Review

Therapeutic Potential of Extracellular Vesicles for the Treatment of Corneal Injuries and Scars

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Infection, trauma, and chemical exposure of the ocular surface can severely damage the cornea, resulting in visually significant stromal scars. Current medical treatments are ineffective in mitigating corneal scarring, and corneal transplantation is the only therapy able to restore vision in these eyes. However, because of a severe shortage of corneal tissues, risks of blinding complications associated with corneal transplants, and a higher rate of graft failure in these eyes, an effective and deliverable alternative therapy for the prevention and treatment of corneal scarring remains a significant unmet medical need globally. In recent years, the therapeutic potential of extracellular vesicles (EVs) secreted by cells to mediate cell-cell communication has been a topic of increasing interest. EVs derived from mesenchymal stem cells, in particular human corneal stromal stem cells, have antifibrotic, anti-inflammatory, and regenerative effects in injured corneas. The exact mechanism of action of these functional EVs are largely unknown. Therapeutic development of EVs is at an early stage and warrants further preclinical studies.

Introduction

The human cornea is often called the front window of the eye, and its optical transparency is essential for vision. The transparent cornea not only serves as a protective barrier of the eye against external insults but also provides 60% of the refractive power for focusing images on the retina. Approximately 90% of the thickness of the cornea is the stroma, which consists mainly of mesenchymal extracellular matrix and keratocytes. The highly organized collagen matrix provides a transparent optical path that transmits light very efficiently. The stroma has a specific level of stiffness and elasticity that maintains the shape of corneal surface to achieve a stable refractive power. Throughout adulthood of vertebrates, keratocytes are quiescent, showing neither apoptotic nor mitotic figures to any significant extent.[1–8]

Wound healing of the corneal stroma is a fibrotic process. Corneal scars result predominantly from injuries such as infectious keratitis, mechanical trauma, or chemical exposure. Corneal blindness is the fourth leading cause of blindness worldwide,[9] with corneal scars being their primary cause.[10] In an analysis of chemical exposures alone, chemical ocular burns were reported to result in 36,000 emergency visits or $26.6 million in emergency charges per year in the United States.[11] Chemical warfare is another cause of severe ocular surface injury. There is no effective treatment that prevents corneal scarring after these injuries, which often result in loss of vision and blindness.

This concise review focuses on the current management of corneal injuries, the recent advances in understanding the function of extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs), and the challenges faced in the development of safe, effective EV-based therapy for corneal injuries and scars.

Clinical Course and Current Management of Corneal Injuries

Chemical injury of the cornea can be caused by acids, alkali, or vesicants. The prognosis of a cornea with a chemical burn is influenced by its severity. Alkaline substances are lipophilic and penetrate the eye more rapidly and more deeply than acids. The alkali substance can deposit within the tissues and lead to saponification within cells; subsequent severe inflammation occurs during the acute phase. Opacification and ulceration of the cornea occur during the first two weeks after exposure.[12] During the acute stage of chemical injury, topical antibiotics are administered to prevent secondary infection, and topical corticosteroids are frequently applied to reduce ocular surface inflammation.[13] Adjuvant therapy such as amniotic membrane grafts have been ineffective in reducing long-term complications such as central corneal neovascularization, stromal scars, and symblepharon.[14,15] In the chronic phase, ocular surface inflammation persists, further worsening these complications.[13] As a result, visual acuity is reduced, often to the level of blindness.

The cornea is highly susceptible to damage from sulfur mustard, which acts as an alkylating agent and causes oxidative damage to cellular structure, eventually causing cell death.[16,17] Upon exposure, there is a latent period of a few hours before the onset of symptoms. Management during the acute phase is similar to that for alkaline and acid burns, which aims to prevent infection, reduce inflammation, and promote re-epithelialization.

The resultant damage from exposure to vesicants or alkaline, or acidic substances are dose-dependent, i.e., a larger amount of chemicals will lead to greater damage to the ocular surface and cornea. In severe cases, the eyes may not recover from the initial injury, leading to persistent symptoms and damage to the anterior segment. Compared with other ocular surface tissues, the corneal epithelium and endothelium are more susceptible to mustard gas injury than the stroma.[18] Persistent inflammation also contributes to late ocular complications, collectively termed mustard gas keratopathy.[19] Corneal stromal scarring and neovascularization, neurotrophic keratitis, corneal edema, and limbal stem cell deficiency are observed in the late stage.[18–21]

Infectious keratitis often results in dense stromal fibrosis. The intense inflammatory response against the infectious organisms during the active infection leads to stromal opacification and even corneal ulceration. Timely aggressive treatment to eradicate the infectious organisms is crucial in preventing corneal keratolysis and perforation. Chronic inflammation often persists after the organisms are eradicated and is accompanied by stromal opacity and loss of vision.[22] Topical corticosteroids have not been found to be effective in reducing corneal scarring.

In summary, medical treatments such as topical corticosteroids and nonsteroidal anti-inflammatory drugs are not effective in preventing corneal fibrosis and reducing stromal scars and are associated with a high rate of ocular complications.[23,24] Corneal transplantation is the only effective sight-restoring therapy to treat visually significant corneal stromal scars. However, there is a severe shortage of tissue worldwide.[9] In addition, blindness can result from corneal transplant–related complications including infection, bleeding, glaucoma, retinal detachment, and graft failure. Therefore an effective and deliverable alternative therapy that can mitigate corneal scarring is a significant unmet medical need and of great interest to vision scientists and clinicians.

Pathophysiology of Corneal Wound Healing

Corneal wound healing is rather complex and is achieved through multiple processes including cell death, migration, and proliferation; myofibroblast differentiation; and extracellular matrix remodeling. The corneal fibrotic process is often accompanied by neovascularization and inflammation regardless of the type of injury. The current understanding of corneal wound healing is based mostly on studies of animal injury models resulting from keratectomy by mechanical wounding or excimer laser, infection, or chemical exposure, although some information comes from limited case series from humans.[25] Upon a mechanical injury not involving the limbus, the corneal epithelium is able to heal itself as a result of functional epithelial limbal stem cells. Limbal stem cell proliferation, migration, and differentiation are critical for wound closure and maintenance of the corneal epithelial homeostasis.[26,27]

EVs were detected within the epithelium, the basement membrane, and the anterior stroma when the Bowman's layer was compromised after epithelial debridement.[28] EVs derived from epithelial cells directly promote in vitro myofibroblast differentiation through the transfer of their cargo.[29] Thus EV-mediated communication between the epithelial and

stromal layers directly influences the development of corneal scars.[29,30]

The integrity of Bowman's layer is important in the healing of stromal wounds.[28,31–33] When trauma breaks the integrity of Bowman's layer and the epithelial basement membrane, the diffusion of factors and EVs into the stroma promotes fibrotic tissue genesis.[30,33] Corneal keratocytes, localized at the wound edge, undergo apoptosis, and neutrophil infiltration starts within hours. Corneal keratocytes transition from a quiescent to an activated state and differentiate to myofibroblasts a few days after wounding.[34–36] Another source of myofibroblasts is bone marrow–derived precursor cells. Myofibroblasts are opaque and produce enormous quantities of disorganized extracellular matrix once they become established in the stroma (Fig. 1).[37,38] The accumulation of activated fibroblasts and myofibroblasts leads to persistent fibrotic activity, which generates disorganized fibrils in the stroma.[39] These disorganized fibrils cannot transmit light; therefore the cornea becomes opaque.[40] Although scarring can be detected within a month in mice and last at least for eight weeks,[41] corneal opacities persist in humans and are often permanent.

Upon corneal injury, disturbance of corneal hydration, lysis of the cell membrane, and cell death liberate mediators of chemotaxis and interleukins; this process leads to an immediate intense immunologic reaction or even necrosis of the cornea.[42] The subsequent progression of the injury and healing may range from a highly active inflammatory process to a hyporeactive process that fails to regenerate corneal structures, or full reconstitution. Epithelial wound healing may be impaired as a result of extensive damage to the limbal stem cells and their niche. The subsequent chronic inflammation and injury continue to fuel the fibrotic process leading to progressive corneal fibrosis.

Stem Cell Therapy

To address the unmet medical need of an effective treatment of corneal scars, multiple approaches are under intense investigation and include gene therapy to deliver antifibrotic genes,[43–45] miRNA therapy to modify biological processes,[46] tissue engineering to create stromal equivalence,[47,48] and synthetic keratoprostheses.[49] In the last decade, interest in stem cell therapies has grown because of their regenerative and reparative properties. As described above, the inflammatory response plays a critical role in corneal wound healing and fibrosis. Of the different types of stem cells, only MSCs possess the immunomodulatory ability. MSCs isolated from different tissues have been explored for their potential in corneal wound healing. For example, corneal transparency can be restored by the transplantation of human bone marrow–derived MSCs (BM-MSCs) cultured on human amniotic membrane onto chemically injured rat corneas during the acute period of injury.[50] The therapeutic effect of the transplanted BM-MSCs may be associated with the inhibition of inflammation and angiogenesis rather than the epithelial differentiation of MSCs.[50] MSCs derived from amniotic membrane and adipose tissues also have antifibrotic effects in animal models of chemical injuries and fungal infection, respectively.[51,52] Subsequently, the Funderburgh research group at the University of Pittsburgh isolated and characterized corneal stromal/mesenchymal stem cells (CSSCs), which are MSCs within the human limbus.[53] CSSCs have the potency of multilineage differentiation and have the highest differentiation potential to keratocytes than do MSCs derived from adipose tissue, umbilical cord, or bone marrow.[53,54] Application of CSSCs topically or via stromal injection in injured mouse corneas prevented and reduced corneal scarring in mouse models of injuries by mechanical wounding or freezing.[40,55] CSSCs have been shown to modulate local inflammation and exert an antiangiogenic effect.[5,56] Results from these MSC studies in animal models support the hypothesis that MSCs have therapeutic potential in preventing fibrosis and reducing corneal stromal scars resulting from different types of injuries. Because CSSCs are progenitors of keratocytes, they may have additional therapeutic potency in reducing corneal fibrosis and regeneration than do MSCs derived from other tissues.[54] The first clinical trials using human CSSCs (https://clinicaltrials.gov/ct2/show/NCT02948023 and https://clinicaltrials.gov/ct2/show/NCT03295292) are being conducted at the L.V. Prasad Eye Institute in India; preliminary results suggest encouraging outcomes in restoring corneal transparency and vision.[57]

Despite numerous studies showing promising therapeutic effects of MSCs in different diseases in animal models and early clinical studies,[58,59] the efficacy of MSCs in humans has yet to be demonstrated in late phases of clinical trials. There are several challenges in using stem cells as a therapeutic agent. Because of safety concerns associated with live stem cells, regulatory requirements are more stringent for the use of live stem cells than for biologics and inorganic compounds. Scalability and cost of manufacturing, stability, storage, and delivery of live cell products are other major hurdles for stem cell therapies.

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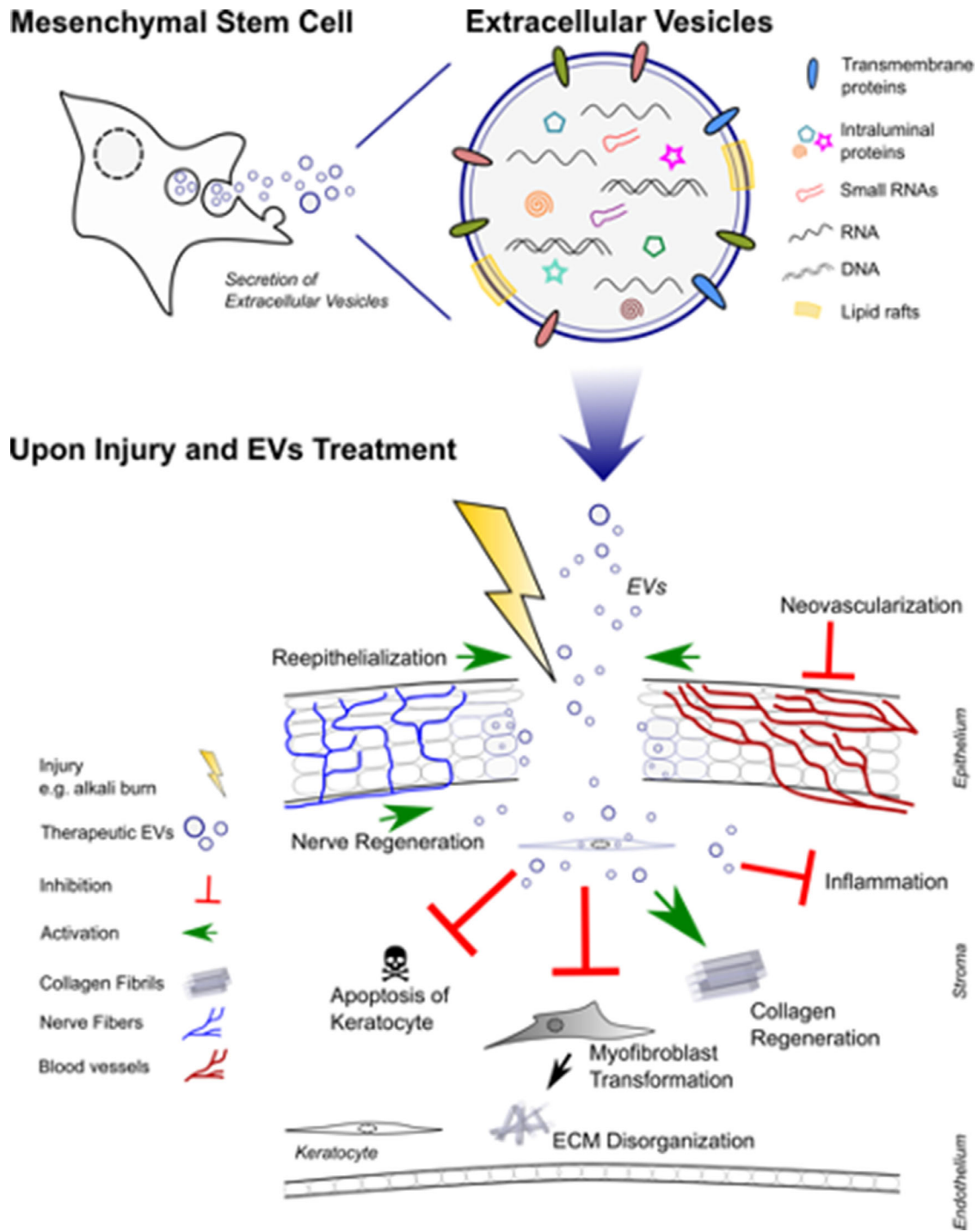
Figure 1. Biological function of EVs derived from corneal stromal stem cells in corneal repair and regeneration. Most of these EVs are exosomes, i.e., bilipid-layer vesicles enriched in small RNAs, proteins, and lipids with a functional role in cellular communication. When applied to the injured cornea, EVs derived from CSSCs promote re-epithelialization while inhibiting inflammation, myofibroblast transformation, and apoptosis of keratocytes. These properties ultimately lead to the regression of inflammation and regeneration of the corneal stroma.

Extracellular Vesicles in Corneal Wound Healing

Recent findings suggest that EVs of stem cells exert effects on target cells/tissues similar to those exerted by their parental stem cells, i.e., effects resulting from paracrine signaling and modification of the host's microenvironment.[60] EVs are lipid bilayer membrane-bound vesicles excreted from cells. Exosomes (40–200 nm), microvesicles (50–1000 nm), and apoptotic bodies (500–2000 nm) are the common subtypes of EVs. EVs can be defined by their biogenesis, size, constituent molecules, function, or method of separation. The apoptotic bodies are the largest vesicles, which result from programmed cell death.[61] The microvesicles originate from the budding of the cell membrane. Exosomes originate from the intracellular budding of endosomes and are released into the extracellular compartment.[62,63] The current most common methods of EV isolation are based on their size, density, or surface markers. Isolation methods include differential ultracentrifugation, density gradient ultracentrifugation, size exclusion chromatography, ultrafiltration, and affinity or immunoaffinity capture. Exosomes and microvesicles share some surface markers, and the range of their sizes overlaps. The current isolation methods are not able to separate the subtypes of EVs based on their different origins. Unless the origin of the vesicles is clearly demonstrated, the term "extracellular vesicle" is used.[64]

EVs shuttle important biomolecules between cells, maintain biological homeostasis, and influence the function of target cells.[65] Because of their ability to shuttle molecules between cells, the therapeutic potential of EVs as drug carriers and delivery vehicles across biological membranes is an active area of investigation.[66] Stem cell–derived EVs are being explored for their potential in regenerative medicine and tissue repair, such as for cardiovascular and neurodegenerative diseases.[67–69] Because MSC-derived EVs have both regenerative capacity and anti-inflammatory properties,[70,71] the effects of MSCs-derived EVs in corneal wound healing and regeneration have gained increasing interest in the vision science community in recent years.

Samaeekia and colleagues[6] report that CSSC-derived EVs promot not only corneal epithelial cell migration and proliferation in a cell scratch assay but also epithelial wound closure in a mouse model of mechanical epithelial wounding. Subsequently, we show that CSSC-derived EVs reduce stromal scarring and promote regeneration of normal corneal collagen in a mouse model of mechanical stromal wounding.[2] Mostly recently, EVs derived from human placenta MSCs promoted wound healing and reduced stromal scarring in a chemical burn mouse model.[3] Collectively, these results provide proof of concept that EVs may serve as a potential treatment of corneal injuries and scarring.

The inflammatory response is a very early process in corneal wound healing and unregulated chronic inflammation results in further corneal scarring. Studies have shown that CSSC-derived EVs could reduce inflammation by reducing early neutrophil infiltration and modulate the inflammatory response by inducing specific macrophage phenotypes.[2,3,72]

Corneal neovascularization occurs frequently after corneal injuries, leading to persistent inflammation and to subsequent reductions in corneal transparency by promoting the fibrotic response. Antiangiogenic effects have been attributed to MSC-derived EVs in alkali burns in mice.[3] A reduction in the expression of angiogenesis markers after EV treatment was observed, such as vascular endothelial growth factor and matrix metallopeptidase 2. Both CSSCs and the conditioned media of CSSCs selectively modulate the phenotype of macrophages that have antiangiogenic activity, thus, reducing corneal neovascularization in chemical burns of mice.[5] It is possible that the antiangiogenic effect of CSSC is due to EVs in their conditioned media. Moreover, EVs isolated from placenta-derived MSCs have been shown to suppress apoptosis of corneal epithelial cells in mouse corneas that have sustained a chemical injury.[3] This finding is of importance to the treatment of corneal injury due to vesicant exposure, such as mustard gas exposure, because this type of injury activates a cascade of reactions leading to corneal cell apoptosis and subsequent chronic inflammation and corneal neovascularization.[20]

The multiple properties of MSCs-derived EVs, namely immunomodulatory/anti-inflammatory, antiangiogenic, and antiapoptotic functions, likely interplay during the corneal wound healing process to favorably shift the fibrotic process to a regenerative pathway (Fig. 1). However, the exact mechanisms of MSCs and their EVs in promoting corneal epithelial and stromal wound healing, and corneal regeneration are largely unknown.

The function of EVs is dictated by their cargos, which include small RNAs, specific proteins, lipids, and metabolites. The microRNA (miRNA) are selectively packaged into exosomes. EVs derived from functional CSSCs contain a unique set of miRNA compared with those contained in EVs derived from HEK293 cells, which do not have a scar-reducing

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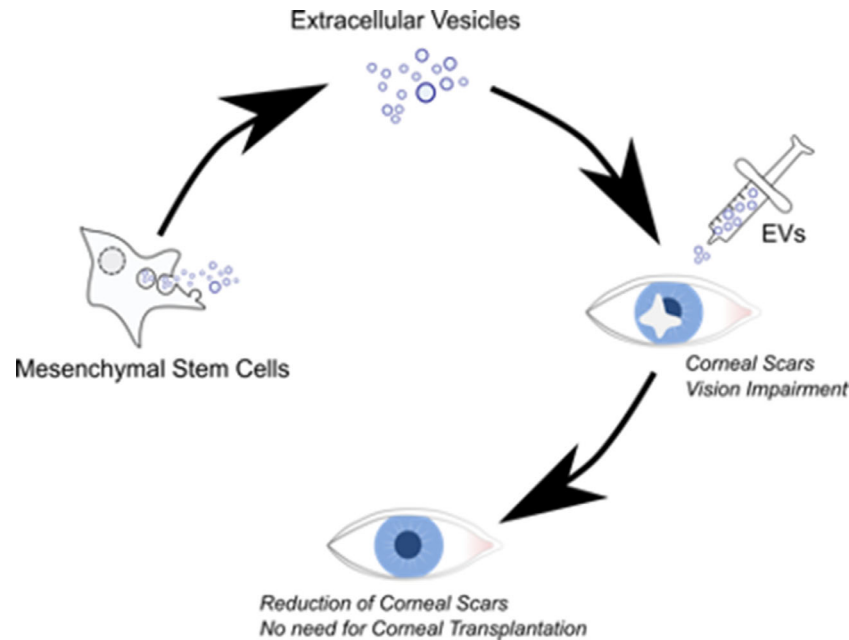
Figure 2. Extracellular vesicles as a treatment of corneal scars. EVs secreted from mesenchymal stem cells are purified by good manufacturing practice-compliant methods and subsequently delivered to the injured cornea as an outpatient procedure to treat corneal scars.

effect.[2] Furthermore, when the packaging of miRNA into exosomes was inhibited by the knockdown of Alix protein, which is required in miRNA packaging to exosomes during exosome synthesis in multivesicular endosomes, the CSSCs that expressed a low level of Alix protein became ineffective in reducing scarring and stromal regeneration.[2] This finding supports the notion that CSSCs reduce corneal stromal scarring via miRNAs delivered by exosomes. Previous studies of other systems demonstrate that miRNAs are key mediators of wound healing and inflammation via post-transcriptional regulation of mRNA.[73,74] The miRNAs that are responsible for the anti-inflammatory, antiangiogenic, antiapoptotic, antifibrotic, and corneal regenerative effects need to be elucidated. Whether other components of the cargo such as proteins (growth factors and cytokine) and lipids also play a role in these complex processes is another question to be addressed.

Extracellular Vesicle–Based Therapy: Bench to Bedside

Cell-free EV-based therapy is emerging as a promising therapy because of its advantages over current treatments in many diseases. Exosome-based therapies have already been tested in early phases of clinical trials for various cancers and are reported to be safe.[75,76] In the treatment of corneal scars, EVs have advantages over corneal transplantation and live stem cell therapy in areas such as safety/complications, quality, regulatory issues, and cost (Fig. 2). EVs require storage conditions that are less stringent than those needed for live tissues and cells and could be administered topically in outpatient settings. The finding that EVs retain their potency after lyophilization and storage at room temperature greatly increases their accessibility by patients worldwide.[77–79] Therefore EV-based therapy could treat large patient populations and be highly accessible, even in developing countries where medical care is scarce.

Development of EV-based therapy for corneal scars is at the early preclinical stage. Many challenges are faced in the therapeutic development of EVs. First and foremost, the potency and scalability must be considered when the source of cells for EV production is selected. Because the specific functional components of EVs that have the corneal regenerative property are unknown and because the functions of EVs mirror those of the parental cells they are isolated from,[2] a reasonable therapeutic approach is to employ EVs derived from MSCs that have the desired therapeutic properties. A master cell bank with the intended function needs to be selected to produce functional EVs. Primary MSCs, which are a source of EVs with corneal regenerative function, have limited lifespans;

therefore alternative sources of EV production are necessary for adequate scalability. One solution to circumvent this limitation is the immortalization of parental cells, which has been shown to be feasible.[80,81]

An equally important aspect in the preclinical development of EV-based therapy is the establishment of the criteria to define the population of functional EVs. These criteria could include physical properties, surface markers, and unique components in their cargos. Before these criteria can be developed, functional EVs need to be comprehensively characterized. Such study will be informative in furthering our understanding of the biology and function of EVs and shedding light on their mechanisms of action.

The conditions for generating functional EVs need to be optimized. Extrinsic factors such as culture medium composition and the extracellular matrix may influence the quantity and potency of EVs. For example, a three-dimensional system has been shown to stimulate EV production,[82] and treatment with proinflammatory cytokines enhances the release of anti-inflammatory EVs from MSCs.[72,83,84] Overexpression of the bioactive molecules in parental cells could result in more potent EVs.[81,85–87] These bioengineered EVs retain their beneficial function in vivo. If the functional components of the EV cargo are available, the potency of EVs could be further enhanced by enriching them with these functional factors. Thus optimized cell culture conditions coupled with bioengineered parental cell lines would offer an expandable source of high-potency EVs with low variation in quality.

Developing a robust, scalable, good manufacturing practice (GMP)–compliant EV purification method is another challenge. Although GMP-compliant protocols for the purification of exosomes including the use of ultracentrifugation and density gradient separation have been reported, the protocols are labor- and time-intensive and are not feasible for large-scale production. Development of a large-scale, GMP-compliant EV manufacturing process is another area of active investigation. Other outstanding questions involve issues of pharmacokinetics, stability, storage conditions, route and timing of delivery, and safety of EVs.

Conclusions

EV-based therapy is a relatively new concept that has gained increasing interest in the vision community because of the potential of EVs to be safer, more accessible, and cost-effective than current treatments and stem cell therapies. The recent finding that MSC-derived EVs, in particular, CSSC-derived EVs, reduced corneal scarring and regenerated corneal transparency after injuries serves as a proof of concept of EV-based therapy as an alternative to corneal transplantation for the treatment of corneal scars (Fig. 2). The mechanisms of action of these functional EVs remains yet to be elucidated. Therapeutic development of EVs is at an early stage and warrants further preclinical study.

Acknowledgments

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