus, which causes the coronavirus disease 2019 (Covid-19) is involved in EVs trafficking is still unknown. However, *in vitro* data show that alveolar cells infected with SARS-CoV-1 present whole virions in the secretory vesicles near the plasma membrane of infected cells (Qian et al., 2013; Mason, 2020). Whether SARS-CoV-2 virions also can accumulate in secreting vesicles has not been validated yet, although electron microscopy images of lung biopsy in autopsy specimen from deceased Covid-19 patient intriguingly show SARS-CoV-2 virion within double membrane vesicles (Dittmayer et al., 2020). Moreover, histopathological analysis of renal specimen from deceased patients with Covid-19 showed that SARS-CoV-2 virions are mostly found in the cytoplasm and some vacuoles containing assembling virions similar to what has been seen with SARS-CoV-1 (Farkash et al., 2020; Su et al., 2020). Due to the size of coronavirus (60–140 nm) it is possible that bigger EVs such as microparticles might mediate whole virion transport, while exosomes might carry viral fragments. During an infection, big apoptotic bodies released from infected/injured cells might be an easy way to carry virions due to the larger size, however apoptotic bodies present surface bridging molecules that triggers phagocytosis, which could contribute to contain rather than spreading SARS-CoV-2 in the body (Arandjelovic and Ravichandran, 2015). However, exosomes and microparticles contain virus receptors that make EVs recipient cells susceptible to virus entry (Earnest et al., 2015). Indeed tetraspanin protein CD9 expressed by EVs formed cell membrane complex of the co-receptor dipeptidyl peptidase 4 (DPP4) and the type II transmembrane serine protease member TMPRSS2, a CoV-activating protease, facilitating proteolytic priming events with cells for MERS-CoV pseudovirus, which is necessary for virus cell entry and can be blocked by CD9 inhibition (Earnest et al., 2015). Similarly, SARS-CoV-2 uses DPP4 (Li Y. et al., 2020; Solerte et al., 2020) and TMPRSS2 (Hoffmann et al., 2020) to facilitate cell entry. Although it might be reasonable to think that also SARS-CoV-2 virulence relies on CD9 activity by clustering and scaffolding receptors and proteases for efficient cell entry, this hypothesis has not been validated yet in current literature.

Thus, coronavirus such as SARS-CoV-1 and MERS-CoV may be directed into the exosomal pathway or released from microvesicles, upon intracellular entry, and its components, such as mRNAs and proteins, including spike protein (Kuate et al., 2007), are packaged into EVs for secretion (Hassanpour et al., 2020).

Although conventional biochemistry techniques allow the detection of viral components such as viral RNA or spike proteins, there is currently no empirical evidence that EVs secreted from infected cells contain specific fragments of SARS-CoV-2. EM images potentially would be a method to investigate this, but purification techniques available at the moment cannot separate virions from EVs due to their overlapping size. Therefore, the isolation procedures need to be implemented in order to identify specific viral components (RNA or proteins) carried by EVs. Whether EVs released from infected cells have harmful or protective roles is controversial: vesicles containing only viral fragments might stimulate the immune system for their antigen priming activity, while the whole virion hidden in bigger vesicles could potentially elicit viral dissemination by escaping the immune recognition and further spread the virus within the body.

In addition to CD9, it has been shown that exosomes can transfer the angiotensin converting enzyme 2 (ACE2) receptor (Wang et al., 2020), which SARS-CoV-2 uses for cell entry. It is reasonable to believe that increased levels of ACE2 might promote more virus entry similarly to what has been observed in cellular models transfected with human ACE2 (Blanco-Melo et al., 2020; Ou et al., 2020). Alternatively EVs containing ACE2 might be used to decoy the SARS-CoV-2 and limit virus spreading (Inal, 2020). The presence of docking station for virus priming such as ACE2 is necessary but not sufficient for virus entry as the proteases activity of TMPRSS2 is critical for fusion and internalization of ACE-2/SARS-CoV2 spike complex (Hoffmann et al., 2020), and co-receptors such as neuropilin-1 (NRP-1) can significantly potentiate SARS-CoV-2 infectivity (Cantuti-Castelvetri et al., 2020). Both NRP-1 and TMPRSS2 have been found in EVs (Munshi et al., 2019; Chi et al., 2020). As the inter-cellular trafficking pathways of viruses and EVs are similar or shared, pharmacological inhibition of

EVs trafficking has been proposed as an important antiviral approach in order to limit the systemic spreading of SARS-CoV-2 (Urciuoli and Peruzzi, 2020).

It has been reported that during SARS-CoV-1 and MERS-CoV infections there were increased levels of circulating exosomes enriched in lung-associated self-antigens as well as viral antigens (Gunasekaran et al., 2020). Thus, it might be possible that SARS-CoV-2 infected cells increase the production of exosomes enriched in viral components. In this way, exosomes containing pathogenic proteins and RNA released from infected cells may induce host humoral and cellular immune response (Gunasekaran et al., 2017) and could potentially play anti-infective roles (Zhang et al., 2018). In contrast, it is conceivable that viral material might also be hidden within EVs vesicles using a sort of "trojan horse" strategy for immune system evasion, which might potentially be linked to the variable detection of viral RNA in testing patients with ongoing, or recovered, Covid-19 (Elrashdy et al., 2020). Although different reasons (including methodological problems) may be underlying false negative PCR tests for viral detection, it is intriguing to speculate that EVs could be a hiding place for viral material.

HOW DO EVs PROMOTE THE INTERPLAY BETWEEN THE RENIN-ANGIOTENSIN SYSTEM (RAS) and SARS-CoV-2?

In the progression of chronic heart failure, compensatory but maladaptive renin-angiotensin system (RAS) activation promotes cardiac remodeling (Zhou et al., 2016). Current literature describes a modulatory role of EVs in RAS through intercellular trafficking by exosomes. It has been shown that increased angiotensin type II (AngII) levels in heart failure, elicit secretion of exosomes by cardiac fibroblasts, which induces neighboring cardiomyocytes to release more AngII and express AngII receptors (AT_1R and AT_2R) through a feedback process that promotes myocardial hypertrophy (Lyu et al., 2015). Moreover, increased AngII in plasma and cardiac pressure overload leads to release of exosomes enriched in AT_1R, which target resistance vessels and cardiomyocytes, aggravating cardiac remodeling (Pironti et al., 2015). Post-mortem autopsy analysis showed that almost 35% of patients who succumbed to SARS-CoV-1 (in 2003) presented detectable viral SARS-CoV-1 genome in heart samples, and this was associated with myocardial fibrosis, inflammation, and reduced myocardial ACE2 expression (Oudit et al., 2009). SARS-CoV-2 viral particles have been found in cardiac biopsies of Covid-19 patients (Dolhnikoff et al., 2020; Tavazzi et al., 2020), and it has been reported that this virus can infect human cardiomyocytes and induce cardiotoxicity (Bojkova et al., 2020).

Both SARS-CoV-1 and SARS-CoV-2 uses ACE2 as docking station to infect target cells and the internalization of virus/enzyme complex leads to loss of enzymatically active ACE2 at the cell surface (Gheblawi et al., 2020; Hoffmann et al., 2020).

As RAS-inhibitors can upregulate expression of ACE2, there were initial concerns that use of RAS-antagonists may increase risk or severity of Covid-19 as more docking stations would theoretically be present. However, the use of RAS inhibitor therapy was not associated with increased infectivity or severity of disease (Li J. et al., 2020; Zhang et al., 2020).

As cells are infected by SARS-CoV-2, the ACE2 expression is down-regulated due to virus/enzyme complex internalization. The reduction of ACE2 enzymatic activity results in an imbalance within the RAS, which stimulates neutrophil infiltration, unopposed AngII accumulation and may promote acute lung injury. Thus, RAS-inhibitors may also be protective. Less attention has been given to the fact that increased circulating AngII levels could elicit the endocytosis of ACE-2 bound to SARS-CoV-2 via a AT1R-dependent mechanism (Offringa et al., 2020), which may promote ACE2 degradation (Deshotels et al., 2014). Furthermore, reduced expression levels of ACE2 in the heart, following SARS-CoV-2 infection, may impair the conversion of AngII to the Ang1-7 heptapeptide, which is anti-inflammatory, anti-fibrotic, and cardioprotective (Patel et al., 2014) and represents the main mediators of the protective RAS signaling activating via AT2R and MasR (Santos et al., 2003). Thus, in addition to downregulating ACE2 and Ang1-7, unbalanced RAS activation through exosomes during Covid-19 may represent a novel detrimental pathway that could be responsible for some of the cardiovascular disease manifestations in Covid-19 (**Figure 1**). Indeed in a yet ongoing promising clinical trial, a non-peptide AT_2R agonist is used as therapy to counterbalance the potentially harmful RAS effects during Covid-19 (Angiotensin II Type Two Receptor Agonist in COVID-19 Trial ATTRACT study, NCT04452435) (Steckelings and Sumners, 2020).

Although EVs release as results of RAS disturbance during SARS-CoV-2 infection has not yet been demonstrated, we here provide a provocative hypothesis of a link between EVs, Covid-19 and RAS based on two demonstrated evidences: (i) SARS-CoV-2 infection causes disturbance of RAS; (ii) RAS-dependent release of exosomes in e.g., hypertension are known to aggravate cardiovascular diseases and myocardial remodeling.

HOW TO COMBAT THE CYTOKINE STORM DURING COVID-19 USING EVs AS THERAPY?

Severe Covid-19 display a systemic pro-inflammatory state often referred to as a cytokine storm with critical illness and manifestations such as acute respiratory distress syndrome (ARDS), pulmonary edemea, thromboembolism, and acute cardiac injury, which come with high mortality (Huang et al., 2020; Leisman et al., 2020).

Mesenchymal stem cells (MSCs) have powerful immuno-modulatory effects and are considered potentially effective therapeutic assets to combat a wide spectrum of inflammatory-driven diseases but also clinically useful in preventing inflammatory activation from acute graft vs. host disease following allogenic hematopoietic stem-cell transplantation

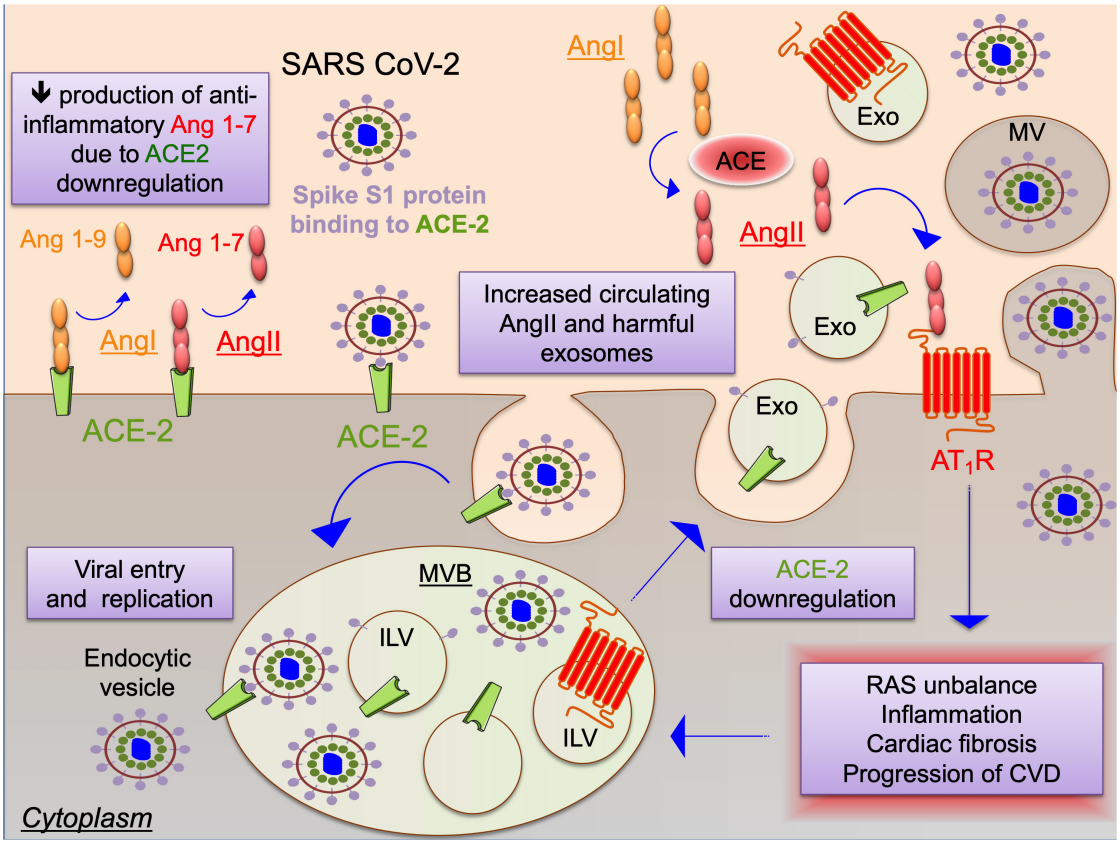

FIGURE 1 | Schematic representation of the potential interplay between the renin-angiotensin system (RAS) and SARS-CoV-2 as mediated by extracellular vesicles. SARS coronaviruses use spike protein S1 to bind ACE-2 as docking station leading to cell entry and also to ACE2 down-regulation. Increased levels of angiotensin type II (AngII) aggravate RAS, promoting inflammation, cardiac fibrosis and progression of cardiovascular diseases (CVD). SARS-CoV-2 infection and RAS trigger the endocytic machinery, leading to internalization of ACE2, virion components and angiotentin II type 1 receptor (AT_1R) into intraluminal vesicles (ILV) within multivesicular bodies (MVB), which are released as exosomes (Exo) upon fusion of the MVB with the cell plasma membrane. SARS-CoV-2 spreading may be enhanced also through release of virions in larger microvesicles (MV) upon plasma membrane budding of infected cells. Exosomes or other EVs might be responsible for manifestations and progression of cardiovascular disease in Covid-19 through maladaptive RAS signaling.

(Hashmi et al., 2016; Bazzoni et al., 2020). Thus, MSCs might be used to prevent or reduce the cytokine storm caused by SARS-CoV-2. Interestingly, EVs released from MSCs is one of the mechanisms whereby MSCs may promote anti-inflammatory effects (Hashmi et al., 2016; Bazzoni et al., 2020). Indeed, several studies have described beneficial effect of MSCs-derived EVs following myocardial injury by increasing angiogenesis (Liang et al., 2016; Gong et al., 2017) improving cardiac contractility (Kang et al., 2015; Ma et al., 2017; Mayourian et al., 2017). Moreover stem cells derived from cardiac tissues, i.e., cardiosphere derived cells (CDC), produce EVs able to reduce inflammation and fibrosis signaling (Gray et al., 2015; Gallet et al., 2017; Rogers et al., 2020). The intercellular communication between macrophages, fibroblasts and endothelial cells orchestrates the progression of chronic heart failure and elicit myocardial fibrosis. Thus, in the context of anti-fibrogenesis, EVs may have a beneficial effect by promoting the trans-differentiation of macrophages from pro-inflammatory M1 into M2-like cells with immunosuppressive phenotype (Silva et al., 2017). This process is important for the resolution of inflammation and might be particularly relevant in the context of therapy meant to combat the systemic pro-inflammatory state in Covid-19.

Interferon gamma (INF-γ) is a major proinflammatory cytokine secreted by activated T cells and natural killer cells, which promotes macrophage activation and mediate host defense against pathogen infection. Decreased plasma levels of INF-γ in the early stage of the disease (before the onset of cytokine storm) were associated with increased risk of pulmonary fibrosis in Covid-19 patients (Hu et al., 2020). Indeed INF-γ has antiviral action, through enhancing the viral antigen processing and presentation to cytotoxic T lymphocytes for virus clearance, and has also anti-fibrotic effect. Thus early intervention of anti-viral infection using IFN-γ could be significant in the inhibition of fibrosis for better functional recovery (Hu et al., 2020). Stem cells exposed to proinflammatory cytokine signaling released EVs enriched in mRNA coding for INF-γ signaling pathway, such as STAT1, JAK 1-2 (Cossetti et al., 2014). Interestingly EVs can recycle and deliver free INF-γ and by that, promoting the

continuation of pro-inflammatory signaling response in target cells (Cossetti et al., 2014).

Therapeutic approaches for Covid-19 based on MSCs-derived EVs aimed to enhancing the healing process following lung injury, have been taken in consideration (Akbari and Rezaie, 2020; Al-Khawaga and Abdelalim, 2020; Pinky et al., 2020; Tsuchiya et al., 2020). However, experimental evidences of their mechanism of action, and whether MSCs-derived EVs therapy can ameliorate cardiovascular manifestations have not been elucidated. Interestingly, the first clinical trial using MSC derived exosomes that were intravenously injected in Covid-19 patients with moderate-to-severe symptoms (Sengupta et al., 2020) (NCT04276987) showed positive results in terms of safety profile, capacity to restore oxygenation, downregulation of cytokines, and reconstitute immunity. In another clinical trial (NCT04276987), inhalation of aerosol enriched in allogenic EVs derived from adipose MSC have been tested for safety and efficacy in Covid-19 patients with severe symptoms (Pocsfalvi et al., 2020).

The timing of an intervention seems to be a critical aspect in Covid-19 disease. The acute phase of Covid-19 illness is characterized by three pathological phases: (i) the early infection phase when SARS-CoV-2 infiltrates and replicate; (ii) the pulmonary phase characterized by respiratory dysfunction; (iii) finally the hyperinflammation phase driven by the host immunity with an exaggerate inflammatory response (Akhmerov and Marban, 2020). The specific stage of disease must be taken into consideration when commencing different therapeutic interventions e.g., with antiviral, immunopotentiating or anti-inflammatory properties (de Simone and Mancusi, 2020). Although each phase can be defined based on clinical and laboratory findings the preciseness of this diagnosing is not complete thus providing a major challenge for timing of intervention. A too early anti-inflammatory intervention might hinder the viral elimination by host immunity, while a too late anti-inflammatory therapy might have a slight efficacy.

Although EVs containing viral components might elicit the adaptive immune response by presenting the antigen in a process similar to a vaccination, EVs secreted from infected/injured cells could activate macrophage secreting cytokines and contribute to the cytokine storm formation during Covid-19 (Perez et al., 2019). Indeed, EVs can carry pathogen-associated molecular patterns (PAMPs) released from stressed or injured cells and therefore triggering inflammation upon interaction with innate immune cells contributing to inflammation induction and persistence (Bhatnagar et al., 2007). Additionally, EVs containing cytokines (Pizzirani et al., 2007), enzymes involved in the biosynthesis of lipid mediators (Cossetti et al., 2014) and other chemotactic signals (Esser et al., 2010; Kriebel et al., 2018) can contribute to the propagation of inflammation. Interestingly, ACE2 enriched EVs derived from engineered MSC has been considered as a possible Covid-19 therapy in order to decoy the SARS-CoV-2 and limit virus spreading (Inal, 2020).

Thus, a therapeutic application of EVs for Covid-19 must include donor cells with anti-inflammatory activity such as mesenchymal stem cells or cardiosphere derived cells. The anti-inflammatory activity of EVs indicate that this platform can work well in xenogenic applications (de Couto et al., 2017; Gallet et al., 2017), which could simplify the large scale production of EVs for therapeutic applications by using MSCs or CDC derived from large animals rather than humans.

CONCLUSION

We have discussed the possible function of EVs in Covid-19 and highlight potential therapeutic application for EVs in modulating the virus infection, the pro-inflammatory state and cardiovascular disease in Covid-19. EVs, such as exosomes could be involved in aggravated cardiovascular manifestations of Covid-19 either by promoting viral docking in cardiac cells or shifting RAS to promote inflammation, coagulation, fibrosis and endothelial dysfunction. Furthermore, EVs derived from engineered MSCs or CDC used as drug delivery system to modulate the cytokine storm of virus spreading represent a promising strategy to combat Covid-19. In summary, understanding the molecular mechanisms behind the entry, replication, and spreading of SARS-CoV-2, in conjunction to EVs trafficking, may provide tools to limit the manifestation of Covid-19.

AUTHOR CONTRIBUTIONS

GP substantially contributed to the conception and design of the perspective article, literature search, drafting the article and revising the article critically for important intellectual content. DA and LL contributed in literature search, drafting the article and revising the article critically for important intellectual content. All the authors approved the final version of the article to be published.

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frontiers in Cell and Developmental Biology

REVIEW
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The Emerging Role of Exosomes in Oral Squamous Cell Carcinoma

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Oral cancer constitutes approximately 2% of all cancers, while the most common type, oral squamous cell carcinoma (OSCC) represents 90% of oral cancers. Although the treatment of OSCC has improved recently, it still has a high rate of local recurrence and poor prognosis, with a 5-year survival rate of only 50%. Advanced stage OSCC tends to metastasize to lymph nodes. Thus, exploring new therapeutic strategies for OSCC is therefore an urgent priority. Exosomes, the small membrane vesicles derived from endosomes, have been detected in a wide array of bodily fluids. Exosomes contain a diversity of proteins, mRNAs, and non-coding RNAs, including microRNAs, long non-coding RNAs, piRNAs, circular RNAs, tsRNAs, and ribosomal RNAs, which are delivered to neighboring cells or even transported to distant sites. Exosomes have been associated with the tumorigenesis of OSCC, promote the proliferation, colonization, and metastasis of OSCC by transferring their contents to the target cells. Furthermore, exosomes are involved in the regulation of the tumor microenvironment to transform conditions favoring cancer progression *in vivo*. In this review, we summarize the crucial role of exosomes in the tumorigenesis and progression of OSCC and discuss the potential clinical application of exosomes in OSCC treatment.

Keywords: oral squamous cell carcinoma, non-coding RNAs, exosomes, tumor microenvironment, extracellular vesicles

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Abbreviations: OSCC, oral squamous cell carcinoma; EVs, extracellular vesicles; MVB, multivesicular body; CHB, chronic hepatitis B; TME, tumor microenvironment; ESCRT, endosomal tethering complexes necessitated for transport; GTPase, Rab guanosine triphosphatase; PEG, Polyethylene glycol; ncRNAs, non-coding RNAs; miRNAs, microRNAs; hnRNPA2B1, heterogeneous nuclear ribonucleoprotein A2B1; CAFs, cancer-associated fibroblasts; PTEN, phosphatase and tensin homolog; PDCD4, programmed cell death 4; Mark1, microtubule-affinity-regulating kinase 1; TGFBR1, type I TGF-beta receptor; SOCS1, suppressor of cytokine signaling 1; TPM1, tropomyosin alpha-1 chain; CHD9, chromodomain helicase DNA binding protein 9; WRN, Werner syndrome gene; DENND2D, DENN/MADD domain containing 2D; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; MMPs, extracellular matrix proteins; NFs, normal fibroblasts; BCL2, B-cell lymphoma 2; Tregs, regulatory T cells; MDSCs, myeloid-derived suppressor cells; TEX, hypoxic tumor-derived exosome; lncRNAs, long non-coding RNAs; circRNAs, circular RNAs; piRNAs, P-element-induced wimpy testis (PIWI)-interacting RNAs; rRNAs, ribosomal RNAs; tsRNAs, transfer RNAs; HMGA1, the high mobility group A1; USP7, ubiquitin-specific protease-7; ARHGAP11A, Rho GTPase activating protein 11A; ATMs, ataxia telangiectasia mutated; AMPK, adenosine monophosphate-activated protein kinase; TRAP1, Tumor necrosis factor receptor-associated protein 1; EGFR, epidermal growth factor receptor; HSP, heat shock protein; PF4V1, platelet factor 4 variant 1; CXCL7, C-X-C motif chemokine ligand 7; F13A1, Coagulation factor XIII A chain; ApoA1, apolipoprotein A1; VEGF, vascular endothelial growth factor; LNM, lymph node metastasis; ROC, receiver operating characteristic.

INTRODUCTION

Oral squamous cell carcinoma is the most common subtype of oral carcinoma, a genetic and epigenetic disease characterized by histopathological differentiation and a propensity for LNM (Bu et al., 2015). Surgical removal of tumors as well as pre- or post-operative chemotherapy, radiotherapy, and adjuvant therapies are the main strategies to increase survival (Coyte et al., 2014; Adel et al., 2016). Although treatment outcomes for OSCC have been improved recently, the prognosis for OSCC is still poor, the 5-year survival rate is reported as 50%, due to late diagnosis which resulting infeasibility of curative resections. Furthermore, the prognosis for this disease is poor due to metastatic invasion, with a propensity for local recurrence and distant metastasis (Huang et al., 2019). OSCC dissemination targets both local tissues via direct invasion and distant sites by seeding pre-metastatic niches through secreted elements, including exosomes (Qiu et al., 2019). Considering the risk of late diagnosis of OSCC, improvements in prevention, early diagnosis, and treatment efficacy are urgently needed (Csõsz et al., 2017).

According to the International Society for Extracellular Vesicles (ISEV), EVs are nano-size lipid bilayer vesicles released naturally from the cells to the ECM (Kalra et al., 2012; Thery et al., 2018; Jabbari et al., 2020b) (**Figure 1**). Generally, EVs are categorized as exosomes, microvesicles, and apoptotic bodies (Ahmadi and Rezaie, 2020; Jabbari et al., 2020a). Exosomes are small, membranous, extracellular microvesicles (∼30–150 nm in diameter) of endocytic origin. The formation of exosomes includes the beginning, endocytosis, MVB creation, and finally exosome secretion (Mellman, 1996; Keller et al., 2006; Raposo and Stoorvogel, 2013; Abak et al., 2018). Previous studies showed that non-coding RNAs (ncRNAs), mRNAs, proteins, and DNA fragments can be carried as "cargo" in EVs, which could serve as novel diagnostic biomarkers for OSCC (Raposo and Stoorvogel, 2013; Abak et al., 2018; Gai et al., 2018; Pourhanifeh et al., 2020). Another study found that exosomes can reprogram signal transduction under pathophysiological conditions and deliver important proteins as mediators (Li et al., 2019d; Amiri et al., 2021). These studies demonstrated that exosomes are present in the saliva of healthy donors, and exosomes have been reported in other body fluids, such as blood, cerebrospinal fluid, serous cavity effusion, and urine (Cao et al., 2020). Exosomes play an essential role in mediating signal transport for intercellular communication or over long distances by transporting microRNA (miRNAs), mRNAs, and proteins (Ohno et al., 2013a; Zhang et al., 2015; Sadri Nahand et al., 2020). Exosomal miRNAs are potential diagnostic biomarkers for various malignancies, regulating protein expression in cell proliferation, tumor metastasis, cell apoptosis, genomic instability, and immune responses. Wang H. et al. (2014) showed that exosomal miR-21 in the serum of OSCC patients is higher than that in chronic hepatitis patients and healthy individuals, suggesting that serum miRNAs may act as diagnostic biomarkers for OSCC. Sohn et al. (2015) detected serum exosomal miRNAs in patients with CHB, liver cirrhosis, and OSCC with CHB and discovered that a panel of eight exosomal miRNAs are significantly different between the OSCC and chronic hepatitis or liver cirrhosis groups. These efforts have revealed that exosomal contents can be transported across biological membranes and that exosomes have a role in OSCC development. Exosomes and their pattern of transfer deserve more attention to clearly define their roles in OSCC diagnosis and therapeutics. This review highlights exosomes in exchanges between normal and OSCC cells as well as the endocrine transport of exosomes from distant cells via the TME. We also review recent advances in exosomes in cancer initiation, progression and their potential clinical relevance to OSCC.

EXOSOME BIOGENESIS, ISOLATION METHODS, AND BIOLOGICAL FUNCTIONS

The Biogenesis of Exosomes

The intraluminal vesicles (ILVs) are formed through inside budding in the multivesicular bodies (MVB) during the maturation of early endosomes to late endosomes which is regulated by ceramide (Nahand et al., 2020a). The cargos are encapsulated into ILVs during budding. However, the mechanism controlling cargo sorting is rather complicated and still unclear (Leidal and Debnath, 2020). EVs have overlapping sizes, similar morphology, and unspecific contents, resulting in difficulties in the isolation of specific subpopulations (Mathieu et al., 2019). The ILVs fuse with the cell membrane and are released into the extracellular space (Nahand et al., 2020b). It has been demonstrated that endosomal-tethering complexes necessitated for transport (ESCRT)-dependent and independent activities play an essential role in MVB formation (Hashemian et al., 2020). The ESCRT members including ESCRT-0, -I, -II, -III, and the related AAA-ATPase Vps4 complex recognize ubiquitinated membrane proteins leading to their internalization within the multivesicular endosome (Asgarpour et al., 2020; Ghaemmaghami et al., 2020). The MVB trafficking and secretion of exosomes are regulated by several members including the GTPase family (Rab11, Rab27a, Rab27b, and Rab35), heparanase, soluble NSF attachment receptor, and cytoskeleton regulatory proteins (Pant et al., 2012; Azmi et al., 2013; Beach et al., 2014; Abak et al., 2018). Furthermore, the dissemination process of the exosomes requires cellular stress, such as oxidative stress, hypoxia, etc (Abak et al., 2018). The released exosomes could transport into the recipient cells by the interaction between receptor-ligand, membrane fusion, and endocytosis through phagocytosis (Abak et al., 2018).

Exosome Isolation Methods

The isolation methods of exosomes mainly include ultracentrifugation, size-based isolation, polymer precipitation, immunoaffinity, and microfluidic separation (Oeyen et al., 2018). Currently, ultracentrifugation is the most commonly used and the gold standard for exosome isolation. The advantage of ultracentrifuge is to treat lots of samples at one time. However, the problems of purity and damage the integrity of exosomes are the main disadvantages of the ultracentrifugation method (Tschuschke et al., 2020). PEG

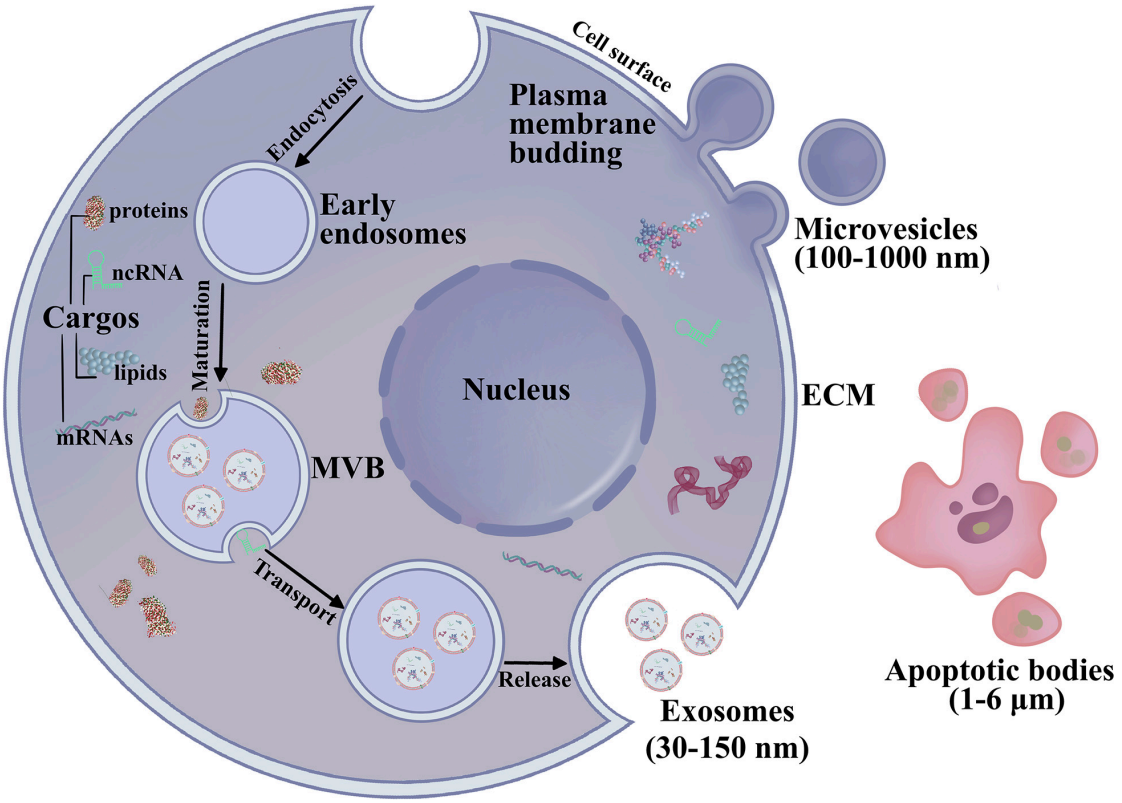

FIGURE 1 | The biogenesis and secretion of EVs. Exosomes (30–150 nm) are generated by membrane endocytosis. Microvesicles (100–1,000 nm) are formed by the plasma membrane budding. Microvesicles are irregular in shape and heterogeneous in population. The largest EVs are apoptotic bodies (1–6 μm) are involved in the pathological condition.

polymer combines the water molecules of the exosomes and reduces the solubility. PEG-based exosome isolation can be done in low-speed centrifugation with high efficiency (Doyle and Wang, 2019). The failure of the selection of exosomes and other EVs is the main drawback of PEG precipitation. Size-based exosome isolation is based on ultrafiltration and size exclusion chromatography. Ultrafiltration membrane isolates the exosomes by cutoff molecular sizes, which may lead to the exosome damage due to the pressure (Li et al., 2017). The size exclusion chromatography can be used to slow down the movement of small molecular, that protect the integrity and activity of exosomes. Magnetic bead and enzyme-linked immunosorbent assay are immunoaffinity-based exosome isolation methods, These methods capture the exosomes according to the antigen recognition of specific antibody (Zhang et al., 2020). Immunoaffinity-based exosome isolation generates pure exosomes with low yield. Microfluidic technology isolates exosomes in a short time with high purity, it differentiates physical and biochemical characteristics of exosomes by phosphatidylserine-specific proteins on exosomes (Jiang et al., 2020).

The Biological Function of Exosomes

Under physiological conditions, exosomes are important mediators of cell-cell and inter-tissue communication. Exosomes exhibit important functions in regulating cellular activities during physiological and pathological conditions. During the cancer progression, different cells such as cancer cells, immune cells in TME generate exosomes that can transfer nucleotides and proteins among cells and participate in the complex pathogenesis of tumor development and metastasis (Ridder et al., 2014; Nazimek et al., 2016). Increasing evidence indicates that tumor cells communicate both with other tumor cells and with normal cells present in the TME via secretion and transfer of exosomal contents. Exosomal contents regulate tumor growth, angiogenesis, metastasis, sensitivity to chemotherapy, and immune evasion. Thus, it is essential to explore the effects of exosomes on OSCC development *in vitro* and *in vivo*.

EXOSOMAL miRNAs ARE ESSENTIAL FOR OSCC DEVELOPMENT

miRNAs in Exosomes

Among the bioactive components of exosomes, miRNAs can epigenetically alter gene function in the recipient cell, thus exerting their essential regulatory function on gene expression (Wang Y. et al., 2014). miRNAs are short ncRNAs of approximately 19–24 nucleotides in length (Chen et al., 2012b) and function to suppress the expression of protein-coding genes

at the post-transcriptional level by degrading or inhibiting the translation of mRNAs (Bartel, 2004; Miska, 2005; Kim et al., 2009). Moreover, miRNAs and their target genes constitute complicated regulatory networks that contribute to the fine-tuning of various biological processes, such as cell proliferation, differentiation, and apoptosis (Miska, 2005; Hwang and Mendell, 2006). It has been demonstrated that miRNAs manipulate more than 30% of human genes, governing all cellular, physiological, and developmental processes. The majority of miRNAs exist intracellularly, while some exist in body fluids, including a variety of extracellular biologic fluids, such as blood, urine, saliva, pancreatic juice, and breast milk (Weber et al., 2010). Under physiological and pathophysiological conditions, exosomes are released through two slightly different mechanisms of "unconventional" exocytosis into the extracellular milieu by several cell types. Whether the packing of miRNAs into exosomes takes place at the pre- or mature-miRNA level has not yet been fully clarified. It has been demonstrated that the sequence motifs present in mature miRNAs can control their sorting into exosomes, and the ubiquitous hnRNPA2B1 recognizing these motifs binds exosomal miRNAs and specifically controls their loading into exosomes (Villarroya-Beltri et al., 2013).

miRNAs dysregulation associated with cancer progression, is common in all human cancers, including OSCC (Lee et al., 2008; Tahiri et al., 2014). Therefore, miRNAs have the potential to be used for the diagnosis and treatment of OSCC (Xu et al., 2016; Božinović et al., 2019; Kirave et al., 2020). miRNAs can be packed into microparticles and exosomes, resulting in transfer of miRNAs to body fluids by a passive release mechanism (Chen et al., 2008; Chim et al., 2008). Exosomes can also be released by tumor cells or circulating microvesicles via shedding as an active secretion mechanism (Shah and Calin, 2013). Exosomal miRNAs from the TME exert diverse effects on tumorigenesis, including the regulation of host immune responses, tumor growth, angiogenesis, metastasis, tumor chemoresistance, and control of the TME. Exosomal miRNAs derived from tumor cells affect the immune activity of several tumor-associated immune cells as well as the transport of signaling molecules among tumor cells, immune cells, and other cell types.

Exosomal miRNAs and OSCC Progression

The contemporary therapies for OSCC yet are inefficient due to the limited understanding of their underlying mechanisms and the difficulties posed for developing accurate diagnostic methods. As important genetic materials transported in exosomes, exosomal miRNAs could serve as potential biomarkers and therapeutic targets for the treatment of OSCC. MiR-24-3p from salivary exosomes has been reported as a potential biomarker for OSCC (He et al., 2020). Furthermore, exosomal miRNAs play an essential role in growth, metastasis, and drug resistance. Exosomal miR-382-5p and miR-34a-5p from CAFs influence the proliferation, migration, and invasion of OSCC (Li et al., 2018; Sun et al., 2019). Li et al. (2018) showed miR-34a targeting AXL through AKT/GSK-3β/β-catenin pathway to promote the OSCC progression. Liu et al. (2017) first acquired cisplatin-resistant OSCC cells and used the conditional medium from resistant cells to treat parent OSCC cells. They further revealed that cisplatin-resistant OSCC cells could transfer miR-21 by exosomes targeting PTEN and PDCD4 to confer the cisplatin-resistance of the parental OSCC cells (Liu et al., 2017). Thus, exosomes may have the function as a vector for resistance transfer in cancer cells, and the resistance-related factors should be considered as therapeutic targets for effective treatment of OSCC.

Differential miRNA contents in OSCC exosomes have been reported in both pre-clinical and clinical studies. Recently, Rabinowits et al. (2017) compared the miRNA content of OSCC-derived exosomes with matching benign tissue and plasma from a patient. They found seven downregulated and nine upregulated miRNAs in tumor tissue compared with adjacent tissues. Furthermore, OSCC cells secreted miR-24-3p, miR-891a, miR-106a-5p, miR-2a-5p, and miR-1908 decreases the T-cell response in the tumor stroma by targeting the Mark1 signaling pathway and subsequently manipulating the proliferation and differentiation of cells (Ye et al., 2014). Moreover, miR-142-3p derived from exosomes were found to reduce TGFBR1 activity and promote OSCC cell proliferation *in vitro* and *in vivo* (Dickman et al., 2017). Exosomal miR-29a-3p derived from OSCC cells enhances tumor growth in a nude mouse model and M2 macrophage polarization by targeting the SOCS1 (Cai et al., 2019). Tachibana et al. (2016) and Xie et al. (2019a) showed that miR-223 and miR-101-3p function as tumor suppressors by inhibiting cell proliferation and inducing apoptosis through the process of exosome secretion, and exosomes secreting miR-338 from OSCC cells were also identified as tumor suppressors. Moreover, it was demonstrated that the overexpression of miR-34a-5p suppresses the proliferation of both CAL-27 and SCC-15 cells (Rabinowits et al., 2017). In addition, based on the colony formation assay, exosomal miR-34a-5p overexpression significantly reduced the colony counts of both CAL-27 and SCC-15 cells (Li et al., 2018).

Increased miRNA expression in exosomes is believed to promote OSCC metastasis. Leukoplakia is a precancerous lesion in OSCC, and it was found that miR-21 secreted from OSCC cells was correlated with low expression of its target genes, TPM1 and PTEN, and was highly expressed in progressive leukoplakia and OSCC to promote disease progression (Liu et al., 2017). Similarly, the involvement of exosome-delivered miRNAs in OSCC metastasis has been reported. Further analysis of six selected miRNAs revealed that miR-200c-3p silences its targets, CHD9 and WRN, as a key exosomal miRNA to promote tumor invasion that significantly accelerates the invasive potential of OSCC cells (Kawakubo-Yasukochi et al., 2018). OSCC-derived exosomes may influence cell motility and angiogenesis that, in turn, can influence OSCC progression. Two oncogenic miRNAs, miR-342-3p and miR-1246, are highly expressed in OSCC exosomes, leading to the metastasis of OSCC and increasing cell motility and invasive ability. miR-1246 directly targets DENND2D to promote the motility of tumor cells (Sakha et al., 2016). Thus, miRNAs in exosomes may be considered as non-invasive biomarkers for OSCC screening. On the contrary, inhibitory miRNAs may be delivered with exosomes to treat OSCC.

Exosomal miRNAs and the OSCC Microenvironment

The TME contains a complex network of non-malignant cells, molecules, structural components, and chemicals that surround cancer cells. Multiple non-malignant cells, including endothelial cells, pericytes, immune cells, and fibroblasts, together with the surrounding ECM, comprise the supportive stroma of the tumor and manipulate the TME. The "seed and soil" hypothesis is widely accepted in the cancer field (Paget, 1989; Fidler and Poste, 2008). The pre-metastatic niche, conceptualized as a fertile soil conducive to the survival and growth of metastatic seeds, consists of diverse cell populations, such as CAFs and various infiltrating immune cells, and non-cell components of the ECM. These niche components influence the fate of disseminated tumor cells in diverse ways, such as cell proliferation and differentiation, and contribute to tumor angiogenesis, invasion, and metastasis (Wu et al., 2018; Peltanova et al., 2019). Exosomes have been identified as a crucial means of cell-to-cell communication, involving both near and distant signal transduction. Thus, tumor-derived exosomes can serve as messengers in the tumor environment, creating favorable environment for tumor growth and metastasis (Bae et al., 2018).

Alteration of the TME is the first step in forming a pre-metastatic niche. As one of the most abundant constituents of the TME, we demonstrated that CAFs perform critical roles during tumor progression and metastasis (Vu et al., 2019). miRNAs from cancer-derived exosomes are crucial messengers in the intercommunication between tumor cells and CAFs within the TME. Bovy et al. (2015) demonstrated that exosomes derived from CAFs enhance OSCC cell metastasis. Besides, fibroblasts in the TME "communicate" with tumor cells through the transfer of miRNAs contained in exosomes (Bovy et al., 2015). Li et al. (2018) found that the expression of miR-34a-5p in CAF-derived exosomes was significant, thereby inducing the EMT and reducing expression of the cancer stem marker AXL to facilitate cancer cell metastasis via the AKT-GSK3β-β-catenin signaling pathway. Furthermore, the miR-34a-5p-AXL axis enhanced nuclear translocation of β-catenin, thereby inducing transcriptional upregulation of SNAIL, which in turn activated the ECM proteins MMP-2 and MMP-9. Besides, it was found that miR-3188 expression by directly targeting to BCL2, is reduced in exosomes and their parental CAFs from OSCC tissues (Wang et al., 2019b). Sun et al. (2019) discovered that exosomal miR-382-5p derived from CAFs and NFs upregulates MMP-3, MMP-9, N-cadherin, and β-catenin in OSCC cells, thus increasing the migration of CAL-27.

The composition and function of the vasculature in the TME exhibits abnormalities, including leakiness, a heterogeneous basement membrane, irregular vessel branching, and poor pericyte coverage. These changes ultimately lead to a hypoxic TME (Hu and Polyak, 2008), and TEX can then be induced to migrate and invade normoxic cells. Li et al. (2016) showed that a hypoxic microenvironment may stimulate OSCC tumors to produce miR-21-rich exosomes, enabling miR-21 to be transported to normoxic regions and drive non-hypoxic cells toward a pro-metastatic phenotype.

The human immune system exerts its defensive functions by innate immunity and adaptive immunity. Innate immunity provides the body with its instinctive defense against the pathogenic infections, while their propagation brings about activation of adaptive immune responses. The infiltration of lymphocytes, including regulatory T cells (Tregs), MDSCs, and tumor-associated macrophages, is common in OSCC. By dampening the immune response and generating immune tolerance, these lymphocytic cells promote immune evasion by tumor cells. Li et al. (2019b) suggested that exosomal miR-21 plays a key role in the regulation of TEX-induced γδ T-cell function by affecting MDSCs. These authors utilized lenti-miR-138 virus γδ T cell-derived exosomes (γδTDEs) as a drug delivery system in the treatment of OSCC (Xie et al., 2019a). Delivery of miR-138 with γδTDEs had a synergistic inhibitory effect on CAL-27 cells *in vitro* (Xie et al., 2019a). In immunocompetent C3H mice, applying miR-138-rich γδTDE as a form of pre-immunization inhibited the growth of OSCC (Xie et al., 2019a). Using differential fluorescence, miR-101-3p was found to be transferred from donor hBMSCs to recipient TCA8113 cells. By targeting COL10A, the transferred miR-101-3p significantly repressed cell invasion and migration (Li et al., 2019c). Further evidence showed that the miR-21-5p that was released from CAL27-derived exosomes was taken up by THP1 monocytes, playing a role in activating the NF-κB inflammatory pathway. The delivery of miR-21-5p by exosomes promotes monocyte migration and infiltration, which in turn participates in the promotion of angiogenesis in OSCC (Momen-Heravi and Bala, 2018).

Interestingly, alcohol treatment (25 mM for 24 h) increases exosome production and alters the subset of oncogenic miRNAs that are specifically enriched in exosomes released from tumor cells (Momen-Heravi and Bala, 2018). Mechanistically, exosome uptake from OSCC cells by monocytes causes activation of the NF-κB pathway and establishment of a pro-inflammatory milieu (Momen-Heravi and Bala, 2018). Thus, TEXs promote changes in the microenvironment, such as oxygen reduction and decreased immune responses, this deterioration of the microenvironment exacerbates the progression of OSCC. However, the presence of exosomes from immune cells in the OSCC microenvironment was not reported.

The traditional therapies for treating OSCC are surgery followed by chemotherapy. Due to the complexity of the TME, conventional drug delivery systems fail to transfer chemotherapeutics in an effective concentration to kill cancer cells and are associated with debilitating side effects. Exosomes have the essential characteristics including biocompatibility, non-cytotoxicity, low immunogenicity, simple to produce and store, long life span, and high cargo loading capacity (Steinbichler et al., 2019). The small size confers exosomes resistant to lung clearance and passing through the blood–brain barrier effectively (Kawikova and Askenase, 2015). Furthermore, exosomes may be used as specific targeting against cancer cells rather than normal cells by receptors in exosomes (Wang et al., 2016). Mounting evidence has provided insights about the crucial role of exosomal miRNAs in controlling the TME, and these insights could be applied to drug delivery. These miRNAs could

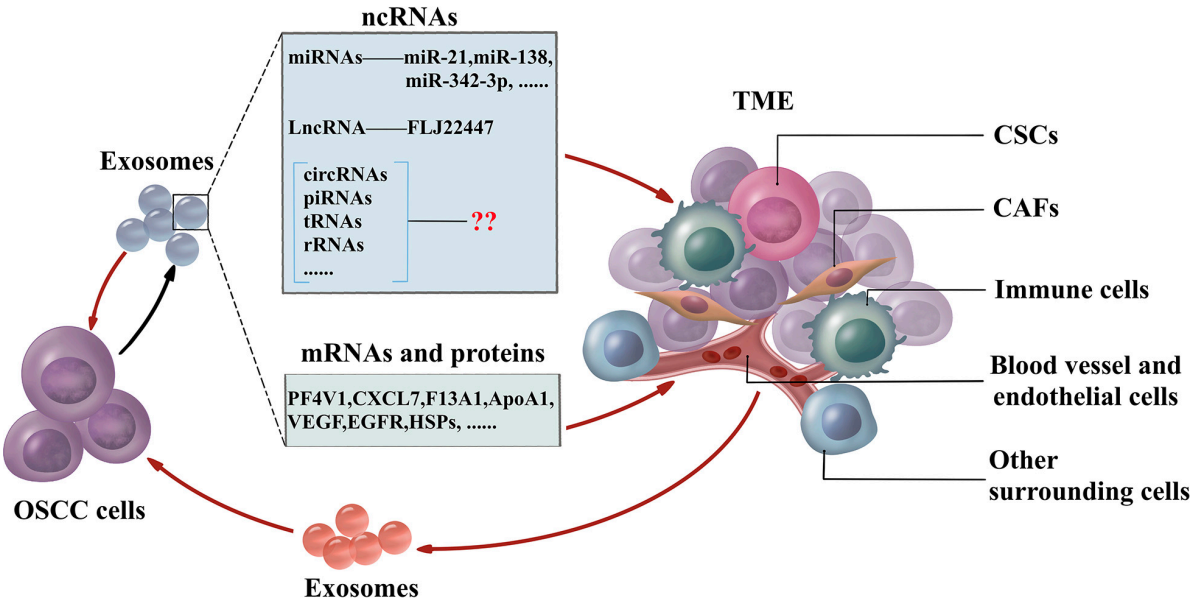

FIGURE 2 | The feedback regulation between OSCC and TME. OSCC cells generate exosomes which include non-coding RNAs (miRNAs, lncRNAs, circRNAs, piRNAs, tsRNAs, and rRNAs), mRNAs, and proteins to influence the TME. CAFs, CSCs, immune cells, blood vessels, endothelial cells, and other surrounding cells are the essential cells in TME. Furthermore, cells in TME influence the function of OSCC cells by exosomes. OSCC cells also interact with adjacent OSCC cells via exosomes. OSCC, oral squamous cell carcinoma; TME, tumor microenvironment; miRNAs, microRNAs; lncRNAs, long non-coding RNAs; circRNAs, circular RNAs; piRNAs, PIWI-interacting RNAs; tRNAs, transfer RNAs; rRNAs, ribosomal RNAs; CAFs, cancer-associated fibroblasts; CSCs, cancer stem cells.

be utilized as therapeutic components delivered to the OSCC microenvironment by exosomes. Exosome-delivered tumor suppressor miRNAs, miR-143 inhibits the growth of prostate cancer, while the let-7a significantly reduces the growth of breast cancer *in vivo*, respectively (Kosaka et al., 2012; Ohno et al., 2013b). Bio-safety has also been confirmed by adverse effects detection in normal prostatic epithelial cells with treatment of exosome-containing miR-143 (Kosaka et al., 2012). Cancer may acquire the drug resistance against chemotherapeutics by drug efflux with transporters. Exosomes could deliver the anti-miR-9 to reduce the transporter level, thus, sensitizes glioblastoma cells to temzolomid to increase cell death (Munoz et al., 2013). The feedback regulation of exosomes and TMEs is shown in **Figure 2**.

EXOSOMAL lncRNAs, CIRCULAR RNAs, AND piRNAs IN OSCC

Aside from miRNAs, ncRNAs also consist of long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), and piRNAs, rRNAs and tsRNAs. lncRNAs are defined as transcriptional products, with a length of more than 200 nucleotides and generally having no protein-coding potential (Jin et al., 2016). However, ncRNAs involve in multiple pathological processes. Gastrointestinal cancer has the same problem as OSCC of resistance to chemotherapy agents, such as cisplatin.

Exosomal lncRNAs

Exosomal lncRNAs mediates the progression and chemoresistance of tumor cells in the TME through diverse mechanisms. The exosomal lncRNA HOTTIP, transmitted from cisplatin-resistant gastric cancer cells to sensitive cancer cells, plays a role in conferring cisplatin resistance to sensitive cancer cells by binding to miR-218 to activate HMGA1 (Wang et al., 2019a). Moreover, this lncRNA is upregulated in the bodily fluids of gastric cancer patients, which indicates that it is a potential biomarker for early diagnosis and treatment. Similarly, Kogure et al. (2013) reported that the exosomal lncRNA TUC339 secreted by hepatocellular cancer cells is absorbed by surrounding cells and thereby promotes the growth of hepatocellular carcinoma. Ding et al. (2018) found that the lncRNA FLJ22447 derived from OSCC exosomes is upregulated in CAFs and activates them to induce the proliferation of OSCC cells through IL-33.

Exosomal CircRNAs

CircRNAs are a kind of single-stranded RNA that is comprised of mostly cytoplasmic exonic particles with linked 3′ and 5′ ends in eukaryotic cells. Several circRNAs have been found in body fluids, such as blood and saliva (Holdt et al., 2018; de Fraipont et al., 2019). circRNAs sorting into exosomes may be controlled by modulation of associated miRNA levels in parental cells and may transfer biological activity to target cells (Li et al., 2020). A recent study has reported that the exosomal circRNAs DB, derived from adipocytes, promotes the growth of hepatocellular carcinoma by sponging miR-34a and activating USP7/Cyclin A2 signaling pathway (Zhang et al., 2019). Zhao et al. (2019) found that the circRNA ATP8B4 acts as miR-766 sponge and plays a role in the development of radiation resistance in glioblastoma. The circRNA CDR1as functions as a miR-7 sponge, regulating insulin

transcription and secretion in pancreatic islet cells via miR-7 targets and downregulates miR-7 to perturb the development of diabetes (Xu et al., 2015). Hansen et al. (2013) introduced miR-7 mimics into HEK293T and MCF-7 cell lines and reported that the level of exosomal CDR1as is significantly decreased while increased in cells due to the ectopic expression of miR-7 in both HEK293T and MCF-7 cells.

Exosomal piRNAs

piRNAs are abundant small, non-coding, single-stranded RNAs 21-30 nucleotides in length, with little conservation of sequence between organisms (Weick and Miska, 2014). Compared with miRNAs, piRNAs function to repress transposons at the transcriptional and posttranscriptional levels to maintain genome integrity, while miRNAs play a role in repressing translation at the post-transcriptional level to regulate gene expression (Iwasaki et al., 2015). Although piRNAs have only 1–4% of all identified sequence content in the exosome, it was found that piRNAs are as abundant as miRNAs in exosomes isolated from plasma (Yuan et al., 2016). piRNAs are regarded as potential biomarkers in breast cancer, colon, and gastric cancer for pathological expression (Cheng et al., 2011; Hashim et al., 2014; Vychytilova-Faltejskova et al., 2018). Mounting evidence shows that piRNAs strongly correlate with tumor cell malignant phenotype and clinical stage. piR-021285 regulates cell proliferation and invasion by DNA methylation. The piR-021285 variant mimics transfection into breast cancer cell lines and weakens pro-invasive and pro-apoptosis gene methylation of ARHGAP11A at the 5′-UTR-first exon CpG site, which results in higher ARHGAP11A expression and increased breast cancer cell invasiveness (Fu et al., 2015). piRNAs are also involved in the development of lung cancer. The tumor promoter RASSF1C upregulates piR-34871 and piR-52200 and downregulates piR-35127 and piR-46545 through the RASSF1C-PIWIL1-piRNA axis, resulting in the promotion of stem cell proliferation and the EMT in lung cancer. These piRNAs changes inhibit AMPK phosphorylation in the ATM-AMPK-p53-p21cip pathway and thereby block cell cycle arrest and enhance cell proliferation (Reeves et al., 2012). Moreover, we developed chemical induced OSCC mouse model and found some piRNAs were significantly changed. And piRNAs has been identified in the exosomes of mesenchymal stem cells (Wang et al., 2020). However, the exosomal function of piRNAs in human OSCC should be further revealed.

Generally, exosomal lncRNAs, circRNAs, and piRNAs are involved in the tumor development. However, there are few reports on the involvement of these exosomal ncRNAs in the functioning of OSCC and their potential as biomarkers in OSCC, which deserves more attention and deeper exploration.

mRNAs AND PROTEINS IN OSCC

Proteins contained inside exosomes have also been evaluated in patients with OSCC, although not as extensively as miRNAs, and studies have shown promising exosomal protein markers for early diagnosis of OSCC, such as TRAP1, EGFR, heat shock protein 90 (HSP-90), and MMP-13, which can affect the intracellular functions of genes (Kaskas et al., 2014; Xie et al., 2019b). Among the proteins contained in exosomes, 23 were identified as potential biomarkers of OSCC (Boldrup et al., 2017). Recent studies found that the contents of free exosomes in blood were correlated with OSCC cells. Proteins in those exosomes, including PF4V1, CXCL7, F13A1, and ApoA1, could be used in the diagnosis of OSCC (Li et al., 2019a). Angiogenesis is generally correlated with tumor growth and metastasis, and exosomes derived from OSCC cells could have an inhibitory or promotional effect on angiogenesis, thereby influencing OSCC metastasis (Zhang et al., 2019). Rosenberger et al. (2019) demonstrated that exosomes manipulate the secretion of VEGF to inhibit the angiogenic activity of endothelial cells, thus reducing tumor metastasis. It was found that exosome treatment inhibits angiogenic activity, including both vessel density and vascular area.

The EMT also plays an essential role in tumor migration and invasion. Overexpression of EGFR is an essential feature of OSCC. It was found that OSCC cells abundantly express EGFR, which is secreted from cells as OSCC exosomes upon EGF stimulation (Fujiwara et al., 2018). Furthermore, OSCC LNM was always found in patients who were diagnosed in the later stages of the disease. Li et al. (2019a) isolated exosomes from the serum of OSCC patients, and found that the exosomal proteins PF4V1, CXCL7, F13A1, and ApoA1 in serum affect OSCC LNM and thereby influence prognosis. ROC analysis is a kind quantification method to acquire desirable levels of sensitivity and specificity (Obuchowski and Bullen, 2018). ROC analysis using the relative abundances of ApoA1, CXCL7, PF4V1, and F13A1 in serum, serum exosomes, and whole blood indicated that exosomal proteins are potentially predictive biomarkers for OSCC with LNM.

Cancer cells often secrete exosomes carrying heat shock proteins, which play a part in tumor progression. It was found that abundant secretion of exosomes rich in HSP-90 was found in OSCC with LNM, indicating a poor prognosis (Ono et al., 2018). Besides, drug resistance also remains a severe problem in most chemotherapy treatments for OSCC (Samuel et al., 2017). Recently, it has been suggested that cancer cell-derived exosomes mediate drug resistance. Khoo et al. (2019) showed the increased exosome production in both *de novo* (H314) and adaptive (H103/cisD2) resistant cell lines compared with sensitive H103 cells. Moreover, differences in the proteomes contained within exosomes indicate that adaptation to cisplatin treatment causes significant changes in the secreted exosomes (Khoo et al., 2019). The cargos in the exosomes were showed in **Table 1**.

DISCUSSION

In 2012, miRNAs were found in the exosomes of invasive tumors, suggesting that tumor-derived exosomes may serve as an important diagnostic tool to avoid metastasis and improve prognosis (Chen et al., 2012a). Therefore, it is essential to determine the involvement of exosomes in maintaining the

aggressive phenotype of OSCC cells and their unique roles in intercellular communication.

Although several miRNAs have been identified in the exosomes of OSCC, more should be analyzed by improving the isolation and purification of exosomes. Furthermore, the function of lncRNAs, circRNAs, piRNAs, mRNAs, and proteins in the exosomes of OSCC also needs to be determined. In fact, tRNAs and rRNAs are infrastructural ncRNAs, they are also involved into the cancer initiation and progression. tRNAs re small conserved RNA molecules that allow the translation of the genetic code into amino acids. Overexpression of initiator $tRNA^{Met}$ ($tRNA_i^{Met}$) is to promote metastasis of melanoma cells, through α5β1 integrin-dependent signaling (Birch et al., 2016). Furthermore, the levels of $tRNA^{Glu}UUC$ and $tRNA^{Arg}CCG$ are increased in metastatic breast cancer cell lines (Goodarzi et al., 2016). Dysregulated rRNA transcription is essential in cancers and may be involved in the initiation stage of hepatocarcinogenesis (Donati et al., 2011; Xie et al., 2018). However, whether rRNAs are existed in exosomes and the exosomal rRNAs should be further revealed.

As a viable alternative to tissue-based sampling in the clinic, it is clear that there is a great deal of interest in non-invasive liquid biopsies (Xue et al., 2019). Liquid biopsies utilize blood and saliva to detect the circulating tumor cells, circulating tumor DNA, and exosomes for the diagnosis and prognosis of oral cancer (Lousada-Fernandez et al., 2018). This method allows repeated sampling to monitor the treatment response, assess tumor heterogeneity, and even use in cancer screening programs. Wu et al. (2017) has shown that an acoustofluidic platform integrating acoustics and microfluidics efficiently isolates exosomes directly from undiluted blood samples and saliva. Saliva collection was rather easy and non-invasive. It has demonstrated that exosomes have a higher amount and larger size in the saliva of patients with oral cancer than healthy individuals (Zlotogorski-Hurvitz et al., 2016; Nair et al., 2018). Furthermore, increased expression of CD63 and lesser expression of CD9 and CD81 are found in the saliva exosomes of oral cancer patients (Zlotogorski-Hurvitz et al., 2016). More efforts should be made to identify new biomarkers in the exosomes of saliva, finally increasing the application in non-invasive cancer diagnosis. Considering the endogenous transport function, exosomes have robust potential to be applied as therapeutic delivery systems. However, efficiency in delivering drugs to the tumor is still a major challenge due to the blood–brain barrier and degradation (Kawikova and Askenase, 2015). Exosomes can be fused with the cell membrane with high reliability, and therefore non-coding RNAs or peptide drugs may be packed into exosomes and delivered to the OSCC tumor. Furthermore, the process of exosomes transfected with ncRNAs may influence the activity of exosomes. Thus, ncRNAs could be transfected into the exosome derived cells. It has demonstrated exosomes from mesenchymal stem cells overexpressed with tumor suppressed miRNAs has inhibitory effect on tumor progression (Che et al., 2019; Xu et al., 2019; Yuan et al., 2019). Furthermore, the fabrication of target-specific exosomes could increase the efficacy of cancer treatment (Wang et al., 2016). The exosomes conjugated with antibodies specifically target cancer cells (Stickney et al., 2016). The exosomes magnetized or modified by pH-sensitive peptide also contributes to the accumulation in the cancer cells (Nakase and Futaki, 2015; Qi et al., 2016). Furthermore, the glycosylation of the surface proteins increases the stability of exosomes (Hung and Leonard, 2015). The therapeutic application of exosomes against OSCC is illustrated in **Figure 3**.

In summary, it is essential to understand the role of exosomes in influencing tumor phenotype, angiogenesis,

TABLE 1 | Cargos in exosomes derived from OSCC.

Biomolecules	Function	Molecules	Exosome origin	References
miRNAs	Suppressor	miR-338, miR-24-3p, miR-891a, miR-106a-5p, miR-2a-5p, miR-1908, miR-101-3p	Cell lines	Ye et al., 2014; Tachibana et al., 2016; Xie et al., 2019a
	Biomarker	miR-223	Plasma	Tachibana et al., 2016
	Metastasis	miR-21, miR-142-3p, miR-29a-3p, miR-342-3p, miR-1246, miR-200c-3p	Cell lines	Sakha et al., 2016; Dickman et al., 2017; Liu et al., 2017; Kawakubo-Yasukochi et al., 2018; Cai et al., 2019
	Accumulation of fibronectin	miR-382-5p, miR-34a-5p, miR-3188	CAFs	Bovy et al., 2015; Liu et al., 2017; Li et al., 2018
	Motility	miR-342-3p, miR-1246,	Cell lines	Sakha et al., 2016
	Angiogenesis	miR-21	Cell lines	Momen-Heravi and Bala, 2018
	Immune regulation	miR-138,	Cell lines	Xie et al., 2019a
Proteins	Biomarker	PF4V1, CXCL7, F13A1, ApoA1	Serum	Li et al., 2019a
		VEGF, EGFR, HSPs	OSCC cells	Fujiwara et al., 2018; Ono et al., 2018; Rosenberger et al., 2019
lncRNAs	Proliferation	Lnc-FLJ22447	OSCC cells	Ding et al., 2018

CAFs, cancer-associated fibroblasts; HSPs, heat shock proteins; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; PF4V1, platelet factor 4 variant 1; CXCL7, C-X-C motif chemokine ligand 7; F13A1, coagulation factor XIII A chain; ApoA1, apolipoprotein A1.

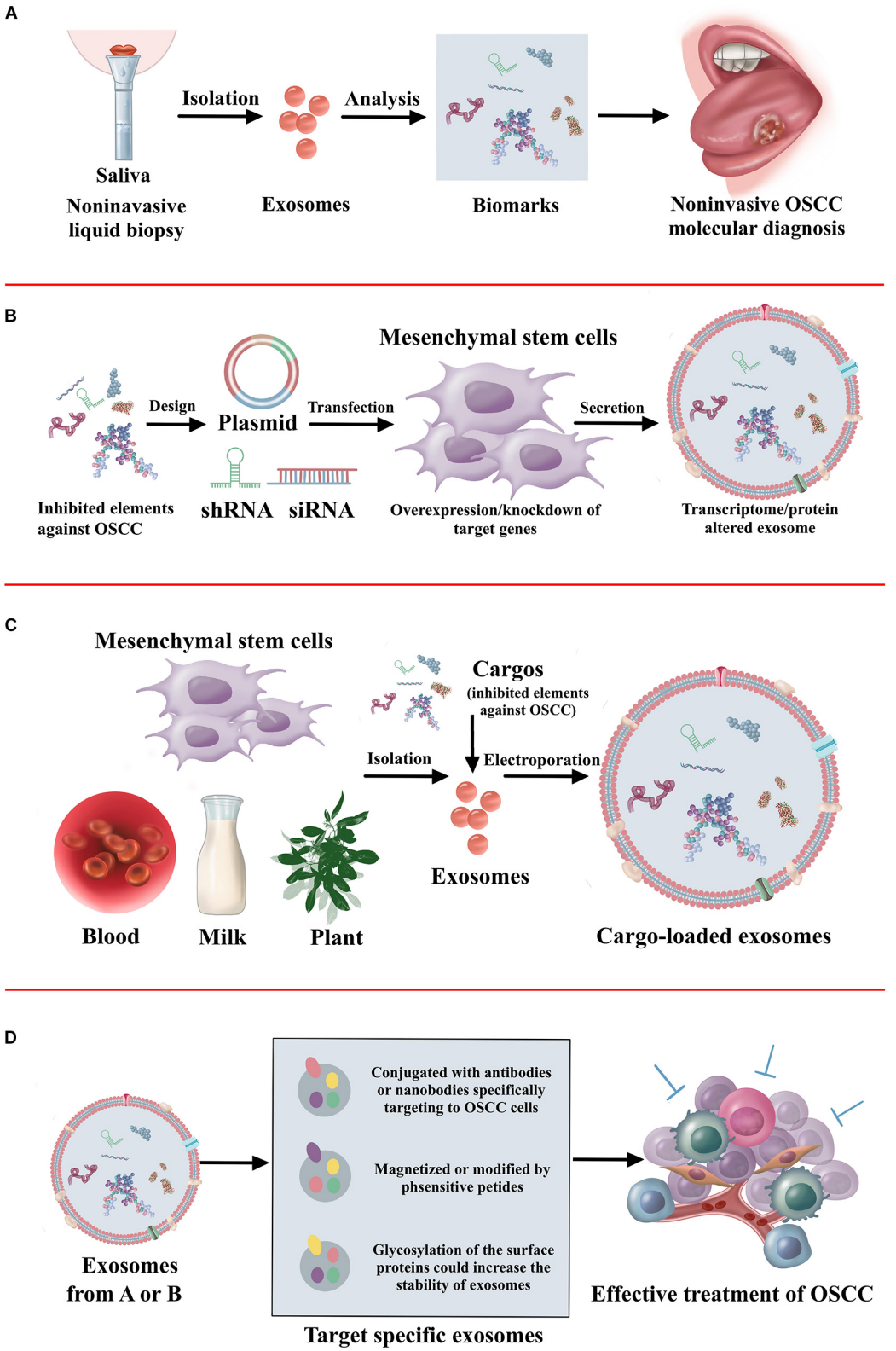

FIGURE 3 | The summary of therapeutic application of exosomes. **(A)** The non-invasive diagnosis and prognosis of OSCC using exosomes. **(B)** Approaches to alter cargo contents in the mesenchymal stem cell-derived exosomes to treat OSCC. **(C)** Loading of exogenous proteins, genes, or signaling molecules in the exosomes to treat OSCC. **(D)** Exosome surface modification for target specific delivery of exosomes toward OSCC.

immune modulation, metastasis, and drug resistance. While studies on the role of exosomes in OSCC have made progress, there are several outstanding questions that need to be further explored. The versatile biological functions of exosomes could be promising tools to apply in the diagnosis, prognosis, and effective treatment of OSCC.

AUTHOR CONTRIBUTIONS

LWu, MZ, and WC concepted the manuscript. YL, ZZ, and YY wrote the original draft. JP, XY, LWa, LWu, and WC revised the manuscript. ZY made the improvement for the figures. All authors contributed to the article and approved the submitted version.

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Extracellular Vesicles From the Human Natural Killer Cell Line NK3.3 Have Broad and Potent Anti-Tumor Activity

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Natural killer (NK) cells are critical mediators of immune function, responsible for rapid destruction of tumor cells. They kill primarily through the release of granules containing potent cytolytic molecules. NK cells also release these molecules within membrane-bound exosomes and microvesicles – collectively known as extracellular vesicles (EV). Here we report the characterization and anti-tumor function of EVs isolated from NK3.3 cells, a well described clonal normal human NK cell line. We show that NK3.3 EVs contain the cytolytic molecules perforin, granzymes A and B, and granulysin, and an array of common EV proteins. We previously reported that the E3 ubiquitin ligase, natural killer lytic-associated molecule (NKLAM), is localized to NK granules and is essential for maximal NK killing; here we show it is present in the membrane of NK3.3 EVs. NK3.3-derived EVs also carry multiple RNA species, including miRNAs associated with anti-tumor activity. We demonstrate that NK3.3 EVs inhibit proliferation and induce caspase-mediated apoptosis and cell death of an array of both hematopoietic and non-hematopoietic tumor cell lines. This effect is tumor cell specific; normal cells are unaffected by EV treatment. By virtue of their derivation from a healthy donor and ability to be expanded to large numbers, NK3.3 EVs have the potential to be an effective, safe, and universal immunotherapeutic agent.

Keywords: natural killer, extracellular vesicle, exosome, leukemia, NK3.3, NKLAM, breast cancer, ubiquitin ligase

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INTRODUCTION

Natural killer (NK) cells are a subset of lymphocytes with the innate ability to selectively recognize and kill cancer cells. They have both activating and inhibitory receptors that control their cytotoxic function. Tumor cells, by their down-regulation of self-recognizing major histocompatibility complex class 1 (MHC-I) molecules and expression of stress-induced proteins, shift the balance toward activation of NK cells. This induces migration and fusion of NK granules to the plasma membrane, followed by degranulation and entry of cytolytic molecules into the tumor cell, resulting in tumor cell death (Moretta and Moretta, 2004).

Granules arise from the formation of a dense core of effector molecules inside large, lipid membrane-bound vesicles within NK cells. Cytolytic proteins - perforin, granzyme A, granzyme B,

and granulysin induce death in target cells by activating caspase -dependent and -independent death pathways (Sanchez-Ruiz et al., 2011). Upon fusion of granules with the plasma membrane, their contents are emptied into the immune synapse formed between the NK cell and the target cell. As a result, a concentrated discharge of potent effector molecules attack the target (Culley et al., 2009).

Within granules, smaller membrane-bound vesicles called exosomes are formed, which are secreted during degranulation. Activated NK cells also release microvesicles - pinched off sections of plasma membrane. All are collectively referred to as extracellular vesicles (EVs). Virtually all cells secrete EVs which function as a means for intercellular communication and modulation of cellular function via the diverse bioactive cargo within them. In addition to components common to all EVs, they also carry proteins, lipids, and RNA, unique to the cell type from which they originate. Exosomes are the smallest vesicles secreted by cells, ranging in size from 30 to 150 nm; microvesicles are larger, measuring between 100 and 1000 nm (Cufaro et al., 2019).

Extracellular vesicles derived from NK cells have been shown to contain nucleic acids (mRNA, micro (mi)RNA, rRNA) and common EV proteins such as CD63, tsg101, Alix, and HSP70. Importantly, NK-derived EVs also contain the cytolytic molecules responsible for NK-mediated anti-tumor activity. Recently, NK-derived EVs have been shown to effectively induce tumor cell death independent of the cell-to-cell contact necessary to activate the anti-tumor mechanisms of NK cells (Lugini et al., 2012; Jong et al., 2017; Zhu et al., 2017, 2019; Neviani et al., 2019; Wu et al., 2019; Federici et al., 2020).

We report the characterization and function of EVs derived from NK3.3. This cloned NK cell line was generated from the peripheral blood of a healthy human donor by our laboratory and is the only published NK cell line of this kind to date (Kornbluth et al., 1982, 1985; Kornbluth and Hoover, 1988). We performed comprehensive proteomic and RNA analysis of NK3.3-derived EVs. They contain the granule-associated proteins necessary for tumor killing, including natural killer lytic-associated molecule (NKLAM/RNF19b), an E3 ubiquitin ligase. Our laboratory identified NKLAM and found that it has two transmembrane domains and resides in the membranes of NK cell granules. Its ligase activity is mediated by three cysteine rich clusters that form a RING - in between RING - RING (RBR) domain. The catalytic activity of the RBR domain enables NKLAM to ubiquitinate its substrates. Studies conducted in our laboratory with NKLAM-deficient mice indicate that NKLAM is required for maximal NK-mediated anti-tumor function and for suppression of tumor dissemination and metastasis *in vivo* (Portis et al., 2000; Fortier and Kornbluth, 2006; Hoover et al., 2012; Lawrence et al., 2020).

Recent published studies have demonstrated tumor killing using exosomes derived from NK-92, an NK tumor line derived from a patient with non-Hodgkin's lymphoma (Zhu et al., 2017). Other investigators used NK cells sorted and expanded from the peripheral blood of healthy human donors to collect NK-derived exosomes (Lugini et al., 2012; Jong et al., 2017). NK3.3-derived EVs reduce the need for identifying and consenting heathy peripheral blood donors as well as eliminating donor variability. They also prevent the potential introduction of tumorigenic elements that might occur using EVs from transformed NK-92 cells. Because use of NK-derived EVs is relatively new, there are a limited number of tumor types that have been examined for susceptibility to EV-mediated killing. These include melanoma (Zhu et al., 2017), neuroblastoma (Neviani et al., 2019; Wu et al., 2019), acute lymphoblastic leukemia (Wu et al., 2019; Di Pace et al., 2020) and glioblastoma (Zhu et al., 2018, 2019).

This study describes the anti-tumor effect of NK3.3-derived EVs. We initiated studies using K562 tumor cells, derived from the bone marrow of a patient with chronic myelogenous leukemia in blast phase, with characteristics of an acute leukemia (Lozzio et al., 1981). K562 cells are highly susceptible to NK-mediated killing because they lack expression of MHC-I (Kornbluth et al., 1985). We show that NK3.3-derived EVs induce morphological changes and altered protein expression in K562 characteristic of death via apoptosis. They inhibit tumor proliferation, and are strongly cytotoxic to K562 cells, while having no effect on the growth or survival of non-tumorigenic, normal cells. We performed proteomic analysis of K562 cells to identify changes in protein expression induced by NK EV treatment. We expanded studies to evaluate the ability of NK3.3-derived EVs to kill other types of tumor cells. We found that NK EVs kill T cell leukemia and breast cancer cells. This indicates that they have the potential to be a safe alternative, or synergistic partner, to current therapeutic modalities for multiple types of cancer, especially for those that lack effective, curative treatments.

MATERIALS AND METHODS

Cell Lines

The human NK cell line, NK3.3, was maintained in RPMI-1640 medium supplemented with 10% FBS, 1% glutamine, 1% penicillin-streptomycin, and 200 IU/ml recombinant IL-2 (rIL-2) (R&D Systems). NK-92 cells (obtained from Dr. Hans Klingemann) were cultured using the NK3.3 culture protocol. Cryopreserved umbilical cord blood (CB) was obtained from the St. Louis Cord Blood Bank. Units were thawed and CD34+ hematopoietic stem cells were isolated using immunomagnetic CD34 antibody coated beads (Miltenyi Biotec). CD34+ cells were expanded in culture using a cocktail of cytokines (G-CSF, GM-CSF, IL-6, SCF, IL-7 and TPO) and aliquots of cells frozen over 2 weeks of culture. Peripheral blood mononuclear cells were obtained from normal donors as described (Kozlowski et al., 1999) and cultured for 3–7 days using the NK3.3 culture protocol. To obtain EV-depleted FBS, FBS was ultracentrifuged at 118,000 × *g* (Type 45 Ti rotor, Beckman Coulter, CA) for 20 h, then filtered through a 0.22 μm filter. Human embryonic kidney cells (HEK293) for EV collection were cultured in DMEM media supplemented with 3% EV-depleted FBS. K562 and Jurkat T cell leukemia cells (ATCC, Manassas, VA) were cultured in RPMI-1640 media supplemented with 7.5% FBS and 1% L-glutamine. HEK293 and breast cancer cell lines MCF7 and MDA-MB-231 (ATCC, Manassas, VA) were cultured in DMEM media supplemented with 1% glutamine, 1% penicillin-streptomycin, and 10% FBS. All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Extracellular Vesicle Generation and Isolation

NK3.3 and NK-92 cells were cultured overnight with IL-2 and then treated with phorbol myristate acetate (PMA) and ionomycin for 5 h to enhance secretion of EVs into the media. HEK293 cells were grown to 90% confluency over 48 h. Media was harvested and centrifuged at 300 × *g* for 10 min to pellet cells. A commercial polyethylene glycol polymer for precipitation of EVs was added to the supernatants for a minimum of 12 h at 4°C (ExoQuick-TC, System Bioscience (SBI)) and were centrifuged at 3000 × *g* at 4°C for 10 min per a modified ExoQuick-TC protocol. EV-depleted supernatants were discarded, and EV pellets were resuspended in PBS. Protein concentrations were assessed via BCA protein assay kit (ThermoFisher Scientific).

NK3.3-derived vesicle fractions were isolated by differential ultracentrifugation (Federici et al., 2020). Briefly, NK supernatants were centrifuged at 300 × *g* for 10 min, followed by centrifugation at 2,000 × *g* for 20 min and then 10,000 × *g* for 30 min at 4°C. This pellet was considered to be the microvesicle (MV) fraction. Additional ultracentrifugation at 100,000 × *g* for 90 min at 4°C was performed to isolate the exosome fraction. All pellets were resuspended in PBS and protein quantitated by BCA.

Transmission Electron Microscopy Imaging

UV treated 300 mesh copper formvar coated grids were loaded with EV solution for 1.5 min, washed with 5 water drops, stained with 1% uranyl formate, blotted, and dried. EVs were imaged using a JEOL JEM1400 Plus transmission electron microscope (TEM) in the Research Microscopy and Histology Core, Saint Louis University.

Nanoparticle Tracking Analysis

Size distribution of EV particles was determined by nanoparticle tracking analysis (NTA) using the NanoSight NS300 analyzer. Measurements were performed in triplicate for each EV preparation using 1 μg/μl EV protein diluted 1000-fold in PBS to obtain between 20 and 100 particles per frame. Samples were introduced at ambient temperature using an automated syringe pump set to 40, with camera level set to 13, detection threshold set to 3. Acquisition time was 60s and at least 200 tracks were completed. Data were obtained and analyzed using NTA 3.3 analytical software (Malvern Instruments).

Proteomic and RNA Analysis

EV-containing preparations from NK3.3 cells were submitted to SBI for proteomic and RNA analysis. EVs were isolated, resuspended in buffer, and then filtered through a purification column (ExoQuick-TC ULTRA, SBI). For proteomic analysis, a 10 μg sample was processed by SDS-PAGE, the migration line excised, and in-gel digestion performed using trypsin. Analysis was performed using nano LC-MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptide sequence data was searched using Mascot software and filtered using Scaffold software.

For RNA analysis of the EV sample, RNA was extracted and purified using the RNA Purification Column kit (SBI). Small RNA libraries were constructed, purified, and then quantified. An 8% TBE gel was used to size select for the 140 to 300 bp region from the pooled libraries. This library was quantified by qPCR, and then high output single-end sequencing was performed.

For proteomic analysis, K562 cells were treated for 24 h with PBS (20% of total volume in culture media), HEK293 EVs or NK3.3 EVs (100 μg/ml). Cells were pelleted, washed, and then cell pellets were flash frozen on dry ice. Frozen pellets were submitted to Creative Proteomics. Cell pellets were lysed using an ultrasonic lysis device. Supernatants were transferred into YM-10 Microcon devices (Millipore) and centrifuged at 12,000 × *g* at 4°C for 10 min. A series of ammonium bicarbonate washes, reductions, and centrifugations were performed, the lyophilized extracted peptides were resuspended in formic acid, and 1 μg of protein was analyzed by LC-MS/MS using the Ultimate 3000 nano UHPLC system (ThermoFisher Scientific). The MS raw files were analyzed and searched against the human protein database based on sample species using Maxquant (1.6.2.6). Significance was defined as fold-change above 1.5 or below 1/1.5 determined by quantitative protein ratios.

Pronase Assay

Twenty micrograms of NK3.3-derived EVs were treated with either PBS, Pronase (Sigma) at a final concentration of 10 μg/ml, or 10 μg/ml Pronase plus saponin (Sigma) at a final concentration of 0.2%, with final equivalent total volumes for all three conditions. Samples were incubated at 37°C for 1 h, mixed with Laemmli buffer, boiled at 95°C for 5 min, then immediately analyzed by immunoblotting.

Cell Growth/Viability Assays

Cells were placed in fresh culture media 24 h before treatment. At the time of set up, cells were resuspended in fresh culture media at a final concentration of 5×10^5 cells/ml – K562 cells, 2.5×10^5 cells/ml – Jurkat cells, or 1×10^5 cells/ml – HEK293, MDA-MB-231, and MCF7 cells. Treatments consisted of PBS only, EVs suspended in PBS, or staurosporine/PBS solution; all treatment volumes were 20% of the total culture volume. NK3.3-, or HEK293- derived EV treatment concentrations were between 0 and 100 μg/ml; the staurosporine concentration was 2.5 μM. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were evaluated morphologically and counted via hemocytometer using a phase microscope or automated cell counter (Countess II FL, Invitrogen) at 24, 48, and 72 h after treatment. Cell viability was assessed by trypan blue dye exclusion.

The WST-1 cell proliferation colorimetric assay (Roche) was used to quantitate the number of viable, metabolically active K562, Jurkat and IL-2 stimulated peripheral blood lymphocytes upon treatment with HEK293 or NK3.3 EVs. Cells were seeded in 96-well plates at 10,000 cells/well in a final total volume of 0.1 ml. Wells were set up in triplicate for each condition. At the desired time point, 10 μl of WST-1 was added to each well. After a 1 h incubation, absorbance was measured using a Gen5 microplate reader (BioTek) with a 450 nm filter.

Crystal violet (Sigma) was used to determine the ability of EVs to affect the viability and growth of adherent cell lines HEK293, MCF-7 and MDA-MB-231. Cells were seeded in 96-well plates at 15,000/well. At the desired time point, media was removed from the wells, wells were washed with PBS, and then incubated with 35 μl of crystal violet stain (0.1%) at room temperature for 10 min. After removal of the stain, 50 μl of lysing solution was added (0.1 M sodium citrate in 50% ethanol, pH 4.2) and incubated for 15 min. Absorbance was measured using a Gen5 microplate reader with a 590 nm filter.

K562 cells were cultured as previously described; CB cells were thawed and allowed to expand for 10 days before use. Metabolic activity was monitored using the RealTime-Glo MT Cell Viability Assay (Promega), with the kit protocol adapted for a 384-well plate with a final volume of 80 μl per well. Wells were seeded with 1,500 K562 or 5,000 CB cells. Final NK3.3 EV concentrations ranged from 25 to 50 μg/ml. Plates were placed in a Gen5 microplate reader and maintained at 37°C; luminescence output was measured at 1 h intervals for 89 h.

Caspase Assay

Caspase-3 and -7 activity was measured using Caspase-Glo 3/7 (Promega). K562, Jurkat, MCF7 and MDA-MB-231 cells were treated with either 2.5 μM staurosporine, or 100 μg/ml of HEK293 EVs or NK3.3 EVs suspended in PBS at 20% total volume in Eppendorf tubes. 5,000 treated cells in 10 μl were added in triplicate to wells of 384-well plates for each condition and time point. At the appropriate time interval, 10 μl Caspase-Glo 3/7 substrate was added to each well. The plate was incubated for 1 h. For long term detection of caspase activity, 2.5 μM staurosporine or 100 μg/ml of HEK293 EV- or NK3.3 EV-treated K562 cells were seeded in 96-well plates at 50,000 cells/well triplicate for each condition and time point. Caspase activity, quantitated using Caspase-Glo 3/7, was determined using the kit protocol adapted for 5000 cells in 30 μl total volume for each reaction in 384-well plates. Luminescence was measured using the Gen5 microplate reader.

Flow Cytometry

Cells were monitored for changes in cell surface CD71 expression using FITC anti-CD71 (M-A712, BD Biosciences). Apoptosis/cell death was determined using PE-Annexin V (#640908, BioLegend) with 7AAD staining (#559925, BD Biosciences). All assays were performed following manufacturers' protocols and cells were analyzed using an LSRII flow cytometer (BD Biosciences). Data were evaluated using FlowJo software.

Immunoblot Analysis

PBS- and EV-treated K562 cells were lysed in RIPA buffer (Pierce) supplemented with a protease inhibitor cocktail (ThermoFisher Scientific). Cell and EV lysates were analyzed by SDS-PAGE and transferred onto PVDF (Bio-Rad). Membranes were blocked using 3% BSA and probed with specific antibodies. Proteins were detected using either Clarity (Bio-Rad) or WesternBright (Advansta) western ECL blotting substrates via chemiluminescence (ChemiDoc XRS+, Bio-Rad). Protein loading was normalized using anti-β-actin antibody (A5441, Sigma). Antibodies used for this study include: Alix (#2171), annexin V (#8555), Bcl-2 (#2872), CD9 (#13174), cleaved caspases -3 (#9664), -7 (#8438), -8 (#9496), and -9 (#9505), granzyme A (#4928), granzyme B (#4275), HSP70 (#4876), HSP90β (#5087), ICAM1 (#4915), LAMP-1 (#3243), phospho-STAT1: Ser727 (#9177) and Tyr701 (#9167), STAT1 (#9172), and VCAM1 (#13662) - (Cell Signaling); cytochrome c (sc-13156), full-length caspases -3 (sc-7272) and -7 (sc-28295), granulysin (sc-271119), MHC-1 (sc-55582), perforin-1 (sc-136994), Tsg101 (sc-136111), DNAM-1 (sc-376736) - (Santa Cruz); CD63 (SBI); rabbit polyclonal anti-LAMP-1 tail antibody; mouse monoclonal NKLAM and MHC-II antibodies made in-house. Horseradish peroxidase-labeled secondary anti-mouse and anti-rabbit antibodies were from Cell Signaling.

In vivo Tumor Studies

GFP-expressing MDA-MB-231 cells (2×10^6) were injected into the 4th mammary fat pad of female athymic nude mice (Charles River). When tumors were palpable, 50 μg of either NK3.3 or HEK293-derived EVs were administered by intratumoral injection every 3–4 days. After 7 injections, mice were euthanized, tumors excised, formalin fixed, and paraffin embedded. Tumor sections were stained for apoptotic cells by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which detects DNA fragmentation. Slides containing tumor sections were scanned and fluorescent pixel intensity quantitated using CellSelect software (Takara Bio). Results are expressed as the ratio of TUNEL positive cells to total nuclei (stained with DAPI). Four control and 5 NK EV-treated mice were evaluated, with 3 tumor sections per mouse.

Statistical Analysis

All data were recorded and analyzed in Microsoft Excel. Reported statistics are expressed as the mean ± SE. Statistical significance was determined at $p < 0.05$ using Student's *t*-test with unequal variance.

RESULTS

Verification of Extracellular Vesicle Isolation

Transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) were used to characterize the size and homogeneity of the vesicles isolated from NK3.3, NK-92, and HEK293 cells. Examination of TEM images of NK3.3-derived vesicles identified a mixed population of membrane-bound spherical structures, which coincide with the defined size ranges of both exosomes and microvesicles (**Figure 1A**). This mixed population will collectively be referred to as extracellular vesicles (EVs).

NTA concurrently identified a mixed population of vesicles produced by NK3.3 cells (**Figure 1B**). While the majority – represented by the highest peak – were 133 nm, consistent with the size of exosomes, the next highest peak signified a

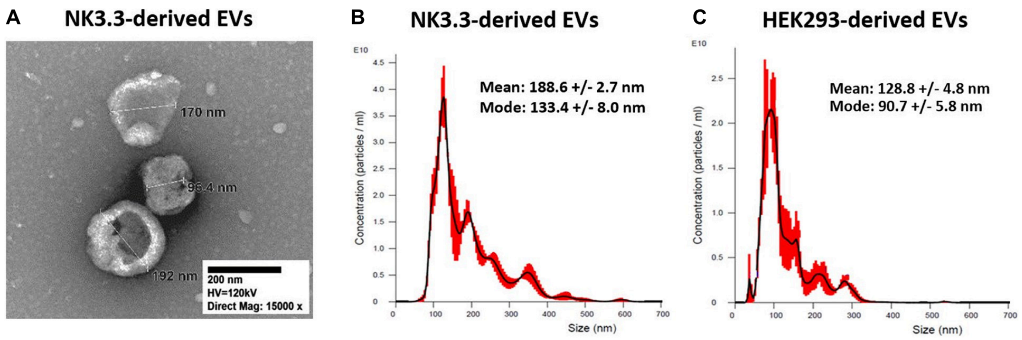

FIGURE 1 | Size characterization of EVs derived from NK3.3 and HEK293 cells. **(A)** TEM was used to visualize and measure the size of EVs recovered from NK3.3 cells. EVs collected from NK3.3 **(B)** and HEK293 **(C)** supernatants were evaluated by NTA to measure vesicle size and particle concentration. Data are representative of 3 measurements each.

population of vesicles of 193 nm in size. For comparison, NTA was performed to evaluate vesicles produced by NK-92 cells (**Supplementary Figure 1A**). There was a less distinct division of size in this sample; the majority of NK-92 vesicles were 155 nm, closely followed by a population of 173 nm. Our measurements of NK-92 EVs corresponded to other published measurements for EVs derived from both NK-92 cells and NK cells isolated from peripheral blood mononuclear cells (Jong et al., 2017; Zhu et al., 2018, 2019; Federici et al., 2020). Vesicles produced by HEK293 cells were more homogeneous, with the majority measuring 91 nm, indicating that HEK293 EVs were primarily exosomes (**Figure 1C**). These measurements were consistent with other published HEK293 exosome measurements (Li et al., 2016). Therefore, NK3.3 EVs were similar in size to other NK-derived EVs. We obtained a high yield of NK3.3 EVs: $1.45 \pm 0.13 \times 10^{12}$ particles per 10^6 cells, and $8.47 \pm 0.98 \times 10^{10}$ particles per μg ($n = 23$). This corresponds to 18.36 ± 1.00 μg per 10^6 cells. A similar number of HEK293 EVs were obtained: $1.08 \pm 0.09 \times 10^{11}$ particles per μg ($n = 23$).

Differential ultracentrifugation was performed, following a standard protocol to separate microvesicles (MV) from exosomes (Federici et al., 2020). By NTA, the exosome fraction was almost identical to that obtained by polyethylene glycol polymer precipitation (**Supplementary Figures 1B,C**). The MV fraction contained larger particles and was more heterogeneous (**Supplementary Figure 1D**).

Characterization of NK3.3-Derived Extracellular Vesicles

Immunoblot characterization of NK3.3- and HEK293-derived EVs indicates that they contained an array of EV-specific proteins, including CD63 and LAMP-1, heat shock proteins HSP70 and HSP90, vesicle structural proteins Alix and Tsg101 and other common EV cargo proteins β-actin and annexin V (**Figure 2A**). Published proteomic analysis of HEK293 EVs corroborates the expression of CD9 and other EV proteins (Li et al., 2016). Cytochrome-c, which resides between the inner and outer mitochondrial membrane, was detected in whole cell lysates; however, it was not found in EVs, making it one indicator of EV purity (**Supplementary Figure 2A**).

NK3.3-derived EVs also contained cytotoxic proteins required for NK anti-tumor activity: NKLAM, perforin, granzymes A and B, and granulysin, as well as immune-associated proteins MHC I and II and DNAX Accessory Molecule (DNAM-1; CD226), an activating receptor on NK cells (Neviani et al., 2019). NK3.3 EVs also expressed adhesion molecules ICAM1 and VCAM1 (**Figure 2B**). HEK293 EVs lacked the effector molecules possessed by NK3.3 EVs that contribute to cytotoxicity or participate in immune functions. For comparison, we assessed the expression of immune-associated proteins in NK3.3- and HEK293- EVs with NK-92 EVs, and in HEK293- and NK3.3-whole cell lysates (**Supplementary Figure 2B**). NK3.3- and NK-92- EVs expressed a similar array of proteins.

Proteomic analysis of NK3.3-derived EVs identified 623 proteins. Their subcellular distribution is shown in **Supplementary Figure 2C**. NK-associated proteins included granzymes A and B, perforin, MHC class I and class II molecules. Other proteins identified by proteomic analysis that were detected by immunoblotting included β-actin, Alix, CD63, various species of HSP70 and HSP90, ICAM1 and VCAM1. CCL5 (C-C motif chemokine 5/RANTES) was identified as a highly abundant protein and the most abundant chemokine. Another protein of interest was FCER1G (high affinity immunoglobulin epsilon receptor subunit gamma). This subunit is involved in signaling through multiple cell surface receptors. Also identified in the proteomic analysis was an array of surface proteins important in NK signaling and/or adhesion that may contribute to NK3.3 EV uptake and function: LFA-1 (CD11b/CD18), ezrin, CD70, CD59, CD53, CD50, CD48, CD47, and CD2 (Robertson et al., 1990; Montaldo et al., 2013). The distribution of proteins by biological process and molecular function is shown in **Figures 2C,D**. Proteins of interest in NK3.3 EVs associated with NK function are listed in **Table 1**. The complete proteomic dataset is included in the supplementary data (**Supplementary Table 1**). Based on the results of immunoblot and proteomic analysis, NK3.3 EVs contain a robust profile of death-inducing effector molecules

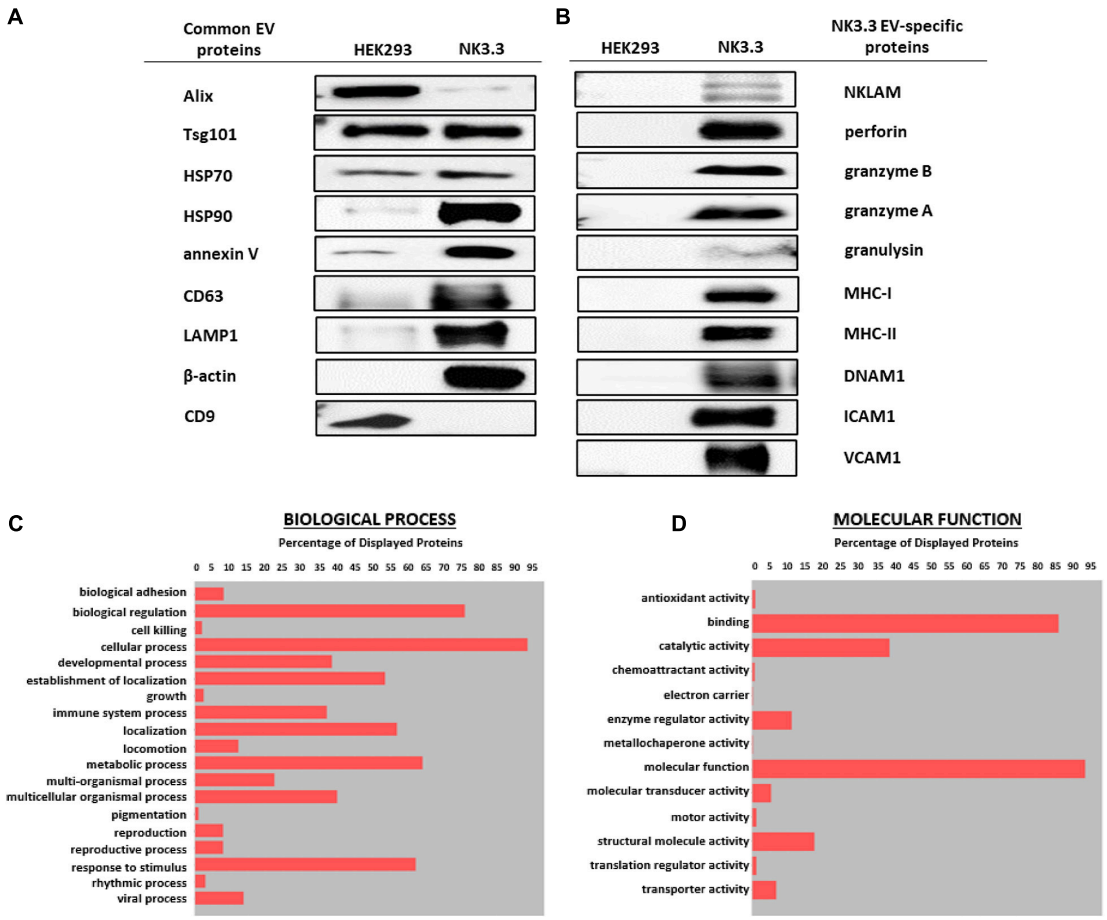

FIGURE 2 | Comparison of EV proteins derived from NK3.3 and HEK293 cells. EV lysates were examined by immunoblot analysis using 30 μg of EV protein for **(A)** common EV proteins and **(B)** proteins specific to NK3.3 EVs. Gene ontology annotation generated from proteomic analysis identified the frequency of **(C)** biological processes and **(D)** molecular function associated with the proteins in NK3.3 EVs.

and receptors that are used by NK cells to recognize, bind and kill tumor cells.

RNA profiling of NK3.3 EVs was also performed. Over 1000 mRNA species were identified in NK3.3 EVs. Among the most highly expressed were transcripts encoding membrane proteins and transport proteins. The next most represented RNA class was micro (mi)RNA; over 450 miRNAs were identified. **Table 2** highlights some of the key miRNAs detected in NK3.3-derived EVs. The most abundant miRNAs were miR-21 and 21-5p, which are also the most abundant miRNAs in NK cells (Fehniger et al., 2010). Other highly expressed miRNAs were miR-155 and 155-5p, the miR-181 cluster, miR-92a-3p, hsa-let-7f, and miR146a-5p (Fehniger et al., 2010; Cichocki et al., 2011; Leong et al., 2012; Trotta et al., 2012; Pesce et al., 2018, 2020). Several of these miRNAs have tumor suppressor function. miR-16 has been identified as a tumor suppression factor in B cell leukemias (Aqeilan et al., 2010). miR-186 has been reported to inhibit neuroblastoma cells (Neviani et al., 2019). miR-146b-5p has been shown to inhibit drug-resistant colorectal cancer (Zhao et al., 2018). miR-34a suppresses acute myeloid leukemia (Wang et al., 2015). Another miRNA found in NK3.3 EVs, miR-3607-3p, inhibits pancreatic cancer cells (Sun et al., 2019). The frequency of the different RNA species is shown in **Supplementary Figure 2D**. The full RNA dataset of NK3.3 EVs is included in the supplementary data (**Supplementary Table 2**).

Orientation of Membrane Proteins in NK3.3-Derived Extracellular Vesicles

We utilized an enzymatic protein digestion assay to determine the orientation of several membrane proteins identified in NK3.3 EVs. NK3.3 EVs were treated with PBS (-), Pronase (+), or Pronase plus saponin (+s), then lysed and analyzed via immunoblotting (**Figure 3A**). Pronase, a combination of non-specific bacterial proteases, was used to digest proteins located on the surface - or cytoplasmic side - of the vesicle membrane. Addition of saponin permeabilized the vesicle membranes to allow Pronase to digest the proteins within the vesicle lumen.

NKLAM has two transmembrane domains. In NK lytic granules, both the N- and C-terminal ends of the protein are cytosolic-facing (Fortier and Kornbluth, 2006; Lawrence et al., 2020). We probed for NKLAM expression in EVs

TABLE 1 | Proteomic analysis of NK3.3-derived EVs listed by abundance.

Protein	Accession ID	Function
Actin	P60709	Cytoskeleton component
CCL5 (RANTES)	P13501	Chemokine
HLA class II DR, DQ	P01903, P01920	Antigen presentation
Granzyme B	P10144	Cytotoxic function
HSP90	P07900	Heat shock protein; chaperone
Granzyme A	P12544	Cytotoxic function
CD70	P32970	Cytokine; TNF family member; immune activation
Ezrin, moesin	P15311, P26038	Actin filament binding proteins
CD45	P08575	Hematopoietic cell membrane phosphatase
HLA class I A, B	P30512, P30495	Antigen presentation
FcRγ	P30273	CD16 signaling adapter protein
CD63	P08962	Tetraspanin EV protein
CD48	P09326	CD2 SLAM subfamily costimulatory molecule
CD59	P13987	Cell surface complement inhibition; CD2 ligand
Perforin	P14222	Cytotoxic function
HSP70	P0DMV8	Heat shock protein; chaperone
CD2	P06729	Costimulatory adhesion molecule
CD47	Q08722	Integrin-associated molecule; "don't eat me"
ICAM1	P05362	Integrin binding transmembrane protein
CD53	P19397	Tetraspanin adhesion molecule
LFA-1 (CD11a/CD18)	P20701, P05107	Integrin; adhesion and costimulatory molecule
TNFSF4	P23510	Cytokine and chemokine regulation
CD82	P27701	Tetraspanin EV protein
CCR7	P32248	Chemokine
TNFRSF18	Q9Y5U5	Cytokine receptor; apoptosis; STAT1 phosphorylation
CD44	P16070	Cell adhesion; immune response
CD86	P42081	CD28 binding costimulatory molecule
CD166	Q13740	Leukocyte adhesion molecule
VCAM1	P19320	Integrin binding adhesion molecule
ICAM3	P32942	Integrin binding adhesion molecule
IL2Rβ	P14784	High affinity IL-2 receptor β chain
PDCD6IP (ALIX)	Q8WUM4	Exosome biogenesis

TABLE 2 | List of abundant miRNAs in NK3.3-derived EVs.

miRNA	Chromosome	Function	References
miR-21-5p	17	Abundant in NK cells; regulates NK cell proliferation	Fehniger et al., 2010
miR-155-5p	21	Regulates IFNγ; tumor suppressor	Trotta et al., 2012
miR-181a-5p	1	NK cell development; promotes IFNγ	Cichocki et al., 2011; Leong et al., 2012
miR-181b-5p	9	NK cell development; promotes IFNγ	Cichocki et al., 2011; Leong et al., 2012
miR-92a-3p	13	NK cell maturation	Pesce et al., 2020
miR-146b-5p	10	Tumor suppressor	Zhao et al., 2018
miR-26b-5p	2	Growth inhibitor	Pesce et al., 2020
let-7f-5p	9	Cell development regulator; activates Toll-like receptors	Pesce et al., 2020
miR-146a-5p	5	Immune activator	Pesce et al., 2018
miR-16-5p	13	Abundant in NK cells; tumor suppressor	Aqeilan et al., 2010
miR-101-3p	1	Growth inhibitor	Pesce et al., 2020
miR-186-5p	1	Inhibits neuroblastoma growth	Neviani et al., 2019
miR-3607-3p	5	Tumor suppressor	Sun et al., 2019
miR-34a-5p	1	Immune activator; tumor suppressor	Wang et al., 2015

using an antibody that binds to an epitope on its C-terminal end (Kozlowski et al., 1999). Pronase treatment significantly diminished the expression of NKLAM; application of Pronase plus saponin completely eliminated antibody binding, indicating that the ubiquitin ligase domain of NKLAM is predominantly cytoplasmic-facing.

Other membrane proteins were evaluated using antibodies known to bind to extracellular or cytoplasmic-facing epitopes. The antibody used to probe for LAMP-1 binds to an epitope on its short C-terminal tail on the cytoplasmic side of membranes. Pronase treatment diminished LAMP1-tail expression. Application of Pronase plus saponin completely eliminated antibody binding, indicating that the majority of LAMP-1 is oriented with the C-terminus on the cytoplasmic face of the EVs. ICAM1 and VCAM1, both single-pass transmembrane adhesion proteins, are oriented on the cytosolic side of cellular membranes (Wai Wong et al., 2012). Both Pronase and Pronase plus saponin resulted in complete digestion of ICAM1 and VCAM1. This indicates that the portion of the proteins to which the antibodies bind is on the cytosolic/surface side of the EV membrane.

The combination of Pronase plus saponin was required for digestion of the luminal proteins examined: granzyme B, β-actin, Alix, and HSP70. Based on these results, we propose a model representing the location and content of components within NK3.3-derived EVs (**Figure 3B**).

NK3.3-Derived Extracellular Vesicles Have a Potent Anti-Tumor Effect

Having established the presence of cytotoxic molecules and tumor suppressor miRNAs via immunoblot, proteomic and RNA analyses, we tested whether EVs derived from NK3.3 cells had an anti-tumor effect. K562 and Jurkat T cell leukemia cells were treated with increasing concentrations of either NK3.3- or HEK293- EVs. Low doses of NK3.3 EVs inhibited growth of K562 and Jurkat cells over 72 h, while high doses of NK3.3 EVs were cytotoxic. Equivalent doses of HEK293 EVs had no effect on K562 or Jurkat cells (**Figures 4A–D**). Therefore, the anti-tumor effect was dose dependent and specific to NK3.3-derived EVs.

We evaluated the effect of NK3.3 EVs on cell survival. The reduction in K562 live cell counts induced by NK3.3 EVs compared to HEK293 EVs was statistically significant beginning at 48 h with concentrations of 10 μg/ml and above. Treatment of Jurkat with concentrations of NK3.3 EVs at 25 μg and 100 μg/ml resulted in a significant reduction in live cells as early as 24 h

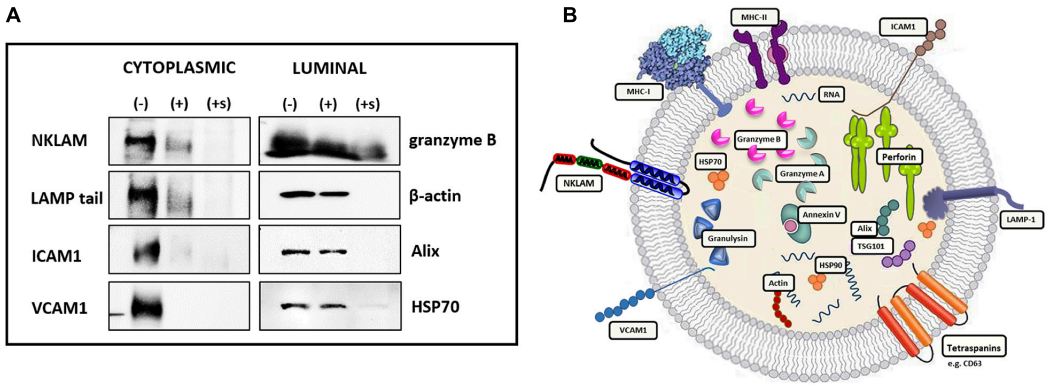

FIGURE 3 | Orientation of transmembrane proteins in NK3.3-derived EVs. **(A)** Twenty micrograms of NK3.3 EVs were treated with either PBS (-), Pronase (+), or Pronase + saponin (+s). Samples were then boiled in Laemmli buffer; immunoblot analysis was performed using antibodies to protein epitopes that have a vesicle membrane, cytoplasmic-facing orientation or contained within the EV lumen. **(B)** Conceptualization of NK3.3 EV composition depicting the localization of proteins in NK3.3-derived EVs. The majority of NKLAM and LAMP-1 proteins are positioned in this orientation.

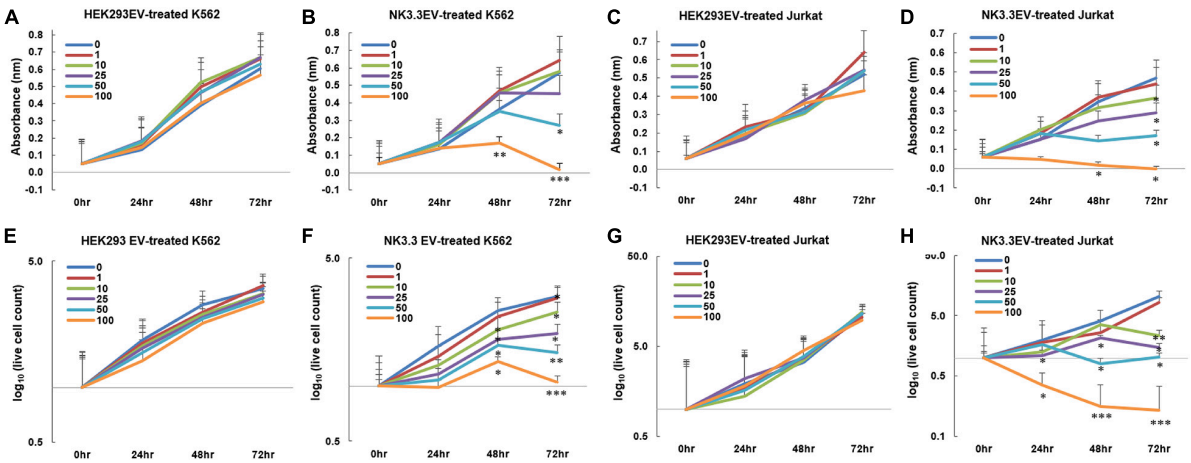

FIGURE 4 | Inhibition of leukemia cell growth and metabolic activity by NK3.3-derived EV treatment *in vitro*. **(A–H)** Dose response analyses were performed using concentrations of EVs ranging from 1 to 100 μg/ml or PBS (0). Absorbance measurements using the WST-1 cell viability assay were assessed in K562 cells treated with either **(A)** HEK293 EVs or **(B)** NK3.3 EVs, and Jurkat cells treated with either **(C)** HEK293 EVs or **(D)** NK3.3 EVs. Mean absorbance ± SE; $n = 3$. Corresponding live cell counts, determined by automated cell counting using trypan blue dye exclusion, were performed at the same time as the WST-1 measurements for K562 cells treated with either **(E)** HEK293 EVs or **(F)** NK3.3 EVs, and Jurkat cells treated with either **(G)** HEK293 EVs or **(H)** NK3.3 EVs. Mean cell numbers ± SE; $n = 3$. *p*-Values determined by comparison of NK3.3 EV-treated cells at each treatment concentration to HEK293 EV-treated cells at the corresponding EV concentration. $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$.

(**Figures 4E–H**). Cells treated with 100 μg/ml NK3.3 EVs had the most rapid and continuous decrease in cell number over time.

NK3.3 EV treatment induced a variety of morphological changes in K562 cells (**Supplementary Figure 3**). Plasma membrane blebbing was observed in the majority of cells treated with NK3.3 EVs. This decreased with time and was replaced by the formation of apoptotic bodies within the cytoplasm, hypotonic swelling, and atypical structural changes and projections. We consistently observed these striking morphological changes that were both dose and time dependent.

We performed differential ultracentrifugation to assess the contribution of NK3.3-derived exosomes and microvesicles (MV) to the inhibition of K562. Using established protocols to separate exosomes from MV, we were not able to isolate the 110–120 nm vesicle population from the 180–220 nm population. However, the MV fraction contained a substantial number of particles in the 250–350 nm range (**Supplementary Figures 1B–D**). K562 cells were treated with each of these preparations; metabolic activity and live cell counts were monitored at 24 and 48 h. The NK3.3 exosome fraction had the strongest anti-tumor activity; the MV fraction was less effective (**Supplementary Figures 4A,B**).

Studies were expanded to evaluate the ability of NK3.3 EVs to inhibit breast cancer cells MDA-MB-231 and MCF7. MDA-MB-231 lack the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), classifying it as a triple-negative breast cancer cell line. MCF7 are ER-positive and PR-positive and are considered to be

less aggressive and invasive than MDA-MB-231 cells (Cailleau et al., 1978). As shown in **Figures 5A–D**, NK3.3-derived EVs reduced the viability of both breast cancer cell lines in a dose and time-dependent manner; comparable amounts of HEK293 EVs had no effect.

NK3.3-Derived Extracellular Vesicles Do Not Affect Survival of Non-Tumor Cells

NK3.3 EVs were examined for their potential toxicity against normal or non-tumorigenic cells. We treated the human embryonic kidney line HEK293 and IL-2 stimulated peripheral blood lymphocytes or cord blood lymphocytes with NK3.3-derived EVs. There was no significant change in metabolic activity or viability with NK EV treatment (**Figures 5E,F** and **Supplementary Figure 4**). This reflects the selective anti-tumor cytotoxic activity of NK3.3 EVs.

NK3.3-Derived Extracellular Vesicles Induce Apoptotic Death of Tumor Cells

To identify the death pathways induced by NK3.3 EVs, we performed an annexin V apoptosis assay. PBS- and NK3.3 EV-treated K562 and Jurkat cells were stained with fluorochrome-conjugated annexin V (PE-Annexin V) and vital dye (7AAD) to identify early apoptosis and late apoptosis/necrosis, respectively. Treated cells were analyzed by flow cytometry over the course of treatment.

Scatter plots from representative experiments show the frequency of live cells in the lower left quadrants, early apoptosis in the lower right quadrants, and late apoptosis/necrosis in the upper quadrants (**Figures 6A,B**). The upper left plot shows PBS-treated cells at 24 h. Greater than 80% of the PBS-treated control cells were alive with minimal, normally occurring death common for these cell lines. Over the time course of NK EV treatment, there was a progressive reduction in live cells, an increase in apoptotic cells, and a shift from apoptosis to cell death/necrosis.

The full data set of the cytotoxic effect of NK3.3 EVs on K562 and Jurkat is presented in **Figures 6C,D**. The most dramatic cell death occurred in cells treated with 100 μg/ml NK3.3 EVs at all time points, with statistical significance beginning as early as 24 h. By 72 h, over 80% of the remaining K562 cells and 40% of remaining Jurkat cells were dead or dying. The results of this analysis indicate that NK3.3-derived EVs induced death of K562 and Jurkat tumor cells in a time and dose dependent manner.

NK3.3-Derived Extracellular Vesicles Affect Expression of Apoptosis-Associated Proteins

Immunoblotting was performed to identify changes in expression of key proteins involved in cell death pathways. K562 lysates were prepared 24 h after treatment with PBS, the apoptosis inducer staurosporine or NK3.3 EVs. Caspases -3, -7, -9, and their cleavage products - essential to intrinsic apoptosis pathway function - were evaluated. Treatment with NK3.3 EVs at 100 μg/ml or staurosporine induced the greatest expression of these three caspase cleavage products (**Figure 6E**). Both treatments increased cleaved caspase-3 by 20-fold, cleaved caspase-7 by 20-fold and 6-fold, respectively, and cleaved caspase-9 by 2.5-fold compared to PBS-treated cells. NK3.3 EV treatment at 25 μg/ml produced the same caspase cleavages, but to a lesser extent. Both NK EV treatments reduced the expression of Bcl-2, an important pro-survival protein associated with mitochondrial involvement in apoptosis (Ben Safta et al., 2015), by 50%.

We also investigated the expression of other proteins associated with apoptosis. Caspase-8 was upregulated in all treated conditions; 100 μg/ml NK3.3 EV treatment induced

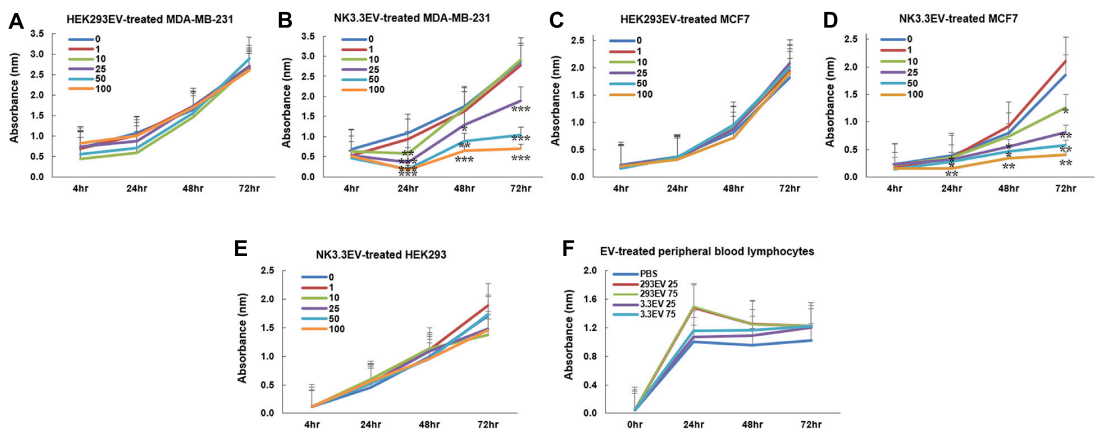

FIGURE 5 | Inhibition of breast cancer cells but not non-tumorigenic cells by NK3.3-derived EV treatment *in vitro*. **(A–D)** Dose response analyses were performed using concentrations of EVs ranging from 1 to 100 μg/ml or PBS (0). Cells were monitored daily using a crystal violet viability assay. MDA-MB-231 cells were treated with either **(A)** HEK293 EVs or **(B)** NK3.3 EVs, and MCF7 cells treated with either **(C)** HEK293 EVs or **(D)** NK3.3 EVs. Mean absorbance ± SE; $n = 3$. **(E)** HEK293 embryonic kidney cells were treated with the same increasing NK3.3 EV concentrations and stained with crystal violet. **(F)** IL-2 stimulated peripheral blood lymphocytes from a healthy donor were treated with 25 or 75 μg/ml of either HEK293 EVs or NK3.3 EVs; metabolic activity was monitored using WST-1. Mean absorbance ± SE; $n = 3$. p-Values determined by comparison of NK3.3 EV-treated cells at each treatment concentration to HEK293 EV-treated cells at the corresponding EV concentration. $^*p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$.

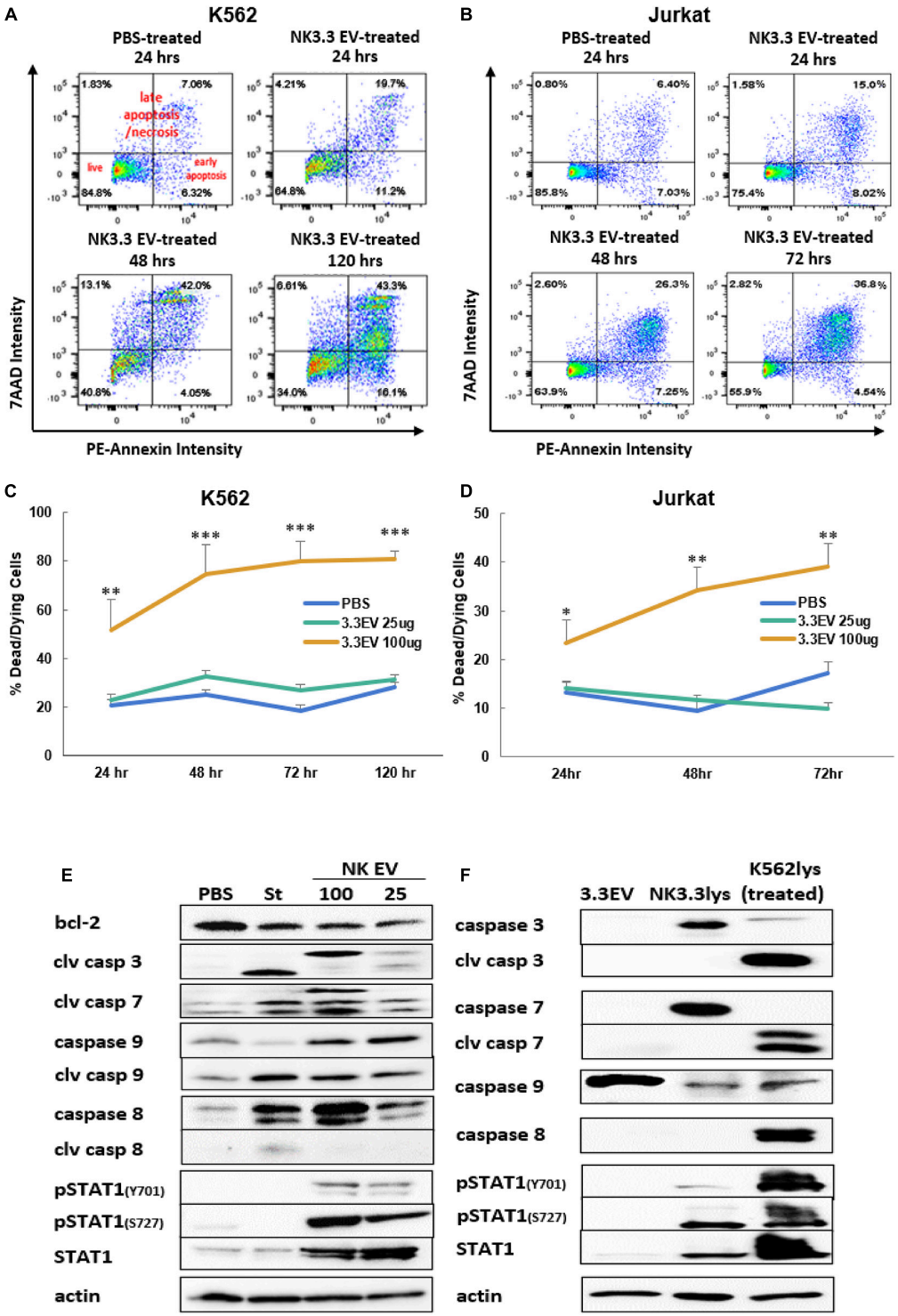

FIGURE 6 | Induction of apoptosis in leukemia cells and alterations in K562 protein expression by treatment with NK3.3-derived EVs. Leukemia cells treated with either PBS, 25 or 100 μg/ml NK3.3 EVs were stained for annexin V and 7-AAD and analyzed by flow cytometry. Representative scatter plots are presented for **(A)** K562 cells and **(B)** Jurkat cells. Graphs of the cumulative apoptosis data collected by flow cytometry are shown for **(C)** K562 cells and **(D)** Jurkat cells. Dying/dead cell frequency is the sum of all annexin V- and 7AAD-positive quadrants. Mean frequency ± SE; $n = 6$. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$. **(E)** Immunoblots of K562 protein lysates from cells treated for 24 h with PBS, 2.5 μM staurosporine (St), 100 μg/ml NK3.3 EVs (100), or 25 μg/ml NK3.3 EVs (25); 25 μg protein per lane was used. **(F)** Comparison of protein expression from NK3.3-derived EVs (3.3EV), NK3.3 cells (NK3.3lys), or K562 cells 24 h post- 100 μg/ml NK3.3 EV treatment (K562lys). Lysates were prepared, and immunoblotting performed to assess the potential contribution of NK EV-associated proteins to NK EV-treated K562 cells (25 μg of protein per lane).

upregulation by more than 40-fold. However, only staurosporine treatment produced an active caspase-8 cleavage product. STAT1, a transcription factor shown to have an association with apoptosis via its interaction with caspases -3 and -7, was phosphorylated at tyrosine701 and serine727 when K562 cells were treated with NK3.3 EVs; no phosphorylation was induced by staurosporine treatment. Total STAT1 was also highly upregulated in cells treated with NK3.3 EVs, while staurosporine-treated cells expressed the same basal level of STAT1 as PBS-treated cells.

To determine the contribution of EV-derived proteins to the results observed, we examined the protein composition of NK3.3 EV lysates, NK3.3 whole cell lysates, and whole cell lysates of K562 cells treated with NK3.3 EVs for 24 h. NK3.3 EVs did not contain caspases -3, or -7, or their cleavage products (**Figure 6F**). NK3.3 EVs also did not contain caspase-8, cleaved caspase-8, or phosphorylated STAT1. There was, however, a small amount of total STAT1 in NK3.3 EVs. NK3.3 EVs did contain a relatively large amount of procaspase-9 compared to that found in NK3.3- and K562- cell lysates. Taken together, these results suggest that NK3.3-derived EVs induced death in K562 cells largely through an intrinsic apoptosis program than by extrinsic apoptosis.

NK3.3-Derived Extracellular Vesicles Induce Significant Caspase 3/7 Activity

We assessed the induction of caspase -3 and -7 activity by NK3.3 EV treatment of K562, Jurkat, MDA-MB-231 and MCF7 cells using the luminescent Caspase-Glo 3/7 assay. NK3.3 EVs induced significant amounts of active caspases 3/7 in all tumor cell lines at all time points (1–24 h) when compared to both PBS- and HEK293 EV-treated cells (**Figure 7**). Caspase 3/7 activity remained high in K562 over 72 h (**Supplementary Figure 4D**).

NK3.3-Derived Extracellular Vesicles Inhibit Proliferation of Tumor Cells

To evaluate the effect of NK3.3 EVs on tumor proliferation, we measured the change in cell surface CD71 expression via flow cytometry. CD71 (transferrin receptor-1) is commonly used as a proliferation marker; its expression decreases when proliferation is inhibited (Caruso et al., 1997). Analysis of mean fluorescence intensity (MFI) indicated that there was a significant reduction in CD71 expression by K562 and Jurkat cells treated with either 25 or 100 μg/ml NK3.3 EVs as early as 24 h after exposure (**Figure 8**). Statistically significant reductions in CD71 expression were seen in both K562 and Jurkat cells treated with either concentration of NK3.3 EVs over 72 h.

Proteomic Analysis of K562 Tumor Cells Treated With NK3.3-Derived EVs

Proteins from K562 cells treated with PBS, 100 μg/ml HEK293- or NK3.3-derived EVs for 24 h were profiled by mass spectrometry. 2769 proteins were identified; 492 proteins changed at least 1.5 fold with NK3.3 EV treatment. **Table 3** is

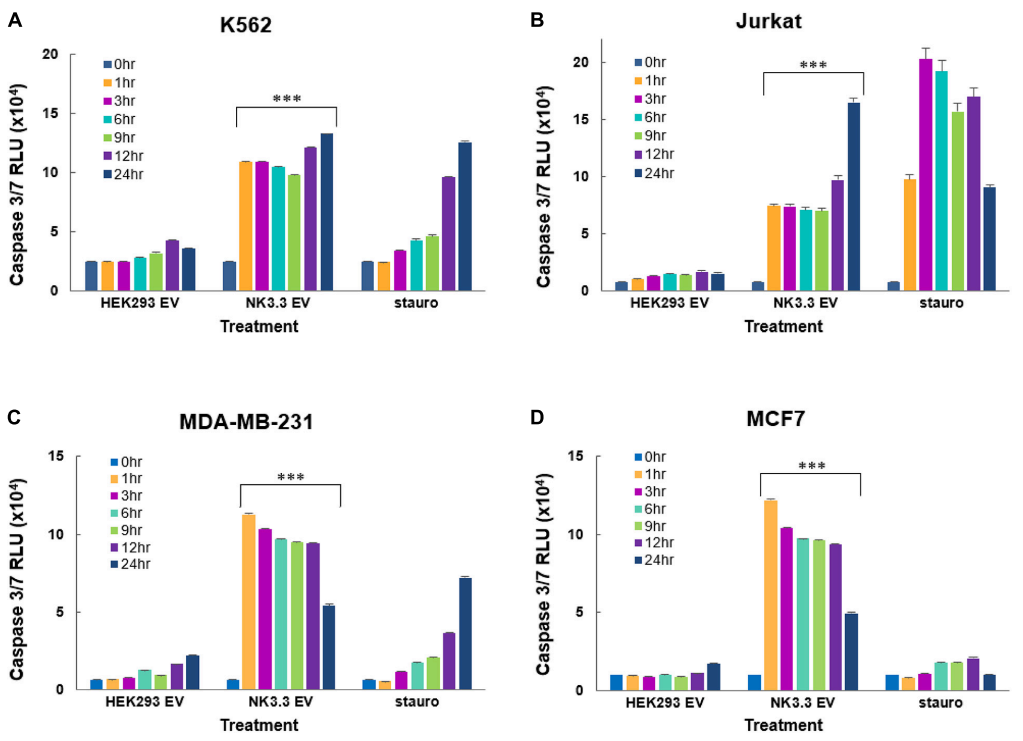

FIGURE 7 | Induction of early caspase-3/7 activity in cancer cells treated with NK3.3-derived EVs. Leukemia cells and breast cancer cells were treated with either 100 μg/ml HEK293 or NK3.3 EVs or 2.5 μM staurosporine. A DEVD pro-luciferin substrate was added to **(A)** K562, **(B)** Jurkat, **(C)** MDA-MB-231, and **(D)** MCF7 cells at the indicated times. A luminescent signal is generated upon cleavage by activated caspases -3 and -7. RLU: relative luminescence unit. Mean luminescence ± SE; $n = 6$. p-Values determined by comparison of NK3.3 EV-treated cells to HEK293 EV-treated cells. ***$p \leq 0.0005$.

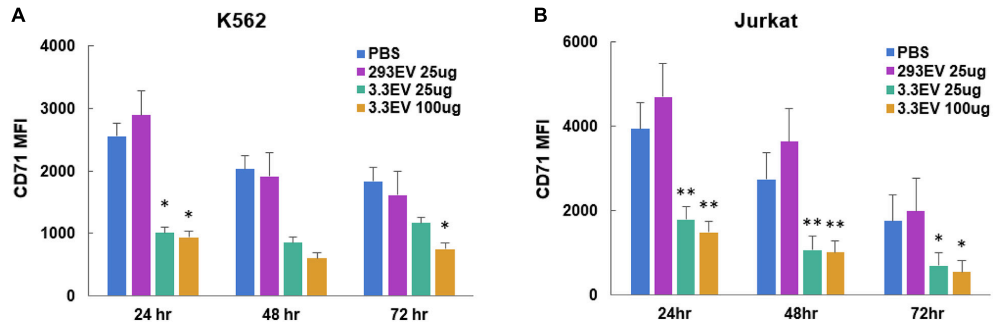

FIGURE 8 | Reduction in expression of leukemia cell proliferation marker CD71 in NK3.3 EV treated cells. **(A)** K562 and **(B)** Jurkat cells were treated with either PBS, 25 μg/ml HEK293 EVs, 25 or 100 μg/ml NK3.3 EVs, stained for proliferation marker CD71, and analyzed by flow cytometry. MFI: mean fluorescence intensity. Mean MFI ± SE; $n = 3$. p-Values determined by comparison of NK3.3 EV-treated cells to HEK293 EV-treated cells. $^{*}p \leq 0.05$, $^{**}p \leq 0.005$.

a list of selected, highly upregulated or downregulated proteins in NK3.3 EV-treated K562 cells when compared to HEK293 EV-treated cells. The full proteomic dataset is included in the supplement (**Supplementary Table 3**).

Granzyme B was one of the most abundantly expressed proteins detected in NK3.3 EV-treated cells. Control-treated cells contained no detectable granzyme B, indicating that granzyme B was most likely introduced by NK EVs. CD47, which functions as a "don't eat me" signal (Takimoto et al., 2019), was highly expressed in NK3.3 EV-treated K562 cells compared to control-treated cells. NK3.3 EVs also contain CD47, which could be, in part, responsible for the increased expression detected in K562 cells. The adhesion molecules CD44, ICAM1, integrin β-3 and integrin α-5, were also highly abundant (Wai Wong et al., 2012; Anderson et al., 2014). Inhibitor of nuclear factor kappa-B kinase-interacting protein, another highly upregulated protein, as well as apoptosis regulator BAX, are associated with promoting apoptosis and cell death (Pawlowski and Kraft, 2000; Hofer-Warbinek et al., 2004). Other upregulated proteins such as STAT1, N-myc-interactor, TGFβ-1, and NF-kappa-B-repressing factor, are associated with inhibition of proliferation and/or transcription (Niedick et al., 2004; Dimco et al., 2010; Kubiczkova et al., 2012; Pruitt et al., 2016).

TABLE 3 | Proteomic analyses of NK3.3 EV-treated K562 cells.

Protein name	Gene name	Change in protein expression
Granzyme B	*GZMB*	UP
Leukocyte surface antigen CD47	*CD47*	UP
Inhibitor of nuclear factor kappa-B kinase-interacting protein	*IKBIP*	UP
Integrin beta-3	*ITGB3*	UP
CD44 antigen	*CD44*	UP
Intercellular adhesion molecule 1	*ICAM1*	UP
Interferon-induced guanylate-binding protein 2	*GBP2*	UP
Signal transducer and activator of transcription 1-alpha/beta	*STAT1*	UP
N-myc-interactor	*NMI*	UP
Integrin alpha-5; heavy chain; light chain	*ITGA5*	UP
Transforming growth factor beta-1	*TGFB1*	UP
NF-kappa-B-repressing factor	*NKRF*	UP
Apoptosis regulator BAX	*BAX*	UP
Mini-chromosome maintenance complex-binding protein	*MCMBP*	DOWN
Sororin	*CDCA5*	DOWN
Geminin	*GMNN*	DOWN
Cyclin-dependent kinase 4	*CDK4*	DOWN
Protein tyrosine phosphatase type IVA 2; type IVA 1	*PTP4A2; PTP4A1*	DOWN
DNA replication complex GINS protein SLD5	*GINS4*	DOWN
Cell division cycle protein 23 homolog	*CDC23*	DOWN
PCNA-associated factor	*KIAA0101*	DOWN
BRCA1-associated ATM activator 1	*BRAT1*	DOWN
DNA polymerase alpha subunit B	*POLA2*	DOWN
Cell division control protein 45 homolog	*CDC45*	DOWN
Cell division cycle protein 123 homolog	*CDC123*	DOWN
Proliferating cell nuclear antigen	*PCNA*	DOWN
Transferrin receptor protein 1	*TFRC*	DOWN

The protein with the most reduced expression was mini-chromosome maintenance complex-binding protein (MCMBP), which is involved in maintaining sister chromatid cohesion and whose increased expression is linked to highly proliferative malignant cells (Quimbaya et al., 2014). A large majority of the other proteins with greatly decreased expression listed in **Table 3**: sororin, geminin, CDK4, PTP4A2/A1, GINS4, CDC23, PCNA-associated factor, BRAT1, POLA2, CDC45, CDC123, PCNA, and CD71, are closely associated with DNA replication mechanisms and proliferation (Caruso et al., 1997; Masai et al., 2010; Quimbaya et al., 2014). These results reinforce the finding that NK3.3-derived EVs induce cell death and inhibit tumor cell proliferation via multiple mechanisms.

NK3.3 EVs Induce Apoptosis of Breast Cancer Cells *in vivo*

To determine whether NK3.3 EVs have cytotoxic activity *in vivo*, GFP-expressing MDA-MB-231 cells were injected into the mammary fat pad of female nude mice. When tumors were palpable, NK3.3 or HEK293-derived EVs were injected intratumorally every 3–4 days. After 7 injections, tumors were excised, formalin fixed and paraffin embedded. Tumor sections were analyzed for apoptotic cells by TUNEL staining, to detect DNA fragmentation, and DAPI, to label nuclei. Representative staining is shown in **Figure 9A**. Fluorescent pixel intensity

was quantitated in tumors from 4 control-treated and 5 NK3.3 EV-treated mice (3 tumor sections per mouse). Results are expressed as the ratio of TUNEL positive cells to total nuclei. As shown in **Figure 9B**, there was significantly more apoptosis in breast tumors treated with NK3.3 EVs than in the control-injected mice.

DISCUSSION

We report here the results of a comprehensive analysis of the characteristics and function of extracellular vesicles derived from NK3.3 cells (Kornbluth et al., 1982). NK3.3 EVs induce robust anti-tumor activity *in vitro*. They activate both anti-proliferative and apoptotic mechanisms in an array of tumor cell lines while having no effect on normal cells. As a first step toward the development of NK3.3 EVs for cancer treatment, we demonstrate that NK3.3 EVs induce apoptosis of triple negative breast cancer cells *in vivo*. We identify key NK3.3 EV proteins by both immunoblotting and proteomic analysis that are cytolytic effector molecules. Next generation RNA sequencing analysis identified miRNA species that have tumor-suppressing activity. We also performed proteomic and immunoblot analysis of tumor cells treated with NK3.3 EVs, which identified multiple pathways associated with proliferation and cell death.

One component with a critical role in NK anti-tumor activity is NKLAM (Kozlowski et al., 1999; Portis et al., 2000; Fortier and Kornbluth, 2006; Hoover et al., 2012; Lawrence et al., 2020). We show for the first time that NKLAM is a component of NK EVs. Pronase digestion experiments indicate that the N- and C-terminal ends of NKLAM in the EV membrane are primarily positioned facing the cytoplasm. Orientation of NKLAM with its ubiquitin ligase domain facing the cytosol indicates that this is where interaction with substrates and ubiquitination occurs. Studies are currently ongoing to identify NKLAM targets of ubiquitination within tumor cells.

In this study, NK-derived EVs were generated from a non-tumorigenic NK cell line, NK3.3, isolated from the peripheral blood of a healthy donor. NK3.3 cells have been thoroughly characterized (Kornbluth et al., 1982, 1985; Kornbluth and Hoover, 1988; Kozlowski et al., 1999). While studies using NK-derived exosomes have emerged in the last few years, none use a cloned, normal human NK cell line. NK-derived EVs have been isolated and evaluated from B6 mice, human NK cells collected from peripheral blood, or tumorigenic non-Hodgkin's lymphoma NK cells (NK-92) (Lugini et al., 2012; Jong et al., 2017; Zhu et al., 2017, 2019; Neviani et al., 2019; Wu et al., 2019; Federici et al., 2020). Although NK-92 -derived EVs have anti-tumor activity, studies have shown that EVs derived from transformed/tumorigenic cells carry cargo specific to those cells that are capable of negatively influencing or altering recipient cells (Hannafon and Ding, 2013; Mashouri et al., 2019). Therefore, NK3.3-derived EVs are likely to be safer and more effective for therapeutic use.

Comparison of NK3.3 EV size to NK-92- and peripheral blood NK cell- derived EVs show strong similarities. By NTA, we identified a major population of NK3.3 EVs in the range of 120–130 nm, with a smaller population in the 170–195 nm range. Our measurements correspond to NK-92 EV measurements taken in our laboratory as well as NK-92- and peripheral blood NK- EV published data (Jong et al., 2017; Zhu et al., 2017). In those reports, NK EVs ranged from 100 to 170 nm, consistent with the size of the vesicles we obtained from NK3.3. It is likely that our EV preparations contain both exosomes and MV. Ultracentrifugation at 10,000 × *g* to isolate MV, with further ultracentrifugation at 100,000 × *g* to obtain exosomes, was performed. MV ranged from 129 to 344 nm. The exosome fraction was identical to that obtained using polyethylene glycol polymer precipitation. Functional assays indicated that the strongest anti-tumor activity was mediated by the exosome fraction. The NK3.3 EV protein profile also closely compares to published NK-92- and peripheral blood NK- EV content. Common EV proteins CD63, tsg101, HSP70, and Alix, and primary effector molecules perforin, granzymes A and B, and granulysin have been identified in all three types of NK EVs (Lugini et al., 2012; Jong et al., 2017; Zhu et al., 2017; Neviani et al., 2019; Wu et al., 2019). We are the first to report full proteomic analysis of NK3.3 EVs, which identified NKLAM

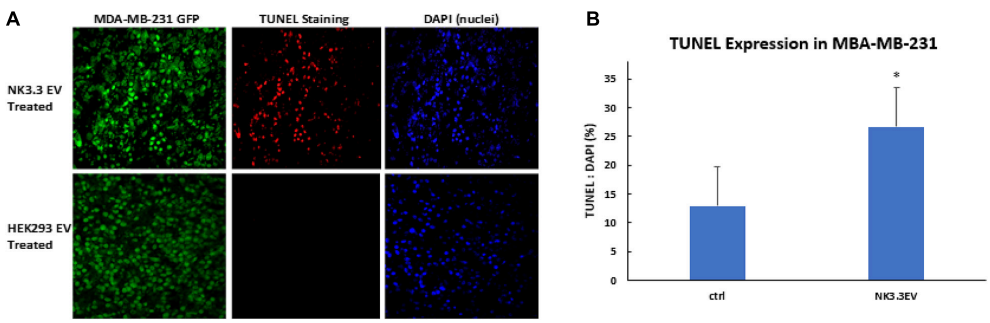

FIGURE 9 | Apoptosis induction in MDA-MB-231 breast cancer cells by intratumoral injection of NK3.3 EVs *in vivo*. **(A)** GFP-expressing MDA-MB-231 tumors in the mammary fat pad of female nude mice were excised, paraffin embedded, sectioned, and subjected to TUNEL staining (red). Tumor cells: GFP+ (green); Nuclei: DAPI+ (blue). EVs from HEK293 human epithelial cells served as a negative control. **(B)** Comparison of TUNEL staining of tumors from control and NK3.3 EV-treated mice. Whole slide scanning was performed, and fluorescence intensity was quantitated using CellSelect software. Results of tumor sections from 4 control and 5 NK3.3 EV-treated mice are presented as % TUNEL/DAPI positive. *$p < 0.05$.

as well as several additional proteins that play a critical role in NK EV function.

NK3.3 EVs are cytotoxic to several different types of tumor cells while having minimal effect on normal cells. Protein profiling of NK3.3 EVs identified high levels of adhesion molecules ICAM1 and VCAM1, which may participate in EV binding and/or uptake. Recent studies have shown that DNAM-1 is expressed by NK exosomes. Blocking of DNAM-1 on the NK exosome delayed tumor apoptosis (Di Pace et al., 2020). Therefore, expression of DNAM-1 on NK3.3 EVs may play a role in their selective anti-tumor activity. Additional candidates within the NK3.3 EV proteome involved in NK cell adhesion and signaling were also identified. CD50 (ICAM3) and ICAM1 are ligands of LFA-1, which contributes to immune cell binding, migration, and signal activation (Montaldo et al., 2013). CD53 amplifies NK cell adhesion by enhancing the activation of LFA-1 (Todros-Dawda et al., 2014). CD48 and CD59 are ligands for CD2 (Deckert et al., 1992). The interaction between CD48 and CD2 has been shown to stimulate signaling for NK cell degranulation. CD2 also promotes the formation of plasma membrane nanotubes which NK cells use to tether to target cells and transmit molecular information (Comerci et al., 2012). Ezrin and CD47 contribute to adhesion and migration (Oldenborg, 2013; Takimoto et al., 2019). CD70 participates in NK cell adhesion through interaction with the CD27 receptor (Yang et al., 1996); both CD47 and CD70 also have a role in apoptosis signaling (De Colvenaer et al., 2010; Oldenborg, 2013). CD47 was one of the most abundantly expressed proteins in NK3.3 EV-treated K562 cells. Determination of the molecules involved in the interaction between EVs and tumor cells is under investigation.

Novel proteins identified in NK3.3 EVs include the chemokine CCL5/RANTES and high affinity immunoglobulin epsilon receptor subunit gamma FCER1G. CCL5 is involved in the recruitment of lymphocytes and monocytes to sites with pathogenic antigens or abnormal cells like tumors (Aldinucci and Colombatti, 2014). In an *in vivo* setting, areas of high concentrations of NK-derived EVs may amplify the immune response within the tumor microenvironment. The same amplification response may hold true for FCER1G in that it can associate with surface receptor complexes to initiate a killing response in recruited immune cells. Further studies of the role of proteins identified in this analysis in the anti-tumor activity of NK EVs are warranted.

NK3.3 EVs upregulate and phosphorylate STAT1 in K562 cells, which may be another mechanism for inhibiting proliferation and inducing killing of K562 cells. NK3.3 EV treatment stimulates phosphorylation of STAT1 at serine-727 and at tyrosine-701. IFN-γ is the primary ligand for STAT1 activation through the induction of tyr701 phosphorylation, which is associated with regulation of pro-apoptotic gene expression (Stephanou and Latchman, 2003). IFN-γ has been shown to be a component of NK EVs (Di Pace et al., 2020). STAT1 has also been shown to inhibit proliferation by reducing cyclin D expression (Dimco et al., 2010). High levels of caspases -3 and -7 activity were found in cells expressing a constitutively active form of STAT1 (Sironi and Ouchi, 2004). Therefore, NK3.3 EV activation of STAT1 in K562 cells might enhance apoptosis in both a caspase -dependent and -independent manner while also upregulating expression of anti-proliferation and/or pro-apoptosis genes. Identification of STAT1 gene targets in NK3.3 EV-treated K562 cells remains to be determined.

RNA sequencing analysis identified a diverse profile of RNA species. Some of the miRNAs identified in NK3.3 EVs are also found in NK cells, where they regulate NK function, granzyme B and perforin expression, and IFN-γ production (Fehniger et al., 2010; Trotta et al., 2012). This profile is markedly different from the miRNA profile reported for HEK293-derived EVs (Li et al., 2016). Other miRNAs identified in NK3.3 EVs are miR-16, miR-3607-3p and miR-186. miRNAs miR-16, miR-3607-3p and miR-186 have been shown to have tumor suppressor activity. One target of miR-16 is Bcl-2 (Yan et al., 2013); miR-186 from peripheral blood NK-derived exosomes has been shown to inhibit proliferation of neuroblastoma cells by targeting TGFβ1 and other proliferation genes (Neviani et al., 2019). miR-3607-3p was found to inhibit proliferation, migration, and invasion of pancreatic cancer cells, potentially by targeting IL-26 (Sun et al., 2019). Of the mRNAs identified, the transcript for HUWE1 was the eleventh most abundant. HUWE1 is a HECT E3 ubiquitin ligase that ubiquitinates and degrades the anti-apoptotic protein Mcl-1, suggesting it might play a role in the anti-tumor activity of NK3.3 EVs (Strappazzon et al., 2019). More extensive mining of the RNA data is underway to identify additional RNA transcripts in NK3.3 EVs that might cause tumor suppression.

NK3.3 EVs induce caspase 3/7 activity in all the tumor lines tested. NK3.3 EVs contain high concentrations of perforin and granzymes B and A. Once taken up by the target cell, granzymes enter the cytoplasm to induce apoptosis via disruption of mitochondrial outer membrane potential and cleavage of caspases -3, -7, and -9 (Metkar et al., 2002; Bots and Medema, 2006). Granzyme B directly targets and cleaves caspases -3 and -7, leading to a rapid initiation of apoptosis. It also induces activation of the intrinsic apoptosis pathway by cleaving BID to tBID, initiating a disruption of the mitochondrial outer membrane potential and release of cytochrome c. This is followed by formation of apoptosome scaffolding that incorporates caspase-9, which is then cleaved into active caspase-9 within the apoptosome. This active structure then moves on to cleave caspases -3 and -7 into active products. Many of these cleavage products translocate to the nucleus where they induce DNA damage (Ben Safta et al., 2015). Our data indicates significant and prolonged involvement of active caspases -3 and -7 in NK3.3 EV treated tumor cells.

Immunoblot analysis demonstrated that while NK3.3 EVs do not carry full-length or cleaved fragments of caspases -3, -7, or -8, they carry full-length caspase-9. By transport of caspase-9 into the target cell, NK3.3 EVs may provide a surplus of enzymatic building material for continuous construction of apoptosome structures to perpetuate caspase-dependent apoptotic activity. Additionally, we show reduced expression of Bcl-2 after NK3.3 EV-treatment of K562 cells. This is consistent with well-documented evidence that granzyme B has a role in initiating and prolonging apoptosis via direct activation of caspases and induction of mitochondrial membrane damage (Ben Safta et al., 2015).

In addition to granzyme B, granzyme A expression is also highly expressed in NK3.3 EVs. Granzyme A primarily induces mitochondrial damage in a caspase-independent manner resulting in production of reactive oxygen species (Lieberman, 2010). Studies have shown granzyme A activity in NK EVs but its role in tumor killing has not been delineated (Wu et al., 2019). Further investigation is ongoing to determine the extent to which granzyme A contributes to killing by NK3.3 EVs.

We also show that granulysin, another cytolytic effector molecule, is packaged in NK3.3 EVs. We identified the 15kDa granulysin precursor, which would need to be modified to its 9kDa active form after target cell entry for function. Granulysin has been shown to damage both the plasma and mitochondrial membranes of several tumor types by causing an influx of Ca^{2+} and an efflux of K^{+} from the cell. ER stress is also induced, resulting in caspase-7 activation followed by apoptosis without mitochondrial involvement (Okada and Morishita, 2012). We are currently investigating the potential role of granulysin in NK EV-mediated tumor death.

NK3.3 EV studies are in progress to determine the best method to obtain EVs with the strongest anti-tumor activity and the least non-specific activity. Treatments with IL-12, IL-15, or IL-21 alone or in combinations with IL-2 have been used to enhance NK cell cytotoxicity and expansion. Most recently, cytolytically enhanced exosomes, isolated from IL-15-treated NK-92MI cells, have been used to treat glioblastoma, breast cancer, and thyroid cancer in mice (Zhu et al., 2019). We are currently investigating the most effective stimulation of NK3.3 cells to produce EVs with maximum cytolytic activity.

NK3.3 EVs have a strong anti-tumor effect on every tumor cell line we tested to date, including K562, Jurkat, MDA-MB-231, MCF7 and several multiple myeloma cell lines. Many tumor cells develop mechanisms to avoid killing by evading recognition by immune cells or by becoming resistant to first-line chemotherapy drugs. Patients with triple negative breast cancer have limited treatment options as there is no molecular therapeutic target. This disease disproportionately affects young women of color (Gupta et al., 2020). Additional *in vivo* studies are ongoing to further analyze the ability of NK3.3- derived EVs to inhibit MDA-MB-231 breast tumor growth, using larger cohorts of mice, different routes of EV administration, and monitoring both primary tumor growth and metastasis. Identification of additional tumors susceptible to NK EV-mediated killing is also in progress. We propose that NK3.3 EVs may be a safe and effective new form of immunotherapy due to their ability to efficiently reduce tumor burden by selectively targeting and killing tumor cells while sparing normal cells.

DATA AVAILABILITY STATEMENT

The data from RNA sequencing of NK3.3 EVs have been deposited in the NCBI Sequence Read Archive (SRA) repository with the accession number PRJNA742047 and run number SRR14934743. The mass spectrometry proteomics data of NK3.3-derived EVs have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD027051 and DOI: 10.6019/PXD027051. The proteomics data for control and EV-treated K562 cells can be accessed by identifier PXD027221 and DOI: 10.6019/PXD027221.

ETHICS STATEMENT

The animal study was reviewed and approved by Saint Louis University and St. Louis VA Animal Care and Use Committees.

AUTHOR CONTRIBUTIONS

AC performed the experiments and data analysis and wrote the manuscript. JK designed the project, reviewed the data and manuscript and supervised the study. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.698639/full#supplementary-material

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miR-4732-3p in Extracellular Vesicles From Mesenchymal Stromal Cells Is Cardioprotective During Myocardial Ischemia

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Extracellular vesicles (EVs) derived from mesenchymal stromal cells (MSCs) are an emerging alternative to cell-based therapies to treat many diseases. However, the complexity of producing homogeneous populations of EVs in sufficient amount hampers their clinical use. To address these limitations, we immortalized dental pulp-derived MSC using a human telomerase lentiviral vector and investigated the cardioprotective potential of a hypoxia-regulated EV-derived cargo microRNA, miR-4732-3p. We tested the compared the capacity of a synthetic miR-4732-3p mimic with EVs to confer protection to cardiomyocytes, fibroblasts and endothelial cells against oxygen-glucose deprivation (OGD). Results showed that OGD-induced cardiomyocytes treated with either EVs or miR-4732-3p showed prolonged spontaneous beating, lowered ROS levels, and less apoptosis. Transfection of the miR-4732-3p mimic was more effective than EVs in stimulating angiogenesis *in vitro* and *in vivo* and in reducing fibroblast differentiation upon transforming growth factor beta treatment. Finally, the miR-4732-3p mimic reduced scar tissue and preserved cardiac function when transplanted intramyocardially in infarcted nude rats. Overall, these results indicate that miR-4732-3p is regulated by hypoxia and exerts cardioprotective actions against ischemic insult, with potential application in cell-free-based therapeutic strategies.

Keywords: extracellular vesicles, mesenchymal stromal (stem) cell (MSC), miR-4732-3p, angiogenesis, myocardial infarction, fibrosis, cardiac function analysis

INTRODUCTION

Coronary artery disease remains the primary cause of death in emerging and developed countries. Its most severe manifestation, acute myocardial infarction (AMI), is characterized by the occlusion of a coronary artery, which interrupts blood supply and oxygen and can lead to sudden death (Shao et al., 2020). Cell therapy is gaining traction as a promising alternative to conventional therapies for many diseases, including cardiac tissue repair. In this setting, mesenchymal stromal cells (MSCs)

have been investigated in-depth, and are believed to act largely in a paracrine manner (Gnecchi et al., 2005; Armiñán et al., 2010). More recently, there has been a shift from cell (MSC)-based therapies to cell-free therapies, based on the use of adult stem cell-derived small extracellular vesicles (EVs), which can recapitulate the therapeutic benefits of parental cells (Rani et al., 2015). A particular strength of this approach is that EV-based therapies have fewer safety issues than cell-based therapies, and handling of these biological products is less complex (Zhang et al., 2021).

Extracellular vesicles are small particles 30–200 nm in diameter delimited by a lipid bilayer that are released from almost every type of cell. EVs are involved in intracellular communication, and transfer information via a diverse cargo of cytokines, membrane-trafficking molecules, chemokines, heat shock proteins and even mRNAs and microRNAs, which trigger myriad responses in target (recipient) cells including cardiac cells (Wu et al., 2020). In the setting of cardiac disease, EVs secreted by MSCs from different tissue origins have been demonstrated to mitigate ischemia/reperfusion (I/R) injury in different experimental models of AMI and I/R (Lai et al., 2010; Bian et al., 2014; Takov et al., 2020; Wu et al., 2020; Zhang et al., 2020).

The cardioprotective effects of MSC-derived EVs are due, at least in part, to different microRNA (miRNA) cargo molecules, which can modulate gene expression in recipient cardiac cells through post-transcriptional mRNA repression. The role of some of these miRNAs have been investigated in pre-clinical models. For instance, intramyocardial infusion of bone marrow-derived EVs overexpressing miR-486-5p from non-human primates was demonstrated to recover cardiac function after myocardial infarction in murine and non-human primates (Li et al., 2021). Similarly, delivery of MSC-derived EVs containing miR-150-5p attenuated adverse myocardial remodeling in a rat model of I/R (Ou et al., 2020). Likewise, MSC-EV-derived miR-21a-5p induced cardioprotection in mice after I/R injury (Luther et al., 2018), and adenovirus delivery of miR-148a prevented ventricular remodeling in mice subjected to pressure-overload (Raso et al., 2019). Mechanistically, miR-210 was found to enhance myocardial vascularization in a rat model of AMI after myocardial transduction by upregulating the expression of hepatocyte growth factor (Fan et al., 2018). Nonetheless, some cargo miRNAs from MSC-EVs can be deleterious to cardiac function, and strategies to reduce or eliminate specific packaged miRNA molecules have been shown to improve the anti-apoptotic and pro-angiogenic functionality of EVs (Zhang et al., 2017; Ning et al., 2021). In this scenario, it would be highly desirable to identify the optimal EV cargo and to design novel "miRNA cocktails" with selected miRNAs for specific applications such as cardioprotection (Jayawardena et al., 2014). With this aim, we sought to identify some key components that contribute to the therapeutic activity of MSC-derived EVs by evaluating several miRNAs contained in EV cargo. Here, we demonstrate that miR-4732-3p contributes to the therapeutic potential of EVs and induces protective mechanisms both in cardiomyocytes and endothelial cells. Furthermore, a miR-4732-3p mimic induced angiogenesis subcutaneously when implanted in a basement membrane plug and exerted cardioprotection when transplanted intramyocardially in a rat model of permanent coronary occlusion. These findings underscore miR-4732-3p as a potentially important player in ischemia-related processes with utility in cardioprotective strategies.

MATERIALS AND METHODS

Ethics Statement

Animal procedures were approved by the institutional ethical and animal care committees according to guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, enforced in Spanish law under Real Decreto 1201/2005 (GVA authorized procedures 2020/VSC/PEA/0123 for the model of angiogenesis in NOD/SCID mice and 2020/VSC/PEA/0122 for the experimental model of myocardial infarction in nude rats).

Primary Cultures

Neonatal rat cardiomyocytes (NRCM) were isolated as described (Armiñán et al., 2009) with some modifications. Briefly, rats aged 1 to 2 days were euthanized by decapitation, and the hearts were extracted, minced and incubated overnight in HBSS (without Ca and Mg) with 0.0125% trypsin (Sigma Aldrich) with gentle agitation at 4°C. Subsequently, the fragments were digested for 20 min at 37°C in an enzyme mixture (0.2% collagenase, 1 mg/mL DNAse) in Leibovitz L15 medium (all from Gibco-Invitrogen, Grand Island, NY, United States). Cell suspensions were collected and centrifuged at 90 × *g* for 5 min at room temperature and the cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% horse serum, 20% M-199 Medium, 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Gibco). The cells were then incubated for 1.5 h under standard culture conditions (37°C in 5% CO_2) to allow fibroblasts to attach to the culture plates. Then, the medium containing unattached cells was centrifugated and cardiomyocytes were pelleted and seeded in 0.1% gelatin-coated plates with DMEM, 4% horse serum, 17% M-199 Medium, 5% FBS, and 1% penicillin/streptomycin. Primary cultures of human umbilical vein endothelial cells (HUVEC; ATCC, Manassas, VA, United States) were grown in Vascular Cell Basal Medium supplemented with Endothelial Cell Growth Kit-VEGF (ATCC).

EV Isolation

The immortal MSC-TERT line was used as previously described (Gómez-Ferrer et al., 2021). Donor cells were cultured in DMEM-low glucose supplemented with 1% penicillin/streptomycin and 10% FBS (Lonza, Basel, Switzerland). FBS was first ultracentrifuged at 100,000 *g* for 16 h to remove contaminant bovine EVs. Cells were incubated in a humidified atmosphere (37°C in 5% CO_2) with EVs-free serum. EVs were collected after 48 h using a serial ultracentrifugation protocol (Gonzalez-King et al., 2017). Briefly, supernatants were first centrifuged at 2,000*g* for 10 min, then at 10,000*g* for 30 min and filtered through a 0.22-μmm filter. EVs were pelleted by ultracentrifugation at 100,000*g* for 70 min at 4°C (Optima L-100 XP, Beckman Coulter, Pasadena, CA, United States), washed with PBS, and pelleted

again as before. Pellets were resuspended in 100 μl of PBS. Isolated EVs (10 mμL) were used for protein quantification with the BCA Protein Assay Kit (Pierce, Thermo Scientific Inc., Rockford, IL, United States). Where indicated, cells were treated with EVs by direct addition to the culture medium to a final concentration of 30 μg/mL shortly before the initiation of the different experimental conditions.

EV Characterization

Western Blotting

Extracellular vesicles were suspended in RIPA buffer (1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate in Tris-buffered saline [TBS]) (Sigma-Aldrich, Madrid, Spain) supplemented with the protease inhibitors PMSF and leupeptin (Roche, Basel, Switzerland). Lysis was completed by five freeze-thaw cycles, followed by centrifugation at 12,000 *g* for 10 min at 4°C. EV protein concentration was determined as above. Equal amounts of samples were mixed with non-reducing Laemmli sample buffer (Bio-Rad, Hercules, CA, United States) and denatured at 96°C for 5 min. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, United States). Membranes were blocked with TBS containing 5% (w/v) non-fat dry milk powder and 0.1% Tween-20. Human primary antibodies used for western blotting were as follows: anti-tubulin (dilution 1/4000; Sigma-Aldrich; T5168), anti-CD9 (Santa Cruz Biotechnology, Santa Cruz, CA, United States; C-4), anti-Tsg101 (dilution 1/200; Santa Cruz Biotechnology; C-2), anti-conexin43 (Cell Signaling Technology, Danvers, MA, United States) anti-ALIX (Santa Cruz Biotechnology), anti-HSP70 (Cell Signaling Technology), anti-cleavage-caspase 3 (Cell Signaling Technology), anti-SMA (Sigma-Aldrich) and anti-col I (Cell Signaling Technology). Detection was carried out using peroxidase-conjugated secondary antibodies and the ECL Plus Reagent (GE Healthcare, Little Chalfont, United Kingdom). Reactions were visualized using an Amersham Imager 600 (GE Healthcare) and quantified with ImageJ software (NIH).

Nanoparticle Tracking Analysis

Extracellular vesicle size distribution and quantification was performed using nanoparticle tracking analysis (NTA) on a NanoSight NS3000 System (Malvern Instruments, Malvern, United Kingdom). EV pellets were suspended in PBS in 200 μl of 0.22-μm-filtered PBS.

Electron Microscopy

Electron microscopy was performed as described (Garcia et al., 2016). Briefly, EVs were diluted in PBS, loaded onto Formwar carbon-coated grids, contrasted with 2% uranyl acetate and finally examined on a FEI Tecnai G2 Spirit transmission electron microscope. Images were acquired using a Morada CCD Camera (Olympus Soft Image Solutions GmbH, Münster, Germany).

EVs Small RNA Sequencing

Libraries were prepared using the SeqMatic TailorMix miRNA Sample Preparation Kit according to the manufacturer's protocol. Briefly, 1 to 5 ng of RNA from EVs were subjected to adaptor 3'and 5' ligation and first strand cDNA synthesis. Library amplification was performed by PCR using Indexed primers supplied in the kit. Final libraries were analyzed using Agilent Bioanalyzer to estimate the quantity and check size distribution and were sequenced on Illumina's HiSeq2500.

miRNA Electroporation of EVs

To incorporate miR-4732-3p into EVs, the later were resuspended in an electroporation buffer after the last ultracentrifugation step of isolation and were incubated with an artificial mimic of the chosen miRNA (MISSION miRNA mimic, Merck) at a concentration of 40 nM. The suspension was loaded into a Gene Pulser/MicroPulser Electroporation Cuvette and treated with 3 pulses of 300 V with 5 s between each pulse. Subsequently, the transduced EVs were incubated at 4°C for 30 min. To eliminate the miRNA not loaded in the EVs, the volume was increased with PBS to 25 mL and the solution was ultracentrifuged as described. The final pellet was resuspended in 100 μL of PBS.

Generation of Lipofectamine miR-4732-3p Complexes

Transfection of miR-4732-3p mimic *in vitro* was assessed using Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, 100 μL of miR-4732-3p mimic 1 μg/μL was mixed with vigorous shaking for 30 s with 200 μL of Lipofectamine. The mixture was incubated for 30 min at room temperature.

Encapsulation of miRNA-4732-3p Mimic

Transfection of miR-4732-3p mimic *in vivo* was assessed using Maxsuppressor *in vivo* RNA-LANCEr-II lipidic reagent (Cosmo Bio Co., Ltd., Japan) following manufacturer's instructions. Briefly, 8.5 μl of semi-dry Maxsuppressor *in vivo* RNA-LANCEr-II was mixed with 16 μl of miR-4732-3p mimic in a total volume of 25 μl of PBS to a final dose of 25 μg/heart in each rat. The mixture was sonicated and used fresh for *in vivo* surgical procedures.

qPCR Detection of miRNA Expression in Cell Cultures

Reverse transcription was performed with the miRCURY LNA Universal RT miRNA PCR Kit (Qiagen, Madrid, Spain). The miRNA was quantified using a Viia TM 7 Real System and the results were analyzed with QuantStudio Real-Time PCR Software (Applied Biosystems, Foster City, CA, United States).

Oxygen/Glucose Deprivation (OGD) Procedure

Oxygen-glucose deprivation was induced on NRCM by culture with DMEM without glucose, glutamine, and phenol red (Thermo Fisher Scientific) in a chamber at 1.5% O_2.

Annexin V Staining

Neonatal rat cardiomyocytes were seeded at 2×10^5 cells/cm^2 in 1% gelatin-coated plates. After OGD, the cells were detached

from the plastic with trypsin/EDTA, and were washed and resuspended in binding buffer with annexin-V-FITC and propidium iodide for 15 min. The percentage of annexin-positive cells was measured by flow cytometry in a FACS Canto II cytometer (BD Bioscience, San Jose, CA, United States).

Lactate Dehydrogenase Assay

Neonatal rat cardiomyocytes were seeded at 2×10^5 cells/cm^2 in 1% gelatin-coated plates. After OGD, the supernatant was tested for lactate dehydrogenase using the Cytotoxicity Detection KitPLUS (LDH) (Roche, Indianapolis, IN, United States).

Cell Rox Staining

Hypoxia-treated cells were washed three times with PBS and incubated with CellRox-FITC® (Thermo Fisher) at a dilution of 1/200 in DMEM with no FBS. After 20 min incubation at 37°C the cells were washed three times in PBS to eliminate the unconjugated stain and were detached with trypsin/EDTA. The fluorescence intensity of the staining was measured by flow cytometry.

NRCM Beating Measurement

To test the effect of OGD and EV treatment on cardiac contraction, we seeded cardiomyocytes at high confluence in gelatin-coated 6-well plates (5×10^5 cells/well). After several days cardiomyocytes start to beat spontaneously. The number of beats per minute was measured using a Leica DM6000 inverted optical microscopy at 10 × magnification. Cells were treated with 30 μg/mL of EVs or transfected with 20 nM encapsulated miR-4732-3p using Lipofectamine as indicated above and were then subjected to OGD for 6 h, and each hour the beats were counted and normalized to non-treated cells.

Cardiac Fibroblast Scratch Assay

Fibroblasts from neonatal rats were seeded in a 48-well plate at 2×10^5 cells/well. Fibroblasts were treated the day before the analysis with 10 ng/mL of TGF-β to stimulate differentiation into myofibroblasts. Cells were then treated with 30 μg/mL of EVs or transfected with 20 nM miR-4732-3p using lipofectamine as indicated above. A straight line in the monolayer of fibroblast was created using a 20-μL pipette tip. Images were taken 24 h after the addition of treatments using a Leica DM600 inverted microscope at 4 × magnification. The area of the scratch wound was then measured using ImageJ.

Immunofluorescence

Cardiac fibroblasts were cultured on glass coverslips at 2×10^4 cell/glass in a 24-well plate. After treatment with TGFβ and EVs, cells were fixed in 4% paraformaldehyde for 10 min, washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 25 min. Cells were then washed with PBS and blocked with 10% FBS at room temperature for 30 min. Next, the cells were incubated with anti-actin, α-smooth muscle-Cy3 (Sigma-Aldrich) or anti-col1A (Cell Signaling Technology) at a concentration of 1/200 in a humidified incubator overnight. After washing in PBS with PBS, and in the case of col1A treated with the Alexa488 Donkey anti-rabbit (BD Bioscience) for 1 h, the cells were washed with PBS and counterstained with DAPI for 15 min and mounted using a drop of FluorSave (CalbioChem, San Diego, CA, United States). Images were captured with a Leica DM2500 fluorescence microscope.

Tube Formation Assay

Tubular-like formations were measured using a described assay (Gonzalez-King et al., 2017). HUVEC cells were counted and seeded at 1.5×10^4 cells/well in a 96-well plate pre-coated with 50 μL of growth factor-reduced Matrigel® (BD Bioscience). Cells were incubated in a starvation medium to simulate the conditions of the infarcted heart (DMEM no glucose, 1% FBS) for 6 h with 30 μg/mL of EVs from MSC-TERT cells or transfected with 20 nM miR4732-3p. Images were taken using a Leica DM600 microscope at 10 × magnification and were analyzed using the online software Wimasis WimTube (WimTube: Tube Formation Assay Image Analysis Solution. Release 4.0[1]).

Matrigel Plug Angiogenesis Assay

Angiogenesis was assayed *in vivo* by measuring blood vessel formation from subcutaneous tissue sprouted into a semi-solid gel of basement membrane containing the test sample, as described (Malinda, 2009). Growth factor-reduced Matrigel (350 μL) was mixed in liquid form at 4°C with 50 μL of EVs or 20 μg of encapsulated miRNA mimic (57 ng/μL). The Matrigel mix was injected subcutaneously into the flanks of 6–8-week-old athymic nude mice. After 14 days, mice were sacrificed and the Matrigel plugs were excised and fixed in 4% formaldehyde in PBS. Plugs were embedded in paraffin, sectioned, and immunostained with anti-CD31 antibodies and examined for growth of blood vessels. As a positive control, 400 μL of Matrigel containing 100 ng/mL fibroblast growth factor (FGF) and 20 U of heparin were injected, and as a negative control 400 μL of Matrigel containing saline vehicle were injected.

Experimental Model of Myocardial Infarction

Animals

Nude rats weighing 200–250 g (HIH-Foxn1 rnu; Charles River Laboratories Inc., Wilmington, MA, United States) were used for infarction studies. The initial number of animals included in the study was 70. Mortality in all groups due to surgical procedures was ∼30%.

Myocardial Infarction and Cell Transplantation

Permanent ligation of the left coronary artery and intramyocardial transplantation was performed as described (Cerrada et al., 2013). Briefly, immediately after permanent left anterior descendent artery (LAD) ligation, rats were transplanted intramyocardially (saline, 3.5×10^{10} EVs or 25 μg miR-4732-3p mimic per animal) in two injections of 10 μL, at two discrete locations of the infarct border zone with a Hamilton syringe).

[1]https://www.wimasis.com/en/WimTube

Functional Assessment by Echocardiography

Transthoracic echocardiography was performed in rats under inhalatory anesthesia (Sevorane) using an echocardiographic system (General Electrics, Milwaukee, WI, United States) equipped with a 10-MHz linear-array transducer, as previously reported (Gandia et al., 2008). Measurements were taken at baseline and post-transplantation (4 weeks). M-Mode and two-dimensional (2D) echocardiography was performed at the level of the papillary muscles in the parasternal short axis view. Functional parameters over five consecutive cardiac cycles were calculated using standard methods (Litwin et al., 1994). Left ventricular (LV) dimensions in end diastole (LVDd) and end systole (LVDs), anterior and posterior wall (AW and PW) thickness in diastole and systole, end-diastolic area (EDA) and end-systolic area (ESA) were measured. Fractional area change (FAC) was calculated as FAC = [(EDA–ESA)/EDA] × 100. Fractional Shortening (FS) was calculated as FS = [(LVDd-LVDs)/LVDd] × 100.

Measurements of Infarct Size

Left ventricle infarct size was measured in 8–12 transverse sections of 7 μm (1 slice each 200 μm of tissue) from apex to base in hearts fixed with 2% paraformaldehyde and stained with Masson's trichrome. The fibrotic zone was determined by computer planimetry (Image-Pro Plus 7.1 software). Infarct size was expressed as percentage of total left ventricular area and as a mean of all slices from each heart.

Statistical Analysis

Data are represented as mean ± SD. Student's *t*-test was used for unpaired samples in the comparison between groups and the Chi-Square test was used when contingency tables were analyzed. When the distribution was not normal the Mann-Whitney *U* test was used. Analyses were conducted with GraphPad Prism 8 software. Differences were considered statistically significant at $p < 0.05$ with a 95% confidence interval.

RESULTS

Identification of miR-4732-3p in Extracellular Vesicles

Extracellular vesicles are postulated to be effective for cardiac repair (Zhao et al., 2015; Forsberg et al., 2020; Nazari-Shafti et al., 2020). However, the complexity involved in producing homogeneous populations in sufficient quantity hampers their clinical use. To overcome this problem, we previously immortalized dental pulp-derived primary MSC cultures by overexpressing human telomerase enzyme (termed MSC-T) using a lentiviral vector that provided resistance to hygromycin and increased telomerase activity. MSC-T exhibit prolonged lifespan and maintain cell division long after equivalent unmodified MSCs reach replicative senescence (Gómez-Ferrer et al., 2021). We harvested EVs from conditioned media of MSC-T cells using ultracentrifugation as described (Gonzalez-King et al., 2017). EVs from MSC-T were characterized by western blotting, nanotracking analysis and electron microscopy. The presence of the tetraspanin markers ALIX, HSP70, TSG101, and CD9 confirmed the presence of EVs (**Figure 1A**). The concentration and size of these vesicles was determined by nanotracking analysis and revealed structures of 100–130 nm (**Figure 1B**). This was confirmed by electron microscopy, which revealed circular membranous structures of 100 nm (**Figure 1C**), and further analysis of tetraspanins by immunogold staining with anti-CD63 antibodies revealed the accumulation of gold nanoparticles in vesicular structures (**Figure 1D**). We next analyzed the EV cargo by RNA-seq analysis and we examined for miRNAs that were regulated by hypoxia in parental MSC-T. Among them, we identified miR-4732-3p (**Figure 1E**), which was also found in cardiac cells at different intracellular abundance. Analysis of absolute copy number of miR-4732-3p per 0.5×10^6 seeded cells was 73.46 ± 119.49 copies in NRCM 434.45 ± 134.51 in human umbilical vein endothelial cells (EC) and 595.02 ± 159.38 in rat cardiac fibroblasts (cFib). When these cultures were independently subjected to oxygen/glucose deprivation (OGD), the levels of intracellular miR-4732-3p were measured by qPCR and normalized to miRNA levels in cells cultured in standard conditions. These levels were significantly upregulated in NRCM and EC but downregulated in cFib (**Figure 1F**), indicating that this miRNA might play a specific role in different cardiac populations.

miR-4732-3p Exerts Cardioprotective Effects on Cardiomyocytes Subjected to Oxygen and Glucose Deprivation

Extracellular vesicles are known to mediate cardioprotection in therapeutic interventions designed to mitigate I/R cardiac injury, and it is believed that EV miRNAs play a pivot role in this process (Barile et al., 2017; Zhang et al., 2017; Wu et al., 2020). To analyze the potential therapeutic effect of miR-4732-3p, we first electroporated EVs with a miR-4732-3p mimic to increase its concentration in EV particles (**Figure 2A**). Of note, electroporation did not affect the concentration or size of the EVs (**Supplementary Figure 1**) and they remained suitable for functional studies although the loss of miRNA cargo due to electroporation process is unknown. As an alternative to electroporation, synthetic miR-4732-3p was formulated into liposomes as a delivery system before transfection. The anti-apoptotic effect of EVs, with or without electroporated miRNA-4732-3p, and also miR-4732-3p liposome complexes, was assessed in NRCMs cultured under OGD conditions. We found that the number of apoptotic NRCMs was significantly reduced to a similar degree by the addition of native EVs or EVs previously electroporated with the miR-4732-3p mimic. Interestingly, a similar level of cardioprotection was achieved with liposomal miR-4732-3p (**Figure 2B**). Of note the control electroporation of EVs with saline reduced their anti-apoptosis action, likely due to the loss of vesicle integrity. In the context of electroporation, EVs that incorporated miRNA-4732-3p showed improved anti-apoptotic effects over electroporated vesicles with no miRNA (EVs_{saline}) or scramble miRNA (not shown). The finding that transfected miR-4732-3p could induce a similar reduction in apoptosis to

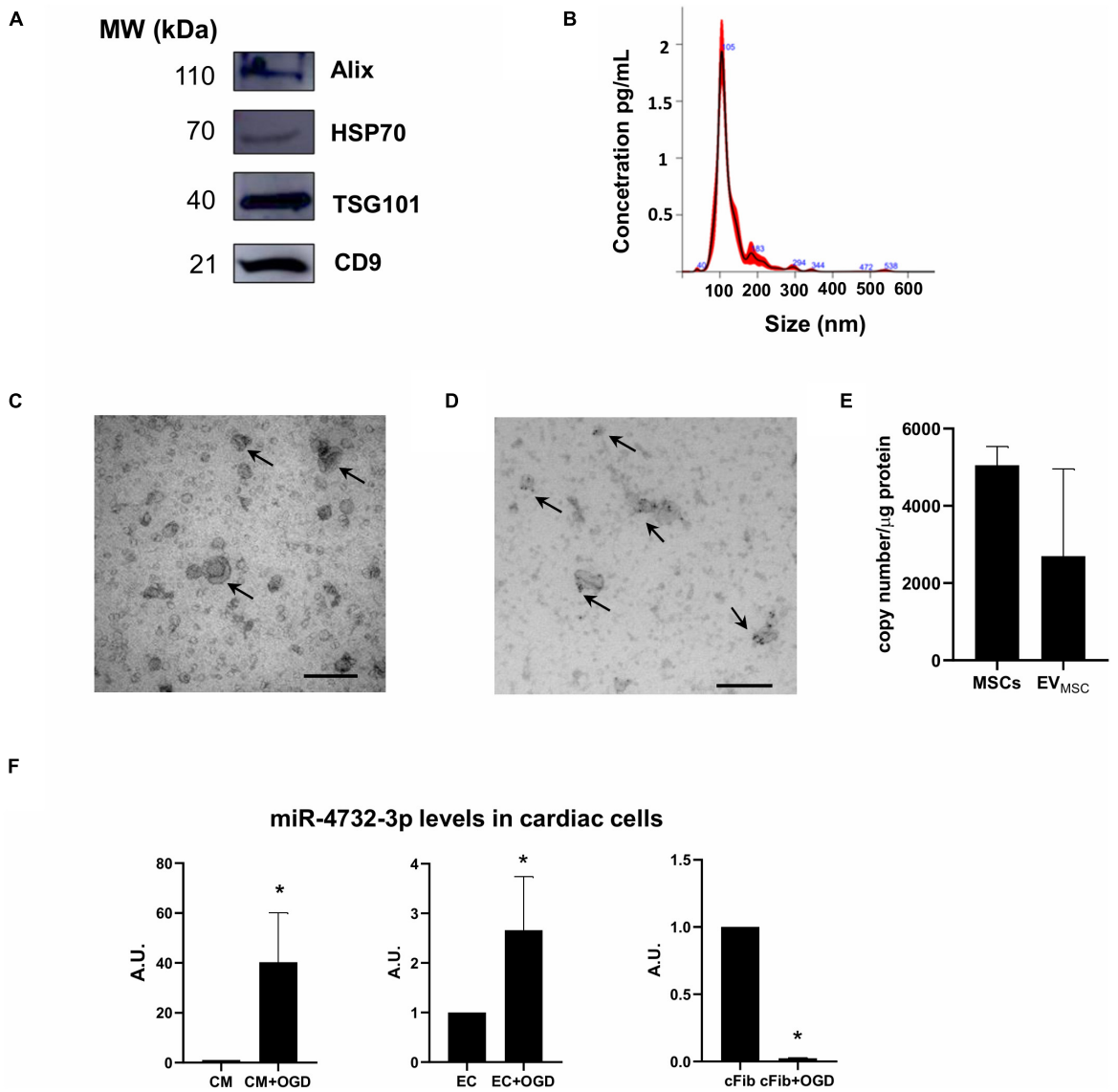

FIGURE 1 | Characterization of EVs from immortalized dental pulp-derived MSC. **(A)** Representative western blot analysis of extracellular vesicle markers. **(B)** Representative histogram of EV size analyzed with the NanoSight NS300 instrument ($n = 3$). **(C)** Representatives image of isolated EVs analyzed by electron microscopy. **(D)** Representative image of EVs stained with immunogold anti-CD63 antibody and analyzed by electron microscopy. **(E)** Levels of miR-4732-3p in MSCs and in EVs released from these cells ($n = 3$). **(F)** Levels of miR-4732-3p in neonatal rat cardiomyocytes (NRCM) ($n = 3$), human umbilical cord blood vein cells (EC) ($n = 4$) and cardiac fibroblasts (cFib) ($n = 4$) in the presence or absence of oxygen glucose deprivation (OGD) (*$p < 0.05$). Scale bar = 200 nm.

addition of EVs underlines the potential role of this miRNA in cardioprotective mechanisms.

miR-4732-3p Recapitulates the Therapeutic Effects of EVs on OGD Injured Cardiac Cells

Analysis of reactive oxygen species production and LDH activity in NRCMs treated with either EVs or transfected with miR-4732-3p mimic before OGD revealed similar levels of cardioprotection (**Figures 2C,D**). While the amount of miR-472-3p delivered by EVs is much lower than the transfected amount, it is likely that other miRNAs in EVs play a role in the cardioprotection. To analyze the global response of miR-4732-3p in comparison with EVs, we tested the contractile profile of cardiomyocytes during ischemia. When we added EVs to NRCM cultures before the OGD episode, we observed that cells maintained contraction capacity in comparison with untreated NRCMs (**Figure 2E**). NRCMs transfected with miR-4732-3p also showed an improvement in their contraction capacity but the effect was not as pronounced (**Figure 2E**). To further explore this mechanism, we analyzed the levels of connexin 43 and caspase 3 by western blotting. Results showed that whereas cleaved caspase 3 was less abundant in NRCMs treated with EVs or

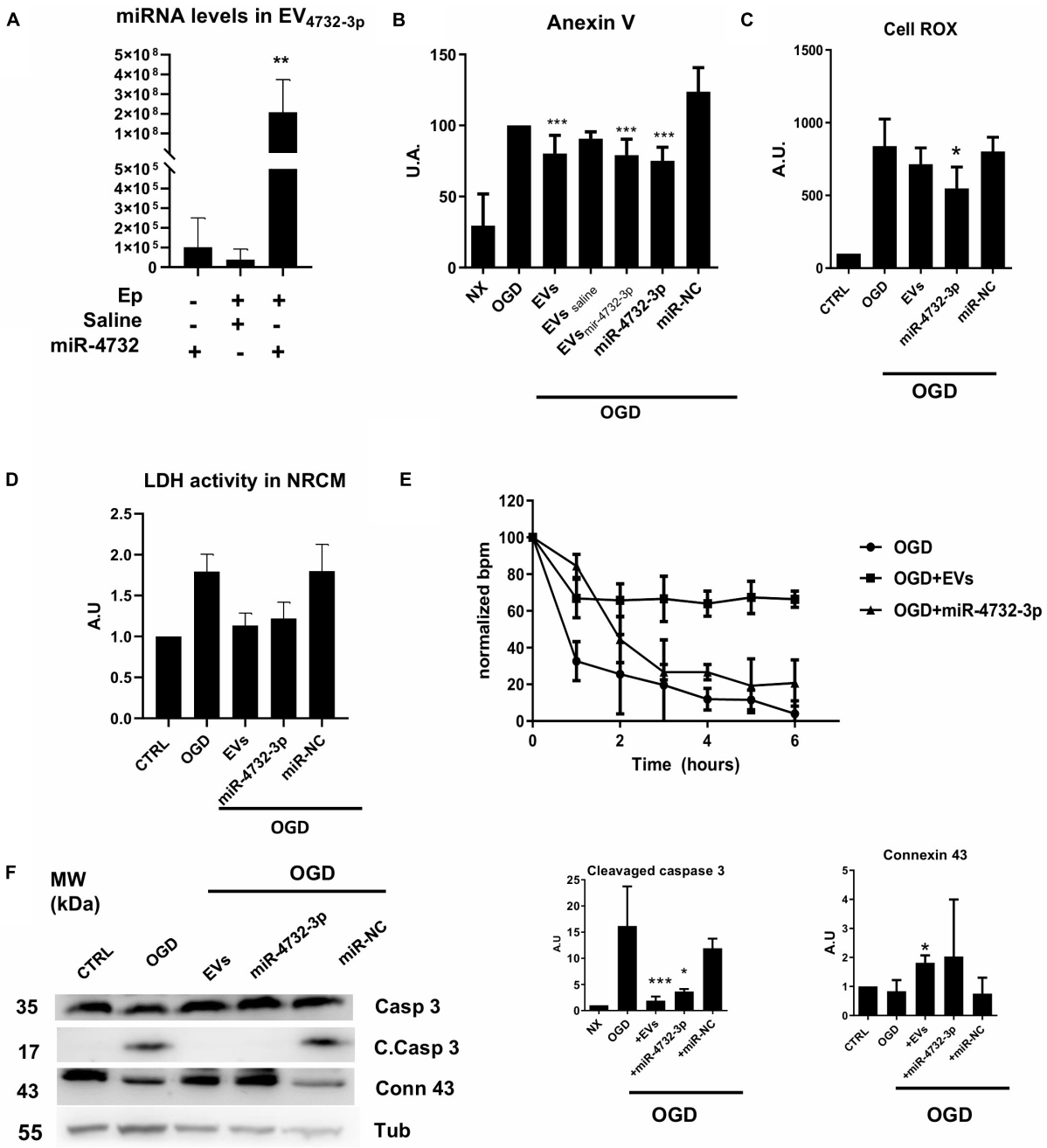

FIGURE 2 | Effect of EVs and miR-4732-3p treatment in NRCM. **(A)** Quantification of miR-4732-3p levels in electroporated EVs (Ep = 3 pulses of 300V) with 40 nM miRNA or saline in the electroporation buffer ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). **(B)** Effect of EVs on NRCM treated with oxygen and glucose deprivation (OGD) for 6 h. NRCM were treated with 30 μg/mL of EVs without electroporation (EVs), EVs electroporated with saline (EVs saline) or EVs electroporated with 20 nM miRNA hsa-4732-3p (EVs4732-3p). In addition OGM-treated NRCM were transfected with miR-4732-3p in max suppressor reagent (see section "Materials and Methods") (miR-4732-3p) ($n = 3$, $^{***}p < 0.001$). **(C)** Quantification of ROS by Cell Rox kit measured in cardiomyocytes under OGD and treated with 30 μg/mL of EVs (EVs)transfected with 20 nM miR-4732-3p (miR-4732-3p) or 20 nM of miR-Negative control (miR-NC) ($n = 3$, $^{*}p < 0.05$). **(D)** Relative quantification of lactate dehydrogenase activity (LDHA) measured by the absorbance in cardiomyocytes after OGD ($n = 3$). **(E)** Relative quantification of the contraction capacity of the cardiomyocytes *in vitro* during the OGD episode. **(F)** Representatives western blot images of cleavaged caspase 3 (C. Cas3) and connexin 43 (Conn 43) and α tubulin (Tub) ($n = 3$, $^{*}p < 0.05$ and $^{***}p < 0.001$).

miR-4732-3p suggesting a possible interaction of the miRNA with this pathway. However, further experiments are needed to confirm this interaction like a Cas3 inhibition assay. EVs exerted a greater upregulation of connexin 43, which could explain why EVs were more effective in preserving cell beating after cardiac injury (**Figure 2F**).

miRNA-4732-3p Modulates the Migration Capacity of Fibroblasts

We next sought to analyze the response of cardiac fibroblasts (cFib) to miR-4732-3p since these cells play an active role in myocardial remodeling after injury. Among the putative target genes of miR-4732-3p are FGFs, angiotensin II and members of the TGFβ family including TGFβ, TGFβR1/3 and SMAD2/4[2] (not shown). To test the anti-fibrotic effect of miR-4732-3p, we added EVs or the miR-4732-3p mimic to fibroblasts treated with TGFβ using a scratch assay. Results confirmed that the migratory capacity was lower in fibroblasts transfected with miRNA-4732-3p and treated with TGFβ than in control TGFβ-treated fibroblasts. By contrast, EV treatment failed to affect the pro-migratory activity of TGFβ (**Figure 3**).

[2]http://www.targetscan.org/vert_72/

miRNA 4732-3p Reduces the Expression of Myofibroblast Markers in Cardiac Fibroblasts

A major consequence of ischemic damage is the formation of a scar in the infarcted area derived from the differentiation of fibroblasts to myofibroblasts (Shinde and Frangogiannis, 2014). After myocardial infarction, a cytokine storm induces myofibroblast differentiation via angiotensin II and TGFβ, among others (Frangogiannis, 2015), Myofibroblasts have a greater contractile and invasion capacity and also a higher production of extracellular matrix components; hence, the excessive differentiation to myofibroblasts results in scar formation with no contraction capacity. To evaluate the effect of miR-4732-3p on myofibroblast induction, we treated cardiac fibroblasts with 10 ng/mL TGFβ to simulate differentiation. Myofibroblast differentiation was characterized by an increase in the expression of smooth muscle actin

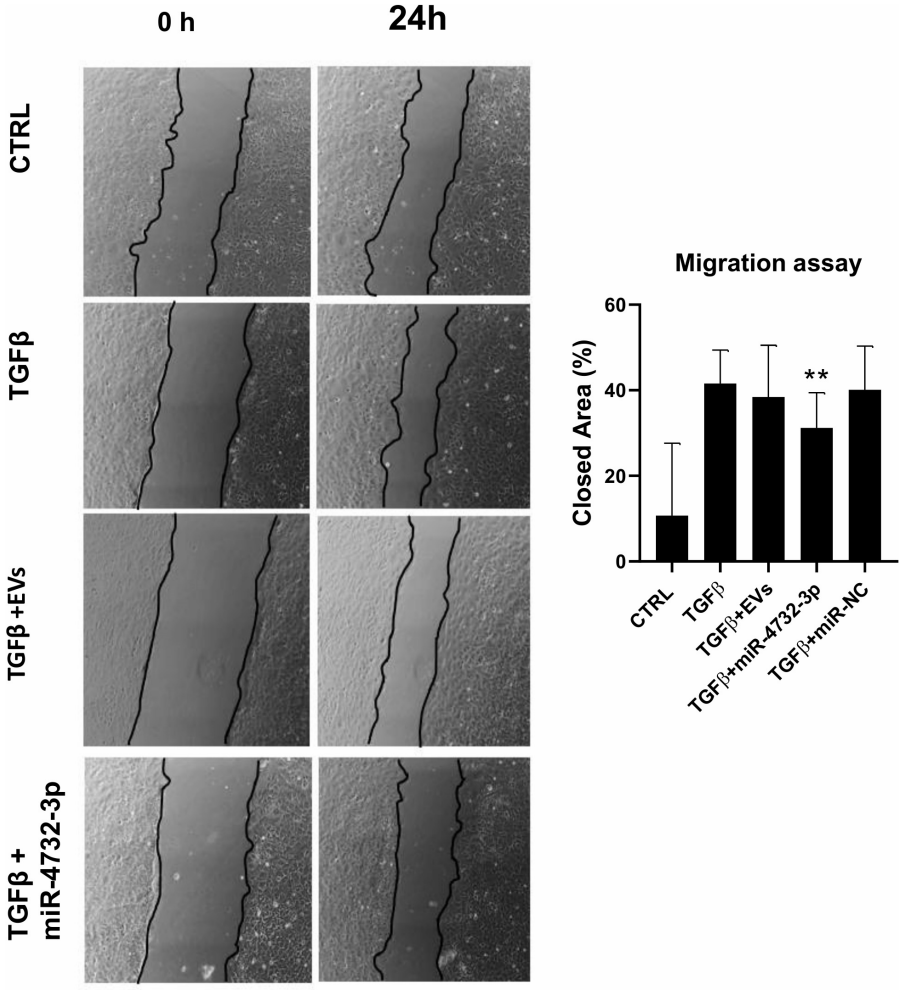

FIGURE 3 | Effect of EVs and miRNA 4732-3p on fibroblast migration. The percentage of closed area was calculated from the difference between the area at baseline and after 24 h. Fibroblasts were cultured in standard culture condition (CTRL) or treated with 10 ng/mL of TGFβ alone or with 30 μg/mL of EVs (TGFβ + EVs) transfected with 20 nM of lipofectamine-miRNA 4732-3p complexes or 20 nM of miR-Negative Control. Images were captured on an inverted microscope at 4 × magnification. The area of the scratch was measured using ImageJ ($n = 6$, $^{**}p < 0.01$).

(α-SMA) and increased production of extracellular matrix proteins like collagen IA (col I-α1) (**Figure 4**). Although both EVs and transfection of miR-4732-3p led to a reduction in α-SMA expression, only miR-4732-3p treatment reduced collagen production. Taken together, the results suggest that miR-4732-3p inhibits myofibroblast differentiation and the production of extracellular matrix, which might control fibrosis in the ischemic area.

miR-4732-3p Has Angiogenic Effects in Endothelial Cells *in vitro* and *in vivo*

We tested the capacity of miR-4732-3p to induce the formation of new vasculature *in vitro*. We did not use OGD treatment since in those conditions tube formation was drastically impaired. Instead, HUVEC were seeded in DMEM without glucose and glutamine (Thermo Fisher Scientific) in growth factor-reduced Matrigel (GD condition). Results showed a significant increase in the formation of tubular structures from HUVEC transfected with the miR-4732-3p compared with untreated cells (**Figure 5A**). Contrastingly, cells treated with EVs showed no significant increase in vascular structures at the doses analyzed. We next sought to test the angiogenic potential of miR-4732-3p *in vivo*. Nude mice were injected subcutaneously with a Matrigel plug containing either saline, FGF (as a positive control), EVs or miR-4732-3p (**Figure 5B**). Plugs were removed 15 days later and were embedded in paraffin for histological analysis (**Figure 5C**). Individual pugs are shown in **Supplementary Figure 2**. Quantification of angiogenesis was performed by measuring plug-infiltrating vessels using anti-CD31 staining. The results showed a potent angiogenic effect in mice treated with miR-4732-3p in comparison to EVs and control conditions (**Figures 5D,E**). Overall, these findings indicate that miR-4732-3p induces angiogenesis both under stress and in physiological conditions.

miRNA-4732-3p Improves Cardiac Function and Reduces Infarct Size

The ability of EVs derived from MSC isolated from different origins has been extensively reported and in most cases therapeutic effects have been attributed to miRNA cargo components (Wang et al., 2017; Luther et al., 2018; Zhu et al.,

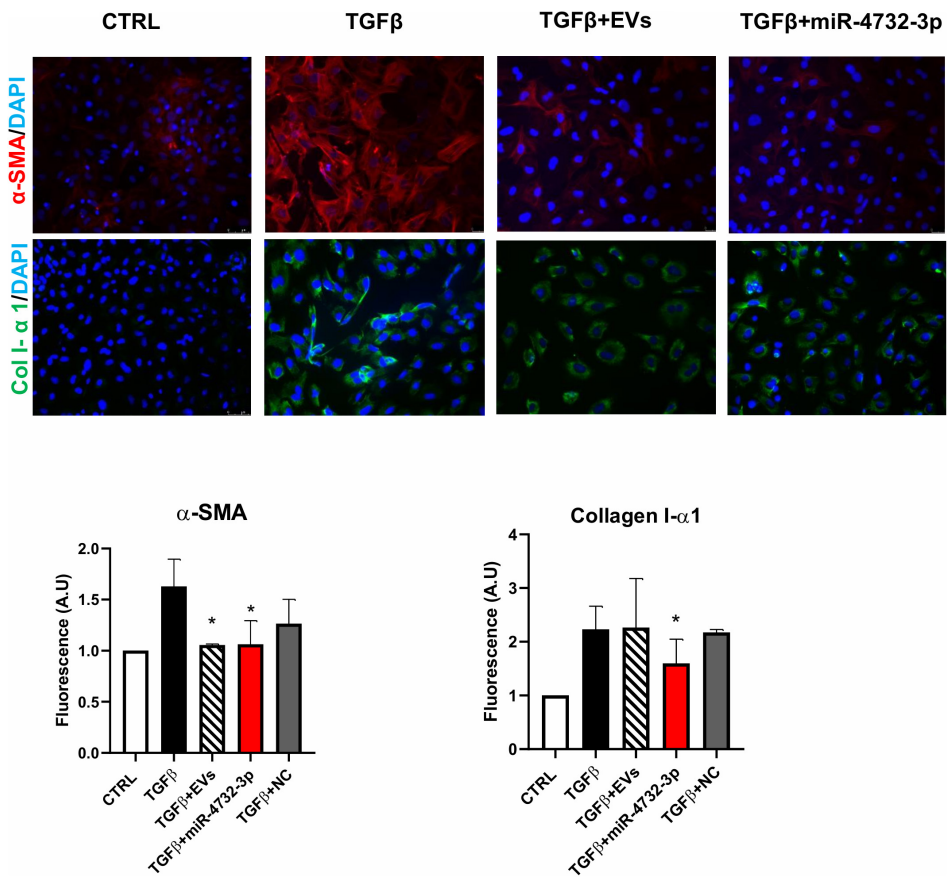

FIGURE 4 | Effect of EVs and miRNA 4732-3p on the expression of the myofibroblast markers α-smooth muscle actin (α-SMA) and collagen 1 α (Col1). Fibroblasts were cultured in standard culture condition (CTRL) or treated with 10 ng/mL TGFβ (TGFβ),with or without 30 μg/mL of EVs (TGFβ + EVs), 20 nM of lipofectamine-miR 4732-3p complexes or 20 nM of miR-Negative Control. Images were taken with a fluorescence microscope and the fluorescence was quantified using ImageJ ($n = 3$, $^*p < 0.05$).

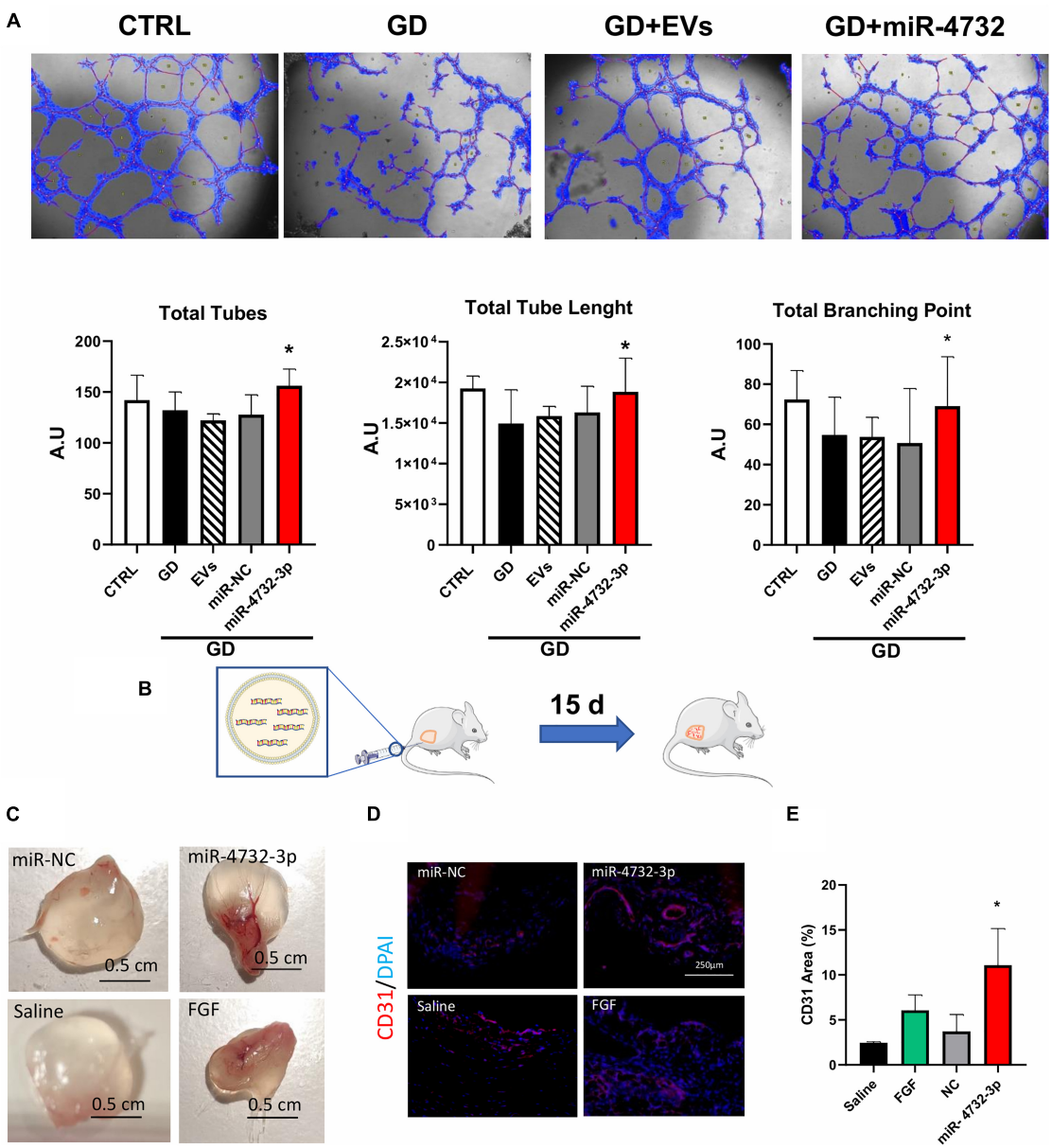

FIGURE 5 | Effect of EVs and miRNA 4732-3p on angiogenesis. **(A)** Representative images and quantification of the tubular structures in endothelial cells in standard culture condition (CTRL) in glucose deprivation (GD), with or without 30 μg/mL of EVs(EVs), 20 nM lipofectamine-miRNA-4732-3p complexes (miR-4732-3p) or 20 nM of a negative control miRNA (miR-NC) and quantification of total tubes, total tube length and total branching points. **(B)** Scheme of Matrigel plug assay in nude mice to measure angiogenesis. Mice were divided into four groups. Matrigel was mixed with saline, 100 ng/mL FGF and 20 U heparin (FGF), 20 μg of negative control miRNA (miR-NC) or 20 μg of miR-4732-3p in Maxsuppressor reagent (miR-4732-3p). Mice were sacrificed 15 days after Matrigel injection. **(C)** Representatives images of extracted plugs (scale-bar = 0.5 cm). **(D)** Representative images of CD31-positive staining in the plugs by immunohistochemistry. **(E)** Quantification of CD31-positive area in the plugs ($n = 5$, $^*p < 0.05$).

2018; Mao et al., 2019; Huang et al., 2020; Peng et al., 2020; Xu et al., 2020).

We tested whether the administration of miR-4732-3p could recapitulate this effect by encapsulating it in liposomes before intramyocardial implantation shortly after permanent LAD ligation in nude rats. Results were compared to saline intramyocardial injections. Four weeks after transplantation, cardiac function parameters were measured to assess the degree of functional recovery. The miR-4732-3p group displayed a significant recovery of systolic function as calculated by the percentage of fractional shortening (FS) and fractional area change (FAC) (**Figure 6A**). Treatment with miR-4732-3p also significantly reduced the area of fibrous scar tissue (**Figure 6B**). These *in vivo* data support the hypothesis that miR-4732-3p can induce cardioprotection against cardiac ischemia.

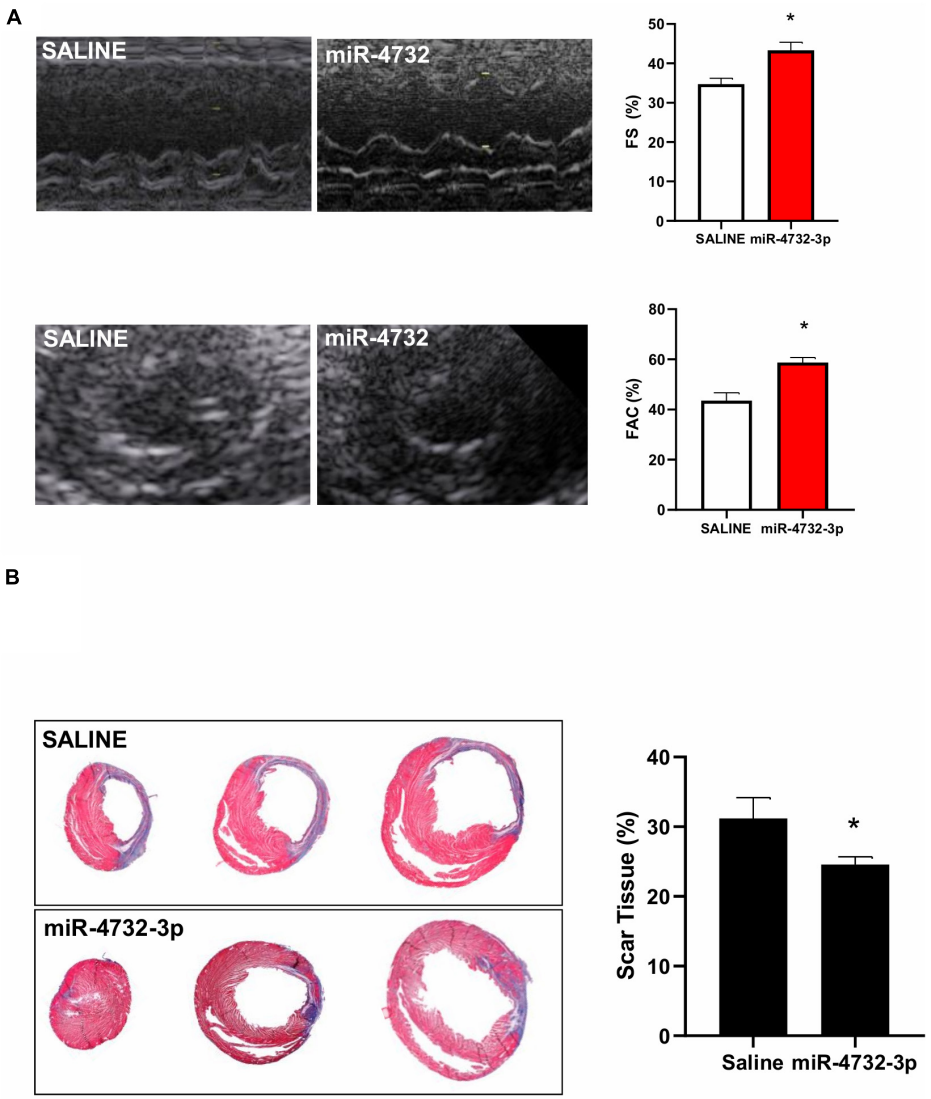

FIGURE 6 | Effect of EVs and miRNA 4732-3p in infarcted nude rats **(A)** improvement of LV function in miR-4732-3p-treated animals. Representative echocardiographic images of M-mode and two-dimensional systolic frame showing differences in wall motion in one animal from saline (n = 6), and miR-4732-3p (n = 8) groups. Quantified values of fractional shortening (FS) area fractional change (FAC) are given. Data are expressed as mean ± SEM (*$p < 0.05$ in both panels). **(B)** Fibrotic area (blue color) in the LV was calculated from Masson's Trichrome stained sections. Animals were euthanized 4 weeks post-transplantation. Values are the mean ± SEM (*$p < 0.05$).

DISCUSSION

The lack of effective regenerative therapies in the treatment of ischemia-related diseases compels the development of new approaches to improve clinical outcomes. Recently, the field of cardiovascular therapies have moved to cell-free-based therapies using EVs derived from MSC. There is evidence to suggest that MSC-EVs isolated from different tissues are able to promote myocardial repair through protection of ischemic cardiomyocytes and preservation of cardiac function (Teng et al., 2015; Chen et al., 2020; Mathew et al., 2020; Takov et al., 2020). These biological products are cardioprotective in preclinical models of myocardial infarction, and increasing evidence indicates that miRNAs are the main cargo components responsible for the therapeutic potential of EVs. Nonetheless, the heterogeneous composition of EVs is a major hurdle for their clinical use, and simpler biological products would be desirable to induce tissue repair. Another concern for the use of MSC-derived EVs in the clinical context is the potential involvement of deleterious processes mediated by specific miRNAs. For example, miR-130b-3p in MSC-derived EVs has been shown to promote lung cancer cell proliferation, migration and invasion (Guo et al., 2021) and exacerbates I/R injury after adoptive transfer in diabetic mice (Gan et al., 2020). With the aim of identifying miRNAs responsible (at least in part) for the beneficial effects attributed to EVs, we

analyzed miRNA content in MSC derived EVs and investigated a miRNA with cardioprotective features when transplanted as a single molecule.

We provide evidence that miR-4732-3p is contained in EVs released from dental pulp-derived MSC (**Figure 1E**). We transfected miR-4732-3p *in vitro* and *in vivo* in liposomes that allowed delivery into cells and tissues and compared its therapeutic effect with that of MSC-EVs. EVs have been shown to reduce oxidative stress and apoptosis and to induce angiogenesis and cardioprotection against I/R injury mediated by specific miRNAs (Arslan et al., 2013; Zhao et al., 2019; Chen et al., 2020; Wen et al., 2020) and so we focused on those mechanisms in this study. We show that miR-4732-3p: (i) reduces apoptosis, ROS levels and LDH activity in NRCMs after OGD; (ii) prevents fibroblast migration and myofibroblast differentiation; (iii) induces angiogenesis both *in vitro* and *in vivo*; and (iv) confers cardioprotection when injected intramyocardially in infarcted nude rats as measured by functional and morphometric studies.

miR-4732-3p is also present in cardiac cells where it is modulated by oxygen in a cell-specific manner. Hypoxia upregulates miR-4732-3p in NRCM and EC, which is in accordance with the cardioprotective responses induced by miR-4732-3p in these cell populations after transfection. However, a decrease in oxygen levels induces downregulation of miR-4732-3p in cFib. Accordingly, transfection of TGF-β-treated cFib with miR-4732-3p reduced their migration capacity and differentiation into myofibroblasts. Interestingly, miR-4732-3p targets the SMAD2 and SMAD4 components of TGF-β pathway (Doss et al., 2015), and so the hypoxia-induced downregulation of miR-4732-3p in response to OGD might translate to an upregulation of the TGF-β pathway, leading to cFib proliferation and myofibroblast differentiation.

Although the doses are not comparable, the paracrine effects observed after miR-4732-3p transfection were more robust than those exerted by EVs. In this context, miRNA therapy offers the advantage of a homogeneous content versus the heterogeneous content of vesicles, which would simplify scale-up of biological products. EVs electroporated in saline solution with no added miRNA did not offer cardioprotection of cultured cells, indicating the importance of the EV cargo. Our data do not dismiss the possibility that other miRNAs, metabolites or proteins from EVs could also induce strong cardioprotective effects but, as mentioned, the complexity of EV cargo together with intra-donor variability limits their exploitation in a clinical scenario. *In vivo* transfer of single miRNAs would overcome this problem. In this context, other studies have reported therapeutic effects of individual miRNAs present in EVs from MSC and other adult progenitor cells. As an example, a combination of miR-1, miR-133, miR-208, and miR-499 induced cardiac reprogramming in the infarcted heart and improved cardiac function in mice subjected to cardiac injury by permanent LAD artery ligation (Jayawardena et al., 2014), and adoptive transfer of miR-181b mimic recapitulated cardioprotection induced by exosomes derived from cardiosphere-derived cells (CDCs) in a rat model of MI (De Couto et al., 2017).

Because hypoxia preconditioning promotes the release of vesicles with enhanced therapeutic potential (Bister et al., 2020), we were interested in those miRNA present in EVs and modulated by hypoxia (unpublished results). miR-4732-3p is regulated by hypoxia in MSC, CM, EC, and cFib. We do not know if this miRNA is directly regulated by Hypoxia Inducible Factor 1 subunit α; however, HIF-1α inhibitor (HIF1AN) is a putative target gene of this miRNA, as assessesed by Targetscan[3], Diana tools[4], and miRDB[5]. miR-4732-3p upregulation by hypoxia could presumably downregulate HIF1AN, leading to stabilization of HIF-1α, as has been reported for other miRNAs including miR-335, which also confers cardioprotective effects (Wu et al., 2018).

The present study has some limitations. First, the therapeutic contribution of miR-4732-3p in comparison with other miRNAs present in EVs is unknown. The absolute copy number of this miRNA is relatively low in MSC-derived EVs so it is plausible that other miRNAs with higher abundance like miR-21a-5p (Luther et al., 2018), miR-26a (Park et al., 2018), miR-181a (Zilun et al., 2019), miR-210 (Wang et al., 2017), or miR-486-5p (Li et al., 2021) have a greater contribution (Nazari-Shafti et al., 2020). Second, no target genes have been validated for miR-4732-3p, but this molecule has been described as a key modulator of TGFβ signaling pathway in human erythropoiesis (Doss et al., 2015). The results presented here point in the same direction and indicate that the TGFβ pathway has a major contribution. Third, we did not perform loss-of-function experiments to analyze the effect of miR-4732-3p inhibition on myocardial repair. We previously observed low levels of miR-4732-3p in the serum of patients that experienced cardiotoxicity after doxorubicin challenge (Hervás et al., 2018). In addition to TGFβ, bioinformatic analysis identified components of Wnt/β-catenin pathways among the putative target genes of miR-4732-3p, supporting the idea that this molecule is cardioprotective and could be involved in cardiac turnover in the adult heart. In this context, in a study of monozygotic twins discordant for congenital heart disease, miR-4732-3p was among the most upregulated miRNAs in those with congenital heart disease, further linking this molecule to heart developmental processes (Abu-Halima et al., 2019).

In conclusion, and in agreement with previous reports, EVs derived from MSC have cardioprotective mechanisms in cardiac cells. We found that miR-4732-3p is present in EVs from MSC and can trigger angiogenic and cardioprotective responses compatible with the effect of EV therapy. When transplanted in murine models of angiogenesis and myocardial infarction, miR-4732-3p induced blood vessel formation, reduced scar formation and prevented cardiac function deterioration indicating that a single miR can recapitulate the therapeutic effects of EVs. These results support the potential paracrine actions of miR-4732-3p, but the mechanism by which this molecule operates in different cardiac cell populations requires further investigation. Additional experiments to identify 3'-untranslated regions in putative miR-4732-3p target genes are needed as a next step to identify the specific molecular pathways modulated by this miRNA.

[3]http://targetscan.org

[4]http://diana.imis.athena-innovation.gr

[5]http://mirdb.org

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the GEO database repository, accession number is GEO:GSE179940.

ETHICS STATEMENT

The animal study was reviewed and approved by the Hospital La Fe Ethics and Animal Care Committee.

AUTHOR CONTRIBUTIONS

RS-S, MG-F, IR, MB, EV-B, HG-K, and IO-O were responsible for design, performance, and analysis and interpretation of the *in vitro* and *in vivo* experiments. EP-M and AH were responsible for cardiac function *in vivo* studies. AD was responsible for conceptualization, investigation, data curation, and review. PS was responsible for conceptualization, data curation, funding acquisition, writing, and review. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.734143/full#supplementary-material

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REVIEW
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Development of Extracellular Vesicle Therapeutics: Challenges, Considerations, and Opportunities

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Extracellular vesicles (EVs) hold great promise as therapeutic modalities due to their endogenous characteristics, however, further bioengineering refinement is required to address clinical and commercial limitations. Clinical applications of EV-based therapeutics are being trialed in immunomodulation, tissue regeneration and recovery, and as delivery vectors for combination therapies. Native/biological EVs possess diverse endogenous properties that offer stability and facilitate crossing of biological barriers for delivery of molecular cargo to cells, acting as a form of intercellular communication to regulate function and phenotype. Moreover, EVs are important components of paracrine signaling in stem/progenitor cell-based therapies, are employed as standalone therapies, and can be used as a drug delivery system. Despite remarkable utility of native/biological EVs, they can be improved using bio/engineering approaches to further therapeutic potential. EVs can be engineered to harbor specific pharmaceutical content, enhance their stability, and modify surface epitopes for improved tropism and targeting to cells and tissues *in vivo*. Limitations currently challenging the full realization of their therapeutic utility include scalability and standardization of generation, molecular characterization for design and regulation, therapeutic potency assessment, and targeted delivery. The fields' utilization of advanced technologies (imaging, quantitative analyses, multi-omics, labeling/live-cell reporters), and utility of biocompatible natural sources for producing EVs (plants, bacteria, milk) will play an important role in overcoming these limitations. Advancements in EV engineering methodologies and design will facilitate the development of EV-based therapeutics, revolutionizing the current pharmaceutical landscape.

Keywords: extracellular vesicle therapeutics, drug and vector delivery, exosome-based therapeutics, nanomedicine, nanovesicles/microparticles, EV hybrids and mimetics, bioengineering, clincal trials and utility

INTRODUCTION

Extracellular vesicles (EVs) are nanosized, membranous cell-derived particles with important roles in exchanging molecular information between cells; they have been shown to contain and transfer proteins and nucleic acids (DNA, mRNA, miRNA) to recipient cells, modulating their functional activity through transcriptional and translational regulation (Luga et al., 2012; Xu et al., 2016; Thery et al., 2018; Flaherty et al., 2019; Kalluri and LeBleu, 2020);

transfer of organelles has also been highlighted by EVs, including mitochondria (Spees et al., 2006), to regulate inflammatory response (Zhang Y. et al., 2021). EVs can be classified by their biogenesis and biophysical/biochemical characteristics. The subtypes include intracellular formed exosomes (50–200 nm) secreted after fusion of multivesicular bodies with the cell surface, microvesicles (100–1,000 nm) formed by outward budding of the plasma membrane, shed midbody remnants released during cytokinesis (200–600 nm), and apoptotic bodies (100–5,000 nm) released during apoptosis (Al-Nedawi et al., 2008; Willms et al., 2016; Xu et al., 2016; van Niel et al., 2018; Jeppesen et al., 2019; Martinez-Greene et al., 2021; Rai et al., 2021). The International Society for EVs (ISEV) recommends the use of "EV" as a broad classifier term for these types of vesicles, due to the difficulty in assigning an EV to a particular biogenesis pathway, and instead recommends classifying EVs by their physical attributes (size, density), their differing biochemical composition, and surface charge (Thery et al., 2018). The nature and relative abundance of EV cargo is selectively determined during EV biogenesis (Palmulli and van Niel, 2018; van Niel et al., 2018; Clancy et al., 2021), and varies according to EV subtype and state/type of the producing cell (Kowal et al., 2016; Xu et al., 2016; Zabeo et al., 2017; Greening and Simpson, 2018; Martin-Jaular et al., 2021). Importantly, EVs, comprising of a lipid membrane and aqueous lumen (Cvjetkovic et al., 2016; Skotland et al., 2019), provide a pathway for the transfer of hydrophobic and hydrophilic components allowing for complex intercellular signaling (Luga et al., 2012; Cossetti et al., 2014; de Couto et al., 2017; Kamerkar et al., 2017; Nabet et al., 2017; Wang and Lu, 2017; Flaherty et al., 2019; Han et al., 2019).

Due to their nanoscale size, stability, biocompatibility, and propensity for cellular uptake, EVs have been recognized as viable vehicles for therapeutic application. Recent studies have highlighted the therapeutic potential of EVs, investigated in clinical trials (phase I/II) for their regenerative capacity (NCT04223622) (Niada et al., 2019), vaccine potential (Gehrmann et al., 2014; Narita et al., 2015; Besse et al., 2016; Coakley et al., 2017; Shehata et al., 2019; Nikfarjam et al., 2020; Andrews et al., 2021), immunotherapeutic activity (Narita et al., 2015; Besse et al., 2016; Chen G. et al., 2018), and application as delivery vectors (NCT01294072). Indeed, pre-clinical and therapeutic applications of EVs across a wide range of pathologies for tissue repair and regeneration are well underway (**Table 1**). Critically, stem cell-derived EVs can ameliorate the effects of various diseases in the liver (fibrosis, hepatitis, inflammation), brain (stroke), heart (myocardial infarction, contractility), kidney (renal ischemia, stenosis), and immune system [reviewed in Wiklander et al. (2019) and Yin et al. (2019)]. EVs have been shown in various mechanisms to regulate the immune system, enhancing or inhibiting the immune response depending on their parental cell source and of the immune context of the application site (Zhou et al., 2020), demonstrating a potential use in immunotherapy. Indeed, EVs exert specific and potent therapeutic effects on recipient cells because of complex bioactive properties and are an effective and efficient system of cell-cell communication, surmounting biological barriers.

The diverse beneficial effects of seemingly identical entities [i.e., mesenchymal stem cell (MSC)-EVs, bone marrow-derived stem cell (BMDSC)-EVs (Kordelas et al., 2014; de Couto et al., 2017; Xue et al., 2018; Han et al., 2019; Lukomska et al., 2019; Williams A. M. et al., 2019)] suggests a complex repertoire of active cargo (Toh et al., 2018) working synergistically, as opposed to a single molecular component. As such, a global view of EV-based therapeutic action is needed. The biological cargo harbored by EVs, including proteins (Al-Nedawi et al., 2008; Yim et al., 2016; Yuan et al., 2017; Zhang G. et al., 2017; Roefs et al., 2020), nucleic acids (Ratajczak et al., 2006; Liang et al., 2016; Xiao G. Y. et al., 2016; Song et al., 2017; Gu et al., 2018; Shi et al., 2018; Gu Y. Y. et al., 2019; Basalova et al., 2020), and lipids (Lindemann, 1989; Fadok et al., 2000; Gurnani et al., 2004; Yuyama et al., 2014) [reviewed in Greening et al. (2017), Skotland et al. (2019), O'Brien et al. (2020)] (**Table 2**), greatly influence their clinical potential. The protein and lipid expression of EVs yield insights into their surface receptor mediated interactions with, and effects on recipient cells, including their fusion and uptake (Christianson et al., 2013; Purushothaman et al., 2016; Berenguer et al., 2018), while their genetic landscape sheds light on the EVs' reprogramming potential through regulation of protein expression (Ratajczak et al., 2006; Skog et al., 2008; Abels et al., 2019). Findings from such studies have inspired strategies for EV-based therapeutic development, such as the modification of their contents to perform a specified function for a specific disease phenotype (**Table 3**) (Al-Nedawi et al., 2008; Hall et al., 2016; Yim et al., 2016; Greening et al., 2017; Yuan et al., 2017; Zhang G. et al., 2017; Chen R. et al., 2019; Shi et al., 2019; Skotland et al., 2019; O'Brien et al., 2020; Roefs et al., 2020). Comprehensive deciphering of EV biochemical and biophysical heterogeneity (Jeppesen et al., 2019), variable composition (Chen et al., 2016; Kowal et al., 2016; Greening et al., 2017; Jeppesen et al., 2019; Martin-Jaular et al., 2021), pharmacokinetic behavior (Gupta et al., 2020), and functional diversity needs to be addressed in order to harness their potential as next generation therapeutics.

Advances and applied technologies which can be used to reproducibly monitor form and function of EVs at molecular and structural levels will be instrumental in future development of EV research knowledge, and therapeutic design and application. Bioinspired engineered EVs/nanovesicles have emerged as an alternative to native EVs to address issues in production, purity, scalability, and economic viability, while maintaining key properties required for *in vivo* trafficking, biological function, and therapeutic efficiency. Recent advances in bioengineering have allowed the refinement of cargo loading, targeting capacity, and pharmacokinetic properties of EV-based therapeutics, both native and mimetic.

Here, we focus on recent advances in clinical utility of EVs, understanding the molecular complexity of bioactive cargo, avenues for bioengineering, and monitoring the form and function of EVs intended for clinical use. For further discussion on topics not covered in extensive detail here, we direct readers

to the following recent reviews and position papers (Lener et al., 2015; Reiner et al., 2017; Colao et al., 2018; Pinheiro et al., 2018; Sluijter et al., 2018; Russell et al., 2019; Whitford and Guterstam, 2019; Wiklander et al., 2019; Gandham et al., 2020; Nelson et al., 2020; Pirisinu et al., 2020; Yin et al., 2020; Grangier et al., 2021; Herrmann et al., 2021; Johnson et al., 2021; Ozturk et al., 2021; Sahoo et al., 2021; Suharta et al., 2021).

CURRENT DEVELOPMENTS IN EV-BASED THERAPEUTICS

Recent years have seen significant development and translation of EV-associated therapeutics, progressing to pre-clinical and clinical studies (**Table 1**). Further, the capacity of EVs to transfer biological and pharmaceutical molecules to specific tissues and cell types has raised considerable interest in their development as biocompatible drug delivery systems [reviewed in Sluijter et al. (2018); Pirisinu et al. (2020); Herrmann et al. (2021); and Rankin-Turner et al. (2021)]. At present, https://www.clinicaltrials.gov lists 224 studies which include "exosome," 84 with "extracellular vesicle," 9 with "nanocarrier," 4 with "engineered exosome," and 2,101 with "liposome." While a portion of these are diagnostic/biomarker studies, most are clinical trials based on pre-clinical therapeutic success in wound healing (NCT04761562, NCT04281901, and NCT04664738) (Jia et al., 2021; Zhao et al., 2021), heart disease (NCT04327635) (Aday et al., 2021; Hu S. et al., 2021), COVID-19 (NCT04657458, NCT04493242, NCT04276987, NCT04747574, NCT04389385, and NCT04969172) (Mitrani et al., 2021), infectious disease (NCT01478347, NCT01717638, and NCT04350138), diabetes (NCT02138331), stroke (NCT03384433), arthritis (NCT04223622), and drug delivery (NCT01294072, NCT02889822, and NCT04217096). While classified as EV therapies, such studies are more often comprised of a variety of secreted components (i.e., secretome containing soluble factors and EVs) than purified EVs (**Table 1**). Various terminology is used in the field (Thery et al., 2018), including "extracellular vesicles," "exosomes," "secretomes," "nanoparticles," or components "enriched in extracellular vesicles." Regardless of terminology or composition, these therapies utilize the functional capacity EVs/secreted components have to mediate a recipient-cell response through the delivery of cargo including siRNAs (Shtam et al., 2013), miRNAs (Li L. et al., 2019), proteins (Garaeva et al., 2021), small molecule drugs (Tian et al., 2014), and molecular toolkits (Ye et al., 2020; Luo et al., 2021; Yao X. et al., 2021).

Immunomodulation

Extracellular vesicles hold the potential for potent immunomodulation, both in eliciting and suppressing immune response (Zhou et al., 2020). EVs share structural similarities to viruses and recent findings demonstrate that viruses exploit mechanisms associated with EV uptake and release (Feng et al., 2013; Altan-Bonnet, 2016; van Dongen et al., 2016; Urbanelli et al., 2019). Previously, the ground-breaking application of EVs in anti-tumor immunotherapy (Zitvogel et al., 1998) led to two clinical trials where EVs activated patient immune response against tumor antigens (Escudier et al., 2005; Morse et al., 2005). Since then, refinements in EV production and modification have led to successful reduction in tumor size in various pre-clinical models (Lee et al., 2012; Mahaweni et al., 2013; Rao et al., 2016; Cheng et al., 2021) and additional clinical trials exploiting their immunomodulatory capabilities to target various cancer types (NCT01550523, NCT01159288, and NCT02507583) (Besse et al., 2016; Andrews et al., 2021). The use of EVs as antigen vehicles is an approach still under development (Cheng et al., 2021; Hu S. et al., 2021), but does represent the most successful translated application. The FDA approved Bexsero bacterial outer membrane vesicle (OMV)-containing meningococcal vaccine is administered to protect against meningococcal group B (Gorringe and Pajon, 2012). This vaccine exploits the complexity of bacterial OMVs to boost immunogenicity of multi-component formulas over those without OMVs (Gorringe and Pajon, 2012). Anti-viral EV-based vaccines are also in development (Sabanovic et al., 2021), with pre-clinical success against COVID-19 (Polak et al., 2020), influenza H1N1 (Rappazzo et al., 2016; Watkins et al., 2017; Shehata et al., 2019), influenza H3N2 (Watkins et al., 2017), and MERS-CoV (Shehata et al., 2019). In addition to immune-priming, EV therapeutics hold the potential for deactivation and suppression of over-active immune responses. Indeed, although results have yet to be released, a clinical trial is underway examining the therapeutic effect of MSC-derived EVs in the autoimmune disease Type I diabetes (NCT02138331). Pre-clinical success in the treatment of sepsis (Song et al., 2017), inflammatory bowel disease (Yang et al., 2015; Mao et al., 2017), and multiple sclerosis (Casella et al., 2018; Laso-Garcia et al., 2018) suggests EV-mediated treatment of inflammatory and autoimmune disorders (Coakley et al., 2017; Sharma et al., 2017; Fujita et al., 2018; Xu H. et al., 2019; Goodman and Davies, 2020; Kahmini and Shahgaldi, 2020; Xu et al., 2020; Horst et al., 2021) may soon expand further into patient trials.

Regeneration and Recovery

Extracellular vesicle therapeutics are also being explored to facilitate/promote recovery and regeneration following injury, surgery, and tissue damage, such as that arising from viral infections [i.e., pulmonary damage in COVID-19 (Borczuk et al., 2020)]. There are currently 13 clinical trials treating COVID-19 with "exosomes" or "extracellular vesicles" (NCT04902183, NCT04798716, NCT04602442, NCT04747574, NCT04491240, NCT04389385, NCT04276987, NCT04657406, NCT04384445, NCT04623671, NCT04493242, NCT04657458, and NCT04969172). Although no large studies have released results, a case report of three critically ill patients administered with amniotic fluid-derived nanoparticles (likely containing EVs and other small extracellular particles) revealed the therapy had no adverse effects and the patients' status improved (Mitrani et al., 2021). There are now clinical trials further evaluating the safety and efficacy of this treatment for COVID-19 (NCT04384445, NCT04657406). Another major application of EV therapies is in accelerated and improved healing and regeneration of damaged tissue (Dalirfardouei et al., 2021;

TABLE 1 | Clinical and preclinical applications of extracellular vesicles.

	EV type/source*	Method of isolation/ components	Application	Dosage	Method of administration	Active component/ mechanism of action	Outcome/Stage	Clinical trial number/ References
Delivery mechanism	Plant vesicles containing curcumin		Method of curcumin delivery to colon tumors		Ingestion, tablet taken daily for 7 days		Phase I, active	NCT01294072
	Paclitaxel liposome		Drug delivery for advanced metastatic pancreatic cancer	175 mg/m^2 (body surface area)	Intravenous infusion, once every 3 weeks		Phase IV	NCT04217096
	Exosomes derived from Lhfpl5 AAV-plasmid transfected HEK293T cells	Ultracentrifugation (20,000 *g*, 100,000 *g*)	Treatment of hereditary deafness in mice		Direct injection into the ear (single dose)	Restore Lhfpl5 protein expression	Exosomes containing the plasmid were able to promote expression of Lhfpl5 and partially restore hearing to mice	Gyorgy et al., 2017
	Exosomes derived from HEK293T cells expressing Lamp2b fused to IL3 fragment, loaded with Imatinib or siRNA	Ultracentrifugation (10,000 *g*, 100,000 *g*)	Treatment of leukemia in mice	10 μg	Intraperitoneal injection, twice a week for 3 weeks	Drug/siRNA	Exosomes targeted IL3 receptor overexpressed on leukemia cells, and inhibited cancer cell growth	Bellavia et al., 2017
	Exosomes containing 5-Fluorouracil and miR-21 inhibitor	Ultracentrifugation (10,000 *g*, 100,000 *g*)	Treatment of tumors in mice	2 mg	Intravenous injection 3 days a week for at least 6–7 weeks	Drug/miRNA inhibitor	Exosomes down regulated miR-21 expression and reduced tumor growth through reversal of drug resistance	Liang et al., 2020
	Platelet-derived extracellular vesicles (whole blood, platelet-rich plasma) loaded with TPCA-1 drug	Platelet activation, ultracentrifugation	Delivery of anti-inflammatory agents to pneumonia (acute lung injury)	12.6 mg/kg	Intravenous injection (one time)	TPCA-1 (drug), anti-inflammatory response, lung (ALI-induced) specific	EVs containing anti-inflammation agent TPCA-1 reduced inflammation (CD45 immune cells infiltration) and cytokine storm syndrome (pro-inflammatory cytokines)	Ma et al., 2020
	HEK293T-derived aptamer-modified exosomes carrying siRNA	Ultracentrifugation (10,000 *g*, 100,000 *g*)	Treatment of orthotopic prostate cancer mouse model		Intravenous injection twice a week for 3 weeks	SIRT6-siRNA, reduced tumor SIRT6 (incl. Notch pathway)	Engineered exosomes impaired the proliferation and metastasis of prostate cancer	Han et al., 2021
Immuno-modulation	Human umbilical cord mesenchymal stem cell derived exosomes	Concentration (100 kDa cut-off), centrifugation with density cushion (100,000 *g*)	Treatment of inflammatory bowel disease in mice	400 μg	Intravenous injection, three times over 9 days	Decreasing the expression of IL-7	Exosomes relieved the severity of inflammatory bowel disease through decrease of inflammatory cytokines and macrophage infiltration	Mao et al., 2017
	Bone marrow mesenchymal stem cell derived EVs	Ultracentrifugation (100,000 *g*)	Treatment of colitis in mice	50-200 μg	Intravenous injection (one time)		EVs attenuated severity of colitis in mice through downregulation of inflammatory cytokines, regulation of antioxidant/oxidant balance, and moderation of apoptotic occurrence	Yang et al., 2015
	IL-1β pre-treated human umbilical cord mesenchymal stem cell derived exosomes	Ultracentrifugation (10,000 *g*, 110,000 *g*)	Treatment of sepsis in mice	30 μg	Intravenous injection (one time)	miR-146a	Exosomes from mesenchymal stem cells increased survival rates of septic mice, possibly through immunomodulation	Song et al., 2017
	Mesenchymal stem cell derived EVs	Ultracentrifugation (18,000 *g*, 120,000 *g*) and filtration (0.22 μm)	Treatment of autoimmune encephalomyelitis in mice	60 μg	Intravenous injection (one time)		EVs attenuated the disease phenotype, T cell proliferative potency, leukocyte infiltration, and demyelination	Jafarinia et al., 2020
	Umbilical cord-blood mesenchymal stem cell derived microvesicles		Type 1 diabetes mellitus		Intravenous infusion, two doses 1 week apart		Phase II/III, no results posted	NCT02138331
Regeneration/ healing	Human mesenchymal stem cell derived extracellular vesicles	Ultracentrifugation (10,000 *g*, 100,000 *g*)	Treatment of a murine multiple sclerosis model	25 μg	Intravenous injection (one time)		EV reduced inflammation and levels of circulating cytokines, reduced brain atrophy, and promoted remyelination and motor function	Laso-Garcia et al., 2018
	Autologous plasma rich in platelets and EVs (PVRP)	None, plasma enriched for platelets and extracellular vesicles	Healing from surgical treatment of chronic middle ear infections		Gelatine sponge soaked in PVRP applied to the wound (one time)		Recruiting	NCT04761562
	Autologous plasma rich in platelets and EVs (PVRP)	None, plasma enriched for platelets and extracellular vesicles	Treatment of chronically inflamed post-surgical bone cavities (ear)		Ear wicks soaked in PVRP (one time)		PVRP patients had improved treatment outcomes (outcomes measured: health related quality of life questionnaire and surface area of inflammation)	NCT04281901

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TABLE 1 | (Continued)

	EV type/source*	Method of isolation/ components	Application	Dosage	Method of administration	Active component/ mechanism of action	Outcome/Stage	Clinical trial number/ References
	Purified exosome product (platelet derived, PEP, Rion™)	Combination of filtration and centrifugation	Treatment (safety) of acute myocardial infarction		Intracoronary infusion (one time), 5%, 10%, or 20% PEP over 5 min		Not yet recruiting	NCT04327635
	Purified exosome product (platelet derived, PEP, Rion™)	Combination of filtration and centrifugation	Treatment (safety) of skin graft donor site wounds		Topical, 10% or 20% PEP, 10% or 20% PEP with TISSEEL (fibrin sealant)		Enrolling by invitation	NCT04664738
	Allogenic mesenchymal stem cell derived exosomes enriched with miR-124		Treatment of acute ischemic stroke		Stereotaxis/Intraparenchymal, (one time)		Phase I/II, recruiting	NCT03384433
	Adipose mesenchymal stem cell derived secretome	Concentration of conditioned media (3 kDa cut-off), whole secretome	Treatment of arthritic osteochondral explants		*ex vivo*		Secretome reduced the pathological phenotype of explanted chondrocytes	NCT04223622 (Niada et al., 2019)
	Mesenchymal stem cell derived exosomes	Sequential ultracentrifugation	Treatment of macular holes	20–50 μg	Intravitreal injection (one time)		Phase I, active	NCT03437759
	Autologous extracellular vesicles from serum		Treatment of venous ulcers		Peri-wound injection, once a week for 3 weeks		Recruiting	NCT04652531
	Allogeneic derived extracellular vesicle from mesenchymal stem cells (AGLE-102, AEGLE Therapeutics)		Treatment of dystrophic epidermolysis bullosa wounds		Up to six administrations		Phase I/II, not yet recruiting	NCT04173650
	Exosomes derived from bone mesenchymal stem cells (BMSC) and BMSCs overexpressing sonic hedgehog (Shh)	Exo Quick-TC kit	Treatment of spinal cord injuries in rats	40 μg	Intravenous injection, three injections total (once every other day)	Increased level of Shh in injured tissue	Both BMSC and BMSC-Shh exosomes promoted repair of spinal cord injuries, with BMSC-Shh exosomes more beneficial	Jia et al., 2021
	Human placental mesenchymal stem cell derived extracellular vesicles	Ultracentrifugation (10,000 *g*, 100,000 *g*)	Skin rejuvenation	75 μg	Chitosan hydrogel loaded with extracellular vesicles or extracellular vesicles alone, three injections over 2 weeks		EV treatment resulted in reversal of senescent stage of fibroblasts in aging skin, stimulating skin regeneration	Zhao et al., 2021
	Mesenchymal stem cell derived exosomes	Filtration (0.22 μm) and ultracentrifugation (100,000 *g*)	Treatment of vasculature during stent placement		Coated onto stent, implanted for 4 weeks		Exosome coated stents accelerated re-endothelialisation and decreased in-stent restenosis. Promoted tissue repair	Hu S. et al., 2021
	Adipose mesenchymal stem cell derived exosomes	ExoQuick kit or ultrafiltration (0.22 μm)	Treatment of osteoarthritis in mice	1×10^8 particles	Intra-articular injection (one time)	Exosomes regulate mTOR signaling through miR-100-5p	Exosomes protected against cartilage damaged and improved mobility	Wu et al., 2019
	Bone marrow mesenchymal stem cell derived exosomes and microparticles/ microvesicles	Filtration (0.22 μm) and ultracentrifugation (18,000 *g*, 100,000 *g*)	Treatment of osteoarthritis in mice	250–500 ng	Intra-articular injection (one time)		Protected cartilage and bone from degradation	Cosenza et al., 2017
	Mesenchymal stem cell derived exosomes	Size exclusion and concentration (100 kDa cut-off)	Treatment of osteoarthritis in rats	100 μg	Intra-articular injection weekly for 2–8 weeks	Exosomes enhanced s-GAG synthesis and suppressed NO and MMP13 production through AKT, ERK, and AMPK signaling pathways.	Exosomes reduced pain levels, reversed degeneration, and alleviated bone deterioration	Zhang S. et al., 2019
	Mesenchymal stem cell derived exosomes	Ultracentrifugation (10,000 *g*, 110,000 *g*)	Treatment of osteoarthritis in mice		Intra-articular injection every 3 days for 4 weeks		Exosomes impeded cartilage destruction	Wang et al., 2017
	Bone marrow mesenchymal stem cell derived exosomes	Exo Quick-TC kit	Treatment of osteoarthritis in rats	250 ng	Intra-articular injection (one time)	Potentially through miR-26a-5p targeting of PTGS2	Exosomes loaded with miR-26a-5p slow osteoarthritic damage in rats	Jin et al., 2020
	Mesenchymal stem cell derived exosomes	Concentration, qEV column, ultracentrifugation (100,000 *g*)	Treatment of spinal cord injuries in rats	100 μg	Adhesive hydrogel onto damaged tissue or intravenous injection		Mitigated inflammation and oxidation, promoting nerve recovery	Li L. et al., 2020

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TABLE 1 | (Continued)

	EV type/source*	Method of isolation/ components	Application	Dosage	Method of administration	Active component/ mechanism of action	Outcome/Stage	Clinical trial number/ References
	Neural stem cell derived extracellular vesicles	Ultracentrifugation with a density cushion (100,000 *g*) followed by concentration	Treatment of spinal cord injuries in rats	200 μg	Intravenous injection	Through induction of autophagy	Reduce neuronal apoptosis, neuroinflammation, and promote functional recovery	Rong et al., 2019
	Neural stem cell derived extracellular vesicles	Filtration (0.22 μm), concentration (100 kDa cut off)	Treatment of stroke in pigs		Intravenous injection, three times over the 24 h following surgery		Extracellular vesicles improved stroked animal outcomes (decreased lesion volume, brain swelling, edema, and preserved white matter integrity, and improved behavioral and mobility outcomes)	Webb et al., 2018a
	Bone marrow mesenchymal stem cell derived exosomes	Ultracentrifugation (10,000 *g*, 100,000 *g*)	Treatment of cerebral ischemia-reperfusion injury (stroke) in rats	80–120 μg	Intravenous injection one time 2 h following reperfusion	Inhibition of neuron pyroptosis	Exosomes reduced the brain infarct area and improved neurological function	Liu et al., 2021
	Hypoxia-preconditioned or normoxic mesenchymal stem cell derived extracellular vesicles (one group overexpressing miR-486-5p)	Ultracentrifugation (10,000 *g*, 120,000 *g*) and filtration (0.22 μm)	Treatment of myocardial infarction in mice and non-human primates	1 mg (non-human primates), 5 μg (mice)	Intracardiac injection (one time)	miR-486-5p promotes angiogenesis *via* downregulation of cardiac fibroblast MMP19-VEGFA cleavage	Improved cardiac function, vascular density, and smaller infarct size	Li et al., 2021d
	Exosomes derived from mesenchymal stem cells overexpressing miR-133b	Ultracentrifugation (10,000 *g*, 100,000 *g*) and filtration (0.22 μm)	Treatment of stroke in rats	100 μg	Intra-arterial injection (one time)	miR-133b	Exosomes from miR-133b overexpressing MSCs improved recovery following stroke beyond that of exosomes from control MSCs	Xin et al., 2017
	Exosomes conjugated to a cardiac homing peptide	Filtration (0.22 μm) and concentration (100 kDa cut-off)	Treatment of myocardial infarction in mice		Intravenous injection (one time)		Exosomes improved cardiac outcomes, with reduced fibrosis and increased angiogenesis	Vandergriff et al., 2018
	Human neural stem cell-derived extracellular vesicles	Filtration (0.22 μm), ultrafiltration (100 K),	Thromboembolic preclinical stroke model without immunosuppression, murine embolic model	$2.7 \times 10^{11} \pm 10\%$ vesicles/kg	Intravenous injection, three times: two, fourteen and 38 h post stroke	Decreased tissue loss (TTC metabolic tissue staining), decreased pro-inflammatory effector Th17 response,	EVs were neuroprotective, reduced lesion volume and improved functional outcomes	Webb et al., 2018b
	Bone marrow mesenchymal stem cell derived extracellular vesicles	Ultracentrifugation (12,000 *g*, 100,000 *g*)	Treatment of a murine model of rheumatoid arthritis	50 mg	Intra-articular injection twice a week	miR-21, suppressing TET1/KLF4	EVs alleviated rheumatoid arthritis progression	Li et al., 2021b
	Purified exosome product (platelet derived, PEP, Rion™)	Filtration, enucleation, and centrifugation	Treatment of ischemic wound healing in rabbits	0.6 mL 20% PEP (100% is 5 × 10e12 particles/mL)	Topical, one group as a "biogel" combined with a fibrin sealant (TISSEAL) (one time)	TGF-β. PEP regulated epithelial transdifferentiation, collagen reorganization, and guided tissue development	PEP biogel facilitated dermal wound healing	Shi et al., 2021
	M2 macrophage-derived exosomes	Ultracentrifugation (10,000 *g*, 100,000 *g*)	Treatment of a murine model of asthma	20 μg	Treatment for 3 days in a row	miR-370, inactivation of MAPK signaling pathway and downregulation of FGF1	Exosomes alleviated asthma progression	Li et al., 2021a
	Human amniotic fluid mesenchymal stem cell derived exosomes	Ultracentrifugation (13,000 *g*, 100,000 *g*)	Treatment of isoproterenol induced cardiac fibrosis in rats	100 μg	Intravenous injection (one time)	Increased levels of HIF-1α and VEGF in the heart	Alleviated extracellular matrix deposition and promoted angiogenesis, reducing the level of cardiac remodeling	Hu J. et al., 2021
Vaccine	Drug treated tumor cells from patients Tumor antigen loaded dendritic cell exosomes	None, cell secretome	Immunotherapy for malignant glioma Immunotherapy for lung cancer		Cells in small diffusion chambers with 0.1 μm exclusion limits, implanted for 48 h Intradermal injections once a week for 4 weeks	Immune activation (conclusion reached through cytokine monitoring)	Safety: well tolerated. Increased progression free survival compared with historical controls. Completed, no results posted	NCT01550523 NCT02507583 (Andrews et al., 2021) NCT01159288
	Bacterial outer membrane vesicles	Deoxycholate extraction	Vaccine against meningococcal B		Intramuscular injection, multi-dose		Phase III, completed, in use globally	NCT01478347 NCT01717638 NCT00944034 (Claassen et al., 1996; de Kleijn et al., 2001; Gorringe and Pajon, 2012)

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	EV type/source*	Method of isolation/ components	Application	Dosage	Method of administration	Active component/ mechanism of action	Outcome/Stage	Clinical trial number/ References
	Dendritic cell derived exosomes	Ultracentrifugation (10,000 *g*, 100,000 *g*)	Treatment of tumors in mice	3–5 μg	Intradermal injection (one time)	Immune activation of T lymphocytes against tumor antigens	Suppression or eradication of established tumors	Zitvogel et al., 1998
	Patient-dendritic cell derived exosomes loaded with peptides for MHC	Filtration (3/0.8 μm) and concentration (500 kDa), density cushion	Treatment of melanoma patients	$0.13–0.40 \times 10^{14}$	Intradermal and subcutaneous injections, four vaccinations over 4 weeks		Phase I, exosome administration was not toxic and had variable immune response in patients	Escudier et al., 2005
	Patient-dendritic cell derived exosomes loaded with antigenic peptides	Filtration (3/0.8 μm) and concentration (500 kDa), density cushion	Treatment of non-small cell lung cancer patients	1.3×10^{13}	Intradermal and subcutaneous injections, four vaccinations over 4 weeks		Phase I, exosome therapy well tolerated and variable immune response in patients	Morse et al., 2005
	E. coli outer membrane vesicles (OMVs) through fusion or attachment of target proteins with surface protein	Filtration (0.45 μm, 0.22 μm), concentration (50-K), and ultracentrifugation (150,000 *g*)	Promotion of immune response against tumors in mice	50 μg	Subcutaneous injection, twice (4 days apart)	OMVs delivered antigens to lymph nodes, stimulated dendritic maturation, promotes cytokine release.	Elicited a strong T-lymphocyte mediated anti-tumor immune response in mice, including immune memory	Hu S. et al., 2021
	Dendritic cell derived exosomes loaded with antigenic peptides	Filtration (3/0.8 μm) and concentration (500 kDa), density cushion	Treatment of patients with non-small cell lung cancer		Intradermal injections, four vaccinations over 4 weeks	Dendritic cell exosomes increased activity of natural killer cells	Phase II, boosted natural killer cell activity against tumors in some patients	Besse et al., 2016
	E. coli OMVs engineered to present influenza antigen-fused proteins	Filtration (0.22 μm) and ultracentrifugation (121,560 *g*)	Vaccine against H1N1/H3N2 influenza A virus	20–40 μg	Subcutaneous injection, two injections 4 weeks apart	Influenza antigens	Mice vaccinated with generated OMVs are protected against H1N1 influenza A virus	Rappazzo et al., 2016; Watkins et al., 2017
	E. coli OMVs engineered to present viral antigen-fused proteins	Filtration (0.22 μm), concentration (100 kDa cut-off) and ultracentrifugation (150,000 *g*)	Vaccine against H1N1 influenza and MERS-CoV viruses	5μg	Intramuscular injection, two injections 3 weeks apart	Viral antigens	H1N1 vaccine protected against H1N1 viral infection. MERS-CoV vaccine raised antibodies *in vivo* which neutralized inactivated MERS-CoV *in vitro*	Shehata et al., 2019
	CT-26 derived exosomes containing miR-124-3p mimic	Isolated using Exocib kit	Treatment of tumors in mice	20 μg	Subcutaneous injection three times with a 3-day interval	miR-124-3p mimic, elicited a strong anti-tumor immune response	Exosomes inhibited tumor growth and increased median survival time	Rezaei et al., 2021
COVID-19	Bone marrow mesenchymal stem cell derived extracellular vesicles (ExoFlo™, Direct Biologics™)		Treatment of COVID-19 associated acute respiratory distress syndrome		Intravenous infusion		Phase II	NCT04657458 NCT04493242
	Allogenic adipose mesenchymal stem cell derived exosomes		Treatment of COVID-19 pneumonia	2×10^8 particles	Inhalation, once a day for 5 days		Phase I completed, results not posted	NCT04276987
	CD24 overexpressing T-Rex™-293 cell derived exosomes		Treatment of COVID-19	1×10^8, 5×10^8, 1×10^9, 1×10^{10} particles	Inhalation, once a day for 5 days		Phase I/II, recruiting	NCT04747574 NCT04902183
	Donor derived COVID-19 specific T-cell derived exosomes		Treatment of COVID-19	2×10^8 particles	Inhalation, once a day for 5 days		Phase I/II, active	NCT04389385
	Zofin™ (Organicell™)—nanoparticles derived from perinatal sources	Contains soluble factors as well as extracellular vesicles and nanoparticles	Treatment of COVID-19 associated acute respiratory distress syndrome	$1–5 \times 10^{11}$ particles	Intravenous infusion, once every 4 days (three doses total)		Phase I/II, recruiting	NCT04384445 NCT04657406
	Zofin™ (Organicell™)—nanoparticles derived from perinatal sources	Contains soluble factors as well as extracellular vesicles and nanoparticles	Treatment of COVID-19 associated acute respiratory distress syndrome in three patients	$2.83 \text{ mg}/3.26 \times 10^{11}$ particles	Intravenous infusion, three-four doses over 9 days		Therapy was safe for all three patients, and all were transferred out of ICU following treatment	Mitrani et al., 2021

**Nomenclature presented is that used in study/trial.*

Hu S. et al., 2021; Jia et al., 2021; Lou et al., 2021; Saludas et al., 2021; Zhao et al., 2021). There are promising results in the utilization of EVs to repair arthritic joints (NCT04223622) (Cosenza et al., 2017; Wang et al., 2017; Wu et al., 2019; Zhang S. et al., 2019; Jin et al., 2020) and spinal-cord injuries (Rong et al., 2019; Li L. et al., 2020; Jia et al., 2021), with tissue restoration in EV treatments exceeding that of untreated animals/tissue. As these are recent developments, mechanism of action and clinical translation are still underway. However, there are many clinical trials addressing the regenerative capacity of EVs in surgical and dermal wound healing and tissue regeneration (NCT04652531, NCT04173650, NCT04664738, NCT04761562, and NCT04281901). The regenerative capacity of EVs is also relevant in rescuing and restoring function in damaged organs, including brain (Galieva et al., 2019; Beard et al., 2020) and heart (Kennedy et al., 2020). Animal models of stroke treated with EVs show improved outcomes (Doeppner et al., 2015; Otero-Ortega et al., 2017; Tian et al., 2018; Webb et al., 2018a; Spellicy et al., 2020; Liu et al., 2021), in particular those derived from MSCs overexpressing miR-133b (Xin et al., 2017). Indeed, overexpression of miRNAs in EVs represents a promising avenue, with a clinical trial administering EVs enriched with miR-124 for stroke recovery underway (NCT03384433). Cardiac damage following myocardial infarction is debilitating and previously un-preventable, however, there has been significant preclinical success using EVs in reducing infarct size and restoring cardiac function in various *in vivo* models (Barile et al., 2014; Bian et al., 2014; Chen C. W. et al., 2018; Vandergriff et al., 2018; Hu S. et al., 2021; Saludas et al., 2021; Zhu et al., 2021b). There is now a clinical trial treating patients with an EV product following acute myocardial infarction (NCT04327635). While we wait for results from these trials to be released, the applications and modulation of EV therapies will continue to be developed for the next phase.

Combination Therapies

The therapeutic and clinical potential of EVs is further elevated through their biocompatibility and capacity to be surface modified and coupled with other remedial approaches. As discussed, protein or nucleic acid landscape can be modulated in parental cells to generate vaccines (Rappazzo et al., 2016; Watkins et al., 2017; Shehata et al., 2019; Polak et al., 2020; Hu S. et al., 2021) or bio-modified (biosimilar) therapeutics (Xin et al., 2017). These combination therapies can also utilize the protection, stability, and targeting capacity that EVs provide to mediate the delivery of drugs or gene vectors which are otherwise degraded or cleared. Trials *in vivo* have proven the efficacy and feasibility of EV-mediated adeno-associated virus (AAV) and siRNA delivery when uptake levels of the component alone are sub-optimal (Gyorgy et al., 2017; Munagala et al., 2021). Indeed, in *Lhfpl5*−/− hereditary deafness, EV-mediated delivery of AAV was more effective in rescuing hearing than AAV treatment alone (Gyorgy et al., 2017). Additionally, EVs delivering siRNA-*KRAS* were able to successfully silence *KRAS* in murine tumor tissues, reducing tumor size; the same technology also enabled the delivery of plasmids to successfully replace/restore genes (Munagala et al., 2021). Furthermore, the incorporation of drugs and molecular factors into EVs improves their bioavailability and delivery, increasing efficacy of treatment (Aqil et al., 2017; Bellavia et al., 2017; Luan et al., 2017; Liang et al., 2020, 2021). EVs containing curcumin (a natural anti-inflammatory compound) significantly inhibited tumor size when compared with either component alone by increasing curcumin's bioavailability (Aqil et al., 2017). Indeed, the success of this treatment *in vivo* has led to an active clinical trial investigating the effect of curcumin-containing plant exosomes in the treatment of colon cancer (NCT01294072). While there are few clinical trials using EV-combination therapies (NCT04747574, NCT01294072, and NCT03384433), the pre-clinical success supports the translational potential of this approach.

Commercial Pursuit of Clinical Translation

Already the revolution of EV therapeutics is underway, with many emerging and established companies now focused on their development and application (Gimona et al., 2017; Zipkin, 2019). Ventures in treatments for dermatological disorders and skin repair (Aegle Therapeutics, XOStem Inc., Exogenus Therapeutics), as well as cancer (Aethlon Medical, Unicyte AG, TAVEC Pharmaceuticals, Puretech Health, EV Therapeutics Inc., Anjarum Biosciences, Codiak Biosciences) and neurological disorders/diseases (Stemcell Medicine Ltd., Puretech Health, Evox Therapeutics, Codiak Biosciences) dominate the commercial EV-based therapeutic landscape. Codiak Biosciences have identified two EV-associated membrane proteins (internal/lumen or external orientated), which they use as scaffolds to link molecules of interest and engineer EVs for therapeutic application; this engineering platform (engEx^{TM}) has been utilized to develop exoSTING (EVs enriched in stimulator of interferon genes in the lumen) (Jang et al., 2021), and exoIL12 (Lewis et al., 2021), currently in Phase 1/2 clinical trial (NCT04592484). Significant efforts in other areas include wound healing (RION Health), metabolic disorders (Evox Therapeutics), fibrotic and immunological conditions (Puretech Health), acute respiratory distress syndrome (Direct Biologics), kidney and liver disease (Unicyte AG), and inflammation (Direct Biologics, Cell-Factory BVBA, Puretech Health). Also in development are treatments for specific debilitating and/or life-threatening disorders (VivaZome Therapeutics), retinal disease (ReNeuron), genetic diseases (Anjarium Biosciences), diabetes (Unicyte AG), stroke (ReNeuron), lymphatic disorders (Puretech Health), and cystic fibrosis (OmniSpirant). With EV therapeutics being developed to combat a wide variety of diseases, understanding these membranous entities, their activities, and their engineering are all critical for achieving full therapeutic potential.

EVs AS NANOCARRIERS OF FUNCTIONAL CARGO

Native EVs are cell-derived, lipid-bound nanoparticles (30–1000 nm) that facilitate intercellular communication through targeted delivery of bioactive cargo to alter recipient cell

phenotype (Al-Nedawi et al., 2008; Skog et al., 2008; Alvarez-Erviti et al., 2011; Peinado et al., 2012; Kamerkar et al., 2017; Wu et al., 2019; Cocozza et al., 2020; Li et al., 2021b) [reviewed in Vader et al. (2016), Murphy et al. (2019), and Russell et al. (2019)]. EVs are involved in transferring molecular cargo, between animal cells (Valadi et al., 2007; Mittelbrunn et al., 2011; Ono et al., 2019), from parasite to its mammalian host (Buck et al., 2014; Coakley et al., 2017), and from plant to fungal cells (Cai et al., 2018). In addition to their role in communication between cells and organs, EVs are excellent therapeutic delivery systems due to their: (i) ability to protect bioactive cargo, (ii) inherent biocompatibility, (iii) small size and negative surface charge, (iv) ability to cross biological membranes, including blood-brain barrier (BBB), and (v) capacity to target specific cells. The rigid structured membranes [resulting from an abundance of sphingomyelin (Niemela et al., 2004; Saeedimasine et al., 2019; Smith et al., 2020) and cholesterol (Needham and Nunn, 1990; Leftin et al., 2014)] of EVs protect biological macromolecules from degradation [i.e., nucleases (Skog et al., 2008; Cheng et al., 2014; Fernando et al., 2017) and proteases (Haney et al., 2015; Cvjetkovic et al., 2016; Sterzenbach et al., 2017; Zaborowski et al., 2019)] and micro-environmental changes [i.e., pH (Parolini et al., 2009; Aoi and Marunaka, 2014) and osmolarity (Fathali et al., 2017)]. Therefore, compared to soluble factors, EVs more efficiently deliver a complex variety of active biomolecules (Obregon et al., 2009) resulting in greater potency (Mitchell et al., 2019; Cocozza et al., 2020; Gurung et al., 2020). As a result of their cellular origin, EVs are associated with low immunogenicity (Zhu et al., 2017; Sharma et al., 2020) and are capable of interacting with innate immune cells through surface-expressed components (Admyre et al., 2003; Tietjen et al., 2014; Rao et al., 2015; Antes et al., 2018) [e.g., anti-phagocytic signals (Rodriguez et al., 2013; Gordon et al., 2017; Kamerkar et al., 2017; Hsu et al., 2018; Tang Y. et al., 2019; Cordonnier et al., 2020; Daassi et al., 2020) and opsonins (Miksa et al., 2009)]. EV-mediated crosstalk may occur unidirectionally or reciprocally or even *via* systemic communication, during which EVs target to various tissues and organs. Importantly, the EV surfaceome also facilitates inherent targeting properties/tropism (Nakamizo et al., 2005; Kidd et al., 2009; Hoshino et al., 2015; Wu et al., 2020) and direct signal transduction (Nazarenko et al., 2010; Rana et al., 2011; Sobo-Vujanovic et al., 2014; Vinas et al., 2018). Further, EV surface composition is important for traversing biological barriers [e.g., BBB (Haney et al., 2015; Hall et al., 2016; Kojima et al., 2018)] through receptor-mediated transcytosis. The mechanisms by which EVs are internalized by target cells are still poorly understood, often uptake is cell and context dependent. Currently known cellular entry routes of EVs are complex mechanisms ranging through receptor-mediated endocytosis, lipid raft interactions, clathrin interactions, phagocytosis, micropinocytosis and possibly direct fusion (Mathieu et al., 2019). As such, uptake and intracellular mechanisms of trafficking and localization requires more in-depth evaluation exploring subcellular analyses using high-resolution microscopy, subcellular composition studies, or novel live-cell reporters (Chen M. et al., 2018; Sung et al., 2020). In this section, we discuss EVs as carriers of functional cargo and their therapeutically promising characteristics.

Cell Reprogramming Using EVs

Preclinical development of EVs have focused on their ability to horizontally transfer bioactive components, eliciting transcriptional and translational modulation (Valadi et al., 2007), antigen presentation (Gurnani et al., 2004; Garcia et al., 2016), and functional regulation in recipient cells (Al-Nedawi et al., 2008; Skog et al., 2008; Alvarez-Erviti et al., 2011; Peinado et al., 2012; Kamerkar et al., 2017; Wu et al., 2019; Zhang Q. et al., 2019; Cocozza et al., 2020; Li et al., 2021b). A landmark study demonstrated that embryonic stem cell derived EVs contribute to epigenetic reprogramming of target cells (Ratajczak et al., 2006); EVs were enriched in key players for early pluripotency, hematopoietic stem cells (Scl, HoxB4 and GATA 2), and induced MAPK/Akt phosphorylation. Here, mRNA could be delivered by EVs to target hematopoietic stem/progenitor SKL cells and translated as Oct-4 protein expression. Of note, the biological effects of EVs were perturbed following heat inactivation or pretreatment with RNAse, indicating major involvement of the horizontal transfer of mRNA/proteins by EVs in the observed phenomena. Recently, adipocytes have been shown to release lipid-filled EVs that serve as the primary source of lipid for adipose-resident macrophages (Flaherty et al., 2019). Adipocyte-derived EVs have been described previously, primarily as regulators of inflammation and systemic insulin resistance (Thomou et al., 2017). The uptake of adipocyte EVs facilitated the direct transfer of lipids, and a macrophage-type specific transcriptional activation of lipid metabolism in bone marrow cells., revealing a form of intercellular communication and nutrient exchange with important implications for obesity-associated pathologies (Flaherty et al., 2019). Thus, EVs exert functional effects on recipient cells by modifying their composition through protein, lipid or nucleic acid transfer. This mechanism of action has also been demonstrated in other diseases (i.e., sepsis, fibrosis, cardiomyopathy) where various induced disease models were used to highlight the protective and regenerative roles of EVs, as well as identify the key EV-associated bioactive molecules involved in such functions (**Table 2**), which provides a strategy to improve the efficacy of EV treatment. In an acute myocardial infarction (AMI) murine model (Gu et al., 2018), EV-mediated transfer of miR-21 protected cardiomyocytes from oxidative stress-induced apoptosis (Xiao J. et al., 2016; Shi et al., 2018) through the inhibition of pro-apoptotic PDCD4 (Xiao J. et al., 2016; Gu et al., 2018) and cell cycle-regulator PTEN (Shi et al., 2018). Treatment of miR-21 mimics *in vitro* and *in vivo* then significantly reduced apoptosis in cardiomyocytes following AMI, and dramatically attenuated infarct size in mouse hearts. As conduits of bioactive molecule transfer, EVs are also exploited as nanocarriers/delivery systems that provides a sustainable mode of miRNA delivery. Transfer of miRNAs from miR-let7c-overexpressing MSCs to renal cells *via* secreted EVs, suppressed the expression of fibrotic genes, thus reducing interstitial collagen production, attenuating renal fibrosis, and improving kidney structure (Wang et al., 2016).

TABLE 2 | Evidence supporting the role of extracellular vesicle cargo in intercellular communication.

Concept	Repro-gramming	Tropism	Donor cell	Recipient cell	Active cargo	Approach used to confirm cargo functionality	Key findings	References
Protein-based	Y	N	EGFRvIII-positive gliomas (U373 (human astrocytoma) cells)	EGFRvIII-negative gliomas (U373) and human umbilical vein endothelial cells (HUVECs)	EGFRvIII	Western blot validation of MAPK and Akt phosphorylation, changes in expression of EGFRvIII-regulated genes (VEGF, Bcl-xL, p27), and functionally, morphological transformation and increase in anchorage-independent growth capacity	(i) Determined that EGFRvIII-positive gliomas release EVs that express EGFRvIII, (ii) demonstrated activation of associated signaling pathways (MAPK and Akt), changes in expression of EGFRvIII-regulated genes (VEGF, Bcl-xL, p27), and (iii) functionally, morphological transformation and increase in anchorage-independent growth capacity	Al-Nedawi et al., 2008
	Y	N	Human U87 glioma cells	Brain microvascular endothelial cells (HMBVECs)	Angiogenin, IL-6, IL-8, TIMP-1, VEGF and TIMP-2	Validated enrichment of angiogenic proteins in EVs compared to cells using western blot, human angiogenesis antibody array, and functional output (increased angiogenesis)	(i) Demonstrated EV protection of RNA content through exogenous treatment of RNAse, (ii) demonstrated that recipient cells can translate EV-associated RNA (i.e., Gluc mRNA) by observing Gluc activity following EV treatment, (iii) mRNA analysis of glioma-derived EVs highlighted their biological role in angiogenesis, (iv) EVs increased HMBVEC angiogenesis upon treatment, (v) identified angiogenic proteins enriched in EVs through human angiogenesis antibody array and angiogenesis assay	Skog et al., 2008
	Y	Y	Organotropic human breast and pancreatic cancer cell lines that metastasise to the lung (MDA-MB-231), liver (BxPC-3 and HPAF-II), or both (MDA-MB-468), and MDA-MB-231 sub-lines that colonize lung, bone or brain (4175-LuT, 1833-BoT or 831-BrT) respectively	6–8-week-old NCr nude (NCRNU-F sp./sp.) females, human lung fibroblasts WI-38, human bronchial epithelial cells HBEpC, human Kupffer cells	Integrins α6β4 and αvβ5	Knockdown of integrin expression using short hairpin RNAs (shRNAs) demonstrated three-fold reduction of EVs in the lung compared with control EVs, and blocking of cognate ligands of specific integrins markedly reduced uptake by recipient cells	(i) Validated natural and efficient homing capacity of EVs derived from three metastatic cancer cell lines to their corresponding primary sites of metastasis (see donor cells), (ii) demonstrated that EV with specific homing can induce metastasis in corresponding target organ, (iii) highlighted association of specific integrins to EV homing capacity, (iv) demonstrated selective uptake of EVs by specific cell types in target organ *via* expression of cognate ligands on recipient cells, (v) observed EV treatment upregulated gene expression, particularly pro-inflammatory *S100* genes involved in metastasis, as demonstrated by an increase in proliferation and migration in recipient cells following EV treatment, (vi) proposed that integrin expression in plasma-derived EVs is good biomarker for organotropism	Hoshino et al., 2015
	Y	Y	Murine microglia cell line (BV-2)	Myeloid cells, primary microglia, and bone marrow and peritoneal macrophages, and female C57BL/6 mice and R26-stop-dtTomato mice	IL-4, MFGE8	Overexpression of IL-4 upregulated anti-inflammatory markers (arg1 and ym1) in myeloid cells *in vitro*, and in primary microglia and peritoneal macrophages, upregulated anti-inflammatory markers (CD206 and arg1), and decreased pro-inflammatory marker iNOS, both at the protein and mRNA levels. MFGE8 inhibition *via* their corresponding integrin αvβ5 significantly reduced the biological effect of IL-4 + EVs as well as downstream mRNA expression	(i) Validated presence of IL-4 in EVs following plasmid transfection, (ii) observed upregulation of anti-inflammatory markers (i.e., arg1, ym1) in myeloid recipient cells and upregulation of anti-inflammatory markers CD206 and arg1, and downregulation of pro-inflammatory marker iNOS in primary microglia and peritoneal macrophages at protein and mRNA levels, (iii) demonstrated that EV-mediated IL-4 signaling requires surface receptor on recipient cell, and STAT6 signaling, (iv) displayed increase in EV delivery *in vivo* following induced MFGE8 expression, particularly in myeloid cells and astrocytes following injection into mouse cisterna magna, (v) demonstrated IL-4 + and MFGE8 + EVs significantly protected mice from clinical and neuropathological symptoms of disease (i.e., neuroinflammation), (vi) demonstrated, using Cre models, that M2 polarization (anti-inflammatory process) induced by EVs is dependent on intracellular cargo delivery	Casella et al., 2018
	Y	Y	Mouse melanoma (B16F10) cells	Mouse bone marrow-derived cells (BMDCs)	MET	Silencing MET using shRNA and crizotinib (MET inhibitor)	(i) Demonstrated EV-mediated contribution to tumor growth and metastasis through transfer of MET to mobilize BMDCs to form a pre-metastatic niche, and (ii) reduced tumor growth using EVs expressing low levels of MET	Peinado et al., 2012
	Y	Y	Pancreatic ductal adenocarcinoma cells (PDACs)—Mouse (R6560B, PAN02) and human PDACs (BcPC-3)	Mouse and human liver Kupffer cells	MIF	Silencing MIF using shRNA	(i) Demonstrated EV-associated MIF homing to and activation of Kupffer cells in liver to release TGFB, and induce fibrotic pre-metastatic niche and recruitment of BMDC, then (iii) inhibited liver pre-metastatic niche initiation by suppressing MIF expression in EVs	Costa-Silva et al., 2015
	Y	N	Primary rat cardiomyocytes (1–2 days old rat pups), H9C2 (embryonic rat heart tissue)	HUVECs and primary adult cardiac rat microvascular ECs (CMVEC)	GLUT1, GLUT4	Western blot validation of GLUT1/4 presence in EVs, demonstrated EV-associated GLUT1/4-mediated glucose uptake by endothelial cells (function), and established EV-association with GLUT1/4 through co-localization of fluorescent EVs and GLUT1/4	(i) Glucose starvation of parental cells increased production of EVs, which contained GLUT1/4 (demonstrated *via* western blot), (ii) established EV-associated GLUT-mediated glucose uptake by endothelial cells through EV and GLUT-inhibitor (FAS) treatment, and (iii) demonstrated EV-associated GLUT1/4 uptake by endothelial cells through fluorescent imaging (co-localization of EV marker CD63 and GLUT1/4)	Garcia et al., 2016

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TABLE 2 | (Continued)

Concept	Repro-gramming	Tropism	Donor cell	Recipient cell	Active cargo	Approach used to confirm cargo functionality	Key findings	References
Glycolipid-based	Y	N	Murine neuroblastoma Neuro2a (N2a), murine microglial cell line (BV-2)	Wild-type C57BL/6 mice, heterozygotic transgenic mice expressing human APP bearing the Swedish and Indiana (KM670/671NL, V717F) mutations (APPswelnd or J20 strain)	Glycosphingolipid (GSL)-glycan	Cleavage of EV-associated GSL glycans sufficiently prevents the association of EVs with Aβ: deglycosylation of GSL-glycans on EVs using EGCase inhibited EV-dependent amyloid fibril formation, cleavage of sialic acids using sialidase demonstrated similar reductions in fibril formation, and steric blocking of GM1 or GM2 ganglioside by cholera toxin subunit B or anti-GM2 antibody significantly suppressed amyloid formation	(i) Demonstrated that N2a-derived EVs are associated with membrane glycolipid GM1 ganglioside, and are incorporated into hippocampal β-amyloid plaques (Aβ) and microglia in murine brain following injection (ii) continuous administration of EVs using osmotic minipumps ameliorated Aβ pathology and synaptic dysfunction caused by APP, (iii) highlighted significant enrichment (∼2,300%) of GSLs in EVs compared to parental cells, (iv) disruption of GSL-glycans on EV surface *via* sialidase to reduce surface interaction, EGCase for cleavage of molecule, prevented their association with Aβ, highlighting their importance in EV-binding to Aβ *in vitro* and *in vivo*	Yuyama et al., 2014
RNA-based	Y	N	Murine embryonic stem cells (ESC)	Murine hematopoietic progenitor cells (HPC), murine Sca-1 + /kit + /lin− (SKL) cells	Oct4	Demonstrated enrichment of Oct-4 mRNA and protein in EVs compared to parental cells, and that upon treatment with EVs, recipient cells displayed increased expression of Oct-4 protein. Further demonstrated Oct-4 mRNA-mediated reprogramming by pre-treating EVs with RNAse, which diminished Oct-4 expression in recipient cells	Demonstrated ESC-derived EVs (i) enhanced survival of murine SKL progenitor, protecting them from them from undergoing apoptosis, (ii) induced phosphorylation of MAPKp42/44 and AKT in murine Sca-1 + cells, (iii) are enriched in mRNAs (Oct-4, Rex-1, Nanog, SCL and GATA-2) and proteins (Wnt3, Oct4) compared to parental cells, and (iv) potentially transfers Oct4 mRNA to increase Oct4 protein expression in recipient cells, which can be diminished by pre-treatment with RNAse	Ratajczak et al., 2006
	Y	N	Colorectal cancer (SW480)	SW480	miR-19b	Inhibition of EV production using sphingomyelinase inhibitor gw4869, and worked with analogous system of inhibition and overexpression of miR-19b	(i) Demonstrated EV-associated miR-19b downregulated oxaliplatin sensitivity in SW480 cancer cells, then (ii) inhibited EV production using gw4869 which restored sensitivity	Gu Y. Y. et al., 2019
	Y	N	IL-1β-treated human umbilical cord-derived mesechymal stem cells	Mouse bone marrow-derived macrophages (BMDMs)	miR-146a	Overexpression of miR-146a showed the same effect as EV treatment whereas the inhibition of this miR (by transfection) abrogated the efficiency of M2 polarization	(i) Inhibition of EV production caused a M1 polarization (ii) EV treatment caused M2 polarization in macrophages. (iii) Exosomal miR-146a was the key player for macrophage M2 polarization leading to an increased survival in septic mice.	Song et al., 2017
	Y	N	Amniotic fluid stem cells (AFSCs)	CTx-damaged granulosa cells	miR-10a and miR-146a	Inhibition of RNA activity through RNAse treatment of AFSC-EVs reduced protective function, attributed function to miR10a and miR-146a by using liposomes as nanocarrier, and demonstrated similar effects to EV treatment, and inhibition of miR-146a and miR-10a by transfection of parental cells suppressed rescue effects in recipient cells	(i) AFSC-CM or EV treatment decreased cytotoxicity and apoptosis of granulosa cells (GCs), and AFSC-EVs preserved healthy follicles in mice, (ii) identified miR-146a and miR-10a enrichment in AFSC-EVs and through KO of these factors, attributed mechanism of function to apoptosis-related downstream targets including IRAK1 and TRAF6, and BIM respectively, (iii) highlighted miR-146a and miR-10a functions using liposome-mediated encapsulation and delivery to damaged GCs	Xiao G. Y. et al., 2016
	Y	N	Adipose derived mesenchymal stem cells (ADMSCs)	Human dermal fibroblasts (HDF)	miR-21 and miR-29c	Transfection of MSC-EV with the corresponding miR mimics dramatically enhanced (more than 10-fold) the transfer of miRs to the fibroblasts, while transfection of MSC-EV with antagomir significantly decreased expression in fibroblasts	(i) Compared to conditioned media and soluble fraction, ADMSC-EVs caused the most significant decrease in α-SMA mRNA expression in recipient cells, preventing HDF differentiation into fibrotic myofibroblasts, (ii) identified pro-fibrotic miR-21 and anti-fibrotic miR-29c as most enriched in ADMSC-EVs, and (iv) demonstrated dramatically enhanced transfer and function of miRNAs in fibroblasts following transfection with mimics and significantly decreased transfer and function of miRNAs following transfection with antagomirs	Basalova et al., 2020
	Y	N	Blood/serum (mice, human)	*In vitro*—primary neonatal rate cardiac myocytes (NRCMs) and *in vivo*—intramyocardial injection	miR-21	Addition of miR-21 mimics to cells induced cardioprotection, and inhibition of PDCD4 (downstream target of miR-21) resulted in the same cardioprotective effect	(i) Demonstrated selective enrichment of miR-21 in EVs compared to supernatant, and its inhibitory effects on PDCD4 expression as well as anti-apoptotic effects in recipient cells, then (ii) demonstrated EV-mediated reduction of infarct size in mice hearts	Gu et al., 2018
	Y	N	Bone marrow mesenchymal stem cells	C-kit + cardiac stem cells	miR-21	Function of miR-21 highlighted through transfection with mimic and inhibitor and observing respective functions, where miR-21 mimic markedly decreased apoptosis and miR-21 inhibition increased apoptosis, also observed expression of downstream regulators pAKT and PTEN which pathway is responsible for regulating apoptosis in recipient cells	(i) Established enrichment of miR-21 in EVs derived from H2O2-treated cells, which prevented oxidative-stress induced apoptosis, and highlighted miR-21 as apoptosis regulator through transfection with mimic and inhibitor and observing marked decrease in apoptosis with mimic and increased apoptosis in inhibitor, (ii) identified key pathway involved in this function as PTEN/PI3K/AKT by studying downstream targets of miR-21, including increased expression of pAKT, and decreased expression of PTEN	Shi et al., 2018
	Y	N	Plant (*Arabidopsis*)	Fungal (*Botrytis cinerea*)	Several dozen different small RNAs (e.g., TAS1c-siR483, TAS2-siR453, IGN-siR1, and miRNA166)	Transferred host sRNAs induce silencing of fungal genes critical for pathogenicity. Host sRNAs were protected from nuclease digestion, tetraspanins associated with their transferring to fungal cells, with transferred host sRNAs silence fungal virulence genes and suppress fungal pathogenicity (directly regulates virulence not growth). Host mutant strains (lacking target sRNAs) showed reduced virulence.	Findings support that transferred host sRNAs (Arabidopsis) contribute to host immunity by silencing fungal pathogen (B. cinerea) genes. Demonstrates cross-kingdom sRNA trafficking	Cai et al., 2018

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TABLE 2 | (Continued)

Concept	Repro-gramming	Tropism	Donor cell	Recipient cell	Active cargo	Approach used to confirm cargo functionality	Key findings	References
	Y	N	Nematode (*Heligmosomoides polygyrus)*	Murine intestinal epithelial (MODE-K) cells	Various microRNAs (miR-193, miR-10 and miR-200), Y RNAs, and nematode Argonaute protein	Microarray analysis identified (128 genes differentially expressed upon treatment) with Il33r and Dusp1 as key suppressed genes, and Dusp1 repressed by nematode miRNAs. validated by RT–qPCR, and control EV (mouse intestinal cell) treatment. Identify miRNAs from filarial nematode in serum of infected mice	(i) miRNA secretion into host tissues is conserved among parasitic nematodes, (ii) secreted nematode miRNAs that have identical seed sites to mouse miRNAs can downregulate DUSP1 through conserved sites in its 3′UTR, (iii) suppress an innate Type 2 response *in vivo*, (iv) mechanism by which helminths manipulate their hosts (combination of RNA- and protein-based regulatory function) and provide a mechanistic framework for RNA transfer between animal species	Buck et al., 2014
	Y	N	Nematode (*Heligmosomoides polygyrus)*	Bone marrow stem cells, bone marrow macrophages, MODE-K cells	N/A	Macrophage internalization of EVs causes downregulation of type 1 and type 2 immune-response-associated molecules (IL-6 and TNF, and Ym1 and RELMα) and inhibits expression of the IL-33 receptor subunit ST2. EV target antibodies abrogated suppression of alternative activation and associated with increased co-localization of intracellular EVs with lysosomes.	(i) Internalized EVs can induce alternative activation and IL-33-mediated signaling in macrophage to counter parasite expulsion, (ii) EV target antibodies increase their co-localization with lysosome in macrophages (iii) targeting EVs by vaccination provide protective immunity against the parasite, highlighting their key role in establishing infection	Coakley et al., 2017
	Y	N	Raji B-cell line, Jurkat-derived J77 T cell line, and primary dendritic cells (DCs) derived from human monocytes	Antigen-presenting cells (APCs)	miR-335 and miR-92a	(i) Demonstrated miRNA transfer *via* immunological synapse (IS) through co-culture of J77 cells overexpressing miR-355 with Raji B cells following treatment of SEE which activates formation of IS, (ii) blocking EV production significantly impaired miRNA transfer *via* EVs, (iii) demonstrated miR-355 synaptic transfer downregulates its target SOX4 in recipient APCs	(i) Profiled and identified enrichment of small RNA species in EVs compared to parental cells, (ii) demonstrated that miRNAs are transferred *via* immunological synapse between immune cells and APCs through overexpression of miR-355, (iii) inhibition of EV production through nSMase2 significantly impaired transfer of EV-associated miRNAs, (iv) using luciferase assays, demonstrated synaptic transfer of miR-335 downregulates miR-335 downstream target SOX4 in the APC	Mittelbrunn et al., 2011
	Y	N	Mast-cell line (MC/9), primary bone marrow-derived mast cells (BMMC) and human mast-cell line (HMC-1)	MC/9, BMMC, HMC-1, mouse CD4 + T cells	mRNA coding for proteins (Cox5b, Hspa8, Shmt1, Ldh1, Zfp125, Gpi1 and Rad23b)	(i) *In vitro* translation assay was conducted, comprising of mRNA isolation and translation, then separation of resultant proteins by two-dimensional PAGE to identify seven new proteins which were excised and analyzed by LC-MS/MS, (ii) demonstrated and quantified EV-mediated mRNA transfer using radioactive labels and scintillation counting, (iii) demonstrated interspecies mRNA translation, where human mast cells translated two mouse proteins corresponding to the EV-associated mouse mRNA	(i) Identified multiple and heterogenous mRNA species within BMMC and MC/9 EVs using microarray assessment, (ii) function of EV-associated mRNA were demonstrated using a rabbit lysate *in vitro* translational assay, identifying seven proteins associated with the mRNA, including Cox5b, Hspa8, Shmt1, Ldh1, Zfp125, Gpi1 and Rad23b, (iii) through radioactive (3H-uracil) labeling of MC/9 EVs, demonstrated mRNA transfer to MC/9 cells and HMC-1 cells, but not CD4 cells, (iv) using species differences (mouse vs. human), identified three new mouse proteins in human recipient cells following EV treatment, two of which mRNAs were present in the microarray, suggesting that they were translated by recipient cells, (v) identified several miRNA species enriched in EVs compared to parental cells, with potential roles in angiogenesis, hematopoiesis, exocytosis and tumorogenesis	Valadi et al., 2007
	Y	N	Adipose derived mesenchymal stem cells (ADMSCs)	HUVECs	miR-125a	Overexpression of miR-125a (i) inhibited expression of downstream target DLL4 (angiogenic repressor), (ii) increased angiogenesis, (iii) upregulated pro-angiogenic genes and downregulated anti-angiogenic genes, whereas inhibition of miR-125a (by transfection) exerted the opposite effect on endothelial cells	(i) Demonstrated that EV treatment increased angiogenesis *in vitro* and *in vivo*, (ii) determined miR-125a enrichment in EVs compared to parental cells, (iii) established miR-125a functional influence on HUVECS to increase angiogenesis, and upregulation of pro-angiogenic and downregulation of anti-angiogenic gene expression (iv) inhibited downstream target of miR-125a (DLL4—angiogenic repressor) and its influence on angiogenesis through endothelial tip formation	Liang et al., 2016

Extracellular vesicles have also been shown to contain and transfer tyrosine kinase receptors (i.e., EGFRvIII) to increase downstream signaling events (MAPK, AKT) and expression of EGFRvIII-regulated genes (*VEGF, Bcl-xL, p27*) involved in tumor vascularization, survival and proliferation (Al-Nedawi et al., 2008). EV-mediated delivery of functional receptors can also be physiological, as demonstrated by the transfer of glucose transporters (GLUT) 1 and 4 between cardiomyocytes and endothelial cells during metabolic stress to increase glucose uptake by endothelial cells and mediate plasma glucose concentration (Garcia et al., 2016). In addition to their functional value, the presence of intact receptors in EVs can facilitate loading of bioactive ligands through their ligand-receptor interaction, increasing the efficacy of EV function on recipient cells. As a cerebral hypoxia/ischemia treatment, EV-bound transferrin receptor was exploited to load apotransferrin into EVs, which, when administered to animal demyelination models, conferred neuroprotection and reduced white matter damage, neuronal loss, and astrogliosis through apotransferrin-activated differentiation of oligodendroglial cells in the brain (Mattera et al., 2020).

This protective effect of EVs have also been shown to rescue adverse side-effects following chemotherapy-induced damage. EVs derived from amniotic fluid stem cells enriched in pro-survival miR-146a and miR-10a were directly injected into murine ovaries, where they promoted the survival and elevated the apoptotic resistance of granulosa cells in mice undergoing chemotherapy; side effects can otherwise cause premature ovarian failure and aberrant fertility (Xiao G. Y. et al., 2016). This is attributed to the apoptosis- and inflammation-related downstream targets of EV-associated and transferred miR-146a and miR-10a, including IRAK1 and TRAF6, and BIM respectively (Xiao G. Y. et al., 2016). However, if these anti-apoptotic EVs are trafficked to off-target sites (i.e., tumors), they can significantly reduce the sensitivity of tumor cells to chemotherapy treatment, as demonstrated by EV-mediated transfer of miR-19b (Gu Y. Y. et al., 2019). Therefore, stringent regulation of EV-associated bioactive cargo and components of interest is critical in developing EVs for therapeutic application.

For further streamlining of functional/therapeutic effect, EV donor cell source is a critical factor for consideration, particularly in the field of regenerative medicine, where MSCs, with unique multipotent differentiation potential (Sasaki et al., 2008; Sarugaser et al., 2009; Dos Santos et al., 2019), are expansively explored in various pathologies and regenerative applications. Importantly, MSC-derived EVs have been shown to alleviate CNS-associated disorders/injuries, including subcortical stroke [i.e., through axonal sprouting, tract connectivity, remyelination and oligodendrogenesis (Otero-Ortega et al., 2017)], and, cerebral ischemic-reperfusion injury [i.e., by conferring neuroprotection through modulation of M1-M2 microglia toward an anti-inflammatory phenotype (Liu et al., 2021)]. They are also an avenue for treatment of physical ailments, such as osteoarthritis [i.e., by attenuating inflammation and regulating extracellular matrix synthesis/degradation (Cosenza et al., 2017; Wang et al., 2017; Zhang S. et al., 2019)], skeletal muscle injury [i.e., through promotion of skeletal muscle cell proliferation, differentiation and migration (Mitchell et al., 2019)], and ageing [i.e., by ameliorating dermal fibroblast senescence and promoting skin rejuvenation (Zhao et al., 2021)]. In models for cardiac injury, MSC-derived EVs have been shown to promote proliferation and differentiation of epicardial-derived cells and improve cardiac morphology (Arslan et al., 2013; Zhu et al., 2021b). In fact, improvements for sustained administration of MSC-EVs have been made to enhance their effects at sites of cardiac (Chen C. W. et al., 2018), spinal cord (Li L. et al., 2020), or hepatic injury (Mardpour et al., 2019), by loading them into biocompatible hydrogels which are then administered to target sites.

For therapeutic development, it must be noted that EV composition and release is influenced by environmental and signaling changes occurring in donor cells {i.e., hypoxia, stress [metabolic (Fan et al., 2020), heat, oxidative], infection (Pegtel et al., 2010) and cell activation (Gao et al., 2019, 2020)} as a mechanism of homeostatic maintenance (Takahashi et al., 2017) [reviewed in Desdin-Mico and Mittelbrunn (2017)], thus, EV donor cell culture conditions may be modified accordingly to suit various clinical applications. For instance, MSCs under high oxygen level-induced oxidative stress release depolarized mitochondria to remove accumulated reactive oxygen species (ROS) generated as a result of oxidative phosphorylation (Phinney et al., 2015). The EV-associated mitochondria are transferred to and metabolically reprogram recipient cells with potent regenerative effects, as observed in cardiomyopathy (Plotnikov et al., 2008; Ikeda et al., 2021), and renal (Plotnikov et al., 2010) and pulmonary (Islam et al., 2012) injury. Another key, yet not fully explored consideration in this study, is the synergistic function of EV cargo. For example, in a pulmonary silicosis-induced murine model, MSC-derived EVs transfer both mitochondria and anti-inflammatory and immunomodulatory miR-451 to macrophages; increasing their metabolic state while inhibiting their activation and protecting them from excessive inflammation caused by accumulated mitochondrial ROS (Phinney et al., 2015). The synergistic function performed in this study by EV-associated mitochondria and anti-inflammatory miRNA is therefore a well-balanced one, highlighting a need for in-depth EV composition characterization during their development as therapeutics. Modifying protein cargo loading into EVs can be manipulated in donor cells directly and exploiting endogenous protein sorting mechanisms. Ubiquitin-like 3 (UBL3) was identified as the protein sorting target which can interact with and post-translationally modify cargo proteins (i.e., Ras and tubulin) to enhance their loading into EVs (Ageta et al., 2018). Through donor cell transfection of mutated, oncogenic *RasG12V*, a binding partner of UBL3, this study demonstrated effective loading into released EVs and subsequent downstream signaling activation in target cells (Ageta et al., 2018). Development of loading proteins into EVs from the cytosol in a free form has been shown *via* optically reversible protein-protein interactions; EXPLORs (Yim et al., 2016). This approach is based on the selective interaction of cargo protein and tetraspanin CD9, allowing cargo proteins to be freely localized in the recipient cells. On the other hand, loading of specific miRNA can be achieved by exploiting the

short sequence motifs over-represented in miRNAs commonly enriched in EVs, and the sumoylation of their binding partner hnRNPA2B1, which is required to facilitate miRNA sorting into EVs (Villarroya-Beltri et al., 2013). Other loading RNA-based methods using RNA binding proteins include EXOsomal transfer into cells (EXOtic) using the interaction between C/D box RNA structure and L7Ae ribosomal protein fused with tetraspanin CD63 (Kojima et al., 2018), and conjugated mRNA sequence with interaction between *trans*-activating response (TAR) sequence and *trans*-activator of transcription (Tat) protein (with membrane protein ARMMs) (Wang Q. et al., 2018), to load specific mRNAs into EVs. Further, this latter study demonstrated a highly versatile platform for packaging and intracellular delivery of therapeutic macromolecules, including protein, RNAs, and the genome-editing CRISPR-Cas9/guide RNA complex (Wang Q. et al., 2018). While the molecular mechanisms of cargo sorting into EVs are still being understood, it may be useful to consider the above characteristics of target molecules and their compatibility with endogenous donor cell cargo sorting machinery to improve their loading efficiency and consistency for EV-based therapeutic development.

Targeted Delivery of EVs

Specific components present in the EV surfaceome (selections of proteins presented on the external side of the lipid bilayer), allow for preferential uptake by specific cells, tissues, and organs (Peinado et al., 2012; Oksvold et al., 2014; Costa-Silva et al., 2015; Hoshino et al., 2015; Belov et al., 2016; Rodrigues et al., 2019; Wu et al., 2020). Although this capacity for tropism is supported by various reports, our understanding of the complexities behind this intrinsic targeting in native EVs remains incomplete, though they are widely attributed to their biological origin. Like their progenitor cells, platelet-derived EVs have an intrinsic affinity with inflammatory sites and atherosclerotic plaques, binding to activated/inflamed vascular walls and plaques through various receptors including CD40L (Mach et al., 1997; Lievens et al., 2010), glycoproteins Iba and aIIb (Guo et al., 2015; Pawlowski et al., 2017), and P-selectin (Dinkla et al., 2016; Pawlowski et al., 2017). Thus, platelet-derived EVs hold great potential to be exploited as nanocarriers of anti-inflammatory reagents to ameliorate inflammatory diseases including pneumonia (Ma et al., 2020) and atherosclerosis (Song et al., 2019). EV homing to specific organs also play roles in disease progression, such as those derived from pancreatic ductal adenocarcinoma cells (PDAC) (a cancer commonly associated with liver metastasis), which demonstrated natural homing to the liver upon *in vivo* administration, where they were selectively taken up by Kupffer cells (Costa-Silva et al., 2015). Though the molecular mechanism behind this liver tropism is unknown, the current hypothesis is that EVs express surface components to facilitate a specific interaction with target cells. Indeed, EVs derived from tumors with metastatic organ tropism (lung, liver, or brain) express different integrin complexes; lung-tropic: α6β4 and α6β1, liver-tropic: αvβ5, brain-tropic: ITGβ3 (Hoshino et al., 2015). EVs containing these integrin complexes bind to S100-A4-positive fibroblasts and surfactant protein C-positive epithelial cells in the lungs, Kupffer cells in the liver, or CD31-positive endothelial cells in the brain (Hoshino et al., 2015), respectively. Lung-tropism is also influenced by non-integrin proteins, with genetic knockdown of SLCO2A1, CD13, and CLIC1 in cervical adenocarcinoma cells (HCA1) resulting in the decreased accumulation of EVs in lung tissue (Wu et al., 2020). EVs' tropism to particular organs and cells may also be mediated by surface ligands that interact with cognate receptors on recipient cells to induce binding and subsequent uptake. This ligand-receptor interaction (i.e., EV chemokine receptor CXCR4 to recipient cell-expressed chemokine SDF-1α) mediated targeting of endothelial colony forming cell-derived EVs to the kidney, thus preventing kidney injury and neutrophil infiltration following ischemic injury through the transfer of PTEN-inhibitor miR-486-5p (Vinas et al., 2018).

Other surface-expressed molecules that mediate EV-cell interaction include glycosphingolipid glycan groups, which are present on neuroblastoma-derived EVs, facilitating their binding to and clearance of β-amyloid aggregates in the brain (Yuyama et al., 2014). Their ability to target the brain highlights the potential of EV-based treatments for brain cancer and Alzheimer's disease. Other mechanisms for brain-tropism also exist, such as the natural homing capacity of EVs derived from specialized immune cells in the central nervous system (CNS) (i.e., microglia) to the brain, where they reduced multiple sclerosis-associated inflammation in myeloid cells and astrocytes through the transfer of anti-inflammatory cytokine IL-4 (Casella et al., 2018). These studies highlight the natural ability of EVs to transverse the BBB, an ability mediated in-part by surface components (i.e., heparan sulfate proteoglycans, mannose 6-phosphate receptor, CD46, integrin complexes αVβ6 and αVβ3, and endothelial- and leukocyte-associated transmembrane protein ICAM-1) which allow receptor-mediated transcytosis (Banks et al., 2020; Joshi and Zuhorn, 2021); a transcellular route allowing EVs to traverse brain endothelial capillary cells toward brain parenchyma. Further characterization of surface components which possess tropic-capacity will allow for refinement and selection of EV therapeutics.

Biodistribution and Clearance of Native EVs

As mediators of inter-organ communication, EVs must evade immune clearance for as long as possible to remain in circulation prior to cellular uptake (Smyth et al., 2015; Charoenviriyakul et al., 2017). The mononuclear phagocyte system (MPS) [previously termed reticuloendothelial system (RES)] encompasses monocytes, macrophages, and other cells present in liver, spleen, and lungs, and contributes to EV sequestration and clearance (Rao et al., 2015; Smyth et al., 2015). Indeed, following *in vivo* administration, EVs accumulate in the liver, spleen and/or lung; an occurrence widely observed in EVs derived from dendritic (Wei et al., 2017), MSCs (Grange et al., 2014), myoblasts (Wiklander et al., 2015; Charoenviriyakul et al., 2017), kidney (Lai et al., 2014), glial (Lai et al., 2014), melanoma (Peinado et al., 2012; Takahashi et al., 2013; Imai et al., 2015; Charoenviriyakul et al., 2017), macrophages (Charoenviriyakul et al., 2017), and placental (Tong et al., 2017; Nguyen et al., 2021) cells. With phagocytosis central to

clearance, EVs avoid engulfment through surface presentation of anti-phagocytic signals including immunomodulatory receptors, most commonly CD47 (Chao et al., 2012; Rodriguez et al., 2013; Kaur et al., 2014; Kamerkar et al., 2017; Tang Y. et al., 2019), PD-L1 (Gordon et al., 2017; Hsu et al., 2018; Cordonnier et al., 2020; Daassi et al., 2020), CD24 (Barkal et al., 2019), CD31 (Brown et al., 2002), and CD44 (Vachon et al., 2007; Amash et al., 2016), that act as "don't eat me" signals to phagocytic cells, potentially prolonging their EV half-life in circulation.

Conversely, internalization of EVs ensures the delivery of EV content into target cells, allowing them to exert their effector functions. Phosphatidylserine (PS) is a lipid located on the outer leaflet of EV membranes, forming lipid-receptor interactions [e.g., with immune regulatory receptor TIM4 (Tietjen et al., 2014)] to facilitate EV engulfment and internalization by recipient cells (Lindemann, 1989; Fadok et al., 2000; Gurnani et al., 2004). EVs also express opsonins [e.g., MFGE8 (Dasgupta et al., 2009; Miksa et al., 2009; Casella et al., 2018), β2-glycoprotein-1 (Abdel-Monem et al., 2010), developmental endothelial locus-1 (Dasgupta et al., 2012)] that facilitate their internalization through PS-dependent phagocytosis. Indeed, increasing the expression of MFGE8 on microglia-derived EVs elevated their uptake by macrophages and microglial cells in the CNS, facilitating the transfer of EV-loaded anti-inflammatory cytokine (i.e., IL-4) to significantly reduce clinical symptoms of neuro-inflammatory multiple sclerosis, and experimental autoimmune encephalomyelitis (EAE) in a murine model (Casella et al., 2018). Other factors that influence EV biodistribution and uptake *in vivo* is the microenvironment from which they are released. Tumor-derived EVs can bind to soluble secreted cytokines and chemokines (i.e., CCL2 and IL-6) in the tumor microenvironment through their surface-expressed glycosaminoglycan (GAG) side chains of proteoglycans, significantly increase their uptake in the liver, spleen, and lung (Lima et al., 2021). Through a similar mechanism, EVs expressing GAGs released by glioblastoma bind to, and are decorated with, chemokine ligand CCL18, facilitating their interaction with cognate receptor CCR8 on recipient glioblastoma cells (Berenguer et al., 2018) to increase uptake and induce a proliferative phenotype (Berenguer et al., 2018). Indeed, pharmacological inhibition of this interaction (*via* CCR8 inhibitor, R243) completely blocked EV-induced tumor growth, thus neutralizing EV-induced phenotypic remodeling (Berenguer et al., 2018).

Despite these insights into EV biodistribution and clearance, attempts to comprehensively define the pharmacokinetics of EVs *in vivo* have remained inconsistent, with results ranging from their rapid clearance within 2–4 min (Takahashi et al., 2013) (as monitored by fluorescence imaging) up to 7 days, with DiR-labeled EVs localized to the liver and spleen a week following administration (Liu H. et al., 2016). This variation may be resultant of EV labeling with lipophilic dyes, which have been found to remain in the system long after EVs have been degraded or recycled (Takov et al., 2017). Regardless of these uncertainties, EVs possess important qualities—highlighting them as promising modalities for therapeutic applications. Further development toward EV-based clinical application involves their engineering/modification to reduce their immune clearance and prolong their half-life in circulation, improve their biodistribution and tropism to sites of interest, and, enhance their functionality on recipient cells, tissues, and organs.

ENGINEERED EVs

Native EVs offer unique advantages for cellular regulation and the efficient delivery of therapeutic payloads. However, several studies have highlighted the intrinsic limitations of native EVs including long-term maintenance of parental cell culture with minimal metabolic/phenotypic variation (Lambshead et al., 2018; Cherian et al., 2020; Escude Martinez de Castilla et al., 2021), biodistribution (i.e., organ targeting) (Escude Martinez de Castilla et al., 2021; Lazaro-Ibanez et al., 2021; Ullah et al., 2021; Witwer and Wolfram, 2021), clearance rates [complement and immune systems (RES-MPS system)] (Wiklander et al., 2015; Ha et al., 2016; Kwon, 2020; Lara et al., 2020; Buschmann et al., 2021; Escude Martinez de Castilla et al., 2021; Lazaro-Ibanez et al., 2021), and crucially, difficulties associated with large scale generation (i.e., processing times, variable potency between batches, good manufacturing practice; GMP) (Whitford and Guterstam, 2019; Cherian et al., 2020; Buschmann et al., 2021; Escude Martinez de Castilla et al., 2021; Ullah et al., 2021; Witwer and Wolfram, 2021). Moreover, the regulatory machinery of EVs and their distinct subtypes associated with production and cellular uptake remains largely unknown. Engineering therapeutics based on native EVs offers an alternative approach, employing their advantages while bypassing limitations. As such, the engineering of EVs for therapeutic application is a field undergoing rapid development with applications for regeneration/repair, immune disorders, wound healing and cancer (Commisso et al., 2013; Kordelas et al., 2014; Xitong and Xiaorong, 2016; Gyorgy et al., 2017; Wu et al., 2019; Li L. et al., 2020; Hu S. et al., 2021; Shi et al., 2021). Here, we discuss several strategies recently developed to address such limitations through modification or engineering of EVs including: (i) generation of mimetic EVs/nanovesicles (M-NVs), EV synthetics (synEVs), and EV hybrids (hEVs), (ii) improvement of targeting of native EVs and (iii) customized cargo loading (into native and engineered EVs) to enhance their functional properties (**Table 3**).

Engineering Alternatives to EVs

M-NVs simulate the biophysical properties of native EVs, including size (50–200 nm), which has been reported to influence half-life in circulation (i.e., nanoparticles smaller than 200 nm are able to evade RES uptake, and nanoparticles larger than 30 nm to avoid rapid renal elimination) (Ha et al., 2016; Murphy et al., 2019; Lara et al., 2020; Buschmann et al., 2021; Dooley et al., 2021; Escude Martinez de Castilla et al., 2021; Lazaro-Ibanez et al., 2021; Witwer and Wolfram, 2021). M-NVs can be generated by either top-down (extruding parental cells into nano-sized fragments) to obtain biological M-NVs, or bottom-up methods (selecting cargo and capsule materials i.e., liposomes or polymer nanoparticles) to obtain chemically-defined synEVs (Nasiri Kenari et al., 2020). Top-down M-NVs

TABLE 3 | Approaches in engineering extracellular vesicles.

	Modification/ Generation technique	Description	Advantages	Disadvantages	Pre-clinical applications	References
Engineered EVs	Surface functionalization	Use of chemical reactions to couple molecules of interest (i.e., peptides, proteins) to the EV surface (i.e., Click Chemistry) to enhance targeted delivery or half-life in circulation.	Targeting to specific organs and areas of interest (i.e., brain, tumor), increase half-life and stability in circulation, binding of negatively charged molecules (i.e., siRNA, miRNA, mRNA, DNA).	(i) May increase overall size of EVs, (ii) reduces natural targeting and delivery capability of EVs, (iii) peptide used must be very specific to the target site.	Targeted therapy.	Alvarez-Erviti et al., 2011; Niu et al., 2021
	Modification of parental cells	Genome engineering of parental cells to modify produced EVs (i.e., surface protein that influences targeting).	Highly modifiable depending on application, can target wide range of luminal molecules for functional modification of EVs, or surface molecules for targeting modification of EVs.	Requires extensive validation of the efficiency and off-target errors of genome engineering strategies, may alter the properties and structure of the EV.	Lentivirus transfected MSCs to overexpress miRNA-let7c, known to possess anti-fibrotic properties, generated exosomes capable of transferring this cargo to renal cells *in vitro*.	Skog et al., 2008; Alvarez-Erviti et al., 2011; Wang et al., 2016
	Microfluidics	Fragmentation of cells/lipid micelles using microfluidic systems (pressure) to generate EV mimetics or liposomes of consistent size.	Fast, efficient, scalable, consistent, less manual handling.	Expensive, complex equipment.	Mimetics were generated from embryonic stem cells and used to deliver drugs/RNA *in vitro*.	Jo et al., 2014a
	Serial extrusion	Generation of EV-mimetics by fragmenting whole cells through micro-sized pore filters of descending size to obtain vesicles of similar size to EVs.	Consistent, scalable generation of EVs from cells of interest, easily modifiable protocol to suit application.	Requires extensive manual handling, additional purification steps, quality assurance of progenitor cells.	Human MSCs treated with ion oxide nanoparticles (IONP) were extruded serially (five times through 10, 5, and 1 μm and 400 nm), and demonstrated their feasibility for spinal cord-injury treatment.	Kim H. Y. et al., 2018
	Centrifugation	Use of centrifugal force to fragment cells through a membrane generating EV mimetics.	Fast, efficient, scalable, less manual handling compared to extrusion.	Relative scalability depending on equipment capacity.	Murine embryonic stem cells were generated by this method and were able to transfer RNAs.	Jo et al., 2014b
Post-EV production engineering	Passive loading	Incubation with molecule of interest for diffusion loading into EVs.	Efficient protocol, easily modified depending on application, scalable.	Low loading efficiency in comparison to all other methods. Depends on Fick's laws and hydrophobicity of the cargo.	Loading of anti-cancer drugs (Doxorubicin, Paclitaxel, Gemcitabine) into various EV systems showed tumor reduction/elimination in murine models.	Jang et al., 2013; Rani et al., 2015; Gao et al., 2017; Li Y. J. et al., 2020
	Serial extrusion	Generation of EV-mimetics encapsulating target molecules by performing serial extrusion of cells in target molecule-containing buffer.	(i) Surface characteristics similar to EVs and parental cells which improves natural targeting ability, (ii) longer half-life in circulation, (iii) little to no immunogenicity, (iv) allows loading of therapeutic molecules, increases targeting capacity toward site of interest, and reduces side effects of certain molecules and drugs.	Requires additional purification process, requires extensive handling.	Comparison of biological EVs and mimetics VS free chemotherapeutic drug (Doxorubicin) for delivery and efficacy. The results showed a better effect and delivery to cancer cells using EVs and mimetics in a murine system.	Jang et al., 2013
	Sonication	Treatment with ultrasound produces transient pores in EV membranes that allow drug diffusion.	Efficient loading of drug molecules.	Requires rigorous optimization on to prevent compromising membrane integrity or the cargo structure, increases EV instability; small RNA or EV aggregation; additional equipment or wash step may be required.	The lysosomal enzyme, tripeptidyl peptidase-1 (TTP1) was loaded into 10^{11} EVs (~70 μg), increasing stability and targeting to the brain in a murine model of brain disease.	Haney et al., 2019
	Microfluidics	Addition of cargo molecules to the system for loading after membrane disruption by forcing the EVs through microchannels.	Fast, efficient, scalable, consistent, less manual handling.	Expensive, complex equipment.	Drug and RNA delivery system.	Meng et al., 2021
	Electroporation	Temporary disruption of the EV membrane using electricity to allow incorporation of cargo.	Fast, well-established protocol, widely used to load small molecule drugs and nucleic acids.	Low loading efficiency (~15–20%), low scalability, requires standardization for cargo and target EVs, requires rigorous optimization to prevent compromising membrane integrity or cargo structure, increases EV instability; small RNA or EV aggregation; additional equipment or wash step may be required.	Utilized to load tumor suppressor miRNAs into fibroblast-derived EVs to treat ovarian cancer. Repeated intraperitoneal injections of miRNA-loaded EVs drastically inhibited peritoneal dissemination and reduced the tumor burden in a xenograft ovarian cancer mouse model.	Kobayashi et al., 2020
	Transient permeabilization	Use of detergents to allow the selective removal of membrane-bound cholesterol, creating transient holes/pores in the EV bilayers, and thus, promoting drug loading.	Simple, efficient, and modifiable protocol, scalable.	Low efficiency, requires standardization, use of detergents may degrade critical biomolecules that influence biological function, requires rigorous optimization on to prevent compromising membrane integrity or cargo structure, increases EV instability; small RNA or EV aggregation; additional equipment or wash step may be required.	Catalase was loaded by saponin permeabilization of macrophage- and monocyte- derived EVs to prevent neurodegeneration and neuroinflammation in mouse models of Parkinson's disease.	Haney et al., 2015

(Continued)

TABLE 3 | (Continued)

	Modification/ Generation technique	Description	Advantages	Disadvantages	Pre-clinical applications	References
	Freeze-thaw	Incorporation of target molecules/drugs into EVs by increasing membrane permeability through multiple freeze-thaw cycles.	Simple and efficient protocol, minimal handling, able to modify according to concentration of target molecule/drug.	Biological EV function, and membrane integrity may decrease or become compromised with increasing number of freeze-thaw cycles.	Curcumin was loaded into mouse embryonic stem cell-derived exosomes by freezing and thawing three successive times promoted neurovascular restoration following ischemia-reperfusion injury in mice.	Kalani et al., 2016
Artificially stimulated	Overexpression of biogenesis-associated genes	Genetic engineering to introduce enhancers, promoters or sequences targeting specific genes for over-expression.	Increases yield of EVs.	Low efficiency and off-target errors of genome engineering strategies, influences proteome and potentially function of EVs, expensive, time consuming.	MSCs with a combination of *N*-methyldopamine and norepinephrine robustly increased exosome production by three-fold without altering the ability of the MSC exosomes to induce angiogenesis, polarize macrophages to an anti-inflammatory phenotype, or downregulate collagen expression.	Wang Y. et al., 2020
	Artificial blebbing	Chemical induction of the blebbing process (paraformaldehyde, dithiothreitol, N-ethyl maleimide).	Efficient and scalable.	Requires standardization, requires further processing for chemicals removal.	No applications thus far.	Thone and Kwon, 2020
Hybrids	EVs and liposomes	Fusing EV membranes with lipids.	Increase half-life and stability in circulation, modifiable based on lipid used, enhanced structural integrity, allows loading of target molecules.	May decrease biocompatibility and influence EV surface proteins important for specific organ targeting.	HEK293FT derived EVs were incubated with Lipofectamine 2000 and selected plasmids.	Lin et al., 2018
	EV-modified scaffolds	Incorporating EVs onto scaffolds comprised of various polymers (i.e., extracellular matrix proteins, chitosan, silk fibroin, hydrogels).	Sustained release of EVs over longer periods of time, higher efficacy than free-EVs, easy to handle, high mechanical/tensile strength and controllable degradability which can be modified based on polymer/s used, higher biocompatibility if ECM-based.	Generally required to be in direct contact with target tissue/organ, which is invasive if targets are internal, toxicity of residual crosslinking agents from scaffold manufacturing, can be pH- or temperature- sensitive and cause plugging of needle during injection, unknown release rate *in vivo*.	Targeted regeneration of bone, heart, liver, skin, and vasculature.	Mardpour et al., 2019; Hu S. et al., 2021; Kim et al., 2021; Shi et al., 2021; Zhao et al., 2021
Synthetics	Silicon	Modification of silicone nanoparticles to display or encapsulate target molecules and drugs for delivery.	Robust production, able to load target molecules, high structural integrity, able to modify porosity and surface chemistry to control degradation/drug release.	Can be toxic, lower biocompatibility, lacks surface proteins for targeting and uptake by recipient cells.	Pre-clinical studies pSivida has shown that BioSilicon is both biodegradable and biocompatible, used for controlled release of substances without penetrating the cells.	O'Farrell et al., 2006
	Iron	Coating of iron nanoparticles with proteins/other to target specific tissues.	Many methods of production to suit application, can cross BBB, increased structural integrity, FDA approved, suitable for anemic patients, allows magnetic guidance for specific site targeting, used as a label for scaffold-based therapies to allow for imaging, surface-modifiable by biocompatible coating.	Can be toxic, lower biocompatibility, depending on application, metal teeth fillings may interfere with imaging, disadvantages associated with production method (i.e., adverse side effects from residual surfactants, large-scale synthesis, cost, temperature regulation).	Medical imaging, cell labeling, iron replacement therapy for patients with kidney failure or anemia, macrophage polarization, magnetic drug targeting, theranostic applications.	Huber, 2005
	Lipids	Bottom-up generation of nanovesicles.	Scalable, time efficient, customizable lipid composition and size depending on application, low cost, consistent generation.	Low targeting capabilities, lack of surface proteins, low uptake, low biocompatibility, faster systemic clearance.	PEG -DOPE nanovesicle generation for chemotherapeutic drug delivery (Doxorubicin and emtansine) in a murine model.	Han et al., 2006; Cao et al., 2016
	Gold	Gold nanoparticles associated to PEG or other lipids by cross-linking reactions.	Robust production, chemically inert, stable, modifiable (size—which influences toxicity and biocompatibility properties), radiosensitizing, and anti-microbial properties.	Can be toxic, largely influenced by size (i.e., biocompatibility, renal clearance, tumor tissue permeability, cell uptake, entry into nuclei), more suitable for theranostic and imaging applications.	Photothermal therapy. Use of PEG-gold nanoparticles and drug adsorption for chemotherapeutics delivery.	Dykman and Khlebtsov, 2011
	Carbon nanotubes	Controlled carbon concatenation reactions depending on the desired shape and size.	Robust production, stable, modifiable (size, tubes, nets, scaffolds—which influences toxicity and biocompatibility properties).	Can be toxic, lower biocompatibility, lacks surface proteins for targeting and uptake by recipient cells.	Drug, protein, and DNA/RNA delivery systems by adsorption methods into biodegradable scaffolds that can penetrate the cells for cancer therapy, neurodegenerative diseases, tissue regeneration in murine and *in vitro* models.	He et al., 2013

are generated by serial extrusion, ultracentrifugation, or pressure-based microfluidic approaches [reviewed in Mentkowski et al. (2018)]. Mechanical extrusion is achieved by forcing the cell suspension to pass through membranes of different pore size to cause cell disruption (Jang et al., 2013). After extrusion of parental cells, the membrane fragments form membrane-derived vesicles due to their physicochemical properties, engulfing the cellular components in suspension and generating mimetic EVs (Jo et al., 2014b). Following this method, M-NVs generated from macrophages through serial extrusion with concurrent loading of catalase (added to the cell suspension before extrusion) demonstrated elevated neuroprotective activity while increasing the yield ∼100-fold, compared to native EVs (Haney et al., 2015).

MN-Vs can also be generated from MIN6 pancreatic β-cells by serial extrusion i.e., five passages through 10, 5 and 1 μm polycarbonate membrane filters using a mini extruder (Oh et al., 2015). In parallel, bone marrow MSCs isolated from femurs and tibias of BALB/c mice were embedded in Matrigel and implanted subcutaneously into NSG mice (NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ). Into these subcutaneous patches, 10 μg/injection of MIN6 mimetics were administered for a total of four injections in 10 days. The authors demonstrated that the MSCs imbedded in Matrigel formed islet-like clusters with extensive capillary networks as a cause of M-NV administration, and that these were able to maintain the glucose levels of the mice for over 60 days (Oh et al., 2015). This work provided a key insight of the effectiveness of mimetics *in vivo* as delivery and signaling systems. To understand composition of M-NVs, Hill and collaborators (Nasiri Kenari et al., 2019) performed proteomic profiling of M-NVs and demonstrated their distinct composition from exosomes and parental cells. Although M-NVs shared many similarities with native EVs (physical attributes, key protein EV markers, proteins that overrepresented the original cell), differences were observed in protein post-translational modifications, specifically phosphorylation, ubiquitination, and thiophosphorylation. This raises an important consideration of using M-NVs as an alternative nanocarrier when spontaneous endosomal sorting of therapeutics is limited or when modulation of donor cells influences native EV generation. An important consideration of M-NV generation is that despite the 100 to 150-fold increase in yield, this method still relies in parental cells as raw materials which requires the surveillance of their long-term maintenance (genome stability, passage number, cell culture technologies) and culture conditions/media type and potential influence of non-model EV source [i.e., bovine-derived (Eitan et al., 2015; Lehrich et al., 2021; Pham et al., 2021)].

A different stream of research to solve upscaling as a key limitation for native EVs' and M-NVs' pharmaceutical use, focuses on the generation of bottom-up particles denominated synEVs. synEVs have demonstrated a high efficacy and high scalability as drug and vaccine-based delivery systems (Park et al., 2021). Generation of liposomes include extrusion over membrane filters [analogous to mimetic generation (Cao et al., 2016; Nele et al., 2019; Shah et al., 2019)], and hydrophilic microchannels [microfluidic systems (Shah et al., 2019; Kotoucek et al., 2020)]. The development of synEVs has gained immense interest due to COVID-19 vaccine research, including mRNA encapsulated in liposomes by Moderna/NIAID, BioNTech/Pfizer, Arcturus/Duke-NUS, PLA/Walvax Biotech, Imperial College London, and CureVac AG (Park et al., 2021). These synEVs (in particular Moderna/NIAID and Pfizer non-viral vaccines) have shown to be a highly efficient system for delivery and immunoregulatory response (Park et al., 2021). Furthermore, the methods for generation and encapsulation of cargo (i.e., mRNA) into synEVs has demonstrated high efficiency and yield; >72% encapsulation rates (Hassett et al., 2019). Although different approaches for generating synEVs with vaccine applications have been employed, in all cases their lipid composition provides key therapeutic advantages, including ability to encapsulate and condensate mRNA, promote delivery to cytosol by increasing cellular uptake (due to their composition compatible with biological membranes i.e., PEGylated lipids, cholesterol and cationic or ionizable lipids), protect mRNA (or any other cargo) from degradation in extracellular spaces, and their components are easily manufactured with GMP in a large scale. The latter demonstrates synEVs represent a unique advantage in combining with production of native EVs, despite issues with their targeted delivery. Unlike cell-derived EVs, synEVs lack targeting and recognition molecules, therefore, their synthesis has been coupled with different functionalization techniques such as bioconjugation (Smyth et al., 2015; Lim et al., 2021) [reviewed in Murphy et al. (2019), Rayamajhi and Aryal (2020), Salmond and Williams (2021), Sharma et al. (2020), and Takayama et al. (2019)] and cargo loading (Haney et al., 2015, 2019) [reviewed in Luan et al. (2017), Nasiri Kenari et al. (2020), Roberts et al. (2020), and Sterzenbach et al. (2017)] to obtain use-specific synEVs (Garcia-Manrique et al., 2018), as well as their fusion with EVs (native or mimetics) to generate EV hybrids (hEVs).

hEVs are a recently developed method to generate vesicles—they comprise native EV components and synthetic liposomes (Gangadaran and Ahn, 2020). Hybrids could be a more effective alternate to both EVs and liposomes as drug delivery systems by combining the advantages of loading versatility (diverse molecular cargo), targeting capabilities (native EV tropism), and stability (structure stabilization, cargo protection, handling stability) [reviewed in Ou et al. (2021)]. Moreover, hEVs can be generated by different methods such as extrusion, sonication, co-incubation or freeze/thaw cycles, which makes them convenient for clinical development and diverse applications (Gangadaran and Ahn, 2020). Freeze/thaw cycle method was used in combination to surface modification techniques to fuse EVs expressing a specific surface protein produced by macrophages and different cancer cell lines (mouse fibroblast sarcoma-derived CMS7-wt, CMS7-HE, and Raw 264.7 macrophages) with liposomes (Sato et al., 2016). Hybrids have also been generated by sonication of an aqueous suspension of macrophage-derived EVs and L-α-phosphatidylcholine/Cholesterol liposomes (Rayamajhi et al., 2019). The resulting hybrids are a promising platform for tumor-targeted drug delivery, releasing Doxorubicin predominantly to macrophages, osteosarcoma cells and breast cancer cells (compared to normal fibroblasts) *in vitro*, demonstrating these hEVs have preferential targeting to parental cells and tumor cells (Rayamajhi et al., 2019). Furthermore,

a variant of hEVs using native EV sources [bone marrow MSC-derived EVs (purified from conditioned media) and platelet mimetics (generated by a combination of freeze/thaw and consecutive sonication)], were generated by co-extrusion and administered to mice (100 μg/mice, once a week for 4 weeks), demonstrated increased targeting and pro-angiogenic activity in a mouse model of myocardial infarction in mice (Li et al., 2021c). Here, tropism of platelets toward activated endothelium (which occurs during injury or stress) was inherited by the resulting hybrids enhancing their targeting and accumulation capabilities to the myocardium (compared to native MSC-derived EVs) allowing the targeted delivery of reparative cargo despite systemic administration.

Improved Targeting: Genome Engineering and Surface Functionalization

Modifying EVs to improve their specific delivery is a key requirement in therapeutic applications. Several engineering approaches have been developed and applied to EVs. Click chemistry, genetic modification, and glycoengineering have proven to be highly efficient methods to increase site-specific retention, thus reducing off target effects (Smyth et al., 2014; Williams et al., 2018; Rayamajhi and Aryal, 2020). EV composition and function are influenced by the cell source, a characteristic which can be exploited during EV-based therapeutic development (Kim et al., 2020). Many technologies can modify progenitor cells to alter the functional capacity of their derived EVs; one of which is genome engineering, which involves either the knockout [i.e., siRNA, CRISPR (Horodecka and Duchler, 2021)] or overexpression [i.e., Lentiviral/Adenoviral, plasmid, or nucleic acids (siRNA, miRNA, anti-miR) transfections (Li et al., 2013; Zhang et al., 2014; Chen S. et al., 2019; Escude Martinez de Castilla et al., 2021)] of genes (Shi et al., 2020). The composition of EVs released from these modified cells are therefore customized, heavily influencing recipient cell function [i.e., immunomodulatory (Trivedi et al., 2016; Gomez-Ferrer et al., 2021), pro-angiogenic (Zuo et al., 2019; Zhang L. et al., 2021), anti-apoptotic (Wen et al., 2020) or anti-cancer (Kim R. et al., 2018)]. Thus, these strategies are applicable to a range of pathologies, including cardiovascular disease, tissue repair/regeneration, cancer, and immunological disorders. However, continuous improvements are required for these techniques due to their low mutation efficiency and potential off-target sequence error (CRISPR-Cas9) (Siddique, 2016) or low efficiency and variable expression levels by transfected cells (transfection) (Di Blasi et al., 2021) (which may influence the biogenesis/yield/content/function of the produced EVs (Carli et al., 2021) or parental cell viability or proliferation (Lambshead et al., 2018; Cherian et al., 2020; Escude Martinez de Castilla et al., 2021).

In this context, genetic manipulation strategies [e.g., plasmid transfection (Cho et al., 2018; Hong et al., 2019; Shi et al., 2020; Feng et al., 2021), pDisplay vector transfection (Ohno et al., 2013), retroviral transfection (Fan et al., 2013)] pre-EV isolation have been shown to modify the surface of EVs to enable site-specific delivery (Wan et al., 2018) (**Table 3**). For instance, overexpression of folate receptor α (FRα) on EV surface facilitated their specific binding to the brain parenchyma, crossing the BBB, demonstrating a mechanism of brain-specific drug delivery *in vivo* after an intraventricular injection (Grapp et al., 2013). Furthermore, genome engineering of cardiosphere-derived cells (CDC) using lentiviral particles to express LAMP2B fused to a cardiomyocyte specific peptide (CMP; WLSEAGPVVTVRALRGTGSW) generated EVs displaying LAMP2B-CMP on their surface, increasing retention time and improving targeted delivery to the heart (Mentkowski and Lang, 2019). Moreover, modification of mouse dendritic cells to express LAMP2B fused to the neuron specific RVG peptide was shown to target EVs with siRNA-BACE1 to neuronal cells (Alvarez-Erviti et al., 2011). The therapeutic potential of this system was demonstrated by mRNA (60%) and protein (62%) knockdown of BACE1, a therapeutic target in Alzheimer's disease. This strategy was also employed to modify BMSCs for the generation of EVs expressing LAMP2B coupled with ischemic myocardium-targeting peptide (IMTP; CSTSMLKAC), which caused a significant increase of EV accumulation in cardiac tissue when compared to peptide sequence (non-targeted) controls (Antes et al., 2018; Wang X. et al., 2018). Altogether, these studies highlight the capacity of genome engineering to generate surface modifications that enhance EV targeting capabilities. On the other hand, off-target errors during genome editing complicate validation and are time consuming/expensive, thus would impede upscaling. It was also observed that despite their targeting capabilities, engineered EVs accumulate in non-targeted tissue/organs, including liver and kidney (Wang X. et al., 2018); determination of the EVs' effects at these sites and an improved understanding of EV-site interaction are required for clinical applications. Engineering methods can also be combined to refine EV-based therapeutics. Decoy mimetic EVs have been generated through mechanical extrusion of genetically engineered parental-cells overexpressing surface receptor ACE2 (Rao et al., 2020). These mimetic EVs were fused with human myeloid mononuclear THP-1 mimetics, to generate hEVs. Upon administration to a murine model of acute pneumonia, these hEVs suppressed immune disorder and decreased lung injury (Rao et al., 2020).

A different approach for surface engineering is modification post-EV isolation. These modifications can be performed through glycoengineering [attachment of glycans to proteins by generation of covalent bonds (Williams et al., 2018; Williams C. et al., 2019; Sharma et al., 2020; Della Rosa et al., 2021; Lim et al., 2021; Martins et al., 2021)] and click chemistry [reactions which involve conjugation of molecules in a modular fashion, for example, the bio-orthogonal copper-free azide alkyne cyclo-addition (Smyth et al., 2014; Ouyang et al., 2018; Murphy et al., 2019; Takayama et al., 2019)], and are used for targeting (Kooijmans et al., 2016) and cloaking strategies (Kooijmans et al., 2016; Suk et al., 2016), also known as "surface functionalization"(Rayamajhi and Aryal, 2020). For example, glycoengineering of anti-EGFR nanobody to the phosphatidylserine (PS) of EVs derived from blood, or Neuro2A cells, promotes their uptake by $EGFR^{+}$ cells in a dose-dependent manner and decreases their non-specific binding to other cells

(Suk et al., 2016). Further, this modification resulted in an increased circulation time of >60 min for engineered EVs in comparison to 10 min for native EVs (Suk et al., 2016). Together, these results provide an effective strategy for cloaking and targeting using glycoengineering.

A combination of glycoengineering and click chemistry has been employed as a highly- efficient and specific approach to modify the EV surface through irreversible bioconjugation (Smyth et al., 2014; Takayama et al., 2019). This allows EV surface functionalization for delivery of small molecules, large biomacromolecules, and polymers without altering particle size or function. Murine MSC-derived EVs were modified by copper-free click chemistry to generate an Ale-EVs system (EVs coupled with Alendronate, a medication used for osteoporosis) administered to an *in vivo* model of ovariectomy (OVX)-induced osteoporosis (Wang Y. et al., 2020). These Ale-EVs had an affinity to bone tissue and promoted regeneration under osteoporotic conditions with low toxicity. Similar methods, including genetic modification of parental cells and/or post-EV isolation methods can also be used to enrich surface-ligands on EVs, which can then induce/inhibit signaling or target specific recipient cells for delivery (Jafari et al., 2020). As a direct approach to modify the surface of MSC-derived EVs by click chemistry conjugation, c(RGDyK) peptide which exhibits affinity to integrin αvβ3 and expressed in reactive cerebral vascular endothelial cells after ischemia, was used for improved delivery (11-fold tropism to the lesion region of ischemic brain) and treatment of stroke (Tian et al., 2018). Additionally, surface modifications using click chemistry can be used to chemoenzymatically label EVs to visualize cellular uptake in real time. EVs isolated from conditioned media of human breast cancer MCF-7 cells were purified and an alkyne group (click chemistry target) substituted for the choline group from native EV phosphatidylcholine by an enzymatic reaction using phospholipase D (Jiang et al., 2020). Using the alkyne-azide click chemistry, fluorescent Cy5 dye was covalently fused and used to track cellular internalization in real time using fluorescent confocal microscopy *in vitro* (Jiang et al., 2020). Furthermore, the surface modification did not alter EV size (compared with native EVs by nanoparticle tracking analysis) (Jiang et al., 2020). This study provides an efficient method for tracking EVs that could be applied to monitor biodistribution, targeting and uptake of engineered EVs.

Cargo Loading Strategies

In addition to cargo loading *via* surface conjugation (Wang Y. et al., 2020), other strategies used to load cargo into EVs include parental cell modification, passive diffusion, and active loading. Overexpression of components in parental cells is an effective way to increase abundance in resulting EVs for greater function. Transfection of MSCs with miR-133b resulted in accumulation (∼2.5-fold higher levels) of miR-133b in EVs (when compared to EVs from non-transfected MSCs), which subsequently improved functional recovery, reduced lesion volume, and increased neuron survival in an *in vivo* model of spinal cord injury (Li et al., 2018). Several other studies have modified either parental cells (pre-EV isolation) or EVs directly (post-EV isolation) for improved therapeutic response [reviewed in Lara et al. (2020), Nagelkerke et al. (2021), Ullah et al. (2021), and Witwer and Wolfram (2021)] such as in cancer (Zhang et al., 2014), neurodegeneration and Parkinson's disease (Haney et al., 2015), and kidney fibrosis (Wang et al., 2016; Tang T. T. et al., 2019).

An alternative to modifying parental cells is the direct incorporation of specific molecules (i.e., miRNA, siRNA, protein, lipids, drugs) into EVs through passive or active methods (Luan et al., 2017; Sterzenbach et al., 2017; Nasiri Kenari et al., 2020; Nazimek and Bryniarski, 2020). Incubation of EVs with active components is a passive method of loading that involves the diffusion of drugs or molecules with varied encapsulation efficiency through the EV membrane. This approach has facilitated loading of siRNAs, miRNAs, proteins (i.e., catalase; an antioxidative enzyme, Parkinson's disease treatment) (Haney et al., 2015), and anti-cancer drugs [i.e., Paclitaxel (Saari et al., 2015; Kim et al., 2016), Doxorubicin (Smyth et al., 2015), Imatinib (Bellavia et al., 2017)] into EVs for delivery. The loading efficiency is dependent on incubation time, cargo concentration and physicochemical properties of the cargo (i.e., solubility, surface area polarity, lipophilicity, hydrophobicity) (Liu C. et al., 2016; Luan et al., 2017; Kim et al., 2020). To address this variability, active loading methods have been implemented (co-incubation with membrane permeabilizers, i.e., saponin) to improve loading efficiency of specific molecules by up to 11-fold (Fuhrmann et al., 2015). This involves temporary and controlled disruption of EV membrane, usually accomplished by sonication, electroporation, membrane permeabilizers, freeze-thaw cycles, or cell extrusion (**Table 3**) to allow entry of cargo into EVs.

Sonication of EVs (using sonic waves) for therapeutic cargo loading is a widely used method for various clinical applications. Using this technique, macrophage-derived EVs were successfully loaded with Doxorubicin and Paclitaxel, allowing resulting vesicles to target cancer cells and inhibit tumor growth. These modified EVs are an attractive therapy for pulmonary metastases and triple negative breast cancer in murine models (Kim M. S. et al., 2018; Haney et al., 2020). Importantly, these studies observed that pH, temperature, and sonication configuration (time, power, probe, or water bath) affected loading efficiency, and required optimization (dependent on the cargo properties). Using an electrical pulse to temporarily disrupt the EV membrane, electroporation is a widely used method for loading small molecule drugs and nucleic acids into EVs to treat Alzheimer's disease (Alvarez-Erviti et al., 2011), and breast (Ohno et al., 2013), lung (Takenaka et al., 2019), and ovarian (Kobayashi et al., 2020) cancers. The loading efficiency of nucleic acids (i.e., siRNA) through electroporation ranges between 15 and 20%, demonstrating a robust method for cargo loading (Jhan et al., 2020).

As an alternative to physical membrane disruption, incubation of EVs with chemical membrane permeabilizers also allows efficient loading of therapeutic cargo into EVs. Incubation of EVs with the detergent-like molecule saponin has been used to incorporate antioxidant catalase and the enzyme tripeptidyl peptidase-1 (TPP1) into bone marrow macrophage-derived EVs (up to 6.3 $\mu g/10^{11}$ particles for catalase, or 50 $\mu g/10^{11}$ particles for TPP1 were incorporated) (Haney et al., 2015, 2019). The loaded EVs significantly inhibit neurodegeneration

and neuroinflammation in mouse models of Parkinson's disease (Haney et al., 2015) and Batten disease (Haney et al., 2019). Other techniques used for EV cargo loading include multiple freeze-thaw cycles (Kalani et al., 2016), and serial extrusion of cell suspension through decreasing pore sizes in a buffer containing cargo of interest (Kim H. Y. et al., 2018). Studies comparing these processes highlight serial extrusion as the most efficient method for cargo loading, resulting in stable EVs with elevated functional effects (Fuhrmann et al., 2015; Haney et al., 2015; Kim et al., 2016; Le Saux et al., 2020). In the context of EV-based therapeutics, all cargo loading methods have intrinsic advantages and disadvantages that must be considered (**Table 3**). The methods applied should be modified according to the clinical/disease/model context (i.e., model and tissues of interest, biological/clinical question). Altogether, these advances in EV engineering provide highly customizable and combinatorial techniques to overcome current limitations of native EV as therapeutics, thus improving delivery, efficacy, and function. These advancements have allowed rapid development of EV-based therapeutics for transition to preclinical and clinical development.

CHALLENGES TO FURTHER DEVELOPMENT OF EV THERAPIES

With increasing attention on EV-based therapeutics, the need for further refinement and standardization of design, production, and clinical administration approaches is critical. Specifically, there remain several fundamental challenges the field must come to terms with; low yield of production, scalable and standardized EV generation, standardized dose and potency monitoring, determination and quantification of molecular bioactivity for regulatory purposes, and unsatisfactory targeting capacity. Fortunately, international efforts to address aspects of these are ongoing (Lener et al., 2015; Reiner et al., 2017; Witwer et al., 2019; Gandham et al., 2020; Nguyen et al., 2020; Nieuwland et al., 2020; Rankin-Turner et al., 2021). In this section, we outline the challenges faced in EV therapeutic development, with an emphasis on the updated research and technologies offering avenues for preclinical and clinical advancement (**Figure 1**).

Scalability and Standardization of EV Production

Clinical translation of EVs demands the development of standard, scalable, and cost-effective approaches for their production. For EV-based therapeutics, manufacturing requires high capacity and scalability without influencing the composition or potency of EVs (Whitford and Guterstam, 2019). A lack of clinical grade purification protocols suitable for large-scale production, and an incomplete understanding and standardization of variables influencing EV production represent main challenges in this area. Selection of an appropriate donor cell for native EVs (Charoenviriyakul et al., 2017) and monitoring variables such as growth state (epithelial, mesenchymal, or adherent/anchorage-dependent) (Tauro et al., 2013; Willms et al., 2016) can assist with this. Owing to significant functional advantages in regenerative medicine including wound healing and tissue repair, and therapeutic advantages in anti-inflammatory and low immunogenicity properties, amniotic and adipose cell-derived EVs are considered as a suitable candidates for therapeutic EV research and translation (Tan et al., 2018; Shukla et al., 2020). Aspects of culture influencing cell state include type of cell culture system [conventional and bioreactor systems (Mitchell et al., 2008; Mendt et al., 2018; Palviainen et al., 2019)] and media/supplements used (product and batch variance) (Quah and O'Neill, 2007; de Jong et al., 2012; Li et al., 2015; Patel D. B. et al., 2017; Thery et al., 2018; Zhu et al., 2021a). Moreover, modifications in culture parameters resultant of scaled-up systems, pH, mass transfer, and hydrodynamic (or shear) force, can result in modified cell state and growth, viability, expression, and activity of cell receptor/signaling, potentially impacting the composition and therapeutic efficacy of derived EVs. Further strategies exist to stimulate EV production and enhance yield have been reported, including *N*-methyldopamine and norepinephrine (Wang J. et al., 2020). However, the extent to which these factors impact EV composition, efficacy or other factors associated with their therapeutic use remains predominantly unknown (Colao et al., 2018; Adlerz et al., 2020) [reviewed in Whitford and Guterstam (2019)]. Therefore, as the therapeutic effects of EVs can be modulated by these variables, there is an emphasis on parental cell characteristics that should be carefully considered to exploit their clinical application (for example, therapeutic potency, immunogenicity, targeting selectivity), including their capacity to be manipulated (i.e., genetic engineering, transfection efficiency, genetic stability) or parental cell immortalization. For primary and immortalized cells, a thorough, risk-based analysis must be conducted, for the cells and their derived EVs, in addition to modified cells [reviewed in Rohde et al. (2019)]. In the context of EVs for therapeutic use, parental cell immortalization has been shown to enable sustainable production of EVs without influencing their therapeutic efficacy or immunosuppressive activity (Chen et al., 2011), however, safety concerns have been raised for the procedures and requirements to monitor oncogenicity and genetic drift [reviewed in Herrmann et al. (2021) and Xunian and Kalluri (2020)]. Recently, the production of therapeutic EVs has been amplified (Cha et al., 2018) by enhancing the biological functions of MSC-aggregates (spheroids) and their large-scale EV production (Cha et al., 2018). This study further highlighted key requirements in quality control (QC) monitoring cell source throughout changes in production and maintenance, monitoring morphological, size, and structural characteristics of derived EVs, their cytokine and miRNA expression and *in vitro* function, demonstrating they retained their stemness and marker gene expression during dynamic culture (Tan et al., 2018; Shukla et al., 2020). A safety concern with stem cells is the use of animal-derived serum for cell growth; the presence of such cross-species factors may cause issues from a regulatory standpoint in the production of therapeutics. Alternatively, using xeno-free culture media components or EV-depleted serum should be considered and influence on the compositions or physiological properties of derived EVs should be monitored. Accumulating evidence indicates that other naturally-derived EV

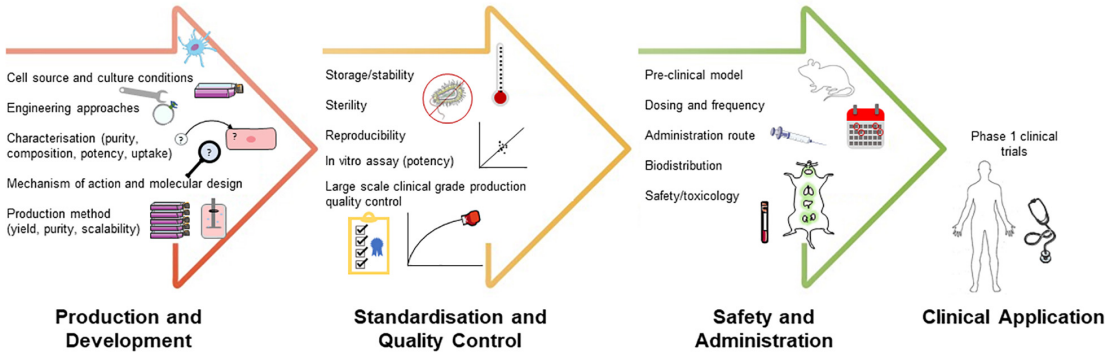

FIGURE 1 | Challenges and translational considerations of development of EV-based therapeutics. Developing rational targeting or therapeutic strategies for these different stages will advance the application of EV-based therapeutics.

source, including plant-based (NCT01668849, NCT01294072) (Dad et al., 2021) and bovine-milk-derived EVs (Grossen et al., 2021) may be sustainable alternatives for large-scale utility of engineered EVs. Bacterial EVs from non-pathogenic or probiotic bacterial source may also be harnessed as potential EV-based delivery carriers for anti-inflammatory function (Kuhn et al., 2020), with further advantages in their versatility in being readily functionalized (Shehata et al., 2019; Cheng et al., 2021) and their scalable production (Gujrati et al., 2014; Cheng et al., 2021).

For EV-based therapeutics to be considered a viable option for clinical applications, large-scale commercial production is required. For small-scale manufacturing, cells can be expanded in multilayered flasks, spinners, wave bags or fixed-bed or hollow-fiber bioreactors depending on their growth characteristics, while for large-scale culture, cells can be grown in large-capacity enclosed (stirred-tank) bioreactors or platform-rocker wave bags (Whitford and Guterstam, 2019). Presently, large-scale production of functional homogeneous stem cell-derived EVs for therapeutic utility has been developed using filtration (0.2 μm) and conventional ultracentrifugation (Mendt et al., 2018), and size-based chromatography fractionation (Reiner et al., 2017). While large-scale production of GMP-grade EVs for clinical trialing employs a combined approach of ultrafiltration coupled with sucrose/deuterium oxide (with ultracentrifugation cushion) (Lamparski et al., 2002; Nikfarjam et al., 2020). Further, GMP-grade EVs from human cardiac progenitor cells for therapeutic application have been isolated from 8 L of bioreactor-derived culture medium using tangential flow filtration (TFF) (without ultracentrifugation) in an integrated closed circuit which encompasses the full downstream process (clarification, concentration, diafiltration, and final sterilizing filtration) (Andriolo et al., 2018). This approach provides a high product yield (≥58%, 3×10^{13} particles), formulated in a clinical grade solution (Plasma-Lyte A) with concomitant consistent reduction of contaminants (total protein removal 97–98%). Importantly, this study employed a characterization QC strategy, including monitoring with suitable sensitivity, precision, and accuracy, according to regulatory guidelines. Commercially, companies including Codiak Biosciences employ TFF perfusion or alternating TFF perfusion to increase cell viability and isolate MSC-derived EVs using sequential filtration (0.8/0.45 μm), while others such as Evox Therapeutics Inc., use a combination of ultrafiltration and size-exclusion liquid chromatography. Larger-scale versions of other methods to isolate and enrich EVs (and subtypes within) may be possible with continuous-flow ultracentrifuges, size exclusion/ligand-activated core-bead technologies using distinct multimodal chemistry, microfluidics, field-flow fractionation, precipitation, continuous chromatography, and other industrial equipment, but feasibility of their use for EVs is limited (Li X. et al., 2019; Whitford and Guterstam, 2019; Yang et al., 2020; Martinez-Greene et al., 2021).

Profiling EV composition is a key strategy in understanding the influence had by modification and production variables. Technologies central to these efforts include mass spectrometry and nucleic acid sequencing. Indeed, proteomics can provide validation of modified (fusion) protein transfection in donor cells and act as a QC metric to evaluate the expression of donor cell and EV proteome and different EV/non-EV markers (Greening et al., 2017; Thery et al., 2018). Furthermore, proteomics can be employed with other biophysical/biochemical approaches to define and monitor the impact of culture media formulation from a single cell source on EV composition and surface protein epitope expression (Zhu et al., 2021a). Additionally, non-targeted metabolite profiling can assess how the metabolomic composition of EVs is influenced by conventional cell culture and bioreactor cell growth conditions (Palviainen et al., 2019). Proteomics and RNA sequencing has also been employed to indicate that cellular architecture (i.e., 2D vs. 3D) modifies transcriptomic and proteomic cargo of EVs (potentially altering overall function), the latter of which may affect efficiency of association and consequently uptake by recipient cells (Rocha et al., 2019). With significant therapeutic implications, the influence of engineered microtissues and immunoisolation devices on EV composition has recently been reported (Kompa et al., 2021; Millan et al., 2021), where microtissue stem cells cultured in a TheraCyte device 3D environment increased EV yield while additional markers (not detectable in EVs secreted by cells cultured in standard 2D conditions) were identified using proteomic profiling. Such findings reveal that cell growth conditions and media formulations influence the

yield, surface epitopes (including tetraspanins), and broad (proteome/transcriptome/metabolome) composition of derived EVs. Thus, these are critical considerations in the scalable manufacture of clinical-grade EV therapeutics, as such variables may have profound impact on their utility. For instance, modifying the abundance of transmembrane proteins such as MHC complexes on their surface may affect their low immunogenicity (Petersen et al., 2011; Robbins and Morelli, 2014; Wahlund et al., 2017), a characteristic important for the utility of EVs as therapeutics.

As complex entities, EV therapeutics will require a suite of QC measures for successful clinical translation. A combination of discovery and pre-selected/targeted "omic" analyses will enhance our understanding of bioactive EV composition, cargo selection/loading; such knowledge could be translated to monitor generation, modification, and manufacture of EV-based therapeutic products with high throughput, sensitivity, and specificity. Indeed, in-depth monitoring is essential as EV preparations for preclinical application; often most studies use a combination of EVs (subtypes) in addition to various cell-derived and extracellular elements (i.e., secretomes) [reviewed in Rohde et al. (2019) and Witwer et al. (2019)]. For the successful translation of EVs to the clinic, the identification of critical quality attributes (e.g., size, purity, molecular composition) that impact the potency and stability of the product is essential (**Figure 1**). Considering the difficulties in standardized isolation and characterization of EVs, another strategy would be to prioritize therapeutic efficacy over purity (Wiklander et al., 2019; Herrmann et al., 2021). Recently, engineered recombinant EVs and reference particles have been suggested as reference materials to monitor technical variability of EV generation and their applications and promote intra- and inter-laboratory studies (Kim M. S. et al., 2018; Varga et al., 2018; Geeurickx et al., 2019, 2021). This could be applied to manufacturing and scalability in generating and monitoring EVs.

Increased and sustainable EV production is essential for the successful application and development of EV-based therapeutics. Recently, cellular nanoporation has been shown to increase production of exosomes as a universal nucleic-acid carrier for applications requiring transcriptional manipulation [up to 50-fold greater exosome yield and >103-fold increase in exosomal mRNA transcripts (Yang et al., 2020)]. Further, M-NVs generated by microfluidic and extrusion techniques have a high and consistent production yield, in comparison to native EVs, overcoming their incumbent production and isolation challenges and represent a promising alternative to native EVs for scalable and clinical application. Considerations of such approaches in terms of physical parameters may present new difficulties in establishing a standardized protocol for their generation, efficiency of molecular incorporation, modification, and storage (Shah et al., 2019; Meng et al., 2021). Further, M-NVs are comprised of diverse, undefined molecules derived from donor cells, which may cause potential safety concerns, similar to native EVs. As such, a careful screening of producer cells is essential—focused on the consistency of long-term cell growth, and specific therapeutic potency and bioactive effect. In addition, the ability of M-NVs post-generation to encapsulate drugs (i.e., hydrophilic, negatively charged macromolecular drugs) remains inefficient (generally < 30% incorporation), highlighting the need to further refine composition to enhance encapsulation efficiency (Yoon et al., 2015; Molinaro et al., 2016).

Key criteria in the development of EV-based therapeutics is the stability, preservation and storage of EV therapies (Jeyaram and Jay, 2017; Kusuma et al., 2018). Recently, the effects of storage conditions on EVs for functional analysis and therapeutic use was investigated (Wu et al., 2021), evaluating temporal EV stability/quantity across various storage temperatures and freeze-thawing cycles. Importantly, storage alters EV size distribution and impacts cellular uptake and biodistribution, with −20°C (short-term) and −80°C (long-term) storage recommended. Further, for GMP-compliant EVs, several studies have reported native EV storage at −80°C in single-dose aliquots (preliminary stability studies indicate no loss of functional activity in up to 7 months) (Andriolo et al., 2018) and modified EVs storage at –80°C for up to 5 months with no change in potency or activity (Mendt et al., 2018).

Overall, challenges of industrial scale-up and specific variables that will impact transition to clinical development must be addressed and monitored in early development. The clinical and commercial demands of EV-based therapeutic production such as high, consistent yield, reproducible composition/purity, and efficiency can eventually be met with modifications to existing technologies for improved scalability (Colao et al., 2018; Adlerz et al., 2020).

Analytical Assessment of EVs: Potency and Molecular Insights for Regulatory Purpose

In delivering EV-based therapeutics dosing remains a significant challenge, with dose selection, assessment, and administration (route, frequency, time window) all factors in achieving therapeutic benefit without adverse effects. Development of appropriate potency assay that employ relevant functional end points (Nguyen et al., 2020), and demarcate *in vitro* potency (dependent on EV property and cell type) and *in vivo* efficacy is required (Willis et al., 2017; Witwer et al., 2019). Such potency assays need to be standardized and context-/disease-/model-dependent, which are unfortunately currently limited in their acceptance and utilitization (Adlerz et al., 2020).

Analytical assessment and monitoring, and rigorous *in vitro* and *in vivo* testing for safety and efficacy must precede approval of EV-based therapeutics [as comprehensively discussed (Reiner et al., 2017; Nguyen et al., 2020)]. Several seminal clinical-grade EV studies have reported specific potency assessments including *in vitro* anti-/pro-activity, sterility, *in vivo* pro- activity, and toxicity or immunogenicity assays (Andriolo et al., 2018; Mendt et al., 2018). Analytical assessments include: (i) molecular fingerprinting (identify bioactive/target molecule/s), monitoring of multiple components and influence of preparation/isolation method on donor and cell-derived product), (ii) potency assays (Nguyen et al., 2020) (monitoring therapeutic effect *in vitro*/*in vivo*), and (iii) mechanistic assays (identify mode of action) (Willis et al., 2017; Colao et al., 2018; Surman et al.,

2019; Witwer et al., 2019). Such analytical assessments may provide quality control in the transition from research-grade to clinical (GMP)-grade EVs [reviewed in Adlerz et al. (2020) and Willis et al. (2017)].

At present there are no standardized methods in quantifying EV concentration and dosage. Moreover, comparative studies of EV monitoring techniques has shown that protein concentration is not relative to particle number (Lobb et al., 2015); consequently such criteria is insufficient to determine dosing. Quantitative EV analytical methods include reporting cell equivalents, protein concentration, and quantitative analytical measurement [dynamic light scattering (DLS), single-particle interferometric reflectance imaging sensing, tuneable resistive pulse sensing (RPS), nanoflow cytometry and nanoparticle tracking analysis (NTA)] (Arab et al., 2021). Significant concerns have recently been presented around existing single-particle analysis capabilities (e.g., sizing, counting, tetraspanin phenotyping) (Arab et al., 2021). Moreover, single-particle technologies will be required to separate heterogeneous EV populations into well-defined and easily recognized subgroups. Therefore, consideration of limitations in some analytical platforms is a key requirement [e.g., resolution and accuracy of the quantitative analysis of EVs using single-particle analysis capabilities (Welsh et al., 2020; Arab et al., 2021)]. In order to fully characterize a therapeutic nanoparticle preparation, it is imperative to consider particle size, particle size distribution, charge, number of particles or concentration, and the molecular composition (Nelson et al., 2020). Absolute values measured for particle or protein concentration thus need to be critically evaluated and compared to control condition, using calibration/standardized measurements at specific dose as required. Current methods have shown to be efficient in quantifying the biophysical parameters [light-scattering methods (multiangle light scattering, DLS, NTA, particle interferometric reflectance imaging sensing), RPS, transmission- and cryogenic-electron microscopy, and small angle neutron scattering], hence providing the tools to validate and standardize nanoparticle therapeutics (Buzas et al., 2017). It should be noted, these technologies are not able to determine the particle type or chemical/molecular makeup of a sample, making it difficult to determine whether the sample contains EVs, protein aggregates, or other non-membranous particles (Arab et al., 2021). This limitation requires optimization of sample purity prior to size measurement.

Expectantly, EV heterogeneity confounds aspects of therapeutic application, with subtypes varying in composition and function. One of the challenges of EV biology is identification of specific EV-subtype markers (Thery et al., 2018). If aiming to purify EVs for stringent biochemical analysis (e.g., define cargo for regulatory purposes, quality control, therapeutic design) and specific functionality (e.g., identification of bioactive cargo, therapeutic screening), then rigorous purification and fractionation strategies are critical [reviewed in Gandham et al. (2020); Xu et al. (2016)]. Current research indicates that further subdivisions of EVs are needed to accurately differentiate subtypes (e.g., biogenesis, size, charge, molecular cargo), which may in turn offer unique therapeutic avenues. A key advantage here is the considerations of quality assessment and quantification of EV preparations based on recommendations of MISEV2018 and EVTRACK (Consortium et al., 2017; Thery et al., 2018). These considerations will be essential for generation of clinical-grade EVs or standardized engineered EVs (Lener et al., 2015; Merino-Gonzalez et al., 2016; Paganini et al., 2019; Meng et al., 2020; Salmond and Williams, 2021) (**Figure 1**).

To establish consistency in the therapeutic assessment of clinically translated EVs, effective dosing harmonization is a key requirement. It is recommended to consider the type of payload, whether it is a drug (Doxorubicin, Aspirin, Gemcitabine), an enzyme/protein (caspase, TTP1) or other biological component (nucleic acids, lipids). It is known these groups possess different pharmacokinetics and thus different units have been assigned to describe their activity or effect i.e., drug dosing is expressed in terms of mass of active substance (μg, mg, g) per pharmaceutical presentation unit (capsule, ampule, particles, grams) (Powell et al., 2021); enzymes and protein dosing is expressed in terms of enzymatic catalytic activity or IU (1 IU = 1 μmol/min = 1/60 μmol/s ≈16.67 nmol/s) (Units of Enzyme Activity, 1979; Iupac, 2018); lastly, biological payload such as nucleic acids and lipids could potentially be expressed in units of mass or activity depending on their function. Another consideration is the function and effect of the native/engineered EVs itself, aside from specified cargo. As vesicles interact with the host, proper controls should be established to determine their protective [i.e., RNAse-mediated degradation effect (Mendt et al., 2018)] and measured effect without the cargo of interest (Kennedy et al., 2020). Studies have shown that dosing variations of purified EV (from 30 to 300 μg of protein) did not present any adverse consequences and still exerted their desired effect in a myocardial infarction model *in vivo* (Barile et al., 2014). This suggests the key for dosing is not the particle itself but the cargo, especially if incorporated post-isolation.

As EVs are biological entities, their complex and variable nature challenges the highly regulated requirements for pharmaceutical production. A solution to this is functional assessment of each batch [cell source consistency (Viaud et al., 2011; Andriolo et al., 2018; Mendt et al., 2018) and EV production for specific patient-derived cell source based on EV yield (Mendt et al., 2018), positive/negative EV markers (Andriolo et al., 2018; Mendt et al., 2018), reviewed in Nguyen et al. (2020)]; identification and quantification of key markers indicating functional competency could aid this process (van Balkom et al., 2019) [i.e., tissue-based immunoregulation (Ma et al., 2020)]. Mapping specifically regulated proteins using microfluidic approaches (Zhao et al., 2016; Fang et al., 2017; Xu et al., 2018) or quantitative proteomics onto known protein networks has highlighted mechanism of action (Martin-Jaular et al., 2021) and is being applied to EV research (manufacture, composition, and function). Proteomic approaches allow researchers to understand protein signatures of native and engineered EVs (Nasiri Kenari et al., 2019; van Balkom et al., 2019), which may have implications in quality control platforms to confirm the identity and test for purity of therapeutic EVs. Proteomics has been used to assess plasma EVs after separation from

lipoproteins (i.e., lipoprotein particle depletion) (Karimi et al., 2018), tissue-derived stress/damage markers following cardiac-EV isolation (Claridge et al., 2021), and oncogenic mutations on EV proteome landscape (Al-Nedawi et al., 2008; Lobb et al., 2017; Emmanouilidi et al., 2019; Chennakrishnaiah et al., 2020; Shafiq et al., 2021; Tawil et al., 2021). Several key omic-based studies have provided direct insight into the composition and (re)classification of EVs, their biogenesis and content (Greening et al., 2015; Kowal et al., 2016; Jeppesen et al., 2019; Kugeratski et al., 2021; Martinez-Greene et al., 2021; Rai et al., 2021). Moreover, an aptamer-based proteomic analysis (SOMAscan) has enabled multiplexed, highly-sensitive, and specific protein detection in human blood and other biomatrices, and applied to profile EVs (Welton et al., 2017). Other high-throughput MS approaches, such as multiple/parallel reaction monitoring MS (MRM/PRM-MS) enable rapid and accurate identification and quantification of protein biomarkers in broad dynamic range (Anwar et al., 2019; Makridakis et al., 2020). Indeed, MRM-MS has been used for identification of *Mycobacterium tuberculosis* peptides in patient derived exosomes (Kruh-Garcia et al., 2014), highlighting the sensitivity and specificity of this approach in EVs. Additionally, RNA profiling has been used to confirm the absence of inhibitory miRNAs (Rohde et al., 2019).

The application of multi-omic technologies, such as transcriptomics and proteomics are emerging as crucial for understanding how EVs perform therapeutic functions (Li et al., 2021d). In the wider drug development field, omics technologies are already in use to better understand mechanisms of action and identify off-target effects (Jia et al., 2019; Friman et al., 2021). Additionally, comprehensive profiling of patient and population proteomes/transcriptomes contribute to *in silico* predictions of drug efficacy [reviewed in El-Khateeb et al. (2019)]. Integration of cellular technologies [microphysiological systems or "tissue chips" (Ronaldson-Bouchard and Vunjak-Novakovic, 2018; Low et al., 2021; Zhang S. et al., 2021)] aimed to closely mimic *in vivo* conditions to assess the potency of experimental biological products and understanding of tissue-specific effect will be important future developments in clinical translation. The development of new technologies, such as machine learning and control algorithms, to interface with microphysiological systems may further advance their use in systems biology and precision medicine (Zhang Y. S. et al., 2017). For clinical translation, the FDA does not require any understanding of the mechanism by which a drug acts, moving directly to clinical trials without this knowledge may be a clear path. However, often limitations in understanding how the drug or biological regulator works may contribute to unfortunate outcomes, off-target/side effects, or poor/ineffective dosing; understanding mechanism of action may help to stratify/focus clinical trials to those patients most likely to respond (No Author, 2010). Significant changes in the FDA (Critical Path Initiative) now support development of patient screens to improve the chances for drug approval, and integration of pharmacogenomic data (proteomics, metabolomics, genome-wide association study data) in developing and evaluating medicines. In the context of EV-based therapeutics, identification of active components as a constituent of the total EV will enable optimization and improvements in dosing and effect (Xin et al., 2017; Jia et al., 2021).

Tissue Targeting and Delivery of Therapeutic EVs

Specific, targeted delivery presents a major challenge for EV-based therapeutics. Sophisticated solutions are required to minimize off-target accumulation and maximize efficacy through the targeted transport and delivery of cargo (Vargason et al., 2021). Indeed, a major obstacle preventing widespread usage of other types of regulatory therapeutics (i.e., nucleic acid polymers/oligonucleotides) is efficient delivery to target organs and tissues, minimizing off-target accumulation [reviewed in Roberts et al. (2020)]. In nanoparticle-based therapeutics, the pharmacokinetics, biodistribution and targeting are all influenced by design and biological factors of the nanoparticle constructs (Haun and Hammer, 2008; Calderon et al., 2009; Haun et al., 2011; Gu W. et al., 2019) [reviewed in Mitchell et al. (2021) and Wei et al. (2018)]. Additionally, different cell types and phenotypes internalize and traffic nano-carriers differently (MacParland et al., 2017; Patel S. et al., 2017; Brownlee and Seib, 2018) [reviewed in Biswas and Torchilin (2014) and Howard et al. (2014)]. This is especially relevant for EV therapeutics, as the heterogeneity of EVs can contribute to differential recognition and binding specificity and target cell uptake (Chivet et al., 2014; Choi et al., 2019) [reviewed in Mathieu et al. (2019)]. Characterization of such mechanisms will be essential in the field's pursuit of accurate, potent, on-target delivery and uptake of EV therapeutics.

Drug targeted delivery can be passive, where circulating nanoparticles [e.g., coated with platelet or red blood cell membranes (Usman et al., 2018)], extravasate through leaky vasculature and accumulate in surrounding tissue *via* the enhanced permeability and retention (EPR) effect (Chan et al., 2010; Hu et al., 2011; Howard et al., 2014; Luk et al., 2016). Alternatively, targeting can be active, relying on membrane moieties to bind protein/peptide receptors/sequences on target cells or tissues (Peer et al., 2007; Bertram et al., 2009; Cheng et al., 2020; Yang et al., 2020). The relatively large surface area to volume ratio of EVs enables highly efficient surface interactions, highlighting the latter form of targeting as a promising avenue for development. Recently, the inflammation-targeting ability of platelet-derived EVs (PEVs) was verified, with drug-incorporated PEVs able to target inflamed lungs *in vivo* to precisely deliver and modify the immune response at the site of inflammation (Ma et al., 2020), revolutionizing the way inflammatory diseases can be treated. While effective, the molecular interactions (perhaps surface-based) behind this functional delivery are not yet understood. However, there are known protein signatures on EV surfaces which direct uptake in specific organs (Hoshino et al., 2015); unfortunately, very few targeting moieties have been identified and characterized on EVs. Defining additional signatures is essential for the refinement of targeted EV therapeutics, and will require analysis of their surfaceome and biodistribution, as well as the molecular signatures of proteins/peptides exposed in the targeted

tissue/cell pathology [reviewed in Howard et al. (2014) and Shao et al. (2018)].

The surface proteome of EV subtypes (exosomes) has been defined using various proteolytic and biotinylation approaches (Cvjetkovic et al., 2016; Williams C. et al., 2019; Xu R. et al., 2019) with their topology determined using similar methods (Jeppesen et al., 2019). This has provided insights into components exposed and available for cell-interaction. Recently, proteins with exposed regions on the EV surface, CD63 and LAMP2B, have been the base for topologically distinct scaffolds for fusion proteins containing targeting sequences, enabling the flexible engineering of EV surface for applications in disease-targeted drug delivery and therapy (Curley et al., 2020). Use of these fusion protein scaffolds of attachment of components with known binding partners enriched at target sites has successfully assisted in increased targeted uptake, but has not abolished non-specific uptake (Munagala et al., 2021). Development of new scaffolds is dependent on the display of a N- or C-terminal on the outer surface of EVs, with topology of the surface proteins essential (Jeppesen et al., 2019). However, the surface signature of EVs is highly heterogeneous, which presents a challenge in designing targeting scaffolds present on all EVs, rather than a subtype, and could reflect different baseline functionalities (Kowal et al., 2016; Xu R. et al., 2019). Furthermore, despite the high lipid content and diversity on EVs, the role of lipids (i.e., glycosphingolipids) in drug delivery, specific tissue-derived EVs (Flaherty et al., 2019), and how other surface moieties facilitate delivery (Zhu et al., 2018; Mentkowski and Lang, 2019) is still poorly understood. Comprehensive multi-omic surfaceome studies of homogeneous EVs will assist in understanding which effectors (e.g., tetraspanins, integrin receptors/ligands, glycoproteins, membrane lipids) facilitate direct functional content transfer and targeting, findings which will be critically important in exploiting EVs as drug delivery systems (Kooijmans et al., 2021; Richter et al., 2021). Combining surfaceome characterization with techniques to study tropism (Hoshino et al., 2015; Wu et al., 2020) and biodistribution (Wu et al., 2020) will assist in identification of components that determine targeting and interaction.

Understanding the biodistribution of EVs is critical for the identification of targeting-moieties and ensuring dosage at the intended site/tissue. While simple, fast, and relatively inexpensive to use, lipophilic dyes can present issues for biodistribution studies. Dyes can form micelles (Puzar Dominkus et al., 2018) and interfere with EV size, charge, uptake, biodistribution and clearance (Lai et al., 2014; Dehghani et al., 2020), raising questions about their suitability for this application (Takov et al., 2017). Indeed, EVs are likely degraded and/or recycled *in vivo* resulting in inaccurate spatiotemporal information (Takov et al., 2017)—a consideration when using such dyes is their capacity to interact with other membranes and remain visible in such tissues. Fortunately, alternatives to dye-based tracking are emerging; recent developments in live cell reporters allow visualization of EV biogenesis, uptake and intracellular trafficking (Sung et al., 2020). Fusing surface proteins to NanoLuc or ThermoLuc allow highly sensitive *in vivo* quantification or real time imaging, respectively, at low cost and in semi-high throughput (Kojima et al., 2018; Gupta et al., 2020). Additionally, other reporter systems have been used to label EVs for *in vivo* imaging, spatiotemporal dynamics, and pharmacokinetic analysis of administered EVs (Takahashi et al., 2013; Lai et al., 2014; Sung et al., 2020; Wu et al., 2020). Imaging technologies have shown EV biodistribution is influenced by route of administration (intravenous, intraperitoneal, and subcutaneous injection) and dosage (Ohno et al., 2013; Takahashi et al., 2013; Wiklander et al., 2015). Mode of delivery also effects the plasma pharmacokinetic patterns of EVs, while different subpopulations of EVs differ in their *in vivo* biodistributions (Gupta et al., 2020). This variation presents a challenge for the clinical application of systemically administered EV therapies, with altered biodistribution complicating potential dosage and off-target effects. However, non-systemic administration of EVs may bypass this challenge.

Delivery of EV-based therapeutics directly to the site of interest overcomes the limitations of current therapeutic delivery strategies and can ensure sequestration of the therapeutic. Lung-based pathologies are employing inhalation as the method of administration to ensure site-specific delivery (NCT04389385, NCT04747574, and NCT04276987). Injections of EVs to sites of damage are another method of focusing delivery, with therapeutic application of EVs directly to arthritic joints (Wang et al., 2017; Wu et al., 2019; Li et al., 2021b), venous ulcers (NCT04652531), inner ears (Gyorgy et al., 2017), eyes (NCT03437759), and heart [damaged myocardium (Yao J. et al., 2021)]. While suitable for individual treatments at injection-accessible sites, ongoing release of EV-based therapeutics at inaccessible sites raises a challenge. The injection or topical application of hydrogels and biogels offer a form of slow-release EV administration directly to the site of injury (Li L. et al., 2020; Shi et al., 2021; Zhao et al., 2021; Zhu et al., 2021b). Furthermore, the recent development of EV-coated scaffolds, such as cardiac stents and cardiac fibrin patches, allow for the therapeutics extended release at the site of damage (Hu S. et al., 2021; Yao J. et al., 2021). As a minimally-invasive direct therapeutic delivery strategy, EVs have been shown to be released in high capacity *in vivo* from human stem cells within a semi-permeable chamber inserted subcutaneously for cardiac therapy (Kompa et al., 2021). Coating implants for expedited healing and recovery is an area with great therapeutic potential, however, refinements and standardization will be required prior to clinical translation. Developments utilizing shape memory materials [e.g., smart or intelligent materials (Huang et al., 2019; Zhao et al., 2019)] and dynamic response composites which can adapt to external stimuli, could offer significant advantages in EV-coated implant/scaffold systems for wound treatment (Melocchi et al., 2021). Further improvements to EV-based therapeutic delivery will be made with additional molecular characterization of EV-tissue tropism, biodistribution, and delivery mechanism designs to ensure the specificity of therapeutic delivery for specific cell types.

CONCLUSION

Extracellular vesicle-based therapeutics hold the greatest clinical promise when a combination of native and engineered aspects are utilized. Native EVs hold innate therapeutic potential—they are biocompatible, stable, and due to their specific targeting, facilitate therapeutic use. However, there are significant challenges associated with their commercialization and clinical development, with engineered EVs allowing modified content, increased production, and targeting for improved therapeutic outcome. Regardless of native or engineered state, there are several aspects which must be considered prior to pharmaceutical translation and clinical application (**Figure 1**), primarily the source, standardized EV generation/isolation and characterization, defining composition/potency/dose/safety, understanding targeting and biodistribution, and elucidating mechanism of action in target cell(s). This review details diverse scalable processes/strategies for EV generation and isolation, and development/integration of methods for research/clinical grade quality control. In developing EV-based therapeutics, a quantitative analysis of pharmacokinetics, biodistribution, and influence of storage conditions on shelf life, are essential. Indeed, several seminal studies have reported a clinically feasible approach for EV production, scalability, and storage for therapeutic application (Gimona et al., 2017; Andriolo et al., 2018; Mendt et al., 2018; Witwer et al., 2019). Interestingly, for EV-based therapeutic development, as discussed (Grangier et al., 2021), the most advanced scale-up strategies preferentially use abundant quantities of available biomaterials (biofluids, blood products including plasma, red blood cells, platelets) and produce engineered EVs or secretome-based products for translation. Such strategies may be the result of the clinical translation of prior regulatory requirements in the established, licensed use of these products as medicines, abundant supply, human origin, and biotechnology and cell therapy industry links to accelerate clinical translation. The use of these products has significantly inherent translational advantages for hemostasis, for regenerative medicine, and as drug-delivery vehicles (Johnson et al., 2021). Once quality control concerns have been addressed and applied by the field, clinical trials will be able to advance further (Gandham et al., 2020). Successful translation of EV therapeutics in immunomodulation, regeneration and repair, and combination therapies will provide desperately needed treatments globally, transforming the current bio-pharmaceutical landscape.

Considering that EVs carry and transfer various functional molecular cargo, quantitative global analyses to understand EV-associated components [e.g., EV-mediated transfer of proteins/lipids/RNAs between specific cell types and organs (Costa-Silva et al., 2015; Hoshino et al., 2015; Flaherty et al., 2019; Rodrigues et al., 2019; Kugeratski et al., 2021; Nguyen et al., 2021)] warrants further investigation. Increasingly, the field is shifting toward systems biology to understand EVs (Xu et al., 2016; Gezsi et al., 2019), integrating different analysis platforms to achieve multi-omic characterization of EVs for therapeutic application—their source (different donor origins, organ/tissue-derived), composition (including core and surfaceome/interactome landscape), and capacity to reprogram target cells and phenotype (Hoshino et al., 2015, 2020; Melo et al., 2015; Kowal et al., 2016; Xu et al., 2016; Figueroa et al., 2017; Greening et al., 2017; Flaherty et al., 2019; Rontogianni et al., 2019; Xu H. et al., 2019; Jung et al., 2020; Bijnsdorp et al., 2021; Kugeratski et al., 2021; Rai et al., 2021). Further development of these technologies will facilitate the advancement and refinement of EV-based therapeutics. Indeed, developments in EV-based function in cross-kingdom delivery [i.e., RNAs in trafficking from plant to regulate fungal pathogens (Cai et al., 2018)] may further develop effective delivery methods of pathogen-targeting regulatory RNAs for therapeutic and agriculture use.

Designing EV-based therapeutics is reliant on identification of components with a beneficial effect at a target site. Given their complex composition, mechanisms by which EVs induce their therapeutic effects remain incompletely understood. As a priority, a major challenge toward therapeutic utility of EVs is their heterogeneity in content and composition inherent in their biogenesis and generation. This heterogeneity currently complicates the design, dose, standardization, regulation, and delivery of EV-based therapeutics. Enrichment strategies that can distinguish between different EVs may help identify the functional sub-population and enrich for active components. Such an approach will not only result in more potent therapeutic applications but allow decoding and translation of molecular insights and mechanisms of action underlying function. The design of simple, effective, and cost-efficient processes to assess the required purity of EVs will facilitate much-needed standardization in the field. Careful consideration and standardized/regulatory requirements for challenges raised in this and other key reviews (Willis et al., 2018; Whitford and Guterstam, 2019; Wiklander et al., 2019; Gandham et al., 2020; Nguyen et al., 2020; Rankin-Turner et al., 2021) will assist in development of EV-based therapeutics, bringing them closer to the clinic (Davis et al., 2008; Chan et al., 2010; Buss and Bhatia, 2020; Li et al., 2021c; Swingle et al., 2021; Yang et al., 2021).

AUTHOR CONTRIBUTIONS

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Engineered Fibroblast Extracellular Vesicles Attenuate Pulmonary Inflammation and Fibrosis in Bleomycin-Induced Lung Injury

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Pulmonary fibrosis is a progressive disease for which no curative treatment exists. We have previously engineered dermal fibroblasts to produce extracellular vesicles with tissue reparative properties dubbed activated specialized tissue effector extracellular vesicles (ASTEX). Here, we investigate the therapeutic utility of ASTEX *in vitro* and in a mouse model of bleomycin-induced lung injury. RNA sequencing demonstrates that ASTEX are enriched in micro-RNAs (miRs) cargo compared with EVs from untransduced dermal fibroblast EVs (DF-EVs). Treating primary macrophages with ASTEX reduced interleukin (IL)6 expression and increased IL10 expression compared with DF-EV-exposed macrophages. Furthermore, exposure of human lung fibroblasts or vascular endothelial cells to ASTEX reduced expression of smooth muscle actin, a hallmark of myofibroblast differentiation (respectively). *In vivo*, intratracheal administration of ASTEX in naïve healthy mice demonstrated a favorable safety profile with no changes in body weight, lung weight to body weight, fibrotic burden, or histological score 3 weeks postexposure. In an acute phase (short-term) bleomycin model of lung injury, ASTEX reduced lung weight to body weight, IL6 expression, and circulating monocytes. In a long-term setting, ASTEX improved survival and reduced fibrotic content in lung tissue. These results suggest potential immunomodulatory and antifibrotic properties of ASTEX in lung injury.

Keywords: extracellular vesicles (EVs), genetic engineering, regenerative therapy, lung injury, inflammation, fibrosis

INTRODUCTION

Pulmonary fibrosis (PF) is a progressive lung disease characterized by alveolar epithelial damage and the accumulation of collagen in the pulmonary interstitium (Barratt et al., 2018). Globally, PF is on the rise with incidence and prevalence nearly doubling (Hutchinson et al., 2015; Strongman et al., 2018) with commensurate increases in resource use and cost (Diamantopoulos et al., 2018). Current strategies include antifibrotic compounds and tyrosine kinase inhibitors such as pirfenidone and nintedanib, respectively (Raghu et al., 2015). Despite these advances, options remain limited as current treatment strategies only function to decelerate disease progression.

Extracellular vesicles (EVs) are nanosized lipid bilayer particles secreted by nearly all cell types and function as a versatile mode of paracrine and endocrine signaling (Sahoo et al., 2021). EVs derived from therapeutic cell types are the principal signaling mediators of cell therapy in a variety of models (Marban, 2018b; Sahoo et al., 2021). Therapeutic EVs have the potential to repair tissue in ways conventional therapeutics cannot. This is due, in part, to the plethora of bioactive signals in their payload which affect multiple pathways (as opposed to a single target) (Marban, 2018a; Popowski et al., 2020). Our group has recently identified pathways involved in cell (and EV) potency in a therapeutic cell type, cardiosphere-derived cells (CDCs), a cardiac progenitor cell type with preclinically and clinically demonstrated tissue-reparative capacity (Smith et al., 2007; Makkar et al., 2012; Gallet et al., 2016). This finding led to engineering these therapeutic properties in an otherwise non-therapeutic cell type, skin fibroblasts. Constitutive activation of beta-catenin and gata4 in skin fibroblast turned them into producers of therapeutic EVs.

We call these engineered fibroblasts and the EVs they produce activated specialized tissue effector cells (ASTECs) or extracellular vesicles (ASTEX). Preclinical data suggest that ASTECs and ASTEX recapitulate the therapeutic effects of CDCs and CDC-EVs. Previous work demonstrates that ASTECs and ASTEX trigger functional improvement in injured mouse hearts (Ibrahim et al., 2019). Furthermore, in a mouse model of Duchenne muscular dystrophy (DMD), ASTEX trigger exercise improvement, attenuation of fibrosis, and preservation of skeletal muscle myofibers (Ibrahim et al., 2019). Further investigation implicated enrichment of miR-92a in ASTEX and downstream signaling of the bone morphogenic protein among the protissue reparative pathways. Bone morphogenic protein (BMP) signaling plays an active role in lung tissue recovery. For instance, upregulation of BMP signaling through derepression of noggin was protective in a mouse model of bleomycin-induced lung injury (De Langhe et al., 2015). Furthermore, single-cell analysis of the injured lung epithelium identified BMP repression and its rescue-attenuated epithelial metaplasia and fibrosis (Cassandras et al., 2020). Finally, epigenetic analysis of lung tissue from pulmonary fibrosis patients identified significant methylation of the miR-17-92 cluster (of which miR-92a is included) (Dakhlallah et al., 2013). Among the targets of miR-92a is WNT1-inducible-signaling pathway protein 1 (WISP1) that signals to the profibrotic transforming growth factor-beta (TGFβ) pathway in pulmonary fibrosis (Berschneider et al., 2014). Given this mechanistic rationale, we investigated the therapeutic utility of ASTEX *in vitro* and in a bleomycin mouse model of lung injury.

RESULTS

Engineered Fibroblasts Secrete Higher Quantities of EVs With Wider Size Distribution and Distinct Marker Expression From Primary Fibroblast EVs

Following transduction of dermal fibroblasts with beta-catenin and gata4, ASTECs exhibited features of cell immortalization including faster growth rate and extended population doubling and increased telomerase (Ibrahim et al., 2019). Nanosight tracking analysis demonstrated that ASTEX had a broader diversity of EV sizes compared with EVs from primary dermal fibroblasts (DF-EVs) as shown by nanosight tracking analysis (DF-EV: **Figure 1A**). Despite this, the average size difference between DF-EVs and ASTEX was nominal (**Figure 1B**). By quantity, ASTECs secrete nearly double the amount of EVs compared with primary fibroblasts (**Figure 1C**). This was expected as immortalization has been shown to increase EV secretion (Yu et al., 2006). Analysis of EV preparations confirmed the presence of conserved EV markers including CD9, CD81, HSP90, and TSG101 and the absence of the contaminating endoplasmic reticulum marker calnexin (**Figure 1D**).

The ASTEX Proteome Is Enriched in Transcription Factors and Deficient in FGF Signaling and Complement Activators Compared to Fibroblast EVs

To compare the protein content of DF-EVs and ASTEX, we performed proteomics on both EV populations. Using FunRich (Pathan et al., 2015), a software tool for functional enrichment and network analysis, we identified clear differences in protein content. ASTEX contained a wider diversity of proteins (**Figure 2A**). ASTEX had lower levels of extracellular matrix and complement factors and were enriched in proteins with enzymatic activity and chaperone proteins (**Figure 2B**). Among the most bioactive protein cargo in EVs are transcription factors as they can affect multiple gene targets in host cells. Analysis of transcription factors in both groups showed that ASTEX contained a wider diversity of transcription factors activated by beta-catenin and gata4 including MAFK (Katsuoka et al., 2000; Cao et al., 2019) and RUNX1 (Su and Gudas, 2008; Ugarte et al., 2015) which are involved in antioxidant response (Nguyen et al., 2000) and regulation of hematopoiesis (Bowers et al., 2010; **Figure 2C**). ASTEX were also deficient in homeobox proteins most notably ALX1, which are downregulated by beta-catenin (Ettensohn et al., 2003). Analysis of the predicted biological activity of EV proteins demonstrated suppression of pathways downregulated by beta-catenin signaling including vascular endothelial growth factor (VEGFR) (Zhang et al., 2001) and fibroblast growth factor signaling (FGFR) (Wang et al.,

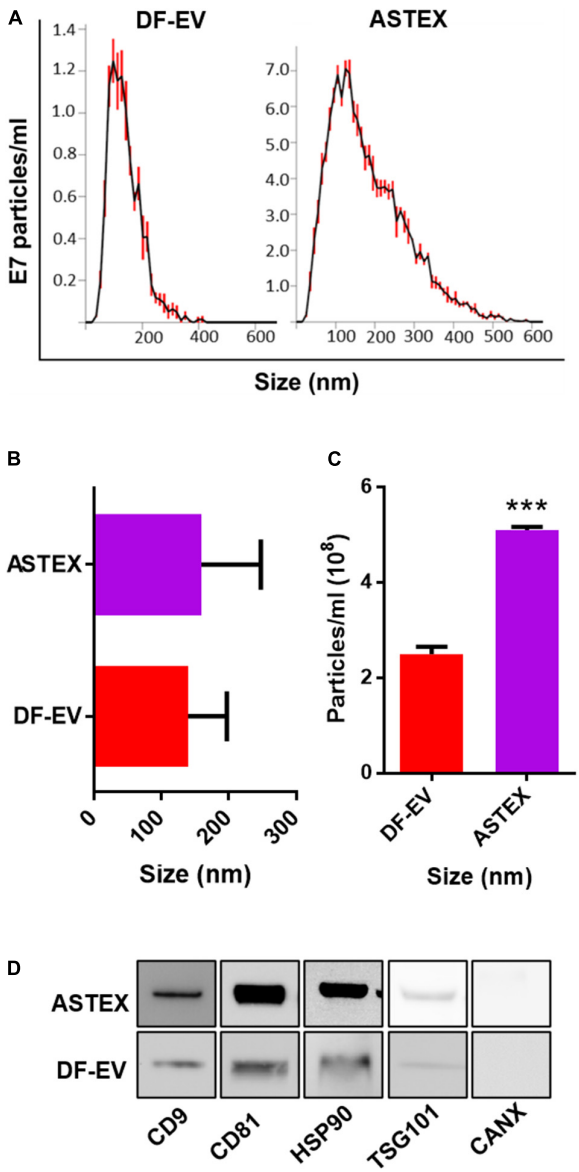

FIGURE 1 | Characterization of DF-EVs and ASTEX. **(A–C)** Nanosight tracking analysis showing size distribution and concentration from conditioned media. **(D)** Western blot of conserved EV markers including tetraspanins (CD9 and CD81), heatshock protein 90 (HSP90), tumor susceptibility gene 101 (TSG101), and absence of the ER protein calnexin (CANX). Statistical comparison was done using independent Student's *t*-test with 95% CI; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2017) in ASTEX compared with DF-EVs. The most prominent biological activity in ASTEX was relevant to mRNA transcription and translation (**Figure 2D**). Beta-catenin activation is associated with broad changes in the transcriptome with a corresponding increase in protein translation (Valenta et al., 2012). Therefore, ASTEX had reduced complement fixation capacity (and immune activation), less extracellular matrix burden, and increased catalytic activity and mRNA transcription. These results suggest fundamental proteomic changes in the beta-catenin/gata4-engineered fibroblasts and, by extension, their EVs.

ASTEX Are Enriched in miRNAs With Anti-Inflammatory and Anti-Fibrotic Function

Sequencing RNA from DF-EVs and ASTEX showed dramatic changes in RNA content. Most strikingly, activation of beta-catenin and gata4 in fibroblasts increased micro-RNA (miR) and Piwi RNA (piRNA) abundance in ASTEX. MiR abundance, in particular, increased nearly threefold compared with DF-EVs. Moreover, the abundance of ribosomal RNA (rRNA) and transfer RNA (tRNA) were reduced in ASTEX (**Figure 3A**). An analysis of differentially expressed miRs identified significant divergence in the miR content of DF-EVs and ASTEX (**Figure 3B**). ASTEX were deficient in proinflammatory and profibrotic miRs including miR-199a, miR-143, miR-382, and miR-134 (**Figure 3C**). MiR-199a promotes fibrosis through suppression of caveolin and induction of the TGFβ pathway (Yang et al., 2020). MiR-143 also promotes fibrosis through targeting sprouty RTK signaling antagonist 3 (Spry3) to activate the p38-ERK-JNK signaling axis (Li et al., 2019). MiR-382 promotes inflammation and fibrosis through targeting of PTEN and potentiation of the NFkB-AKT axis (Wang et al., 2020). MiR-134 promotes inflammatory activation in macrophages through targeting angiopoietin-like 4 (Lan et al., 2016). Conversely, ASTEX were enriched in immunoregulatory and antifibrotic miRs including miR-183, miR-182, miR-19a, and miR-92a (**Figure 3D**). MiR-183 and miR-182 target epithelial to mesenchymal transition and myofibroblast development through targeting snail family transcriptional repressor 1 (SNAIL) (Li et al., 2018) and C/EBP homologous protein (CHOP), respectively (Yao et al., 2017; Burman et al., 2018). MiR-19a regulates inflammation through targeting of suppressor of cytokine signaling 1 (SOCS1), a potentiator of macrophage activation (Gao et al., 2019). Finally, miR-92a, a miR identified (and discussed earlier) as a driver of beta-catenin-mediated therapeutic potency (Ibrahim et al., 2019), regulates macrophage inflammation through attenuation of mitogen-activated protein kinase kinase 4 (MKK4) (Lai et al., 2013) and fibrosis through targeting of the TGFβ mediator, WISP1 (Berschneider et al., 2014). Finally, we validated the overexpression of these therapeutic miRs by qPCR to demonstrate significant enrichment in ASTEX compared with DF-EVs (**Figure 3E**). Therefore, ASTEX contain miRs that target inflammatory and profibrotic pathways compared with DF-EVs.

ASTEX Exert Immunomodulatory and Anti-Fibrotic Effects *in vitro*

Macrophages including tissue-resident and monocyte-derived (circulating) macrophages play distinct roles in lung tissue homeostasis and disease (Grabiec and Hussell, 2016). Tissue-resident macrophages are depleted in injury and are replaced by circulating monocytes that differentiate into macrophages with a spectrum of phenotypes (Morales-Nebreda et al., 2015). We decided to focus on macrophages derived from circulating monocytes as these drive pulmonary inflammation and fibrosis and persist in pulmonary tissue throughout the disease (Misharin et al., 2017). Therefore, we sought to assess the effect of ASTEX on bone marrow-derived macrophages (BMDMs). Exposure of

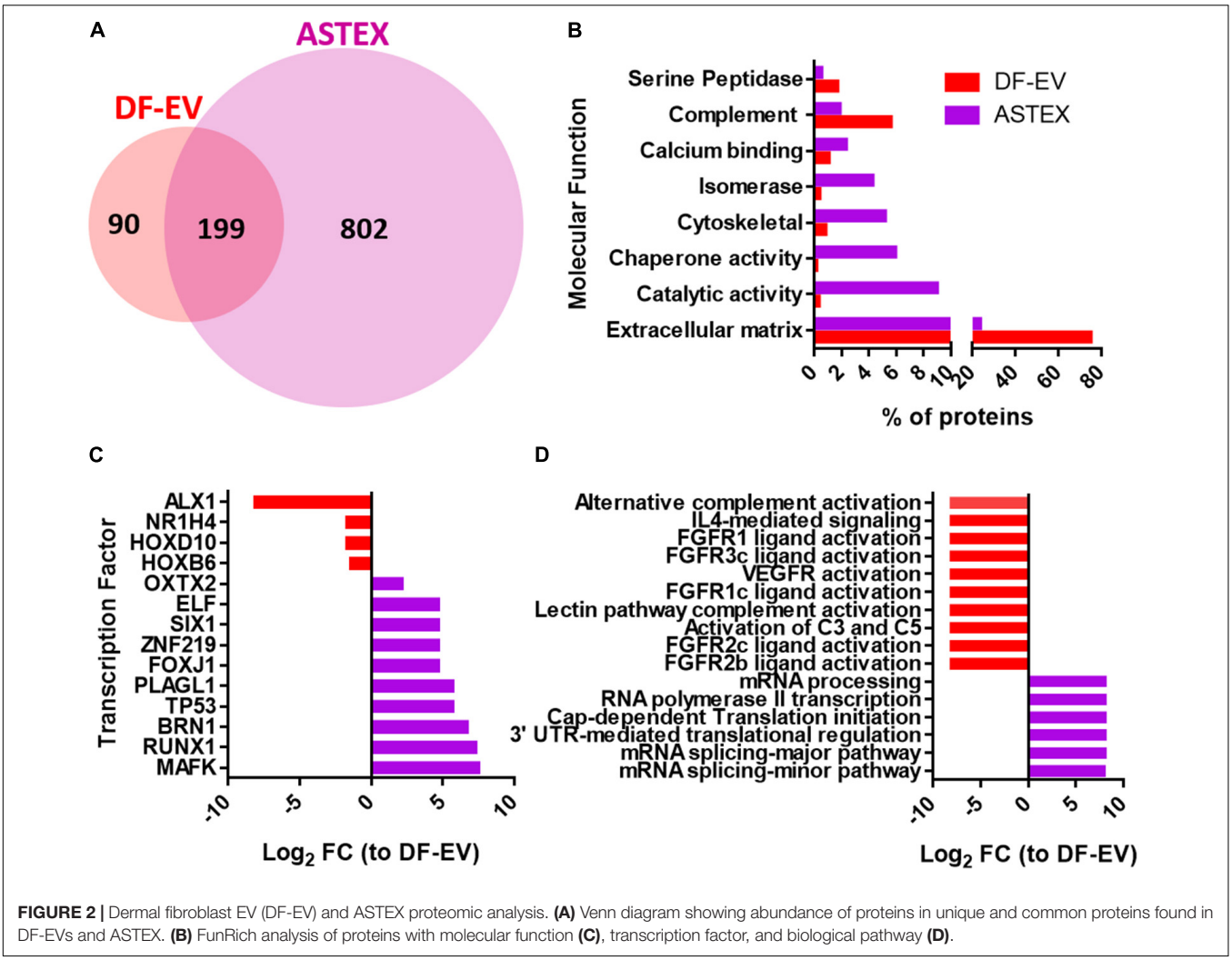

FIGURE 2 | Dermal fibroblast EV (DF-EV) and ASTEX proteomic analysis. **(A)** Venn diagram showing abundance of proteins in unique and common proteins found in DF-EVs and ASTEX. **(B)** FunRich analysis of proteins with molecular function **(C)**, transcription factor, and biological pathway **(D)**.

BMDMs to ASTEX reduced expression of the proinflammatory cytokine IL6 and increased expression of the anti-inflammatory cytokine IL10 (**Figures 4A,B**). Indeed, reduction in IL6 alone is therapeutic in pulmonary fibrosis (Le et al., 2014). Another driver of pulmonary fibrosis is fibroblast activation followed by myofibroblast transdifferentiation (Kendall and Feghali-Bostwick, 2014). A primary signal for myofibroblast transdifferentiation is transforming growth factor β (TGFβ) (Chen et al., 2009). Treatment of human primary lung fibroblasts (HLF) with recombinant TGFβ induced expression of the myofibroblast marker smooth muscle actin (SMA) (Zhang et al., 1996). We decided to use SMA as a functional readout as it is necessary for focal adhesion maturation of myofibroblasts. Studies of smooth muscle actin inhibition demonstrate decreased myofibroblast development (Hinz et al., 2003; Sousa et al., 2007). Cotreatment of HLF with TGFβ and ASTEX reduces SMA expression (**Figures 4C,D** and **Supplementary Figure 1A**). Endothelial cells are another cell type that transdifferentiates into myofibroblasts upon exposure to insult; a process called endothelial to mesenchymal transition (EndMT). This pathogenic cell type is also characterized by SMA expression. To investigate the effect of ASTEX on EndMT, we exposed human umbilical vein endothelial cells (HUVECs) to IL1b and TGFβ. HUVECs exposed to these cytokines upregulated the expression of SMA (**Figures 4E,F**). This effect was attenuated in cells cotreated with ASTEX. Morphologically, HUVECs treated with IL1b and TGFβ had a hypertrophied tube-shaped structure typical of myofibroblasts which were attenuated in cells treated with ASTEX (**Supplementary Figure 1B**). Taken together, these findings demonstrate that ASTEX inhibit IL6, a major driver of inflammation in lung inflammation. Furthermore, ASTEX attenuate fibroblast activation and EndMT as seen by inhibition of smooth muscle actin. By targeting markers of inflammation and fibrosis, these findings suggest that ASTEX may exert therapeutic bioactivity in pulmonary injury.

ASTEX Exhibit a Favorable Safety Profile in Healthy Animals

Before evaluating the therapeutic efficacy of ASTEX in lung injury, we sought to evaluate the safety of intratracheal instillation of ASTEX into healthy C57BL/6 mice (**Figure 5A**). At 3 weeks

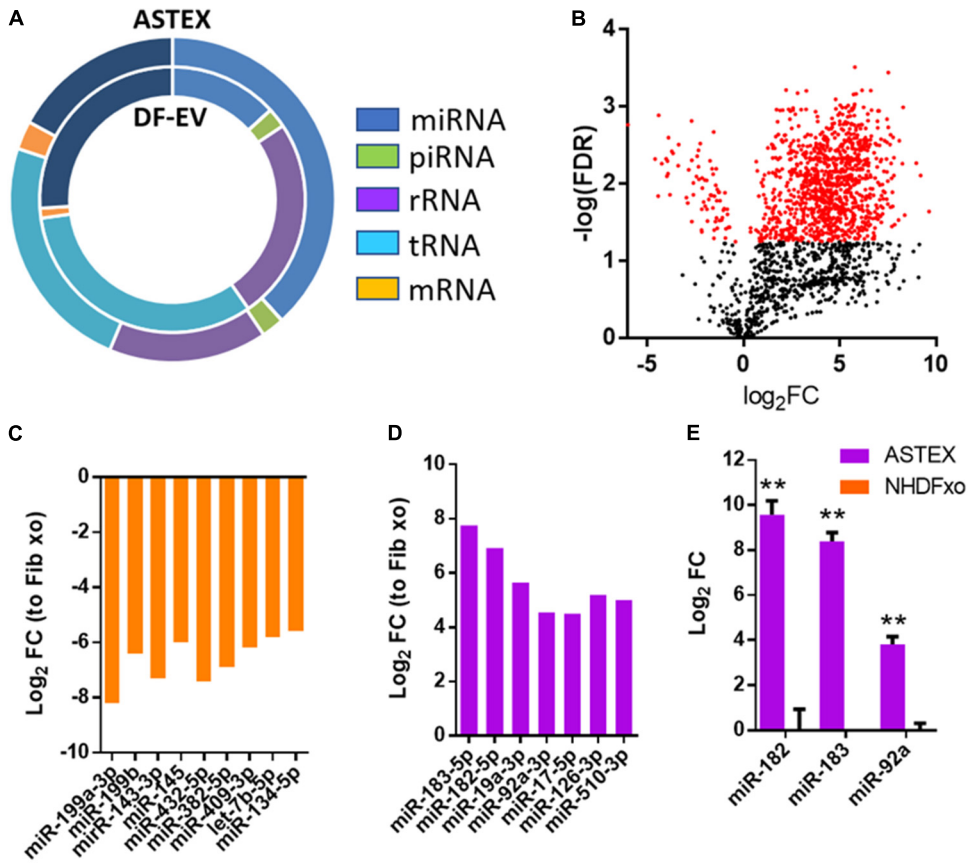

FIGURE 3 | Dermal fibroblast EV (DF-EV) and ASTEX proteomic analysis. **(A)** Distribution of RNA classes in EV-RNA of DF-EV and ASTEX. **(B)** Volcano plot showing differential expression of miRs expressed as fold change of ASTEX over DF-EV. **(C)** MiRs enriched in DF-EVs compared with ASTEX and **(D)** miRs enriched in ASTEX compared with DF-EVs. **(E)** QPCR validation of miRs enriched in ASTEX. Statistical comparison was done using independent Student's *t*-test with 95% CI; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

post-ASTEX instillation, animals exposed to a range of doses showed no weight loss (**Figure 5B**). Furthermore, no changes were seen in lung weight to body weight ratio indicating lack of inflammatory reaction (e.g., pulmonary edema; **Figure 5C**). Animals treated with ASTEX developed no fibrotic burden in the lung compared with naïve animals (**Figure 5D**). Finally, histological analysis including hematoxylin and eosin (H&E) and Masson's trichrome staining showed no remarkable signs of inflammation or fibrosis as evaluated by the Ashcroft score in a blinded manner (**Figures 5E,F**). These findings demonstrate a highly favorable safety profile of ASTEX.

ASTEX Exert Immunomodulatory and Antifibrotic Effects in a Mouse Model of Bleomycin-Induced Lung Injury

Having shown that ASTEX contain immunomodulatory and antifibrotic miRs, exert salutary effects in three relevant cell types *in vitro*, and exert no apparent toxic effects *in vivo*, we then investigated the therapeutic bioactivity of ASTEX in bleomycin-induced lung injury. Intratracheal (IT) instillation of bleomycin is a well-established model of lung injury. The hyperinflammatory period sustains until day 5 postexposure with an active profibrotic and injury resolution phase that sustains for 3 weeks thereafter (Kolb et al., 2020). We sought to investigate the effect of ASTEX on both short-term inflammation and long-term fibrotic development. In the first experiment, animals were given intravenous injections (retroorbital administration) of ASTEX, DF-EV, or vehicle at days 2 and 5 postbleomycin exposure and sacrificed on day 7 for analysis (**Figure 6A**). None of the treatments affected the weight loss seen with bleomycin exposure (**Supplementary Figure 2**). However, animals receiving ASTEX had attenuated lung inflammation as demonstrated by lower lung weight to body weight ratio (**Figure 6B**). Cell blood count (CBC) measurements from blood smears at baseline (day 2) and endpoint (day 7) showed no significant difference in overall leukocyte count (though all bleomycin-treated groups trended toward lower leukocyte count; **Supplementary Figure 3A**). Similarly, no differences were observed in neutrophil count (**Supplementary Figure 3B**). However, ASTEX-treated animals had significantly lower levels of circulating monocytes compared with vehicle-treated animals at day 7 and comparable with uninjured animals (**Supplementary Figure 3C**). As observed in the macrophage *in vitro* assay (**Figure 4A**), IL6 expression was

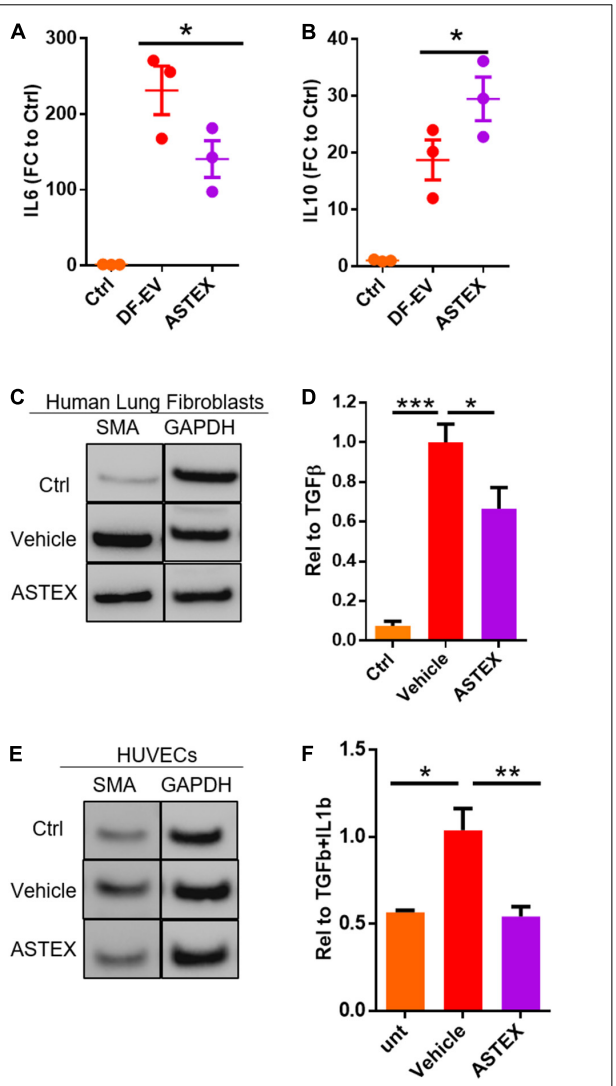

FIGURE 4 | ASTEX are immunomodulatory and antifibrotic *in vitro*. Macrophages incubated with ASTEX have reduced levels of IL6 expression **(A)** and increased IL10 **(B)** (each point represents one rat donor in technical duplicates) compared with DF-EVs. **(C,D)** Attenuation of fibroblast activation in human lung fibroblasts by ASTEX as shown by reduced expression of smooth muscle actin (n = 3 biological replicates). **(E,F)** Attenuation of endothelial to mesenchymal transition in human umbilical vein endothelial cells (HUVECS; n = 3 biological replicates). Statistical analysis done using one-way ANOVA with Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar: 100 μm.

reduced in lung tissue compared with ASTEX-treated animals compared with DF-EV or vehicle (**Figure 6C**). Inflammatory cytokine array analysis of lung tissue lysates identified no statistically significant differences between the groups. However, a trend toward lower proinflammatory cytokine levels including eotaxin 2, IL1a, and IL6 was observed in ASTEX-treated lung tissue compared with vehicle and DF-EV (**Supplementary Figure 4**). The lack of statistical significance may be due, in part, to the patchy nature of bleomycin injury in lung tissue and that in some animals one lobe is preferentially affected over the other giving rise to high variability. It may also be due to the later time point of lung collection (day 8) where inflammation is resolved, and the differences are more subtle. In a second study, animals received a single IT administration of ASTEX or vehicle at the beginning of the fibrotic phase and followed for 28 days postbleomycin exposure (**Figure 6D**). Animals receiving ASTEX had significantly better survival (**Figure 6E**) and lower fibrotic burden as shown by hydroxyproline content in lung tissue (**Figure 6F**). Taken together, this suggests an immunomodulatory and antifibrotic capacity of ASTEX in a bleomycin model of lung injury.

DISCUSSION

Pulmonary fibrosis is a progressive and deadly disease for which no curative strategy exists. EV therapy represents an advance in regenerative medicine as it is a cell-free therapeutic. ASTEX were developed based on previous investigation into the mechanism by which cells and their EVs exert therapeutic potency. Augmenting therapeutically inert fibroblasts with beta-catenin and gata4 alters the proteomic and RNA content, and the therapeutic utility of the EVs. Here, we demonstrate *in vitro* and *in vivo* evidence of the therapeutic bioactivity of ASTEX in models relevant to lung injury. As discussed earlier, the miR-92a-BMP signaling axis has been implicated in the mechanism of action of ASTEX. BMP activation has been shown to drive the salutary effects observed in cardiac, skeletal, and pulmonary injury models where ASTEX were tested. BMP activation in macrophages inhibits inflammatory activation through, in part, IL6 inhibition (Xia et al., 2011; Talati et al., 2014; Wei et al., 2018). It has been proposed that the onset of familial pulmonary arterial hypertension involves a two-hit event comprising a BMP receptor loss of function mutation and dysregulation of IL6 (Hagen et al., 2007). These findings corroborate our observation of the inhibitory effect of ASTEX on IL6 in macrophages and SMA expression in TGFβ-activated fibroblasts. Of course, miR-92a operates in a combination of other miRs and small RNAs which might function additively or synergistically.

While the present findings and mechanistic rationale suggest a therapeutic utility of ASTEX in PF, this study remains preliminary. The proposed mechanism for ASTEX function (*via* miR-92a transmission and BMP activation) has yet to be validated. Furthermore, the full anti-inflammatory effect of ASTEX in the inflammatory response remains to be fully elucidated. Another limitation of this study is the limited scope of the investigation. For instance, we have not investigated the effect of ASTEX on resident (bronchioalveolar) macrophages. These findings will also need to be validated *in vivo* to ensure the generalizability of *in vitro* findings. Finally, while we have investigated the anti-inflammatory and antifibrotic properties of ASTEX, the effect of this therapeutic on the alveolar progenitor niche has not been investigated. Therefore, future studies will explore the current findings in-depth and broaden the scope of the effect of ASTEX on pulmonary tissue and, more specifically, therapeutically relevant pathways. Increasing understanding of how EVs like ASTEX function to repair pulmonary tissue offers

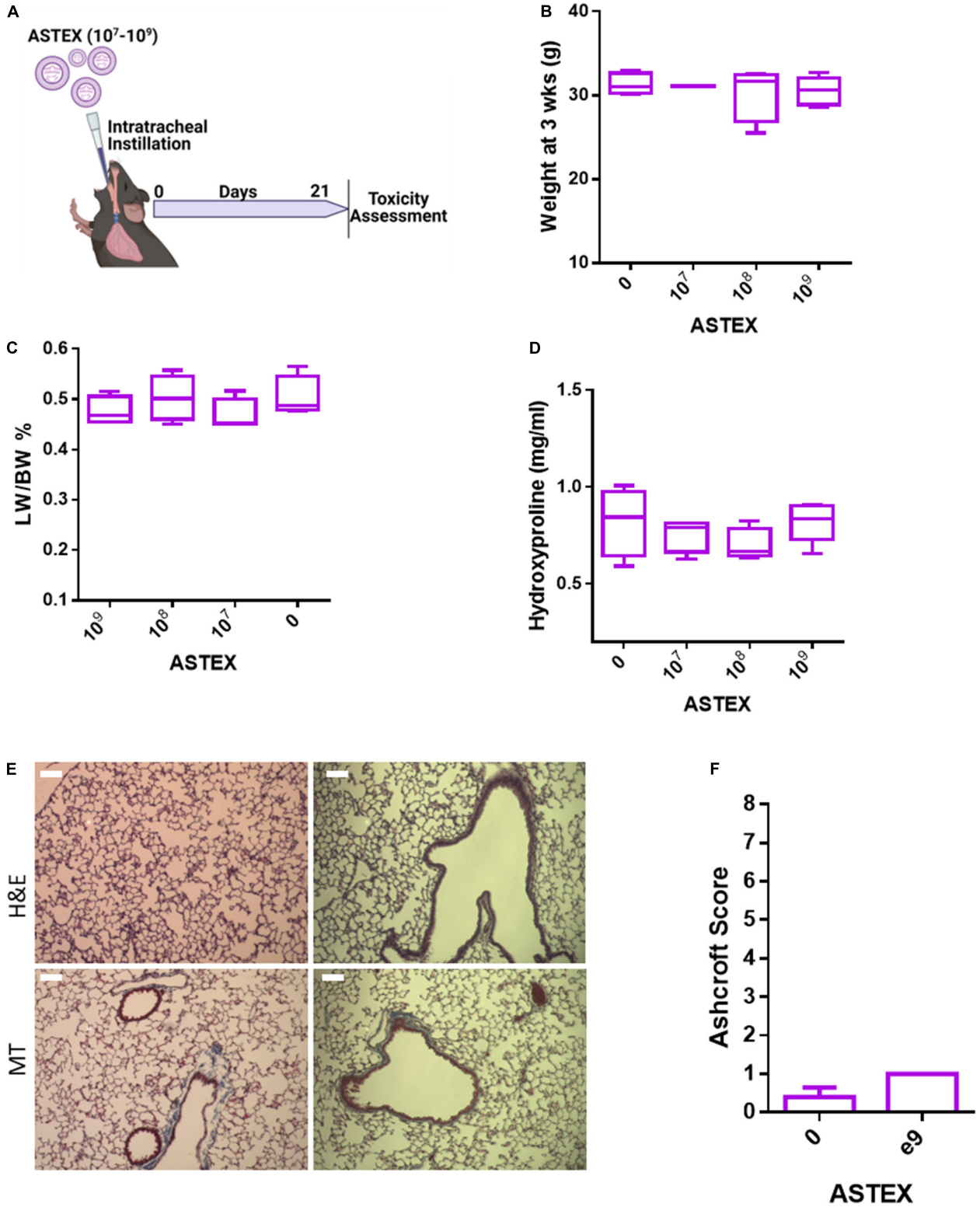

FIGURE 5 | ASTEX have a favorable safety profile in healthy mice. **(A)** Design of safety study. **(B)** Animal weight at 3 weeks postintratracheal instillation of different doses of ASTEX. **(C)** Lung weight to body weight ratio of animals at 3 weeks postexposure. **(D)** Lung fibrotic content as measured by hydroxyproline context in tissue 3 weeks postexposure. **(E)** H&E staining and Masson's trichrome of lung tissue and **(F)** tissue scoring (n = 4–5 animals per group) Statistical analysis was done using one way ANOVA with Tukey's multiple comparisons test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

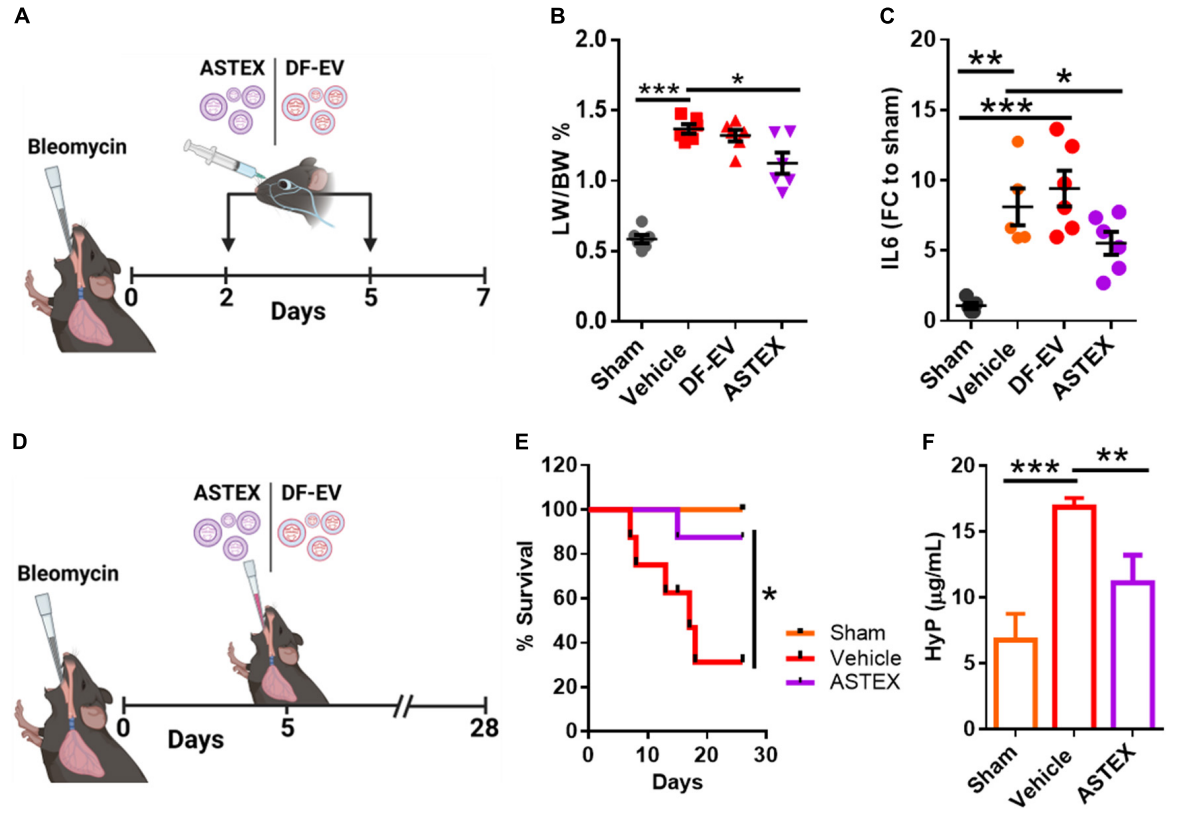

FIGURE 6 | ASTEX are therapeutic in a mouse model of lung injury. **(A)** Design of the short-term bleomycin study. **(B)** Lung weight to body percent and **(C)** IL6 expression in lung tissue. **(D)** Study design for the long-term bleomycin study. **(E)** Kaplan–Meier curve showing survival of bleomycin-treated groups and sham animals ($n = 6$ animals per group) and lung fibrotic content of animals as measured by hydroxyproline content **(F)**. Statistical analysis was done using one-way ANOVA with Tukey's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

a new avenue of therapeutic development for this and other diseases for which no curative strategy exists.

MATERIALS AND METHODS

Animal Subjects

All procedures related to animals have been conducted under approved Institutional Animal Care and Use Committee protocols.

Activated, Specialized Tissue Effector Cells Preparation

Engineered fibroblasts (ASTECs) and their EVs (ASTEX) were prepared as described in detail previously (Ibrahim et al., 2019). Briefly, primary neonatal skin fibroblasts cultured in 10% FBS, 50 µg/ml gentamicin, and 2 mmol/l L-glutamine in Iscove's modified Dulbecco's medium (complete medium). Cells were transduced (MOI: 20) with lenti-viruses containing transgenes for ctnnb1 (Santa Cruz Biotechnology, Dallas, TX, United States) and gata4 (AMSBIO, Abingdon, United Kingdom). Following 1 week in selection (puromycin and blasticidin for ctnnb1 and gata4, respectively), cells are expanded and banked for future use.

Activated Specialized Tissue Effector Extracellular Vesicles Preparation

To produce EVs from ASTECs (ASTEX), cells are expanded in a tri-layer flask (Thermo Fisher Scientific, Waltham, MA, United States) in complete media. When cells reach confluence, complete media are removed, and cells are washed with Iscove's modified Dulbecco's medium (IMDM) to remove FBS contaminants. Cells are then conditioned in IMDM for 15 days. Prior to ASTEX isolation, conditioned media are cleared from cellular debris through a 0.45-µm vacuum filter. ASTEX are isolated using ultrafiltration using a 100-kDa centrifugal filter.

Bleomycin Model

Fourteen-week-old male C57BL/6 mice were instilled with 0.7 U/kg (retro-orbitally for short-term study) or 1.5 U/kg (intratracheally) for long-term study of bleomycin (MilliporeSigma, Burlington, MA, United States) exposure. Animals were sacrificed at day 7 or 28 for the long- and short-term studies, respectively.

Lung Fibroblast Model

Primary adult human lung fibroblasts [American Type Culture Collection (ATCC)] were maintained in fibroblast basal medium

(ATCC) and supplemented with fibroblast growth kit-low serum (ATCC). Cells were expanded for three passages before performing TGFβ exposure assay. Briefly, cells were exposed to 5 ng/ml of recombinant TGFβ (Thermo Fisher Scientific). Cells were also cotreated with ASTEX at a cell to ASTEX ratio of 1:100. Cells were incubated for 48 h prior to Western blot or flow cytometry analysis.

Human Umbilical Vein Endothelial Cell Endothelial to Mesenchymal Transition Assay

Human umbilical vein endothelial cells (HUVECs: ATCC) were maintained in endothelial cell medium supplemented with endothelial growth kit (ATCC). Cells were expanded for three passages before exposure to 5 ng/ml of recombinant TGFβ (Thermo Fisher Scientific) and 1 ng/ml of IL1b (Thermo Fisher Scientific) for 5 days. Cells were treated at day 0 with vehicle or ASTEX at a cell to ASTEX ratio of 1:100.

Macrophage Isolation Assay and Model

Macrophages were prepared by isolating bone marrow cells from 3-month-old female Wistar Kyoto rats. Monocytes were purified and differentiated into BMDM by culturing with recombinant M-CSF (20 ng/ml; Life Technologies, Carlsbad, CA, United States). Whole bone marrow cells were collected *via* aspiration with ice-cold phosphate-buffered saline (PBS) and filtered using a 70-μm cell strainer and centrifuged at 400 × *g* for 10 min. Red blood cells were lysed out using 10 ml ACK buffer (Gibco, Waltham, MA, United States) for 30 s followed by quenching with complete media (IMDM + 10% FBS + 20 ng/ml M-CSF). Cells were centrifuged and resuspended in complete media and cultured for 5 days at 37°C with 20% O_2 and 5% CO_2. Cells were then seeded into six-well plates at 6–8.0*e*10 (Diamantopoulos et al., 2018) cells/well. Cells were incubated. Fresh complete media were exchanged on day 5, and cells were monitored for confluence. EVs were administered once BMDM cultures reached ∼75% confluence. Serum concentration was reduced to 1% during assays to allow EV uptake. BMDMs were cultured with EVs over night before RNA was isolated for analysis.

EV Proteomics

Chemicals and Instrumentation

DL-Dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA), and acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Trypsin from bovine pancreas was purchased from Promega (Madison, WI, United States). Ultrapure water was prepared from a Millipore purification system (Billerica, MA, United States). An Ultimate 3000 nano-UHPLC system coupled with a Q Exactive™ HF mass spectrometer (Thermo Fisher Scientific) with an ESI nanospray source.

Sample Preparation

(1) Take 100 μg protein per sample in the ultrafiltration tube. Transfer the solution into Microcon devices YM-10 (Millipore). The device was centrifuged at 12,000 rpm at 4°C for 10 min. Subsequently, 200 μl of 50 mM ammonium bicarbonate were added to the concentrate followed by centrifugation and repeat once.
(2) After reduced by 10 mM DTT at 56°C for 1 h and alkylated by 20 mM IAA at room temperature in the dark for 1 h, the device was centrifuged at 12,000 rpm at 4°C for 10 min and wash once with 50 mM ammonium bicarbonate.
(3) Add 100 μl of 50 mM ammonium bicarbonate and free trypsin into the protein solution at a ratio of 1:50, and the solution was incubated at 37°C overnight.
(4) Finally, the device was centrifuged at 12,000 rpm at 4°C for 10 min; 100 μl of 50 mM ammonium bicarbonate was added into the device and centrifuged, and then repeat once.
(5) Lyophilize the extracted peptides to near dryness. Resuspend peptides in 20 μl of 0.1% formic acid before liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis.

Nano-LC-MS/MS Analysis

Nano-LC

Nanoflow UPLC: Ultimate 3000 nano-UHPLC system (Thermo Fisher Scientific, United States).

Nanocolumn: trapping column (PepMap C18, 100Å, 100 μm × 2 cm, 5 μm) and an analytical column (PepMap C18, 100 Å, 75 μm × 50 cm, 2 μm).

Loaded sample volume: 1 μg.

Mobile phase: A: 0.1% formic acid in water; B: 0.1% formic acid in 80% acetonitrile.

Total flow rate: 250 nl/min.

LC linear gradient: from 2 to 8% buffer B in 3 min, from 8 to 20% buffer B in 56 min, from 20 to 40% buffer B in 37 min, then from 40 to 90% buffer B in 4 min.

Mass Spectrometry

The full scan was performed between 300 and 1,650 *m*/*z* at the resolution 60,000 at 200 *m*/*z*, the automatic gain control target for the full scan was set to 3e6. The MS/MS scan was operated in Top 20 mode using the following settings: resolution 15,000 at 200 *m*/*z*; automatic gain control target 1e5; maximum injection time 19 ms; normalized collision energy at 28%; isolation window of 1.4 Th; charge sate exclusion: unassigned, 1, >6; dynamic exclusion 30 s.

Data Analysis

Six raw MS files were analyzed and searched against human protein database based on the species of the samples using Maxquant (1.6.2.14). The parameters were set as follows: the protein modifications were carbamidomethylation (C) (fixed), oxidation (M) (variable); the enzyme specificity was set to trypsin; the maximum missed cleavages were set to 2; the precursor ion mass tolerance was set to 10 ppm; and MS/MS tolerance was 0.6 Da.

EVs were lysed using RIPA buffer followed by acetone precipitation and reduction using 1 mM tris(2-carboxyethyl)

phosphine (TCEP). Samples were then alkylated with 5 mM iodoacetamide and digested with ∼1:40 trypsin-LyC (Promega, Madison, WI, United States), desalted, and speed vac to total dryness. Liquid chromatography MS/MS was done using a Dionex Ultimate 3000 Nano-LC coupled with an Orbitrap Q Exactive™ mass spectrometer (Thermo Fisher Scientific, USA) with an ESI nanospray source. Mobile phase A comprised 0.1% aqueous formic acid and mobile phase B of 0.1% formic acid in acetonitrile. Peptides were then loaded onto the analytical column [100 μm × 10 cm in-house-made column packed with a reversed-phase ReproSil-Pur C18-AQ resin (3 μm, 120 Å, Dr. Maisch GmbH, Germany)] at a flow rate of 600 nl/min using a linear AB gradient composed of 6–30% B for 38 min, 30–42% B for 10 min, 42–90% B for 6 min, and 90% B elution for 6 min. The temperature was set to 40°C for both columns. Nanosource capillary temperature was set to 270°C and spray voltage set to 2.2 kV. The Orbitrap Q Exactive™ operated in a data-dependent mode, where the five most intensive precursors were selected for subsequent fragmentation. Resolution for the precursor scan (m/z 300–1,800) was set to 70,000 at m/z 400 with a target value of 1 × 106 ions. The MS/MS scans were also acquired in the orbitrap with a normalized collision energy setting of 40 for HCD.

Raw MS files were then analyzed and cross-referenced against the human protein database based on the species of the samples using Maxquant (1.5.6.5). The parameters were set as follows: the protein modifications were carbamidomethylation (C) (fixed) and oxidation (M) (variable); the enzyme specificity was set to trypsin; the maximum missed cleavages were set to 2; the precursor ion mass tolerance was set to 10 ppm; and MS/MS tolerance was 0.6 Da. Only high confident identified peptides were chosen for downstream protein identification analysis. The listed common secreted proteins were classified using Panther (Protein Analysis Through Evolutionary Relationships[1]). To glean relevant biological processes, transcription factors, and FunRich (Functional Enrichment Analysis Tool) to explore protein classification, molecular function, and pathways.

RNA Isolation and qRT-PCR

Total RNA was isolated using miRNeasy Mini Kit (Qiagen, Hilden, Germany) for cells or Urine Exosome RNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada) for exosomes. Reverse transcription was performed using High-Capacity RNA to cDNA (Thermo Fisher Scientific) or Taqman® microRNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, United States). Real-time PCR was performed using Taqman Fast Advanced Master Mix and the appropriate TaqMan® Gene Expression Assay (Thermo Fisher Scientific). Samples were measured using Applied Biosystems 7800HT fast Real-Time PCR system. Each reaction was performed in triplicate samples and adjusted using hprt1 and u6 for mRNA and miRs, respectively. The gene expression assays/microRNAs used in this study were as follows (Thermo Fisher Scientific):

Assay names	Species	Assay numbers
hsa-miR182-5p	Human	002334
hsa-miR183-5p	Human	002269
hsa-miR-92a-3p	Human	000431
IL6	Rat	Rn01410330_m1
IL10	Rat	Rn01483988_g1
IL6	Mouse	Mm00446190

Flow Cytometry

Cells were harvested and counted (2×10^5 cells per condition) and washed with 1% bovine serum albumin (BSA) in 1 × PBS and stained with the appropriate antibody (BD Pharmingen, San Diego, CA, United States) for 1 h at 4°C. The cells were then washed again and resuspended in 1% BSA in 1× PBS. BD Cytofix/Cytoperm™ kit was used for cell permeabilization before staining. Flow cytometry was performed using the Sony SA3800 Spectral Cell Analyzer.

Exosome Preparation and Collection

Exosomes were harvested from transduced fibroblasts at passage 16 under hypoxia conditioning. Briefly, cells were grown in 636 cm^2 CellStack chambers (Corning, Corning, NY, United States) until confluence. The flask was then washed three times with phenol-free IMDM and conditioned in the same for 15 days under 20% O_2. Conditioned media were collected, pooled, and filtered through 0.45 μm filter to remove apoptotic bodies and cell debris and frozen at −80°C for later use. Extracellular vesicles were isolated using centrifuge-based ultrafiltration with a molecular weight cutoff of 100 kDa (MilliporeSigma). Isolated EVs were quantified using Malvern Nanosight NS300 Instrument (Malvern Instruments, Malvern, UK) with the following acquisition parameters: camera levels of 15, detection level less than or equal to 5, number of videos taken 4, and video length of 30 s.

Hydroxyproline Assay

Hydroxyproline content in lung tissue was quantified chemically using a Hydroxyproline Assay Kit (Sigma-Aldrich) per manufacturer's instructions.

Mouse Inflammatory Cytokine Array

Expression of inflammatory markers in lung tissue lysates from animals exposed to bleomycin (including those also treated with ASTEX, DF-EVs, or vehicle) or sham control (day 8 postexposure) were measured using a Quantibody® Mouse Inflammation Array 1 Kit (RayBiotech, Peachtree Corners, GA, USA) per manufacturer's instructions.

Western Blot

Membrane transfer was performed using the Turbo Transfer System (Bio-Rad, Hercules, CA, United States)

[1]http://pantherdb.org

after gel electrophoresis. The following antibody staining was then applied and detected by SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). To prove for SMA, antimouse human/mouse/rat antibody (MAB1420-SP; R&D Systems, Minneapolis, MN, United States) was used.

Exosome RNA Sequencing

Library Preparation and Sequencing: miRNAs

The miRNA sequencing library was prepared using the QIASeqTM miRNA Library Kit (Qiagen). Total RNA was used as the starting material. A preadenylated DNA adapter was ligated to the 3′ ends of miRNAs, followed by ligation of an RNA adapter to the 5′ end. A reverse-transcription primer containing an integrated Unique Molecular Index (UMI) was used to convert the 3′/5′-ligated miRNAs into cDNA. After cDNA cleanup, indexed sequencing libraries were generated *via* sample indexing during library amplification, followed by library cleanup. Libraries were sequenced on a NextSeq 500 (Illumina, San Diego, CA, United States) with a 1 × 75 bp read length and an average sequencing depth of ∼10 M reads/sample.

Data Analysis

The demultiplexed raw reads were uploaded to GeneGlobe Data Analysis Center (Qiagen) at https://www.qiagen.com/us/resources/geneglobe/, for quality control, alignment, and expression quantification. Briefly, 3′ adapter and low-quality bases were trimmed off from reads first using cutadapt (version 1.13) with default settings, then reads with less than 16 bp insert sequences or with less than 10 bp UMI sequences were discarded (Martin, 2011). The remaining reads were collapsed to UMI counts and aligned to miRBase (release v21) mature and hairpin databases sequentially using Bowtie v1.2 (Langmead et al., 2009). The UMI counts of each miRNA molecule were counted, and the expression of miRNAs was normalized based on total UMI counts for each sample.

Total RNA Sequencing

Library construction was performed using the SMARTer® Stranded Total RNA-Seq Kit (Takara Bio USA, Inc., Mountain View, CA, United States). Briefly, total RNA samples were assessed for concentration using a Qubit fluorometer (Thermo Fisher Scientific) and for quality using the 2100 Bioanalyzer (Agilent Biotechnologies, Santa Clara, CA, United States). Total RNA was converted to cDNA, and adapters for Illumina sequencing (with specific barcodes) were added through PCR using only a limited number of cycles. The PCR products were purified, and then ribosomal cDNA was depleted. The cDNA fragments were further amplified with primers universal to all libraries. Lastly, the PCR products were purified once more to yield the final library. The concentration of the amplified library was measured with a Qubit fluorometer, and an aliquot of the library was resolved on the Bioanalyzer. Sample libraries were multiplexed and sequenced on a NextSeq 500 platform (Illumina) using 75 bp single-end sequencing. On average, 50 million reads were generated from each sample.

Data Analysis

Raw reads obtained from RNA-Seq were aligned to the transcriptome using STAR (version 2.5.0) (Dobin et al., 2013)/RSEM (version 1.2.25) (Li and Dewey, 2011) with default parameters, using human GRCh38 (or mouse CRCm38) transcriptome reference downloaded from http://www.gencodegenes.org. Expression counts for each gene [transcripts per million (TPM)] in all samples were normalized by the sequencing depth.

Statistical Analysis

Statistical comparisons between two groups were made using an independent two-tailed independent Student's *t*-test with a 95% CI. Comparisons between three or more groups were done using one-way ANOVA with Tukey's or Dunnett's test for multiple comparisons. Calculations were done using Graphpad Prism 6.0 software.

DATA AVAILABILITY STATEMENT

The data presented in this study are deposited in Exocarta under accession ID ExoCarta_312.

ETHICS STATEMENT

All procedures animal-related work was conducted under approved Institutional Animal Care and Use Committee (IACUC) protocols at Cedars Sinai Medical Center.

AUTHOR CONTRIBUTIONS

AI and AC conceived the idea, performed the experiments, and wrote the manuscript. AA, CL, KP, AMo, and KJ-U performed the experiments and analyzed the data. AMa analyzed the data. EM conceived the idea and analyzed the data. AGI conceived the idea, performed the experiments, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.733158/full#supplementary-material

Supplementary Figure 1 | (A) Flow cytometry of human lung fibroblasts treated with TGFβ only (vehicle) or TGFβ with ASTEX. **(B)** Phase contract images of HUVECs, treated with TGFβ + IL1b only (vehicle) or TGFβ + IL1b with ASTEX six days postexposure (scale bar: 100 μm).

Supplementary Figure 2 | Change in animal weight in animals treated with bleomycin (vehicle, DF-EV, or ASTEX) and sham (n = 5 animals/group). Statistical analysis was done using one-way ANOVA with Dunnett's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Figure 3 | Cell blood count of animals exposed to bleomycin (vehicle, DF-EVs, and ASTEX) or sham. **(A,B)** Total white blood cell count, **(C,D)** neutrophils, and **(E,F)** monocytes (n = 5 animals per group). Statistical analysis was done using one-way ANOVA with Dunnett's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Figure 4 | Inflammatory cytokine array of mouse lung tissue lysates. Lysates were collected from lung tissue of bleomycin and sham-treated groups 8 days postbleomycin exposure (n = 4 mice per group). Statistical analysis was done using one-way ANOVA with Tukey's multiple comparisons test.

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REVIEW
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Exosomes: Emerging Cell-Free Based Therapeutics in Dermatologic Diseases

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Exosomes are lipid bilayer vesicles released by multiple cell types. These bioactive vesicles are gradually becoming a leading star in intercellular communication involving in various pathological and physiological process. Exosomes convey specific and bioactive transporting cargos, including lipids, nucleic acids and proteins which can be reflective of their parent cells, rendering them attractive in cell-free therapeutics. Numerous findings have confirmed the crucial role of exosomes in restraining scars, burning, senescence and wound recovery. Moreover, the biology research of exosomes in cutting-edge studies are emerging, allowing for the development of particular guidelines and quality control methodology, which favor their possible application in the future. In this review, we discussed therapeutic potential of exosomes in different relevant mode of dermatologic diseases, as well as the various molecular mechanisms. Furthermore, given the advantages of favorable biocompatibility and transporting capacity, the bioengineering modification of exosomes is also involved.

Keywords: exosomes, cell-free therapy, cell communication, bioengineering, dermatologic diseases

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INTRODUCTION

The skin is composed of hair, sweat glands, sebaceous glands and other accessory structures, as well as enriched blood vessels, nerve muscles and lymphatics (Wong et al., 2016). The direct contact with the environment leads to various external and internal damage to it. External factors including sunlight, infection, trauma and chemical media invasion caused by antigen or irritant, and internal factors including genetic factors, psychological factors, medical diseases, drug reactions and so on, which jointly bring significant discomfort to patients in appearance, functionality and

Abbreviations: MVB, multivesicular body; MSC, mesenchymal stem cells; ILV, intraluminal vesicles; EV, extracellular vesicle; ESCRT, endosomal sorting complex required for transport; TSG101, tumor susceptibility gene 101 protein; ALIX, ALG-2 interacting protein X; VPS, vacuolar protein sorting-associated protein; SNARE, soluble NSF Attachment Protein Receptor; DPC-Ex, dermal papilla cells derived exosomes; MAPK, mitogen-activated protein kinase; EMT, epithelial-to-mesenchymal transition; MSC, mesenchymal stem cell; hucMSC, human umbilical cord derived MSC; BM-MSC, bone marrow derived MSC; hiPSCs, human induced pluripotent stem cells; MMPs, matrix metalloproteinases; 3D, three-dimensional; TIMP-1, tissue inhibitor of metalloproteinases-1; SHSs, sponge sp. spicules; DFU, diabetic foot ulcer; GLUT, glucose transporter; MDSC, myeloid-derived suppressor cell; SCC, squamous cell carcinoma; TF, tissue factor; IGF-1, insulin-like growth factor-1; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; HSP90α, heat shock protein 90α; AdMSC, adipose derived MSC; DIM, 3,3′-diindolymethane; MISEV, minimal information for studies of extracellular vesicles; FDA, Food and Drug Administration.

even psychology. Some intractable skin diseases and severe burns still lack of effective treatments or control measures. For decades, "cell-based" therapy, especially mesenchymal stem cell (MSC) is holding tremendous promise for tissue regeneration. Emerging evidence shows that the cells like MSCs restore the tissue function mainly by paracrine manner, in which exosomes stand out (Phinney and Pittenger, 2017; Phan et al., 2018). Exosomes shed by MSC are safer and more stable than their donor cells concerning the management of live cells (Nikfarjam et al., 2020). These bioactive vesicles are becoming a leading star in cell-cell communication for delivering message from their cells of origin to the recipients (Wortzel et al., 2019; Li C. J. et al., 2020). Study have indicated that MSC-Exo may emerge as a promising therapeutic approach of SARS-CoV-2 pneumonia and in ischemic diseases (Akbari and Rezaie, 2020; Babaei and Rezaie, 2021). The basis, transformation and application of exosomes derived from stem cells and cells of various skin tissues are springing up and obvious progress has been made (Kalluri and Lebleu, 2020). In this review, the regulatory roles of exosomes in skin differentiation, development and the repair of injury will be further elaborated.

BRIEF DESCRIPTION OF EXOSOMES

Biogenesis and Characterization

Numerous cells types release exosomes into the extracellular environment after fusing with the cytomembrane. Exosomes are membrane vesicle with a size of 30-150 nm. (Kalluri and Lebleu, 2020). Generally, exosomes can be classified as natural and engineered exosomes. The engineered exosomes are artificially designed and modified while natural exosomes can be natively derived from animals and plants. Ulteriorly, animal-derived exosomes, depending on the normal and tumor culture conditions, consist of two parts, normal exosomes and tumor exosomes (Zhang Y. et al., 2020). The heterogeneity of vesicles which originate from various cells are reflective in density, lipid, protein, density and subcellular origin (van der Pol et al., 2012). Multiple signaling pathways are involved in regulating the exosome biogenesis. Exosomes are acknowledgedly released by microvesicle body (MVB) signaling. At the very beginning, the endosome is formed as a small intracellular body by wrapping little intracellular fluid. The endosome is distinguished as early and late endosomes. After the intraluminal vesicles (ILV) formed, the early endosome develops into the late endosome, namely MVB. The MVB can degrade the contents by fusing with the lysosome or release its ILVs by fusing with the plasma membrane in an exocytotic manner, which are exosomes (Théry et al., 2002; Trajkovic et al., 2008; McAndrews and Kalluri, 2019). During this progress, endosomal-sorting complexes required for transport (ESCRT)-dependent and independent pathways are both involved (**Figure 1**).

However, after exosomes released, the nomenclature and characterization still not come into a consensus. According to the Minimal Information for studies of Extracellular Vesicles (MISEV), three key areas were addressed: EV isolation/purification, EV characterization and EV functional studies (Lotvall et al., 2014). Regarding the characterization of exosomes, at least three positive protein markers (such as CD9, CD63, CD81, Alix, Hsp70), meanwhile no less than one negative protein marker (such as albumin, calnexin) should be testified. Two different but complementary techniques should be used to characterize single vesicles, such as an electronic or atomic force microscope (which can display near and wide fields) and single particle analyzers (Théry et al., 2018).

Secretion

The secretion of exosomes is a process in which MVB fusing with the plasma membrane and different mechanisms are involved (Record et al., 2011; Azmi et al., 2013; Beach et al., 2014; He et al., 2018; Yang et al., 2019). Several ESCRT and relevant proteins including Rab, Sytenin-1, TSG101, ALIX, VPS4, actin and SNARE are participants in exosome release (Baietti et al., 2012; Colombo et al., 2013; Friand et al., 2015; Ciardiello et al., 2016; Jackson et al., 2017; Guo et al., 2020). The Rab family proteins regulate the exosome secretion in a large extent, in which Rab27a and Rab27b are widely reported to regulate exosome secretion (Ostrowski et al., 2010). Mutant Rab11S25N inhibiting exosome release confirms that Rab11 is involved in their release (Savina et al., 2002). Rab35 localized on the cell surface of oligodendroglia can regulate the density of exosomes (Hsu et al., 2010). SNARE proteins and synaptotagmin family members also help the docking and fusing of vesicles with the cytomembrane and to release the contained exosomes into the extracellular environment (Beach et al., 2014).

Other alternative ways to affect exosome secretion is certain kind of inducement or stimulation of the origin cell. An increase of intracellular Ca^{2+} and activation of protein kinase C promotes both the generation and delivery of exosomes (Nguyen et al., 2016). In cancer cells, the acute elevation of calcium also causes a quintuple increase in the amount of exosome$^{ALIX+CD63+CD9+}$ (Messenger et al., 2018). Moreover, Transferrin (Tf)-stimulated exosome release is in a calcium ion -dependent manner (Savina et al., 2003). Heparanase involves in almost every related step from biogenesis to release of the exosomes as well as regulating the exosomal protein cargo (David and Zimmermann, 2020; Purushothaman and Sanderson, 2020). Diabetic sera are also found to affect exosome release by disrupting the normal MSC-derived exosome signaling pathway *in vitro* (Rezaie et al., 2018b) (see more stimulation methods in **Table 1**). Though some different mechanisms or ways of exosome biogenesis and release are discussed currently, they are not all-inclusive and there still remains plenty room for fully uncovering exosome pathways in all situations.

Transporting Cargos

Transporting cargos in their lipid bilayer membrane, exosomes successfully pass on the message from parent cells to the recipient cells, which includes proteins, nucleic acids and lipids (Tkach and Théry, 2016; van Niel et al., 2018). The mechanisms of how exosome uptake by target cells remains unclear (Villarroya-Beltri et al., 2014), it is more likely to be a dynamic process. Exosomes may enter cells directly through different mechanisms like the participation of calveolae, clathrin, lipid rafts, direct fusion with cell membrane, receptor-ligand interaction, endocytosis by

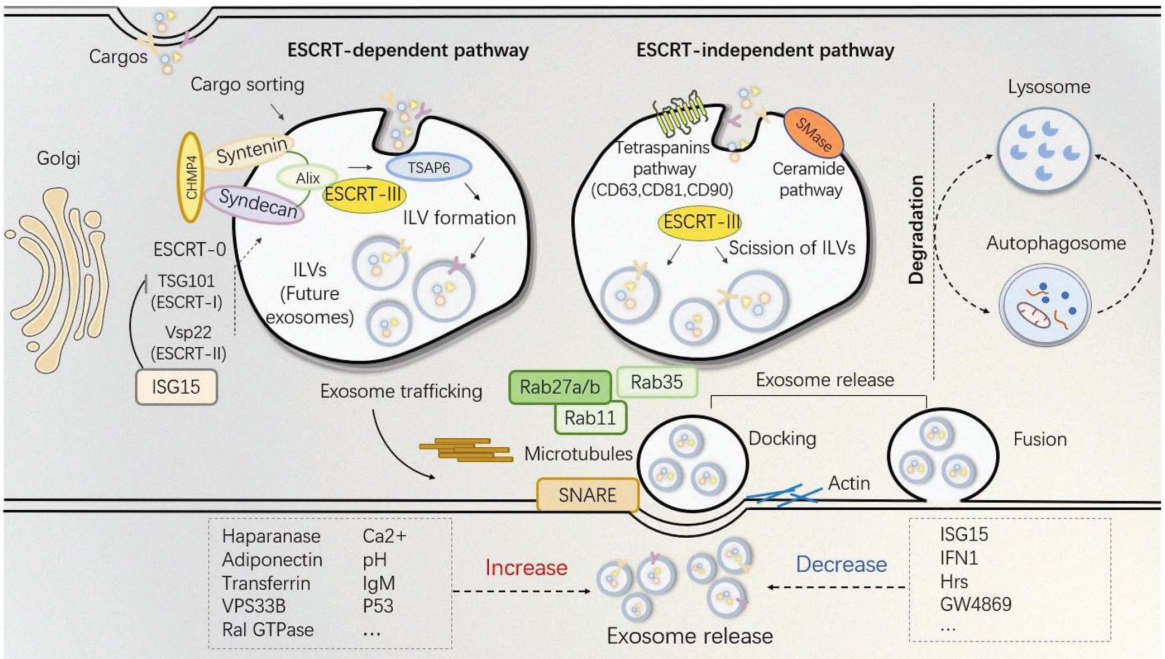

FIGURE 1 | Exosomes biogenesis and release. Cytomembrane lipids and membrane-associated proteins are clustered in micro-domains for MVB formation. Soluble components, such as cytosolic proteins and RNAs are recruited for exosome cargo sorting. Other cargos can be specifically sorted via the trans-Golgi network into MVBs. Mechanisms of exosome biogenesis are well-accepted by different pathways, basically distinguished as ESCRT-dependent and ESCRT-independent manner. Notably, ESCRT-III is required for the final formation of the ILVs, while cargo sorting and membrane budding can be regulated by either pathways. MVBs will either undergo degradation by lysosomes and autophagosome (finally lysosomes) or to dock or fuse with the cytomembrane. Docking and fusion are the two final processes required for exosome release whereby Rab family members, SNARE, as well as actin are involved. Treatments such as haparanase, adiponection, transferrin, VPS33B, Ral GTPase, Ca^{2+}, IgM, pH, p53 will help to increase exosome production while ISG15, IFN1, Hrs, GW4869 can block exosome secretion by different mechanisms.

phagocytosis and also micropinocytosis (Kalluri and Lebleu, 2020). However, at the meantime, the endocytosis process also gives rise to the *de novo* formation of exosomes by cells. Whether the release of exosomes which generate endogenously or take up occurs separately or together is still hard to tell.

Previously, exosomes are considered as vehicles transporting cargos to discard and unnecessary for their cells of origin, but now accumulating evidence confirms that these vesicles as well as their contents are playing full roles in the physiological and pathological process (Phinney and Pittenger, 2017; Bebelman et al., 2018; van Niel et al., 2018; Kalluri and Lebleu, 2020). The comprehensive exosome-mediated crosstalk between different cell types are now attracting increasing attention, exosomes are recognized as a new "cell-free" therapeutic strategy in regenerative medicine and the roles of exosomes in skin development and cutaneous wound healing will be discussed in detail below.

EXOSOMES AND DERMATOLOGIC DISEASE

Under pathological conditions like diabetes or severe burns, skin function is not fully restored via the normal wound healing process, leading to potentially serious complications such as ulcers or infections (Nuschke, 2014). Therefore, it is crucial to complete wound healing with the least delay after injury. Stem cells, particularly MSCs, present great clinical application prospects in skin tissue regeneration for their advantages of extensive sources, easy expansion *in vitro* and low rejection after transplantation *in vivo*. It is well accepted now cells like MSCs releasing repair promoting factors in a paracrine manner. Paracrine is the dominating way to facilitate damage repair, whereby exosome outstands (Phinney and Pittenger, 2017; Mehryab et al., 2020; Rahmani et al., 2020). The findings open up the alternative avenue of cells such as MSCs to exosomes for safer, potent and "cell-free" based therapeutic application. A good deal of preclinical experiments has confirmed the multiple roles of exosomes in various skin injury repair (**Figure 2** and **Table 2**).

Exosomes and Laceration

Exosomes present favorable repair activity in skin lacerations and full-layer resection. After partial resection of back skin in 12-week-old C57B/6 mice, human umbilical cord MSC derived exosomes (hucMSC-Ex) effectively promote wound angiogenesis and epidermal regeneration *in vivo*. Further exploration shows that the mechanism is related to miR-21-3p conveyed by exosomes, which regulates cell function by inhibiting its downstream PTEN and SPRY1 target genes

TABLE 1 | Approaches to affect exosome release.

Agent	Mechanisms	Models	Production	References
Rab27a/Rab27b	Multivesicular endosomes docking		increase	Ostrowski et al., 2010
Rab11	Rab11S25N mediated exosome release	K562	increase	Savina et al., 2002
Rab35	GTP-dependent localization	Oligodendroglia cells	increase	Hsu et al., 2010
Ca^{2+}	a. Activation of protein kinase C b. .Munc13-4/Rab11 dependent pathway	Human RBCs MDA-MB-231	increase	Nguyen et al., 2016; Messenger et al., 2018
PH	a. Low PH induced high rigidity and sphingomyelin content b. .MVB acidification	Melanoma cells Human cells	increase	Parolini et al., 2009; Sung et al., 2020
Transferrin	Ca^{2+} dependent manner	K562	increase	Savina et al., 2003
P53	a. Regulation of TSAP6 b. .IGF-1/AKT/mTOR signaling	BMDCs cells respond to stress	increase	Lespagnol et al., 2008; Feng, 2010
IgM	BCR activation	CLL cells	increase	Yeh et al., 2015
Haparanase	Accumulation of syndecan-1	MCF-7	increase	David and Zimmermann, 2020
Adiponectin	Binding to T-cadherin	MSCs	increase	Nakamura et al., 2020
VPS33B	GD12/Rab11a/Rab27a pathway	HSCs, LICs	increase	Gu et al., 2016
Ral GTPase	colocalization with SYX-5	nematodes, mammary tumor cells	increase	Hyenne et al., 2015
ISG15	Targeting TSG101 mediated plasma membrane formation	HEK293	decrease	Nabhan et al., 2012; Choudhuri et al., 2014
IFN1	ISG15 triggered MVB-lysosome fusion	HEK293T	decrease	Villarroya-Beltri et al., 2016
GW4869	nSMAse-independent mechanism	myeloma cells SKBR3 cells	decrease	Stoffel et al., 2016; Menck et al., 2017; Vuckovic et al., 2017

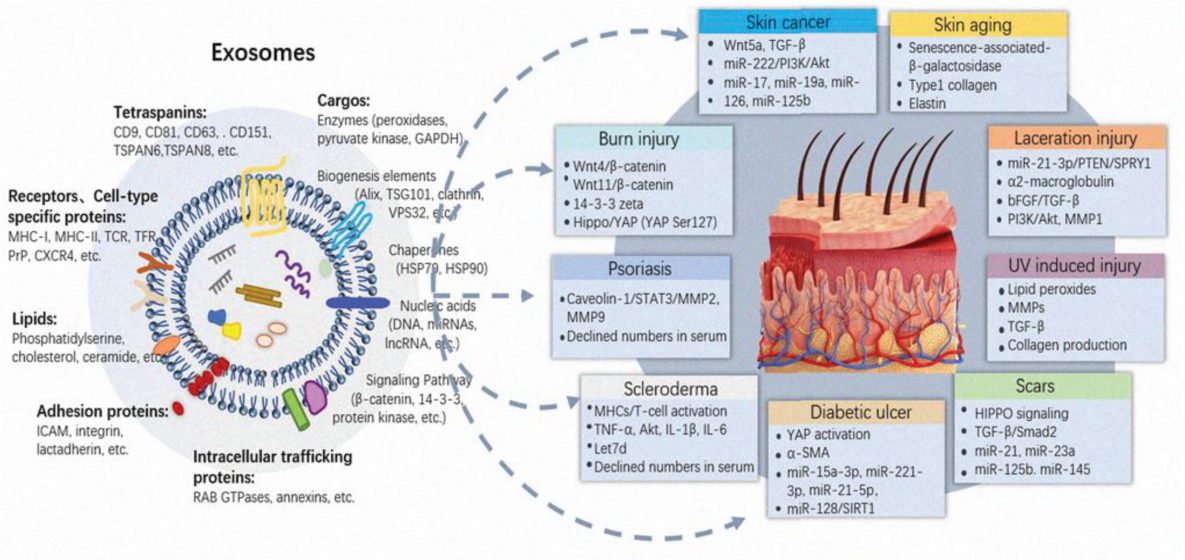

FIGURE 2 | Main composition of exosomes and their functions on skin injuries. Despite the different origin and mode of biogenesis, exosomes display a similar appearance, and common composition. Exosomes are featured by tetraspanins, receptors or cell-type specific proteins, lipids, adherin proteins, intracellular trafficking proteins. Deep analysis of extracellular vesicle composition reveal that they convey various cargoes, including nucleic acids, proteins (biogenesis elements, chaperones and signaling pathway molecules) and lipids, which all vary widely between cells and conditions. The vast information will directly affect the fate and function of exosomes in skin injuries. Exosomes participate in multiple cutaneous diseases including laceration, burn, aging, diabetic wound healing, scar formation, skin autoimmune disease and skin cancers, through different biological pathways.

(Hu et al., 2018). Protein spectrum reveals that hucMSC-Ex is enriched in a large amount of alpha-2-macroglobulin improve the activity and migration ability of skin fibroblasts (Bakhtyar et al., 2018). Bone marrow MSC (BM-MSC) derived exosomes can accelerate the healing of wound by promoting the neovascularization in the dermal laceration model of large animal dogs, and the mechanism of this repair effect is also related to the fact that BM-MSC exosomes contain various active factors related to angiogenesis (El-Tookhy et al., 2017). Adipose-derived MSC (AdMSC) exosomes are internalized and

TABLE 2 | Functions of exosomes derived from different origin in cutaneous disease.

Origin	Recipient	Mechanisms	Functions	References
hucMSC	fibroblasts and endothelial cells	miR-21-3p/PTEN/SPRY1	promote proliferation and migration	Hu et al., 2018
hucMSC	fibroblasts	enriched of alpha-2-macroglobulin	improve the activity and migration ability of skin fibroblasts	Bakhtyar et al., 2018
BM-MSC	dermal vascular endothelium	various active factors related to angiogenesis	formation of new blood vessels	El-Tookhy et al., 2017
AdMSC	fibroblasts	bFGF and TGF-β PI3K/Akt signaling pathway	increased proliferation and migration	Zhang W. et al., 2018
autologous human epidermal cells	dermal fibroblasts	activating matrix metalloproteinase-1	cell proliferation and cause the degradation extracellular matrix	Zhao et al., 2017
hucMSC	dermal fibroblasts epidermis cells	Wnt4/β-catenin Akt signaling activation	promote the proliferation of skin cells re-epithelialization of epidermis orchestrate the distribution collagen Enhance angiogenesis	Zhang et al., 2015a, 2016
hucMSC	dermal fibroblasts epidermis cells	14-3-3ζ/ LATs/ YAP/ HIPPO	restrict the scar formation	Zhang et al., 2016
hucMSC	fibroblasts	miR-21, miR-23a, miR-125b and miR-145 targeted TGF-β/SMAD2	reduce the expression of α-SMA and collagen deposition control scar formation	Fang et al., 2016
hiPSC	human dermal fibroblasts	down-regulate senescence-associated β-galactosidase	protective and anti-aging effect	Oh et al., 2018
human umbilical cord blood	epidermis cells	regulating the expression and distribution of collagen and elastin	a certain degree of anti-aging effect	Kim et al., 2017
iPSC	human dermal fibroblasts	reduced the expression level of SA-β-Gal and MMP-1/3	protect skin from ultraviolet damage	Oh et al., 2018
3D fibroblasts spheroids	not mentioned	expressed a higher level of TIMP-1 differentially expressed miRNA cargos.	inducing collagen synthesis	Hu et al., 2019
Platelet-rich plasma	endothelial cells and fibroblasts	YAP activation	regeneration of chronic skin wounds in diabetic rats	Guo et al., 2017
iPSC	fibroblasts	not clear yet	accelerates diabetic wound healing	Kobayashi et al., 2018
gingival MSC	not mentioned	not clear yet	promote the healing and nerve repair	Shi Q. et al., 2017
melanoma cell	MDSC lymphatic endothelial cells	enhance the tolerance capacity of lymph gland to tumors	immune evasion	Hood, 2016
melanoma cell	skin cells	miR-222/ PI3K/AKT miR-125/ c-Jun	tumor migration and progression	Alegre et al., 2014; Felicetti et al., 2016
SCC cell	microenvironment cells	TGF-β activation	tumor growth and metastasis	McAndrews and Kalluri, 2019
donor MSC	recipient BM-MSC	inhibition of IL4Rα down-regulation of mTOR pathway increase osteogenic differentiation reduce adipogenic differentiation.	caused the rescue of osteopenia impaired BM-MSCs, tight skin, and immune disorders in scleroderma mice	Chen et al., 2017

ingested by skin fibroblasts to accelerate the proliferation and migration of target cells. Expression levels of repair related factors such as collagen, bFGF and TGF- beta are increased, and the PI3K/Akt signaling pathway is activated by AdMSC exosomes in damaged skin cells to accelerate the repair process (Zhang W. et al., 2018). Additionally, dermal fibroblasts can also internalize autologous human epidermal cells derived exosomes in order to promote the proliferation of cell and cause the

degradation of some extracellular matrix by activating matrix metalloproteinase-1(MMP-1). Exosomes derived from human epidermal cells are applied in the full-layer skin excision model of rats, whereby the epidermal regeneration is basically completed within two weeks, which also have significant healing effect (Zhao et al., 2017).

Exosomes and Burn

Burns is a common injury in daily life, which occurs in 50 to 100 cases per 10,000 people annually (Lewis, 2013). At present, the treatment of large-scaled scald is mainly through systemic support, wound cleaning, scab excision and surgical skin grafting (Calota et al., 2012), which are difficult to grasp the scab depth and easy to be infected, and in source tension of skin grafts (Eisenbeiß et al., 2012; Lootens et al., 2013; Nacer Khodja et al., 2013).The skin deep burn and scald within 3 weeks is difficult to repair completely by itself, and is easy to form scar tissue (Cuttle et al., 2006; Ribeiro et al., 2009). Thus, shortening the wound healing time is particularly important. In our previous study, we successfully established the deep secondary burn injury model in SD rats, and found that hucMSC-Ex accelerated the cutaneous wound recovery by facilitating the proliferation of skin tissue cells and the re-epithelialization of epidermis, as well as orchestrating the rational distribution of collagen in skin tissue and enhancing angiogenesis (Zhang et al., 2015a,b). Interestingly, long-term (more than 4 weeks) observation showed that hucMSC-Ex conversely inhibited the proliferation of skin cells and the activation of β-catenin, meanwhile, hucMSC-Ex restricted the amplification of skin stem cells, the expression of α-SMA in dermal cells and the deposition of collagen III. Along with the healing process, the cell density varies from low to high, which triggers the Hippo signaling activation. Mechanically, hucMSC-Ex could deliver 14-3-3 zeta protein to combine YAP and LATs, thus speeding up the phosphorylation of YAP to limit the continuous activation β-catenin (Zhang et al., 2016). The dynamic regulation pattern prevents excessive hyperplasia of skin tissue and scar formation after injury (**Figure 3**).

Exosomes and Scars

During the proliferation period of skin injury repair, fibroblasts, epidermal cells and vascular endothelial cells jointly promote the formation of vascular network, and in the meantime, cells migrate from the edge to the surface of the wound, reducing the area of the wound surface to promote the smooth progress of epidermal regeneration. In this process, excessive activation and recruitment of fibroblasts under pathological conditions often lead to scar formation and fibrosis (Wynn and Ramalingam, 2012; Van De Water et al., 2013). In our studies, hucMSC-Ex can inhibit tissue over-proliferation and scar formation by activating Hippo signaling pathway in the later stage of burn injury repair, providing a guarantee for the security of exosome *in vivo* (Zhang et al., 2016). In addition, it is worth noting that the TGF-β family plays a positive regulatory role in cell differentiation, proliferation and metabolism during tissue injury and repair, while the pathological continuous activation of TGF-β activates the SMAD2 signaling pathway, which can easily lead to fiber deposition and scar formation (Frei et al., 2015). HucMSC-Ex could effectively inhibit the activation of fibroblasts in mice after full-layer resection of dorsal skin. A key group of miRNAs, miR-21, miR-23a, miR-125b and miR-145, could inhibit the activation of fibroblasts by targeting the TGF-β/SMAD2 signaling pathway, reduce the expression of α-SMA and collagen deposition, so as to control scar formation (Fang et al., 2016). At the same time, researchers also propose to conduct gene transfection modification on hucMSC-Ex based on this group of miRNA molecules, so as to acquiring a better and safer therapeutic strategy.

Exosomes and Aging

Skin aging is mainly for the genetic factors and easily found in exposure and the exposed parts, with wrinkles and sagging as the main characteristics. Pathologically, the skin epidermis and the dermis thickness become thinner, dermal collagenous fibers and collagen content declines with neatly arranged collagen bundles, clinically characterized by skin with fine wrinkles. hiPSC exosomes are used to confront aging skin, the expression of senescence-associated-β-galactosidase, a natural aging marker, is significantly down-regulated, and the expression of type I collagen in aging fibroblasts increased, thus playing a good protective and anti-aging effect on human dermal fibroblasts (Oh et al., 2018). Human umbilical cord blood derived exosomes penetrate into the epidermal layer of skin within 15 h, and reach a certain degree of anti-aging effect by regulating the expression and distribution of collagen and elastin in skin cells (Kim et al., 2017) and MSCs derived exosomes can also ameliorate aging and age-related diseases (Ahmadi and Rezaie, 2021).

Exosomes and Ultraviolet Damage

Ultraviolet damage of skin is the accumulation of ultraviolet damage in the process of skin aging, which is the result of the interaction of endogenous aging and ultraviolet radiation, featured tissue disintegration, fragmentation and collagen bundle dispersion (Bellei et al., 2018). Matrix metalloproteinases (MMPs) specifically degrade almost all extracellular matrix (ECM) components and play a vital role in photoaging of skin. Photoaging is found to gradually impair the function of human dermal fibroblasts (HDFs), which generate collagen and matrix metalloproteinase in dermis (Jeong et al., 2015; Zorin et al., 2017; Hu et al., 2019). The iPSCs-Ex can increase the expression level of collagen type I in the photo-aged HDFs and reduce the expression level of SA-β-Gal, MMP-1/3 and restore the collagen type I expression in senescent HDFs (Oh et al., 2018). Another interesting research compared exosomes derived from monolayer culture of HDFs (2D HDF-XOs) with that from the three-dimensional spheroids (3D HDF-XOs). The result showed that there was a notably higher level of TIMP-1 and differentially express miRNAs in 3D HDF-XOs (Hu et al., 2019). 3D HDF-XOs mainly work through the downregulation of TNF-α and the upregulation of TGF-β to induce collagenation and antiaging. Additionally, an interdisciplinary study showed that marine sponge spicules (SHSs) significantly enhanced the skin targeted delivery of hucMSC-Ex, which also revealed anti-photoaging effects in mice, including reducing micro-wrinkles

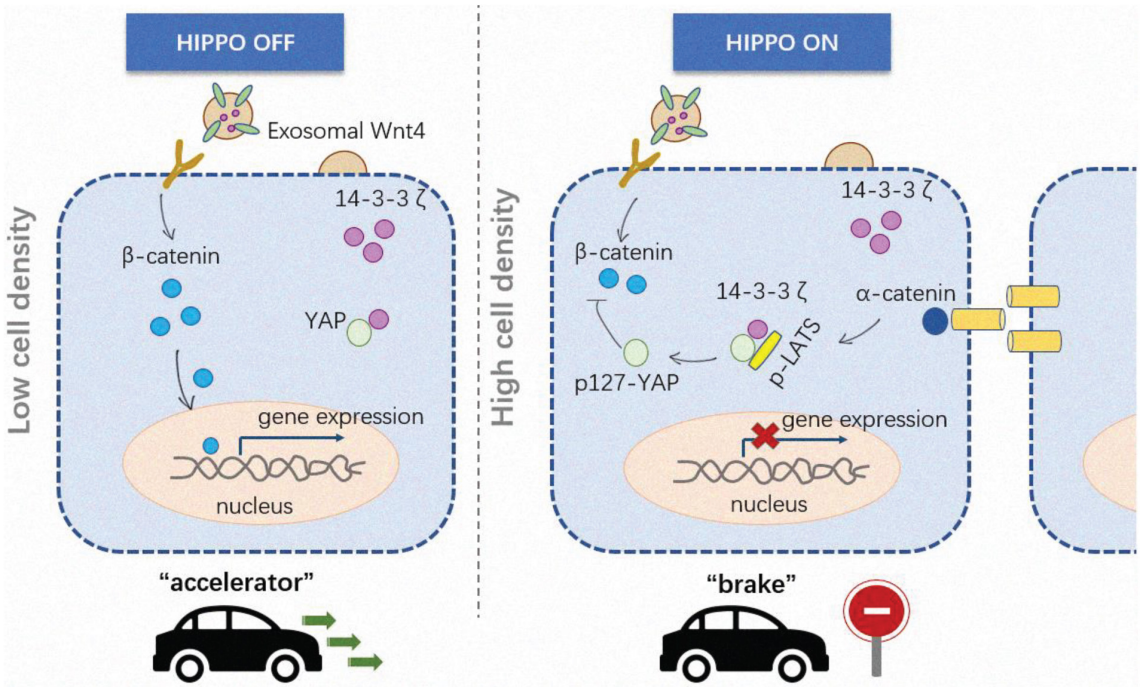

FIGURE 3 | The dynamically regulatory role of exosomes in burn injury. In the early stage of repairing, cells are in a low density, exosomal Wnt4 activates β-catenin in skin cells to promote proliferation and migration, meanwhile stimulates vascular endothelial cell to enhance angiogenesis. With the progression of wound healing, cell density becomes from low to high, the cell-cell contact triggers the activation of Hippo signaling, leading to p-LATS accumulation. 14-3-4ζ released by exosomes works like a "table" to facilitate the combination of YAP and LATS, so as to accelerate the phosphorylation of YAP, the phosphorylated YAP shows a intracellular retention rather than nuclear translocation to suppress the down-stream gene expression and finally inhibit the cell proliferation and migration. The exosomes mediated dynamic regulation in different stage of wound healing, works as an "accelerate" to promote wound healing in early stage, while as a "brake" to prevent excessive proliferation and scar formation.

and enhancing the expression level of extracellular matrix, and facilitating the skin to recover shortly (Zhang K. et al., 2020).

Exosomes and Diabetic Wound Healing

Diabetic foot ulcer (DFU) is one of the most common and major complications of diabetes. At present, the pathogenesis of DFU is considered to be the result of the interaction between peripheral blood vessels and peripheral neuropathy. The complexity of the pathogenesis and the difficulty of wound healing make it one of the problems that threaten the life and health of diabetic patients (Iversen et al., 2009; Goodall et al., 2020). Our previous research has confirmed that hucMSC-Ex can alleviate insulin resistance in type 2 diabetic individuals, increase the distribution of GLUT on the surface of liver, muscle, fat and other tissue membranes, and improve the amount and activity of glucose metabolism enzymes in tissues such as liver, muscle and fat, promote the body's intake and utilization of glucose in the blood to reduce blood sugar in individuals with type 2 diabetes. Specific mechanisms such as exosome directly carrying GLUT molecules to receptor cell (liver, muscle, and fat) membranes, which facilitate extracellular glucose transportation, provide a basic mechanism for exosomes to treat diabetes-related complications (Sun et al., 2018). Conversely, study indicated that diabetes could decrease angiogenic and restorative effect of stem cells *in vitro* (Rezaie et al., 2018a). In diabetic mice, human fibroblast-derived exosomes can promote the acceleration of wound repair (Geiger et al., 2015). The platelet-rich plasma-derived exosomes can activate YAP and promote the regeneration of chronic skin wounds in diabetic rats (Guo et al., 2017). iPSC exosomes enhance fibroblast migration and proliferation, accelerates wound healing and local nerve regeneration in diabetic foot (Kobayashi et al., 2018). The gingival MSC exosome and the synovial MSC exosome have also been reported to promote the healing and nerve repair of diabetic ulcer wounds, however, the specific mechanism requires to be refined (Shi Q. et al., 2017).

Exosomes and Skin Cancer

Exosomes are closely related to tumorigenesis, such as U87 MG human glioblastoma, colorectal cancer, skin cancer (Ahmadi et al., 2021; Soraya et al., 2021). Study showed that exosomal cargos modulate autophagy and play pivotal roles in neoplasis, tumor heomostasis and metastasis (Hassanpour et al., 2020; Jafari et al., 2020; Salimi et al., 2020). Exosomes in skin cancer microenvironment are secreted and enriched, provide cancerous cell the platform to metastasize and attacking the host's immune system (Gowda et al., 2020). Melanoma is the most malignant of skin neoplasms, and melanoma cell-derived exosomes affect the sentinel lymph node genes in a variety of ways to promote tumor progression. In melanoma cells, Wnt5a promotes the release of immunoregulatory and proangiogenic factors from exosomes

(Ekström et al., 2014), while melanoma cell-derived exosomes can regulate lymphatic endothelial cells and myeloid-derived suppressor cells (MDSCs) to enhance the tolerance capacity of lymph gland to tumors, finally achieving immune evasion (Hood, 2016). Metastatic melanomas release exosomes carrying PD-L1, which also be able to suppress $CD8^{+}$T cells function and facilitates tumor growth (Chen et al., 2018). Besides, exosomes can also contain miRNAs, such as miR-222, activates PI3K/AKT signaling pathways in skin cells to promote tumorigenesis (Felicetti et al., 2016). Human metastatic melanoma serum exosomes show a high expression level of miRNA-17, miRNA-19a, miRNA-126 (Pfeffer et al., 2015). MiRNA-125b is selectively expressed by tumor cells due to its ability to inhibit tumor-associated c-Jun protein expression (Alegre et al., 2014). Due to tissue-specific miRNAs can be encapsulated in EVs and distributed in multiple biological fluids, they may serve as a tumor biomarker in neoplasia early progression (Jafari et al., 2019).

In squamous cell carcinoma (SCC), exosomes also serve as an important vehicle for tumor and microenvironment communication, and reactivate tumor-associated signals such as TGF-β in order to promote metabolism and growth of cancer cells (McAndrews and Kalluri, 2019).

Exosomes and Other Skin Lesions

Scleroderma is characterized by inflammatory, degeneration, thickening, and fibrosis, which in turn is hardened and atrophied, thus causing multiple system damage. Studies have reported a significant reduction in exosome levels in peripheral blood serum from patients with scleroderma, which is highly likely to be associated with impaired exosome transport system caused by vascular abnormalities (Nakamura et al., 2016a). This decrement of exosome production in scleroderma patients' serum may be attributed to the disordered transport of exosomes which is from the skin epithelium to the blood flow, resulting in the down-regulation of collagen I that delays wound repair, leading to a higher susceptibility to cutaneous ulcer (Wermuth et al., 2017). Recently, it has been reported that miR-151-5p is a diagnostic and therapeutic potential biomarker of scleroderma. Chen et al. (2017) found that exosomal miR-151-5p released from donor MSCs could inhibit the expression level of IL4Rα and suppress the activation of mTOR pathway, which leads to an increase in osteogenic differentiation and decrease in adipogenic differentiation. Further, miR-151-3p delivered systemically protected the scleroderma mice from immunologic derangement, tight skin, osteopenia and impaired bone marrow MSCs (Chen et al., 2017). Wermuth et al. (2017) found that exosomes derived from scleroderma patients cell culture supernatant were associated with the stimulation of genes expression, including collagen type III alpha 1 chain, COL1A1 and fibronectin-1 encoding ECM components (Chen et al., 2017). In profiles of neutrophils exosomes derived from scleroderma patients, 281 dysregulated lncRNAs and 22 dysregulated miRNAs are identified as well as further analysis were conducted of their target genes and downstream signaling pathways, which could be potential therapeutic targets and biomarkers in scleroderma (Li L. et al., 2020).

Psoriasis is an autoimmune disease of the skin, often accompanied by excessive proliferation of keratinocytes, while iron reserves often affect the working state of the patient's immune system. Some researchers have found that important proteins regulating iron reserve such as hepcidin and soluble transferrin receptor are at low expression levels in serum exosome of patients with psoriasis, presumably affects immune function and thus affects the course of the disease (El-Rifaie et al., 2018).

EXOSOMES PARTICIPATE IN VARIOUS SKIN DAMAGE REPAIR MECHANISMS

Coagulation

Exosomes can be extracted from saliva, mononuclear macrophages, and platelet secretions. After skin trauma, obstruction of coagulation function affects the repair time of wounds, and the blocked coagulation function is relevant to the change of tissue factor (TF) conformation or the presence of TF inhibitory molecules. Saliva-derived exosomes can shorten wound clotting time by transporting active TF in combination with factor VII. At the same time, these different sources of exosomes secreted into the intracellular space can also activate coagulation by transmitting TF, for example, exosome secreted by monocytes can bind to platelets and release their own TF into platelets to activate downstream cascades to promote thrombin and fibrin clot formation (Biró et al., 2003; Del Conde et al., 2005).

Proliferation

Proliferation of skin cells plays a vital role in skin damage repair. At present, exosomes from various tissue and cell regulate the proliferation of skin cells, such as MSC exosomes (Du et al., 2014; Shabbir et al., 2015; Jiang et al., 2020), mouse ESC exosomes (Jeong et al., 2014), endothelial progenitor cell-derived exosomes (Li et al., 2016) and fibroblast-derived exosomes. These exosomes functioning in regulating cell proliferation in two ways, activate cell cycle related genes or increase growth factor expression levels. The exosomal RNAs or proteins are delivered to the target cell, such as Wnt4 transportation to motivate the β-catenin signaling pathway in the epidermal cell (Zhang et al., 2015a), or delivering the small RNAs to target the downstream Akt, Erk, MAPK signaling pathway, etc. (Zhang et al., 2016; Guo et al., 2017), ultimately attributed to the regulation of cell cycle-related genes such as cyclinA1, cyclinD1, cyclinD3 to promote cell proliferation. Additionally, exosomes can also activate interleukin-6 (IL-6), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) and other growth factors, which stimulate damaged skin cells proliferation. The proliferative effect of exosomes can be caused both by paracrine affecting surrounding cells and autocrine to *de novo* affect itself.

Migration

Proliferation and migration of cells are pivotal for wound healing. With the onset of the clotting period, immunoinflammatory cells such as macrophages and neutrophils are first recruited to the

damaged site to get rid of necrotic tissue fragments as well as phagocytose infected microbe. Subsequently, epidermal cells and dermal fibroblasts begin to migrate to the injured sites to fill the vacancies and promote wound healing (Singer and Clark, 1999). In this process, exosomes from various cells are involved in the regulation of cell migration, such as MSCs, keratinocytes, endothelial cells and fibroblast-derived exosomes (Cheng et al., 2008). Keratinocyte-derived exosomes can promote their own migration by transporting heat shock protein 90α (HSP90α) in an autocrine manner. In addition, keratinocyte-derived exosomes can also promote the migration of vascular endothelial cells, thereby promoting the regeneration and repair of blood vessels (Leoni et al., 2015). Moreover, keratinocyte-derived exosomes enhance the migration of dermal fibroblasts by transporting HSP 90α, which mechanically relate to its interaction with cell surface receptor LRP-1/CD91 (McCready et al., 2010). MSC exosomes regulate fibroblast migration by activation of Erk1/2 signaling pathway, resulting in up-regulating the expression of IL-6, cyclin1, MMP-1, N-cadherin and other factors in target cells (Huang et al., 2015; Hu et al., 2016).

Vascularization

Angiogenesis provides oxygen for repairing tissues and supply nutrients. The proliferation of vascular endothelial cells is an essential constituent of it. Moreover, it also requires interaction with angiogenic, endothelial cells and the surrounding extracellular matrix environment (Liekens et al., 2001). The wound area exhibits chemotactic effect on vascular endothelial cells, enabling endothelial cells to penetrate the vascular basement membrane, enter the extracellular matrix, form a lumen-like structure and continuously extend and branch, and finally generate a new vascular network. In this process, human MSC and endothelial cell-derived exosome can increase the density of neovascularization in the wound area, which is related to the promotion of endothelial cell proliferation and migration. In addition, endothelial progenitor cells and platelet-rich plasma-derived exosomes can activate the expression of pro-angiogenic genes in target cells, such as IL-8, IL-6, fiber growth factor 2, E-selectin, etc. The expression of these factors will further activate Erk1/2 and other related signaling pathways, activate downstream VEGFA, VEGFR-2, c-Myc, cyclinD1 and other target genes, thereby further promoting neovascularization (Hromada et al., 2017). In our previous studies, we found that hucMSC-Ex repaired wounds by promoting Wnt4/β-catenin signaling pathway in vascular endothelial cells (Zhang et al., 2015a). Conversely, some researchers find exosomes to inhibit the formation of blood vessels by binding to CD36 on the surface of target cells (Ramakrishnan et al., 2016). Therefore, despite of a great potential in regulating angiogenesis, it is still necessary to specifically analyze the source, type and specific pathological conditions when exosomes are used.

Skin Stem Cell Mobilization and Participation

Stem cells are the important reason to explain the super self-repair ability of skin. Epidermal stem/progenitor cells generally locate in the basal layer of the epidermis to meet the daily keratin renewal metabolism or the need to fulfill new cells after skin damage. The proliferation and differentiation of skin stem cells are affected by multiple factors, and finally achieve the balance between proliferation and differentiation (Li and Sen, 2016; Yang et al., 2020). In the model of deep second-degree scald repair in rats, we confirm that hucMSC-Ex can activate CK19 and α6 integrin-positive epidermal stem cells in the early stage of repair, and make the epidermal stem cells return to resting state after the repair is completed. The mechanism may be related to the dynamic regulation between Wnt/β-catenin signaling pathway and Hippo/YAP signaling pathway (Zhang et al., 2016). In addition, epidermal stem cells are involved in epidermal regeneration in the early post-traumatic period, but do not play a role in late and normal epidermal renewal. Exosomes from autologous stem cells are also effective in promoting proliferation, angiogenesis and wound healing of damaged skin cells (Lu et al., 2019).

Immunoregulation

After skin trauma and infection, a great quantity of immune cells are recruited to the lesion region, Exosomes transport pathological nucleic acids, proteins and lipids, and take part in antigen-promoting, immune response-related receptors activation, and defense induction, which therefore arouse interests to consider exosomes as good immune-based therapeutics and vaccine candidates (Schorey and Harding, 2016).

Skin and immune cells mediate the corresponding immunological effects by secreting exosomes. Keratinocytes expedite the stimulation and maturation of dendritic cells by secreting exosomes, enhance the expression of CD40, and produce a large number of cytokines such as IL-6, IL-10, IL-12 (Kotzerke et al., 2013). Exosomes from circulating immunological cells such as monocytes can also promote the expression of MMP-1 in dermal fibroblasts by transporting 14-3-3 protein, thereby promoting cell migration to the wound area (Medina and Ghahary, 2010). Moreover, MSC exosomes are involved in autologous skin transplantation by regulating the inhibitory immune cell Treg (Zhang B. et al., 2018; Showalter et al., 2019), and exosomes from sweat can also regulate skin immunity, which is the donor exosomes trigger alloreactive T cell responses after transplantation (Marino et al., 2016). Furthermore, the M2 Macrophage-derived exosomes (M2-Exo) induce M1-M2 conversion *in vitro* to promote wound healing by facilitating neovascularization, collagen deposition as well as re-epithelialization (Kim et al., 2019). In recent years, immune cell such as dendritic cell-derived exosomes are used to stimulate T cells to activate host anti-tumor immunity, and have entered Phase I and Phase II clinical trials (Besse et al., 2016; **Figure 4**). Conversely, some studies have found that after skin transplantation, some donor-derived exosomes unexpectedly trigger an immune response and attack receptor tissue cells by activating autologous T-cell immunity, which reminds us the immune system in skin is quite a complex network that every type of cells inside is possibly to communicate with other cell types nearby or in a distance, some of the "cross-talk"

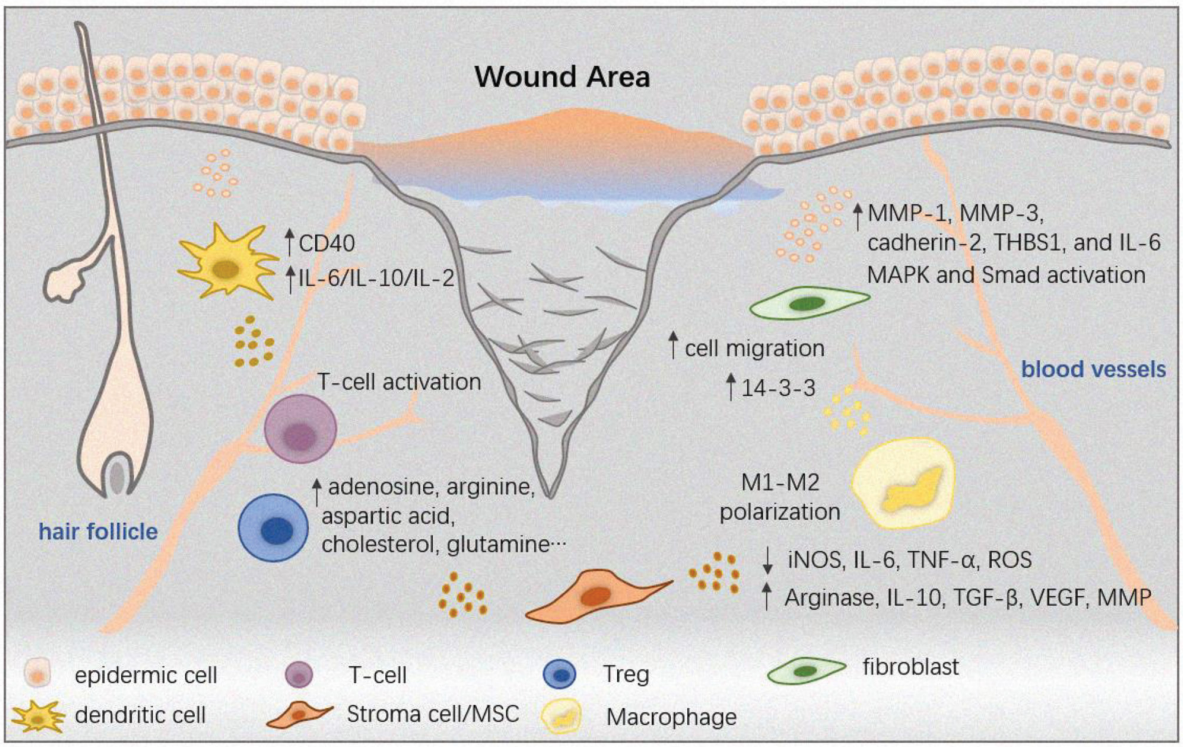

FIGURE 4 | The immunoregulatory roles of exosomes in skin diseases. The skin relies on the immune system for essential functions, such as thermoregulation and infection control. The wound healing process is a complex one with at least three stages, while immune cells participate in them all. Neutrophils and macrophages infiltrate into the wound healing as soon as the inflammatory stage begins. The wound area is a complicated microenvironment that cells inside of it like epidermic cells, fibroblasts, stroma cells can interact with immune cells including T-cells, Tregs, dendritic cells and macrophages by releasing specific bio-molecules, jointly affect the fate of disease.

mechanisms are unlocked while some are not, the function and application of exosomes in immune regulation remains to be more fully lucubrated (Morelli et al., 2017; Benichou et al., 2020).

Extracellular Matrix Remodeling

Exosomes are involved in the hemorrhagic, inflammatory and proliferative phase of skin damage repairing as well as the tissue remodeling. Exosomes from various cells have shown an effective regulatory function on extracellular matrix. Elastin is an important component of the extracellular matrix maintenance structure, and exosomes can promote elastin expression during tissue remodeling (Zhang et al., 2015c). Collagen mainly secreted by fibroblasts also takes part in tissue remodeling. Adipose-derived MSC exosomes can effectively promote the proliferation and secretion capacity of dermal fibroblasts (Fang et al., 2019). Exosomes are also able to promote collagen formation in the extracellular matrix through its membrane surface LOXL2 protein (de Jong et al., 2016). Other identical studies revealed that exosomes could be isolated in fiber networks, demonstrating their involvement in the formation and functional regulation of extracellular matrices (Huleihel et al., 2016). In addition, exosomes can accelerate the repairing process and restrict scab size by interacting with the Annexin A1 and formyl peptide receptors to complete tissue remodeling (Nakamura et al., 2016b).

APPLICATION PROSPECT AND OPEN QUESTION

Exosomes and Scar Free Treatment

Skin wound healing is often accompanied by scab and scar formation, when the skin tissue is seriously damaged, exceeding its self-healing capacity, the wound area will be replaced by fibrous scars. The atrophy period of scar formation will bring physiological discomfort to the patient, and after the scar formation will certainly affect the local appearance and function. Our previous findings indicate that hucMSC-Ex retain YAP in the cytoplasm by transporting the active 14-3-3 molecule at the later stage of repairing, thus inhibiting the excessive proliferation of skin cells and the formation of fibrosis (Zhang et al., 2016). HucMSC-Ex also inhibit scar formation by inhibiting TGF- beta/SMAD2 pathway activation in fibroblasts by transshipping mir-23a, mir-125b and mir-145b, etc., thereby inhibiting myofibroblast activation (Chen J. et al., 2019). Meanwhile, AdMSC exosomes can reduce scar area and inhibit the differentiation of fibroblasts into myofibroblasts,

mechanically related to the inhibition of TGF- beta 1 signaling and as well as ERK/MAPK signaling activation in fibroblasts (Wang et al., 2017). However, scars such as hypertrophic scars, resulting from alterations in the normal processes of cutaneous wound healing are finally featured by excessive deposition of collagen, persistent inflammation and fibrosis. New therapies including exosomes are designed to no more than minimizing scarring and accelerating wound healing immediately to avoid scar formation at an early stage. The hypertrophic scar treatments and even the keloid treatments still call for intensive studies.

Exosomes as Bio-Markers for Cutaneous Diseases

Exosomes are carrying a variety of bioactive nucleic acids or proteins, which have been widely studied and reported as diagnostic markers of various diseases. Both the quantity and contents may alter when pathological conditions occur. In comparison with healthy controls, serum derived exosomes are more secretion in cutaneous diseases, especially tumors such as melanoma. Moreover, exosomes derived from melanoma patients are proved to convey an increased level of S100B (Alegre et al., 2016; Isola et al., 2016). Due to EV-related miRNAs are tissue-specific and exist in most biological fluids, they may serve as a tumor biomarker in early diagnosis, treatment response and prognosis of cancer (Mirzaei et al., 2016; Chen L. et al., 2019; Rahbarghazi et al., 2019). Exosomal miR-17, miR-19a, miR-21, miR-126, and miR-149 were increased in serum of patients diagnosed with melanoma (Chen L. et al., 2019). The expression level of exosomal miR-222 derived from melanoma samples was consistent with the metastatic capacity of melanoma cells (Felicetti et al., 2016). With the exception of skin tumors, the numbers of exosomes in the serum of patients with scleroderma is also remarkably higher than healthy group, which was of certain diagnostic value.

Recently, by using mass spectrometry and RNA sequencing, vast information has been acquired, which can be reference to the disease of interest. For example, profiles of neutrophils derived exosomes revealed 281 dysregulated lncRNAs and 22 dysregulated miRNAs as well as the predicted target genes and reactome pathways such as Wnt, IL-23, AMPK, and NOTCH signaling in systemic sclerosis, which could be high diagnostic value in the disease (Li L. et al., 2020). A mass spectrometry-based proteomic characterization of melanoma exosomes identified 1507 tumor-related proteins, most of which participated in cell proliferation, migration, EMT and neovascularization, which indicated a potential for further research identifying new biomarkers (Surman et al., 2020). Nevertheless, to be a competent diagnostic marker, more comprehensive case data and criteria are needed to promote its transformation from basic science to clinic. In order to realize the diagnostic and prognostic potential of exosomes containing distinct biological markers, it is essential to evolve the proper exosome isolation methods. Effective and clean without contamination separation of exosomes in circulation should be conducted to gain valid information for clinical application, the selected cargos should be specific and sensitive.

Tailored Exosome as New Therapeutics for Cutaneous Disease

The positive role of exosomes in cutaneous wounds has been continuously recognized. At present, a craze of engineering modification of exosomes has been set off in the research field, which maximizes the advantages of exosome by gene manipulation of origin cells, drug or therapeutic molecular loading by physical incubation, the freeze-thaw cycling, electroporation, sonication and other techniques (**Figure 5**). The well-designed exosomes could erase the limitations of natural exosomes and increase the targeting capability, half-life in circulation as well as the cargo concentrations.

The endogenous way to get proteins encapsulated into exosomes is mainly by protein fusion which depends on the available cellular machinery for classifying therapeutic proteins into exosomes (Nasiri Kenari et al., 2020). Proteins such as lactadherin, lamp2b, which exist on the membrane of exosomes are used to deliver therapeutic siRNA, miRNA to the site of interest, tetraspanin family members CD9, CD63, CD81 are mostly used as a candidate for fusion of fluorescent proteins to trace exosomes *in vitro* and *in vivo*. In addition, tailored exosomes by transferring certain specific miRNA or siRNA has also been explored for disease treatment. By gene manipulation of synovial MSCs, exosomes express a high level of miR-126 to promote the angiogenesis in skin wound (Tao et al., 2017). Other pre-treatment of the origin cells like chemical agents, hypoxia is also effective for exosome modification. Melatonin-pretreated MSC-derived exosomes can enhance diabetic wound healing by regulating macrophage M1-M2 conversion (Liu et al., 2020). In our previous study, we found that small molecule drugs 3,3′-diindolymethane (DIM), as a natural small-molecule drug extracted from cruciferous plants, can activate hucMSC-Ex and enhance the dry and paracrine ability of MSC by carrying active Wnt11 molecules, so as to enhancing the repairing effect of deep second-degree scald wounds (Shi H. et al., 2017).

Exosomes also can be modified exogenously after their isolation process. Approaches including electroporation, incubation, sonication, extrusion, hypotonic dialysis, freeze-thaw cycles, saponin, $CaCl_2$ and lipofectamine reagent are used for exosomes loading. Sources such as platelet derived exosomes loaded with curcumin on polysaccharide, can more effectively promote diabetic foot ulcer wound repair (Brennan et al., 2020). MSC derived exosomes assembled in a chitosan/silk hydro gel (Shiekh et al., 2020) and in a self-healing, anti-bacterial polypeptide hydro gel (Wang et al., 2019) promoted diabetic wound healing. It can be predicted that exosome modification will make it play its maximum advantages in cutaneous wound healing. Meanwhile, it remains to be determined the specific mechanism of cargo loading, release, uptake, and destiny of vesicle in recipient tissue.

Future Application Prospects and Problems of Exosomes

Exosomes can significantly repair cutaneous damage, provide new therapeutic strategies for the treatment of various diseases,

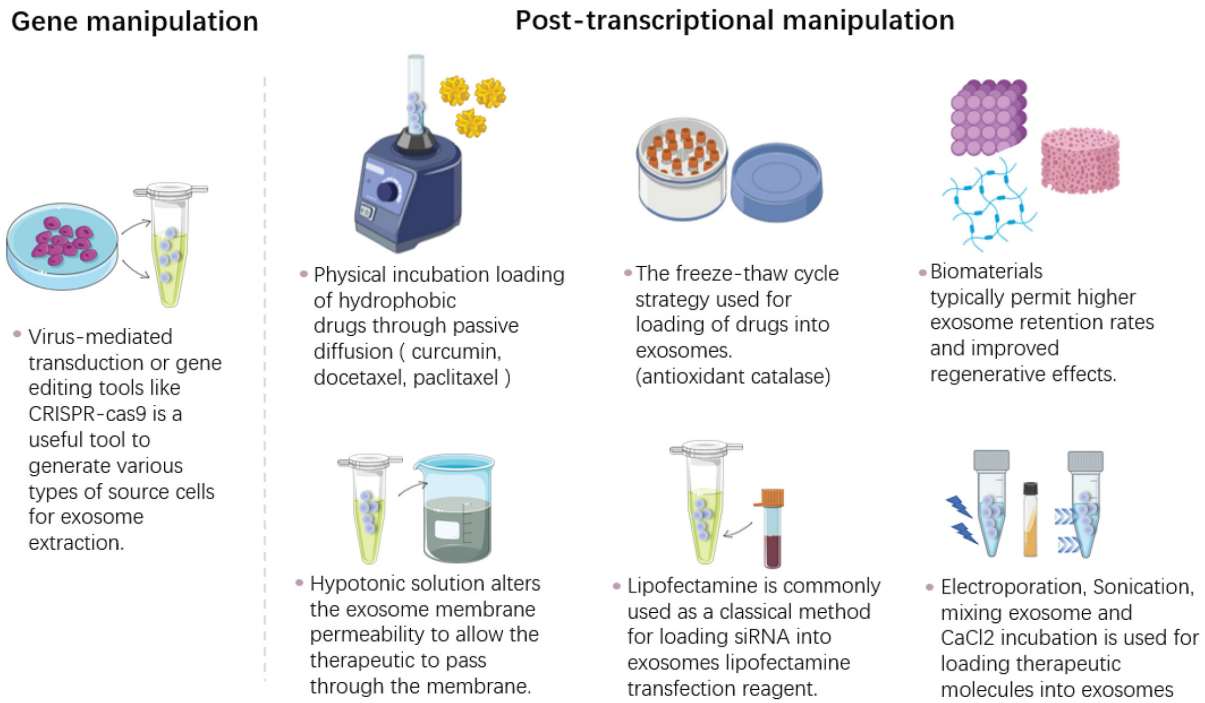

FIGURE 5 | The bioengineering modification of exosomes. This is a simplified illustration of methods for modification of therapeutics into exosomes. The methods are basically divided into two parts, the gene manipulation modification and the post-translational modification. The first one is to use virus mediated system or gene editing techniques to modify the parental cells before exosome extraction. The other way is to directly modify exosomes after the isolation process by various method including incubation, electroporation, sonication, extrusion, hypotonic dialysis, freeze-thaw cycles, saponin, $CaCl_2$ and lipofectamine reagent.

and lay an important foundation for biopharmaceuticals based on exosome modification. However, there still exists many unanswered questions for exosome utilization from basic science to clinic: (1) The scalability of exosomes is a major challenge currently. The future clinical use for exosomes depends on a mass-produced and GMP-compliant production process. New approaches like tangential flow filtration can concentrate large quantities of cell-conditioned media for exosome enrichment (Heinemann et al., 2014). (2) There is an urgent need for precise and accurate characterization of exosomes to better evaluate their heterogeneity, their cargo, and functions. A new criterial for exosome identification is put forward by MISEV (Théry et al., 2018). (3) The use of supraphysiological numbers of exosomes still needs to be re-considered, for example, the dose, time duration as well as the route of administration. (4) It is still unclear that how the multiple information conveyed by exosomes can be fully used to develop more precise or early-phase biomarker to help clinical decision making. (5) The application of exosomes as therapeutics relies on producing homogeneous exosomes on a large scale by finding optimal producer cells, culture conditions as well as stimulation conditions, subsequently, getting the approval by the FDA for clinical applications. A large amount of studies has demonstrated the role of exosomes, especially MSC-exosomes in tissue regeneration, immunomodulation and tumor. However, exosomes under different conditions may have opposite effect, for example, exosomes from aged MSC shows no effect on skin regeneration. Further investigation is still required to uncover the multifaceted function of exosomes.

Ultimately, although we have already acquired certain stock of knowledge on exosomes and their functions on skin biology, there still much room for exploration on the potential of exosomes in dermatologic disease is diagnose and treatment.

AUTHOR CONTRIBUTIONS

HS did the conceptualization. HS and YS wrote. MW and DY collected the data. HQ and WX reviewed and edited. All authors contributed to the article and approved the submitted version.

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Extracellular Vesicles Secreted by TDO2-Augmented Fibroblasts Regulate Pro-inflammatory Response in Macrophages

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Extracellular vesicles (EVs) are secreted lipid bilayer vesicles that mediate cell to cell communication and are effectors of cell therapy. Previous work has shown that canonical Wnt signaling is necessary for cell and EV therapeutic potency. Tryptophan 2,3-dioxygenase (TDO2) is a target gene of canonical Wnt signaling. Augmenting TDO2 in therapeutically inert fibroblasts endows their EVs with immunomodulatory capacity including attenuating inflammatory signaling in macrophages. Transcriptomic analysis showed that macrophages treated with EVs from fibroblasts overexpressing TDO2 had blunted inflammatory response compared to control fibroblast EVs. *In vivo*, EVs from TDO2-overexpressing fibroblasts preserved cardiac function. Taken together, these results describe the role of a major canonical Wnt-target gene (TDO2) in driving the therapeutic potency of cells and their EVs.

Keywords: extracellular vesicles, exosomes, inflammation, immunoregulation, macrophages, fibroblasts, TDO2

INTRODUCTION

Extracellular vesicles (EVs) are nano-sized lipid-bilayer vesicles secreted by nearly all cell types and represent an evolutionarily conserved mechanism of cell–cell communication (Yáñez-Mó et al., 2015; Zaborowski et al., 2015; Mathieu et al., 2019). EVs are broadly classified by their biogenesis (Meldolesi, 2018). Exosomes are smaller (30–100 nm) (Raposo and Stoorvogel, 2013) EVs that arise from the late endosome processed by the endosomal sorting complexes required for transport (ESCRT) pathway. Ectosomes which include microvesicles and apoptotic bodies, in contrast, are passively shed from the plasma membrane (ectosomes) (Mathieu et al., 2019; Sahoo et al., 2021). EVs are laden with potent signaling molecules including lipids, proteins, and RNA (Yáñez-Mó et al., 2015; Zaborowski et al., 2015; Mathieu et al., 2019). EV signaling plays a critical role in development, health, and disease (Ibrahim and Marban, 2016; Malloci et al., 2019).

Emerging evidence also implicates EV secretion and signaling in the therapeutic effect of cell therapy (Ibrahim and Marban, 2016; Marban, 2018; Yin et al., 2020). Cardiosphere-derived cells (CDCs) are a population of cardiac stromal progenitors with demonstrated therapeutic bioactivity in cardiac and skeletal muscle indications. Early studies implicated CDC-EVs as mediators of the CDC therapeutic effect (Ibrahim et al., 2014; Gallet et al., 2017; Rogers et al., 2019). CDCs, through CDC-EVs, modulate several pathways of tissue healing and repair (Ibrahim et al., 2014), most notably, immunomodulation (de Couto et al., 2017, 2019). Furthermore, we and others identified macrophages as major functional recipients of CDC-EVs and mediators of therapy (de Couto et al., 2017, 2019). Macrophages are pivotal players in tissue injury and resolution. Recent mechanistic investigation by our group further implicated Wnt-β-catenin signaling activation as necessary for the secretion of therapeutic EVs by CDCs (Ibrahim et al., 2019, 2021). However, the specific downstream target genes of β-catenin signaling, and their effect on EV-cargo, remain poorly described. Here, we identify a β-catenin-upregulated target gene tryptophan 2,3-dioxygenase (TDO2). TDO2 is an enzyme involved in the metabolism of tryptophan into various metabolites including kynurenine with well-described roles in immunomodulation. Here we investigate the role of TDO2 activation in modulating macrophage inflammatory activation.

MATERIALS AND METHODS

Neonatal Human Dermal Fibroblasts

Neonatal human dermal fibroblasts (nHDFs) were sourced from ATCC (PCS-201-010). Cells were cultured in IMDM (GIBCO), 10% FBS (Hyclone), 2 mM L-glutamine (GIBCO), and gentamicin (GIBCO). Cells were maintained at 37°C 20% O_2/5% CO_2 in complete medium with medium exchanges every 3–4 days as needed. Cells were grown until near confluent and passaged using TrypLE (GIBCO).

Lentiviral Transduction

Neonatal human dermal fibroblasts were plated in T25 flasks and transduced with TDO2 activation lentiviral particles (Santa Cruz Biotech) at MOI:20 in complete medium. After 24 h of transduction, the virus was removed, and fresh complete medium was added for cell recovery for a further 24 h. Cells were then subjected to selection by 5.0 μg/mL puromycin for approximately 3–4 days. Following selection, complete medium was replaced and cells were grown and passaged.

EV Preparation and Isolation

Extracellular vesicles were harvested from primary nHDFs at passage 5-7, from normal and transduced cells using a 15-day serum starvation method previously described (Walravens et al., 2021). Briefly, cells were grown to near confluence (∼90%) at 20% O_2/5% CO_2 at 37°C. Cell bed was washed 2x with warmed phosphate-buffered saline (PBS) and then incubated in IMDM without serum supplementation for 15 days in the same environment. Conditioned medium was collected, centrifuged at 3,000 × *g* for 10 min to remove dead cells and debris, then filtered through a 0.45-μm PES filter to remove apoptotic bodies and protein aggregates, and frozen for later use at −80°C. EVs were purified using centrifugal ultrafiltration with a 100-kDa molecular weight cutoff filter (Sigma-Millipore). EV preparations, before and after concentration were analyzed by NTA using the Malvern Nanosight NS300 Instrument (Malvern Instruments) with the following acquisition parameters: camera levels of 15, detection level less than or equal to 5, number of videos taken = 5, and video length of 30 s.

Size-Exclusion Chromatography

Extracellular vesicles were collected and prepared as described above. After 100 kDa ultrafiltration, EVs were further purified using size exclusion chromatography (SEC) columns (SBI). Briefly, 1.0 mL of concentrated EVs was added to each chromatographic column and incubated at room temperature with rotation for 30–35 min. EVs were eluted from the column by centrifugation at 500 × *g*. EV size and concentration were analyzed by NTA as described above. Protein content of EV preparations was quantified using a BCA assay (Pierce).

Bone Marrow-Derived Macrophages

Bone marrow-derived progenitor cells were collected from 3-month-old female Wistar Kyoto rats and differentiated into bone marrow-derived macrophages (BMDMs) by culturing with 20 ng/mL recombinant M-CSF (Life Technologies). Briefly, whole bone marrow cells were collected via aspiration with ice-cold PBS. Cells were filtered using a 70-μm cell strainer and centrifuged at 400 × *g* for 10 min at 4°C to pellet. The cell pellet was resuspended in 10 mL ACK buffer (GIBCO) for 30 s. ACK was quenched with IMDM + 10% FBS, and cells were centrifuged as described above. Cells were resuspended in complete medium; IMDM + 10% FBS + 20 ng/mL M-CSF and counted. Cells were seeded into six-well plates at $8.0e10^4$ cells/well, or equivalent. Cells were incubated at 37°C with 20% O_2 and 5% CO_2. Fresh complete medium was exchanged on day 3 and cells monitored for confluence. Test compounds were administered once BMDM cultures reached ∼75% confluence. Serum concentration was reduced to 1% during assays to facilitate EV uptake.

Bromodeoxyuridine (BrdU) Assay

Primary bone marrow macrophages were collected as previously described and plated in 96-well plates at a density of $4.0e^4$ cells/well in complete medium (IMDM + 10% FBS + human recombinant M-CSF 20 ng/mL). After attachment and maturation (∼3 days), complete medium was removed and replaced with IMDM w/1% FBS for all test conditions. $4.0e^6$ EVs were added ∼1 h after medium change along with lipopolysaccharide (LPS) (10 ng/mL, Sigma). Control conditions were cultured in IMDM w/10% and 1% FBS. Cells were grown overnight after EV & LPS addition and proliferation quantified using a bromodeoxyuridine (BrdU) Cell Proliferation ELISA (Abcam, ab126556).

Cell Migration (Modified Boyden Chamber Assay)

Bone marrow-derived monocytes were seeded onto 100 mm cell culture dishes and differentiated into mature macrophages using IMDM + 10% FBS + 20 ng/mL human recombinant M-CSF (Life Technologies). Upon reaching 75% confluence, BMDMs were lifted using ice-cold PBS + 2 mM EDTA. Cells were quantified and re-seeded onto 8.0-μM pore size transwells (Costar). Cells were recovered overnight at 37°C, 5% CO_2 with complete medium (IMDM + 10% FBS). One hour before EV administration, complete medium was removed, cells were gently washed 1x with serum-free IMDM, and medium was replaced with IMDM + 1% FBS. Cells were incubated with EVs overnight and then fixed with 4.0% PFA. Cells were gently removed from the upper side of the transwell using a cotton swab. The underside of the transwell was stained for 20 min at RT using Crystal Violet. After staining, cells were gently washed several times with PBS until the wash ran clear. Ten images were captured of each transwell at 10x magnification (three per condition). Quantification of cell migration was done using ImageJ.

RNA Isolation and RT-qPCR

Total cell RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. Total EV RNA was isolated using the miRNeasy Advanced Serum Plasma Kit (Qiagen). Total cell RNA was quantified using NanoDrop and diluted using diH_2O. Total EV RNA was quantified by Qubit (Thermo Fisher Scientific). Cellular RNA Reverse Transcription was performed using the High-Capacity RNA-to-cDNA kit (Life Technologies) with 1 μg RNA per reaction. PCR reactions were performed on the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) using TaqMan Fast Advanced Master Mix (Life Technologies, 4444556) and TaqMan primers. Each reaction was performed in triplicate. The gene expression assays used for this study are summarized in **Supplementary Table 1**.

Cell Lysate and Protein Assay

Cell lysates were collected for ELISA and western blot from six-well plates. Cells were washed 1x with ice-cold PBS. Cells were lysed in-well with 75 μL 1 × lysis buffer with phospo/protease inhibitors (Thermo Fisher Scientific). The cell lysate was incubated on ice for 15 min, sonicated twice for 10 s each, and centrifuged at 15,000 × *g* for 15 min at 4°C. The supernatant was collected and frozen for later use at −80°C. Protein lysates were quantified using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific).

Electrophoresis and Western Blot

Electrophoresis was conducted using NuPage 4–12% Bis-Tris protein gels (Life Technologies) using 25 μg protein per well. HPVD Membrane transfer was performed using the Turbo Transfer System (BIO-RAD) after gel electrophoresis. Blocking was performed using 5% non-fat milk in TBS + 20% Teen, 1 h at RT. Primary antibody staining was done overnight at 4°C. Secondary HRP antibody staining was done for 90 min at RT and then detected by SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). Antibodies used in this study are summarized in **Supplementary Table 2**.

ELISA

Interleukin-6 ELISA (R&D Systems, Quantikine ELISA) was performed according to the manufacturer's protocol. Samples concentration for testing was 1.5 mg mL^{-1}.

RNA and miRNA Sequencing

Cell and EV RNA samples were sequenced at the Cedars-Sinai Genomics Core. Total RNA and Small RNA were analyzed using an Illumina NextSeq 500 platform for cell and EV samples, respectively.

Proteomic Analysis of Extracellular Vesicles

Proteomics of nHDF-EV and $nHDF^{TDO2}$-EV was conducted by Creative Proteomics (Shirley, NY, United States) using 200 μg protein per sample. Data analysis and processing were done using FunRich (Pathan et al., 2015).

Animal Study

All animal studies were conducted under approved protocols from the Institutional Animal Care and Use Committee protocols.

Mouse Acute Myocardial Infarction Model

Acute myocardial infarction was induced in 3-month-old male C57/B-L6 mice as described previously (Ibrahim et al., 2014). Within 10 min of left anterior descending artery ligation, a total of 1 × 10^5 cells (or vehicle) were administered via 3 × 8μL injections intramyocardially.

Echocardiography

Echocardiography was performed in the mouse model of acute myocardial infarction at 1 day (baseline) and 21 days after surgery using Vevo 3100 Imaging System (Visual Sonics) as described (Ibrahim et al., 2019). The average of the left ventricular ejection fraction was analyzed from multiple left ventricular end-diastolic and left ventricular end-systolic measurements.

Statistics

GraphPad Prism 9.0 (GraphPad Software) was used to analyze the data. A comparison of three or more groups was performed using two-way or one-way ANOVA followed by Sidek's *post hoc* multiple comparison test for paired groups. Two-group comparisons were analyzed using two-tailed unpaired *t*-tests with a confidence interval of 95%. RNA sequencing data were analyzed for differential expression, fold change, and unsupervised PCA using DESeq2 (Anders and Huber, 2010; Love et al., 2014).

RESULTS

TDO2 Augmentation Results in Broad Gene Expression Changes in Fibroblasts

Normal human dermal fibroblasts (HDFs) treated with the beta-catenin activator 6-bromoindirubin-3'-oxime (BIO) lead to upregulation of TDO2 more than any other gene (**Supplementary Figure 1A**). Therefore, to examine the role of TDO2, we transduced neonatal human fibroblasts (nHDFs) with lentivirus containing the TDO2 transgene under the control of a constitutive promoter. Upon transduction and subsequent selection by puromycin, expression of TDO2 was 50-fold higher than non-transduced cells (**Supplementary Figure 1B**). TDO2 activation had a significant impact on the nHDF transcriptome. Transcriptomic sequencing identified 3,000 differentially expressed genes and over 400 unique genes in TDO2 transduced cells (**Supplementary Figures 1D–E**). Increased expression of TDO2 was further confirmed by the sequencing data as well (**Supplementary Figure 1F**).

TDO2-Expressing Fibroblasts Attenuate Macrophage Activation

To explore the effect of TDO2 activation on the immunomodulatory capacity of nHDFs, rat BMDMs were co-cultured with TDO2-transduced nHDFs ($nHDF^{TDO2}$), un-transduced nHDFs ($nHDF^{UNT}$), or LPS as an activation control. Examination of macrophage polarization and inflammatory markers showed no changes in arginase 1, IL-6, and IL-1B expression following co-culture (**Figures 1A–C**). Nos2 expression was significantly decreased compared to both LPS- and nHDF-co-cultured BMDMs (**Figure 1D**). Decreased expression of the protein, iNOS, was further confirmed by western blot (**Figures 1E,F**). Interestingly, while no changes were observed in IL-6 transcription (**Figure 1B**), secreted IL-6 in the conditioned culture medium was lower as observed by ELISA which suggests potential post-translational regulation (**Figure 1G**). To investigate the potential mediator of the immunoregulatory effect of TDO2-activated nHDFs, we assessed the levels of secreted kynurenine. TDO2 is a rate-limiting enzyme in the conversion of L-tryptophan to *N*-formyl-L-kynurenine. Kynurenine and its downstream metabolites play roles in anti-inflammation and vascular relaxation (Nguyen et al., 2010; Wang et al., 2010). However, subsequent analysis of conditioned medium by ELISA revealed no increase in kynurenine (**Supplementary Figure 1C**). Therefore, TDO2 activation endows nHDFs with immunomodulatory capacities as shown by their ability to blunt IL-6 and Nos2 expression in co-cultured macrophages. Furthermore, this effect is not driven by the synthesis of kynurenine.

TDO2-Augmented Fibroblasts EVs Are Enriched in Small Non-coding RNA

Having ruled out the role of kynurenine, we investigated changes in EV payload post TDO2 activation in nHDFs. $nHDF^{TDO2}$ ($nHDF^{TDO2}$-EVs) and control nHDF EVs (nHDF-EVs) were conditioned using a 15-day serum-starvation protocol described by us previously (Ibrahim et al., 2014). EVs were isolated using ultrafiltration with 100 kDa molecular weight cut-off followed by buffer exchange with PBS. The

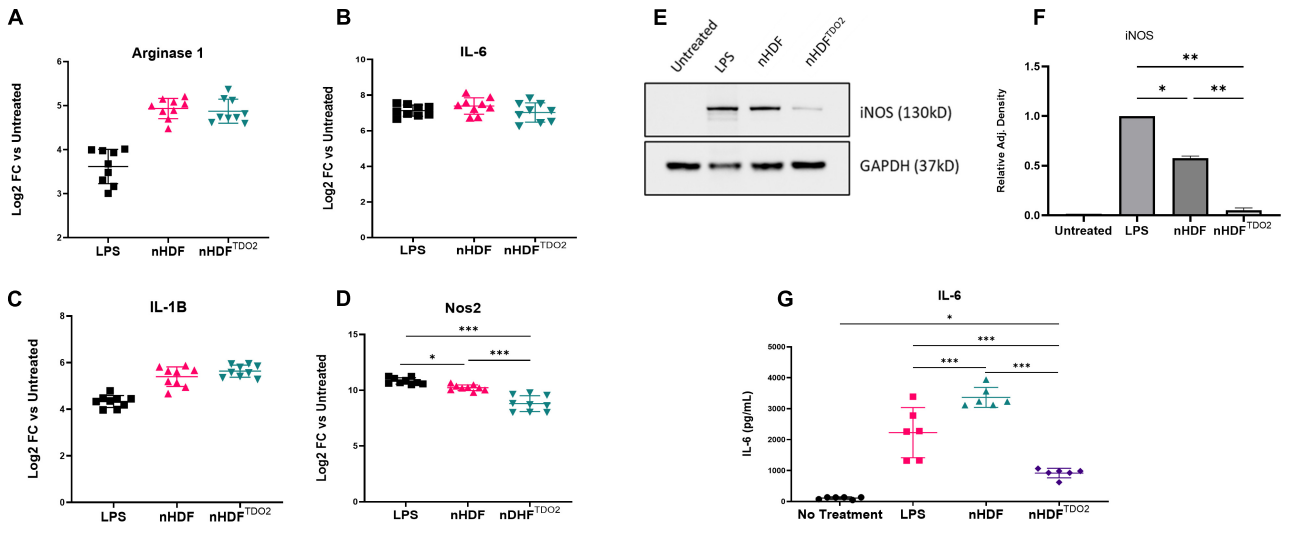

FIGURE 1 | Transwell co-culture of bone marrow-derived macrophages. **(A–D)** Gene expression of inflammatory genes after co-culturing of cells with nHDF or $nHDF^{TDO2}$ cells (n = 3 triplicates from three independent experiments). **(E)** Representative image of western blot detection of iNOS in BMDM lysates after overnight co-culture with nHDF or $nHDF^{TDO2}$ cells and pooled data (n = 2 biological replicates). **(F)** Analysis was done using one-way ANOVA with Sidak's multiple comparison test. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

size and concentration of EVs were similar between the nHDF and nHDF^{TDO2} groups (**Figures 2A–C**). Further characterization was completed demonstrating the presence of conserved EV markers such as HSP70, CD81, and CD63 (**Figure 2D**). The absence of the endoplasmic reticulum (ER) protein Calnexin in both preparations demonstrates equivalent purification during EV concentration (**Figure 2D**). Total RNA and protein content were comparable in both groups (**Figures 2E,F**). Subsequent RNA sequencing of the vesicles revealed a slight enrichment of total small RNAs in the TDO2 group (**Figure 2G**). This increase appeared to be primarily driven by increases in piRNA and tRNA (**Figure 2H**).

EVs From TDO2-Augmented Fibroblasts Attenuate Macrophage Activation

To investigate the function of EVs from nHDF^{TDO2}, BMDMs were treated directly with EVs from each group and assayed for the same markers of macrophage and inflammatory markers described earlier. Significant reductions were observed in the expression of Arg-1, Nos2, IL-1B, and Nos2, and ADAM17 was observed after overnight treatment with EVs (**Figures 3A–D** and **Supplementary Figure 4A**). Additional inflammation markers were investigated but were not observed to have significantly changed expression levels (**Supplementary Figure 4B**). Reduced iNOS levels in BMDM were confirmed by western blot (**Figures 3E,F**). Reductions in IL-6 secretion were observed by ELISA (**Figure 3G**). Both EV groups also stimulated BMDM proliferation equally as shown by BrdU incorporation (**Figure 3H**). The effect of EVs on migration was also evaluated using a modified Bowden's Chamber assay. Interestingly, nHDF^{TDO2}-EVs enhanced macrophage migration compared to other groups (**Figure 3I**). Taken together, these data demonstrate a potent immunomodulatory effect of nHDF^{TDO2} in macrophages. This effect was reflected by inherent changes in the inflammatory profile of the cells rather than impairing proliferation or infiltration. To rule out bioactivity from extra-vesicle proteins, EV preparations were further purified using SEC. SEC purification yielded preparations with nearly 10-fold less protein compared to the ultrafiltration only preparation (**Supplementary Figure 2A**). Particle numbers were equivalent between both groups (**Supplementary Figures 2B,C**). Equivalently to the ultrafiltration product, SEC-purified EVs attenuated Nos2 and IL6 (**Supplementary Figures 2E,F**) which suggests that the immunomodulatory effects on macrophages are mediated by the EVs. Additionally, very few differences were observed when comparing the proteomic composition of nHDF and nHDF^{TDO2} derived EVs (**Supplementary Figure 3**). These data suggest that EV-associated proteins are not responsible for the immunomodulatory effects observed.

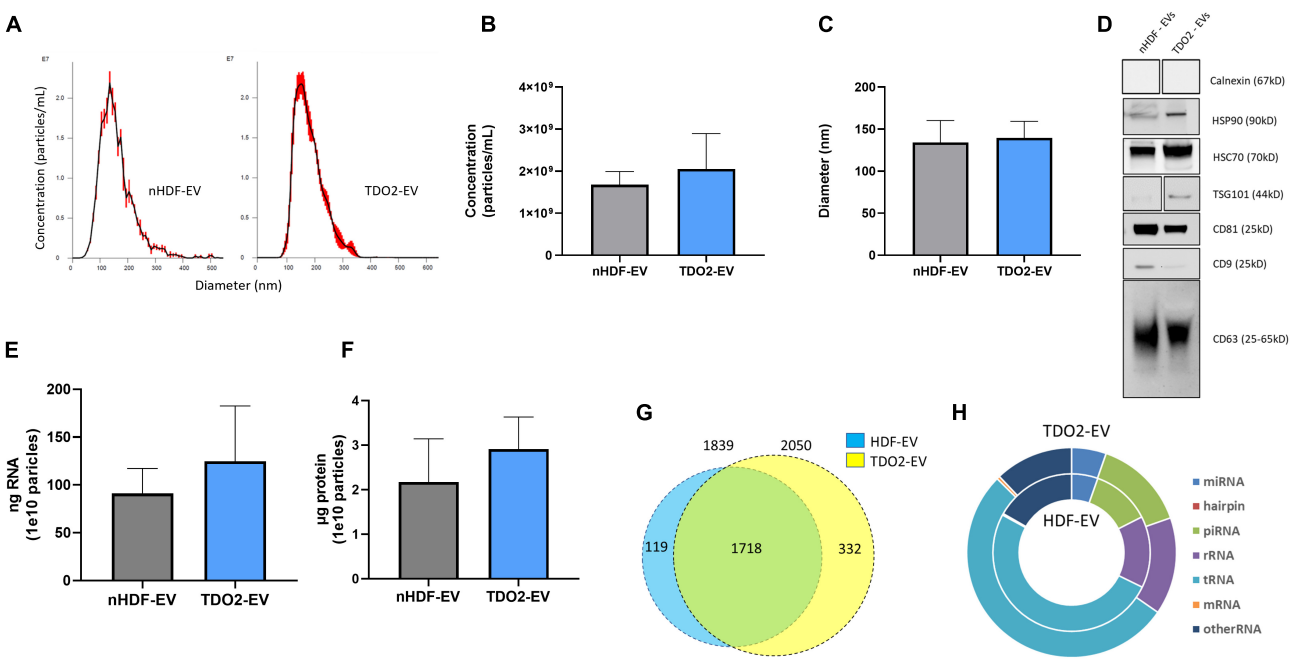

FIGURE 2 | nHDF and nHDF^{TDO2} derived extracellular vesicles share a similar phenotype. **(A)** Nanosight NS300 analysis of 15-day, serum-starved, conditioned medium, from nHDF and nHDF^{TDO2} cultures (n = 3 technical replicates). **(B,C)** Particle concentration and particle diameter as observed by NTA (n = 3 triplicates from three independent experiments). **(D)** Western blot of common EV markers. **(E)** Total RNA was extracted from 1.0e^{10} EVs and quantified using Qubit (n = 4 from two biological replicates). **(F)** EV protein content was analyzed by micro-BCA from 1.0e^{10} particles per sample (n = 4 from two biological replicates). **(G)** miRNA sequencing aligned 2,169 miRNA sequences with annotated miRNA. **(H)** Ratio of RNA species found during sequencing. Sequencing data were derived from three biological replicates from each nHDF and nHDF^{TDO2} derived EV.

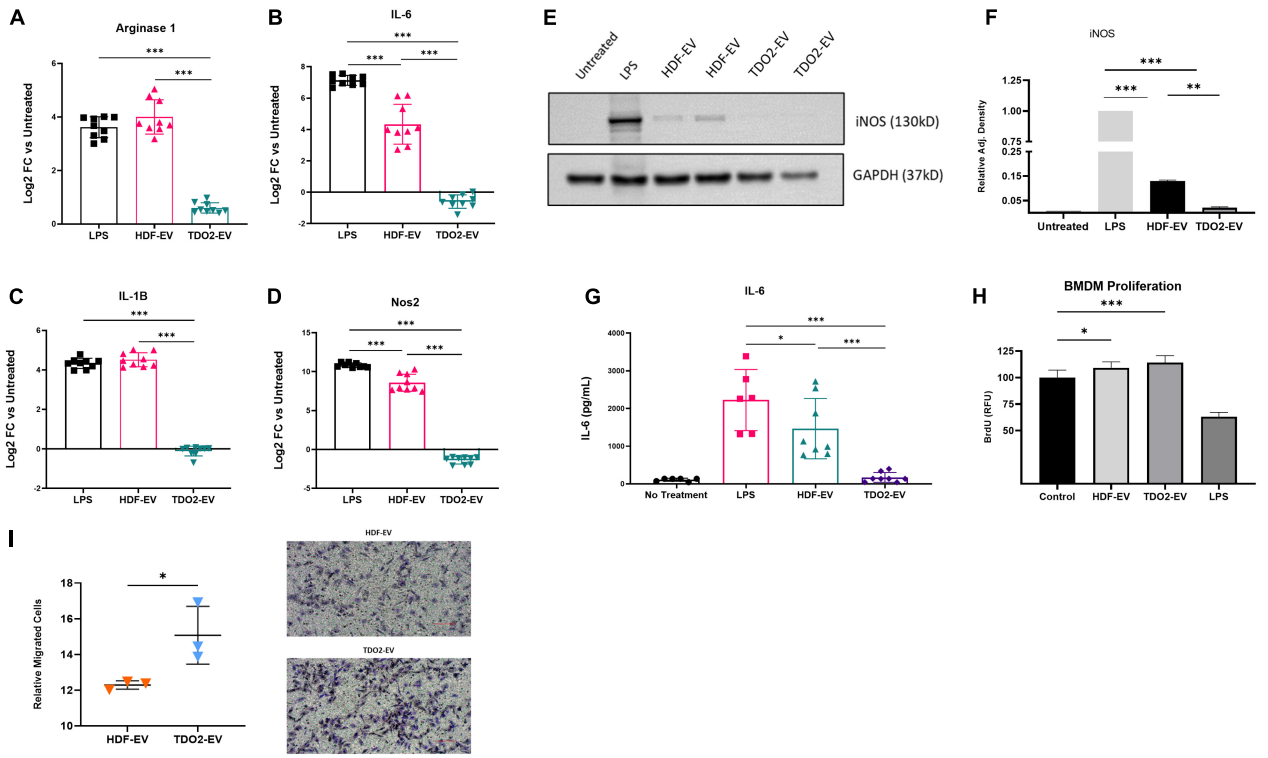

FIGURE 3 | nHDF^{TDO2}-EVs show anti-inflammatory function in bone marrow-derived macrophages. **(A–D)** Gene expression of inflammatory genes in BMDM cells after overnight treatment with nHDF or nHDF^{TDO2} EVs results graphed as log2 fold change versus untreated cells (not shown) (n = 3 triplicates from three independent experiments). **(E)** Representative image of western blot detection of iNOS in BMDM cell lysates (n = 2 from biological replicates). **(F)** Quantification of iNOS western blot images. **(G)** ELISA quantification of IL-6 in the cell culture conditioned medium of BMDMs treated overnight with nHDF-EV or nHDF^{TDO2}-EVs (n = 3 triplicates from three independent experiments). **(H)** Proliferation of BMDM cells analyzed by colorimetric BrdU incorporation assay (n = 8 technical replicates from a single experiment). **(I)** Results of modified Bowden-chamber assay for cell migration (n = 3, 10 technical replicates; images from three independent experiments). **(A–H)** Analysis was done using one-way ANOVA with Sidek's multiple comparisons test. **(I)** Analysis was done using an unpaired, two-tailed, t-test. All error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

EVs From TDO2-Augmented Fibroblasts Induce Macrophage "Anergy" via Suppression of NFκB

To investigate the mechanism by which EVs from nHDF^{TDO2} regulate macrophage activation, we performed transcriptomic analysis on macrophages treated with nHDF^{TDO2}-EVs and nHDF-EVs. Macrophages treated with nHDF-EVs had significant transcriptomic changes including the activation of several pro-inflammatory genes (**Figure 4A**). In contrast, macrophages treated with nHDF^{TDO2}-EVs had a much more muted effect (**Figure 4B**). This is further reflected using principle component analysis where nHDF^{TDO2}-EV-treated groups are more proximal to the untreated group than the nHDF-EV-treated group (**Figure 4C**). Indeed, nHDF^{TDO2}-EVs nearly reverse the inflammatory effects of nHDF-EV treatment (**Figure 4D**). Given this major reversal of inflammatory phenotype, we interrogated nuclear factor-kappa b (NFκB) genes as it is a central regulator of inflammation (Taniguchi and Karin, 2018). Analysis of the NFκB inflammatory complex shows potent abatement compared to the nHDF-EV-treated group and equivalent to the untreated group (**Supplementary Figure 4G**). Direct comparison of canonical inflammatory markers and pathways shows universal upregulation in macrophages treated with nHDF-EVs versus those treated with nHDF^{TDO2}-EVs (**Figures 4E–J**) including decreased expression of all constituents of the NFκB complex (**Figure 4I**). This led us to suspect whether the nHDF^{TDO2}-EVs might be inert and incapable of signaling to macrophages. To test this hypothesis, we performed a sequential exposure experiment whereby macrophages were treated first with nHDF^{TDO2}-EVs followed by exposure to nHDF-EVs. If the nHDF^{TDO2}-EVs are truly inert, then the nHDF-EVs should still induce inflammatory activation. If the nHDF^{TDO2}-EVs induce anergic modulation in macrophages, then macrophage activation by nHDF-EVs will be attenuated. Indeed, pre-treating macrophages with nHDF^{TDO2}-EVs blunted their ability toward inflammatory activation by nHDF-EVs. This is shown by decreased levels of Arg-1, Nos2, and IL-6, and a significant increase in IL-10 expression after treatment with nHDF-EVs (**Supplementary Figures 4C–F**). Taken together, these data demonstrate a unique ability of nHDF^{TDO2}-EVs to induce immunomodulation through inducing anergia in

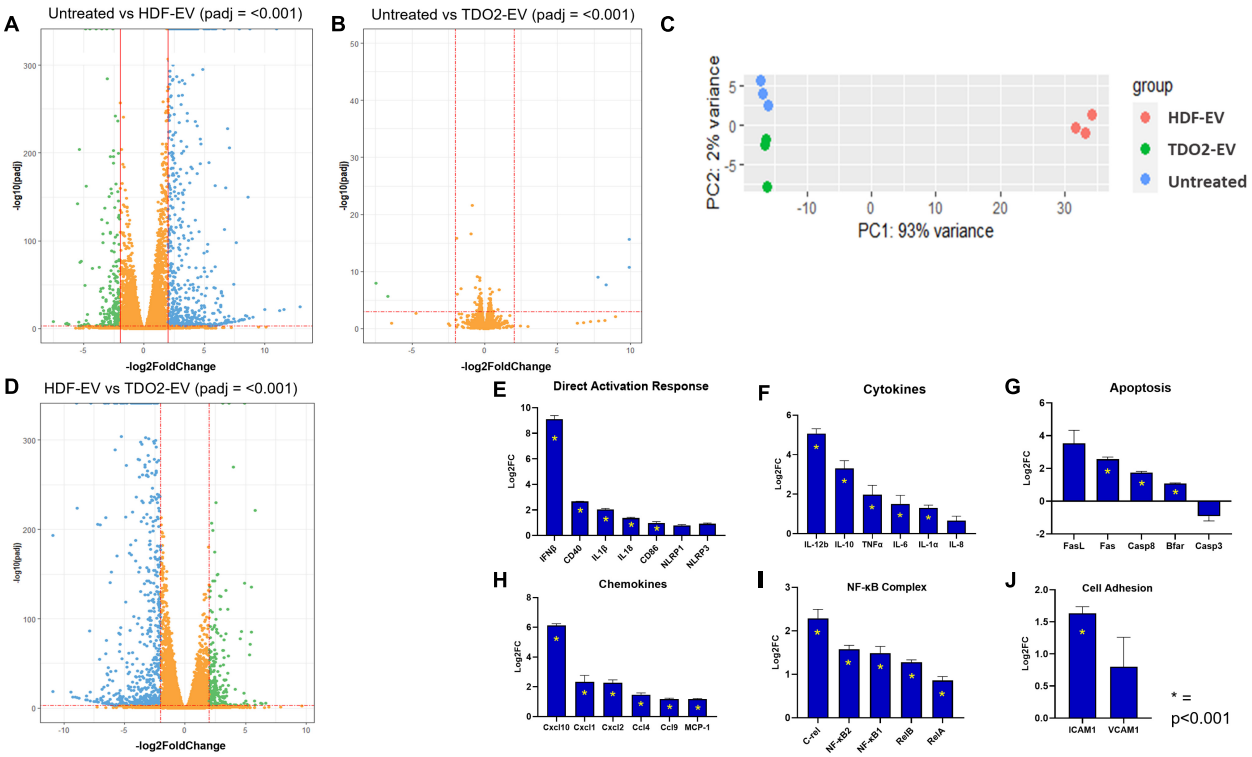

FIGURE 4 | RNA sequencing of EV-treated bone marrow-derived macrophages (BMDMs) reveals activation of inflammatory pathways by nHDF-EV. Differential expression analysis was calculated from $n = 3$ from two biological replicates. **(A)** Volcano plot visualizing the changes in gene expression in BMDM cells after treatment with nHDF-EVs compared to the untreated group. **(B)** Volcano plot visualizing the changes in gene expression in BMDM cells after treatment with nHDF^{TDO2}-EVs compared to the untreated group. **(C)** Principal component plot of the top 300 differentially expressed genes ($p < 0.001$). **(D)** Volcano plot visualizing the changes in gene expression in BMDM cells after treatment with nHDF^{TDO2}-EVs compared to the nHDF-EV treated group. All volcano plots use −log10 adjusted *p*-values and log2 fold-changes. Genes with adjusted *p*-values less than 0.001 and fold-changes greater than twofold are highlighted. **(E–J)** Log2 fold change gene expression changes in inflammatory markers when BMDMs are treated with nHDF-EV vs nHDF^{TDO2}-EV. Log2FC values calculated from FPKM count values, $n = 3$, error bars represent standard deviation, and starred plots indicate $p < 0.001$.

macrophages. This effect is mediated in part through blunting NFκB activation.

TDO2-Augmented Fibroblasts Are Cardioprotective in Acute Myocardial Infarction

Having observed these profound effects in macrophages, we sought to investigate the effect of nHDF^{TDO2} in a cardiac injury model where macrophages play an active role in injury and resolution. We investigated the therapeutic capacity of these cells in a well-established mouse model of acute myocardial infarction used by our group to establish therapeutic potency of cells and EVs (**Figure 5A**). Results showed significant improvement of cardiac function at 21-day post-injury in nHDF^{TDO2}-treated hearts (compared to nHDF-treated hearts; **Figure 5B**). Observation of both B-mode M-mode images reveals an observable difference in left ventricular wall contractility (**Figure 5C**). Changes in end-diastolic and systolic volumes were decreased in the nHDF-TDO2-EV treated hearts, indicating preservation of end-systolic volumes and maintenance of ejection fraction (**Figures 5D,E**).

DISCUSSION

Understanding the mechanism of action of cell therapy is a cornerstone of regenerative medicine. Previous work by us and others suggests that cell therapy functions primarily through the secretion of EVs (Barile et al., 2014; Ibrahim et al., 2014; Hirai et al., 2020) which deliver molecules like small RNAs with salutary effects that modulate the transcriptome of the injured microenvironment, notably macrophages (de Couto et al., 2017, 2019). The further mechanistic investigation implicated the Wnt-β catenin pathway in driving the therapeutic effect of CDCs and their EVs (Ibrahim et al., 2014, 2021). The work presented here represents a continuation of this mechanistic dissection. We show that TDO2 is a major target gene of β catenin activation. Indeed, it was the single highest upregulated gene in fibroblasts with augmented β catenin activation. TDO2 activation in otherwise therapeutically negative cells (skin fibroblasts) resulted in a secretome capable of regulating macrophage inflammatory activation. This effect was independent of kynurenine synthesis and secretion but instead was mediated by changes in the cargo of EVs. EVs isolated from the conditioned medium of TDO2-augmented cells were potently immunomodulatory in macrophages. Deeper transcriptomic analysis of macrophages

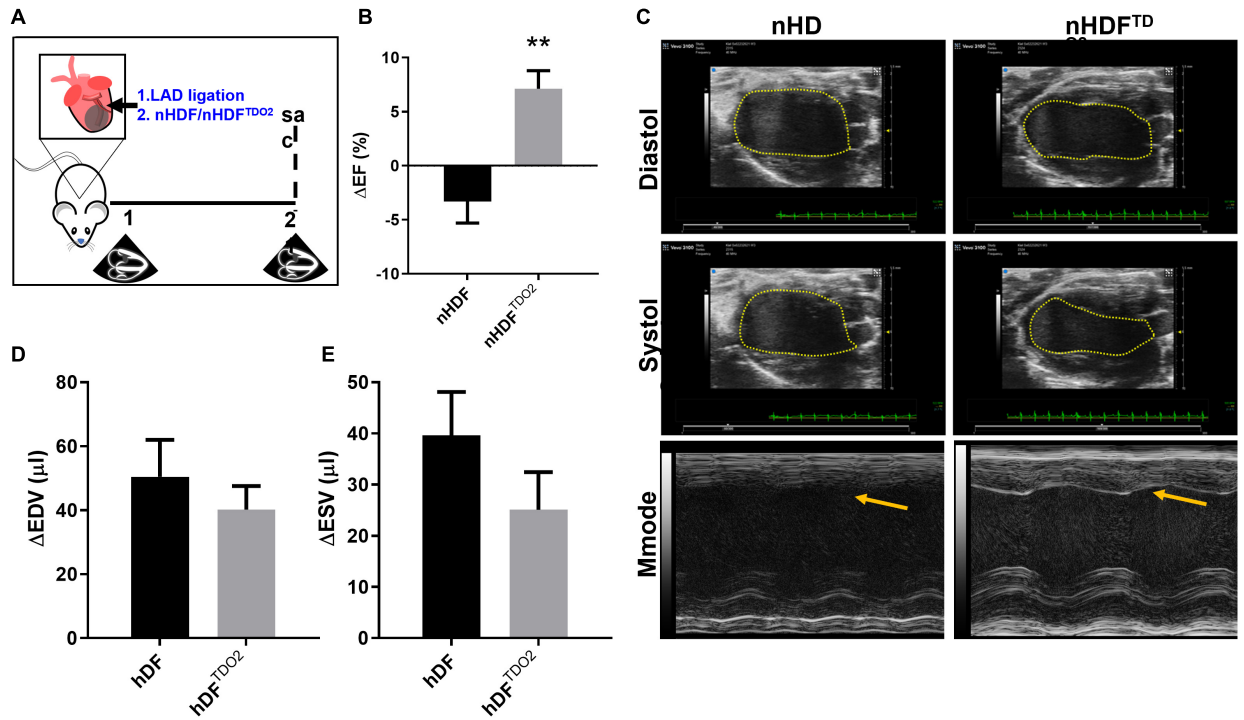

FIGURE 5 | Neonatal human fibroblasts transduced to overexpress TDO2 increase ejection fraction in a 3-week mouse model of MI. **(A)** In an acute model of MI, BL6 mice underwent MI and hearts were injected with nHDF cells (n = 10) and nHDF^{TDO2} cells (n = 8). **(B)** Treatment with nHDF^{TDO2} cells increased ejection fraction in mice 21-day post-infarct. **(C)** Representative B-mode images of systole and diastole illustrating the ventricular-volume tracing method used to measure ejection fraction. M-mode images of left ventricular contraction. Improved contraction in the left ventricular wall after treatment with nHDF^{TDO2} cells is observed by the inward contraction of the left ventricular wall (yellow arrow). **(D)** Average change in left ventricular end-diastolic volumes. **(E)** Average change in left ventricular end-systolic volume. Statistical analysis was done using an unpaired t-test. $^{**}p < 0.01$.

treated with nHDF^{TDO2}-EVs revealed potent silencing of pro-inflammatory properties of nHDF EVs including activation of a master regulator of inflammatory signaling NFκB. This effect was not due to rendering EVs non-reactive but rather an active anti-inflammatory signaling process. Finally, we show that fibroblasts augmented with TDO2 were therapeutically bioactive in a model of cardiac injury. In conclusion, these results shed light on one target gene (TDO2) of β catenin activation and its role in the therapeutic effect. Future work will focus on changes in the cargo of EVs upon TDO2 activation that endow them with immunomodulatory capacity. For instance, it will be important to interrogate changes in the non-coding RNA including micro RNAs (miRs) and their immune-relevant targets. This includes members of the NFκB pathway. This investigation would not be confined to miRs but rather other RNA classes with less described mechanisms that may contribute significantly to the observed effects. Of note are the Piwi-RNAs (piRNAs) which increased in EVs following TDO2 activation. Furthermore, the effect of these EVs on the adaptive immune response was not evaluated here and merits investigation as TDO2 activation has been shown to induce immunological tolerance. Understanding the comprehensive effect of EVs from TDO2-activated cells further advances our growing knowledge of therapeutic signaling by cells and the EVs and more broadly, the relevant mechanisms in tissue healing and repair.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ExoCarta database under the accession number: ExoCarta_313.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee, Cedars-Sinai Medical Center.

AUTHOR CONTRIBUTIONS

KP was the primary contributor, conducted all *in vitro* experiments and bioinformatics, authored the first draft of the manuscript, created all figures, and performed all statistical testing. LL contributed animal surgery support and captured echocardiographs. AC and CL contributed to the method development and study design. AM provided analytical support. EM and AI provided conceptual study design and project oversight. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.733354/full#supplementary-material

Supplementary Figure 1 | Augmented expression of TDO2 has a global effect on the gene expression profile of neonatal human fibroblasts. **(A)** Sequencing of cardiosphere-derived cells (CDCs) treated with BIO revealed TDO2 to be the gene most upregulated. **(B)** TDO2 expression 2 passages after lentiviral transduction and selection by puromycin. **(C)** Secreted kynurenine levels from nHDF versus nHDF^{TDO2} cell cultures as tested by ELISA ($n = 5$). **(D)** Visualization of mRNA sequencing data showing global gene expression in nHDFs before and after transduction. Including significantly ($p \leq 0.001$) differentially expressed genes (direction delineated by colored arrows). **(E)** Heat map visualizing differentially expressed genes ($p < 0.001$) post TDO2 augmentation. **(F)** Volcano plot visualizing the expression of TDO2 in the group of genes upregulated post-transduction. Unpaired, two-tailed *t*-test used for analysis. Error bars represent standard deviation. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Supplementary Figure 2 | Removal of extra-vesicle protein does not change inhibit immunomodulatory function in BMDM. **(A)** Total protein concentration of ultrafiltration and SEC purified EV preparations. **(B)** EV concentrations normalized by volume. **(C)** EVs particle per μg of protein. **(D–F)** Arg1, Nos2, and IL-6 expression in BMDMs treated with different preparations of TDO2 and HDF EVs. Two-group comparisons were analyzed using an unpaired, two-tailed *t*-test. Multiple comparisons were tested by one-way ANOVA with Sidek's multiple comparison test. Error bars indicate standard deviation. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Supplementary Figure 3 | Proteomic analysis of nHDF and nHDF^{TDO2} derived extracellular vesicles. **(A)** Visualization of the ratio of differentially expressed proteins (576) and the number of proteins mapped from each EV type, including unique sequences from nHDF (32) and nHDF^{TDO2} (13). **(B)** Comparison of the ratio of proteins based on their biological processes and molecular function. **(C)** Proteins significantly up- or downregulated in the TDO2-EV population delineated by their biological processes.

Supplementary Figure 4 | Pre-treatment with nDHF^{TDO2}-EVs reduces the inflammatory reaction in BMDM. **(A)** Gene expression of ADAM17 in macrophages after treatment with EVs. **(B)** Expression of genes indicating M1 and M2 polarization. Fold change calculated versus untreated cells. **(C–F)** Gene expression of inflammatory markers after standard treatment with EVs or nHDF^{TDO2}-EV ($n = 3$). **(G)** Heat map illustrating the gene expression of the NFκB inflammatory complex and TLR adapter proteins MyD88 and TRIF after EV treatment versus controls ($n = 3$ triplicates from two independent experiments). Two-group comparisons were analyzed using an unpaired, two-tailed *t*-test. Multiple comparisons were tested by one-way ANOVA with Sidek's multiple comparison test. Error bars indicate standard deviation. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Increasing the Therapeutic Efficacy of Extracellular Vesicles From the Antigen-Specific Antibody and Light Chain Perspective

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Due to their exceptional properties, extracellular vesicles (EVs) receive special attention as next generation biotherapeutics and vehicles for drug delivery. However, despite having many advantages over cell-based therapies, EVs usually exert lower therapeutic efficacy. This results from a number of hurdles that are faced by the EV-based approaches. Administered EVs could be rapidly cleared by the mononuclear phagocytes as well as can randomly distribute within various tissues, making tissue penetration and cell targeting insufficient. However, recent research findings imply that these limitations could be overcome with the use of antigen-specific antibodies and light chains. Major histocompatibility complex (MHC) class II-expressing EVs have been shown to form aggregates after co-incubation with antigen-specific antibodies, which greatly enhanced their biological efficacy. On the other hand, EVs could be coated with antibody light chains of chosen specificity to direct them towards desired target cell population. Both findings open up a promising perspective to achieve the highest efficacy of the EV-based approaches. Herein we discuss the opportunities for enhancing extracellular vesicle's biological activity by using specific antibodies and light chains in the context of the challenges faced by such therapeutic approach.

Keywords: antibody, biotherapeutics, exosomes, extracellular vesicles, treatment efficiency

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INTRODUCTION

Extracellular vesicles (EVs) encompass all classes of lipid-membrane vesicles that differ in the formation pathway but are then released by virtually all cells to their surrounding microenvironment (Yáñez-Mó et al., 2015; Pironti et al., 2021). As a newly discovered mood of intercellular communication, they currently receive special attention as next generation biotherapeutics with likely very limited adverse effects of administration (Kalluri and LeBleu, 2020), and multiple advantages over synthetic liposomes (Vader et al., 2016; Nazimek and Bryniarski, 2020b). Accordingly, EVs, exosomes especially, are considered promising vehicles for drug delivery due to their biocompatibility and exceptional stability in biological fluids (Akuma et al., 2019). In this aspect, however, it should be stressed that EVs themselves are very complex and thus their components should be taken into consideration as additional active drug constituents (Lener et al., 2015). Furthermore, EVs could be used by pathogens for infection spreading, and thus one can speculate that they may transfer virulence factors, which has to be taken into consideration while manipulating EVs for therapeutic applications (Pironti et al., 2021). On the other hand,

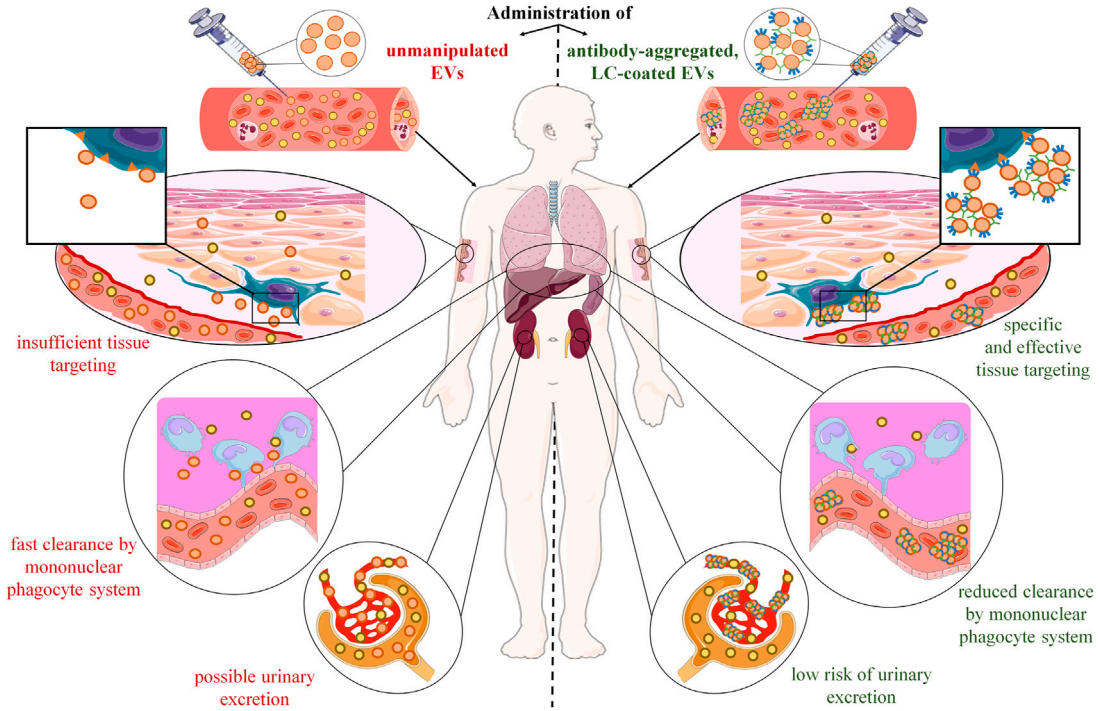

FIGURE 1 | Postulated advantages of extracellular vesicle (EV) aggregation with antigen-specific antibodies and coating with antigen-specific light chains (LC). After systemic administration, therapeutic EVs disperse and mix with their counterparts in the circulation, from which they pass into the tissues. However, their tissue distribution is at least partly random with the preferential accumulation in mononuclear phagocyte-enriched organs, such as liver, spleen, and lungs, where they are rapidly cleared by macrophages. In addition, injected EVs could likely be excreted in urine due to their putative ability to penetrate kidney's glomerular filtration barrier. Altogether, these hurdles make desired tissue penetration and cell targeting insufficient to induce the expected therapeutic effect. However, recent research findings showed that these limitations could be overcome with the use of antigen-specific antibodies and light chains. Incubating EVs with antigen-specific IgG antibodies leads to their aggregation, which enhances their biological efficacy by increasing the amount of EVs that target desired cell population, and by lowering the risk of urinary excretion. In addition, coating EVs with antigen-specific LCs directs them towards target cells, which augments the selectivity of tissue targeting, and limits the unwanted clearance by phagocytes.

attempts to solve the challenges of stem cell therapy allowed to discover that EVs are the main paracrine factors that actually mediate the effects induced by administration of their parental cells (Johnson et al., 2021; Wang et al., 2021). However, despite having many advantages over such approaches, EV-based therapeutics usually achieve lower or almost the same therapeutic efficacy than releasing cells (Kalluri and LeBleu, 2020). Our current research findings suggest that some of these limitations could be overcome by aggregating EVs with antigen-specific antibodies and by increasing the specificity of cell targeting with antibody light chains (LCs). As discussed below, such approaches offer a promising perspective in enhancing EV's therapeutic activity (**Figure 1**).

"POOR IS THE PUPIL WHO DOES NOT SURPASS HIS MASTER"[1]

Recent studies and clinical trials uncovered a number of hurdles that are faced by the cell-based therapies. The widely described obstacles affecting the efficacy of stem cell therapies result mainly from a lack of standardized treatment procedures, low percentage of cells that reach the desired tissue/organ, the poor survival of engrafted cells that often rapidly undergo apoptosis in targeted tissues, and finally from the diversity of yielded cell populations between individual donors (Hassanzadeh et al., 2021; Johnson et al., 2021; Wang et al., 2021). In addition, stem cell therapies raise concerns about possible adverse events of the treatment that are associated with the risk of tumorigenesis, the possibility of disrupted or abnormal maturation of infused cells as well as with their eventual differentiation in undesirable tissue (Lukomska et al., 2019). On the other hand, the main obstacle related to immunotherapy with dendritic cells results from the risk of their phenotype switching under the influence of the tissue microenvironment, especially at the site of tumorigenesis (Gardner et al., 2020). The risk of losing the desired phenotype may also apply to bone marrow-derived macrophages (Cao et al., 2014), and likely other types of therapeutically administered living cells. Finally, cytokine release syndrome is a major severe complication of the very promising anti-tumor therapy with chimeric antigen receptor (CAR)-T cells that results from the uncontrolled activation of

[1]Leonardo Da Vinci (quote translated by Jean Paul Richter)

recipient cells by transferred CAR-T lymphocytes (Cosenza et al., 2021).

Abovementioned obstacles and concerns prompted researchers to search for safer alternatives that could efficiently replace the living cells for therapeutic purposes. Consequently, EVs released by these cells become considered main promising candidates for cell-free therapies (Tang et al., 2015; Johnson et al., 2021; Wang et al., 2021; Yao et al., 2021).

Along these lines, unlike releasing cell, EVs do not contain all components of cellular machinery required for proper functioning of the living cell, but still they can substitute for its activity. The latter is likely possible due to the contained cargo, which enables EVs to perform a specific function. This assumption is supported by the fact that the cargo is sorted into EVs, exosomes especially, in a very sophisticated manner, and represents a unique content of proteins, RNAs, and lipids, usually differing from the parental cell (Wei et al., 2021). Thus, stem cell-derived EVs cannot differentiate but can promote immune tolerance (Wang et al., 2021), and tissue regeneration (Johnson et al., 2021) instead of tumorigenesis (Lukomska et al., 2019), since they do not carry full genomic DNA material. Moreover, EVs can carry and present antigens as do dendritic cells, but they are unable to switch their phenotype (Yao et al., 2021). Finally, EVs can induce antigen-targeted cytotoxicity against cancer cells without the risk of cytokine release syndrome development (Tang et al., 2015).

It is worth noting that, although EV-based strategies also do not have standardized protocols yet, other concerns and hurdles are either not relevant or could be easily avoided, since EVs are much more manipulable than their parental cells. The latter EV feature together with still expanding knowledge about their biology and functions opened up a new research area attempting both to improve the properties of cell-derived EVs and to design and manufacture their artificial counterparts (García-Manrique et al., 2018) in order to design the most appropriate therapeutic modality, for example in personalized medicine. Such possibilities support the consideration of either manipulated or engineered EVs as next generation biotherapeutics with almost unlimited therapeutic activities and indications that would be easy to produce, handle, and distribute (Johnson et al., 2021). Obviously, as discussed below, selecting the best cellular source of EVs for each considered application is the first crucial step on the way to personalize the EV-based therapeutics (Campanella et al., 2019; Logozzi et al., 2021a). However, before it will happen, researchers have to learn how to improve EVs to surpass both their current therapeutic efficacy and parental cell activities.

CHALLENGES IN THERAPEUTIC APPLICATION OF EXTRACELLULAR VESICLES

Among other concerns, EV-based approaches avoid the risk of gene mutation, uncontrolled cell division and differentiation as well as immune rejection (Hassanzadeh et al., 2021). Therefore, EVs are considered safer, more controllable as well as much less immunoreactive and immunogenic than the therapeutically administered cells (Buschmann et al., 2021). Other undoubted advantages over the cell-based therapies result from EV's high stability and resistance to harsh conditions *in vivo,* biocompatibility, ability to cross physiological barriers as well as from their rapid uptake into tissues (Johnson et al., 2021). These features entail a significant increase in interest in the possibility of their therapeutic application, which is still fraught with many challenges (Claridge et al., 2021).

At present, the challenges of standardizing the protocols of EV's generation, characterization, loading with a cargo, dosing and administration are brought to the fore to meet good manufacturing practice requirements (Lener et al., 2015; Buschmann et al., 2021; Thakur et al., 2021). Obviously, choosing the right EV source (i.e., either proper releasing cell subtype or biological fluid), the best loading strategy and the optimal dose administered via the most efficient route would produce the highest efficacy of treatment. But very likely these choices would vary across the specific clinical indications or even patients. The current state-of-the-art technologies and methodologies have been widely summarized elsewhere (Akuma et al., 2019; Doyle and Wang, 2019; Li et al., 2019; Gowen et al., 2020; Klyachko et al., 2020; Gupta et al., 2021; Johnson et al., 2021). However, the use of EV-based therapeutics requires still a very flexible approach for optimization, as it is built on the basis of constantly growing knowledge and clinical evidences.

Although the stem cell therapy achieved promising efficacy with minimal number of reported adverse events in patients with immune-related and inflammatory diseases, stem cell-derived EVs have recently emerged as the easier-to-use treatment modality (Wang et al., 2021) with no risk of stem cell-related coagulopathy (Askenase, 2020) and occlusions (Zhang et al., 2014).

On the other hand, despite promising results in basic and preclinical studies, EVs showed lower than expected therapeutic activity in some cancer clinical trials. Along these lines, dendritic cell-derived EVs demonstrated low clinical efficacy as maintenance immunotherapy in patients bearing inoperable but not progressive non-small cell lung cancer (Besse et al., 2016). This could be due to the fact that EVs were collected from immature dendritic cells and then administered to patients with advanced cancer that likely had impaired immunoreactivity (Yao et al., 2021). This clearly shows that the immune status of patients in target group has to be taken into account while designing appropriate EV-based drug or vaccine.

Another problem is related to pharmacodynamics and pharmacokinetics of therapeutically administered EVs, insufficient tissue penetration and cell targeting especially. Among other possible causes, these limitations could be due to both the dispersion of EVs in biological fluids, and their rapid clearance from circulation by the mononuclear phagocyte system (**Figure 1**).

Accordingly, after systemic administration, EVs can disperse fast in lymph and blood plasma, where they mix with their counterparts from other cellular sources (Nazimek et al., 2016) and form colloid suspension. Interestingly, EV's colloidal stability in biological fluids seems to be affected by their natural tendency

to aggregate (Hood et al., 2014). It is worth noting that EV's aggregation could be enhanced by electroporation (Hood et al., 2014), which may impede their further processing for therapeutic application. However, one can speculate that formation of such "colloid aggregates" may increase EV's half-life in circulation by dampening their random passage to tissues. In addition, aggregated EVs would be less likely to overcome the glomerular filtration barrier and become excreted in urine (Erdbrügger et al., 2021). Moreover, it can be hypothesized that EV's aggregation together with directing them towards desired target tissue or cell population will greatly enhance their therapeutic efficacy. As discussed below, our results showed that this could be achieved by incubating EVs with antigen-specific antibodies (Nazimek et al., 2021) (**Figure 1**).

On the other hand, injected EVs were found to be rapidly cleared by macrophages of the mononuclear phagocyte system. Therefore, they preferentially accumulate in mononuclear phagocyte-enriched organs, such as liver, spleen, and lungs (Chen et al., 2021). Furthermore, resident macrophages are considered to clear EVs also in other tissues. For instance, microglia can phagocytose and thus remove EVs from the brain parenchyma (Schnatz et al., 2021). Thus, fast clearance by macrophages not only hampers EV's delivery to desired tissue and target cell population, but also may either affect the expected therapeutic action or cause some side effects. The latter could be observed if EV's are not cleared in an immunologically silent process but instead when it leads to macrophage activation. Such activation most likely depends on the EV-contained cargo and the state of the donor cell, as discussed recently in the case of microglia (Schnatz et al., 2021). Additionally, one can speculate that macrophages in inflamed tissue are possibly more prone to undergo unexpected activation after EV's engulfment. This seems to particularly apply to the EV-based therapeutics targeting the cells at the site of tissue injury or ongoing inflammation. However, the unwanted clearance could likely be limited by decorating therapeutic EVs with molecules providing "don't eat me" signals (Belhadj et al., 2020), especially in a combination with strategy increasing the specificity and selectivity of cell targeting. And, following the commonly known slogan "simplicity is the ultimate sophistication," antigen-specific antibodies and their derived LCs appear most promising candidates for the latter strategy (**Figure 1**).

ANTIGEN-SPECIFIC ANTIBODIES AND LIGHT CHAINS IN THE FIGHT TO ENHANCE EXTRACELLULAR VESICLES THERAPEUTIC EFFICACY

In *in vivo* conditions, EVs gain most of their attributes at the time of the release by parental cell. However, some of them can be acquired later in the extracellular space. Accordingly, recent study demonstrated the spontaneous formation of a protein corona around EVs in blood plasma (Tóth et al., 2021). Very likely the acquired proteins impact EV's properties and activity, which requires further investigation (Tóth et al., 2021). Similarly, our observations suggested the ability of EVs to bind freely circulating miRNAs (Bryniarski et al., 2015). This could likely be applied in small interfering RNA (siRNA) delivery, as can recently developed EV-liposome hybrid nanoparticles (Evers et al., 2021). These findings also imply that EVs may constitute physiological nanocarriers for removing other molecules from the circulation. Conversely, proteins adhered to EV membrane may redirect the vesicles towards other target.

Along these lines, we found that mouse suppressor T (Ts) cell-derived EVs are coated with B1a cell-secreted antibody LCs (Bryniarski et al., 2013; Wąsik et al., 2019; Nazimek et al., 2020). Detailed investigation of this surprising observation allowed us to discover that LC coating ensures the antigen-specificity of Ts cell-released EV's action (Nazimek et al., 2018). Furthermore, coating EVs *in vitro* with LCs of chosen specificity enables the antigen-specific suppression of immune response (Bryniarski et al., 2013; Nazimek et al., 2018, 2020) (**Figure 2**). Finally, self-tolerant EVs become capable of suppressing hapten-induced contact hypersensitivity after co-incubation with hapten-specific LCs (Nazimek et al., 2019).

We proposed that LCs stick to EV membrane lipids and form multiple adjacent arrangements of high avidity (Nazimek et al., 2016), sufficient for binding to antigenic peptides presented by antigen-presenting cells (APCs) (Nazimek et al., 2020). These findings imply the important role of LCs in specific cell targeting by EVs (Nazimek and Bryniarski, 2020a), thereby increasing their therapeutic potential (**Figure 2**). Especially that LCs could be coated onto EVs by simple co-incubation, which is a technically easy approach. As mentioned above, protein corona can be acquired by EVs during similar co-incubation with plasma samples (Tóth et al., 2021). Thus, one can assume that the ability to become coated with proteins, including antigen-specific antibodies and LCs, is a general property of EVs that could be widely used for manipulating them for therapeutic purposes. It is also worth noting that the delivery of therapeutic antibodies by EVs can increase their stability and bioactivity, as recently shown in the case of immunoglobulin-bearing EVs produced by OKT3 hybridoma (Logozzi et al., 2021b).

Accordingly, considering the immune checkpoint therapy, one can assume that it could be reinforced by co-administration of EV-based therapeutics. In this case, decorating therapeutic EVs with antibodies specific for programmed death receptor-1 ligand (PD-L1) would direct them towards PD-L1-expressing tumor cells, while anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies would support regulatory T cell targeting (Nazimek and Bryniarski, 2020b). Hence, such combined strategy would allow to block immune checkpoints and induce EV-mediated therapeutic effect at the same time, as recently reported (Fan et al., 2021). However, the possibility that therapeutic EVs decorated with anti-PD-L1 antibodies could be bound by PD-L1-expressing tumor EVs to hamper tumor cell targeting cannot be excluded as well, by analogy to the therapies with immune checkpoint inhibitors (Nazimek and Bryniarski, 2020b). Especially that tumor cells are known to potently release EVs into circulation (Logozzi et al., 2021c).

On the other hand, almost 20 years ago it was observed that cross-linking of major histocompatibility complex (MHC) class

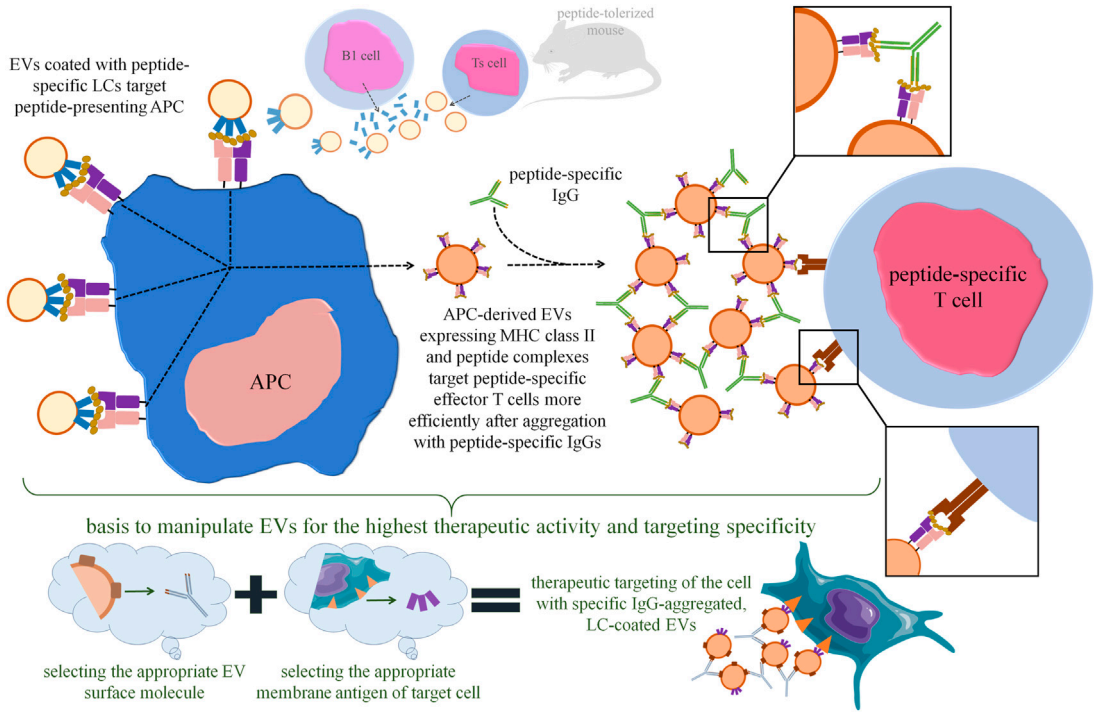

FIGURE 2 | Perspectives in increasing the therapeutic efficacy of extracellular vesicles (EVs) with the use of antigen-specific antibodies and light chains (LCs). Our research findings demonstrated that tolerizing mice with antigen-coupled syngeneic erythrocytes activates suppressor T (Ts) cells to release EVs containing immune regulatory miRNA-150. These EVs are then coated with B1 lymphocyte-secreted, antigen-specific LCs, which allows EVs to specifically bind the antigenic peptide presented by antigen-presenting cell (APC). Importantly, Ts cell-derived EVs could also be *in vitro* coated with LCs of chosen specificity by simple co-incubation. The Ts-cell-EV-targeted APC releases its own EVs expressing major histocompatibility complex (MHC) class II and antigenic peptides that target peptide-specific effector T cells by interacting with T-cell receptor (TCR). Incubation of APC-derived, MHC-expressing EVs with peptide-specific IgG antibodies leads to vesicle aggregation, which greatly enhances their immune suppressive activity by increasing the amount of EVs targeting each effector T cell. Based on these observations, one can assume that therapeutic EVs in general could be manipulated to achieve the highest activity by aggregating them with IgG specific for the selected EV surface molecule, and then by selectively directing them towards target cell with LCs specific for the selected cell surface antigen.

II-expressing EVs with latex beads greatly augments their biological effectiveness (Vincent-Schneider et al., 2002). Similar enhancement was recently demonstrated by us after aggregating MHC class II-expressing EVs with antigen-specific antibodies (Nazimek et al., 2021). These findings imply that EV's aggregation may increase not only their half-life in circulation, as mentioned above, but also the amount of vesicles targeting desired cell population (**Figures 1**, **2**). The latter fits in with the consideration of the optimal EV's dosing at a single cell scale and at the body scale (Gupta et al., 2021). Proposed by us EV's aggregation with antigen-specific antibodies takes an advantage over the latex beads, since antibodies could be safely degraded intracellularly, could limit the unwanted clearance of EVs by phagocytes, and may increase the specificity of cell targeting (Nazimek et al., 2021). Besides, one can assume that EV's could be similarly aggregated with antibodies directed against their surface proteins, which greatly extends the application spectrum of this phenomenon (**Figure 2**). However, chosen surface molecules bound by aggregating antibodies cannot be involved in EV's cellular uptake to not disturb this process. The latter is implied by the observation that the attempts to use anti-CD9 antibodies abolished the immune activity of EVs (Nazimek et al., 2021).

Additionally, some other aspects of this phenomenon require further investigation. Technically, EV's aggregation could be controlled *in vitro* with transmission electron microscopy and nanoparticle tracking analysis after standardizing and optimizing the protocols of obtaining the aggregates. Whereas the methods to track the stability and bioactivity of EV's aggregates in *in vivo* conditions have yet to be developed. Moreover, since aggregation was firstly proved for MHC class II-expressing EVs, the possible MHC-restriction of their activity has to be elucidated with regard to the possibilities to use EVs from allogeneic donors. On the other hand, one can speculate that autologous dendritic cell-derived EVs loaded *in vitro* with dedicated peptides in a direct or indirect manner (Morse et al., 2005) could be considered optimal for aggregation to greatly enhance their vaccine-like activity for instance in cancer immunotherapy (Yao et al., 2021).

CONCLUSION

Last 2 decades have resulted in tremendous advances in the knowledge of the exceptional functions of EVs, which made them promising candidates for various therapeutic applications. Numerous current research findings have

provided the basis for attempting to address the challenges faced by such therapies. Herein discussed possibilities to enhance EV's biological activity and specifically direct them towards desired cells with the use of antibodies and LCs constitute crucial steps forward to achieve the highest efficacy of EV-based therapeutics.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Microglia Polarization: A Novel Target of Exosome for Stroke Treatment

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The vast majority of cells in the human body are capable of secreting exosomes. Exosomes have become an important vehicle for signaling between cells. Exosomes secreted by different cells have some of the structural and functional properties of that cell and thus have different regulatory functions. A large number of recent experimental studies have shown that exosomes from different sources have different regulatory effects on stroke, and the mechanisms still need to be elucidated. Microglia are core members of central intrinsic immune regulatory cells, which play an important regulatory role in the pathogenesis and progression of stroke. M1 microglia cause neuroinflammation and induce neurotoxic effects, while M2 microglia inhibit neuroinflammation and promote neurogenesis, thus exerting a series of neuroprotective effects. It was found that there is a close link between exosomes and microglia polarization, and that exosome inclusions such as microRNAs play a regulatory role in the M1/M2 polarization of microglia. This research reviews the role of exosomes in the regulation of microglia polarization and reveals their potential value in stroke treatment.

Keywords: exosomes, microglia polarization, stroke treatment, microRNA, neuroinflammation, neuroprotection

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INTRODUCTION

According to a report published by the World Health Organization in 2020, stroke is the second leading cause of death worldwide. The lethal and disabling nature of stroke greatly increases the burden on society and individual families. The main treatments are intravenous thrombolysis and mechanical thrombectomy. Both of therapeutic strategies are limited by the recommended treatment time window and the effect is still not ideal (Thiebaut et al., 2018; Ozaki et al., 2019). In order to achieve better treatment, research on new therapeutic targets is necessary.

Ischemic stroke is the main type of stroke pathogenesis, mainly due to impaired blood supply to the brain, which occurs followed by a series of ischemia-reperfusion injuries, such as inflammation and oxidative stress, while persistent neuroinflammation damages neurons and the blood-brain barrier, which in turn leads to disease progression (Zhuang et al., 2017; Xu et al., 2018; Azedi et al., 2019). In the pathological process of stroke, injury are mainly induced by ischemia and/or ischemia-reperfusion, and microglia play an important role in this process. When microglia are activated, they transform into two main M1 and M2 phenotypes. M1 is the pro-inflammatory type, which secretes substances such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor (TNF) that promote inflammation and neurotoxic substances that aggravate brain damage (Shu et al., 2016; Zhu et al., 2019). M2 phenotype is anti-inflammatory, which secretes anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10), transforming growth factor-β (TGF-β) and some neurotrophic

factors, which facilitate brain function recovery and improve the prognosis of stroke (Hu et al., 2012; Liu et al., 2016; Shu et al., 2016).

Exosomes are involved in cell-to-cell regulation through their various inclusions (Braccioli et al., 2014). It not only regulates normal physiological processes, but also assumes an important role in the development of some diseases such as cancer (Pan and Johnstone, 1983). According to recent studies, it was found that exosome transport of miR-124-3p, a substance that promotes M2 microglial polarization, reduced brain damage and improved the outcome of stroke (Huang et al., 2018). This study will summarize the mechanisms of communications between microglia and stroke and elucidate roles of different sources of exosomes in regulating stroke by targeting microglia polarization, thus providing new therapeutic strategies for stroke.

EXOSOME

Almost all prokaryotes and eukaryotes can release extracellular vesicles (EVs) for communication with other cells (Kalluri and LeBleu, 2020). The classification of EVs is constantly improving. Currently, the EVs are mainly divided into two categories: ectosome and exosome (Cocucci and Meldolesi, 2015; Théry et al., 2018). Exosome is a special type of secretory vesicle with small volume in EVs, which is wrapped with a phospholipid bilayer outside. And its diameter is about 50 nm to 1 um (Kalluri and LeBleu, 2020). Exosomes contain proteins, lipids, deoxyribonucleic acid, messenger RNA (mRNA), microRNA and so on (Braccioli et al., 2014). Exosomes emerge from the plasma membrane and endosomal membrane, diffuse into the intercellular fluid, fuse with the recipient cells, release their contents, and exert regulatory effects (Mittelbrunn and Sánchez-Madrid, 2012). they can be produced from a variety of cells such as reticulocytes, antigen-presenting cells, tumor cells, skeletal cells, etc., and the exosomes secreted by different cells have different biological effects and different intensity of effects (Harding et al., 1983; Anderson et al., 2005; Braccioli et al., 2014) (Fordjour et al., 2019). In terms of morphological structure, the exosomes have phosphatidylethanolamine (PE) and phosphatidylserine (PS) on the bilayer membrane, which is a distinctive feature of the exosome plasma membrane and also makes the exosomes have a high membrane curvature, which is conducive to maintaining the stability of the exosome membrane (Booth et al., 2006).

Exosome-mediated cell-to-cell interaction involves many physiological processes such as reproduction and development of mammals, immune response, metabolism, and many pathological changes such as neurodegenerative diseases, cardiovascular diseases and tumorigenesis (Kalluri and LeBleu, 2020). Exosomes play an important role in the physiological activities of cells, e.g., during fertilization, Juno receptors on the ovum are shed as exosomes, thus preventing other sperm from fertilizing (Bianchi et al., 2014). Exosomes, which originate from the embryonic trophectoderm, can transmit their characteristic high resistance to viral infection to other cells (Delorme-Axford et al., 2013). Exosomes, with their PS-rich outer membrane, play an important role in the osteogenesis process, and osteoblasts secrete exosomes to initiate the process of mineralization *in vivo* (Anderson et al., 2005). They also have an important regulatory role in the pathological processes of cells. In tumor cells, the exosome secretion pathway is held hostage by tumor cells to accomplish a variety of pathological activities, and exosomes contain substances that stimulate tumor proliferation (Skog et al., 2008). The exosomes contain substances that stimulate tumor proliferation. Metastatic melanoma cells suppress cellular immunity by releasing exosomes with Programmed Cell Death-Ligand 1 (PD-L1) on their surface and upregulating the intracellular PD-L1 concentration in target cells (Chen et al., 2018). HIV virus can also fuse with normal cells in the form of exosomes, thus infecting normal cells (Melikyan, 2014) (Pegtel et al., 2010). In summary, exosomes are used as a means to protect the normal cells. In conclusion, exosomes play an important role in the mutual regulation of cells as messengers of intercellular signaling.

Exosomes have an important role in the development of many diseases, especially neurological diseases. mRNA mutants and miRNAs characteristic of glioma can be detected in serum exosomes of glioblastoma patients, which may become a new target for the diagnosis and treatment of this disease (Skog et al., 2008). Prions are essentially a misfolded form of protein, and their spread *in vivo* is mainly in the form of exosomes (Fevrier et al., 2004). A large number of recent studies have shown the applicability of exosomes for the treatment of stroke. One reason is that exosomes can easily cross the blood-brain barrier and have the potential to be used as a vehicle for brain-targeted modulation or drug administration (Rufino-Ramos et al., 2017; Khan et al., 2021). Second, exosomes were found to transport miRNAs such as miR-124-3p, which can promote microglia polarization toward the anti-inflammatory M2 phenotype, thereby inhibiting neuroinflammation (Huang et al., 2018). These suggest that exosomes may become important therapeutic targets in the stroke.

In view of the heterogeneity of exosomes in source, content, function and size, exosomes have a good prospect in the diagnosis and treatment of different clinical diseases. The research shows that exosomes may become diagnostic markers of many diseases, including cardiovascular diseases (Zhang et al., 2017a; Jansen and Li, 2017), cancer (Fitts et al., 2019), liver-related diseases (Masyuk et al., 2013), central nervous system diseases and so on (Kanninen et al., 2016). In the mouse model, compared with the use of liposomes, exosomes are able to enter cells more effectively with less immune clearance *in vivo* (Ferguson and Nguyen, 2016; Barile and Vassalli, 2017; Liao et al., 2019). Exosomes play an important role in cardiovascular function regulation and cardiovascular and cancer therapy (Li et al., 2021a; Lim, 2021; Nasser et al., 2021; Robson, 2021). With the development of technology, artificial exosomes have been used to deliver various nanomedicines (Li et al., 2021b). Therefore, the clinical transformation of exosomes in the

diagnosis and treatment of different diseases deserves further research.

EXOSOME AND MICROGLIA

Overview of Microglia

Microglia are resident cells of the Central nervous system (CNS) and belong to the monocyte macrophage system, but microglia, unlike other individuals in the monocyte macrophage system, develop initially from c-KitloCD41lo progenitor cells produced in the yolk sac (YS) around embryonic day 7.25 (E7.25), well before the appearance of other glial cells (Ginhoux et al., 2010). Microglia exhibit different states and have different functions during developmental and adult stages. During development, microglia are "phagocytic" and mobile amoeba-like, reflecting the phagocytic role of microglia in removing dead cells and remodeling neural tissue (Frost and Schafer, 2016; Matcovitch-Natan et al., 2016; Hagemeyer et al., 2017). Microglia have multiple effects on synapses, removing non-functional synapses and remodeling synaptic circuits, as demonstrated by Paolicell et al., 2011, in which C-X3-C motif chemokine receptor 1 (CX3CR) 1-deficient animals exhibit a defective number of microglia and transient defects in synaptic connections during development (Paolicelli et al., 2011; Wake et al., 2013). Microglia at the adult stage appear to be quiescent, but in fact they are constantly scanning and monitoring their surroundings with their characteristic branches and interacting with neighboring cells. Their ability to clear cells is also very strong and does not even require activation (Hambardzumyan et al., 2016).

In the case of stroke, the activated microglia will promote neuroinflammation and thus further brain tissue damage (Jiang et al., 2020). Conversely, induction of M2 microglia polarization facilitates stroke recovery. Multiple pathways can induce different microglia polarization. Among transcription factors, Nuclear factor kappa B (NF-κB), signal transducer and activator of transcription (STAT) family members, and thrombospondin A2R receptor can promote microglial cell conversion to M1 type. Yang et al. used safranin which reduces the expression of M1 markers, inhibits microglial cell conversion to M1 type by reducing IκBα phosphorylation and NF-κB/p65 nuclear translocation (Yang et al., 2019a). Elena Butturini et al. showed that signal transducer and activator of transcription 1 (STAT1) gene silencing can counteract the hypoxic-M1 microglia phenotype, thus suggesting that STAT1 can promote microglia polarization to the M1 phenotype (Butturini et al., 2019).

In ischemia/reperfusion mice, increased Thromboxane A2 receptor (TXA2R) expression was detected in microglia/macrophages by double staining with immunofluorescence, and the TXA2R antagonist SQ29548 inhibited M1 microglia activation and subsequent inflammatory response (Yan et al., 2016). The transcription factors nuclear factor erythroid 2-related factor 2 (Nrf2) and translocation of proliferator-activated receptor gamma (PPARγ) promote microglial cell transition to the M2 phenotype (Jiang et al., 2020). It has been shown that l-F001, a novel multifunctional rho-related protein kinase inhibitor, can increase the expression level of the M2 microglia marker CD206 by activating the Nrf2 signaling pathway *in vitro* (Chen et al., 2017). A study by Liu et al. found that 10-o- (n,n-dimethylaminoethyl) -ginkgolide B methanesulfonate, a new derivative of ginkgolide B, could convert polarized BV2 microglia from M1 phenotype to M2 phenotype by promoting the translocation of PPARγ from the nucleus to the cytoplasm (Liu et al., 2018). Multiple ways have been found capable of inducing microglia polarization. At the same time, it has been found that some substances can convert polarized M1 microglia to the M2 phenotype. This suggests that modulating the conversion of microglia to M2 phenotype may be a practicable therapeutic strategy for stroke.

Exosome-Derived miRNA and Microglia Polarization

Recent studies have shown that different miRNAs transported by exosomes contribute to the polarization of microglia into different phenotypes. miRNAs are endogenous hairpin-loop structured non-coding RNAs, which mainly bind to mRNAs to block their translation and regulate gene expression. Exosomes containing miR-192-5p, miR-183, miR-378, miR-140-3p, miR-222 were found to inhibit the expression of TNF or/and IL-1β expression, thereby promoting microglia M2 polarization (Das et al., 2014; Ti et al., 2015; Eirin et al., 2017; Thomi et al., 2019; Bian et al., 2020). NF-κB is an important signaling hub that drives microglia M1 polarization (Taetzsch et al., 2015). It has been reported that a variety of miRNAs contained in exosomes can regulate the expression of microglia Toll-like receptors (TLRs), thus acting on NF-κB to regulate microglia polarization (Hajinejad and Sahab-Negah, 2021). miR-223, miR-101-3p, miR-26a -5p, miR-326, miR-182, miR-17-5p, miR-140-5p, miR-9, miR-let7, and miR-181c play a role in inhibiting M1 microglia polarization by downregulating TLR expression including TLR2 and TLR4 (Kumar et al., 2015; Wang et al., 2015; Li et al., 2016; Li et al., 2017; Qian et al., 2017; Li et al., 2019; Li et al., 2020a; Yang et al., 2020a; Chaurasiya et al., 2020; Huang et al., 2020). In addition, miR-26b-5p inhibited the TLR signaling pathway by regulating the reduction of CH25H protein expression, which in turn inhibited microglia M1 polarization (Li et al., 2020b).

In addition to regulating TLR expression, miRNAs can also directly regulate NF-κB expression levels. Studies have reported that miR-34a reduces the secretion of pro-inflammatory cytokines such as TNF and IL-1β by decreasing the expression of NF-κB and Notch-1 proteins (Domenis et al., 2018). miR-21 reduces the expression of TNF by inhibiting NF-κB pathway, increases the production of anti-inflammatory factor IL-10 and promotes microglia polarization to M2 phenotype (Das et al., 2014). miR-146a-5p downregulates the expression of M1-type microglia-related genes by inhibiting Interleukin-1 receptor-associated kinases (IRAK1) and Nuclear factor of activated T cells 5 (NFAT5) and thus downregulating the expression of genes characteristic of M1-polarized microglia (Duan et al.,

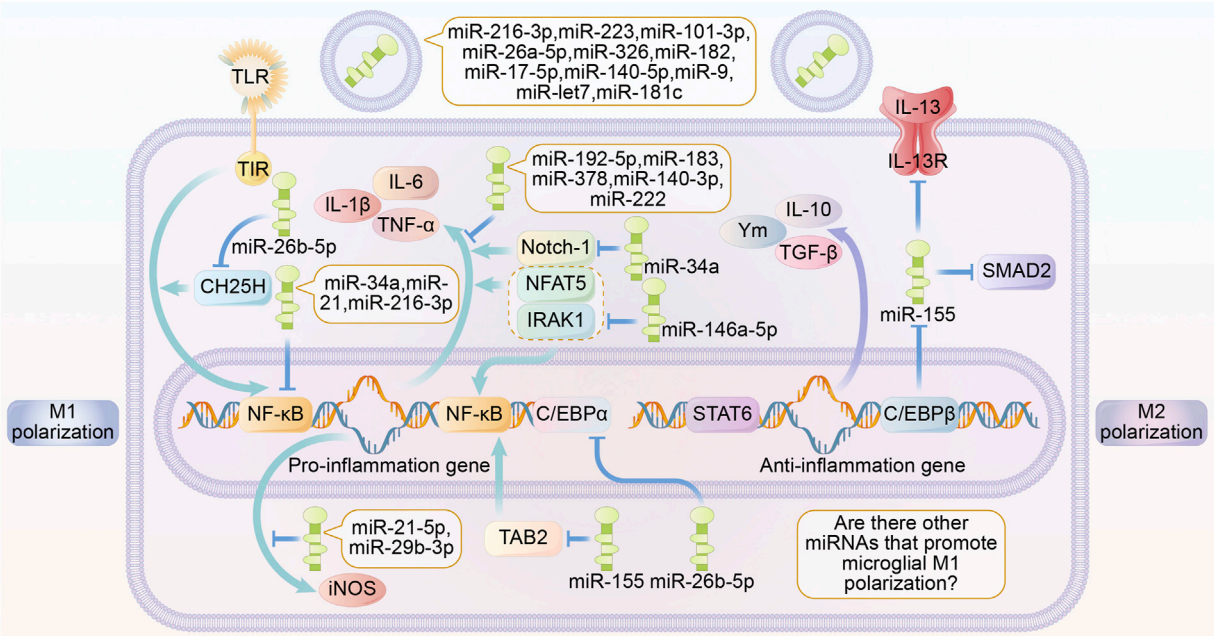

FIGURE 1 | Roles of exosome miRNA in microglia polarization. Different miRNAs secreted by exosomes are able to play a role in regulating microglia polarization through multiple signaling pathways. miR-216-3p, miR-223, miR-101-3p, miR-26a-5p, miR-326, miR-182, miR-17-5p, miR-140-5p, miR-9, miR-let7, miR-181c are able to inhibit TLR expression. miR-192-5p, miR-183, miR-378, miR-140-3p, miR -222 are able to directly inhibit the expression of inflammatory factors. miR-26b-5p inhibits the TLR signaling pathway by suppressing the expression of CH25H. miR-34a, miR-21, miR-216-3p are able to directly inhibit the expression of NF-κB. miR—21-5p, and miR-29b-3p inhibits the expression of the pro-inflammatory factor iNOS. miR-155 inhibits NF-κB activation by suppressing TAB2. miR-26b-5p inhibits C/EBPα expression. miR-146a-5p inhibits the expression of IRAK1 and NFAT5, thereby suppressing the expression of inflammation-associated genes and products. miR-34a inhibits the expression of Notch-κB. MiR-34a is able to inhibit Notch-1. The above miRNAs promote microglia M2 polarization and anti-inflammatory factor production. miR-155 promotes microglia M1 polarization and inflammatory factor production by inhibiting the expression of IL-13 receptor, SMAD2 and C/EBPβ. Abbreviations: TLR, toll like receptor; iNOS, inducible nitric oxide synthase; TGF β, transforming growth factor β; Ym, chitinase-like proteins; C/EBPα, CCAAT/enhancer-binding protein α; C/EBPβ, CCAAT/enhancer-binding protein β; TNF-α, tumour necrosis factor α; STAT6, signal transducer and activator of transcription 6; NF-κB, Nuclear factor kappa B; NFAT5, nuclear factor of activated T cells 5; IRAK1, interleukin-1 receptor-associated kinase1; TAB2, TGF-Beta-Activated Kinase 1-Binding Protein 2; SMAD2, Sma- And Mad-Related Protein 2.

2020). miRNAs have also been found to regulate both TLR and NF-κB expression. miR-216-3p shifts microglia from M1 to M2 phenotype by inhibiting the TLR/NF-κB signaling pathway (Liu et al., 2020). In addition, exosomes containing miR-21-5p and miR-29b-3p promote microglia polarization from M1 to M2 phenotype and inhibit M1 polarization by suppressing Inducible nitric oxide synthase (iNOS) mRNA expression and reducing the production of pro-inflammatory factors (Tsai et al., 2019; Jiang et al., 2020). It was found that miR-124 promoted microglia polarization to M2 type by inhibiting the transcription factor CCAAT/enhancer binding protein-α (C/EBP-α) and its downstream target protein Purine Rich Box-1 (PU.1) (Zha et al., 2021).

As mentioned above, exosomal miRNAs that act on the TLR/NF-κB pathway and its downstream signaling pathway can inhibit microglia M1 polarization. Uniquely, miR-155 was reported to promote M1 polarization in microglia. The mechanism may be related to the suppression of IL-13R, SMAD2 and CCAAT/enhancer-binding protein β (CEBPβ) expression (Allen et al., 2013; Dickey et al., 2017). However, miR-155 has been reported to promote M2 polarization by inhibiting TGF-Beta-Activated Kinase 1-Binding Protein 2 (TAB2) (Zhang et al., 2018). Most studies have reported the inhibitory roles of miRNAs in M1 polarization, while studies on miRNAs that inhibit M2 polarization-related pathways are still lacking and deserve further exploration (**Figure 1**).

MICROGLIA IN STROKE

Microglia and Neuroinflammation

Acute cerebral ischemia is the initiating event of stroke. Inadequate blood supply leads to hypoxia and glucose shortage, disruption of homeostasis, which leads to inflammation, glutamate excitotoxicity, and mitochondrial dysfunction (Mo et al., 2020). Post-ischemia inflammation is mainly caused by necrotic tissue and activated inflammatory cells (Zhang et al., 2020). Microglia are the major intrinsic immune cells of the CNS and dominate the regulation of central inflammation. Damps (damage-associated molecular proteins) include high mobility group box 1tbox1 protein (HMGB-1), extracellular peroxiredoxin (Prx) family proteins and galectin-3 (Gal3), which are closely associated with microglia activation (Amruta et al., 2020). During stroke, hypoxic necrosis of brain

cells leads to the production of various DAMPs (Xiong et al., 2016). HMGB-1 stabilizes nucleosome structure and participates in several physiological processes as a signaling factor. In stroke, HMGB-1 acts as a pro-inflammatory cytokine that activates microglia through the receptor of advanced glycation end-product (RAGE) and TLR-MyD88 pathways, producing immunosuppressive and lymphotoxic effects that further aggravate brain injury (Liesz et al., 2015; Jayaraj et al., 2019). Lin et al. found that in a mouse model of cerebral hemorrhage, heme activates TLR4 which in turn activates the MyD88/TRIF signaling pathway, ultimately allowing activation of the NF-κB pathway (Lin et al., 2012). Activated M1 microglia can release pro-inflammatory cytokines, reactive oxygen species (ROS) and Matrix metalloproteinase (MMP) to disrupt the blood-brain barrier and exacerbate neuroinflammation. After necrotic tissue is cleared, M2 microglia secrete the anti-inflammatory cytokine IL-10 and the neurotrophic factor IGF-1 to promote neural repair and reduce brain damage (Iadecola and Anrather, 2011). High expression of CD206, a marker of M2, and high expression of MHCII, a marker of M1, have been reported at about 3 and 7 days after stroke separately (Mirza et al., 2015). Microglia phenotypic shift and neuroinflammation are closely associated with the progression of stroke.

Many studies have now shown anti-neuroinflammatory effects in mouse models of stroke by modulating microglia polarization. These studies usually target TLR and Notch-related signaling pathways. In mouse/rat middle cerebral artery occlusion (MCAO) and oxygen-glucose deprivation (OGD) models, polyinosinic-polycytidylic acid activates TLR3/IRF3 signaling pathway and inhibits TLR4/NF-κB signaling pathway to reduce brain edema and improve prognosis (Wang et al., 2014). In hypoxia rat models, N-[N- (3,5-difluorophenacetyl) -1-alany1-S-phenyglycine t-butyl ester (DAPT) inhibited NF-κB/p65 expression by suppressing the Notch signaling pathway and TLR4/MyD88/TNF receptor associated factor 6 (TRAF6) pathway. Ultimately the expression levels of various inflammatory mediators such as TNF, IL-1β and iNOS proteins were reduced (Yao et al., 2013). In ischemia-reperfusion, NOSH-NBP, a novel mixture, can suppress the TLR4/MyD88/NF-κB pathway which leads to reduction of pro-inflammatory factors and microglia conversion to M1 phenotype. It also enhances the nuclear translocation of PPARγ and promotes the M2 polarization of microglia (Ji et al., 2017). cAMP-responsive element-binding protein (CREB) competes with NF-κB signaling pathway for the same coactivator molecule. By selectively activating CREB, the expression of M2-related genes may be increased and stroke related brain injury may be improved (Xia et al., 2015).

Microglia and Oxidative Blast

Oxidative stress is one of the important mechanisms that cause brain damage after stroke. Studies have shown that activated M1 glial cells such as microglia in the semidark zone in stroke will generate large amounts of oxidants including superoxide anion, hydrogen peroxide and peroxynitrite or nitrogen dioxide (Orellana-Urzúa et al., 2020). First, ROS lead to membrane damage and calcium overload, which in turn leads to mitochondrial dysfunction and ATP deficiency (Orellana-Urzúa et al., 2020). ATP deficiency will lead to sodium-potassium-ATPase and calcium pump dysfunction, ultimately exacerbating the disorder. In the case of stroke, oxidative stress is further exacerbated by the activation of enzyme xanthine oxidase enzyme (XO) enzymes, which further produce uric acid, superoxide radical anion and hydrogen peroxide by oxidizing hypoxanthine (Zhang et al., 2017b). In the mouse stroke model, the expression levels of antioxidants such as Superoxide dismutase (SOD) and Glutathione (GSH) are significantly reduced, followed by the exposure of proteins, lipids, and nucleic acids to ROS attack leading to neuronal cell death (Chamorro et al., 2016). Microglia located in the ischemic semidark zone of stroke are a major source of ROS (Chamorro et al., 2016). Ischemia and hypoxia lead to neuronal damage and release of DAMPs, which induce microglia to polarize to type M1. STAT family members play an important role in microglia polarization, with interferon γ (IFN-γ) activating STAT1 to promote M1 phenotype and IL-13 and IL-4 activating STAT6 to promote microglia polarization to type M2 (Allen et al., 2013). In stroke, activation of the TLR/NF-κB signaling pathway is associated with the induction of M1 microglia by IFNγ secreted by T helper 1 (Th1) cells (Zhao et al., 2017). NADPH oxidase 2 (NOX2) signaling activation is main pathway for ROS release from microglia. The regulatory part of NOX2 moves to the membrane and binds to the membrane-bound xanthochrome subunit upon induction by inflammatory stimuli such as IFN-γ, thus becoming a complex with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, which eventually promotes ROS production (Radak et al., 2017).

Microglia and Neuroregeneration

M2 phenotype of microglia are thought to be beneficial for neuronal regeneration. Microglia activated to M2 phenotype by IL-4, TGF-β will release brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) (Hu et al., 2015; Spejo et al., 2018). BDNF is able to promote nerve survival, recovery, and regeneration (Allen et al., 2013). In a female mouse model, BDNF was found to interact with TrkB receptors expressed by oligodendrocytes to promote myelin formation, increase oligodendrocyte differentiation and myelin thickness after demyelination injury (Fletcher et al., 2018). Currently, various methods to polarize microglia M2 to promote neuronal regeneration are vigorous. Ultrasound intervention is a new approach that has recently emerged. This approach involves engineering platelet membrane (PM) decorated resting microglia, liposome-encapsulated IL-4 (here called CPIL4) and ultrasound to specifically polarize microglia to an anti-inflammatory phenotype. Intravenous injection of this specific microglia, followed by ultrasound irradiation to remotely control the local destruction of liposomes and release of IL-4, allows local conversion of microglia to the M2 phenotype, thereby inducing regeneration of vascular and neuronal structures in mice with ischemic stroke (Liu et al., 2021). The purinergic receptor (P2X4R) has a regulatory role in the phenotypic conversion of

microglia. Ivermectin (IVM) promotes myelin regeneration by enhancing the P2X4R signaling pathway to promote the phenotypic conversion of microglia to the M2 phenotype (Zabala et al., 2018). In addition, miR-124-3P-containing exosomes secreted by M2 microglia were found to promote neurite outgrowth (Huang et al., 2018). Some drugs that inhibit M1 polarization of microglia have also been found to promote neurite regeneration under pathological conditions. In a mouse model of depression, Minocycline attenuated the inhibitory effect of neurotoxic M1 microglia on neurogenesis under chronic stress (Bassett et al., 2021). M1 microglia can exert synaptic phagocytic pruning by recognizing the complement component subunit 1q (C1q) complex at neuronal synapses (Allen et al., 2013). Short-Chain Fatty Acids promote post-stroke recovery by inhibiting microglia activation and thereby mitigating synaptic elimination (Kano et al., 2019). Collectively, promoting neuronal regeneration by facilitating microglia M2 polarization has great potential for application.

Microglia and Neuron Apoptosis

Brain tissue ischemia will result in insufficient supply of glucose and oxygen, which will disrupt the mitochondrial electron transport chain and lead to reduced ATP production (Ahsan et al., 2021). Dysregulation of ion concentrations caused by ATP deficiency will lead to a series of sequential events such as glutamate excitotoxicity. Excess glutamate will lead to calcium overload in peripheral cells, which induces neuronal cell death (Simpson and Oliver, 2020). Animal studies have shown that the expression of TNF, TNF-related inducing ligand (TRAIL), and Fas ligand (FasL) are all upregulated after ischemia, and these molecules worsen stroke prognosis to some extent (Radak et al., 2017). Microglia trigger neuronal cell death mainly through two pathways including death receptor pathway and mitochondrial pathway (Fricker et al., 2018). M1 microglia activate the death receptor pathway through the release of TNF. Through a cascade of reaction, TNF promotes activation of Bcl-2-associated X (Bid) and causes mitochondrial outer membrane permeabilization (MOMP). Ultimately, cytochrome c and caspase-9 activate downstream effectors to cleave cellular proteins and thus promote neuron apoptosis (Micheau and Tschopp, 2003; Haase et al., 2008). Many factors can activate the mitochondrial pathway, such as toxins, radiation and ROS released from M1 microglia. ROS can directly induce mitochondrial outer membrane permeabilization, which activates caspase-9 and acts downstream on caspase-3 and caspase-7, ultimately inducing apoptosis in neurons (Circu and Aw, 2010).

Microglia Autophagy

Autophagy affects stroke progression by regulating microglia polarization. Peroxisome proliferator-activated receptor γ (PPARγ) is a class of ligand-activated transcription factors that are important for inflammation regulation (Guo et al., 2017). It was shown that the PPARγ antagonist T0070907 could inhibit the expression of M1 markers such as iNOS and promote the expression of M2 markers such as CD206. This result was shown to be associated with enhanced cellular autophagy induced by LKB1-AMPK activation (Ji et al., 2018). In addition, it has been shown that Oxiracetam (ORC) promotes microglia autophagy through the AMPK/mTOR (AMPK inhibits mTOR) pathway, thereby promoting microglia M2 polarization (Wang et al., 2021a). It has also been demonstrated that peripheral macrophage-derived exosomes (PM-Exos) can locally induce M2 microglia production by promoting autophagy (Pandey et al., 2021). Furthermore, in the mouse oxygen glucose deprivation/reperfusion (OGD/R) model, microglia autophagic flux was inhibited by suppression of NF-κB pathway. Meanwhile, rise in M1 markers and decrease in M2 markers were detected when OGD/R reached 72 h (Xia et al., 2016). The above studies suggest that autophagy plays a role in the inhibition of microglia M2 polarization and neuroinflammation, and that inhibition of microglia autophagy would exacerbate neuroinflammation. However, miR-30d-5p-capsuling exosome secreted by adipose stem cells (ADSCs) was found to significantly reduce the area of infarct injury by inhibiting autophagy and promoting M2 microglia in AIS patients and rat models and *in vitro* models of hypoxia-glucose deprivation primary microglia (Jiang et al., 2018a). Heterogeneity of these results remains to be clarified.

Microglia Pyroptosis

In stroke, inflammasome is activated and then activates caspase-1 to shear gasdermin D, the nitrogen terminal of which form pores in the plasma membrane, finally leading to cell rupture and inflammatory responses (Tuo et al., 2021) (Fricker et al., 2018). Most recent studies have showed adverse effects of pyroptosis on the prognosis of ischemic stroke. Inhibition of platelet-activated factor receptors can inhibit the activation of down-regulated inflammasomeand thus reduce pyroptosis, while reducing ischemia-reperfusion injury (Zhao et al., 2021). In the Transient middle cerebral artery occlusion (tMCAO) mouse model, Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) stimulated by medioresinol acts on the PPARα/Glutamate Oxalo (GOT1) axis to significantly reduce the expression of pyroptosis-related proteins and improve ischemic brain injury (Wang et al., 2021b). Microglia pyroptosis was found in ischemia-reperfusion injury (Wang et al., 2020). Bone marrow mesenchymal stem cell-derived exosomes (BMSCs-Exos) are neuroprotective by downregulating the expression of neuronal NLR family pyrin domain containing 3 (NLRP3) inflammasome and pyroptosis-related proteins (Liu et al., 2021). In pMCAO mice, dexmedetomidine inhibited microglia pyroptosis by acting on the P2X7R/NLRP3/Caspase-1 signaling pathway and play a protective role in ischemic brain injury (Sun et al., 2021). In retinal ischemic injury, a NOD-like receptor named NLR caspase recruitment domain (CARD) containing 5 (NLRC5) was identified, which binds to the inflammasome NLRP3 and NLR-family CARD-containing protein 4 (NLRC4) and mediates microglial cell pyroptosis (Deng et al., 2021). In addition, in spinal cord injury, CD73 may inhibit the activation of the NLRP3 inflammasome complex through the adenosine-A2B adenosine receptor-phosphoinositide 3-kinase (PI3K)-AKT-Foxo1 cascade, reducing the activation of gasdermin D and thus microglia

pyroptosis (Xu et al., 2021). Microglia pyroptosis and stroke nerve injury are closely related. However, reports on microglia pyroptosis in stroke models are still lacking and need to be further added. Furthermore, despite the lack of studies linking microglia polarization and pyroptosis, it is known that NLRP3 activation is associated with both microglia M1 polarization and the occurrence of pyroptosis. Therefore, the relationship between microglia pyroptosis and their pro-inflammatory polarization deserves further exploration.

Microglia and Glutamate Excitotoxicity

Glutamate excitotoxicity is an important mechanism associated with neuronal death in stroke. Ischemia and hypoxia lead to impaired ATP synthesis, which leads to sodium-potassium pump dysfunction and eventually causes depolarization of the cell membrane and activation of some calcium channels. This process will lead to increased release and decreased reuptake of glutamate (Fricker et al., 2018). Excessive concentrations of glutamate bound to N-methyl-D-aspartate (NMDA) receptors will lead to calcium overload and thus induce peripheral neuronal death (Yang et al., 2019b). After microglia activation, large amounts of glutamate are released through two glutamate transport systems, the glutamate transporter 1 (GLT-1) transport system and the xCT transport system (Belov Kirdajova et al., 2020). It has been shown that Post-synaptic density 93 (PSD-93) binds to CX3 chemokine ligand 1 (CX3CL1) and thereby activates microglia, mediating ischemia-induced glutamate excitotoxicity in acute ischemic stroke (Zhang et al., 2021a). Glutamate excitotoxicity can also be reduced by controlling the signaling pathway of glutamate release of microglia. Inhibition of endoplasmic reticulum inositol 1,4,5-trisphosphate (InsP3) receptor activation with vitamin C improves the permeability of microglia gap junctions and inhibits glutamate release (Socodato et al., 2018). Microglia reduce glutamate excitotoxicity by modulating neuronal calcium metabolism and reducing calcium overload (Szalay et al., 2016). In addition, G protein-coupled receptor 30 (GPR30) has been demonstrated to be neuroprotective and reduce glutamate-induced excitotoxity. G1, a stimulator of GPR30, can promote microglia polarization to M2 phenotype and thus promotes the neuroprotection (Yang et al., 2021).

Microglia and Blood Brain Barrier Protection

Disruption of the blood-brain barrier is also one of the pathological features of stroke, mainly characterized by structural disruption of tight junction protein complexes and increased permeability, which usually implicates poor prognosis (Profaci et al., 2020). P2RY12 (purinergic receptor P2Y, G-protein coupled, 12) can mediate the movement of microglia to the site of blood-brain barrier damage. These aggregated microglia can not only patch up small breaches, but they can also temporarily assume the function of the blood-brain barrier (Lou et al., 2016). When microglia are polarized to the M2 phenotype, they can better protect the blood-brain barrier by producing IL-10 and TGF-β1, as well as VEGF, IL-8 and pro-MMP-9 to promote vascular remodeling (Jiang et al., 2018b). The use of tetramethylpyrazine (TMP) in experimental autoimmune encephalomyelitis animal models protects the blood-spinal cord barrier by activating the signal transducer and activator of transcription 3 (STAT3)/Suppressor of Cytokine Signaling 3 (SOCS3) signaling pathway and promoting microglia polarization from M1 to M2 phenotype (Zhang et al., 2021b). M1-type microglia not only secrete pro-inflammatory cytokines, but also secrete chemokines such as chemokine (C-C motif) ligand 2 (CCL2) and Chemokine C-X-C ligand 10 (CXCL10) to recruit more immune cells and compromise the integrity of the blood-brain barrier (Jiang et al., 2018b). Minocycline may be an ideal therapeutic agent because of its good blood-brain barrier permeability, high safety profile, and targeting of M1 microglia. In a transient MCAO model, the use of minocycline promoted microglia M2 polarization significantly reduced TNF and IL-1β levels and increased TGF-β, IL-10 and chitinase-like proteins (YM1) levels, thereby reducing cerebral infarct size, promoting vascular remodeling, and improving stroke prognosis (Yang et al., 2015).

Interplay Between Microglia and Astrocyte

IL-1β released from M1 microglia can promote astrocyte activation. In addition, IL-1α, TNF and C1q can also differentiate astrocytes into the A1 phenotype, which is detrimental to stroke recovery (Liddelow and Barres, 2017). Under physiological conditions, astrocytes can render microglia dormant, but in ischemic conditions, the ability of astrocytes to spread calcium ions over long distances may allow microglia to be activated even far from the infarct area (Nedergaard and Dirnagl, 2005). It has been shown that the use of antagonists of purinergic receptors can effectively block this transmission and slow down the expansion of the infarct volume (Xie et al., 2011). *In vitro* cultures of microglia and astrocytes were mixed. Astrocytes stimulated substantial proliferation and M2 polarization of microglia, while astrocytes appeared to differentiate to the beneficial A2 type (Kim and Son, 2021). In the stroke simulation experiment, M2 microglia and A2 astrocytes were highly susceptible to pro-inflammatory cytokines. Secretion of TNF and IFN-γ by microglia induced transition of A2 astrocytes to A1, and secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) by astrocytes caused M2 microglia polarization to M1 (Kim and Son, 2021). In a rat model of tMCAO, microglia-derived macrophage-like cells (MG-MΦ) upregulate astrocyte aquaporin-4 (AQP4) expression and exacerbate brain edema (Murata et al., 2020). In chronic recurrent experimental autoimmune encephalomyelitis, astrocyte-secreted TGF-β2 was found to exacerbate cerebral edema by possibly downregulating major histocompatibility complex class II expression and costimulatory/adhesion molecules, thereby affecting the antigen-presenting function of microglia. Besides, TGF-β2 secreted by prompted astrocytes appear to promote neuroinflammation (Siglienti et al., 2007). Complement C3 secreted by astrocytes can acts on neurons and microglia, resulting in impaired phagocytosis and subsequent abnormal

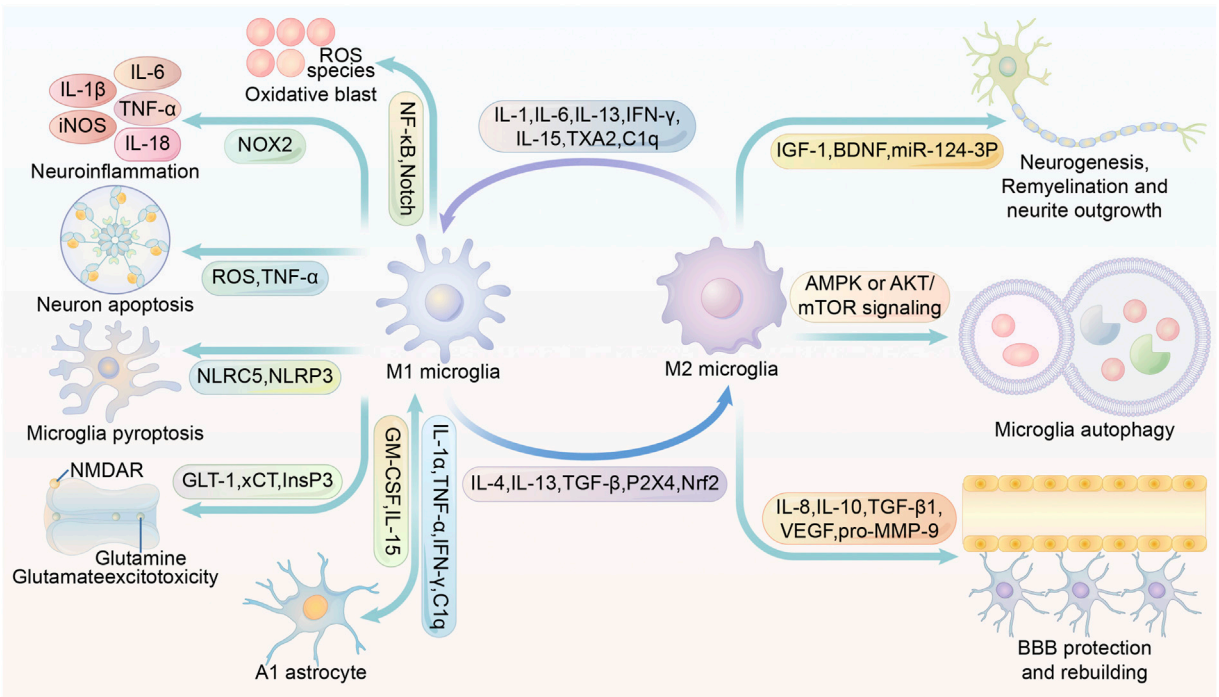

FIGURE 2 | Multiple roles of M1/M2 microglia in stroke modulation. IL-1, IL-6, IL-13, IFN-γ, IL-15, TXA2 and C1q promote microglia M1 polarization or M2 microglia conversion to M1. IL-4, IL-13, TGF-β, P2X4 and Nrf2 promote microglia M2 conversion or M1 microglia conversion to M2. Activation of NF-κB and Notch in M1 microglia promotes inflammatory factor production. activation of NOX2 in M1 microglia promotes ROS production. M1 microglia are able to secrete ROS and TNF and thus induce neuronal apoptosis. activation of NLRP3 and NLRC5 in M1 microglia may contribute to microglia scorching. The inhibition of InsP3 receptors facilitates the inhibition of glutamate release. M1 microglia secrete IL-1α,TNF-α,IFN-γ, C1q to promote the A1 phenotype of astrocytes. A1 phenotype astrocytes secrete GM-CSF and IL-15 to promote microglia M1 polarization. glial cell M1 polarization. IL-4, IL-13, TGF-β, P2X4, Nrf2 induce microglia M2 polarization. M2 microglia promote neurogenesis, remyelination and neurite outgrowth by secreting IGF-1, BDNF and miR-124-3p-containing exosomes. Increased AMPK or AKT/mTOR signaling promotes microglia autophagy, and enhanced autophagy is closely associated with microglia M2 polarization. M2 microglia promote BBB protection and rebuilding by secreting IL-8, IL-10, TGF-β1, VEGF and pro-MMP-9. Abbreviations: iNOS, inducible nitric oxide synthase; TNF-α, tumour necrosis factor α; ROS, reactive oxygen species; NF-κB, Nuclear factor kappa B; NMDAR, N-methyl-D-aspartic acid or N-methyl-D-aspartate receptor; NOX2, NADPH oxidase 2; NLRC5, NOD-, LRR- and CARD-containing 5; NLRP3, NOD-like receptor protein 3; GLT-1, glutamate transporter-1; xCT, cystine/glutamate antiporter; InsP3, inositol 1,4,5-trisphosphate; GM-CSF, granulocyte-macrophage colony-stimulating factor; TXA2, thromboxane A2; TGF-β, tumor growth factor-β; Nrf2, Nuclear factor erythroid 2-related factor; IGF-1, insulin-like growth factor 1; BDNF, brain-derived neurotrophic factor; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; VEGF, vascular endothelial growth factor; BBB, blood-brain barrier.

synaptic pruning of microglia (Lian et al., 2016). In cerebral hemorrhage, IL-15 secretion by astrocytes is significantly upregulated and contributes to the microglia polarization to M1 phenotype, exacerbating brain injury (Shi et al., 2020).. (**Figure 2**)

EXOSOME TREATMENT ASSOCIATED WITH MICROGLIA POLARIZATION IN STROKE ANIMAL MODEL

Recently, a large number of studies have demonstrated that exosomes from different sources exert a significant regulatory effect on stroke-induced brain injury by targeting microglia polarization. Exosomes from endotoxemia mouse serum contain a large number of inflammation-associated miRNAs, which are phagocytosed by microglia and ultimately lead to increased neuroinflammation by promoting microglia activation and M1 polarization (Li et al., 2018). Young rat serum exosomes contain more CD46 and fewer complement components such as C1q. It was shown that serum exosomes from young rats could improve short- and long-term neurological outcomes in older rats with ischemic stroke by reducing M1 microglia and increasing M2 microglia (Zhang et al., 2021c). In acute ischemic stroke (AIS) patients and rat models, exosomes from different sources promote M2 polarization of microglia by releasing hsa-miR-124-3p and miR-30d-5p, respectively, thereby reducing brain damage caused by neuroinflammation (Jiang et al., 2018a; Qi et al., 2021). Exosomes derived from mesenchymal stem cells (MSCs) containing miR-223-3p were found to reduce infarct size and improve neurological deficits and learning memory in a mouse model of stroke by inhibiting the inflammatory response induced by M1 phenotype microglia (Zhao et al., 2020). Further studies found that exosomes from bone marrow mesenchymal stem cells (BMSCs) in Intracerebral hemorrhage (ICH) and MCAO mice reduced M1 microglia polarization and increased M2 polarization, thereby reducing neuroinflammation and neuronal apoptosis caused by ischemia

TABLE 1 | Therapeutic effects of exosome on stroke by targeting microglia polarization.

Exosome	Objectives	Contents	Significance	Mechanism	References
Exosomes from serum of young rats	Aged ischemic rats	More CD46, less C1q, C3a, C3b	Improved short- and long-term functional outcomes after ischemic stroke and reduced synaptic loss	Reducing Iba1$^+$CD86$^+$ microglia but increasing Iba1$^+$CD206$^+$ microglia	147
EVs from serum	AIS patients	hsa-miR-124-3p	Reduced serum pro-inflammatory cytokines and the NIHSS score	Reversing the LPS-induced inflammatory effect in BV2 microglia by inhibiting the expression of GRB2 and AKT3 gene involved in pro-inflammatory signaling pathways	148
Exosome from ADSCs	AIS rats	miR-30d-5p	Reduced cerebral injury	Suppressing autophagy and promoting M2 microglia polarization	119
Exosomes from serum	Endotoxemia mice	miR-15a, miR-15b, miR-21, miR-27b, miR-125a, miR-146a, and miR-155	Increased systemic pro-inflammatory cytokine production, and elevated CNS expression of pro-inflammatory cytokine mRNA and the inflammation-associated miR-155	Inducing the expression of Iba-1 and microglial uptake of exosomes derived from serum containing inflammation-related miRNAs	146
Exosomes from BMSCs	ICH rats	miR-146a-5p	Improved neurological function and reduced neuronal apoptosis	Inhibiting microglial M1 polarization by downregulating the expression of IRAK1 and NFAT5	75
Exosomes from hUMSCs	Ischemic mice	miR-146a-5p	Improved recovery of function, attenuated microglia-mediated inflammation	Decreasing IBA-1$^+$CD16$^+$ cells and increasing IBA-1$^+$CD206$^+$ cells by suppressing IRAK1/TRAF6 signaling pathway	151
EVs from neural progenitor cell	MCAO mice	let-7g-5p, miR-99a-5p, let-7i-5p, miR-139-5p, miR-98-5p, miR-21-5p and let-7b-5p	Suppressed inflammation response	Inhibiting the expression of Iba-1 and MAPK of an inflammation related pathway	152
Exosome from BMSCs	MCAO rats	NR	Attenuated cerebral ischemia-reperfusion injury-induced neuroinflammation and pyroptosis	Shifting M1-polarized microglia shifted toward M2-polarized microglia	104
Exosomes from macrophage	pMCAO rats	Edaravone	Enhanced neuroprotection	Promoting the polarization of microglia from M1 to M2	155
Exosomes from plasma	pMCAO rats	melatonin	Decreased infarct volume, improved recovery of function and reduced microglia pyroptosis	Inhibiting TLR4/NF-κB pathway mediated microglial inflammation and NLRP3-mediated microglia pyroptosis	156
Exosomes from hUMSCs	tMCAO rats	CCR2	Enhanced oligodendrogenesis and remyelination	Decreasing CD16 and IL-1β mRNA expression and increasing CD206 and Arg-1 mRNA expression	150
Exosomes from LPS stimulated macrophage	MCAO/R rats	NR	Increased neuroprotection and functional improvement	Enhancing the microglial polarization from M1 phenotype to M2 phenotype	153
Exosomes from ADSCs	MCAO/R rats	miR-126	Improved neurogenesis and functional recovery	Inhibited microglial activation and the expression of inflammatory factors	154
Exosomes from MSCs	MCAO/R rats	miR-223-3p	Decreased cerebral infarct volume, improved neurological deficits, learning and memorizing abilities	Inhibiting microglial M1 polarization mediated pro-inflammatory response	149
Exosomes from human embryonic kidney cells	Photothrombotic ischemic mice	NGF and NGF mRNA	Reduced ischemic injury via reducing inflammation and cell death	Reducing CD16$^+$ M1 microglia but increasing CD206$^+$ M2 microglia	157

Not reported, NR; middle cerebral artery occlusion and reperfusion, MCAO/R; bone marrow mesenchymal stem cells, BMSCs; Intracerebral hemorrhage, ICH; mesenchymal stem cells, MSCs; Cysteinyl leukotriene receptor 2, CysLT$_2$R; acute ischemic stroke, AIS; adipose-derived stem cells, ADSCs; C-C chemokine receptor type 2, CCR2; transient middle cerebral artery occlusion, tMCAO; permanent middle cerebral artery occlusion, pMCAO; extracellular vesicles, EVs; lipopolysaccharide, LPS; central nervous system, CNS; complement component 1q, C1q; National Institutes of Health Stroke Scale, NIHSS; oxygen-glucose-deprivation, OGD; nerve growth factor, NGF; Intracerebral hemorrhage, ICH; Signal transducer and activator of Transcription3, STAT3; human umbilical cord mesenchymal stem cells, HUC-MSCs; ubiquitin-specific protease 14, USP14; interleukin 1 receptor associated kinase 1,IRAK1; NFAT5 nuclear factor of activated T cells 5; toll like receptor, TLR; nuclear factor-κB, NF-κB; NLR family pyrin domain containing 3, NLRP3; human umbilical cord mesenchymal stem cells, hUMSCs; TNF receptor associated factor 6, TRAF6;

TABLE 2 | Roles of exosomes derived from polarized microglia in stroke treatment.

Exosome	Objectives	Contents	Significance	Mechanism	References
Exosomes from microglia	ICH rats	miR-383-3p	Increased necroptosis and aggravated ICH injury	Inhibiting ATF4 expression by transferring inflammation-related miR-383-3p	158
Exosomes from M2 microglia	tMCAO mice	miR-124	Attenuated ischemic brain injury and promoted neuronal survival	Inhibiting the downstream target USP14	159
Exosomes from M2 microglia	tMCAO mice	miR-137	Decreased infarct volume and behavioral deficits	Downregulating the expression of Notch-1	160
Exosomes from M2 microglia	MCAO mice	miR-26a	Promoted angiogenesis	NR	161
Exosomes from microglia	OGD conditioned microglia	miR-424-5p	Increased cerebral endothelial cell injury	Downregulating FGF2/STAT3 pathway	162
Exosomes from vinpocetine-treated microglia	OGD conditioned microglia	NR	Alleviated OGD-induced neuronal damage and influenced neuronal viability and neurite morphology	Inhibiting the OGD-Induced M1-BV2 Activation and Promoting M2 Phenotype by enhancing autophagic flux	163

Not reported, NR; oxygen-glucose-deprivation, OGD; Intracerebral hemorrhage, ICH; transient middle cerebral artery occlusion, tMCAO; C-C chemokine receptor type 2, CCR2; Fibroblast growth factor-2, FGF-2; Signal transducer and activator of transcription 3, STAT3.

or hemorrhage (Duan et al., 2020; Liu et al., 2021). In addition, human umbilical cord mesenchymal stem cells (hUMSCs) (Yang et al., 2020b; Zhang et al., 2021d), neural progenitor cells (Tian et al., 2021), lipopolysaccharide-stimulated macrophages (Zheng et al., 2019) and ADSCs (Geng et al., 2019) were found to alleviate ischemic injury caused by MCAO and promote neuroprotection and neurogenesis. These effects were shown to be closely related to exosome-induced microglia M2 polarization.

In addition to their own contained components that can exert stroke modulating effects, nanoscale exosomes can also act as carriers to transport relevant drugs or chemical molecules to modulate microglia polarization and thus influence stroke progression. Exosomes encapsulated with edaravone or melatonin can promote microglia M2 polarization and inhibit neuroinflammation (Li et al., 2020c; Liu et al., 2020). Exosomes derived from human embryonic kidney cells loaded with nerve growth factor (NGF) and its mRNA cause a decrease in the M1 phenotype and an increase in the M2 phenotype of microglia, thereby reducing ischemic damage caused by inflammation and cell death (Yang et al., 2020c). The above evidence provides a solid theoretical basis for the application of exosomes targeting microglia polarization in the treatment of stroke (**Table 1**).

Post-polarized microglia can secrete exosomes to influence stroke progression. Exosomes from microglia can promote neuronal necrosis and ICH injury by transmitting the pro-inflammatory miR-383-3p in ICH rats that sacrificed within 7 days (Wei et al., 2021). It was shown that exosomes derived from M2 microglia can reduce the volume of MCAO-induced cerebral infarcts, behavioral defects and promote neovascularization and neuronal survival by releasing miR-124, miR-137 and miR-26a within 3 days after ischemic attack (Song et al., 2019; Tian et al., 2019; Feng et al., 2021). OGD treated microglia can secrete exosomes containing miR-424-5p, which inhibit the fibroblast growth factor 2 (FGF2)/STAT3 signaling pathway and worsen endothelial cell damage (Xie et al., 2020). According to the *in vivo* study, activated microglia in the peri-infarct ischemic region were significantly increased in the first day and peaked at the 14th day after MCAO. The use of vinpocetine promotes microglia M2 polarization by enhancing microglia autophagy, ultimately attenuating neuronal damage caused by OGD treatment (Zang et al., 2020). The exosomes secreted by polarized microglia may also be an effective target for therapeutic modulation of stroke (**Table 2**).

CONCLUSION AND OUTLOOK

Stroke remains to date a fatal and disabling serious neurological disease. Given the specificity of its lesion location and tissues and the complexity of its pathological mechanisms, the clinical outcome of stroke is still not particularly satisfactory. This is especially true for patients treated beyond the thrombolytic window. Therefore, research on new treatment modalities and therapeutic targets is particularly important.

Recent studies have shown that microglia polarization is strongly associated with stroke progression, and that M1 microglia will exacerbate stroke-induced brain injury by promoting neuroinflammation, oxidative stress, neuronal apoptosis, glutamate excitotoxicity, and astrocyte differentiation to the A1 phenotype through multiple signaling pathways. In addition, M1 microglia may further exacerbate inflammatory injury by promoting microglia pyroptosis. In contrast, M2 microglia exert a significant neuroprotective effect and promote neurological recovery. M2 microglia may promote neurogenesis, myelin regeneration, neurite growth and blood-brain barrier protection, thus exerting an important central protective function. Additionally, increased autophagic flux of microglia can promote their M2 polarization and thus

reduce brain injury. Therefore, targeting microglia polarization and promoting the expression of M2 phenotype may become an important target for treatment and improving the prognosis of stroke.

Most cells are capable of secreting exosomes. Numerous studies have found that exosome pairs from different sources are able to regulate microglia polarization. This suggests that targeting the secretion of some specific exosomes may be able to regulate microglia polarization. In addition, the ability of exosomes to cross the blood-brain barrier makes them potentially an ideal vehicle for CNS drug delivery. The transport of specific drugs or molecules from exosomes to the CNS to regulate microglia polarization and improve the neurological environment has shown great potential for development. In animal models of stroke, the use of endogenous exosomes or exosomes as carriers to transport some drug molecules to promote the M2 polarization of microglia has achieved good therapeutic effects, and M2 polarized microglia can also play a corresponding neuroprotective role by secreting unique exosomes, and significantly inhibit the progression of stroke. Thus, targeting exosomes that regulate microglia and exosomes secreted by polarized microglia may be a new measure to treat stroke and improve its prognosis. However, the vast majority of exosomes have poor targeting properties. Currently, most studies have taken to implant molecules with targeting properties on the lipid membrane of exosomes, thus enhancing the targeting of exosomes. This will further increase the cost of exosome production and development, thus hindering their clinical application. Therefore, promoting the development of exosome acquisition and production technologies and increasing research on reagents that enhance exosome targeting will facilitate the clinical translation of relevant researches.

AUTHOR CONTRIBUTIONS

TW designed the article. TW, YH, and XG wrote the manuscript. WW and WG helped research and collect the materials. TW and XG prepared figures and tables. TW and WG critically revised the manuscript for important intellectual content. TW translated the article into English. All authors read and approved the final manuscript and agree to be accountable for all aspects of this work. Data authentication is not applicable.

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