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## **Human retinal secretome: A cross link between mesenchymal and retinal cells**

Donato L *et al.* Cross-link between retinal and mesenchymal secretomes

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### **Abstract**

In recent years, mesenchymal stem cells have been considered the most effective source for regenerative medicine, especially thanks to released soluble paracrine bioactive components and extracellular vesicles. These factors, collectively called secretome, play crucial roles in immunomodulation and in improving survival and regeneration capabilities of injured tissue. Recently, there has been a growing interest in secretome released by retinal cytotypes, especially retinal pigment epithelium and Müller glia cells. The latter trophic factors represent the key to preserving morphofunctional integrity of the retina, regulating biological pathways involved in survival, function, and response to injury. Furthermore, these factors can play a pivotal role in onset and progression of retinal diseases, after damage of cell secretory function. In this review, we have delineated the importance of crosstalk between mesenchymal stem cells and retinal cells, focusing on common/induced secreted factors, during experimental therapy for retinal diseases.

**Key Words:** Secretome; Mesenchymal stem cells; Retinal cells; Extracellular vesicles; Retinal diseases

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**Core Tip:** Recently, mesenchymal stem cell secretome, a solution rich of paracrine bioactive factors and extracellular vesicles, acquired a significant role in immunomodulation and survival induction of damaged tissues. A secretome is also released by retinal cells, physiologically or following pathological stimuli. One of the most promising therapeutic frontiers is represented by a possible “cross-talk” between mesenchymal stem and retinal cells through secretome, in order to improve the knowledge on released factors mechanisms of action during their potential beneficial role.

## INTRODUCTION

In recent years, mesenchymal stem cells (MSCs) have been indicated as the most effective source for cell-based therapy, particularly in regenerative medicine. In particular, MSCs produce major therapeutic effects releasing soluble paracrine bioactive components and extracellular vesicles (EVs) constituting the so called secretome. These secreted factors play crucial roles in modulating immunity and improving survival and regeneration capabilities of injured tissue<sup>[1]</sup>.

Secreted trophic factors are also key to preserving the morphofunctional integrity of the retina, regulating biological pathways involved in survival, function, and response to injury<sup>[2]</sup>. Additionally, these factors can play a fundamental role in onset and progression of retinal diseases, after damage of cell secretory functions<sup>[3]</sup>. In this review, we try to link the secretome of MSCs to the retinal cell secretome, in order to highlight the state of the art of secreted factor involvement in retinal diseases.

### *Main features of MSCS*

One of the most recent fields of therapy research concerns MSCs, multipotent non-hematopoietic stem cells that originate from the mesoderm. They can reach a pathological site following the release of different biologically active immunomodulatory and regenerative factors related to different diseases<sup>[4]</sup>. There are multiple sources of MSCs, including umbilical cord blood, placenta, adipose tissue, skin and bone marrow tissue, with the latter representing the most widely used source<sup>[5]</sup>. MSC isolation is a noninvasive process, and this represents a fundamental advantage also from an ethical and/or legal point of view<sup>[6]</sup>. What probably represents the real key point in MSC use is their low immunogenicity, permitting allogeneic transplantation in the medical setting<sup>[7]</sup>. Moreover, recent studies have shown that MSCs can produce an immune response, mediated by T cells regulated by IFN- $\gamma$ <sup>[8]</sup>. When activated, MSCs can reach the correct pathological site to exert reparative functions, triggered by a huge number of secreted factors from the injured cells, such as cytokines, chemokines and growth factors<sup>[9]</sup>. Among the latter, placental growth factor plays a pivotal role, along with VEGF, EPO,

SDF-1, ANG2, G-CSF, stem cell factor, PDGF, EGF, HGF and IGF-1<sup>[10]</sup>. Regarding cytokines and chemokines, the former include TNF- $\alpha$  and interleukins such as IL-1b, IL-2, IL-3, IL-6, IL-8, the latter comprehend, among others, CCL5 and CCL22<sup>[11]</sup>. Various studies have confirmed that human MSCs evade allorecognition, affect T lymphocytes and dendritic cell activities, and produce a local immunosuppressant microenvironment by releasing the already cited cytokines<sup>[12]</sup>. Moreover, MSCs can be genetically manipulated easily, with elevated metabolic activity and low mutation rate, and can efficiently secrete a wide number of proteins<sup>[13]</sup>. Today, preclinical and clinical trials using MSCs have been performed in different kinds of pathologies with promising results, such as autoimmune disease, joint reconstruction, vascular disease, nerve injury, organ transplantation, degenerative disease and severe infection<sup>[14]</sup>. The protective activity of MSCs was initially linked to their direct differentiation and replacement of injured tissues, as evidenced by human MSCs becoming hepatocyte-like cells or rat MSCs turning into neuron-like cells<sup>[15]</sup>. However, today the protective action of MSCs is well known to be primarily mediated through paracrine properties, exerted by what is defined MSC secretome.

### ***MSC secretome***

The secretome released by MSCs consists of a condition medium (CM) made up of soluble elements (cytokines and growth factors) and a vesicular part made up of exosomes and microvesicles, which are fundamental for protein and genetic material transfer towards other cells<sup>[16]</sup>. The most recent *in vitro* and *in vivo* studies on the features of MSC secretome have highlighted its role in facilitating cell survival, proliferation, differentiation and physiological processes<sup>[17]</sup>. A huge number of secreted growth factors is well known today, including VEGF, SDF-1, TGF- $\beta$ , IGF-1, fibroblast growth factor (FGF), nerve growth factor-beta (NGF- $\beta$ ), HGF, G-CSF and EGF<sup>[18]</sup>. With regard to MSC secreted cytokines and chemokines, the most investigated are CCL2, CCL5 and CXCL12 (SDF-1)<sup>[19]</sup>. One of the most useful aspects of MSC secretome is the possibility to tailor or modify its composition depending on the desired cell-specific therapeutic effects. This

promising possibility depends on MSC tissue sources or on the number of passages, allowing the creation of distinct secretory profiles and exosomal compositions<sup>[20]</sup>. However, several controversial studies have already been published. It was shown, for example, that the impact of MSCs extracted from adipose tissue was more noticeable on axonal growth than MSCs coming from bone marrow, while cell passaging did not influence the secretome content/activities supporting post-natal neuronal survival and axonal growth<sup>[21]</sup>. During the last few years, it has been revealed that MSCs are able to modify the microenvironment by releasing EVs, primarily distinct into apoptotic bodies, micro-vesicles and exosomes<sup>[22]</sup>. The latter subtype consists of a bi-layered lipid film of 30-120 nm, originated by convex membranes in late endosomes, determining the production of multi-alveolar bodies<sup>[23]</sup>. Different and various proteins are typical markers of exosomes, such as tetraspanin (CD9, CD63, CD81), annexin, heat shock proteins, caveolin and clathrin, as well as protein characteristics of source cells<sup>[24]</sup>. Furthermore, exosomes present specific lipids, comprising lipid raft portions, ceramides, sphingomyelin, cholesterol, GM1 ganglioside and phosphatidylserine. Additionally, they can contain nucleic acids, mRNA and ncRNA<sup>[25]</sup>. MSC-derived exosome biosynthesis and secretion are complex pathways which differ in microenvironmental stimuli, like inflammation or hypoxia<sup>[26]</sup>. mTOR and Wnt involving pathways seem to play a pivotal role in exosome release<sup>[27]</sup>. Interestingly, recent studies have shown that MSC-derived exosomes may be involved in antigen presentation and immunologic response, coagulation, angiogenesis and apoptosis, as confirmed by the expression of antigens such as CD9, CD44, and CD89 on their surface<sup>[28]</sup>. Thus, the secretome obtained from the culture of MSCs would appear to promote tissue repair and modulate immune response *in vitro* and *in vivo*, showing a translational impact on regenerative medicine<sup>[29]</sup>. The use of CM could present diverse advantages if compared to the original MSC implantation, such as: (1) Removal of the inherent risks of cell transplantation; (2) Simpler storage, transport, and conservation requirements; and (3) Possible application as a ready-to-go biologic product<sup>[30]</sup>.



### *Secretome preconditioning modulated by MSC cultural microenvironment*

Over recent years, it has been shown how preconditioning approaches for improving paracrine secretion, such as hypoxia, biochemical stimuli and 3D microenvironment, can increase the viability, proliferation and paracrine features of MSCs, thus expanding the therapeutic potential of these cells and their derived products<sup>[31]</sup>. In detail, dynamic culture conditions, such as 3D aggregate culture and fluid flow, could noticeably impact cellular behavior<sup>[32]</sup>. Boosted levels of growth factors and cytokines were detected in 3D MSC cultures grown on rotatory orbital or shaking platforms, in stirred systems, such as stirred tank reactors or spinner flasks, and in microgravity bioreactors<sup>[33]</sup>. Nevertheless, little is still known about the dynamic culture conditions and procedures for 3D aggregate MSC cultures as a scalable and reproducible plan for secretome production. However, the possibility of culturing cells under 3D conditions in a way to better mimic the *in vivo* environment has emerged<sup>[34]</sup>. A dynamic crosstalk between the cells could permit them to constantly modify their secretome following received stimuli, generating a microenvironment able to promote secretome enrichment for specific applications. Additionally, enhancing the manufacturing process allows to obtain MSC cell populations which can be cryopreserved for clinical applications, trying to expand clinical efficacy<sup>[35]</sup>. Recently, the use of matrix-conjugated hydrogel cell culture materials allowed to normalize a culture of induced pluripotent stem cell-derived MSCs (iPSC-MSCs), leading to a well-defined secretory profile able to promote enhanced neovascularization both *in vitro* and *in vivo*<sup>[36]</sup>. Using such innovative biomaterials, it was possible to stimulate reproducible secretion of pro-angiogenic and immunomodulatory cytokines from iPSC-MSCs that improve tubulogenesis of endothelial cells in Geltrex and neovascularization in chick chorioallantoic membranes<sup>[37]</sup>. Treatment with both IFN- $\gamma$  and TNF- $\alpha$  permitted to greatly optimize MSC secretome. Recently, a unique supernatant of MSCs from human umbilical cord-derived MSCs (hUCMSCs), pretreated with TNF- $\alpha$  (S-IT MSCs), was discovered to be more powerful in promoting macrophage migration, M2 polarization and phagocytosis, thanks to the induced high levels of CCL2 and IL-6<sup>[38]</sup>. Another way to overcome the expansion limitation of MSCs is to work with MSCs

derived from human-induced pluripotent MSCs (hiMSCs), serving as a reproducible and sustainable cell source. In a way similar to human bone marrow-derived MSCs (hBMMSCs), hiMSCs can release EVs with *in vitro* immunomodulatory properties, with an increased expression of well-known immunomodulatory genes such as *HLA-DRA*, *IDO1* and *CXCL8/IL8*, and at least another 100 regulated by NF $\kappa$ B signaling, known to play a pivotal role in immune response<sup>[39]</sup>. Interestingly, it has recently been seen that hypoxic preconditioning appears to induce adipose-derived stem cell-secretome (ASCs) to release a secretome with enhanced anti-apoptotic effects by promoting the autophagic process of ASCs<sup>[40]</sup>. Furthermore, the specific content of EVs can be modulated by hypoxia, with their source cell responding by triggering the HIF at low O<sub>2</sub> levels. The pleiotropic effects of HIF permit to regulate the expression of many genes involved in pathways such as inflammation, angiogenesis, migration, differentiation, metabolism, proliferation and apoptosis. Expression of these genes is reflected in the interior of secreted EVs, which showed a greater regenerative ability than those achieved under normal oxygen conditions<sup>[41]</sup>. Moreover, the preconditioning of MSCs in an oxidative stress (OS) environment provides the release of many proteins, growth factors, cytokines and exosomes that could increase the antioxidant ability of MSCs against OS, enforcing secretome as an encouraging, novel, cell-free tissue regeneration approach<sup>[42]</sup>.

#### **Characterization of MSC secretome EVs**

A detailed analysis of the secretome structure might <sup>5</sup> contribute to the improvement of secretome application for regenerative purposes and allow the discovery of novel biomarkers, also circulating in patients' blood, improving pathology diagnosis and discovering new therapeutics targets<sup>[43]</sup>. As already anticipated, secretome fractions consist of lipids, proteins <sup>4</sup> and non-coding RNAs able to impact the physiology of target cells. MSC-EVs were shown to present a significant number of miRNAs, <sup>5</sup> such as miR-210, miR-200b-3p and miR-4732-3p, involved in improving myocardial function<sup>[44]</sup>. BM-MSC-EVs, MSC-EVs-PD-L1 and hUCMSCs-EVs also exhibited a healing role in autoimmune conditions<sup>[45]</sup>. miR-146a and miR-27a/b, up-regulated in ASC derived EVs, were able to



induce neo-angiogenesis pathways, while <sup>5</sup>miR-122-5p, miR-27a, miR-206 and lncRNA MALAT1 played a relevant role in osteogenic regenerative processes<sup>[46]</sup>. Other studies identified specific factors from the secretome released by tumor cells that might be actively involved in cancer progression, thus representing optimal biomarkers<sup>[47]</sup>. Additionally, a customized secretome could be rich in pro-apoptotic factors that are helpful against cancer, <sup>5</sup>or higher levels of pro-angiogenic and pro-osteogenic factors suitable for regenerative applications. An interesting case is represented by human fetal MSCs, producing a secretome rich <sup>5</sup>in anti-apoptotic factors as well as pro- and anti-angiogenic and osteogenic differentiative proteins<sup>[48]</sup>. On the contrary, the multipotent fetal dermal cell secretome was enriched in up-regulated <sup>5</sup>proteins involved in wound healing processes, angiogenesis and cellular metabolism<sup>[49]</sup>. Such data underlined that fetal MSC secretome could be more beneficial for regenerative purposes if compared to the adult cell one and, in agreement with reports on the secretome derived from three-dimensional cultured cells, that <sup>5</sup>fetal cells cultured under 3D conditions might further improve the therapeutic abilities of their secretome<sup>[50]</sup>. Unique immunomodulatory properties emerged for amniotic MSCs. Their secretome was able to reduce <sup>4</sup>the polarization of T cells toward inflammatory Th subgroups, inducing regulatory T cells; to decrease <sup>4</sup>the proliferation of activated peripheral blood mononuclear cells (PBMC); to affect <sup>4</sup>monocyte polarization to antigen-presenting cells stimulating the synthesis of anti-inflammatory macrophage (M2) markers and to reduce the activation of B lymphocytes into plasma cells<sup>[51]</sup>. The most intriguingly aspect of secretome EV fractions probably deals with functional mitochondria release from human mesenchymal stromal cells<sup>[52]</sup>. Recent studies in non-orthopedic tissues proposed that MSCs can rescue damaged cells by donating mitochondria, repairing mitochondrial activity in target cells, preserving cell viability, and stimulating tissue repair. To obtain this goal, MSCs might be able to package mitochondria for export into EVs, and such "mitoEVs" could provide a delivery approach for cell-free mitochondria-targeted therapy<sup>[53]</sup>.

### *Therapeutic role of secretome in central nervous system pathologies*

The MSC secretome is a significant element of the paracrine and autocrine cell signaling mechanism, playing a crucial role in the regulation of many physiological and pathological processes. In particular, its effects on immunomodulation, neuronal survival and regeneration, thanks to the action of soluble and vesicular factors, are pivotal in reducing or even arresting neuronal disease evolution and in promoting repair<sup>[54]</sup>. Thus, the various MSC secreted factors and vesicles seem to be an effective tool for the protection and survival of neuronal and glial cells<sup>[55]</sup>. Traumatic brain injury (TBI) is determined by external mechanical forces able to cause physical, cognitive, and emotional impairments<sup>[56]</sup>. In this case, MSC-derived secretome may be used to control the secondary injury mechanisms of TBI, modulating the abnormal inflammatory cascade, reducing pro-inflammatory cytokines, and stimulating neural stem cell proliferation and differentiation<sup>[57]</sup>. Moreover, EVs released by MSCs reduced neuroinflammation and supported neurogenesis and angiogenesis, rescuing spatial learning and motor damage in TBI animal models<sup>[58]</sup>. Spinal cord injury (SCI) is characterized by long-term functional deficits following the loss of neurons and glial cells, inflammation, and demyelination<sup>[59]</sup>. The paracrine factors secreted into the lesion site by MSCs, such as HGF, BDNF and NGF could promote immunomodulation, glial scar reduction, axonal regeneration and neurite outgrowth. Additionally, ASC-derived secretome reduced the production of TNF- $\alpha$  by M1 macrophages while it improved TGF- $\beta$ 1 and IL-10 production by M2 macrophages<sup>[60]</sup>. Regarding secretome vesicles only, MSC exosomes could stimulate anti-inflammatory and pro-angiogenic effects and axonal regeneration, and suppress glial scar formation and cell apoptosis, reducing lesion size and improving functional recovery after traumatic SCI<sup>[61]</sup>. Ischemic stroke (IS) is a cerebrovascular pathology induced by blood vessel occlusion or injury, leading to a blood supply defect, determining focal tissue loss and endothelial and neuronal cell death<sup>[62]</sup>. The use of MSC secreted factors such as IGF-1 and BDNF could induce neuroprotection by impeding neuronal damage and tissue loss and reduce astrocyte injury by GFAP downregulation<sup>[63]</sup>. Parkinson's disease (PD) is a neurodegenerative pathology characterized by the progressive degeneration of dopaminergic neurons. In PD, it has

already been seen that the addition of MSC secretome can promote a partial reversion of PD histological impairments and gains in animals' motor ability by the secretion of immunomodulatory, anti-inflammatory, neurogenic, neurodevelopmental, neurorescuing or anti-apoptotic factors<sup>[64]</sup>. Recent evidence highlighted the particular ability of MSC secretome to reduce one of the hallmarks of the disease, the alpha-synuclein aggregates, through a MMP-2-based mechanism<sup>[65]</sup>.

### **MAIN FEATURES OF RETINAL CELLS**

Even if peripherally localized, the retina represents an important part of the central nervous system (CNS). Though it presents the same types of functional elements and neurotransmitters sited in other portions of the CNS, the retina includes five classes of neurons: photoreceptors (rods and cones), bipolar cells, amacrine cells, horizontal cells and ganglion cells. Light absorption by the photopigment in the outer segment of rods and cones, the two photoreceptors, starts a cascade of events that changes the receptor membrane potential, and therefore the quantity of neurotransmitter released by the rod and cone synapses onto the adjacent bipolar cells, in the outer plexiform layer. Then, in the inner plexiform layer, the short axonal processes of bipolar cells realize a synapse with the dendritic processes of ganglion cells whose axons form the optic nerve. Horizontal and amacrine cells, instead, present their cell bodies within the inner nuclear layer and are mainly involved in lateral interactions between already described retinal cells, impacting on the visual system's sensitivity to light contrast over a wide range of intensities. Amacrine cell processes, which ramify laterally in the inner plexiform layer, are postsynaptic to bipolar cells and presynaptic to ganglion cells, while the processes of horizontal cells, instead, extend in the outer plexiform layer. The existence of different subgroups of amacrine cells that play a distinct role within visual pathways is relevant. Furthermore, the neural retina and the choroid are connected by a monolayer of cells constituting the retinal pigmented epithelium (RPE). Light absorption, epithelial transport, spatial buffering of ions, visual cycle regulation and phagocytosis of rod and cone outer segment membranes represent the main functions exerted by RPE<sup>[66]</sup>.

### *RPE cell secretome and retinal diseases*

The RPE is characterized by a polarized nature, with molecules expressed by these cells either secreted to the apical or basolateral membrane, respectively by the Na<sup>+</sup>/K<sup>+</sup>-ATPase associated channel or by anion ones. These products, mainly growth, anti/proangiogenic and neurotrophic factors, are critical for the correct functioning of the neuroretina and choroid. Among them, the most characterized are VEGF<sup>[67,68]</sup>, TGF-β<sup>[67,69]</sup>, PEDF<sup>[70]</sup>, MMPs<sup>[71]</sup>, NGF<sup>[72]</sup>, FGF-1, FGF-2, and FGF-5<sup>[73]</sup>, IGF-I<sup>[74]</sup>, BDNF<sup>[75]</sup>, PDGF<sup>[76]</sup>, CTGF<sup>[77]</sup>, LEDGF<sup>[78]</sup>, interleukins<sup>[79]</sup>, tissue inhibitor of matrix metalloproteases<sup>[80]</sup>, PIGF<sup>[81]</sup>, angiogenin<sup>[82]</sup>, EPO<sup>[83]</sup>, somatostatin<sup>[84]</sup> and apolipoprotein A1<sup>[85]</sup>. These factors could also play a fundamental role in the etiology of several retinal diseases such as diabetic retinopathy (DR), age-related macular degeneration (AMD) and retinopathy of prematurity (ROP)<sup>[86]</sup>. Deep proteomic analyses of RPE cells cultivated in these pathological condition microenvironments suggested that previously described molecules could be involved in membrane and cytoskeleton dynamics, mitochondrial trafficking, protection/induction of cellular stress, apoptosis, differential modulation of multidrug resistance-associated proteins, and in other metabolic events already during the first stages of the diseases<sup>[87]</sup>. In physiological conditions, RPE cells release EVs characterized by proteins associated with biological pathways involved in AMD etiology, including drusen composition. Recently, first evidence that drusen-associated proteins are secreted as cargo of EVs produced by RPE cells in a polarized apical to basal way has recently been seen. Remarkably, drusen-associated proteins revealed differential regulation of polarized secretion in homeostatic conditions and in response to AMD stressors<sup>[88]</sup>. Findings suggested that a finely-tuned mechanism is pivotal to regulate directional sorting and secretion of drusen-associated proteins *via* RPE secretome EVs, supporting the influential role of vesicles as a strategic source of drusen proteins and critical elements to drusen development<sup>[89]</sup>. OS changed the release of several factors implicated in neovascularization and AMD, stimulating a pro-angiogenic microenvironment by increasing the secretion of VEGF, PTN, and CRYAB, and reducing



the production of anti-PEDF and CFH. Apical secretion was influenced more than basolateral for PEDF, CRYAB and CFH, while the directional way of secretion was impacted more for VEGF, which may have implications for choroidal neovascularization<sup>[90]</sup>. VEGF-A is an important proangiogenic factor released by different retinal cytotypes (endothelial cells, Müller cells, ganglion cells and pericytes), but primarily by the basolateral side of RPE in homeostatic conditions, shifting to apical during pathological conditions<sup>[91]</sup>. In particular, VEGF over-expression was highlighted in hypoxic and hyperglycemic conditions, by both *in vitro* and *in vivo* studies, also demonstrating that in pathological conditions, VEGF causes alteration of tight junction proteins and transepithelial resistance<sup>[92]</sup>. It has been confirmed that VEGF R2, placed in the apical side of RPE cells, can induce disruption of the RPE barrier by promoting VEGF signaling<sup>[93]</sup>. Considered together, such findings led to the development of anti-VEGF therapies to treat retinal neovascularization in patients with DR and other related diseases. However, many associated complications are still present, such as repeated injection requirements, increased ocular pressure, macular edema, subconjunctival hemorrhage, pain, uveitis, and the compromised viability of RPE, photoreceptors, choriocapillaris and Müller glia (MG)<sup>[94]</sup>. One of the most interesting relates to splice variants of VEGF, such as VEGF165b, expressed by RPE cells. It would appear to act as a powerful anti-angiogenic isoform of VEGF with significant results in treating induced choroidal neovascularization, and was also decreased in DR<sup>[95]</sup>. Nevertheless, while inner retinal barrier and Müller cell association with VEGF is well known, the outer retinal barrier properties of RPE in relation to VEGF in diabetes and other ocular neovascularization-related diseases should be better investigated. One of the most significant glycoproteins of the RPE secretome is PEDF, a serine protease inhibitor with neuroprotective, anti-angiogenic and anti-inflammatory features. In homeostatic conditions, PEDF is apically released from the RPE and preserves retinal and choriocapillaris integrity by preventing endothelial cell proliferation<sup>[96]</sup>. It was seen that PEDF was down-expressed in human hyperglycemic RPE cells, as well as in patients affected by proliferative DR (PDR), diabetic macular edema (DME), ROP, Retinitis

Pigmentosa and Leber Congenital Amaurosis<sup>[97]</sup>. Thus, PEDF is primarily considered for its therapeutic potential, showing positive effects in photoreceptor survival, morphology and function, also reducing vascular permeability in correlation with reduced levels of angiogenic factors (VEGF, VEGFR-2), cytokines and chemokines<sup>[98]</sup>. Additionally, recent animal studies have proven that in an oxygen induced retinopathy model and in rat model of choroidal neovascularization, PEDF up-regulation blocked retinal neovascularization and inflammation<sup>[99]</sup>. Another pro-survival cytokine able to stimulate fibroblast chemotaxis/proliferation and preserve pericyte viability and physiological vascularization of the retina is PDGF. Similar to VEGF, it can promote pathologic neovascularization in PDR and DR, and in a hypoxia regulated microenvironment<sup>[100]</sup>. PDGF receptor activation suggests an autocrine mechanism in epiretinal membrane development and retinal wound repair<sup>[101]</sup>. Today, one of the most promising research fields deals with understanding the crosstalk of PDGF with other signaling pathways, in order to identify the best molecular targets for combinatorial therapies. This idea arose from several animal studies which established that an antagonism to PDGF-BB (a homodimeric form of the PDGF family), together with anti-VEGF, enhanced the arrest of retinal neovascularization<sup>[102]</sup>. An important co-factor of VEGF is PIGF. It can alter retinal fibrovascular integrity and RPE permeability by interaction with VEGF and activation of Akt and HIF-1 pathways<sup>[103]</sup>. Thus, it was found at high levels in AMD and PDR patients<sup>[104]</sup>. The use of anti-PIGF monoclonal antibody in different animal models revealed reduced inflammation and vascular leakage with no adverse effects in retinal ganglion cell (RGC) viability<sup>[105]</sup>. However, novel strategies that avoid the weaknesses observed in repeated intraocular injections should consider PIGF as a valid therapeutic target. RPE cells cultured in high glucose medium also showed an elevated expression of CTGF, one of the main fibrogenic factors involved in fibroblast proliferation and extracellular matrix synthesis, which could control the microenvironment around the distal retinal/RPE/Bruch's membrane complex and protect against neurodegenerative diseases<sup>[106]</sup>. Increased retinal CTGF levels might play an essential role in DR, probably by reducing VEGF levels<sup>[107]</sup>. Thus, the combined use of anti-CTGF and anti-VEGF in



treating complications of DR could exert more beneficial effects than a monotherapy drug<sup>[108]</sup>. CTGF is corroborated in its activity by the more well-known FGF, which play a crucial role in stimulating vascularization, angiogenesis and cell survival, acting as autocrine factors. FGF1, FGF2, and FGF5 are principally released in the RPE, reaching their highest levels in non-proliferative retinopathy, PDR with active proliferative retinopathy and diabetic conditions, respectively<sup>[109]</sup>. Recently, targeting of retinal FGFs exhibited worthy results in improving visual acuity of DME and exudative AMD patients, even if further studies are mandatory to determine long-term effects<sup>[110]</sup>. The secretome produced by human RPE cells also presents IGF-1 and 2, natural proteins promoting growth and insulin-like metabolic effects, together with their receptor (IGF-R) and binding protein (IGFBP-2)<sup>[111]</sup>. Both growth factors seem to play a pivotal role for RPE autocrine/paracrine-mediated modulation of proliferation<sup>[112]</sup>. Recent evidence showed that another IGFBP family member, IGFBP-3, was able to reduce DR by considerably decreasing TNF- $\alpha$  levels and pro-apoptotic markers<sup>[113]</sup>. Among secreted factors, TGF- $\beta$  represents one of the main elements which can modulate main cellular physiological processes, like growth, differentiation, proliferation and apoptosis<sup>[114]</sup>. However, there is scant information on its efficacy and potential mechanisms in relation to retinal homeostasis or pathology. In detail, comparable secretion levels of TGF- $\beta$  from polarized RPE, differentiated from human embryonic stem cells (hESC) and human RPE, promoting retinal homeostasis and sustaining the potential of hESC-RPE in replacement therapies, have recently been highlighted<sup>[115]</sup>. Human stem cell-derived RPE treated with reactive oxygen species (ROS) for 1 or 3 wk released more than 1000 proteins, many of which showed relevant changes due to induced stress. In particular, secreted APOE was decreased 4-fold, as well as TGF- $\beta$ , and urotensin-II, one of the most effective vasoconstrictors, doubled, similar to BMP1<sup>[116]</sup>. The glycoprotein EPO represents one of the most promising molecules found in RPE secretome. It acts as an erythropoiesis regulator with different additive features such as vessel integrity, recruitment of endothelial progenitor cells, neuroprotection and anti-oxidative properties<sup>[117]</sup>. High levels of EPO were recently found in DR, PDR and DME patients<sup>[118]</sup>. Especially in

hyperglycemic conditions, EPO seems to protect the RPE barrier, reducing retinal vasculogenesis, downregulating VEGF and VEGFR expression and protecting tight junctions by increasing the flow of  $\text{Ca}^{2+}$  ions in blood-brain barrier animal models<sup>[119]</sup>. However, the administration of EPO in the late stage of a hypoxia-induced murine retinopathy model worsened retinal neovascularization, suggesting that EPO might play a protective role in early DR and a pathologic one in late DR<sup>[120]</sup>. This dual nature of EPO could be related to its action mechanism, whose first step is its hypoxia-modulated binding to cell surface receptor EPOR. Thus, it can be predicted that in the first stages of DR, EPO exerts neuroprotective functions while in the advanced stage of DR, EPO acts as a neovasculogenesis inducing molecule which is regulated by hypoxia<sup>[121]</sup>. MMPs, apically secreted by RPE, are calcium-dependent endopeptidases involved in angiogenesis, which is fundamental for ocular extracellular matrix and photoreceptor outer segment homeostasis<sup>[122]</sup>. Recently, it has been seen that a basolateral secretion of MMP is related to AMD, and increased levels of MMP-2 and MMP-9 were also observed in the Bruch's membrane of AMD and DR patient eyes<sup>[123]</sup>. Thus, inhibitors of both cited MMPs might also exert an advantageous role by blocking capillary cell apoptosis, growth of vessels and reduce inflammatory-mediated permeability<sup>[124]</sup>. In addition to angiogenic and anti-angiogenic factors, numerous inflammatory chemokines and cytokines were elevated in retinal diseases, such as PD and PDR. Among them, the most investigated were MCP-1, IL-6 and IL-8. It was seen that MCP-1 and IL-8 secretion levels are directly correlated to blood glucose levels, suggesting a crucial role in altered blood retinal barrier (BRB) activities of DR affected patients<sup>[125]</sup>. MCP-1 carries out chemo-attractant activity for monocytes and lymphocytes to promote endothelial proliferation, and may limit the impairment of neurosensory retina<sup>[126]</sup>. IL-6 and IL-8, instead, were over-expressed in cultured RPE cells stimulated with IL-1b or TNF- $\beta$ , suggesting that polarized release of growth factors/cytokines is favored in retinal diseases<sup>[127]</sup>. Additionally, it is interesting to cite the recently discussed role of somatostatin as a neuromodulator of retinal homeostasis, as hypothesized by its downregulation in the RPE of diabetic eyes<sup>[128]</sup>. Finally, several substrates of the serine protease HTRA1 were found in the RPE

secretome, proposing a link between it, complement modulation and amyloid deposition in AMD etiopathogenesis. In detail, a cleavage of fibromodulin (90%), CLU (50%), and vitronectin (54%), involved in regulation of the complement pathway was seen, along with a cleavage of 2-macroglobulin (55%) and ADAM9 (54%) related to amyloid deposition, as well as some cell surface protein cleavages including talin-1 (21%), fascin (40%), and chloride intracellular channel protein 1 (51 %)<sup>[129]</sup>.

### ***MG cell secretome and retinal diseases***

Regarding the RPE, Müller cells can modulate trophic secretion depending on the healthy or pathological status of the retina<sup>[130]</sup>. Müller cell physiological secretome mainly contains molecules that are crucial to increase BRB tightness, like thrombospondin-1 and PEDF<sup>[131]</sup>. In pathological circumstances, factor synthesis and secretion both shift towards an inflammatory environment. Under hyperglycemic conditions, IL-1b release by Müller cells is increased, leading to vascular impairment and cell death via a paracrine mechanism. Thus, by inhibiting IL-1b or knocking down its receptor it was possible to decrease inflammation and photoreceptor/retinal vessel disruption in murine models, exerting a possible therapeutic role for ocular dystrophies related to chemokine expression and/or diabetes<sup>[132]</sup>. Furthermore, also the proinflammatory IL-6 and TNF- $\alpha$  can be secreted by Müller cells, determining a possible promotion of both vascular dysfunction and angiogenesis, even if IL-6 may exert protective effects toward photoreceptor cells<sup>[133]</sup>. Stimulation of porcine and human Müller cells with IL-4, IL-6, IL-10, VEGF, INF- $\gamma$ , TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TNF- $\alpha$  resulted in a primarily pro-inflammatory phenotype with release of cytokines and factors of the complement system<sup>[134]</sup>. Additionally, Müller cells expressed proteins linked to biosynthesis and maturation of phagosomes. These findings, thus, underlined the relevance of Müller cell signaling in chronic retinal inflammation<sup>[135]</sup>. Additionally, under hyperglycemia and hypoxic conditions, Müller cells shift PEDF secretion to VEGF, contributing to ocular vascular diseases<sup>[136]</sup>. Therefore, inhibition or knockdown of Müller cell-derived VEGF could reduce ischemia-induced impairment of the BRB, prevent ischemia-induced retinal

neovascularization and decrease vascular leakage<sup>[137]</sup>. Recent evidence highlighted that, in the diabetic retina, expression of VEGF could be regulated by increasing the activity of the receptor for retinoic acids alpha, which also stimulate the expression of glial cell line-derived neurotrophic factor, with a final significant decrease of vascular leakage<sup>[138]</sup>. Nevertheless, in recent years, the neuroprotective effects of VEGFR-2 in Müller glia have also been described, suggesting its significance for cell survival and consequential viability of neuronal cells in the diabetic retina<sup>[139]</sup>. Interesting, the secretome of Müller cells also contains type 2 and 9 MMPs, increased, respectively, in patients with PDR and AMD<sup>[140]</sup>. It was proposed that the stabilization of HIF-1a could raise the level of VEGF, inducing MMP-2 expression in neighboring endothelial cells, with consequent retinal neovascularization<sup>[141]</sup>. As MMPs regulate crucial cellular pathways through angiogenesis and apoptosis, their targeting could represent an important therapeutic strategy for ocular diseases. Moreover, new evidence has proven that Müller glia release neurotrophic factors that support RGC survival, such as CLU, osteopontin and basigin. The latter two significantly enhanced RGC survival *in vitro*, suggesting that the survival-promoting activity of Müller cell secretome is multifactorial<sup>[142]</sup>. Recently, it was showed that human iPSC-derived multinucleated giant cells hiMGCs could represent an alternative to primary MGCs (pMGCs) in understanding glial cell involvement in retinal disorders, including DR. Under culture with palmitate, a major free fatty acid with elevated plasma levels in diabetic patients, hiMGCs and pMGCs expressed low transcript levels of AQP4, RLPB1, SLC1A3, KCNJ1 and KCJN10. Furthermore, the analysis of palmitate-treated hiMGC secretome evidenced an upregulation of proangiogenic factors powerfully related to DR, including ANG2, endoglin, IL-1b, CXCL8, MMP-9, PDGF-AA, and VEGF<sup>[143]</sup>. One of the most interesting pieces of evidence regarding Müller cell secretome was linked to the production of different EVs from endfeet and microvilli of retinal Müller cells in adult mice. In particular, VAMP5 was identified as a Müller cell-specific snap receptor member which is part of EVs and responsive to ischemia, with relevant changes between the secretomes of Müller cells and neurons *in vitro*<sup>[144]</sup>.



### *Other glial cell secretome and retinal diseases*

Undifferentiated rat RGC line RGC-5 can secrete numerous protein markers of RGCs, even if they are unable to react to glutamate or N-methyl-D aspartate. Furthermore, it has recently been highlighted that human nonpigmented ciliary epithelial (HNPE) cells could release several neuroproteins also located in the aqueous humor, many of which can influence the activity of neuronal cells. Recent works identified about 130 unique proteins from the HNPE cell-conditioned SF-medium, most of them involved in cell differentiation. These results allowed to hypothesize that a differentiation system of HNPE cell-conditioned SF-medium with RGC-5 cells can promote a differentiated phenotype in RGC-5 cells, functionally close to primary cultures of rat RGCs<sup>[145]</sup>. The secretome of retinoblastoma, the solid malignancy of the developing retina, is immunosuppressive and induces a protumoral phenotype. This conclusion was the result of complex analyses that identified the cytokine extracellular matrix metalloproteinase inducer and macrophage migration inhibitory factor (MIF), both characterized by detected immunosuppressive activity and secreted at high levels in retinoblastoma primary cell cultures. In addition, macrophages derived from PBMC increased the expression of M2-like polarization markers following exposure to retinoblastoma-conditioned medium or recombinant MIF<sup>[146]</sup>.

### **CROSS-LINK BETWEEN RETINAL AND MESENCHYMAL SECRETOMES: DIFFERENT MSC-DERIVED SECRETOMES AS INNOVATIVE APPROACHES TOWARDS RETINAL DISEASES**

MSC secretome is currently studied extensively for the treatment of several retinal diseases. Its therapeutic potential lies in its richness of immunomodulatory, antiangiogenic and neurotrophic factors, preventing retinal degeneration and improving retinal morphology and function. Additionally, exosomes secreted by MSCs showed anti-inflammatory and anti-apoptotic effects (Figure 1 and Table 1). Based on MSC origins and their particular secreted factors, several promising preclinical and clinical studies

were initiated to explore the potential advantages of MSC secretome for the treatment of retinal diseases.

#### ***Novel evidence on the role of MSC secreted factors in retinal disease etiopathogenetic pathways***

Novel evidence showed that MSC-CM inhibits abnormal neovascularization and decreases vaso-obliteration (promoting revascularization) in retinopathies by restoring neuronal Sema3E levels which reduce pathological concentrations of IL-17A (and associated proinflammatory factors, such as IL-1b) in myeloid cells<sup>[147]</sup>. Among MSC released factors, PDGF secretion may play a crucial role in MSC-mediated RGC neuroprotection. These results were obtained from the arrest of PDGF signaling by small molecule PDGF inhibitors, neutralizing antibody or downstream phosphatidylinositol 3 kinase, which blocked RGC neuroprotection conferred by MSC co-culture. Furthermore, intravitreal injection of PDGF led to relevant optic nerve neuroprotection *in vivo* after experimental induction of high intraocular pressure<sup>[148]</sup>. Application of CM obtained from MSCs protected against A $\beta$ 1-42 oligomer-induced retinal pathology in RGCs of both rat and ARPE-19 cells, thanks to proteins associated with SIRT1/pAKT/pGSK3 $\beta$ / $\beta$ -catenin, tight junction proteins and apoptosis pathway<sup>[149]</sup>. Furthermore, in recent years, the administration of EVs in models of neurological disorders has highlighted a relevant improvement of neurological dysfunctions. In particular, miRNAs from MSC-EVs, as one of the central mediators that control various genes and decrease neuropathological change, have been identified in various neurological pathologies<sup>[150]</sup>.

#### ***Bone marrow MSC-derived secretome regulates retinal cell neuroprotection***

BMMSC secretome protects retinal morphology, regulates autophagy-, pro-apoptotic- and pro-necroptotic-related gene and protein expression, and promotes the activation of antioxidant machinery, exerting neuroprotective ability during retinal degeneration<sup>[151]</sup>. A recent expression analysis of about 1000 proteins exhibited high levels of <sup>12</sup> paracrine factors secreted by hBMMSCs that might be fundamental in the neuroprotective effect of



the stem cell secretome over *in vitro* retinal degeneration. These results support the hypothesis that the paracrine effect of hBMMSCs may slow photoreceptor death and be a therapeutic possibility in retinal photoreceptor degenerative diseases<sup>[152]</sup>. Additionally, rat BMMSCs cultured with secretome from neonatal rat retinal cells were able to differentiate into RGC-like cells, exhibiting protein expression patterns similar to those of isolated RGCs such as Map2, nestin and Thy1.1<sup>[153]</sup>.

### *Adipose MSC-derived secretome regulates retinal cell regeneration*

Recent evidence showed an important therapeutic effect of human adipose mesenchymal stem cells (hADSCs) and their secretome (hADSC-CM) on an *in vivo* model of sodium iodate retinal neurodegeneration. The studies highlighted that hADSC-CM effects were particularly striking, especially in terms of photoreceptor regeneration and retinal function, as underlined by increased expression of retinal regeneration markers such as Pax6, Chx10, S-Op sin (Opn1sw), Nrl, Crx and GFAP<sup>[154]</sup>. Oxidatively stressed ARPE-19 cells treated with adipose MSC based on CM (aMSC-CM), and/or combined with nicotinamide, vasoactive intestinal peptide, or both factors showed an improved recovery from the damaged status. Additionally, the same treatment could determine better protection of the neuroretinal architecture, mainly rods and cones, and a lower degree of glial cell activation<sup>[155]</sup>. The preclinical efficacy of adipose-derived stem cell concentrated conditioned medium (ASC-CCM) was recently tested in repetitive ocular blast injury mice, highlighting a significant rescue from retinal injury and a significant restoration of visual function, also associated with a significant reduction of neuroinflammation markers, retinal GFAP and OS. Furthermore, *in vitro*, oxidatively stressed Müller cells pre-incubated with ASC-CCM exhibited normalized levels of GFAP, viability, and catalase activity<sup>[156]</sup>. Intravitreal injection of ASC-CCM was safe and efficient against the visual impairments of mild TBI. Blast mice treated with ASC-CCM exhibited improved vision at 5 mo but minimal effects at 10 mo, associated with alterations of GFAP and proinflammatory gene expression in retina. Thus, the unchanged

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glial response and the risk of retinal injury with live cells suggested that ASC-CCM might have better safety and efficacy than live cells for visual dysfunction therapy<sup>[157]</sup>.

### *Human uterine cervical and Wharton's jelly MSC-derived secretomes regulate retinal cell immunomodulation*

Interesting, the treatment of oxidative stressed ARPE-19 cells with human uterine cervical stem cells-conditioned medium evidenced a significant increase of VEGFA, HO-1, HSPB1, GCLC, PDGFA, and PDGFB mRNA expression, highlighting a potential stimulation of detoxifying genes, protection from damage by OS and better vascularization<sup>[158]</sup>. Recent studies demonstrated that RPE cell viability and the expression of anti-apoptotic Bcl2 were reduced significantly in a CM secreted by human Wharton's jelly MSC (WJMSCs) treated RPE cells, while expression of pro-apoptotic biomarkers of Bax and IL-1b was not significantly changed. WJMSCs are a subgroup of MSCs isolated from the Wharton jelly of the umbilical cord characterized by high potential of proliferation and a secretome rich in trophic factors and immunomodulatory cytokines. Moreover, previously described experiments showed that WJMSC secretome could induce apoptosis in RPE cells through activating apoptosis pathways, being a potential therapeutic target for pathologies like proliferative vitreoretinopathy<sup>[159]</sup>.

### CONCLUSION

Current research on the retinal secretome represents a growing field of study due to its elevated potential for treatment of retinopathies. However, many challenges are still open, such as the specific characterization of secretome released factors to further target therapy to the pathology profile, better manipulation of retinal secretome or from other cell sources for noteworthy therapeutic effect, improving methods for intraocular administration of secretome factors, and developing personalized combinations of trophic factors involved in different pathological pathways (inflammation, ROS, angiogenesis, proliferation) to evaluate the collective therapeutic potential. Nevertheless, the possible improvement of new efficient pharmaceutical formulations related to the

secretome of MSCs and retinal cells, with the addition of exogenous factors or drugs without the necessity to deliver cells into the eye may represent a novel milestone towards a personalized approach to retinal disease.

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