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PhD THESIS

Title:

Extracellular vesicles as a therapeutic strategy for regenerative medicine

Scientific supervisor

Professor Benedetta Bussolati

PhD student

Dr. Lola Buono

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Abstract

The general aim of my PhD work is to evaluate the effects of extracellular vesicles (EVs) on different models of human pathologies. EVs are released directly from the cell membrane and are present in almost all biological fluids. EVs can mediate cell–cell communication and are involved in many processes, including immune signaling, angiogenesis, stress response, senescence, proliferation, and cell differentiation. The vast number of processes that EVs are involved in and the different manners in which they can influence the behavior of recipient cells make EVs an interesting source for both therapeutic and diagnostic applications. EVs can affect cell phenotype, recruitment, proliferation, and differentiation in a paracrine manner. These paracrine effects of EVs have a potential benefit in regenerative medicine. Given the role of EVs in processes that greatly affect tissue regeneration, my studies focus on the pro-regenerative role of EVs on two different pathological models. The first model is represented by the human cornea, more specifically by the endothelium of the cornea. The corneal endothelium is the inner layer of the cornea, main regulator of eye transparency, and most likely impaired by several factors. In particular, we focused on corneal endothelial damage due to nutrient deprivation, and we demonstrated that the treatment with mesenchymal stem cell-derived EVs was able to induce the wound healing of this layer, the proliferation and the inhibition of the apoptosis of the cells forming this layer. Further studies allowed us to establish that the pro-regenerative effects of EVs observed, were linked to the recovery from the endoplasmic reticulum stress of the cells, by specific miRNA transfer to target cells. Moreover, during the last part of my PhD, we tested the efficacy of renal progenitor-derived EVs on a model of kidney glomerular disease. We focused on a particular cell type forming the glomerular filtration barrier of the kidney, the podocyte. Podocytes serve as the final barrier to protein loss in the kidney during filtration of blood by the glomerulus. Hence, the podocyte plays a central role in maintaining the glomerular filtration barrier and preventing protein loss into the urine. For instance, we recovered podocytes from the

urines of children affected by glomerular diseases and resistant to classical therapies. We cultured and expanded the urine-derived cells *in vitro.* In our *in vitro* model we were able to show a pro-regenerative effect of renal progenitor-derived EVs on urine-derived podocytes. EVs were indeed able to reduce podocyte permeability to albumin, thus recovering from the damage.

Even though we contributed to showing the great potential of EVs as tissue-regenerative molecules, much remains unknown about the pathways that determine the content of EVs, and many tissue-specific functions of EVs remain to be uncovered. Future studies will provide new insights in EV function and biogenesis and reveal the roles of proteins and miRNAs in EVs function.

Chapter 1 – Extracellular vesicles in regenerative medicine

Extracellular vesicles (EVs) are membranous vesicles released by cells of prokaryotic, eukaryotic, and plants, in an evolutionarily conserved manner [1]. Vesicles are heterogeneous in size, flotation density and composition. EVs are membranous vesicles containing active proteins, lipids, and different types of genetic material such as miRNAs, mRNAs, and DNAs related to the characteristics of the originating cell [2]. The importance of EVs in regenerative medicine involves their ability to transfer biologically active molecules and genetic information to other target cells, influencing their function. In the last decade, many studies have characterized new mechanisms of cell-to-cell communication, capable of influencing the phenotype of target cells through the release of bioactive factors [3]. Among all soluble mediators of paracrine communication, EVs possess a leading role in both physiological and pathological conditions.

Regenerative medicine aims at the functional restoration of a damaged, malfunctioning, or missing tissue. Utilizing autologous stem, progenitor, and tissue-specific cells to restore damaged tissues may bypass the problem of immunogenic responses [4]. Following recent research has increasingly focused on the paracrine hypothesis, investigating the stimulating factors released by these stem- and progenitor cells, including growth factors, cytokines and EVs [5].

EVs composition and biogenesis

EVs are very heterogeneous and based on their origin and size. We can distinguish small-size EVs, medium and large-size EVs. Cells release several types of vesicles with different physiological properties, content and function. As a result of their different mechanisms of generation, and include exosomes, microvesicles, and apoptotic bodies. In the past, vesicle nomenclature was mainly based on the tissue of their origin. More recently, the field has started to shift toward a terminology that focuses rather on the mechanisms of generation of these vesicles [6]. Vesicles in the first category,

exosomes, originate in multivesicular bodies (MVB). When MVB fuse with the plasma membrane, the intraluminal vesicles are released from the cell and are from thereon referred to as exosomes [2]. Exosomes are reported to be between 40 and 150 nm in size. The most common markers used are tetraspanins such as CD9, CD63 and CD81. Microvesicles are shed directly from the plasma membrane and can be a lot larger than exosomes (50–1000 nm) [7]. There is, however, an overlap in size between these two populations. Microvesicles also contain mRNAs and miRNAs, as well as soluble and transmembrane proteins. Like exosomes, microvesicles are able to transfer functional genomic and proteomic content to target cells. Apoptotic bodies originate at the cell membrane as cells undergo apoptosis [8]. Even though these vesicles are of interest in biomarker research and have been shown to have effects on other cells, research on these vesicles in intercellular communication is limited. Generally, the term EV is used when discussing exosomes or microvesicles, or a combination of these vesicle populations, depending on isolation techniques [6].

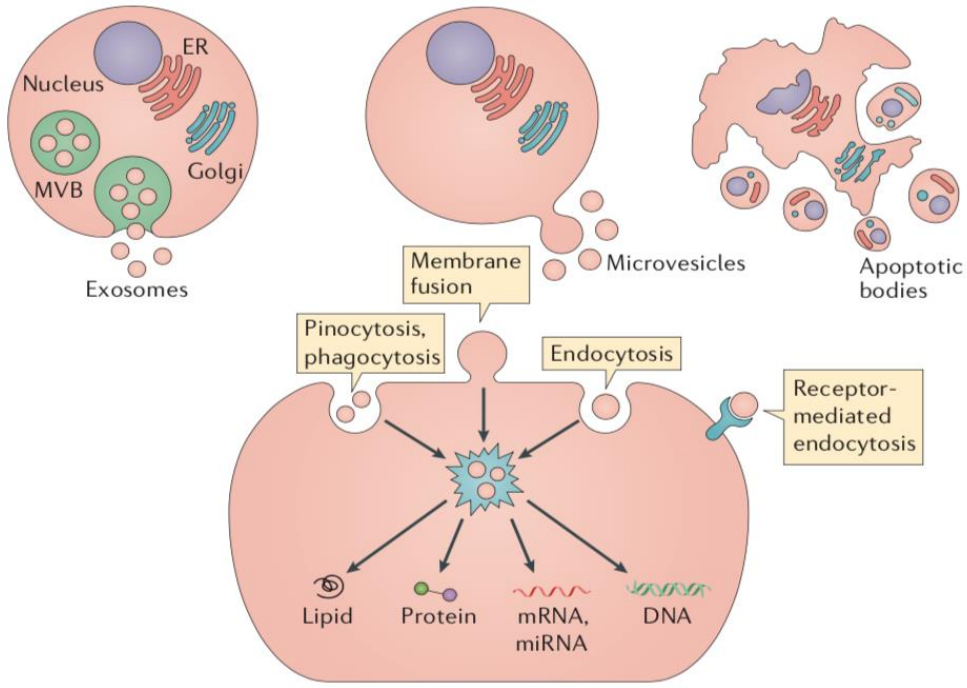

Figure 1. Release and uptake mechanisms of extracellular vesicles. Extracellular vesicles can be classed as exosomes, microvesicles and apoptotic bodies, based on the mechanism by which they are

released from cells and differentiated based on their size and content. Extracellular vesicles are taken up by cells by endocytosis, phagocytosis, pinocytosis, or membrane fusion [9].

EVs mechanisms of communication

Extracellular vesicles can communicate with target cells through several mechanisms. Firstly, transmembrane proteins on the EV membrane can interact with receptors on the cell membrane [5]. These receptor–ligand interactions can then activate signaling cascades to affect target cells. EV can also fuse with their target cells to release their cargo, either by direct fusion with the cell membrane or by endocytosis, after which mRNAs, miRNAs, and proteins are released into the cytosol [2,10]. Fusion of EV with target cells can either occur directly at cell membrane, or after endocytosis. After fusion, mRNAs transferred by EVs can be translated in to protein, and delivered miRNAs inhibit mRNA translation and affect cellular processes. The cargo and function of EV depends on their producing cells, and it has been shown that also cellular stress affects EV content, suggesting that intercellular communication through EVs is a dynamic system [11].

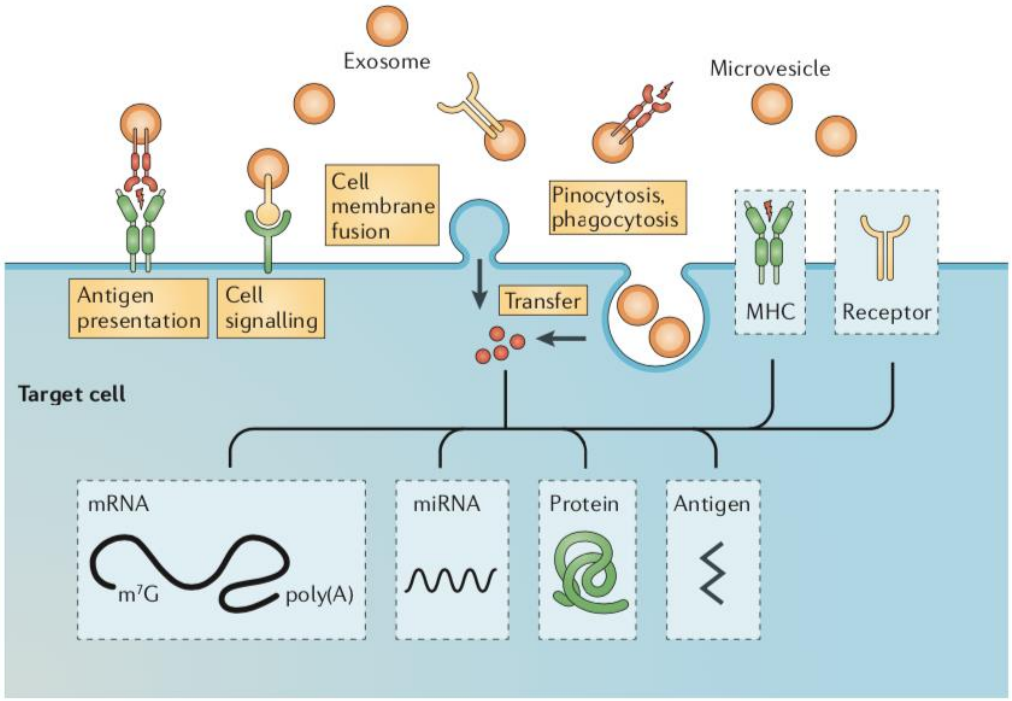

Figure 2. Extracellular vesicles interactions with recipient cells. Extracellular vesicles can directly activate cell surface receptors via protein and bioactive lipid ligands, transfer cell surface receptors or deliver effectors including transcription factors, oncogenes and infectious particles into recipient cells5. In addition, various RNA species including mRNAs and small regulatory RNAs (for example, microRNAs (miRNAs) and non-coding RNAs) are contained in extracellular vesicles and functionally delivered to recipient cells [3].

EVs and tissue regeneration

Prevention of cell death and cell senescence is essential in optimizing the efficiency of regenerative medicine, both in cell therapies as well as in tissue engineering. Bruno et al. showed that the administration of MSC-derived EVs decreased apoptosis in an acute kidney injury model and *in vitro* in cisplatin treated human epithelial cells, through up-regulation of anti-apoptotic genes and downregulation of several apoptotic genes [12].

MSC-derived EVs have also been found to increase proliferation. Indeed, bone marrow MSC-derived exosomes induced proximal tubular epithelial cell proliferation in an acute kidney injury model [13]. Interestingly, Zhang et al. also observed that treatment with these EVs in a rat skin burn model resulted in accelerated epithelialization [14]. Indeed, MSC-derived vesicles show positive effects on tissue repair through various pathways, even reducing apoptosis. EVs have also been demonstrated to stimulate *in vitro* and *in vivo* vessel formation by endothelial cells. For example, adipose MSC-derived EV, which could be increased in function and number by PDGF stimulation [15] as well as bone marrow MSC-derived EV, promoted angiogenesis in a rat myocardial infarction model [16]. Similar effects on hypoxia were observed in microvesicles from human umbilical cord MSC, which promoted angiogenesis *in vitro* as well as *in vivo* in a rat hindlimb ischemia model [16,17]. These studies indicate that EVs play a role in local tissue repair through regulation of cell proliferation. The

capacity of EVs to regulate cell senescence, apoptosis, and proliferation, parameters that greatly affect tissue engineering and cell therapy outcome, suggest a therapeutic potential in regenerative medicine [1]. All in all, EVs show a great potential role in regenerative medicine because of their role in cell recruitment, differentiation, and immunomodulation. Further studies in EV-mediated paracrine signaling and exploration of new methods to utilize EVs may lead to the discovery of novel regenerative therapeutics, as well as methods to improve current techniques.

Chapter 2 – Corneal endothelium

The endothelium of the cornea is a monolayer of cells on the posterior corneal surface that transports water from the stroma into the anterior chamber [18]. The stroma of the cornea has a tendency to swell and, therefore, it is necessary to maintain the cornea transparent [19]. Corneal transparency is the result of many factors such as its structural anatomy and the physiology of its components. The cornea is a fine example of natural engineering and each layer is important in maintaining eye transparency (Figure 3).Indeed, any accumulation of fluid can affect stromal transparency and health, a mechanism for maintaining stromal deturgescence is required [20]. This role is up to endothelium. Human corneal endothelial cells (HCECs) act by functioning both as a barrier to fluid movement into the cornea and as an active pump that moves ions and draws water osmotically, from the stroma into the aqueous humor [19].

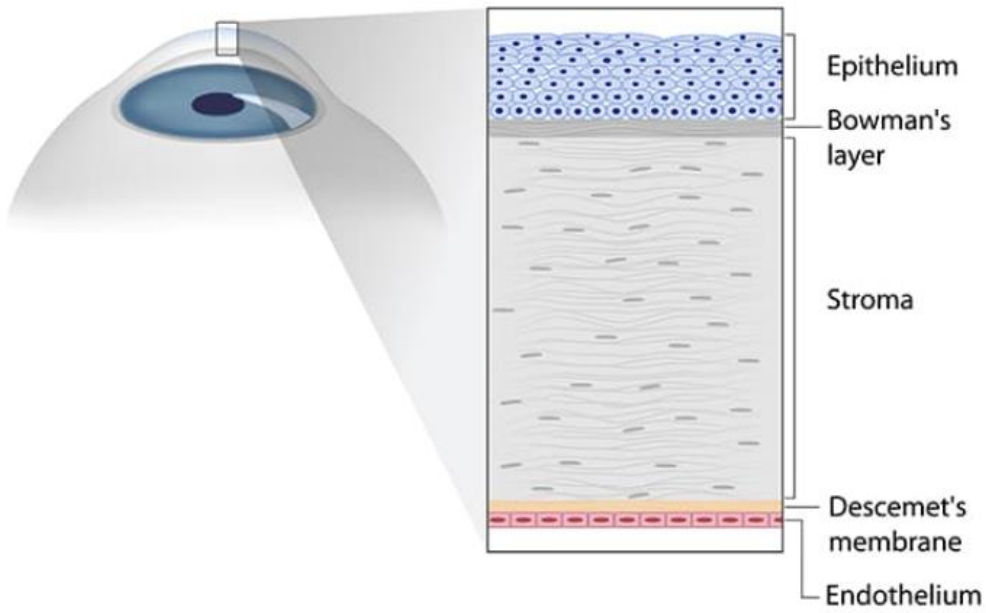

Figure 3. Structure of the cornea. The cornea consists of five layers including the epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium. (Premier Eye Care, premieridaho.com)

Corneal endothelial dysfunction

Since corneal endothelial cells (HCECs) have no mitotic activity *in vivo,* HCECs are prone to corneal endothelial dysfunction that eventually could lead to blindness. The causes of HCECs decrease are age, trauma, inflammation, corneal diseases, and surgical procedures. There are few diseases affecting primary the corneal endothelium. Fuchs' endothelial corneal dystrophy (FECD) is the most common [21]. In FECD, the pump function of the endothelial cells decreases, followed by a reduced barrier function so that the endothelium becomes unable to maintain fluid balance and consequently corneal clarity [21]. Other endothelial diseases are the posterior polymorphous dystrophy [22], congenital hereditary endothelial dystrophy [19], and iridocorneal endothelial syndrome [23] . Moreover, being the cornea the outer part of the eye, it is most likely to sustain damage due to various sources of stress, causing secondary corneal endotheliopathies [24]. Main kinds of insults are external trauma, metabolic damage, hypoxia caused by contact lens wear, or hyperglycemia [25]. Toxic causes of endotheliopathies of the cornea are, for example, due to drugs or their preservatives, related to alterations in pH or osmolarity and can be associated to surgical procedures, in particular related to phacoemulsification and corneal transplantation [26].

2.2 Corneal endothelial cell regeneration

The wound healing of endothelium has certain proper characteristics. The endothelium of the cornea, indeed, mostly heals by cell migration and increased cell spreading, but cell proliferation plays a secondary role [27]. Endothelial cells do not undergo mitosis *in vivo*, so in the first step, the injured cells have to be quickly replaced by migration and extension of the adjacent surviving cells, in order to form a temporary barrier with reduced pump activity [27]. Surviving cells can "stretch" over the space of the degenerated cells, growing in size and losing their typical hexagonal shape. Endothelial

wound healing is associated with a transient acquisition of fibroblastic morphology and actin stress fibers by migrating cells, which is consistent with endothelial-mesenchymal transformation [28]. Whatever it is the mechanism of damage, at present, the only treatment for this dysfunction is a corneal transplantation, or keratoplasty, from donor cadavers [29]. In keratoplasty, a wide part of the central cornea is replaced with a graft, an allogeneic cornea which has been removed *postmortem* from a donor. Due to keratoplasty limits and complications, along with a global shortage of donor corneas, it is important to find alternative strategies of treatment in order to overcome corneal transplantation [30].

Corneal endothelial cells and extracellular vesicles

Recent studies highlight that stem cell–derived EVs play a relevant role in stem cell-induced regeneration by reprogramming injured cells and by inducing pro-regenerative pathways [31,32] A particularly promising area of investigation seems to be the use of extracellular vesicles derived by mesenchymal stem cells (MSC-EVs) that could be able to promote regeneration of damaged endothelium [33] [34]. MSC-EVs have been widely studied in various disease models and in the last decade, they have been of interest in many ophthalmologic pathologies [35]. In 2018, indeed, it was evaluated the effect of MSC-EVs on corneal wound healing, and it was shown that human corneal MSC-EVs significantly increased the proliferation of human corneal epithelial cells *in vitro* and accelerated corneal wound closure in a murine epithelial mechanical injury model [31]. Moreover, our group recently reported the proangiogenic effect of MSC-EVs in an in vitro model of corneal endothelium damage due to serum deprivation. MSC-EVs induced the proliferation and survival of damaged HCECs, and promoted their wound closure [36].

Chapter 3 – The podocyte in the glomerular filtration barrier

The main function of the glomeruli is to filter fluids and electrolytes from the blood, while retaining plasma proteins [37]. This activity happens at the level of the glomerular filtration barrier (GFB) and is coordinated by the interaction of two highly specialized glomerular cells, the fenestrated endothelium, and the podocytes. These two cell types are separated by a thin layer of glomerular basement membrane. Podocytes are highly dynamic and terminally differentiated cells that interact with the glomerular basement membrane and communicate through signalling at the slit diaphragm [38]. The glomerular slit diaphragm represents the junction structure that links the foot processes from neighbouring podocytes and consists of a transmembrane-bridging proteins networking with a juxtaposed cytoplasmic platform of protein complexes, which in turn is linked to the actin cytoskeleton [39]. Within this architecture, ZO-1 protein is located at the cytoplasmic face of the slit diaphragm [40] and is one of its functional molecules. Podocytes serve as the final barrier to protein loss in the kidney during filtration of blood by the glomerulus. Hence, the podocyte plays a pivotal role in maintaining the glomerular filtration barrier and preventing protein loss into the urine[37]. Podocyte damage is a causal factor in the progression of multiple variants of kidney diseases.

Podocyte dysfunctions

An increasing number of genes have been identified as essential for unique structures and functions of podocytes, including those encoding nephrin, podocin and cluster of differentiation 2-associated protein (CD2AP). Nephrotic syndrome is a heterogeneous disease characterized by increased permeability of the glomerular filtration barrier for macromolecules [41]. Podocytes play critical role in ultrafiltration of plasma and are involved in a wide number of inherited and acquired glomerular diseases.

Mutations in different podocyte proteins can target the function of the podocyte through distinct pathologic mechanism by affecting the structure of the slit diaphragm, by directly or indirectly perturbing the intricate podocyte, cytoskeleton, by breaking cell-matrix interactions, or by blocking important signaling pathways (Figure). Systemic or locally produced toxins, viral infection, and local activation of the renin-angiotensin system can also be the cause of podocyte injury. Abnormalities of cytoskeleton structural proteins α-actinin-4 and synaptopodin can adversely affect cytoskeletal dynamics. For example, hereditary autosomal dominant focal segmental glomerulosclerosis is caused by mutation in α-actinin-4, increasing the affinity for actin and resulting in a change in cytoskeletal fluidity of the podocyte. All these mechanisms result in a common final disease pathway characterized by podocyte foot processes effacement, proteinuria, and ultimately disruption of the glomerular filtration barrier.

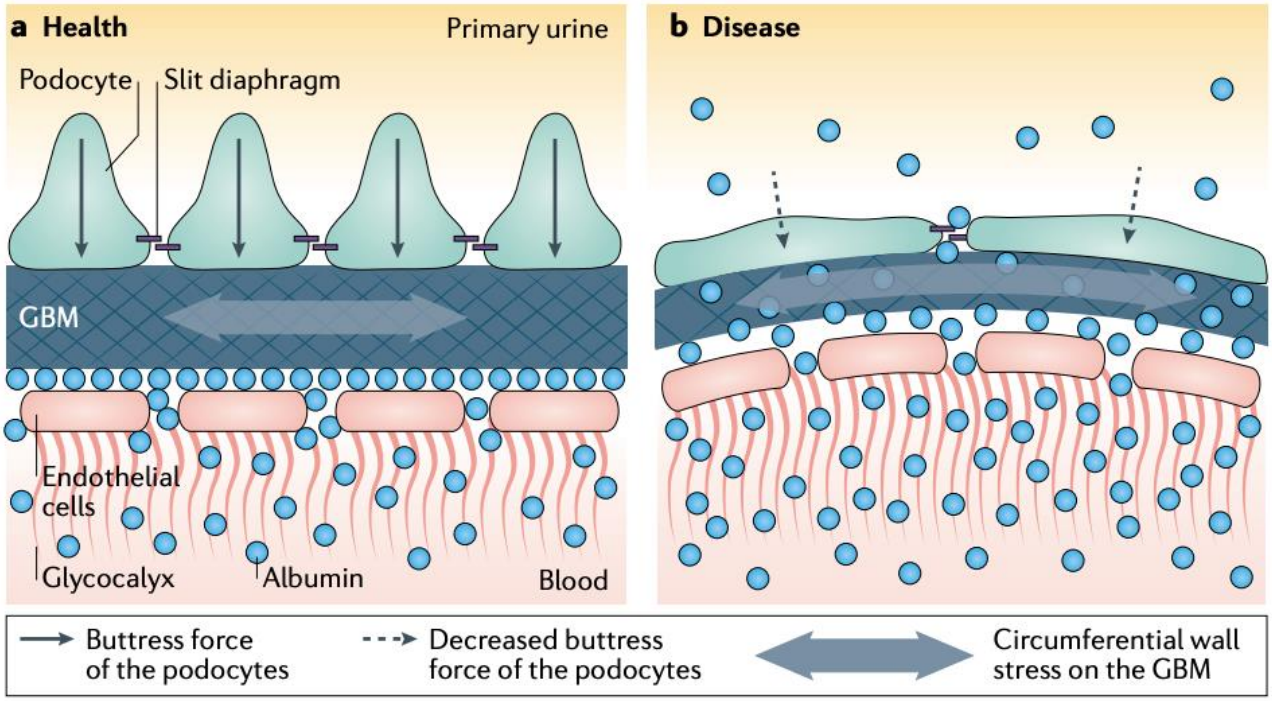

Figure 4. Mechanisms coupling slit diaphragm density and proteinuria. In the extended permeation model, podocyte foot processes are required to compress the glomerular basement membrane (GBM), so that it is impermeable to albumin. In disease states, podocytes and foot processes may be

lost and the GBM is therefore distended by the hydrostatic pressure so that it becomes permeable to albumin [42].

Extracellular vesicles in renal diseases

Extracellular vesicles may be biomarkers of renal disease, as well as mediators of inflammation, thrombosis, adhesion, immune suppression, growth and regeneration. MSCs have been reported to induce tissue regeneration after injury in numerous studies. The beneficial effects of MSCs may be mediated in a paracrine manner via the transfer of extracellular vesicles containing immune modulators to injured tissue [43] . The regenerative potential of EVs has been evaluated in preclinical studies, using various organs including the heart, lungs and kidneys [44]. In rodent models of AKI, an improvement in renal parameters was previously documented [45,46]. Furthermore, *in vitro* studies have demonstrated the potential of extracellular vesicles to transfer mRNA, miRNA and proteins to renal cells [47] and to produce and secrete anti-apoptotic, mitogenic and proliferative growth factors, as well as factors that promote angiogenesis, such as vascular endothelial growth factor, hepatocyte growth factor, insulin-like growth factor 1, adrenomedullin and stromal cell-derived factor 1 [48–50]. These factors can be transferred by MSC-derived extracellular vesicles to tubular cells. For example, MSC-derived exosomes transfer, insulin-like growth factor 1 to tubular cells resulted in a pro-regenerative effect [13]. The EVs accumulate in the kidney [51], resulting in tubular cell proliferation and renal recovery by horizontal transfer of genetic material and modification of gene expression [46,52]. The beneficial effect on nephron recovery may also be related to the uptake in endothelial cells and enhanced vascular permeability [53]. Similar effects to those induced by MSCs were reported in rats injected with EVs derived from endothelial progenitor cells [54] and in mice injected with human liver stem cells or extracellular vesicles [55]. Given all these recent reports, the use of

extracellular vesicles in a model of glomerular disease, seems to be a particularly promising area of investigation.

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Research Article

Effect of Stem Cell-Derived Extracellular Vesicles on Damaged Human Corneal Endothelial Cells

Raffaele Nuzzi ,[1] **Lola Buono** ,[2] **Simona Scalabrin** ,[1] **Marco De Iuliis** ,[1] **and Benedetta Bussolati**[2]

[1]*Eye Clinic, Department of Surgical Sciences, University of Turin, AOU Città della Salute e della Scienza, Turin, Italy*
[2]*Department of Biotechnology and Health Sciences, University of Turin, Turin, Italy*

Correspondence should be addressed to Raffaele Nuzzi; prof.nuzzi_raffaele@hotmail.it

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Purpose. Human corneal endothelial cells (HCECs) are essential to visual function; however, since they have limited proliferative capacity *in vivo*, they are prone to corneal endothelial dysfunction. At present, the only treatment is a corneal transplantation from donor cadavers. Also, due to a global shortage of donor corneas, it is important to find alternative strategies. Recent studies highlight that stem cell–derived extracellular vesicles (EVs) play a relevant role in stem cell-induced regeneration by reprogramming injured cells and inducing proregenerative pathways. The aim of this work is to evaluate whether EVs derived from mesenchymal stem cells (MSC-EVs) are able to promote regeneration of damaged HCECs. *Methods*. We isolated HCECs from discarded corneas in patients undergoing corneal transplantation or enucleation ($N = 23$ patients). Bone marrow mesenchymal stem cells (MSCs) were obtained from Lonza, cultured, and characterized. MSC-EVs were obtained from supernatants of MSCs. In order to establish a valid *in vitro* damage model to test the regenerative potential of EVs on HCECs, we evaluated the proliferation rate and the apoptosis after exposing the cells to serum-deprived medium at different concentrations for 24 hours. We then evaluated the HCEC migration through a wound healing assay. *Results*. In the selected serum deprivation damage conditions, the treatment with different doses of MSC-EVs resulted in a significantly higher proliferation rate of HCECs at all the tested concentrations of EVs (5-20×10^3 MSC-EV/cell). MSC-EVs/cell induced a significant decrease in number of total apoptotic cells after 24 hours of serum deprivation. Finally, the wound healing assay showed a significantly faster repair of the wound after HCEC treatment with MSC-EVs. *Conclusions*. Results highlight the already well-known proregenerative potential of MSC-EVs in a totally new biological model, the endothelium of the cornea. MSC-EVs, indeed, induced proliferation and survival of HCECs, promoting the migration of HCECs *in vitro*.

1. Introduction

Corneal transparency is the result of many factors such as its structural anatomy and the physiology of its components [1]. The cornea is a fine example of natural engineering: as any accumulation of fluid would affect stromal transparency and health, a mechanism for maintaining stromal deturgescence is required. This role is up to endothelium. Human corneal endothelial cells (HCECs) can do their job by functioning both as a barrier to fluid movement into the cornea and an active pump that moves ions and draws water osmotically, from the stroma into the aqueous humor [2, 3]. The combined leaky barrier and fluid pump is called a pump-leak mechanism [4].

Since endothelial cells have no mitotic activity in vivo (although they can be induced to divide in cultured corneas) [5, 6], HCECs are prone to corneal endothelial dysfunction that eventually could lead to blindness. However, the human corneas at birth are characterized by a considerable endothelial cells reserve; in fact during the first months of life, endothelial cell density is around 6.000 cells/mm^2, while dur- ing eighth decades of life, endothelial cell density is approxi- mately 2.600 cells/mm^2 [7, 8], while the percentage of cells with a hexagonal shape decreases from 75% to 60% [9]. The causes of endothelial cell decrease are age but also trauma, inflammation, corneal disease, and surgical procedures.

There are few diseases affecting primary the corneal endothelium [10]. Fuchs' endothelial corneal dystrophy (FECD) is the most common. In FECD, the pump function of the endothelial cells decreases, followed by a reduced barrier function [11] so that the endothelium becomes unable to maintain fluid balance and consequently corneal clarity. Other endothelial diseases are posterior polymorphous dys- trophy [12], congenital hereditary endothelial dystrophy [13, 14], and iridocorneal endothelial syndrome [15]. More- over, being the cornea the outer part of the eye, it is most likely to sustain damage due to various sources of stress, causing secondary corneal endotheliopathies. Main kinds of insults are external trauma; metabolic damage, such as hyp- oxia, for example, caused by contact lens wear [16], or hyperglycemia; toxic, for example, due to drugs or their preservatives; related to alterations in pH or osmolarity; associated to surgical procedures, in particular related to phacoemulsification and corneal transplantation [17–19]. The wound healing of endothelium has certain proper characteristics: endothelium mostly heals by cell migration and increased cell spreading and may undergo epithelial- mesenchymal transformation in this process, but cell proliferation plays a secondary role [20]. More in detail, the pro- cess of resurfacing an injured area is characterized by 3 steps. Endothelial cells do not undergo mitosis in vivo, so in the first step, the injured cells have to be quickly replaced by migration and extension of the adjacent surviving cells, in order to form a temporary barrier with reduced pump activity. So, surviving cells have the ability to "stretch" over the space of the degenerated cells, growing in size (polymegathism) and losing their typical hexagonal shape (pleomor- phism). Endothelial wound healing is associated with a transient acquisition of fibroblastic morphology and actin stress fibers by migrating cells, which is consistent with endothelial-mesenchymal transformation [21] [22]. In the second stage, the number of tight junctions and pump sites returns to normal levels; the endothelial cells form irregular polygons; the corneal thickness typically returns to normal, and transparency is restored. The third stage that can last for many months involves remodeling of the endothelial cells to form more regular hexagonal shapes. Anyway, the endothelium has a limited response to stress: mild injury may result only in changes in cell size and shape, while greater stress may result in cell loss that leads to irreversible alterations in the endothelial morphology and biological function [23]. When endothelial cell decreases below 500 cells/mm^2, the eye becomes at risk for the development of corneal edema, with loss of corneal clarity [24].

Whatever it is the mechanism of damage, at present, the only treatment for this dysfunction is a corneal transplantation, or keratoplasty, from donor cadavers. In keratoplasty, a wide part of the central cornea is replaced with a graft, an allogeneic cornea which has been removed *post mortem* from a donor. It can be classified on the basis of the transplantation technique [25]: from the replacement of full- thickness cornea with a healthy donor cornea, called full-thickness penetrating keratoplasty (PKP), to the replacement of only selective diseased layers, called partial lamellar corneal keratoplasty. The last one can be divided in anterior lamellar keratoplasty or posterior lamellar keratoplasty (PLK). In case of endothelial pathology, PKP or PLK may be indicated. Despite recent improvements in surgery techniques, corneal transplantation presents some limits and difficulties. The surgical techniques are hard to perform, and complications are possible, including choroidal hemorrhage, glaucoma, loose sutures, suture infiltrates, suture-associated astigmatism, dislocation of the graft, and graft infection [26–29]. The visual outcome is generally good, but the graft failure is not uncommon, making necessary a long-term immunosuppressive topical therapy [30, 31]. Any- way, the main problems of keratoplasty are represented by the necessity of a graft from a donor and by the legal, ethical and cultural issues related to the complex process of organ donation and transplantation. It is fundamental a good coor dination between medical officers, nurses, technicians, forensic experts, and the legal system in order to support graft availability. The whole process is not easy, and the need for donor corneas is increasing, contributing to a demand-supply shortage, especially in developing countries.

Due to keratoplasty limits and complications, along with a global shortage of donor corneas, it is important to find alternative strategies of treatment in order to overcome corneal transplantation. Considering all these issues, different strategies have been studied.

Recent studies highlight that stem cell–derived extracellular vesicles (EVs) play a relevant role in stem cell-induced

regeneration by reprogramming injured cells and by inducing proregenerative pathways. A particularly promising area of investigation seems to be the use of extracellular vesicles derived by mesenchymal stem cells (MSC-EVs) that could

be able to promote regeneration of damaged endothelium. MSC-EVs have been widely studied in various disease models [32, 33], and in the last decade, they have been of interest in many ophthalmologic pathologies[34, 35]. In 2018, indeed, it was evaluated the effect of MSC-EVs on corneal wound healing, and it was shown that human corneal MSC-EVs significantly increased the proliferation of human corneal epithelial cells in vitro and accelerated corneal wound closure in a murine epithelial mechanical injury model [36]. The aim of our study is, therefore, to investigate whether EVs, released by human bone marrow MSCs, may be beneficial in reducing ER-stress and HCEC apoptosis induced by an in vitro dam- age model caused by nutrient deprivation.

2. Material and Methods

2.1. Isolation and Characterization of HCECs. We isolated HCECs from discarded cornea patients undergoing corneal transplantation or enucleation ($N = 23$ patients) due to different pathologies (Table 1).

Briefly, the Descemet's membrane and corneal endothelial cells were stripped from the posterior surface of the

TABLE 1: Clinical and biological information of patients undergoing penetrating keratoplasty. In the table are listed the clinical and biological aspects of patients from which we received corneal buttons.

Sex	Age	Diagnosis	Surgical procedure	Medical history	Ophthalmological therapy
F	75	Ocular hypertonia of traumatic etiology	Enucleation	Allergies (indomethacin, tramadol, ciprofloxacin), arterial hypertension	Topical therapy: timolol, diclofenac
F	35	Keratoconus	PKP		Topical therapy: hydrocortisone
F	66	Fuchs' endothelial corneal dystrophy (FECD)	PKP	Smoking, diabetes mellitus type II, arterial hypertension	Topical therapy: loteprednol (od)
F	47	Corneal leukoma	PKP	Postsurgical hypothyroidism	
M	75	Corneal leukoma	PKP	Arterial hypertension, benign prostatic hypertrophy	Systemic therapy: acetazolamide topical therapy: brinzolamide, timolol
M	78	Corneal leukoma	PKP	Left eye trauma at 20 years old, arterial hypertension, diabetes mellitus type II, prostatic cancer	
F	83	FECD	PKP	Allergies (penicillin, metamizole), arterial hypertension, hypercholesterolemia, hypothyroidism, depression	Topical therapy: indomethacina, bromfenac, edenorm
F	47	FECD	PKP	Postsurgical hypothyroidism	
M	81	FECD	PKP	Arterial hypertension COPD, diabetes mellitus type II, benign prostatic hypertrophy	Topical therapy: netilmicin, dexamethasone, ofloxacin
M	40	Keratoconus	PKP	Allergies (pollen)	
F	40	Pellucid marginal degeneration	PKP	Allergies (pollen)	
F	77	FECD	PKP	Diabetes mellitus type II, diabetical neuropathy, acute myocardial infarction, kidney failure treated with transplant, HCV+	Topical therapy: brinzolamide, timolol, brimonidine
M	80	FECD	PKP	Arterial hypertension, keratoconus in both eyes	Topical therapy: cloramphenicol dexamethasone, bluyal a
F	70	Corneal leukoma	PKP	Pontomesencephalic cavernoma	
F	72	Corneal leukoma	PKP	Arterial hypertension, asthma, ulcerative rectocolitis	Topical therapy: trehalose, clobetasone
F	31	Keratoconus	PKP		
M	71	FECD	PKP		
M	67	FECD	PKP		
M	75			Late failure of transplanted cornea	
M	78			Late failure of transplanted cornea	

		Corneal leukoma (chemical burn) reflux disease, benign prostatic hypertrophy, biliary stones, paranoid psychosis		ute myeloid leukemia in remission, seasonal affective disorder
			PKP	Arterial hypertension, benign prostatic hypertrophy PKP
				Diabetes mellitus type II
		A c	PKP	Seasonal affective disorder
F	66	Corneal leukoma	PKP	Facio-scapular-humeral muscular dystrophy

COPD: chronic obstructive pulmonary disease; FECD: Fuchs' endothelial corneal dystrophy; HCV: hepatitis C virus; PKP: penetrating keratoplasty.

peripheral corneoscleral tissue using a scalp and afterwards digested with collagenase A (2 mg/ml). The digested membrane and cells (HCECs) were then placed on a Petri dish previously coated with fibronectin; HCECs migrated out of the Descemet's membrane and were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2 and cultured in ENDOGRO 10% FBS for seven passages. Once cells reached confluency, they were passaged at 1 : 2 ratios using 0.25% trypsin 0.02% ethylenediaminetetraacetic acid solution (Sigma-Aldrich, USA).

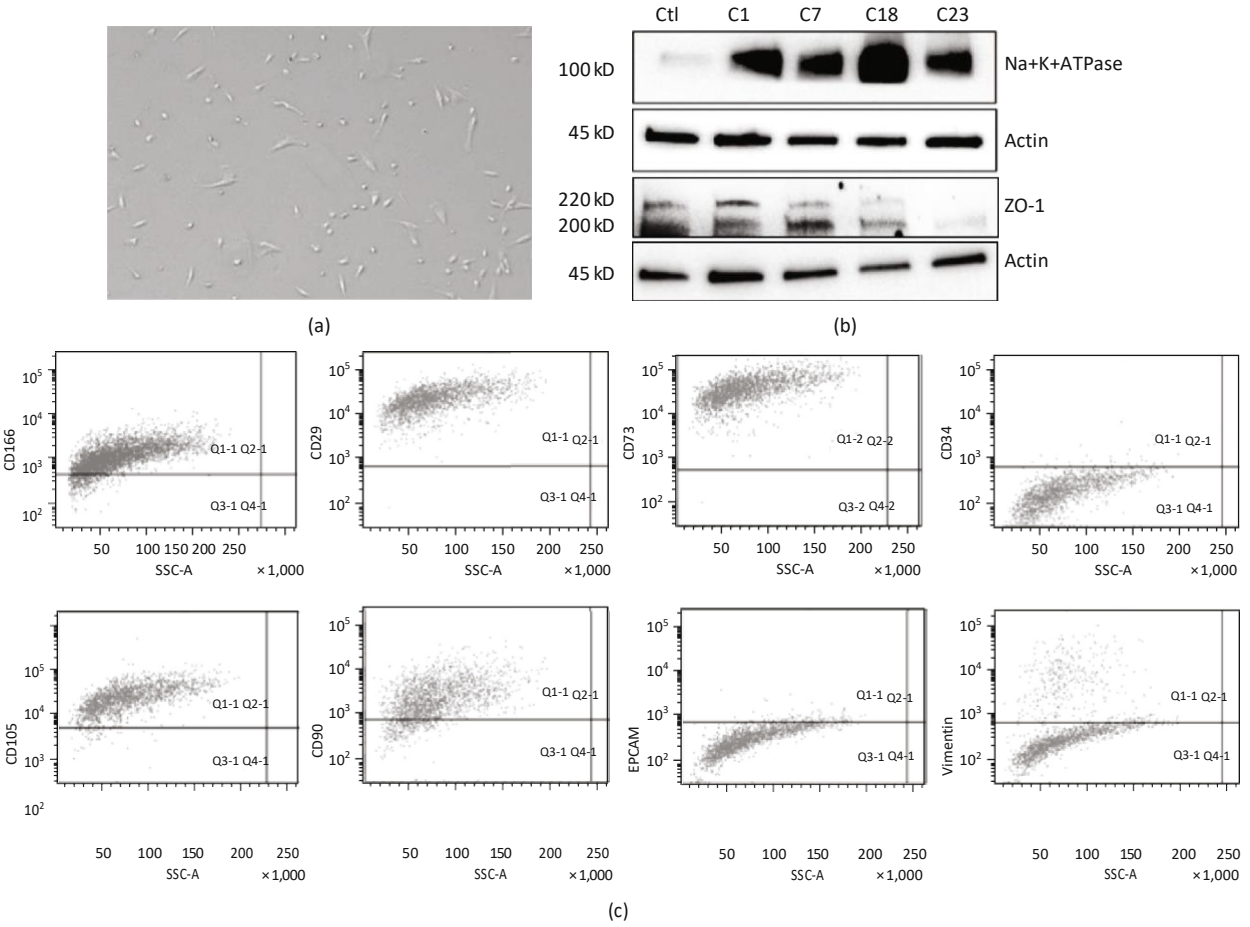

FIGURE 1: Isolation and characterization of corneal endothelial cells. (a) Representative micrograph of HCEC at passage 3. (b) Representative micrographs of western blot on four HCEC independent cell lines deriving from different patients (C1, C7, C18, and C23). The control (Ctl) is represented by the renal HK2 cell line, negative for Na + K + ATPase and positive for ZO-1. Actin was used as an endogenous loading reference. (c) Representative flow cytometry analysis of HCECs showing the expression of CD166, CD105, CD29, CD90, CD73, CD34, EPCAM, and Vimentin on HCECs.

From each patient we isolated a cell line of HCECs, each cell line was kept in culture up to the sixth passage; all experiments were performed between passages 2 and 4 (Figure 1(a)). HCECs deriving from three independent cell lines were then characterized by the expression of HCEC main marker ATP1A1 (Na + K + ATPase) and ZO-1 (Figure 1(b)) and by presence of the surface markers CD166 [37], CD105, CD29 [38], CD90, and CD73 and by the absence of CD34 [39], of the epithelial marker EPCAM, and of the stromal marker Vimentin (Figure 1(c)).

For protein analysis, HCECs were lysed at 4°C for 30 min in RIPA buffer (20 nM Tris·HCl, 150 nM NaCl, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.8) supplemented with protease and phosphatase inhibitor cocktail and PMSF (Sigma-Aldrich). Aliquots of the cell lysates containing 25 μg protein, as determined by the Bradford method, were run on 4-20% (BioRad) SDS- PAGE under reducing conditions and blotted onto PVDF membrane filters using the iBLOT system (Life Technologies). For western blot analysis, Na + K + ATPase (Abcam), ZO-1 (Invitrogen), and Actin (Santa-Cruz Biotechnology) antibodies were used for the characterization of HCECs.

For cytofluorimetric analysis, cells were detached using a nonenzymatic cell dissociation solution (Sigma-Aldrich) and resuspended in PBS 0.1% BSA (Sigma-Aldrich) and incubated with antibodies. The following antibodies, conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC), were used: CD166, CD34 (BD Biosciences), EPCAM, Vimentin, CD90, CD105, CD73, and CD29 (Miltenyi Biotech).

2.2. Isolation and Characterization of MSC-EVs. MSC-EVs were obtained as previously described [40]. In brief, bone marrow mesenchymal stem cells (MSCs) were obtained from Lonza, cultured, and characterized [41]. MSCs derived from five preparations were used up to the sixth passage of culture. MSC-EVs were obtained from supernatants of MSCs cultured overnight in RPMI deprived of FCS. After removal of cell debris and apoptotic bodies by centrifugation at 3000 g for 20 min, EVs were purified by 2 h ultracentrifugation at 100,000 g at 4°C (Beckman Coulter Optima L-90 K; Fullerton). EVs were used freshly or stored at -80°C after resuspension in RPMI supplemented with 1% dimethyl sulfoxide (DMSO). Analysis of size distribution and enumeration of EVs were performed using NanoSight LM10 (NanoSight Ltd.) equipped with a 405 nm laser and the Nanoparticle Tracking Analysis (NTA) 2.3 software (Figure 2).

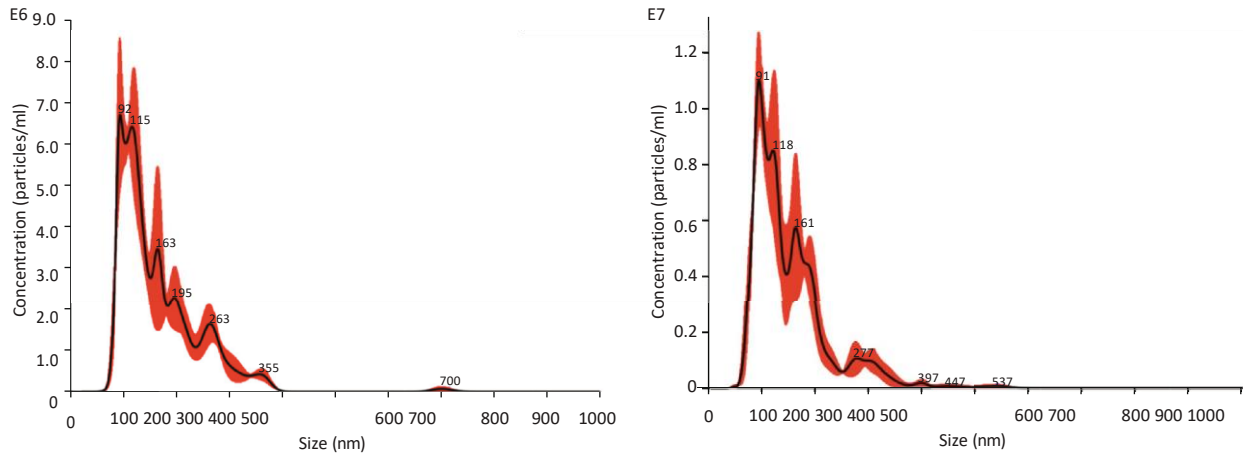

FIGURE 2: EV quantification using Nanosight Nanoparticle Tracking Analysis. MSC produced EVs in cell culture with a mean size of 163.3 nm, with a homogenous EV population.

2.3. Establishing a Serum Deprivation Damage Model on HCECs. In this study, serum deprivation culture was used to mimic the nutrient deficient environment in order to establish a valid *in vitro* damage model to test the regenerative potential of EVs on HCECs. We evaluated the proliferation rate and the apoptosis after exposing the cells to serum-deprived medium at different concentrations for 24 hours (Figure 3). Serum deprivation significantly inhibited HCEC proliferation in all the different concentrations of FBS (Figure 3(a)), and the survival of HCECs was inhibited at both 1% and 2% FBS presence (Figure 3(b)). We chose the 2% FBS concentration to go on with the experiments.

2.4. Evaluation of Regenerative Potential of MSC-EVs: Proliferation and Apoptosis Assay. For proliferation assay, cells were plated in growth medium at a concentration of 5000 HCEC-cells/well in a 96-multiwell plate and left adhere overnight. The day after the culture medium was removed and a new medium containing different concentrations of FBS (1-5%) was added to the cells to induce a damage. After 24 hours of serum deprivation, differential concentrations of MSC-EVs ($5\text{-}20 \times 10^3$ MSC-EV/cell) were added to the medium for further 24 hours. DNA synthesis was detected after 4 hours of incorporation of 5-bromo-2-deoxyuridine (BrdU) using an enzyme-linked assay kit (Chemicon). Data are expressed as the $\text{mean} \pm \text{SD}$ of the media of absorbance of at least three different experiments, normalized to control (not treated cells).

To evaluate apoptosis, Annexin V assay was performed using the MuseTM Annexin V and Dead Cell Kit (Millipore), according to the manufacturer's recommendations and following the methods of Brossa et al. [42]. Briefly, 30×10^3 cells in a 24-well plate were incubated with different concentrations of FBS for 24 hours, and different amount of MSC- EVs was added to the medium for further 24 hours. Cells were then detached and resuspended in MuseTM Annexin V and Dead Cell Kit, and the percentage of apoptotic cells (Annexin V+) was detected.

2.5. Evaluation of HCEC Migration: Wound Healing Assay. For the wound-healing migration assay, 30×10^3 HCECs in a 24-well plate kept in damage conditions for 24 hours and treated for further 24 hours with 10×10^3 MSC-EV/cell (2% FBS) were scratched using a 10 µl pipette tip once the cell confluence reached approximately 90%. Then, the detached cells were washed and removed. Representative photographs were taken under a light microscope (Olympus Life Science) at 0 h and 6 h after wounding. The scratch length was measured three times for each photograph; 10 photographs per condition were taken.

2.6. Statistical Analysis. Statistical analysis was performed by using one-way ANOVA with Tukey's multicomparison tests, with a single pooled variance. A p value of $p < 0{:}05$ was considered significant.

3. Results

3.1. MSC-EVs Induce Proliferation and Survival of HCECs. In the selected serum deprivation damage conditions, the treatment with different doses of MSC-EVs resulted in a significantly higher proliferation rate of HCECs at all the tested concentrations of EVs ($5\text{-}20 \times 10^3$ MSC-EV/cell) (Figure 4(a)). We then evaluated the percentage of apoptotic cells after serum deprivation and following the treatment with MSC-EVs. 20×10^3 MSC-EVs/cell induced a significant decrease in number of total apoptotic cells (Figure 4(b)) after 24 hours of serum deprivation (Figure 4).

3.2. MSC-EVs Induce HCECs Migration. The wound healing assay showed a significantly faster repair of the wound after HCEC treatment with MSC-EVs (10×10^3 EV/cell) in the serum deprivation model. We can see in Figure 5 that in damage conditions, after 6 hours from the scratch in presence of MSC-EVs (T6 $2\% + \text{MSC} - \text{EVs}$), the length of the scratch is significantly shorter than the scratch in absence of MSC-EVs (T6 2%).

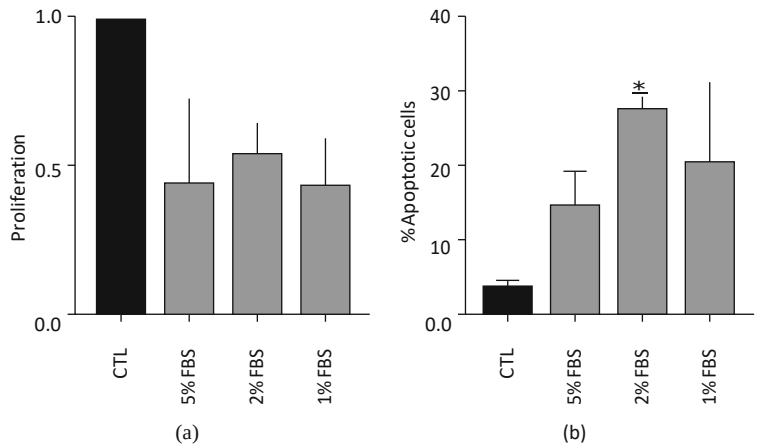

FIGURE 3: Damage setting by serum deprivation of HCECs. (a) Proliferation levels at different concentration of FBS (5-1%) of HCECs after 24 h of treatment. Data are represented as mean ± SD of three independent experiments normalized to CTL. (b) Percentage of apoptotic HCECs at different concentration of FBS (5-1%) of HCECs after 24 h of treatment. Data are represented as mean ± SD of three independent experiments normalized to CTL. One-way ANOVA analysis with Tukey's multicomparison tests was performed among FBS and CTL ($^{*}p < 0{:}05$, $^{**}p < 0{:}001$).

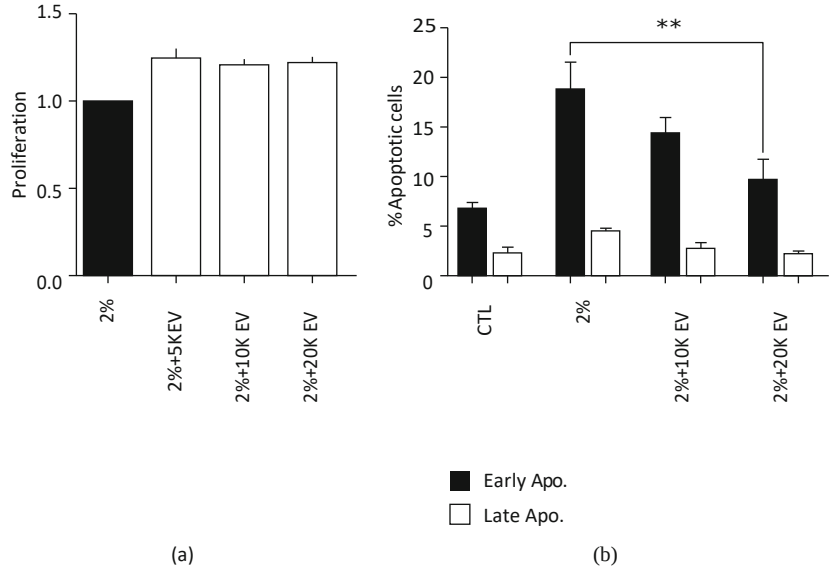

FIGURE 4: Proliferation and apoptosis of HCECs in damage conditions, treated with different doses of MSC-EVs. (a). Proliferation levels of HCECs maintained in 2% FBS for 24 hours and treated for further 24 hours with different doses of MSC-EVs (5-20 × 10^3 MSC-EV/cell). Data are represented as mean ± SD of four independent experiments normalized to CTL. (b). Percentage of early apoptotic (black columns) and late apoptotic (grey column) HCECs cultured either in ENDOGRO 10% (CTL) or in 2% FBS for 24 hours (2%) and treated for further 24 hours with different doses of MSC-EVs (10 and 20 × 10^3 MSC-EV/cell). One-way ANOVA analysis with Tukey's multicomparison test was performed among 2% FBS alone and 2% + EVs ($^{*}p < 0{:}05$, $^{**}p < 0{:}001$).

4. Discussion

MSCs, isolated in many tissues, have been originally described as multipotent stem cells with the potential to differentiate into different cell types [43]. This ability represented the initial drive for their therapeutics use, but this original rationale has then become gradually weaker. In fact, many studies reported that despite functional improvement after MSC transplantation, MSC engraftment and differentiation into proper cell types were infrequent [44, 45]. More- over, efficacy of MSCs did not seem to be dependent on the physical proximity of the transplanted cells to the target tis- sue [46]. These evidences led to the idea that MSCs may exert their therapeutic effects thanks to a paracrine action [47]. Nowadays, MSCs are increasingly seen as stromal support progenitor cells with the potential to differentiate into stromal support cells and secrete factors able to limit cellular injury to support the stroma or other cells, by maintaining a microenvironmental niche that equilibrate the quiescence of stem [48, 49]. At the beginning, efforts to characterize MSC secretion focused on small molecules such as growth factors, chemokines, and cytokines, but no one could sufficiently account for the efficacy of MSCs, until a study con- ducted in 2009 demonstrated that microvesicles, a class of EVs, secreted by MSCs protected against acute tubular injury [50]. Since then, EVs have been more and more reported as the therapeutic driving force of MSCs. MSCs have been reported to secrete different classes of EVs: microvesicles, microparticles, and exosomes [51, 52]. These classes are currently defined by physical and biological parameters [53]. However many criteria are not exclusive to any specific class so that the presence of distinct biological entities is not sure [54].

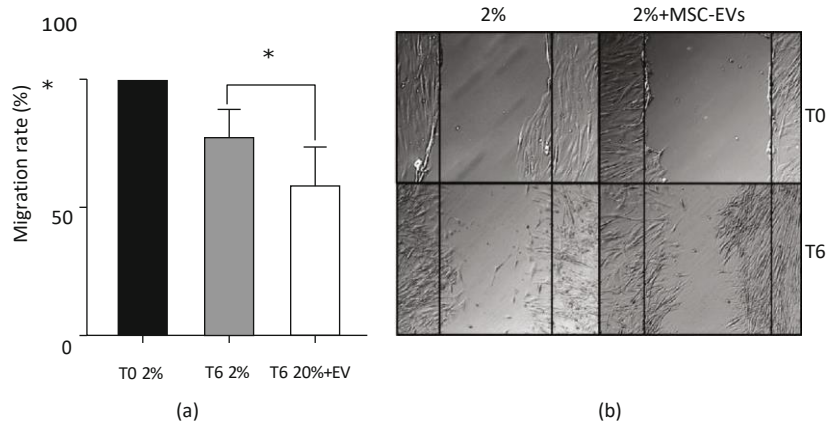

FIGURE 5: Wound healing assay of HCECs in damage conditions treated with MSC-EVs. (a) Wound healing assays were performed to evaluate cell motility of the HCECs in damage conditions (2% FBS); the migration rate was measured by the length of the scratch at different timings (T0 and T6), in absence (T6 2%) or in presence of 20×10^3 MSC-EV/cell (T6 2% + EV). Data are represented as mean ± SD of nine independent experiments normalized to T0 2%. One-way ANOVA analysis with Tukey's multicomparison test was performed among 2% FBS and 2% + EVs ($^{**}p < 0{:}001$). (b) Representative pictures of HCECs in damage conditions (2% FBS) cultured in the presence or absence of 20×10^3 MSC-EV/cell (2% + EV). Pictures were taken at time 0 (T0) and after 6 hours from the scratch (T6).

EVs act as one-way conveyors of cellular material, including nucleic acids, proteins, and lipids, from a secreting cell to a target cell to modulate its activity. They represent an inter- cellular communication vehicle secreted by MSCs to exert a stromal support function by regulating cellular processes such as communication, structure and mechanics, inflammation, tissue repair and regeneration, and metabolism. In addition, EVs are able to mediate MSC interactions with several cell types in immediate but also remote areas; in fact thanks to their biophysical features, they can be easily transported through the blood and other biological fluids, acting in para- and endocrine manner. Their final aim is the maintenance of a dynamic and homeostatic microenvironment. This role is particularly important when tissue microenvironment is altered by an injury or a disease [55]. As a consequence, extensive research is focusing on the potential therapeutic applications of EVs in several areas [56]. More in general, the idea of MSC-EVs as stromal support mediators provides a rationale for the therapeutic efficacy of MSCs and their secretions in a wide spectrum of diseases, also in ophthalmic area, in order to restore tissue homeostasis and enhance tis- sues recovery, reparation, and regeneration.

Many functions of EVs have been identified. Currently, the best described stromal support function of MSC secretions is the preservation of hematopoietic stem cell homeo- stasis [57] and the tumor microenvironment [58]. On the other hand, investigations about EV functions in many specialized tissues of the eye are just at the beginning. However, the etiology of several eye pathologies, including age-related macular degeneration (AMD), diabetic retinopathy, glaucoma, and corneal angiogenesis, involves activation of immune cells, inflammation, fibrosis, cell degeneration, and neovascularisation, with exosomes being probable mediators of these processes. As a consequence, their employment may be a therapeutic strategy for such eye disorders [59–66]. Cell death is part of the pathology process in all eye diseases, for example, trabecular meshwork (TM) cells and retinal ganglion cells (RGCs) in glaucoma [67, 68], retinal pigment epithelium cells and photoreceptors in AMD with geographic atrophy [69], and corneal surface and endothelial cells in corneal diseases [70], so stem cell-based strategies are being studied in order to obtain cell replacement [71–73]. In practice, up to date, stem cell therapies have shown limited success. For example, differentiation of stem cells into RGC- like cells has only been accomplished in culture [74]. In addition, another limit of this strategy is the potential tumorigenic and immunogenic risks [75].

Anyway, as discussed before, being the main therapeutic effect of stem cells probably due to their paracrine action [76], another strategy may be exosome employment that may have several possible applications in ophthalmic pathology. Scientific research has recently focused on the use of exosomes derived from MSCs in various models of retinal damage in vitro and in vivo as they, compared to MSCs, have similar functions and at the same time have different advantages such as greater stability and handling, a lower chance of immunological rejection, and no risk of malignant transformation, being a cell-free approach. The treatment of pathologies and retinal damage with MSC-EVs typically takes place through intravitreal injection, allowing their direct action on the retinal cells and avoiding potential adverse effects towards other organs. Their use has been successfully studied in several diseases of the retina, such as retinal cell degeneration, refractory macular holes, and retinal detachments [35– 77]. In all these cases, EVs have demonstrated a therapeutic effect, encouraging the realization of further studies. Exosomes from MSCs may also be able to enhance neural repair, and this action may be useful to restore retinal ganglion cell glaucoma [78]. They can also stimulate proliferation in a number cell types [79, 80], being a possible strategy to induce proliferation of TM cells in glaucoma [81]. Finally, the immunomodulatory effects of exosomes could be used to decrease inflammation and fibrosis for treatment of inflammatory eye diseases [82, 83] but also dry eye disease mediated by activation of immune cells [84].

Nevertheless, research about EVs in corneal pathology has been limited. Among corneal pathologies, Samaeekia et al. recently evaluated the effect of MSC-derived EVs on corneal wound healing and showed that human corneal MSC-EVs significantly increased the proliferation of human corneal epithelial cells *in vitro* and accelerated corneal wound closure *in vivo*[36]. Moreover, it was recently reported that the coculture of corneal stromal cells with MSC-EVs resulted in enhanced viability and proliferative ability and increased plasticity [85]. Starting from this preexisting data, we established an *in vitro* model to study the effect of MSC-EVs on a different corneal layer, the corneal endothelium.

In our work, the results highlight the already well-known proregenerative potential of MSC-EVs in a totally new bio- logical model. In the selected serum deprivation damage conditions, the treatment with different doses of MSC-EVs resulted in a significantly higher proliferation rate of HCECs at all the tested concentrations of EVs. MSC-EVs/cell induced a significant decrease in number of total apoptotic cells after 24 hours of serum deprivation. Finally, the wound healing assay showed a significantly faster repair of the wound after HCEC treatment with MSC-EVs.

Our group is working on further research to provide more insights into understanding of multiple aspects of MSC-EVs in HCEC protection. An important facet to be defined is the mechanisms of the regenerative potential of MSC-EVs on HCECs. As previously described, many studies have shown that MSCs can protect tissues from damage through the paracrine actions of EVs. The content of an EV is dependent on its origin, size, and the route of biogenesis. EVs are rich with protein such as platelet-derived growth fac- tor (PDGF), organelles, lipids, mRNAs, microRNAs, mitochondria, and cytokines [86]. The presence of a complex cargo within EVs results in a multilevel

modulation of cell functions in the recipient cells [87].

For example, PDGF is a putative antiapoptotic factor that seems to be able to stimulate HCEC growth [88]. PDGF stimulates the proliferation of megakaryocytes, erythrocytes, leukocytes, and their progenitors, presumably through the multiple endogenous growth factors released from mesenchymal stem/stromal cells [89]. Joyce et al. have provided evidence that HCEC in vivo is arrested in G1-phase of the cell cycle [90], but they have also demonstrated that HCECs can proliferate in vitro in response to growth-promoting agents [88]. Their study compared the effect of several growth-promoting agents on proliferation of HCECs from young and older donors. Nerve growth factor did not enhance proliferation above basal levels, regardless of donor age. Epidermal growth factor (EGF) moderately stimulated proliferation in cells from younger donors but not in HCECs from older donors. On the other side, PDGF and bovine pituitary extract stimulated proliferation above the level induced by EGF, while the combination of these two agents had a strong additive effect, notably increasing cell proliferation above that achieved with either factor alone. Of the growth- promoting agents tested, fetal bovine serum was the one that stimulated the greatest proliferation of HCECs, in both younger and older donors. Proliferation in the presence of multiple mitogens ceased when confluence was reached, indicating the formation of a contact-inhibited monolayer.

Another component that may be involved in proliferative potential of EVs is encapsulated mitochondria. MSCs are shown to transfer mitochondria to the recipient cells in different ways: encapsulated within EVs, via cell-to-cell direct communication through tunnelling nanotubes, or through direct release of "naked" mitochondria into the extracellular microenvironment [86]. The organelle incorporates into the endogenous mitochondrial network of the damaged cell that needs to be rescued, reestablishing its bioenergetic homeostasis [91]. The incorporation of mitochondria within released microvesicles has been widely studied in MSCs, where once internalized, mitochondria containing microvesicles can res- cue cells from damage or act as reprogramming agents [90]. Recent findings suggest that mitochondrial transfer may have a key role in protection of ocular cells, including corneal epithelial cells and RGCs. Jiang et al. demonstrated that MSCs can efficiently donate functional mitochondria and protect corneal epithelial cells from oxidative stress-induced damage through tunnelling nanotube (TNT) formation. Further- more, oxidative inflammation improved the efficiency of mitochondrial transfer from MSCs to stressed corneal epithelial cells and increased TNT formation that is regulated by the NF-κB signaling pathway [92]. Jiang et al. more recently provided evidences that intravitreal transplanted induced pluripotent stem cells-derived MSCs (iPSC-MSCs) can effectively donate functional mitochondria to RGCs and protect against mitochondrial damage-induced RGC loss [93]. Mitochondrial transfer from MSCs could provide a novel mechanism of protection also for the corneal endothelium.

Additionally, it seems that a proinflammatory environment can enhance MSC-EV production. As discussed, MSCs displayed a high potential due to secretion of therapeutic fac- tors, both free and conveyed within EVs, and collectively termed secretome. Ragni et al. tried to characterize adipose- derived MSC- (ASC-) secreted factors and EV-miRNAsand their modulation after IFNγ preconditioning in joint disease [94]. Given the assortment of soluble factors and EV-miR- NAs, ASC secretome showed the ability to promote cell motility and modulate inflammatory and degenerative pro- cesses. Preconditioning is able to increase this ability, suggesting inflammatory priming as an effective strategy to obtain a more potent clinical product which use should always be driven by the molecular mark of the target pathology.

In summary, theoretical framework and results of our study in the corneal endothelium setting are promising, but further research will be needed in order to better understand the key components of MSC-EVs that are acting on the protection and damage restoration of HCECs.

For future clinical application of our results, it should be considered that conventional manufacturing approaches of BM-MSCs are challenged by their limited capacity to proliferate and their loss of differentiation potential. Indeed, human iPSC-MSCs may represent a better option to overcome these limitations and be more indicated for tissue regeneration [95]. iPSC-MSCs may therefore represent an unlimited cell source for the production of EVs in large scale [96].

5. Conclusions

In conclusion, in our study, we showed that *in vitro* MSC-EV administration on HCECs has been able to induce proliferation and migration of damaged HCECs and was effective in inhibiting cell apoptosis. This work could represent a valid starting point to explore the effect of MSC-EVs on this type of model in order to make it possible in the future to exploit MSC-EV treatment *in vivo* in patients with corneal endothelial dysfunctions. Our finding suggests that human MSC-EVs may represent a novel therapeutic approach that could lead to an increasing independence of eye banks that could be of great importance to reduce the number of worldwide corneal blindness. Anyway, more studies are useful to determine their possible therapeutic value and the mechanisms involved, so they should be actively pursued in the future.

Abbreviations

AMD: Age-related macular degeneration EGF: Epidermal growth factor
EVs: Extracellular vesicles
FECD: Fuchs' endothelial corneal dystrophy HCECs: Human corneal endothelial cells
iPSC-MSCs: Induced pluripotent stem cells-derived MSCs MSCs: Mesenchymal stem cells
MSC-EVs: Extracellular vesicles derived from mesenchymal stem cells
PDGF: Platelet-derived growth factor PKT: Penetrating keratoplasty
PLK: Posterior lamellar keratoplasty RGCs: Retinal ganglion cells
TM: Trabecular meshwork
TNTs: Tunnelling nanotubes.

Data Availability

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

Ethical Approval

The study was approved by the Intercompany Ethics Committee of *A.O.U. Città della Salute e della Scienza* (Turin), on 21st February 2020 with a reference number 00184/2019.

Consent

Informed written consent was obtained from all tissue owners.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

All the authors have equally contributed to the design of the work, the data collection and analysis, and the search of the literature and cooperated to write the article. The manuscript, in the present form, has been approved by all authors.

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Chapter 5 – Paper "Mesenchymal Stem Cell-Derived Extracellular Vesicles Protect Human Corneal Endothelial Cells from Endoplasmic Reticulum Stress-Mediated Apoptosis"

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Article

Mesenchymal Stem Cell-Derived Extracellular Vesicles Protect Human Corneal Endothelial Cells from Endoplasmic ReticulumStress-Mediated Apoptosis

Lola Buono [1], Simona Scalabrin [2], Marco De Iuliis [2], Adele Tanzi [1], Cristina Grange [3], Marta Tapparo [3], Raffaele Nuzzi [2,†] and Benedetta Bussolati [1,*,†]

1 Department of Biotechnology and Health Sciences, University of Turin, 10126 Turin, Italy; lola.buono@unito.it (L.B.); adele.tanzi@edu.unito.it (A.T.)

2 Eye Clinic, Department of Surgical Sciences, University of Turin, AOU Città della Salute e della Scienza, Ophtalmic Clinic, 10126 Turin, Italy; scalabrin.simona@gmail.com (S.S.); marco.deiuliis@edu.unito.it (M.D.I.); raffaele.nuzzi@unito.it (R.N.)

3 Department of Medical Sciences, University of Turin, 10126 Turin, Italy; cristina.grange@unito.it (C.G.); marta.tapparo@unito.it (M.T.)

* Correspondence: benedetta.bussolati@unito.it; Tel.: +39-011-6706453

† These authors contributed equally to this work.

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Abstract: Corneal endothelial dystrophy is a relevant cause of vision loss and corneal transplantation worldwide. In the present study, we analyzed the effect of mesenchymal stem cell (MSC)-derived extracellular vesicles (MSC-EVs) in an in vitro model of corneal dystrophy, characterized by endoplasmic reticulum stress. The effects of MSC-EVs were compared with those of serum-derived EVs, reported to display a pro-angiogenic activity. MSC-EVs were able to induce a significant down- regulation of the large majority of endoplasmic reticulum stress-related genes in human corneal endothelial cells after exposure to serum deprivation and tunicamycin. In parallel, they upregulated the Akt pathway and limited caspase-3 activation and apoptosis. At variance, the effect of the serum EVs was mainly limited to Akt phosphorylation, with minimal or absent effects on endoplasmic reticulum stress modulation and apoptosis prevention. The effects of MSC-EVs were correlated to the transfer of numerous endoplasmic reticulum (ER)-stress targeting miRNAs to corneal endothelial cells. These data suggest a potential therapeutic effect of MSC-EVs for corneal endothelial endoplasmic reticulum stress, a major player in corneal endothelial dystrophy.

Keywords: exosomes; corneal endothelium; corneal dystrophy

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Introduction

Corneal endothelium, a monolayer of endothelial cells attached to the Descemet mem- brane [1], is required to maintain corneal transparency and clarity [2]. A loss of corneal endothelial cells leads to vision impairment and corneal edema. Fuchs' endothelial corneal dystrophy, a common cause of corneal vision loss, is characterized by endothelial damage as a result of sporadic or autosomal dominant non-inflammatory endothelial dystrophy [3]. So far, there is no definitive conservative non-surgical treatment [4],and this pathology is among the leading causes of primary corneal transplant surgery in Europe and in the United States. Endothelial corneal dystrophy is believed to be caused primarily by endoplasmic reticulum (ER) stress [5]. In particular, the activation of ER stress is known to induce corneal endothelial cell apoptosis, leading to progressive detachmentof the cells from the Descemet membrane, starting from the center and spreading to the periphery [5,6]

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Intracellular transduction pathways activated by ER stress, collectively

termed as unfolded protein response, are a cellular counteraction to damage aimedat restoring protein folding homeostasis [7]. The ER stress pathways are triggered by three main receptors (PERK, IRE1, and ATF6), which are normally maintained inactivated in the ER through the binding of the chaperone protein GRP78. During ER stress, GRP78 detaches from those receptors, causing their activation and the induction of ER stress related genes [8].

At present, as endothelial corneal dystrophy is only cured by transplantation from a cadaveric donor cornea, it is of relevant importance to find alternative strategies to induce regeneration, survival, and to inhibit ER stress activation in corneal endothelial cells. Extracellular vesicles (EVs) are a new promising therapeutic tool for tissue regeneration. EVs are present in all biological fluids, including blood [9,10]; they are released by almost all cell types; and they play a central role in cell-to-cell communication through the transfer of their cargo to target cells [11–13]. Among different EV sources, mesenchymal stem cell (MSC) derived EVs possess unique anti-inflammatory, anti-apoptotic, tissue repairing, proangiogenic, and immunomodulatory properties, similar to their parental cells [14,15]. In the last decade, MSC-EVs have been of interest in many ophthalmologic pathologies, from retinal damage to corneal diseases, with promising emerging findings [14,16–19]. For instance, corneal MSC-EVs ameliorated corneal epithelial damage, showing an increased proliferation of human corneal epithelial cells in vitro, and accelerated corneal wound closure in a murine epithelial mechanical injury model [17]. Our group recently reported the proangiogenic effect of MSC-EVs in an in vitro model of corneal endothelium damage due to serum deprivation. MSC-EVs induced the proliferation and survival of damaged human corneal

endothelial cells (HCECs) and promoted their wound closure [20].

In the present study, we first aimed to study MSC-EVs' effects on HCECs, investigating their effect in in vitro models of ER stress induced apoptosis, as well as the possible mechanisms involved. In particular, we used both serum starvation and tunicamycin, a specific ER-stressor, to examine the effects of MSC-EVs on ER stress signaling pathways. Moreover, we compared the effects of MSC-EVs with that of blood serum-derived EVs (SER- EVs), isolated from healthy donors, already reported to induce angiogenesis in vitro and repair from acute ischemia in vivo [21]. Finally, we examined the MSC-EV microRNA cargo involved in the modulation of the ER stress pathways, and its transfer to the target cells.

1. Results

1.1. Isolation and Characterization of Human Corneal Endothelial Cells and EVs

HCECs were isolated and characterized as previously described [20]. In brief, we isolated HCECs from discarded corneas of patients undergoing corneal transplantation or enucleation (N = 23 patients; Supplementary Table S1). A cell line of HCECs was isolated from each patient; all of the experiments were performed between passage 2 and 4. The HCECs were characterized by morphology, showing cells with an organized cytoskeletal structure and with the presence of the typical corneal endothelial markers [1]: CD166, Na^+K^+ATPase, and ZO-1 (Figure 1A–C) [22].

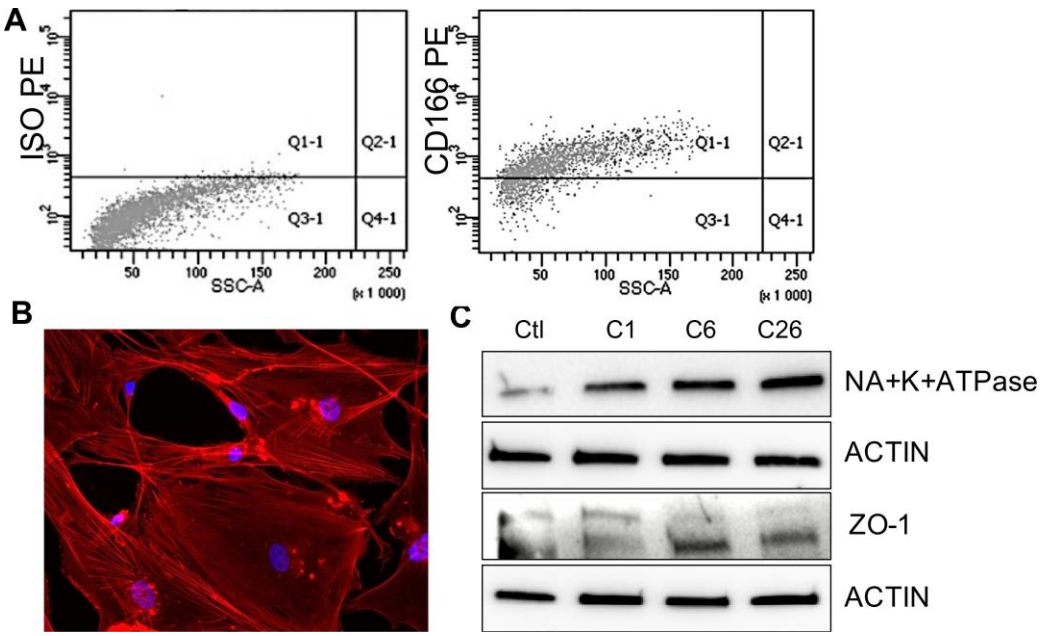


Figure 1. Isolation and characterization of human corneal endothelial cells. (**A**) Representative flow cytometry analysis of human corneal endothelial cells (HCECs) showing the negative staining of a control isotype (ISO PE) and the positive expression of CD166. (**B**) Representative immunofluorescence micrographs of HCECs stained with phalloidin (red), showing an elongated morphology. Blue = nuclear stain DAPI, original magnification: ×20. (**C**) Representative Western blot images of three HCEC independent cell lines positive for Na^+K^+ATPase and ZO-1. The renal HK2 cell line was used as a reference (Ctl). Actin was used as an endogenous loading reference.

MSC-EVs were isolated from the supernatants of MSCs cultured overnight in FCS- deprived RPMI, and SER-EVs were isolated from 100 mL of serum obtained from a blood pool of five healthy donors. The MSC supernatant and serum were then centrifuged for the removal of cell debris and apoptotic bodies at 3000g for 20 min. EVs were purified by 2 h of ultracentrifugation at 100,000g at 4 °C [21,23]. EVs were analyzed by size distribution and were quantified using the nanoparticle tracking analysis. MSC-EVs and SER-EVs appeared as homogenous EV populations with a mean size of 159.6 nm and 238.9 nm, respectively(Figure 2A). The EV size, morphology, and tetraspanin expression

were assessed using super resolution fluorescence microscopy (Figure 2B), confirming the simultaneous presence of tetraspanins on the surface of EVs derived from both sources. CD9 and CD81 appeared in a clustered, uneven distribution in the EV membrane, at a variance of CD63. This could be related to the reported ability of tetraspanins to homodimerize and form large complexes [24]. In addition, EVs were characterized by their surface marker expressions, including tetraspanins, mesenchymal, endothelial/platelet, and immunological markers, using a MACSPlex Exosome analysis kit after bead-based immunocapture (Figure 2C–E). The levels of tetraspanins CD9, CD63, and CD81 were high in both of the EV types (Figure 2E). The EVs isolated from the serum expressed immune system markers such as CD40, CD8, CD24, and HLA-DR (Figure 2E), while MSC-EVs showed higher levels of class-I HLA-ABC. MSC typical markers CD105, CD146, CD44, SSEA4, CD29, and CD49e were confirmed in the MSC-EVs (Figure 2E). Endothelial and platelet markers CD62P, CD42a, CD69, CD31, and CD41b were in turn typically expressed in the EVs derived from the serum (Figure 2E).

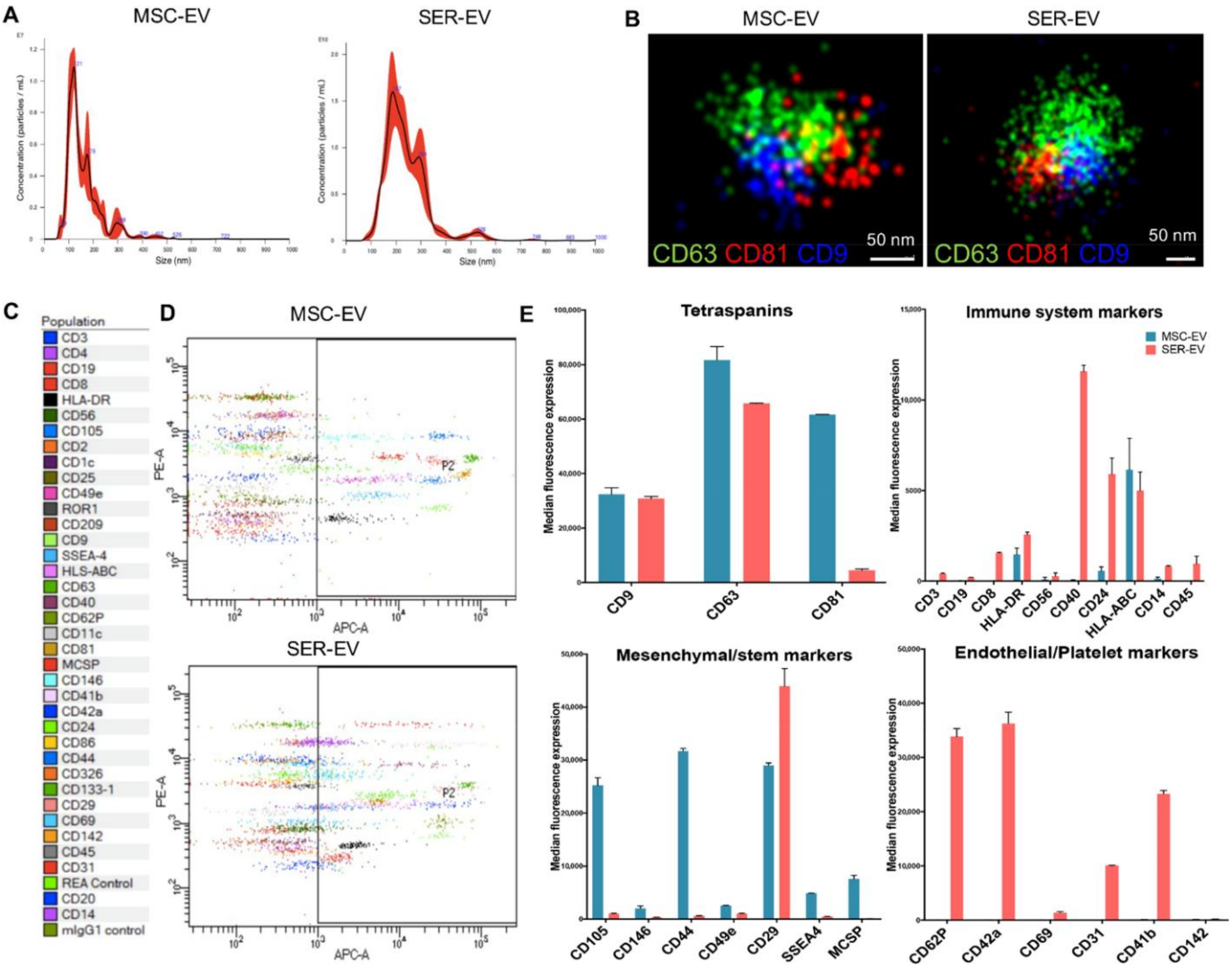


Figure 2. Characterization of mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) and blood serum-derived EVs (SER-EVs). (**A**) Representative nanoparticle tracking analysis showing the EV size distribution. (**B**) Super resolution microscopy micrographs showing the pattern distribution of CD63 in green, CD81 in red, and CD9 in blue for one MSC-EV and SER-EV. Scale bar: 50 nm. (**C**) Legend showing the 39 antibodies used in the assay and their respective colors in the dot plots. (**D**) MACSPlex representative dot plots showing the MSC-EV and SER-EV distribution of allophycocyanin (APC)-stained bead populations; captured EVs are counterstained with APC-labeled detection antibodies using a mixture of anti-CD9, anti-CD63, and anti-CD81 (pan tetraspanins) antibodies. (**E**) Representative quantification of the median APC fluorescence positive values for the bead populations after background correction, clustered in different graphs according to their classification: tetraspanins, immunological, mesenchymal, and endothelial markers.

1.2. ER Stress Induction by Serum Deprivation and Tunicamycin in HCECs

In order to set the model of ER stress in HCECs, we first exposed HCECs to different doses of tunicamycin. In fact, this drug is known to inhibit the first step in the N-linked glycan biosynthesis of proteins, thus inducing misfolded proteins and an unfolded protein response [25]. When tunicamycin was applied to HCECs, the expression levels of the main ER stress-related proteins GRP78 [26] and C/EBP homologous protein (CHOP) [27] were significantly increased in a dose-dependent manner (Figure 3). In parallel, we used the serum deprivation model of HCEC damage already set for previous experiments [20]. In this model, ER stress related genes were upregulated in a comparable manner to the tunicamycin treatment, confirming nutrient deprivation as an inducer of ER stress. The GAPDH levels were similar between the samples in each experiment.

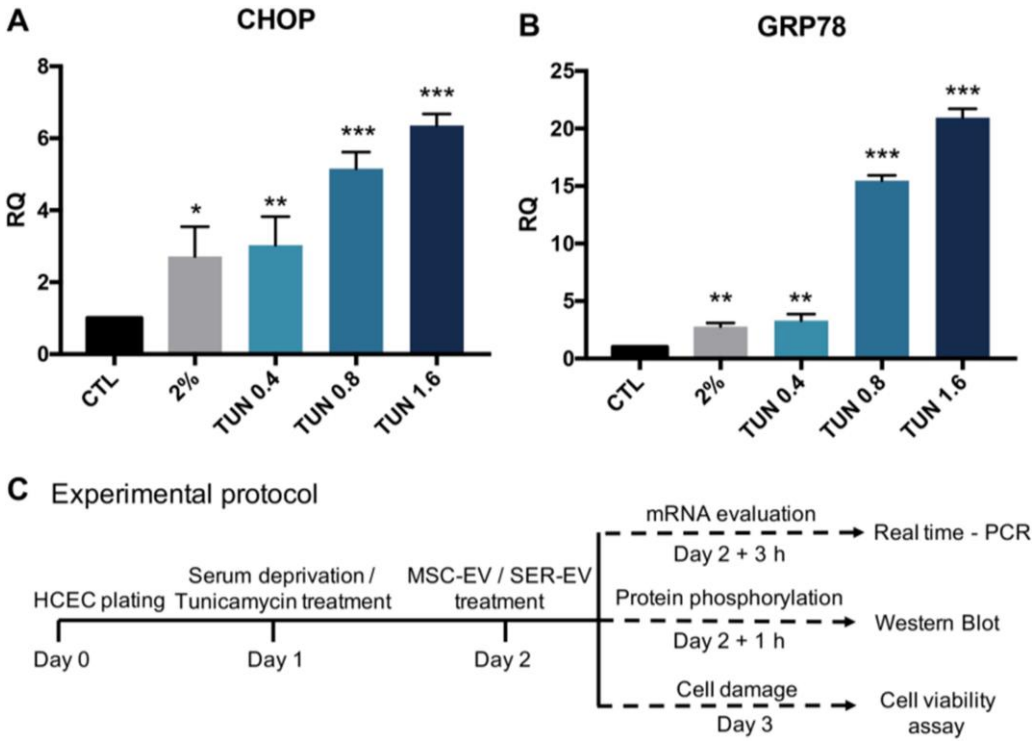


Figure 3. (**A**) Tunicamycin and (**B**) serum deprivation both induce ER-stress. HCECs were treated with 2% Fetal bovine serum (FBS) (2%) and with increasing doses of tunicamycin (TUN 0.4–1.6 ng/mL) for 24 h. The mRNA

levels of C/EBP homologous protein (CHOP) and GRP78 significantly increased in all of the damage conditions, when compared with the untreated cells (CTL). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous normalizer. Data were further normalized to CTL, set as 1, and used as a reference sample for each experiment. The graphs show the RQ average ($2^{-\Delta\Delta Ct}$) of at least three independent experiments ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed after the normalization of each experiment to CTL; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$ with respect to CTL. (**C**) Experimental protocol used in the different experiments involving MSC- and SEV-EV treatments.

1.3. ER Stress Induction by Serum Deprivation and Tunicamycin in HCECs

We subsequently analyzed the effect of MSC-EVs and SER-EVs on the ER stress dam-age model described above, by evaluating the typical ER stress-related genes ATF4, GRP78, XBP1t, and CHOP [5,25,26]. Cells were deprived of serum or treated with tunicamycin for 24 h, and subsequently stimulated with MSC-EVs or SER-EVs for a further 3 h (Figure 3C). The lower concentration of tunicamycin (0.4 ng/mL) was used, as the results were comparable to those obtained with serum deprivation. As expected, a significant upregulationof these ER stress-related genes was observed when the HCECs were deprived of serum or treated with tunicamycin in comparison with the untreated HCECs (CTL; Figure 4). Moreover, MSC-EVs were able to induce a significant down-regulation of most ER stress-related genes. In particular, ATF4, GRP78, XBP1t, and CHOP were all reduced in HCECs after exposure to serum deprivation and tunicamycin (Figure 4). At variance, SER-EVs only partially recovered the ER stress damage, modulating ATF4 in serum deprivation and GRP78 in tunicamycin exposure (Figure 4). The GAPDH levels were similar between the samples in each experiment.

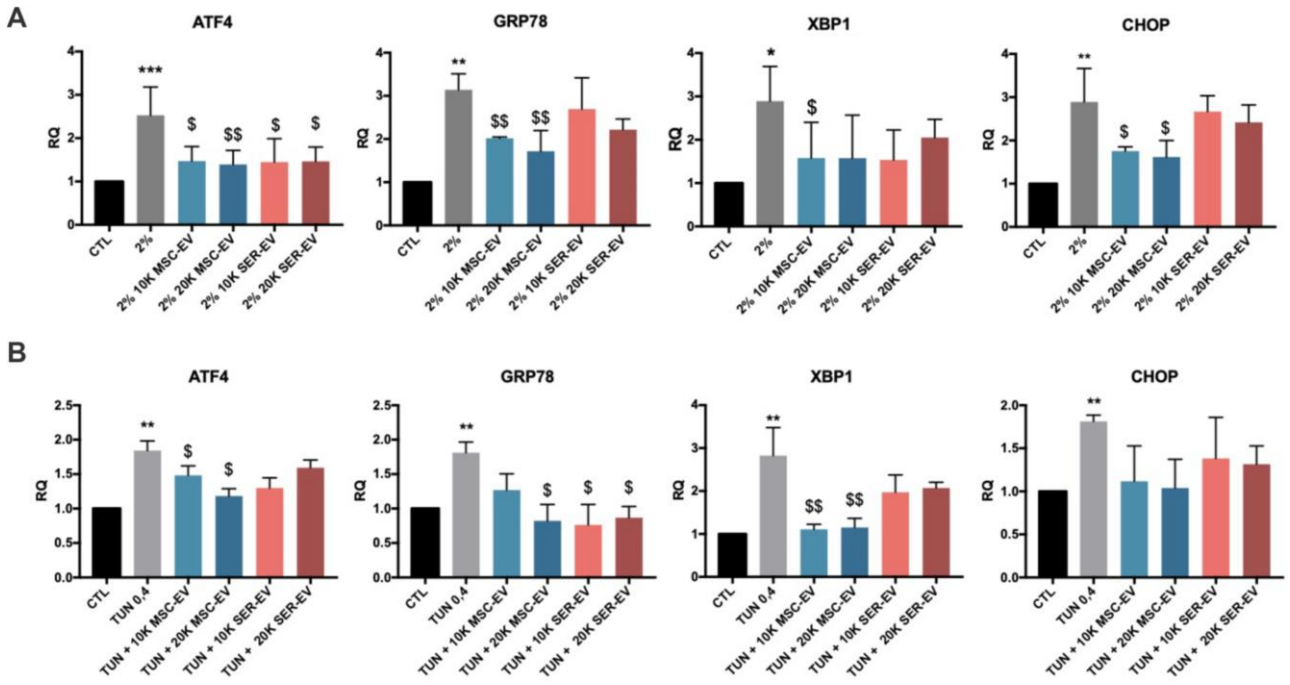


Figure 4. Regulation of the expression of ER stress related genes. HCECs were exposed to serum starvation or tunicamycin for 24 h and were further stimulated with MSC- or SER-EVs for 3 h. Real time analysis showing the expression of ATF4, GRP78, XBP1, and CHOP with HCECs in normal culture conditions (CTL), in serum deprivation (2%) (**A**), or treated with 0.4 ng/mL of tunicamycin (TUN 0.4) (**B**), with or without 10–20 $\times 10^3$ MSC-EV/cell (10K/20K MSC-EV) or 10–20 $\times 10^3$ SER-EV/cell (10K/20K SER-EV). GAPDH was used as an endogenous normalizer. Data were further normalized to CTL, set as 1, and used as a reference sample for each experiment. The graphs show the RQ average ($2^{-\Delta\Delta Ct}$) of at least three independent experiments ± SD. One-way ANOVA with Tukey's multiple comparisons test was performed after the normalization of each experiment to CTL; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$ with respect to CTL, \$ $p < 0.05$, \$\$ $p < 0.01$ vs. 2% (**A**) or TUN (**B**).

We then evaluated the regulation of EIF2a, an activator of the ER stress pathway [28], in the model of serum deprivation. We selected serum deprivation over tunicamycin treatment because of the more consistent results obtained. EIF2a phosphorylation was significantly induced by serum

deprivation and inhibited by the presence of both MSC-EVs and SER-EVs (Figure 5A).

Finally, we considered that the ER stress pathway is linked to Akt regulation, with a reciprocal inhibition, resulting in reduced cell proliferation and induced apoptosis [29]. Given this, we analyzed the levels of Akt phosphorylation during serum deprivation and subsequent treatment with MSC-EVs and SER-EVs (Figure 5B). The significant reduction of Akt phosphorylation after HCEC exposure to serum deprivation was significantly restored by both MSC-EVs and SER-EVs treatment (Figure 5B). Altogether, these data may suggest a differential effect of MSC-EVs and SER-EVs on ER stress gene and Akt pathway modulation.

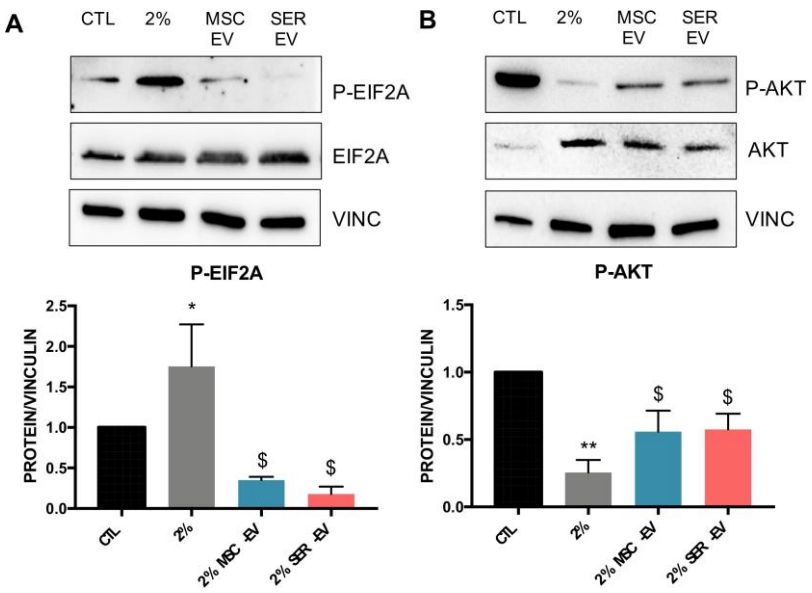


Figure 5. Western blot analysis on HCECs of ER stress related protein phosphorylation. HCECs were exposed to serum starvation for 24 h and further stimulated for 1 h with MSC- or SER-EVs. (**A**,**B**) Representative images of Western blot on HCECs in normal conditions (CTL), in serum deprivation (2%), and in serum deprivation in the presence of 10×10^3 MSC-EV/cell (2% MSC-EV) or in the presence of 10×10^3 SER-EV/cell (2% SER-EV). (**A**) Protein levels and the quantification of phospho- EIF2a normalized to total EIF2a and vinculin (VINC).

(**B**) The protein levels and the quantification of p-AKT normalized to total Akt and vinculin (VINC) and to CTL. CTL, set as 1, was used as a reference sample for each experiment. The graphs show the average of at least three independent experiments ± SD. One-way ANOVA with Tukey's multiple comparisons test was performed after the normalization of each experiment to CTL; * $p < 0.05$ vs. CTL, ** $p < 0.001$ vs. CTL, $ $p < 0.05$ vs. 2%.

1.4. *MSC-EVs, but Not SER-EVs, Reduce HCECs Apoptosis*

We previously showed that serum deprivation could induce HCEC apoptosis and inhibit cell proliferation and demonstrated that MSC-EVs were able to restore this type of damage [17]. Here, we compared the effect of SER-EVs to that of MSC-EVs. Serum deprivation, as expected, resulted in a significant, although not massive, increase in the total apoptotic cells (Figure 6A and Supplementary Figure S1) and a decrease in live cells (Figure 6B and Supplementary Figure S1). Treatment with MSC-EVs re-established the basal levels of the control cells (Figure 6A and Supplementary Figure S1) and increased cell viability (Figure 6B and Supplementary Figure S1). Similar results were obtained with tunicamycin exposure. Accordingly, only the treatment with MSC-EVs was able to reduce the levels of the pro-apoptotic caspase-3 in stress conditions (Figure 6C,D).

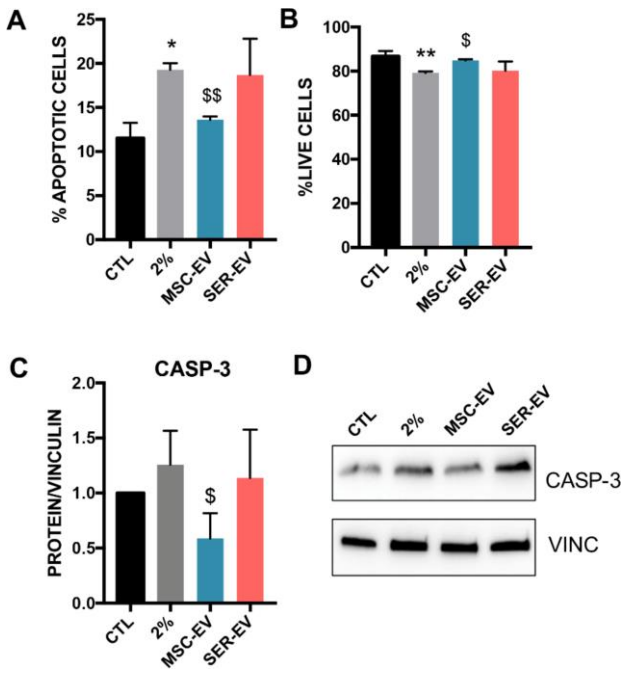
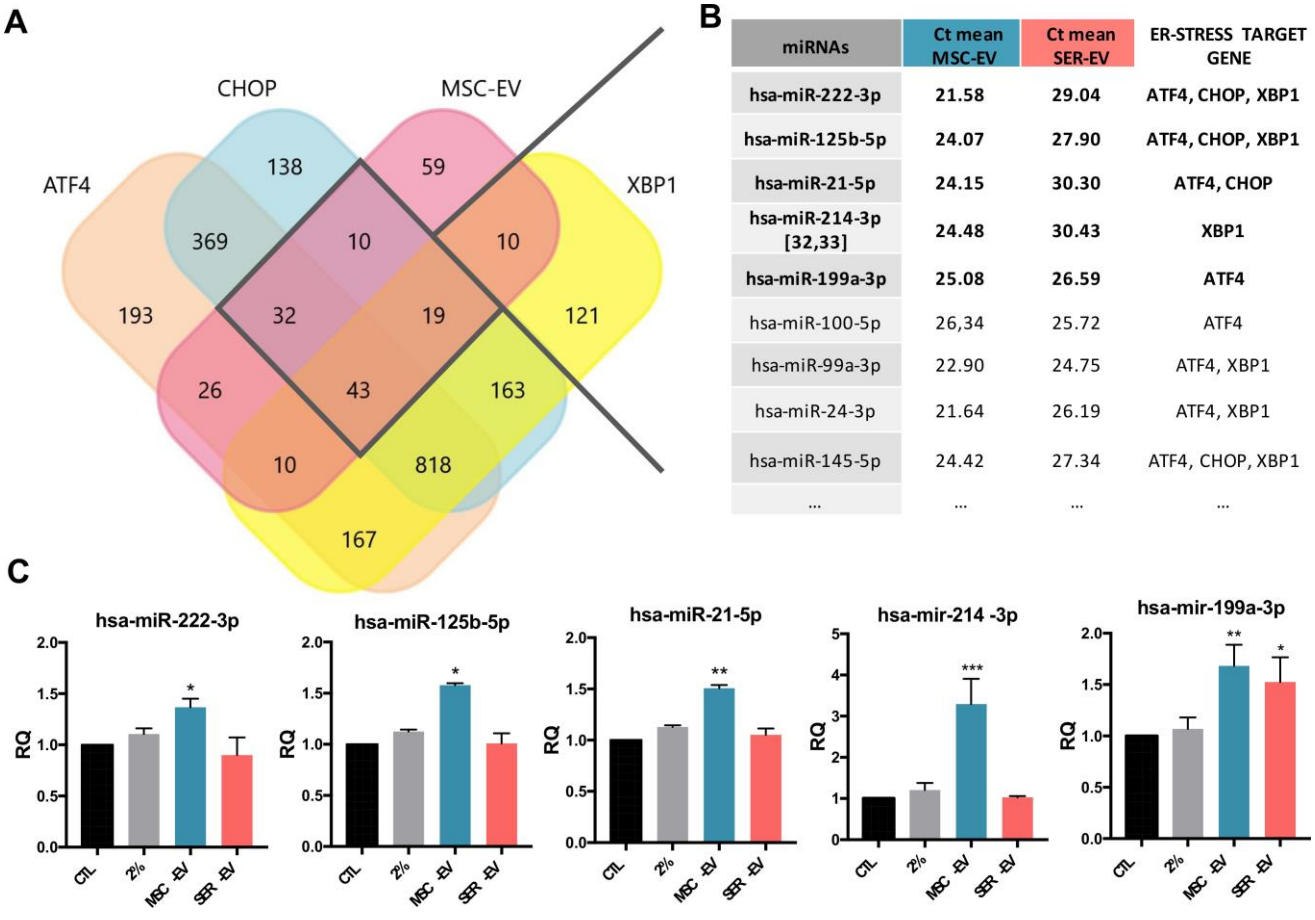


Figure 6. Apoptosis regulation HCECs after serum deprivation and EV treatment. HCECs were exposed to serum starvation for 24 h and further stimulated with MSC- or SER-EVs for 24 h. (**A**) Percentage of total apoptotic HCECs cultured either in normal conditions (CTL) or in serum deprivation for 24 h (2%), and treated for further 24 h with MSC-EVs or SER-EVs (20×10^3 EV/cell). (**B**) Percentage of total live HCECs cultured either in normal conditions (CTL) or in serum deprivation for 24 h (2%), and treated for a further 24 h with MSC-EVs or SER-EVs (20×10^3 EV/cell). (**C**) Protein levels of caspase-3 (CASP-3) and its quantification normalized to total vinculin (VINC). Vinculin was used as the endogenous control. CTL, set as 1, was used as the reference sample for each experiment. The graphs show the average of at least three independent experiments ± SD. One-way ANOVA with Tukey's multiple comparisons test was performed after the normalization of each experiment to CTL. * $p < 0.05$ vs. CTL, ** $p < 0.001$ vs. CTL, $ $p < 0.05$ vs. 2%, $$ $p < 0.001$ vs. 2%. (**D**) Representative image of Western blots on HCECs blotted with caspase-3 and the endogenous control vinculin.

1.5. *MSC-EV miRNAs Transfer to HCECs*

We previously analyzed the miRNA content of both MSC-EVs and SER-EVs [30,31]. In order to explore the mechanisms that account for the MSC-EV

biological activity, we performed a Funrich analysis, combining the mostly expressed miRNAs together with the miRNAs predicted to target the main ER stress genes ATF4, CHOP, and XBP1. We found that several miRNAs targeting the studied genes were also part of the MSC-EVs cargo (Figure 7A). Among those, we focused on the miRNAs with the highest expression in MSC-EVs [30,32] and targeting all three ER-stress genes (Figure 7B), and on miR-214-3p, which was not predicted by miRwalk but was observed by extended literature to target key genes of ER stress [33,34]. The expression levels of the most relevant miRNAs were found to be lower in SER-EVs, except for miR199a-3p (Figure 7B). We subsequently evaluated the possible transfer of ER stress targeting miRNAs to serum deprived HCECs treated with MSC-EVs and SER-EVs, in the presence of amanitin, in order to block endogenous transcription. Figure 7 shows that the miRNA levels found in HCECs were significantly higher in cells exposed to MSC-EVs (Figure 7C). miR-199a-3p was significantly upregulated in both EV treatments, in accordance with its comparable Ct value in both EV sources (Figure 7B).



miRNAs	Ct mean MSC-EV	Ct mean SER-EV	ER-STRESS TARGET GENE
hsa-miR-222-3p	**21.58**	**29.04**	**ATF4, CHOP, XBP1**
hsa-miR-125b-5p	**24.07**	**27.90**	**ATF4, CHOP, XBP1**
hsa-miR-21-5p	**24.15**	**30.30**	**ATF4, CHOP**
hsa-miR-214-3p [32,33]	**24.48**	**30.43**	**XBP1**
hsa-miR-199a-3p	**25.08**	**26.59**	**ATF4**
hsa-miR-100-5p	26,34	25.72	ATF4
hsa-miR-99a-3p	22.90	24.75	ATF4, XBP1
hsa-miR-24-3p	21.64	26.19	ATF4, XBP1
hsa-miR-145-5p	24.42	27.34	ATF4, CHOP, XBP1
...	...	...	...

Figure 7. Target miRNAs prediction of MSC-EVs and ER stress related genes. (**A**) Representative Venn diagram showing the numbers of the target miRNAs predicted for CHOP, ATF4, and XBP1, and those described in the literature for MSC-EVs [30]. The numbers displayed in the diagram represent the number of miRNAs that were found to target the indicated transcripts. (**B**) Table showing the miRNAs and Ct means of the most expressed miRNAs in MSC-EVs [29], compared with the Ct values of the same miRNAs in the SER-EVs [31]. Data are displayed as the mean CT value. The ER stress target gene column highlights the target genes of each miRNA among the genes used (ATF4, CHOP, and XBP1). (**C**) Real time PCR analysis of miRNAs transferred by MSC-EVs in HCECs. HCECs were treated with EVs in the presence of amanitin (50 µg/mL). CTL—HCECs in normal medium; 2%—HCECs in serum deprivation; MSC-EVs and SER-EVs—HCECs treated with MSC-EVs and SER-EVs (20×10^3 EV/cell), respectively. RNU6B was used as the endogenous normalizer. Data were further normalized to CTL, set as 1, and used as reference sample for each experiment. The graphs show the RQ average ($2^{-\Delta\Delta Ct}$) of at least three

independent experiments ± SD. One-way ANOVA with Tukey's multiple comparisons test was performed after the normalization of each experiment to CTL; * $p < 0.05$ vs. CTL, ** $p < 0.001$ vs. CTL, *** $p < 0.0001$ vs. CTL.

2. Discussion

In the present study, we analyzed the effect of MSC-EVs and SER-EVs on the activation of ER stress genes in endothelial cells derived from human corneas. We found that MSC-EVs had a profound effect on the ER stress pathway at different levels, including GRP78, ATF4, CHOP, and XBP1 induction, as well as EIF2a phosphorylation, and prevented cell apoptosis. SER-EVs, at variance, showed a minor effect on the ER stress pathway, and only a specific effect on Akt activation, without preventing cell apoptosis.

ER stress is a relevant pro-apoptotic player in corneal endothelial cells [5] and has a pathogenic role in genetic corneal endothelial dystrophy, one of the most frequent needs for corneal transplantation worldwide [35]. The cornea is an ideal organ for regenerative cell therapy, because of its immune-privileged and avascular na- ture [36]. Therefore, MSCs and their bioproducts, such as EVs, have received much attention among ophthalmologists and visual scientists as an alternative way to manage corneal diseases [22,37,38]. The in vivo treatment with MSC-EVs has been successfully studied in several diseases of the retina, such as retinal cell degeneration, refractory macular holes, and retinal detachments [18,39]. MSC-EV administration typically takes place through intravitreal injection, allowing their direct action on the target cells.

In the present study, in an in vitro model of ER stress, we found that MSC-EVs werehighly active, at both of the doses studied, on all the most typical genes induced by ER- stress, namely: ATF4, GRP78, XBP1, and CHOP

[5,6,25,40]. Moreover, we found that MSC- EVs significantly reduced the apoptosis of corneal endothelial cells in parallel with EIF2a inhibition and caspase-3 downregulation. These data suggest that the anti-apoptotic effect of MSC-EVs in our in vitro models could be directly related to the lowering of ER stress. Our results are in line with the well-established effect of MSC-EVs on the amelioration of ER stress activation shown in a number of different cell types, including tubular epithelial cells, neural cells, and pancreatic beta cells [41–43]. Moreover, as described in other models [44–46], we found that MSC-EVs could act in combination with the Akt pathway activation in corneal endothelium. The restoration of the levels of Akt phosphorylation resulted in a significant downregulation of the pro-apoptotic caspase-3, in line with the reduced number of apoptotic HCECs following treatment with MSC-EVs.

Furthermore, a recent study showed that exosomes from blood plasma could activate Akt to regulate angiogenesis and promote the expression of anti-apoptotic proteins [47]. In accordance with these recent findings, we analyzed the activity of EVs derived from serum, a very accessible source. We were able to confirm that EVs derived from the blood serum of healthy donors could restore the levels of Akt impaired by ER stress, resulting in the regulation of one ER stress related gene, ATF4. However, the modulation of ER stress genes was far lower than that of MSC-EVs. Moreover, SER-EVs, as well as MSC-EVs, were able to significantly reduce the levels of EIF2, a key protein phosphorylation in ER stress known to be a counter-regulator of Akt. However, SER-EVs were not able to restore the levels of caspase-3, nor to reduce the apoptotic number of HCECs. These data highlight a possible therapeutic effect of MSC-EVs, and not of SER-EVs, on endothelial corneal ER stress activation.

The functional effects of MSC-EVs on tissue repair are mainly ascribed to the transfer of their miRNA cargo [32]. Here, we found that a large number of miRNAs, previously involved in the regulation of the different ER stress related genes [33,41,43], were present in MSC-EVs, suggesting a strict connection of those miRNAs with the observed ER stress regulation. Indeed, the highest expressed MSC-EV miRNAs involved in ER stress were foundto be transferred in HCECs. The absence of transfer by SER-EVs confirms the specificity of MSC-EV miRNA transfer, and of the related effect on the apoptosis of corneal endothelialcells. Other studies have shown that miRNAs may act to reduce the phosphorylation of EIF2a, decreasing the translation of ATF4, which inhibits the expression of CHOP, thereby resulting in cell survival and apoptosis reduction [48]. Our results showing the upregu- lation of several miRNAs due to MSC-EV treatment suggest that the observed miRNA transfer is responsible for the MSC-EV mediated reduction of the levels of ER stress and apoptosis. However, the mechanisms underlying the miRNA-dependent regulation of ER stress genes have not been fully elucidated yet.

In conclusion, our study reveals a novel effect of MSC-EV on corneal ER stress- induced cell death, with possible implications in corneal endothelial dystrophy. These data may pave the road for designing more effective therapeutic strategies to battle corneal endothelial pathologies, possibly through direct intra-aqueous humor injection.

3. Materials and Methods

3.1. Isolation and Characterization of HCECs

Human corneal endothelial cells (HCECs) were isolated and characterized as previously described [20]. The study was approved by the Intercompany

Ethics Committee of A.O.U. Città della Salute e della Scienza (Turin), on 21 February 2020 (reference num-ber 00184/2019). Informed written consent was obtained from all of the tissue donors. In brief, we isolated HCECs from the discarded corneas of patients undergoing corneal transplantation or enucleation (N = 23 patients). The Descemet's membrane and corneal endothelial cells were stripped from the posterior surface of the peripheral corneoscleral tissue using a scalpel, and afterwards, was digested with collagenase A (2 mg/mL). The digested membrane and cells were then placed on a Petri dish previously coated with fibronectin. HCECs migrated out of the Descemet's membrane and were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, and were cultured in ENDOGRO (MilliporeSigma™, Burlington, MA, USA) supplemented with 10% FBS (not deprived of EVs). Once confluency was reached, they were passaged at a 1:2 ratio using a 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid solution. From each patient, we isolated a cell line of HCECs, each cell line was kept in culture, and all of the experiments were performed between passage 2 and 4. The cells were characterized by immunofluorescence using phalloidin antibody (ThermoFisher Scientific, Waltham, MA, USA) and DAPI for nuclear staining, for the expression of HCEC main marker Na+K+ATPase, ZO-1, and by presence of the surface marker CD166, as described [20].

3.2. *Isolation and Characterization of MSC-EVs and SER-EVs*

Human bone marrow-derived MSCs were obtained from Lonza (Switzerland), characterized as described [22], and cultured until the sixth passage. All of the cell preparations used were positive for the typical MSC markers (CD105, CD29, CD73, CD44, and CD90). MSC-EVs and SER-EVs were obtained and

characterized as previously described [23,31]. Briefly, MSC-EVs were obtained from the supernatants of MSCs cultured overnight in RPMI deprived of FCS. SER-EVs were obtained from a total of 100 mL of serum isolated from a blood pool of five healthy donors. Informed consent was obtained by the Blood Bank of "Città della Salute e della Scienza di Torino" from all of the participants. MSCsupernatant and serum were then further centrifuged for the removal of cell debris and apoptotic bodies at 3000g for 20 min. EVs were purified for 2 h ultracentrifugation at 100,000g at 4 °C (Beckman Coulter, Brea, CA, USA). EVs from both sources were usedfreshly or stored at 80 °C after resuspension in RPMI supplemented with 1% dimethyl sulfoxide. MSC and serum-derived EVs were characterized by MACSPlex flow cytometry for the expression of CD63, CD81, and CD9, and the main mesenchymal, platelet, andendothelial surface markers. Analysis of the size distribution and enumeration of EVs were performed using NanoSight NS300 (NanoSight Ltd, Malvern, UK) equipped with a 405 nm laser and the Nanoparticle Tracking Analysis (NTA) 2.3 software (NanoSight Ltd., Malvern, UK).

3.3. *MACSPlex Analysis*

Different sample types were subjected to bead-based multiplex EV analysis by flowcytometry (MACSPlex Exosome Kit, human, Miltenyi Biotec, Auburn, CA, USA), and the EV-containing samples were processed as previously described [49]. Samples were diluted with ə MACSPlex buffer (MPB) to a final volume of 120 µL. Then, 15 µL of MACSPlex Exosome Capture Beads (containing 39 different antibody-coated bead subsets) were added to each sample. Generally, particle counts quantified by NTA, and not protein amount, were used to estimate the input EV amounts. The samples were then incubated on

an orbital shaker overnight (14–16 h) at 450 rpm at 4 °C, and were protected from light. To wash the beads, 1 ml of MPB was added and removed after several centrifugations (3000g, 5 min). For counterstaining the EVs bound by capture beads with detection antibodies, 135 µL of MPB and 5 µL of each APC-conjugated anti-CD9, anti-CD63, and anti-CD81 detection antibodies were added to each sample and were incubated on an orbital shaker at 450 rpm protected from light for 1 h at room temperature. Next, to wash the beads, 1 mL of MPB was added and removed after one centrifugation (3000g, 5 min). This was followed by another washing step with 200 µL of MPB, incubation on an orbital shaker at 450 rpm protected from light for 15 min at room temperature, and then the MPB was removed. Subsequently, 150 µL of MPB was added to each sample and flow cytometric analysis was performed using FACS Celesta (BD Biosciences, Franklin Lakes, NJ, USA).

3.4. *Super-Resolution Microscopy*

Super-resolution fluorescent microscopy analyses were performed using a Nanoim-ager S Mark II microscope from ONI (Oxford Nanoimaging, Oxford, UK) equipped with 405 nm/150 mW, 473 nm/1 W, 560 nm/1 W, 640 nm/1 W lasers, and dual emission channels split at 640 nm, as previously described [50]. For the preparation of the sample, 10 µL of Poly-l-Lysine (Sigma-Aldrich, St. Louis, MO, USA) was placed on coverslips, in culture wells (Grace Bio-Labs, Sigma-Aldrich, St. Louis, MO, USA), and left at 37 °C in a humidifying chamber for 2 h. After removal of the excess, MSC-EVs and SER-EVs were left to attach overnight at 4 °C on the coverslips. The next day, non-attached EVs were removed and 10 µL of blocking buffer (PBS-5% Bovine Serum Albumin) was added into the wells for 30 min. Then, mouse anti-CD63, anti-CD81, and anti-CD-9

antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) were conjugated with Alexa Fluor 555, 647, and 488 dyes, respectively, using the Apex Antibody Labeling Kit (Invitrogen, Carlsbad, CA, USA), ac- cording to the manufacturer's protocol. The antibodies were left for overnight incubation at 4 °C. The samples were washed twice with PBS and a 10 µL ONI BCubed Imaging Buffer was added for the EV imaging. Two-channel dSTORM data were acquired sequentially at 30 Hz (Hertz) in the total reflection fluorescence (TIRF) mode. Single molecule data were filtered using NimOS (Version 1.7.1.10213, ONI, Oxford, UK), based on the point spread function shape, photon count, and localization precision to minimize background noise and remove low-precision localizations.

3.5. *Cytofluorimetric Analysis*

For the cytofluorimetric analysis, the cells were detached using a non-enzymatic cell dissociation solution (Sigma-Aldrich, St. Louis, MO, USA), resuspended in PBS 0.1% BSA (Sigma-Aldrich, St. Louis, MO, USA), and incubated with antibodies. For each staining, 100,000 cells were incubated for 20 min at 4 °C with PE-conjugated CD166 antibody (BD Biosciences, Franklin Lakes, NJ, USA). PE-conjugated mouse IgG (Miltenyi Biotec, Bergisch Gladbach, Germany) was used as a negative control. FACS analysis was performed by FACS Celesta cytofluorimeter (BD Biosciences, Franklin Lakes, NJ, USA).

3.6. *Apoptosis Assay*

Apoptosis was evaluated by Muse™ Annexin V and Dead Cell Assay (Millipore, Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The assay was based on the detection of

phosphatidylserine on the surface of apoptotic cells, using fluorescently labeled Annexin V in combination with the dead cell marker, 7-AAD. Briefly, 30 10^3 cells in a 24-well were deprived of serum (2% FBS) or were incubated with 0.4 ng/mL of tunicamycin for 24 h. MSC-EVs or SER-EVs (20 10^3 EV/cell) were added to the medium for a further 24 h. The cells were then detached and resuspended in Muse™ Annexin V and a Dead Cell kit, and the percentages of the total live cells (negative for Annexin V and dead cell marker) and of the apoptotic cells (Annexin $V^{(+)}$ and dead cell $marker^{(-)}$) were detected.

3.7. RNA Isolation and Real Time PCR

To perform the mRNA evaluation of the ERstress markers, HCECs under serum deprivation (2% FBS) or tunicamycin (0.4 ng/mL, ENZO Life Sciences, Farmingdale, NY, USA) for 24 h were treated with MSC-EV or SER-EV (10–20 10^3/ cell). After 3 h of EV exposure, the total RNA was isolated using Trizol Reagent (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA was then quantified spectrophotometrically (Nanodrop ND-1000, Wilmington, NC, USA). For the gene expression analysis, quantitative real-time PCR was performed. Briefly, one-strand cDNA was produced from 200 ng of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Real-time PCR experiments were performed in a 20-µL reaction mixture containing 5 ng of cDNA template, the sequence-specific oligonucleotide primers (purchased from MWG-Biotech, High Point, NC, USA), and the Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA). GAPDH mRNA were used tonormalize the RNA inputs.

The fold change expression with respect to the control was calculated for all of the samples. The specific sequences of the primers used are listed in the supplementary material (Supplementary Table S2).

3.8. *Protein and Western Blot*

For the protein analysis, HCECs were lysed at 4 °C for 30 min in a RIPA buffer (20 nM Tris HCl, 150 nM NaCl, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.8), supplemented with a protease and phosphatase inhibitor cocktail and PMSF (Sigma- Aldrich, St. Louis, MO, USA). Aliquots of the cell lysates containing 25 µg protein, as determined by the Bradford method, were run on 4–20% gel (Biorad, Hercules, CA, USA) under reducing conditions and blotted onto PVDF membrane filters using the iBLOT system (Life Technologies, Waltham, MA, USA). The following antibodies were used: Na^+K^+ATPase (#ab2871, Abcam, Cambridge, UK); EIF2a (#9722s), pEIF2a (#9721), Akt (#9272s) and pAkt (#4058s) antibodies, all purchased from Cell signaling, Danvers, CA, USA, ZO-1 (#sc-33725), caspase-3 (#sc-7148), actin (#sc-1616), and vinculin (#sc-7648), all purchased from Santa-Cruz Biotechnology, Dallas, TX, USA.

3.9. *miRNA Target Prediction*

The predicted miRNA targets were obtained from miRWalk (http://mirwalk.umm. uni-heidelberg.de/, accessed on 1 March 2021). As datasets, the list of miRNAs predicted to target ATF4, CHOP, and XBP1 was used. Published data of our laboratory were utilized for comparison with the MSC-EVs miRNA [30] and SER-EVs miRNA content [31]. Data were further analyzed using Expression Suite and Funrich V3 Software (Bundora, Australia).

3.10. *miRNA Isolation and Analysis*

For the miRNA analysis, miScript SYBR Green PCR Kit (QIAGEN, Hilden, Germany) was used. Cells under 2% FBS starvation were treated for 3 h with MSC-EVs or SER-EVs in the presence of the transcriptional inhibitor α-amanitin (50 µg/mL). The RNA samples of the HCECs were reverse transcribed using the ± miScript Reverse Transcription Kit (QIA- GEN, Hilden, Germany), and cDNA samples were used to quantify the miRNAs of interest. Experiments were run using 10 ng of cDNA for each reaction, as described by the manufacturer's protocol (QIAGEN, Hilden, Germany). RNU6B was used as the endogenous control. Untreated cells (CTL), set as 1, were used as the reference sample for each experiment. The analysis shows the RQ average ($2^{-\Delta\Delta Ct}$) of at least three independent experiments SD. One-way ANOVA with Tukey's multiple comparisons test was performed. The sequence-specific oligonucleotide primers used are listed in the supplementary material (Supplementary Table S2).

3.11. *Statistical Analysis*

One-way ANOVA analysis with Tukey's multiple comparisons test was performed. A p value of < 0.05 was considered to be significant (GraphPad, San Diego, CA, USA).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/ 10.3390/ijms22094930/s1, Figure S1: Representative dot plots showing viability (Dead Cell Marker 7-AAD) vs. apoptosis (Annexin V) of stained HCECs, Table S1: Clinical and biological

information of patients undergoing penetrating keratoplasty, Table S2: Primer sequence list for mRNAs and miRNAs tested in the study.

Author Contributions: B.B. designed the research; L.B. isolated the cells and performed all of the in vitro experiments; A.T. performed the experiments for Figure 3; M.T. performed the miRNA prediction analysis; C.G. performed the super resolution analysis of EVs; R.N., S.S. and M.D.I. provided the human samples and the clinical data; L.B. and B.B. wrote the paper. All of the authors were involved in revising the manuscript and gave final approval of the version to be published. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was approved by the Intercompany Ethics Com- mittee of A.O.U. Città della Salute e della Scienza (Turin), on 21 February 2020 (reference number 00184/2019).

Informed Consent Statement: Informed written consent was obtained from all of the tissue donors. Human serum from healthy blood donors (*n* = 5) was provided by the Blood Bank of "Città della Salute e della Scienza di Torino", after informed consent and approval by the internal Review Board of the Blood Bank.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. **Acknowledgments:** We thank Sergio D'Antico, S.C. Banca del Sangue e del Plasma, Dipartimento di Medicina di Laboratorio, A.O.U. Città della Salute e della Scienza di Torino for providing human sera.

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RESULTS

Podocyte isolation and characterization

Freshly collected urine derived from three different patients (RG, MC and LUR) and from healthy pregnant women (HC) were centrifuged and pelleted cells were kept in culture up to third passage (Table 1).

Table 1. Clinical features of patients

Patient	Sex	Mutated gene/ Complicancies
LUR	M	NPHS2 (Podocin)
MC	M	IFT172, KIF14
RG	F	Type 1 diabetes mellitus
AS	F	COL4A3

After two weeks of culture, urine-derived cells were characterized by the presence of podocyte markers such as podocin and CD2AP (Figure 1A) and by morphology, showing cells with an organized cytoskeletal structure similar to already well-characterized podocytes (Figure 1B). Urine-derived podocytes from patients with Alport syndrome were previously collected and characterized [19].

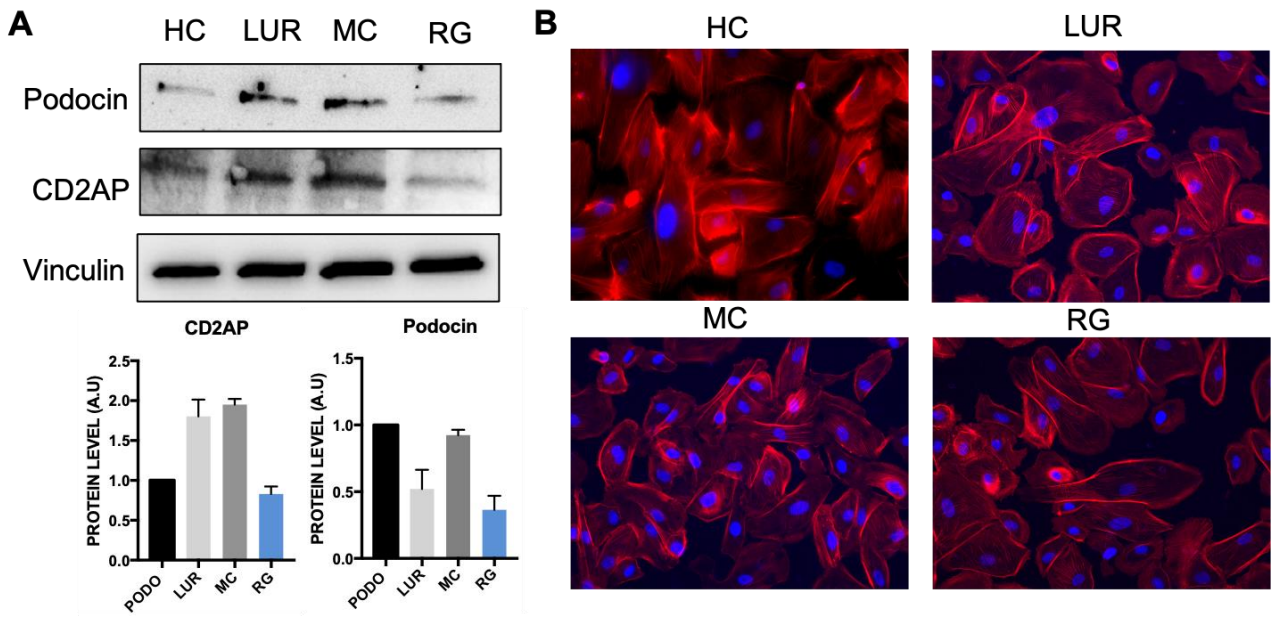


Figure 1. Isolation and characterization of podocytes from urine. A. Representative western blot analysis of podocytes derived from urine of three different patients (LUR, MC and RG) and of healthy pregnant women-derived podocytes positive for podocin and CD2AP. **B.** Representative micrographs of podocytes deriving from urine of HC, LUR, MC and RG stained with phalloidin (red) and blue nuclear stain DAPI. Original magnification: X20.

RNA sequencing of urine-derived podocytes

RNA sequencing was performed on podocytes isolated from urine of the three patients and of Alport Syndrome patients in order to investigate the characteristics of their transcriptome, using urine-derived podocytes from pregnant women (HC) as a control. A specific signature composed of 68 podocyte-typical genes, previously described by Lu et al. [20], was investigated (Figure 2). AS podocytes were used as control as they were previously characterized [19], transcript per million results (TPM) of each gene were normalized as fold change respect to AS. HC, LUR, MC and RG podocyte lines did not reveal significant differences from previous characterization [19] confirming the podocyte phenotype of all cell lines (Figure 2).

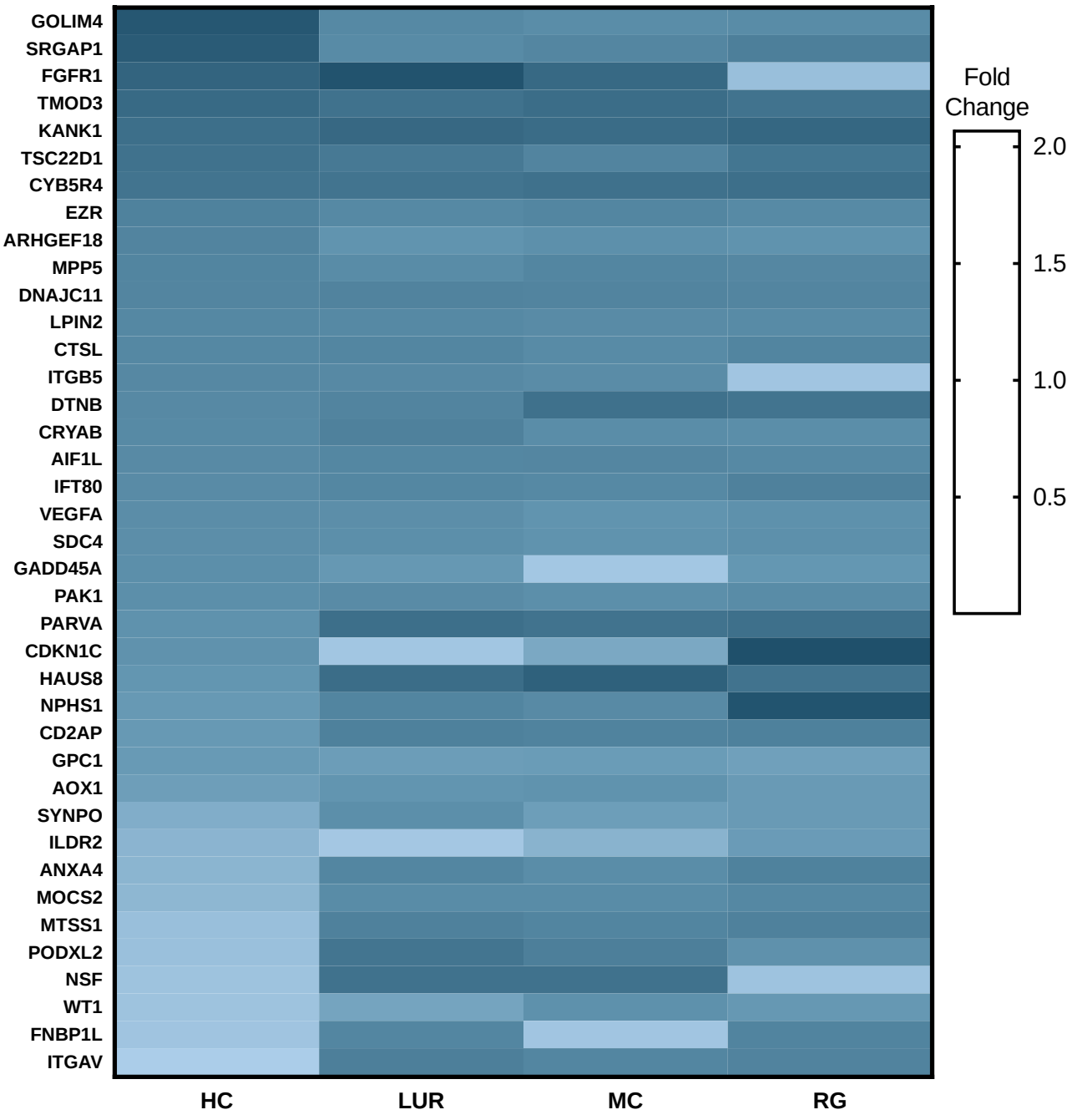


Figure 2. RNA-sequencing analysis of urine-derived podocytes. Heatmap of podocyte signatures showing the fold change (FC) of podocyte-specific genes in HC, LUR, MC and RG podocytes respect to AS podocytes.

Renal progenitor derived EVs extraction and characterization

EVs were isolated from renal progenitor immortalized cells deriving from the urine of preterm infants (PT1PA) [21], following previously described protocols [8].

PT1PA-derived EVs were analyzed by size distribution and quantified using the nanosight tracking analysis showing the appropriate EV mean size (Figure 2A). In addition, EVs were characterized using a MACSPlex Exosome analysis kit after bead-based immunocapture (Figure 2B and C), with the aim of dissecting their profile. The levels of the pan tetraspanins CD9, CD63 and CD81 were high. PT1PA-EVs showed high levels of class-I HLA-ABC and of the mesenchymal typical markers CD146, CD44 and CD29. Endothelial and platelet markers CD142 was found highly expressed in PT1PA-EVs (Figure 2C).

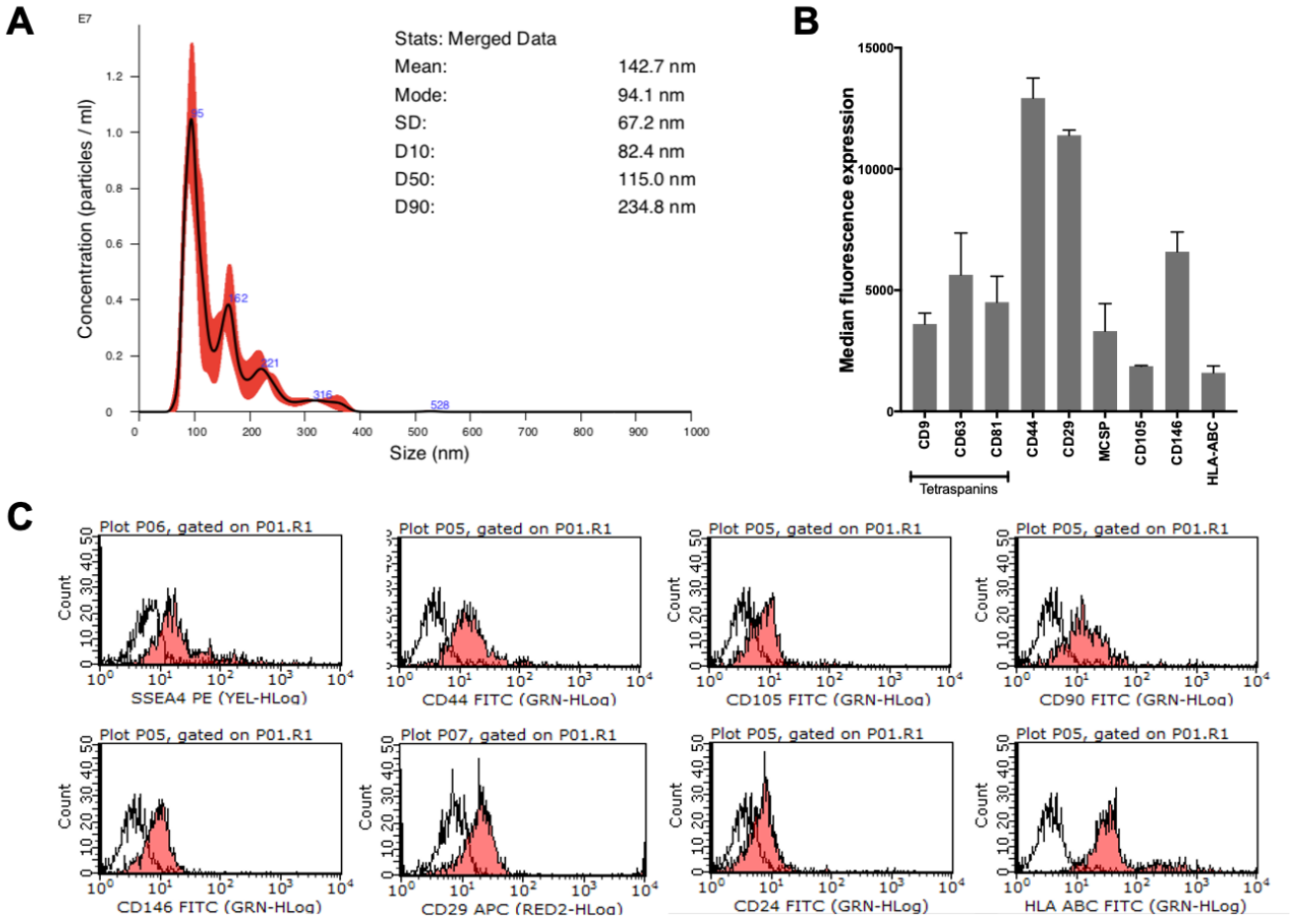


Figure 3. Characterization of renal progenitor-derived EVs. A. Representative nanoparticle tracking analysis showing the EVs size distribution, EVs mean size is 142.7 nm. **B.** MACSPlex representative dot plots showing the EV distribution of allophycocyanin (APC)-stained bead populations; captured EVs are counterstained with APC-labeled detection antibodies using a mixture of anti-CD9, anti-CD63, and anti-CD81 (pan tetraspanins) antibodies. **C.** FACS analysis of renal progenitor-derived EVs for the presence of SSEA4, CD44,CD105,CD90, CD146, CD29, CD24 and HLA ABC surface markers.

Permeability analysis of podocyte cultures

To study the functionality of podocytes recovered from the three different patients *in vitro*, a culture system was assembled. Podocytes were seeded on the upper side of PET inserts and a filtration assay was performed by measuring the transit of FITC-BSA from the lower compartment to the upper podocyte compartment in different experimental conditions (Figure 3). The treatment with the renal progenitor-derived EVs (PT1PA-EVs) was able to significantly reduce FITC-BSA permeability in all the different patient-derived podocytes tested. The filtration rate was evaluated in the same manner treating both with EVs or with different drugs commonly used to treat renal diseases, such as methylprednisolone (URB), ciclosporin (SAND), tacrolimus (TAC), rituximab (RITUX) and colchicine (COLCH). We observed that the podocytes deriving from patient RG responded to three of the drugs tested as well, reducing their permeability to albumin. (Figure 3).

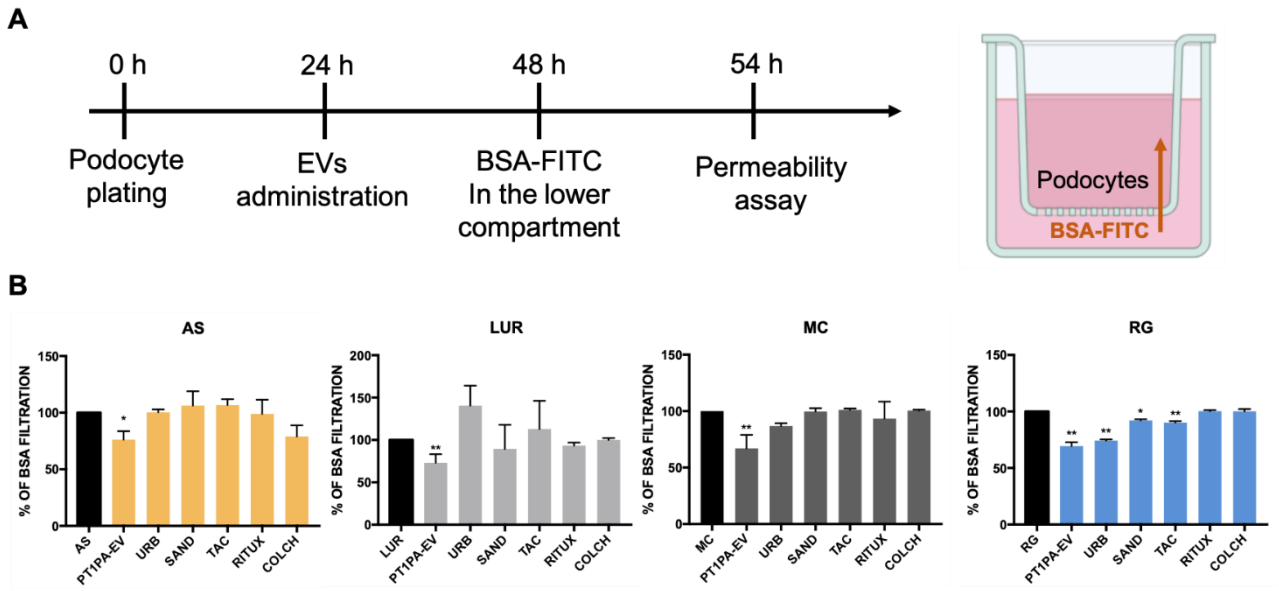
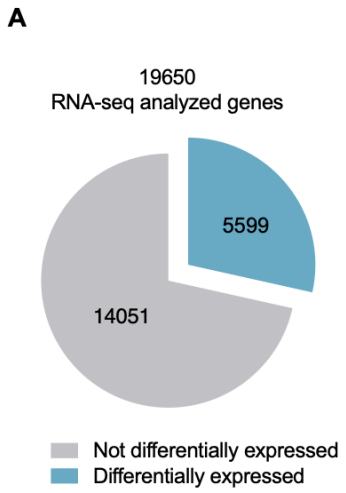


Figure 3. Permeability assay in podocyte cultures treated with EVs and drugs. A. Schematic experimental design and graphic representation of culture set-up. **B.** FITC-BSA permeability indicating albumin passage from the lower compartment to the podocyte compartment in all the different treatment conditions. Podocyte filtration of each patient-derived cell line was used as control for each experiment and set as BSA filtration rate of 100%. Control conditions were compared to FITC-BSA filtration percentages of podocytes treated with renal progenitor-derived EVs (PT1PA-EVs) with the different drugs methylprednisolone (URB), ciclosporin (SAND), tacrolimus (TAC), rituximab (RITUX) and colchicine (COLCH). Data are expressed as the mean amount of filtered BSA-FITC of four different experiments using at least three inserts for each condition in each experiment. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed after the normalization of each experiment to CTL (untreated podocytes); * $p < 0.05$, ** $p < 0.01$, respect to AS, LUR, MC and RG respectively.

RNA-sequencing analysis of urine-derived podocytes treated with EVs

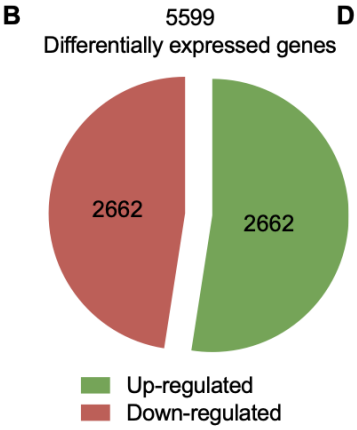
Total RNA sequencing was performed on podocytes isolated from the urines of the three patients to investigate the modifications occurring in the transcriptome following the treatment with renal progenitor derived EVs. We used the transcripts per kilobase million (TPM) normalization method to compare the gene expression profile changes between samples. We detected a total of 2876 upregulated genes (log_2 fold change >1) and a total of 2574 downregulated genes (log_2 fold change <-1), following the treatment with EVs. Among these, five genes were upregulated in all the urine-derived podocytes following the treatment with EVs and five genes were downregulated by the EVs in all the samples (Figure 4). Up/down regulated genes were further analyzed using Expression Suite and Funrich V3 Software (Bundora, Australia).



C

BIOLOGICAL PROCESS – UPREGULATED GENES BY EVS

	ID	Name	Source	pValue
1	GO	98609	cell-cell adhesion	1,43E-06
2	GO	7155	cell adhesion	2,18E-06
3	GO	22610	biological adhesion	2,93E-06
4	GO	9581	detection of external stimulus	4,78E-05
5	GO	9582	detection of abiotic stimulus	6,21E-05
6	GO	98916	anterograde trans-synaptic signaling	1,21E-04
7	GO	7268	chemical synaptic transmission	1,21E-04
8	GO	99537	trans-synaptic signaling	1,88E-04
9	GO	60191	regulation of lipase activity	2,36E-04
10	GO	98742	cell-cell adhesion via plasma-membrane adhesion molecules	2,38E-04



D

CELLULAR COMPONENT – UPREGULATED GENES BY EVS

	ID	Name	Source	pValue
1	GO	31226	intrinsic component of plasma membrane	2,59E-14
2	GO	5887	integral component of plasma membrane	4,22E-14
3	GO	99240	intrinsic component of synaptic membrane	6,35E-05
4	GO	99699	integral component of synaptic membrane	7,53E-05
5	GO	98936	intrinsic component of postsynaptic membrane	3,59E-04
6	GO	99055	integral component of postsynaptic membrane	3,99E-04
7	GO	9986	cell surface	8,34E-04
8	GO	98948	intrinsic component of postsynaptic specialization membrane	1,43E-03
9	GO	99634	postsynaptic specialization membrane	1,89E-03
10	GO	99060	integral component of postsynaptic specialization membrane	2,29E-03

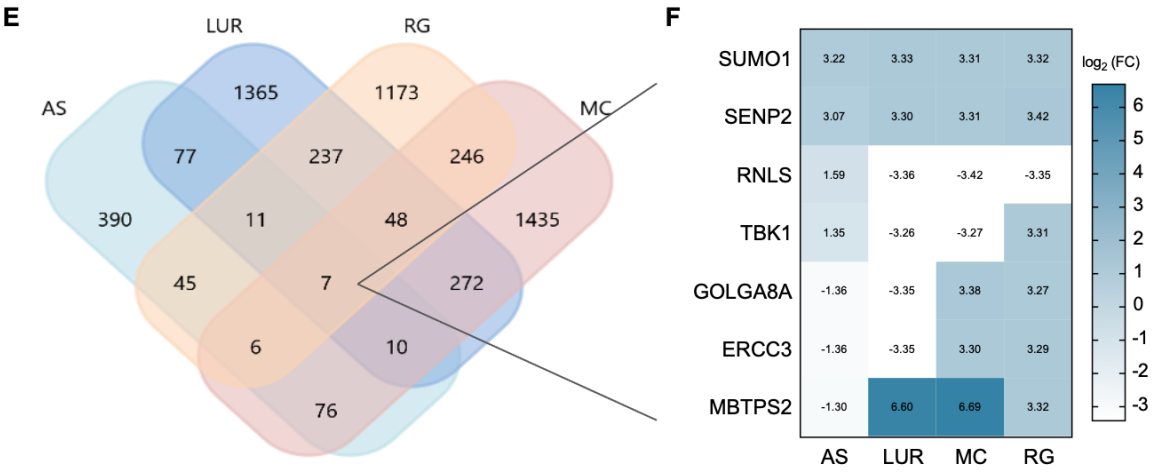


Figure 4. Cross-analysis for the identification of regulated genes common to the three patient-derived podocytes. A. Pie chart representation of common to the different patient-derived podocytes untreated differentially expressed transcripts compared with patient-derived podocytes treated with EVs. **B**. Pie chart representation of up-regulated (red) and down-regulated (green)

differentially expressed transcripts in different patient's urine-derived podocytes untreated and treated with EVs. **C and D.** Gene Ontology (GO) analysis of differentially expressed genes in different patient's urine-derived podocytes untreated and treated with EVs: biological process and cellular component. In each table, the identification (ID) number, the name, and the P value associated with the GO are given. **E.** Representative Venn diagram showing the numbers of the genes that resulted up-regulated or down-regulated by the treatment of the podocytes with renal progenitor-derived EVs by total RNA sequencing analysis, data were analyzed using Expression Suite and Funrich V3 Software. **F.** Heatmap showing similar the levels of expression of commonly EV-regulated genes in urine-derived podocytes from the four different patients (AS, LUR, MC and RG).

The differential expression identified several kidney-related genes, such as SUMO1 which was recently found be correlated in the tight orchestration of nephrin turnover at the slit diaphragm [22]. Interaction network analysis showed that SUMO1 and SENP2 directly interact [23,24]. The EV-induced up-regulation of both SUMO1 and SENP2 in urine-derived podocytes was significantly confirmed by RT-qPCR in 2 out of 4 patients, while we could observe a stable trend of up-regulation all the urine-derived podocytes tested (Figure 5).

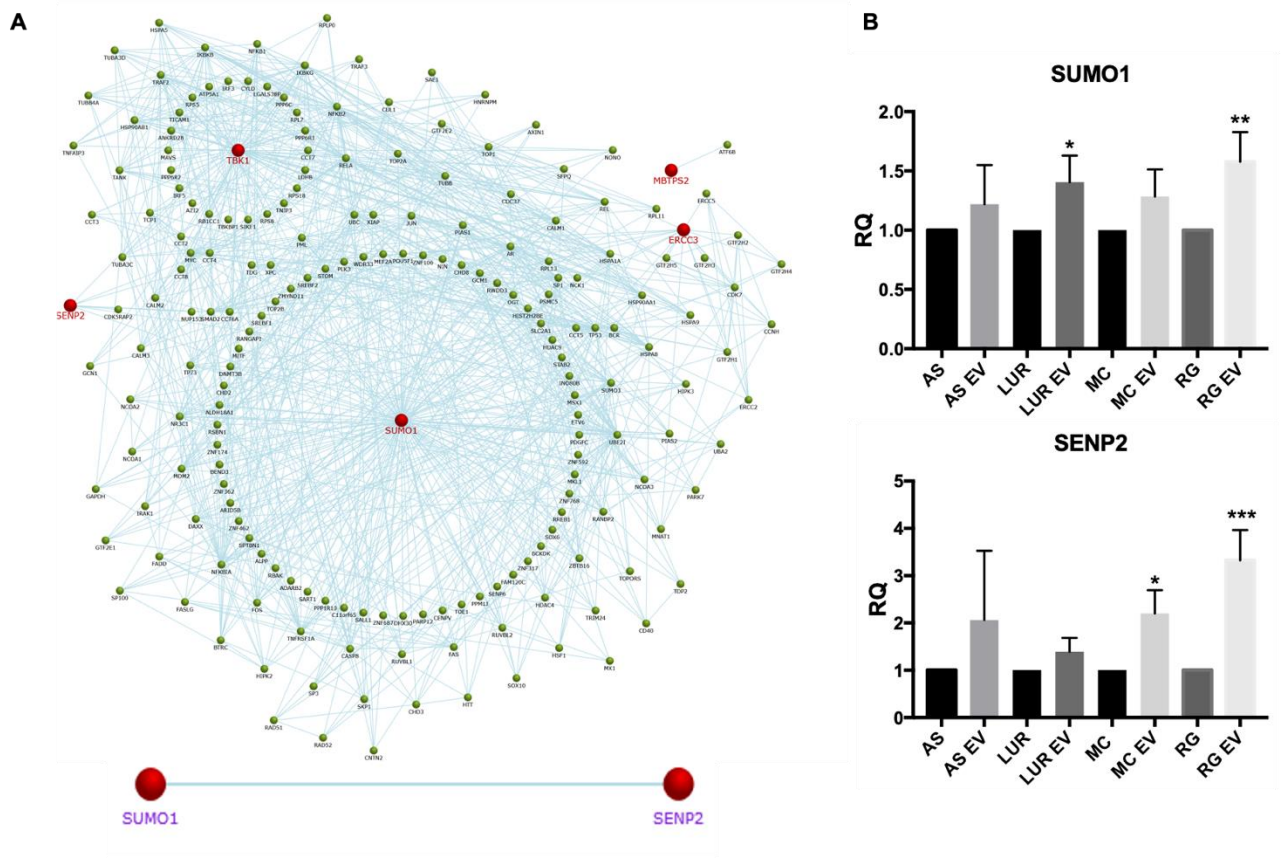


Figure 5. Validation of differentially expressed genes in urine-derived podocytes treated with EVs.

A. Interaction network analysis showing biological pathways of the commonly regulated genes by the EVs. The only direct interaction is represented by SUMO1 and SENP2. **B.** mRNA expression of genes that were found to be up-regulated and directly interacting (SUMO1 and SENP2). Data are shown as relative quantification, normalized to GAPDH and to each untreated control respectively set as 1. The graphs show the RQ average ($2^{-\Delta\Delta Ct}$) of at least three independent experiments ± SD. One-way ANOVA with Tukey's multiple comparisons test was performed after the normalization of each experiment to its untreated condition (AS, LUR, MC, RG); * $p < 0.05$ vs. CTL, ** $p < 0.001$ vs. CTL, ***$p< 0.0001$.

DISCUSSION

The current study brings to light a novel approach to retrieve nephron-derived cells directly from the urine of single patients, and to culture them in order to test the effects of several compounds directly on the cells. In particular, we demonstrated the ability of renal progenitor derived EVs to ameliorate the filtration ability of podocytes derived from urine of patients with different kidney pathologies. Following EVs treatment, indeed, podocytes showed an improvement in the filtration ability, as reflected by a significant reduction of albumin filtration through the podocyte barrier *in vitro.*

Over the last decade, the role of stem-cell derived EVs, as an alternative strategy for tissue regeneration, has greatly evolved. In particular, MSC-EVs have been extensively studied in various experimental disease models [25]. For instance, in a chronic kidney disease model of ureter urinary obstruction, intravenous administration of MSC-EVs not only improved renal function, but also alleviated tubular injury and fibrosis (He et al., 2015). Interestingly, in a *in vivo* model of renovascular disease, a single dose of MSC- EVs alleviated renal inflammation through the regulation of pro and anti-inflammatory cytokines, contributing to the recovery of renal function and to the restoring of a normal glomerular filtration rate [26]. Moreover, urine derived MSC-EVs reduced podocyte and tubular epithelial cell apoptosis and increased proliferation of glomerular endothelial cells, therefore exhibiting an overall regenerative effect in a rat model of diabetic nephropathy [27]. Alternative sources of EVs have also proven to be effective. For instance, our group previously reported the therapeutic effects of multiple sources of stem-cell derived EVs, in a mouse model of streptozotocin induced diabetic nephropathy and showed that the administration of EVs derived from two different sources, significantly ameliorated kidney function [25]. The number of studies on the use of EVs for the treatment of renal pathologies is continuously increasing, and EVs are considered a promising approach for tissue regeneration. The focus of our study is to test the

regenerative potential of renal progenitor derived EVs on podocytes, principal players of the glomerular filtration activity of the nephron.

The availability of human podocytes in culture is, at present, limited. Podocyte-like cells have been recently obtained from induced pluripotent stem cells of patients [28] and this may represent a promising approach. However, it might be difficult to obtain a pure population of podocytes, as differentiated induced pluripotent stem cells cultures contained only 30–50% cells with podocyte-like morphology [29]. Human urine has been previously used as a non-invasive valuable source of podocytes [30]. In patients with active glomerular diseases, podocytes are shed from the glomerulus as a response to local environmental factors. Urine-derived podocytes from both patient and healthy subjects appear positive for podocyte markers, viable, and able to grow in culture [30].

In this scenario, our approach of retrieving podocytes directly from the urine of a single patient in order to test the efficacy of pro-regenerative EVs or chemical compounds in an *in vitro* model of glomerular permeability, represents a very interesting perspective of personalized medicine. In our *in vitro* model of glomerular filtration barrier, we were able to assert that renal progenitor derived EVs were able to reduce podocyte permeability to albumin, ascribing these EVs as a potential therapeutic molecule for glomerular diseases.

Finally, total RNA-sequencing analysis of EV-exposed podocytes and untreated podocytes brought to the identification of several EV-regulated genes common to the three different patients. Among the regulated genes, we confirmed the regulation of several genes that have been recently described to have a role in kidney physiology. For instance, SUMO1, was recently identified as stabilizer of nephrin in the slit diaphragm of the podocyte, thereby increasing nephrin level of expression on the plasma membrane of the cells composing the slit diaphragm [22]. Finally, we observed that the levels of SUMO-specific protease 2 (SENP2) were up-regulated in all the patient-

derived podocytes treated with the EVs. SENP2 is a known regulator of SUMO1 [23], our data suggest that SENP2 may be indirectly involved in nephrin expression on the slit diaphragm as well.

In conclusion, we reported a human *in vitro* model that functionally reproduces the podocyte filtration layer of the nephron and we identified renal progenitor derived EVs as regulators of podocyte permeability, indicating that their administration could significantly ameliorate podocyte function, decreasing their permeability to albumin. Total RNA sequencing allowed to determine the regulation of a pathway by renal progenitor-derived EVs, involved in podocyte stabilization in the slit diaphragm of the nephron. Our data may pave the road for establishing a standard non-invasive *in vitro* model for the screening of regenerative compounds directly on patient-derived podocytes.

MATERIALS AND METHODS

Generation and characterization of podocyte cell lines

Freshly collected urine from HC, LUR, MC and RG (Table 1) were centrifuged at 1500 × g for 10 min and the pellet was resuspended in DMEM/F-12 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen, Carlsbad, CA, USA), 50 IU/ml penicillin, 50 g/ml streptomycin, 5 mM glutamine, 5 g/ml insulin, 5 g/ml transferrin, and 5 mg/ml selenium (all from Sigma-Aldrich, St Louis, MO, USA) and grown at 37°C up to the third passage. AS podocytes were immortalized and sub-cloned using a temperature-sensitive Simian virus 40 large T (SV40T) and human telomerase reverse transcriptase vectors, as described previously [31]. AS podocytes were grown at 33°C and transferred to 37°C for 10–14 days to obtain fully differentiated podocytes. The cells were characterized for the expression of podocin (sc-21009) and CD2AP (sc-25272) using vinculin (sc-7648) as endogenous control, and by immunofluorescence using phalloidin antibody (ThermoFisher Scientific, Waltham, MA, USA) and DAPI for nuclear staining.

RNA sequencing analysis

Total RNA was isolated using Trizol Reagent (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA was then quantified spectrophotometrically (Nanodrop ND-1000, Wilmington, NC, USA). Libraries for RNA-seq were generated using a TruSeq RNA stranded sample preparation kit v2 (Illumina Inc, San Diego, CA, USA) following the manufacturer's instructions, using 1 µg of total RNA as input material. Libraries were pooled and sequenced with a NextSeq 500 sequencer (Illumina Inc) generating 75-bp paired-end sequences. Further analyses were performed using transcript per million (TPM) tables and genes with a $\log_2$ FC >1 and <-1 were considered as differentially expressed.

Isolation and Characterization of PT1PA-EVs

EVs were isolated from renal progenitor immortalized cells deriving from the urine from a 30-week preterm neonate urine (PT1PA) [21], following previously described protocols [8]. PT1PA-EVs were obtained from the supernatants of PT1PA cultured overnight in RPMI deprived of FCS. SER-EVs were obtained from a total of 100 mL of serum isolated from a blood pool of five healthy donors. Informed consent was obtained by the Blood Bank of "Città della Salute e della Scienza di Torino" from all of the participants. PT1PA supernatant and serum were then further centrifuged for the removal of cell debris and apoptotic bodies at 3000g for 20 min. EVs were purified for 2 h ultracentrifugation at 100,000g at 4 ◦C (Beckman Coulter, Brea, CA, USA). EVs from both sources were used freshly or stored at −80 ◦C after resuspension in RPMI supplemented with 1% dimethyl sulfoxide. PT1PA-derived EVs were characterized by MACSPlex flow cytometry for the expression of CD63, CD81, and CD9, for SSEA4, CD105, CD90, CD24, CD29, CD44, CD146 AND HLA-ABC by FACS analysis. Analysis of the size distribution and enumeration of EVs were performed using NanoSight NS300 (NanoSight Ltd, Malvern, UK) equipped with a 405 nm laser and the Nanoparticle Tracking Analysis (NTA) 2.3 software (NanoSight Ltd., Malvern, UK).

MACSPlex Analysis

PT1PA-EVs were subjected to bead-based multiplex EV analysis by flow cytometry (MACSPlex Exosome Kit, human, Miltenyi Biotec, Auburn, CA, USA), and the EV-containing samples were processed as previously described [32]. Samples were diluted with a MACSPlex buffer (MPB) to a final volume of 120 µL. Then, 15 µL of MACSPlex Exosome Capture Beads (containing 39 different antibody-coated bead subsets) were added to each sample. Generally, particle counts quantified by NTA, and not protein amount, were used to estimate the input EV amounts. The samples were then

incubated on an orbital shaker overnight (14–16 h) at 450 rpm at −4 ◦C, and were protected from light. To wash the beads, 1 ml of MPB was added and removed after several centrifugations (3000g, 5 min). For counterstaining the EVs bound by capture beads with detection antibodies, 135 µL of MPB and 5 µL of each APC-conjugated anti-CD9, anti-CD63, and anti-CD81 detection antibodies were added to each sample and were incubated on an orbital shaker at 450 rpm protected from light for 1 h at room temperature. Next, to wash the beads, 1 mL of MPB was added and removed after one centrifugation (3000g, 5 min). This was followed by another washing step with 200 µL of MPB, incubation on an orbital shaker at 450 rpm protected from light for 15 min at room temperature, and then the MPB was removed. Subsequently, 150 µL of MPB was added to each sample and flow cytometric analysis was performed using FACS Celesta (BD Biosciences, Franklin Lakes, NJ, USA).

Permeability assay

Permeability assays were performed after 24 hours treatment with EVs/drugs by measuring BSA filtration from the lower to the upper compartment. Complete medium (500 µl) containing or not FITC-BSA (1 mg/ml, Sigma) was placed in the lower compartment and upper podocyte compartments, respectively. To measure the podocyte filtration ability in basal to apical direction, 100 µl of medium was taken after 6 h from the podocyte compartment and the passage of FITC-BSA was determined by fluorimetry in triplicate. FITC signal was measured in triplicates using a fluorimeter. Data are expressed as the mean amount of filtered BSA-FITC of four different experiments using at least three inserts for each condition in each experiment. Renal progenitor derived EVs were used at a concentration of $2X10^5$ EV/cell. Methylprednisolone (Urbason, Sanofi) was used at a concentration of 40 µg/mL, ciclosporin (Sandimmun, Novartis) was used at a concentration of 25 µg/mL, tacrolimus (Prograf, Panacea Biotec) was used on the cells at a

concentration of 1 ng/mL, rituximab (Mabthera, Roche) was used at a concentration of 12 □g/mL and colchicine (Zydus Synovia) was used at a concentration of 5 ng/mL.

RNA Isolation and Real Time PCR

To perform the mRNA evaluation of SUMO1 and SENP2, the total RNA of podocytes untreated or treated for 24 h with PT1PA-EVs (20×10^3EV/cell) was isolated using Trizol Reagent (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA was then quantified spectrophotometrically (Nanodrop ND-1000, Wilmington, NC, USA). For the gene expression analysis, quantitative real-time PCR was performed. Briefly, one-strand cDNA was produced from 200 ng of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Real-time PCR experiments were performed in a 20-µL reaction mixture containing 5 ng of cDNA template, the sequence-specific oligonucleotide primers (purchased from MWG-Biotech, High Point, NC, USA), and the Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA). GAPDH mRNA were used to normalize the RNA inputs. The fold change expression with respect to the control was calculated for all of the samples.

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