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Roles of Exosomes in the Normal and Diseased Eye

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Abstract

Exosomes are nanometer-sized vesicles that are released by cells in a controlled fashion and mediate a plethora of extra- and intercellular activities. Some key functions of exosomes include cell-cell communication, immune modulation, extracellular matrix turnover, stem cell division/differentiation, neovascularization and cellular waste removal. While much is known about their role in cancer, exosome function in the many specialized tissues of the eye is just beginning to undergo rigorous study. Here we review current knowledge of exosome function in the visual system in the context of larger bodies of data from other fields, in both health and disease. Additionally, we discuss recent advances in the exosome field including use of exosomes as a therapeutic vehicle, exosomes as a source of biomarkers for disease, plus current standards for isolation and validation of exosome populations. Finally, we use this foundational information about exosomes in the eye as a platform to identify areas of opportunity for future research studies.

Keywords

exosome; extracellular vesicle; age-related macular degeneration; proteome; glaucoma; biomarker

1. Exosomes: a brief overview

The endocytic pathway consists of compartments involved in the internalization of extracellular ligands or cellular components, recycling of those components to the plasma membrane, and/or their degradation (Gould & Lippincott-Schwartz 2009, Klumperman & Raposo 2014). During the maturation process of early endosomes into late endosomes (Stoorvogel et al. 1991), intraluminal vesicle (ILVs) accumulate. Because of their appearance, these late endosomes are generally referred to as multivesicular endosomes (MVE) or multivesicular bodies (MVBs). Loading of proteins, lipids, and cytosol into these

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ILVs is achieved by inward budding of the early endosomal membrane and specific sorting. MVEs fuse with lysosomes in most cases, leading to the breakdown of their content. MVEs carrying the lysosomal-associated membrane proteins LAMP1 and LAMP2, the tetraspanin CD63, and other molecules in late endosomes, can however also fuse with the plasma membrane and disseminate their content into the extracellular space (Jaiswal et al. 2002, Raposo et al. 1996; Lo Ciero et al). The most well-known mechanism for formation of MVEs and ILVs is carried out by “the endosomal sorting complex required for transport” (ESCRT). Approximately thirty proteins that assemble into four complexes make up ESCRT (ESCRT-0, -I, -II and -III) and several conserved proteins from yeast to mammals (VPS4, VTA1, ALIX also called PDCD6IP) associate with ESCRT (Hanson & Cashikar 2012). In the early 1980s, small (30–100 nm) vesicles of endosomal origin secreted by reticulocytes, were described for the first time using the term, “exosome” (Harding et al., 1983; Pan et al., 1985). The definition of *bona fide* exosomes is at present somewhat fluid but in this review, we adhere to the recent definition put forth by Kowal and colleagues. According their recent paper, small vesicles (diameter ≈ 30–150 nm) with a low density by gradient ultracentrifugation and carrying CD81, CD63, CD9, Syntenin-1 and TSG101 proteins, qualify as *bona fide* exosomes (Kowal et al., 2016).

In this review, we will use the terms “exosome” and “exosomes” interchangeably to denote vesicles released extracellularly from late endosomes, and the use of this terminology should not be confused with the term “exosome complex” (often just called “the exosome”), which is a multi-protein intracellular complex capable of degrading various types of RNA (Kilchert et al., 2016). For a more detailed description of exosome biogenesis and an overview of the diversity of different cargoes (e.g. lipids, proteins, DNA, RNA) found in exosomes, please see the excellent recent review by Colombo and colleagues (Colombo et al., 2014).

It has become increasingly clear that exosomes have specialized functions and play a key role in intercellular signaling, and cellular waste management (van der Pol et al., 2012). Based on these known roles of exosomes released from cells in other organs, exosomes and other extracellular vesicles (EVs) released from different cell types in the eye are likely involved in similar pathways. However, currently very little is known about exosomes and other EVs released from cells in the eye. Exosomes make up the smallest sized subset (diameter ≈ 30–150 nm) of EVs (diameter ≈ 30–1,000 nm). Their biogenesis and extracellular release is distinct from other EVs such as larger microvesicles (diameter ≈ 150–1,000 nm) that bud directly from the plasma membrane (Raposo and Stoorvogel, 2013), blebs (diameter ≈ 400–800 nm) (Marin-Castano et al., 2005), and apoptotic bodies (diameter ≈ 800–5,000 nm) (Hristov et al., 2004). Other terms used for EVs, sometimes interchangeably, are ectosomes, shedding vesicles, and microparticles (Carver and Yang, 2016; Cocucci et al., 2009; Crescitelli et al., 2013; Gyorgy et al., 2011; Hess et al., 1999; Holme et al., 1994). Exosomes and microvesicles are also functionally distinct in many respects; therefore, to avoid confusion and to narrow the focus, this review will only discuss the current state of exosome(s) and small EV research in the eye and their potential role in ocular health and disease (Kowal et al., 2016; Lotvall et al., 2014).

The field of exosome and small EV research has figuratively exploded in recent years. A recent search with the keyword “exosomes” in NCBI’s PubMed database of scientific

publications returned 4,584 articles (Dec. 14, 2016), 1,205 of which were published in 2016 and 36 slated to be published in 2017, at the time this review was written (Fig. 1). The cancer field in particular has taken the lead investigating exosomes and other EVs for novel approaches to therapy, new mechanistic understanding of tumorigenesis, tumor signaling, novel biomarkers, and modulation of metastasis (Dhondt et al., 2016; Lopatina et al., 2016; Srivastava et al., 2016). For example, modulation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs), annexins and proteoglycans on exosomes have been shown to increase metastasis (Hakulinen et al., 2008; Sakwe et al., 2011; Stepp et al., 2015; You et al., 2015). This ECM-modulating exosomal activity may also play an important role in eye diseases where pathological ECM remodeling is an integral part of the disease mechanism, such as in glaucoma and AMD (Bowes Rickman et al., 2013; Roy Chowdhury et al., 2015).

2. Methods for exosome isolation – pros and cons

A number of methods can be used for isolating exosomes and small EVs. However, the EV preparations resulting from the different methods span a wide range of purities and properties. Thus, it is very important to choose the isolation method that is appropriate for the downstream analysis methods that will be used or the experiments that will be done with the isolated EVs. In particular, complex biological fluids such as plasma, serum and urine pose difficulties for EV isolation.

Following is a brief discussion of the most common exosome isolation methods with regard to their pros and cons:

2.1. Differential ultracentrifugation

Differential ultracentrifugation is considered to be the gold standard for exosome and small EV isolation (Thery et al., 2006). This technique is based on a scheme of sequential centrifugations of the exosome-containing fluid and the resulting supernatants: 200 *g*, 2,000 *g*, 10,000 *g*, and 100,000 *g*. The resulting 100,000 *g* pellet, which contains exosomes, other small to medium-sized EVs, lipoprotein particles and large protein aggregates, is resuspended in PBS and centrifuged again at 100,000 *g* to control for artifactual trapping of materials. The final pellet is resuspended or lysed depending on the downstream analysis method. It is a relatively simple methodology; however, the resulting pellet, although enriched for, does not purely consist of exosomes. Other drawbacks include the need for access to an ultracentrifuge and a relatively low yield compared to PEG precipitation as discussed below.

2.2. Polyethylene glycol (PEG) precipitation

Precipitation of viruses by PEG solutions is a method that has been used for over 40 years (Adams, 1973; Lewis and Metcalf, 1988; Yamamoto et al., 1970). Recent adaptations of PEG precipitation for isolation of EVs have shown that the most efficient protocols for exosome precipitation utilize PEG polymers with average molecular weights of 6,000 or 8,000 Da (PEG-6000 and PEG-8000) (Antes et al., 2013; Vlassov et al., 2013). PEG precipitation-based kits such as ExoQuick (System Biosciences) and Total Exosome

Isolation reagent (TEI; ThermoFisher Scientific) have become increasingly popular due to their ease of use and high yield. Unfortunately, PEG precipitation will not only isolate exosomes but also larger EVs, large protein aggregates, lipoprotein particles (HDL, LDL, VLDL and Chylomicrons), and viruses (Adams, 1973; Iverius and Laurent, 1967; Lewis and Metcalf, 1988; Vikari, 1976; Yamamoto et al., 1970). Thus, PEG precipitation should only be used for exosome and small EV isolation if there is an additional isolation method used on the precipitated EVs and/or if the downstream analysis methods can distinguish exosomes from the other components of the precipitate. In addition, if using commercial kits, the cost of precipitating large volumes of EV-containing solutions is quite high. However, several EV PEG precipitation protocols have been published (Rider et al., 2016; Weng et al., 2016), making the procedure simple and inexpensive if in-house PEG solutions are prepared.

2.3. Sucrose and Iodixanol density ultracentrifugation

Density ultracentrifugation is perhaps the best compromise of methods for EV isolation. It provides purity and specificity sufficient for a highly exosome-enriched preparation, but does not require the method optimization needed for immunoaffinity capture (IAC), as discussed below. Iodixanol (OptiPrep™) rather than sucrose has become the preferred density media due to its superior osmotic characteristics and better separation of EV subpopulations (Kowal et al., 2016). Yields from density ultracentrifugation are much lower than that from PEG precipitation and even standard differential centrifugation, but purity is much higher. A recent study from Théry's laboratory used proteomic analysis to investigate in detail the identity and characteristics of EVs isolated by density ultracentrifugation and IAC (Kowal et al., 2016). The study highlighted the importance of using preparations of relatively high enrichment for small EVs (100,000 *g* pellet) to load into the density ultracentrifugation to be able to generate highly pure exosome preparations.

2.4. Immunoaffinity (IAC) capture

For defining *bona fide* exosomes, small EVs, and other EV populations, IAC is the preferred method (Kowal et al., 2016). IAC provides higher yield than density ultracentrifugation but lower than differential ultracentrifugation (Greening et al., 2015). It is a useful method if robust profiling has been done to validate that the antibody target chosen is present on the majority of EVs in question. The success of the method is dependent on the specificity of the antibody or antibodies used for capture and optimization of capture conditions. Of the three canonical small EV tetraspanin markers (CD9, CD63, and CD81) that have traditionally been used, a recent in-depth study suggests that CD81 may be the best target in most situations for isolation of the majority of the *bona fide* exosome population of small EVs, see (Kowal et al., 2016) and Fig.2. Due to elution conditions of EVs from IAC columns and/or beads, this method may not be the optimal for EV isolation if biological activity or physical integrity is to be retained.

2.5. Size exclusion chromatography

Size exclusion chromatography of EVs has been performed in both fast-protein liquid chromatography (FPLC; (Vickers et al., 2011)) and gravity flow settings (Hong et al., 2016; Lobb et al., 2015). It has not been the preferred method for exosome isolation due to a

relatively low yield and, at least previously, a lack of careful characterization of the resulting EV preparations. However, a renewed interest in the method for direct EV isolation from viscous and complex biological fluids such as plasma, serum and urine; has resulted in a number of reports showing its utility (de Menezes-Neto et al., 2015; Lozano-Ramos et al., 2015; Muller et al., 2014). Size exclusion chromatography is in our opinion the preferred method if retaining the biological activity of exosomes is the primary goal.

In conclusion, the choice of method for EV isolation is dependent on scheduled downstream analyses. In the case of protein and proteomics-based analyses, and most likely lipidomic analyses, methods with relatively high yield are necessary; nucleic acid-based detection methods, which contain amplification steps, can work well on preparations with much smaller yields or amounts. To aid in choosing the optimal exosome isolation method for a particular experiment, we have summarized the most relevant characteristics of each method in Table 1. Recent studies have carried out in-depth analyses and extensive subfractionations of EVs to be able to identify *bona fide* exosome-specific protein markers (Keerthikumar et al., 2015; Kowal et al., 2016) as shown in Fig. 2. The results show that very few proteins are specific for exosomes, in fact only two proteins hold up to scrutiny across different cell types at present: Syntenin-1 and TSG101.

Both of these proteins, and many of the other highly enriched proteins in exosomes and small EVs such as Alix and clathrin for example, are involved in the formation of exosomes by budding from the lumenal side of the membrane in multivesicular endosomes (Baietti et al., 2012). Thus, these markers do not only serve to identify exosomes but also demonstrate the distinct intracellular origin of exosomes compared to other microvesicles that are released directly from the cell surface (Colombo et al., 2014). Going forward, it would therefore be prudent to analyze proteomic datasets and characterize EV preparations with regard to their syntenin-1 and TSG101 content to assess purity and composition in a meaningful way. Since the composition of exosomes reflect the cell-type specific origin (Colombo et al., 2014), unique cell- and tissue-specific exosomal markers can be identified by analyzing the proteins that co-enrich with the pan-cell/tissue exosome markers syntenin-1 and TSG101. Thus, these validated protein markers are extremely valuable to verify purity of exosomal preparations when characterizing protein content in fractions from density gradients in combination with size and quantity determination by nanoparticle tracking analysis and/or transmission electron microscopy.

3. Exosomes and their role in immune regulation

Much of the early seminal work describing, defining and characterizing exosomes was done with exosomes released from immune cells (Blanchard et al., 2002; Escola et al., 1998; Raposo et al., 1996; Skokos et al., 2001; Thery et al., 2006). Over the past decade, the role of exosomes in the regulation and maintenance of immune function has become an area of intense research, displaying substantial promise for novel diagnostic and therapeutic approaches (Robbins and Morelli, 2014). We focus on two areas that appear to have the most potential for future investigations into exosome-mediated immune regulation in the eye.

3.1. Immunomodulation

Considerable work has been aimed at elucidating and harnessing the immunoregulatory function of exosomes in diseases spanning conditions as diverse as cancer (Whiteside, 2016a), infectious diseases (Cheng and Schorey, 2013; Hosseini et al., 2013), inflammatory diseases and autoimmunity (Tran et al., 2015), organ transplant tolerance (Kaipe et al., 2014; Monguio-Tortajada et al., 2014), and neurodegeneration (Hajivalili et al., 2016), to name a few. For example, dendritic cells (DCs), which are professional antigen presenting cells, secrete exosomes expressing functional Major Histocompatibility Complex class I and class II, and T cell co-stimulatory molecules. A groundbreaking study demonstrated that exosomes from DCs loaded with tumor-derived peptides prime specific cytotoxic T lymphocytes *in vivo* and eradicate or suppress growth of tumors in a T cell-dependent manner (Zitvogel et al., 1998). A recent study showed increased efficacy of vaccines based on exosomes from DCs that were treated with TLR agonists while being loaded with tumor antigens (Damo et al., 2015). In addition, it was recently shown that T cell responses are independent of exosomal MHC/peptide complexes if whole antigen is present (Hiltbrunner et al., 2016). This study suggests that exosomes stripped of personal antigens may be used in designing therapeutic approaches, greatly increasing the feasibility of future trials in humans. Taken together these studies show the powerful immunomodulatory potential of exosomes and pave the way for the use of exosome-based cell-free vaccines as an alternative to adoptive transfer of immune cells for cancer treatment and infectious diseases.

To date no work has explored the immunomodulatory potential of exosomes to treat eye diseases. However, a recent *in vitro* study found that small EVs released from cultures of the spontaneously immortalized retinal pigmented epithelium (RPE) cell line ARPE-19, promoted an immunoregulatory phenotype in monocytes (Knickelbein et al., 2016). Interestingly, when ARPE-19 cultures were stimulated with inflammatory cytokines, they released EVs that induced monocyte death. Thus, under inflammatory conditions, EVs released from RPE cells may diminish a potentially harmful inflammatory response via monocyte-killing. However, under non-stimulated homeostatic conditions, EVs from ARPE-19 cells induced monocytes to switch to a non-inflammatory phenotype. These findings suggest that RPE-derived EVs under homeo-static conditions downregulate immune activity in the immediate vicinity of the cells. Consequently, an exosome-based approach to immunomodulation in the eye might be to target and kill infiltrating monocytes or to reprogram their phenotype. Infiltrating and/or local monocytes have been implicated in a wide range of eye diseases such as choroidal neovascularization (CNV) (Espinosa-Heidmann et al., 2003), uveitis (Lee et al., 2014), corneal inflammation (Cursiefen et al., 2004; Cursiefen et al., 2011; Koch et al., 1992), diabetic retinopathy (McLeod et al., 1995; Schroder et al., 1991; Serra et al., 2012), and glaucoma (Alvarado et al., 2010; Howell et al., 2012). To elucidate how exosomes modulate ocular immune functions in health and disease, similar studies need to be carried out in *bona fide* RPE cell culture models (*e.g.* primary cultures of polarized and pigmented human or porcine RPE monolayers) with exosome-specific isolation methods. The importance of choosing an EV isolation method that is exosome-specific and retains biological function was discussed in detail in *Section 2* above.

Human RPE cells release αB-crystallin, a chaperone protein, in association with exosomes from their apical side (Gangalum et al., 2011; Sreekumar et al., 2010). This is noteworthy because αB-crystallin has been implicated as a negative regulator of both innate (Shao et al., 2013) and cellular immunity (Ousman et al., 2007) in the CNS, thus suggesting a potential role in maintaining immune homeostasis in the outer retina. Further studies are needed to clarify whether αB-crystallin is a negative regulator of the immune system in the outer retina.

Finally, a recent report suggests that EVs and possibly apoptotic blebs are responsible for cell surface removal of complement immune regulators including CD46, CD55 and CD59 from RPE (using the ARPE-19 cell model) under conditions of oxidative stress, making them more vulnerable to complement attack (Ebrahimi et al., 2013, 2014). This is particularly relevant to the AMD disease process since genetic, immunohistochemical, and proteomic studies have identified dysregulation of the alternative complement pathway as an important driver of AMD (please see (Anderson et al., 2010), for review). However, the EV isolation methods used in these two studies were not exosome-specific and the methods of analysis did not distinguish exosomes from other EVs. Although intriguing, these studies need validation with differentiated RPE cells and exosome-specific isolation and analysis methods in order to clarify a potential role for RPE-derived exosomes in the modulation of AMD-related complement immune processes.

3.2. Immune privilege

Interesting recent work investigated exosome-mediated immune privilege of the fetus during pregnancy (Mincheva-Nilsson and Baranov, 2014; Stenqvist et al., 2013). Parenchymal cells of immune-privileged tissues secrete CD95L (Fas ligand; FasL) via extracellular vesicles as a mechanism of immune escape (Andreola et al., 2002). Thus, the placental expression of FasL has been implicated as the basis of placental immune privilege, triggering local deletion of activated maternal lymphocytes that recognize placental paternal antigens and express the FasL receptor (Fas, CD95) (Kauma et al., 1999). *In vitro* studies of cultured trophoblast cells showed that FasL is secreted in association with exosomes (Stenqvist et al., 2013), suggesting that one of the mechanisms by which the placenta promotes a state of immune privilege may therefore be by secretion of the exosome-associated form of FasL (Frangsmyr et al., 2005). However, the topographical location in exosomes was not investigated in this study, thus it is not clear if FasL was on the external face or inside the exosomes. Similarly, exosomes released from the two components of the blood-retinal barrier (retinal vascular endothelium and retinal pigmented epithelium) may play an important role in regulating immune privilege in the eye, which is considered an immune-privileged site akin to the placenta and CNS (Perez and Caspi, 2015). There has been very little research focused on the potential role of exosome-mediated immune privilege in the eye, but one study reported RPE-released exosomes carrying FasL *in vitro* (McKechnie et al., 2006), supporting this notion. In order to expand on and solidify these findings, polarized RPE cell and retinal vascular endothelial monolayers will be required in future experiments in combination with mechanistic studies of the potential immunotolerogenic effect of FasL-carrying exosomes. We see this area as particularly significant for further

inquiry as it may hold considerable potential for novel therapeutic targets and approaches in eye diseases.

4. Exosomes and extracellular matrix (ECM)

4.1. Invadosomes in the trabecular meshwork (TM) and lamina cribrosa (LC)

Exosomes have recently been shown to facilitate interactions between the cell and ECM by acting as key components of cellular structures called invadosomes (Hoshino et al., 2013; Mu et al., 2013). This term encompasses specialized cell structures that range from podosomes to invadosomes where focal turnover of ECM takes place (Saltel et al., 2011). A specialized subpopulation of exosomes is likely released into the pericellular space at or near invadosomes where active ECM remodeling is taking place (Hoshino et al., 2013). The precise role that exosomes play in the function of invadosomes is unclear; however, inhibition of exosome genesis blocks the formation of invadosomes and subsequent matrix degradation (Hoshino et al., 2013). This condition can be rescued by addition of exogenous exosomes (Hoshino et al., 2013). Similarly, exosome secretion is critical for cell migration via podosomes. When exosome formation is inhibited, podosomal protrusions decrease, total cell migration slows and migration directionality is disturbed (Sung et al., 2015). Again, these effects were reversed by application of exogenous exosomes. Taken together, these results demonstrate an essential role for exosomes in the pericellular space where they facilitate proper cell matrix interactions that ultimately control cell behavior. Importantly, invadosome activity has been implicated in normal and pathological remodeling in glaucoma (Aga et al., 2008; Han et al., 2013).

Mechanistically, exosomes contribute to mediating these cell-ECM interactions by binding to ECM components and/or expressing proteases on their surface that cleave ECM proteins. Exosomes bind ECM components via a number of surface proteins. For example, exosomal α4β1 integrins bind fibronectin (Rieu et al., 2000). Non-integrin binding of fibronectin to the exosome surface has also been reported, utilizing fibronectin affinity for heparin/heparan sulfate (Balaj et al., 2015). In these cases myeloma exosomes expressed heparan sulfate proteoglycans of the syndecan family (Purushothaman et al., 2016) or TM-derived exosomes used a surface heparin/heparan sulfate receptor to bind heparan sulfate bound fibronectin (Dismuke et al., 2016). These observations suggest that exosomes bind heparan sulfate proteoglycans or possibly other proteoglycans in the ECM. The TM is enriched in proteoglycans (Keller et al., 2011; Tanihara et al., 2002; Tawara et al., 1989) and such binding may be important in TM physiology, but needs to be explored further. Interestingly, TM exosomes released from glucocorticoid-treated TM tissues display changes in expression of the heparin/heparan sulfate binding protein annexin A2 (Dismuke et al., 2016; Shao et al., 2006). This change correlates with a decreased affinity for fibronectin binding (Fig. 3) via a heparan sulfate bridge and may account for the aberrant accumulation of ECM material in patients with steroid-induced glaucoma.

Exosomes also mediate extracellular protease activity. For example, exosomal Hsp90α is reported to activate plasminogen (McCready et al., 2010), which when converted to plasmin activates multiple other proteases such as MMPs (Santala et al., 1999). A large number of MMPs, ADAMs and ADAMTSs have been identified on exosomes and have been shown to

be catalytically active (Shimoda and Khokha, 2013). Thus far in the eye, only two studies have identified MMPs as exosomal proteins, MMP-14 with corneal fibroblast exosomes (Han et al., 2015) and MMP-2 with trabecular meshwork exosomes (Stamer et al., 2011).

Together, these examples of ECM protein binding and ECM degrading enzyme activity on exosomes likely explain their requirement for invadosome function. TM cells *in vivo* and *in vitro* form invadosomes to turn over their local ECM (Aga et al., 2008). Formation/activity of TM invadosomes has also shown to be affected by TFGβ2 (Ebrahimi et al., 2013), a cytokine prominently implicated in the pathogenesis of open angle glaucoma (Prendes et al., 2013; Wordinger et al., 2007). The data showing dexamethasone-treated TM tissue release exosomes with a reduced affinity for fibronectin may also connect exosomes with the TM fibrosis, increased outflow resistance and high intraocular pressure in patients with steroid-induced glaucoma (Dismuke et al., 2016). However, to date, the study of the functional role of exosomes in TM ECM homeostasis is sparse. Further, while invadosomes are thought to mediate the majority of cell-matrix interactions (Saltel et al., 2011), no study to date has looked for this cellular structure in LC cells. Exosomes are a key component of invadosomes (Hoshino et al., 2013) and future studies are needed to determine their precise role in ECM homeostasis in the healthy TM and LC and to determine whether exosomes are involved in the ECM changes in these tissues in glaucoma patients.

Study of the ECM in the TM and LC of the eye is important because in many forms of glaucoma the structure of these two cribriform tissues are preferentially affected. Both of these tissues display signs of fibrosis and/or changes in biochemical, morphological and mechanical properties of the ECM (Hernandez et al., 1990; Overby et al., 2014; Paula et al., 2016; Sigal and Ethier, 2009; Tektas and Lutjen-Drecoll, 2009; Vranka et al., 2015). For example, patients with glaucoma often have changes in the architecture of the LC seen as a posterior displacement of the LC or “cupping” that is due to changes in the mechanical properties of the ECM (Downs, 2015; Roberts et al., 2010; Yang et al., 2011). This posterior displacement reduces the empty spaces between the laminar beams where the RGC axons are located (Yang et al., 2011). Such remodeling appears to compress the unmyelinated RGC axons resulting in RGC stress, death and vision loss (Howell et al., 2013; Nuschke et al., 2015). The cause of this posterior displacement of the LC is not clear, however, elevated IOP is the most common risk factor for developing glaucoma and IOP is likely the mechanical force responsible for the posterior displacement of the LC (Burgoyne et al., 2005; Yang et al., 2011). Thus, artificial elevation of IOP in animal models, including non-human primates, results in posterior displacement of the LC, RGC axon loss and blindness.

Accordingly, lowering IOP is the only therapeutic intervention shown to slow or prevent vision loss in glaucoma patients (The AGIS Investigators, 2000). High IOP is due to idiopathic reductions in the drainage of aqueous humor through the conventional drainage pathway which consists of the TM and Schlemm’s canal (Grant, 1951). One of the hallmarks of open-angle and steroid-induced glaucoma observed in post mortem eyes is changes in the morphology and ultrastructure of the ECM in the TM (Tektas and Lutjen-Drecoll, 2009). These changes are thought to affect 1) the mechanical properties of the TM, reducing the ability of TM cells to modulate aqueous flow pathways and 2) accumulation of/alterations in ECM materials resulting in reduced outflow facility (Vranka et al., 2015; Wang

et al., 2016). Taken together, changes in the ECM of the LC and TM are fundamental to the permanent vision loss in glaucoma. Understanding the defects in matrix homeostasis possibly involving exosomes in these two tissues will give us insights into the pathogenesis of glaucoma.

4.2. Myocilin glaucoma

The first gene variant to be linked to glaucoma was MYOC which codes for the protein myocilin (Stone et al., 1997). Currently there are over 70 amino acid substitutions in the myocilin protein that are linked to high pressure glaucoma and many of these mutations result in early onset glaucoma (Resch and Fautsch, 2009). However, the function of myocilin is still unclear. Myocilin is found as a soluble dimer intra- and extracellularly, in a membrane-associated protein complex and associated with the TM ECM (Dismuke et al., 2012; Hardy et al., 2005; Stamer et al., 2006; Ueda et al., 2002).

Initially myocilin was thought to be secreted in the classical sense, via a novel amino terminal signal sequence (Mertts et al., 1999). However, a large proportion of intracellular myocilin is not associated with secretory vesicles, its “secretion” did not involve trafficking through the golgi (Hardy et al., 2005) and its proposed N-terminal signal sequence fused to GFP failed to be secreted (Stamer et al., 2006). These data suggest that myocilin leaves the cell not by secretion but by an alternative route. A rational explanation for these data was that myocilin exits cells in association with vesicles identified as exosomes (Hardy et al., 2005). Myocilin interacts with the exterior surface of exosomes, as evidenced by sensitivity to protease digestion (Hardy et al., 2005). Myocilin is abundant in aqueous humor and bound to exosomes demonstrating that its association is not a cell culture artifact (Perkumas et al., 2007), (Fig. 4). In other studies, myocilin was released from the RPE *in situ* associated with exosomes and this release was under the control of a G-protein-coupled receptor (Locke et al., 2014). Finally, proteomic analysis of exosomes released from primary cultures of human TM cell monolayers contained an abundance of myocilin (Stamer et al., 2011).

The majority of myocilin mutations are located in the C-terminal olfactomedin domain; a 5-blade, beta-propeller (Donegan et al., 2015). However, this domain does not mediate myocilin’s dimerization or membrane association (Dismuke et al., 2012; Stamer et al., 2006). Thus, myocilin mutations likely do not affect the proteins ability to associate with exosomal membranes but may disrupt other protein-protein interactions, such as those required for biogenesis, targeting or release. Consistent with this idea, most disease-causing forms of myocilin protein are not secreted (Gobeil et al., 2006; Jacobson et al., 2001). Clearly, myocilin’s role in the exosome pathway requires further study.

4.3. Bruch’s Membrane and RPE basal deposits

Bruch’s membrane (BrM) is a pentalaminar basement membrane complex abutting the basal side of the RPE that includes its basolateral cell membrane. It separates the RPE cell monolayer from a fenestrated capillary bed of the systemic blood circulation (choriocapillaris) and thus plays a crucial role in mediating influx of oxygen, electrolytes, nutrients, and cytokines destined for the RPE and photoreceptors, and efflux of waste products and signaling molecules (Curcio, 2013). The pathogenesis of early age-related

macular degeneration (AMD) is characterized by thickening of BrM due to lipid and protein accumulation that lead to formation of sub-RPE deposits that occur as discrete accumulations, called drusen; which can be hard or soft, or as continuous accumulations of basal laminar deposits. The lipid buildup is thought to primarily interfere with the fluid, and likely exosome efflux from the RPE across BrM, thereby inflicting stress on the RPE (Curcio, 2013). Cells under stress are known to increase the release of exosomes (Atienzar-Aroca et al., 2016b; King et al., 2012), and it is possible that this process is in part responsible for the deposits in the sub-RPE region.

One of the more common AMD risk-associated single nucleotide polymorphisms, which was identified in genome-wide association studies, is in the promoter region of a gene coding for *High-Temperature Requirement A Serine Peptidase 1* (HTRA1) (Yang et al., 2006). The risk-associated nucleotide change correlates with increased expression of HTRA1, which is a secreted serine protease involved in ECM remodeling (Tiaden and Richards, 2013).

Experimental studies that over-expressed (Nakayama et al., 2014; Vierkotten et al., 2011) or deleted (Hasan et al., 2015) HTRA1 in mice, suggest that ECM remodeling in BrM plays an important role in the AMD disease process. Supporting this notion, we recently identified *A Disintegrin and Metalloproteinase Domain-Containing Protein 10*, also known as ADAM10, as a major component in highly purified exosomes released basolaterally from polarized RPE cultures (Klingeborn et al., 2017). Members of the ADAM family are transmembrane proteinases with a unique structure possessing both adhesion and catalytic domains. Although ADAM MMPs function primarily to cleave membrane proteins at the cellular surface they have also been shown to remodel ECM (White, 2003). Further studies of basolaterally released exosomes from stressed RPE cells may identify additional proteases involved in pathogenic ECM changes.

The source/process of protein and lipid deposition in the sub-RPE region in Bruch's Membrane (BrM) and subsequent pathognomonic drusen formation in AMD, remain unclear. Progress toward understanding deposit formation, accumulation and biophysical properties of protein plus lipid aggregates may provide novel targets for therapeutic intervention. Interestingly, several proteins found in drusen and sub-RPE deposits, such as annexins and CD63, are also found in exosomes and other EVs (Hageman and Mullins, 1999; Hageman et al., 1999; Mullins et al., 2000; Wang et al., 2009a, b). Furthermore, a recent study revealed an interesting role for Apolipoprotein E (ApoE) and exosomes in regulating pigment granule formation and processing in pigmented cells (van Niel et al., 2015). Perturbation of this pathway in RPE cells may be relevant for AMD since ApoE is one of the major components found in drusen and sub-RPE deposits (Li et al., 2006).

Exosomes released from RPE cells under normal conditions are likely involved in cell-cell communication (on both the apical and basal sides), and lipid homeostasis. Cells under stress are known to increase the release of membranous vesicles including exosomes (King et al., 2012), and this has also been suggested to be the case in RPE cells (Atienzar-Aroca et al., 2016a). Studies have shown that exosomes released by stressed RPE exhibit changes in signaling phosphoproteins (Biasutto et al., 2013), and are coated with complement

components (Wang et al., 2009a, b), including the terminal membrane attack complex, C5b-9 (Pilzer et al., 2005), suggesting exosomes play a role in modulating complement activation in the immediate extracellular milieu of the RPE cells. Moreover, severe oxidative stress of polarized primary human RPE cultures resulted in barrier breakdown and a loss of the apical-specific release of exosome-associated αB-Crystallin (Sreekumar et al., 2010). Furthermore, drusen proteins such as enolase and ATP synthase have been found in exosomes (Olver and Vidal, 2007), supporting an exosomal origin for some drusen components. Although these studies support a role for RPE-derived exosomes in the AMD disease process, they were limited by several factors: (i) the use of ARPE-19 cells (ARPE-19 is a spontaneously immortalized RPE-*like* cell line, lacking many important hallmarks of *bona fide* RPE cells, discussed in detail in a recent publications (Beebe, 2013; Rizzolo, 2014)), (ii) superficial exosome characterization severely lacking in detail, and most important, (iii) cells were grown on plastic, not on permeable supports (i.e. Transwell, ThinCert, Millicell etc.) to facilitate proper epithelial polarization. Several different RPE culture models on permeable supports have been described previously, e.g. (Maminishkis et al., 2006; Toops et al., 2014).

Culture of primary RPE cells on permeable supports is essential to study basolaterally released exosomes, which could be involved in basal deposit formation, intercellular and systemic signaling and to distinguish them from apically released exosomes. An exhaustive characterization of EVs released both apically and basolaterally from RPE cells under normal and pathophysiological conditions is critical to elucidating the potential role of exosomes in the AMD disease process. Interestingly, a recent study showed that under serum-free conditions of exosome collection from polarized ARPE-19 cultures, the total exosome release was about twofold higher on the apical vs basolateral side (Gangalum et al., 2016). Our own studies using polarized primary RPE cultures show similar results under FBS-supplemented culture conditions. However, under culture conditions using a serum supplement instead of FBS, exosome release is shifted to roughly equal apical and basolateral release (Klingeborn et al., 2017), suggesting that serum components also mediate polarized release of exosomes.

In addition to exosome abundance in these studies, we performed a mass spectrometry-based proteomic analysis of apically and basolaterally RPE-derived EVs by quantitatively profiling hundreds of proteins in EV preparations of increasing purity. This approach, termed *protein correlation profiling* (PCP) (Andersen et al., 2003; Skiba et al., 2013), permits the analysis of any sub- or extracellular components/complexes that can be enriched by fractionation but not purified to homogeneity. PCP provides a powerful approach to both identify *bona fide* resident proteins and to exclude contaminating proteins from a proteome dataset. We found that the vast majority of RPE-derived exosomal proteins that were highly co-enriched with the exosome-specific marker Syntenin-1 differed between the apical and basolateral side (Klingeborn et al., 2017), (Fig. 5), which was not unexpected if exosomes contribute to different apical versus basolateral signaling and pathways in these polarized cells. See supplementary tables S1 and S2 for the identity of the apically and basolaterally released RPE-derived exosomal proteins. These findings emphasize the importance of studying exosomes released from both sides of polarized cell types since an apical only approach risks missing important basolaterally released exosomal proteins. Perhaps even more

troubling is the risk of mistakenly using findings in the apical RPE exosome proteome to guide research into basolateral-specific biological processes such as lipoprotein particle flux, waste disposal, and nutrient transport, all of which may play a role in sub-RPE deposit and drusen formation under pathological conditions. The next step in these studies will be to study potential changes in the protein cargo in exosomes derived from RPE cells under conditions of stress relevant to the AMD disease process, such as photo-oxidative stress and dysregulation of lipid metabolism, to mention a few. Identified proteomic changes may result in novel targets for therapy.

5. Exosomes in ocular angiogenesis

5.1. Angiogenesis in Cancer

The involvement of exosomes and small EVs in angiogenesis in cancer has been the focus of intense research in the last couple of years (Kalluri, 2016; Whiteside, 2016b). Exosomes and small EVs have been shown to modulate angiogenesis in tumors by both pro- and anti-angiogenic pathways (Ribeiro et al., 2013). For example, exosome release is increased by hypoxia that often occurs in tumors, and these exosomes stimulate angiogenesis, when taken up by endothelial cells (Hong et al., 2009; Park et al., 2010; Skog et al., 2008; Umezu et al., 2013). The vascular remodeling induced by tumor-derived exosomes likely affects not only tumor growth, but also metastasis. One of the ways tumor cell-derived exosomes may impair the structural integrity of endothelial cells and cause leakiness, is via the exosomal microRNA, *miR-105*, which downregulates the expression of the tight junction associated protein, ZO-1. Downregulation of ZO-1 enhances vascular permeability and thereby metastatic dissemination (Zhou et al., 2014). Two recent studies determined the miRNA content of exosomes derived from uveal melanoma (UM; an ocular cancer) (Eldh et al., 2014; Ragusa et al., 2015). Ragusa and colleagues showed that an exosome-associated miRNA (miR-146a) was upregulated in the vitreous humor of UM patients compared to controls. They also demonstrated that miR-146a was upregulated in serum exosomes from those same patients. Interestingly, the study by Eldh et al. found that UM-derived exosomes from UM metastases in the liver could be recovered from the liver circulation (liver perfusates). Both of these studies exemplify approaches to recover eye-specific exosomal diagnostic markers from tissues and/or fluids that are peripheral to the eye, and thus more easily accessible. It represents encouraging proof-of-concept for identifying eye disease-specific biomarkers outside the eye.

In the eye, both the cornea and the retina maintain very strict homeostatic control over angiogenesis, when left unchecked neovascularization severely affects vision (Abdelfattah et al., 2016; Eichler et al., 2004). Exosomes may play an important role in maintaining this homeostasis under normal conditions.

5.2. Cornea

The transparency of the cornea is critical for vision. The healthy cornea is an avascular tissue and during normal wound healing, repair occurs within hours without the formation of new blood vessels (Chang et al., 2001). In some cases however, imbalance between pro- and anti-angiogenic factors during wound healing can lead to corneal neovascularization. These

blood vessels promote a loss of transparency in the cornea and therefore an impairment of visual clarity. Corneal wound repair is a complex multiphase process that involves apoptosis (Netto et al., 2005), proliferation (Cursiefen et al., 2006), cellular transformation (Mimura et al., 2009), migration (Cursiefen et al., 2006) and ECM remodeling (Mimura et al., 2009). A critical component throughout this process is the transmembrane matrix metalloproteinase-14 (MMP-14). Corneal fibroblasts release exosomes with MMP-14, which are taken up by endothelial cells (Han et al., 2015). Exosomal MMP-14 activity is critical for the accumulation and activation of MMP-2 in the exosomes (Han et al., 2015). This process likely plays a role in the multistep corneal wound healing and represents a therapeutic target to prevent or reverse corneal neovascularization. Thus far, investigation into cornea-derived exosomes has been limited. Additional studies need to determine what role corneal exosomes play in other aspects of neovascularization such as VEGF/PEDF signaling.

5.3. Neovascular AMD and diabetic retinopathy

In contrast to the extensive research studying the involvement of exosomes and small EVs in cancer angiogenesis (see above), very little research has been done aimed at studying the role of exosomes in the development and disease process of neovascular AMD including CNV, or other diseases with aberrant retinal angiogenesis such as diabetic retinopathy. As mentioned above, cancer-cell derived exosomes containing certain microRNAs affect the integrity of endothelial cells in blood vessels by downregulating expression of tight junction-associated protein ZO-1 (Zhou et al., 2014). Thus, there may also be a potential role for exosome-induced ZO-1 downregulation in the RPE monolayer allowing for increased access of nascent choroidal blood vessels into the retina during the CNV disease process. In a pathological state, sources of exosomes inducing increased permeability could include choroidal vasculature and oxidatively stressed and/or hypoxic RPE cells. As the immature leaky blood vessels formed by CNV are responsible for much of the vision loss in patients with exudative AMD (Shao et al., 2016), ways to decrease the infiltration of choroidal vessels into the retina are sorely needed. In the case of diabetic retinopathy, where the disease process is mainly localized to the retinal vasculature, potential impact of exosome-induced downregulation of tight junction proteins in endothelial cells which make up the inner blood-retinal barrier, may be significant (Klaassen et al., 2013). Investigating roles of exosomes in this process may identify novel therapeutic targets. There is a delicate balance of pro- and anti-angiogenic signaling in the retina, RPE and choroid. The role of exosomes in this signaling balance was highlighted by a study demonstrating that exosomes released from retinal astrocytes contain anti-angiogenic components that inhibit laser-induced CNV in a mouse model (Hajrasouliha et al., 2013). Further studies utilizing mass spectrometry and nucleic acid-sequencing to determine the content of exosomes released from astrocytes, Müller cells, photoreceptors and other retinal cells, including apical side exosome release from RPE cells, will be crucial to identify new therapeutic targets to control aberrant retinal neovascularization in a range of retinal diseases.

Exosomes released from cells in the eye in neovascular AMD patients also have the potential to serve as biomarkers. Exosomal biomarkers could be useful not only to stratify neovascular AMD patients according to severity of disease in a way that may be more predictive of

disease progression than current methods (e.g. OCT and fluorescein angiography); but they could also provide novel insight to the pathophysiology of the disease. Interestingly, Kang and colleagues recently identified changes in EV proteins found in the aqueous humor from individuals with neovascular AMD compared to individuals without the disease (Kang et al., 2014). This study also compared results to EVs from ARPE-19 cell cultures, which as discussed above lacks many hallmarks of *bona fide* RPE cell cultures (Rizzolo, 2014) and the methods used for EV isolation were not specific for exosomes or small EVs. Furthermore, no evidence was presented in this study to show that EVs found in aqueous humor originated from RPE cells. Thus, currently it is still unclear if exosomes and/or small EVs in aqueous humor from patients with neovascular AMD can be used as biomarkers of the disease. Interestingly, ongoing studies in our own laboratory recently identified Pigment Epithelium-Derived Factor (PEDF) in highly purified apically released exosomes from polarized RPE cell monolayers, but to a much lesser extent in basolaterally released exosomes (Klingeborn et al., 2017). PEDF is secreted primarily from RPE cells on their apical side to maintain an anti-angiogenic milieu in the outer retina (Ablonczy et al., 2011; Tombran-Tink et al., 1995). Although the majority of secreted PEDF is most likely not released in an exosome-mediated manner, our use of protein correlation profiling (Andersen et al., 2003; Skiba et al., 2013) unequivocally shows that this is a genuine association with exosomes, not due to contamination during the purification procedure. Thus, there may be targeted anti-angiogenic signaling carried out by exosomes that is different from the majority of apically secreted PEDF.

Approaches being developed in the cancer field to use exosomes as carriers for pro and/or anti-angiogenic factors could be adapted to target eye diseases involving dysregulated angiogenesis (Ribeiro et al., 2013; Whiteside, 2016b). For example, high heparanase activity and expression correlate with an aggressive tumor phenotype in many cancers. Heparanase action in cancer results in a large up-regulation of growth factors and increased shedding of syndecan-1, a transmembrane heparan sulfate proteoglycan. A large body of work suggests that syndecan-1 and heparanase together regulate cell behaviors and drive growth factor signaling that enhance tumor growth and angiogenesis. Encouragingly, targeting of the heparanase/syndecan-1 interaction has shown promise in blocking the aggressive behavior of cancer in pre-clinical and clinical studies (Ramani et al., 2013). In addition, both heparanase and syndecan-1 are involved in exosome biogenesis and regulation of exosome release (Thompson et al., 2013). Furthermore, a recent study by Gangalum and colleagues (Gangalum et al., 2016) reported that shRNA knockdown of αB-Crystallin in ARPE-19 cells severely inhibits both apical and basolateral exosome and/or small EV release. Thus, approaches attempting to regulate exosome release in the affected cell types, as well as targeting proteoglycans found on exosomes and in the ECM of the retinal vasculature and BrM at the choriocapillarismay be able to reduce neovascularization in neovascular AMD and diabetic retinopathy.

In conclusion, much research remains to be done to elucidate the role of exosomes in eye diseases with aberrant angiogenesis; nonetheless, the potential for important novel findings is sizeable. Findings may include new drug targets and novel biomarkers for improved diagnostic and prognostic tests. Thus, it appears that there may be significant potential for

therapeutic intervention concerning aggressive neovascularization in eye diseases by attempting to regulate exosome release in the affected cell types.

6. Stem cells and exosomes

Cell death of largely post-mitotic cells is part of the disease process in all eye diseases; therefore stem cell-based approaches aimed at cell replacement are being actively studied for therapy and/or intervention. For example, patients with ocular hypertension and glaucoma have fewer TM cells (Alvarado et al., 1984; Gottanka et al., 2006; Rodrigues et al., 1976) and vision loss in glaucoma is due to death of retinal ganglion cells (Quigley, 1993). Patients with late dry AMD known as geographic atrophy have areas of RPE death which then leads to death of photoreceptors (Bonilha, 2008). A number of ocular surface diseases involve loss of cells on the surface and endothelium of the cornea (Ahmad, 2012). For all of these ocular diseases, researchers have proposed that stem cell-based therapy could be used to restore tissue health and function (Abu-Hassan et al., 2015; Al-Shamekh and Goldberg, 2014; Erbani et al., 2016; Mead et al., 2015; Nakamura et al., 2016; Roubeix et al., 2015; Zhu et al., 2016). One strategy for replacing lost cells is to transplant stem cells into the affected areas where they differentiate into the desired cell type and restore tissue/organ function (Blenkinsop et al., 2012). Another approach under investigation for repairing RPE damage is to differentiate stem cells into RPE monolayers and then transplant the differentiated monolayers into the patient (Carr et al., 2013). Therapeutic stem cell strategies to treat the retina tested both strategies, but with limited success. To date, differentiation of stem cells into RGC-like cells has only been accomplished in culture (Phillips et al., 2012). However, it is now widely accepted that a major therapeutic effect of stem cells is due to their secretion of paracrine factors (Tran and Damaser, 2015). In line with this idea, another strategy uses mesenchymal-derived stem cells to secrete neurotrophic factors for neuroprotection or axonal regeneration of retinal cells (Johnson et al., 2010; Johnson et al., 2014; Mead et al., 2013). A recent study demonstrated that intravitreal injections of exosomes from mesenchymal-derived stem cells partially prevents axonal loss and degeneration following mechanical injury (Mead and Tomarev, 2017). Interestingly, the investigators find that RNA exosomal cargo is responsible for these protective effects. Embryonic stem cell (ESC) derived precursors and induced pluripotent stem cells (iPSCs) have also been transplanted to replace degenerated photoreceptors and RPE cells (Gonzalez-Cordero et al., 2013; Meyer et al., 2009). In the trabecular meshwork, iPSCs have been used to repopulate the meshwork and/or provide trophic factors that induce proliferation in endogenous cells (Abu-Hassan et al., 2015; Zhu et al., 2016).

Pluripotent stem cells express a number of transcription factors that contribute to their undifferentiated phenotype. These transcription factors, including HoxB4, Nanog, Oct-4 and Rex-1 have been detected in stem cell derived EVs where they can be transferred to adjacent resident cells (Ratajczak et al., 2006). In addition to transcription factors, stem cells are known to secrete several signaling molecules including WNTs (Clevers et al., 2014), β-catenin (Clevers et al., 2014), TGF-β1 (Watabe and Miyazono, 2009) and VEGF (Gerber et al., 2002), which have also been found to associate with exosomes and other EVs (Gross et al., 2012; Luga et al., 2012). With this in mind, the mechanism behind the therapeutic effect of stem cells on tissue repair is not fully understood. As discussed above the trophic factors

released by stem cells release appear to mediate a substantial portion of their biological effect (Ratajczak et al., 2012). Exosomes and other EVs function as delivery vehicles for these trophic factors either by sequestering signaling molecules on their surface or transferring transcription factors and miRNAs to resident cells (Ratajczak et al., 2006). In spite of the promising therapeutic potential of stem cell transplantation another potential problem with this approach is that tumorigenic and immunogenic risks still remain (Mousavinejad et al., 2016). Importantly by harvesting and transplanting stem cell exosomes and other EVs, such risks are alleviated yet much of the therapeutic benefit remains (Kishore and Khan, 2016). This cell-free approach is promising for treatment of many eye diseases, but requires further investigation.

7. Exosome biomarkers for eye diseases

Interest in utilizing exosomes and other EVs to identify biomarkers of disease has increased exponentially in recent years (Gonzalez and Falcon-Perez, 2015). It is easy to understand why the perceived potential for development of exosome-based diagnostic assays is so large. Exosomes and EVs have several unique features that make them ideal as targets for finding new biomarkers: (i) the lipid bilayer provides protection for RNA, DNA, and proteins inside the exosome from nucleases and proteases in the extracellular milieu, (ii) exosomes contain tissue-, cell-, or disease-specific proteins and nucleic acids, and (iii) the relative hardiness of exosomes make it possible to use a wide range of methods for isolation and enrichment from a range of body fluids (i.e. plasma, serum, urine, saliva, semen, breast milk, aqueous humor and cerebrospinal fluid). Studies from the cancer, cardiovascular disease and diabetes research fields report promising findings for the utility of exosome biomarkers for diagnosis, risk assessment, and choice of therapy (Joyce et al., 2016; Lawson et al., 2016).

7.1. Tear fluid

Theoretically, there is a significant potential for identification and characterization of exosomal biomarkers of eye disease in tear fluid. Particularly appealing is the noninvasive nature of collecting tear fluid, but a potential drawback is the relatively small volumes that can be collected. To date, tear fluid as a source for exosomal biomarkers has not been extensively investigated, as evidenced by the fact that we only found one publication investigating exosomes in tear fluid (Grigor'eva et al., 2016). Proteomic biomarker studies of tear fluid have identified a number of proteins (e.g. annexins and heat shock proteins) which are most certainly exosome-associated, although not investigated as such (Aass et al., 2015; Matheis et al., 2015). With the recent advances in exosome isolation techniques, protein identification methods, and nucleic acid sequencing, the diagnostic and therapeutic potential of tear-derived exosomal biomarkers appear to be considerable. Certainly, this is a wide-open area of research.

7.2. Aqueous humor (AH)

Aqueous humor (AH) has been used for protein, nucleic acid, and lipid biomarker analyses in a wide range of eye diseases (Ji et al., 2015; Murthy et al., 2015; Wecker et al., 2016). Some of the most prevalent eye diseases such as glaucoma (Agnifili et al., 2015; Goyal et al., 2014), neovascular AMD (Kang et al., 2014; Liu et al., 2016; Park et al., 2014), diabetes-

induced eye diseases (Hillier et al., 2016; Vujosevic et al., 2015, 2016), and uveitis (Haasnoot et al., 2016; Kalinina Ayuso et al., 2013), to name a few, have been investigated for biomarker content in the AH. Although the vast majority of nucleic acid and lipid biomarkers, and some of the protein biomarkers identified in AH were most likely exosome-associated, very little attention has been directed to the exosome-specific biomarkers. The reason for the uncertainty regarding whether identified proteins and nucleic acids are exosome-associated is based on limitations in the studies cited above. The proteins identified in these studies most likely originated from both soluble secreted proteins (and possibly nucleic acid) and proteins, lipids and nucleic acids in and on exosomes and other EVs. The technical reason that there was a mixture of soluble as well as EV-associated proteins and nucleic acids in these preparations is that the methods used for AH sample preparation did not include steps to separate the soluble fraction from the membrane fraction (*i.e.* exosomes and EVs).

There have been studies where EVs were isolated from AH. For example, Kang and colleagues isolated AH EVs from patients with neovascular AMD (Kang et al., 2014), however the methods used for EV isolation were not specific for exosomes or small EVs, raising doubts about whether the findings truly represent exosomal biomarkers. Specifically, the PEG precipitation procedure used results in a preparation that likely contains a mixture of exosomes, ectosomes, blebs, lipoprotein particles, and protein aggregates, as was discussed in *Section 2.2* above. Katome and coworkers reported an increase in exosome-associated peroxisome proliferator-activated receptor gamma (PPARγ) in AH from patients with proliferative diabetic retinopathy compared to controls (Katome et al., 2015). However, the methods used for EV isolation (PEG precipitation) in this study were again not exosome-specific. Thus, it is unclear if these findings represent true exosomal biomarkers of eye disease. Using centrifugation steps to isolate exosomes from mixtures, our laboratory has carried out several studies focused on exosomes in AH and their potential role in glaucoma (Dismuke et al., 2015; Hardy et al., 2005; Hoffman et al., 2009; Perkumas et al., 2007; Stamer et al., 2011), one of which was specifically aimed at generating data which can be used to identify exosomal biomarkers (Dismuke et al., 2015). Finally, it is not currently known if exosomes or other small EVs released from RPE, Müller cells, vascular endothelial cells or other retinal cells can make their way to the AH. To aid in answering this question and potentially identifying novel AH exosomal biomarkers, foundational characterization of the composition of exosomes released from these cell types *in vitro* is needed. Ongoing studies in our laboratory aimed at careful characterization of exosomes and other EVs released from RPE (Klingeborn et al., 2017), can be used as a resource to identify and validate potential exosomal biomarkers in AH.

In summary, it appears clear that future studies focused on exosomal and small EV biomarkers of eye disease in AH must utilize appropriate exosome- and/or small EV-specific methods for isolation; and that there is a considerable need for characterization of protein and nucleic acid composition of exosomes from several different retinal cell types.

7.3. Vitreous humor

Ample volumes of AH are easily accessible during cataract surgery, however vitreous humor (VH) is much less accessible during most standard procedures. That said, small volumes of VH can be obtained with no more discomfort for the patient than routine anti-VEGF and anti-PDGF injections. Thus, if robust vitreal exosomal biomarkers can be identified and validated, their practical use could be substantial.

VH has a high potential to contain biomarkers for diseases such as neovascular AMD, geographic atrophy, early-stage AMD, diabetic retinopathy, glaucoma, and a number of other retinopathies. Several recent studies using proteomic, nucleic acid, and lipidomic approaches identified interesting potential vitreal biomarkers in retinal vein occlusion (Reich et al., 2016), neovascular AMD (Menard et al., 2016; Nobl et al., 2016), diabetic retinopathy (Jin et al., 2016), and primary open-angle glaucoma (Agnifili et al., 2015). However, none of these studies explored or discussed the role of exosomes or other EVs in the transport to and presence of biomarkers in the VH. Some of the biomarkers identified in these studies, such as PEDF in the case of neovascular AMD (Nobl et al., 2016) and keratin 1 and PEDF in the case of diabetic retinopathy (Jin et al., 2016) are proteins that have been identified in association with exosomes (discussed in *Section 4.3* above). The promise in an exosome-specific approach to identifying relevant biomarkers lies partly in the specificity that can be achieved as opposed to a global approach as was used in the cited studies. Proteomic identification carried out on proteins in the vitreous, which contain a mixture of proteins that are exosome-associated and proteins that are not, risks masking correlations of disease-associated proteins in either fraction. Thus, by isolating EVs specifically, the soluble (non-exosome/EV associated) proteins can be removed and potentially confounding results can be avoided. An inkling of this potential can be seen in the few (three) studies that to date have focused on EVs in vitreous as a source of disease biomarkers (Biasutto et al., 2013; Katome et al., 2015; Ragusa et al., 2015).

7.4. Blood

Blood fractions such as plasma and serum represent perhaps the most promising avenue for identifying exosomal biomarkers in eye diseases. This is in large part due to the facts that (i) it is easier and less invasive to collect than AH and VH, and that (ii) much larger volumes can be collected. A possible disadvantage of using blood as a source of exosomes from the eye is that these are likely to make up a very small fraction of the total exosomes found in the systemic circulation and will therefore be difficult to detect and analyze. For successful identification and isolation of ocular exosomes or small EVs from blood, foundational descriptive characterization of exosomes released from ocular cells under healthy and pathological conditions is essential. Once exosome-associated markers specific for ocular cells have been identified, they can be used to isolate ocular exosomes from the vast excess of non-ocular EVs present in blood. Without an enrichment step targeting ocular exosomes, the task to develop blood-based biomarkers of eye disease may be untenable. Currently, there are immunoaffinity-based commercial kits available aimed at isolating exosomes directly from human plasma and serum (Diagenode, MBL International, and System Biosciences), supporting the feasibility of this approach. However, in many cases there is still a need to develop and validate in-house immunoaffinity methods if samples are from

other species than humans (since most commercial kits have only been developed for human samples), or if targets other than those offered in commercial kits are important, as discussed in more detail in *Section 2.4* above. Only two published reports address this area of research so far (Eldh et al., 2014; Ragusa et al., 2015). In one of these studies, Ragusa and colleagues showed that an exosome-associated miRNA (miR-146a) was upregulated in the VH of uveal melanoma patients compared to controls. They also demonstrated that miR-146a was upregulated in serum exosomes from those same patients. This study represents an encouraging proof-of-concept for identifying eye disease-specific biomarkers in the systemic circulation, as further outlined in Fig. 6.

8. Exosomes as therapeutic agents

Today the two leading causes of irreversible blindness in Western societies are AMD and glaucoma. By 2020 it is estimated that 196 million people worldwide will have AMD (Wong et al., 2014) and 79.6 million people worldwide will have glaucoma (Quigley and Broman, 2006). The goal of current therapeutic approaches to treat these late onset diseases is not to reverse the disease course, but only to halt further progression of tissue damage and vision loss. Additionally, many of the therapeutic interventions for these diseases involve monthly administration by a physician in a clinical setting (i.e. injection of anti-VEGF biologics for exudative AMD), repeated daily administration of eye drops by patients where administered doses can vary drastically by individual (glaucoma) or no substantial intervention exists at all (dry AMD). While research has advanced our understanding of the pathogenesis of these diseases and identified targetable pathways that could lead to preservation or even reversal of vision loss, these discoveries have not translated well to the clinic because delivery of drugs, active enzymes/proteins and small RNAs to tissues in the eye remains a steep challenge (Rawas-Qalaji and Williams, 2012). Here we discuss, in detail, the substantial progress made in the use of exosomes for the targeted, effective and safe delivery of these therapeutic molecules.

Exosomes are natural vehicles for the transfer of small RNAs and proteins, as highlighted in previous sections. Cells possess mechanisms to take up exosomes (Mulcahy et al., 2014) and extract the contained microRNAs for use (Zhang et al., 2015). This makes exosomes ideal delivery vehicles for gene therapy involving microRNAs or small interfering RNAs (siRNAs) as they both facilitate uptake (Kooijmans et al., 2012) and protect the RNAs from extracellular degradation (Zhang et al., 2013). Recently exosomes have also been shown to transfer functional proteins to recipient cells. For example, the tumor suppressor, PTEN can be transferred to recipient cells via exosomes where it functioned as a phosphatase (Putz et al., 2012). A number of transmembrane proteins are also transferred from exosomes to recipient cells, including the tyrosine kinase receptors MET (Peinado et al., 2012) and KIT (Atay et al., 2014) as well as αvβ6 integrins (Fedele et al., 2015). Transcription factors are also thought to be transferable between stem cells and recipient cells as discussed above, which has been shown to result in stem cell-like properties such as proliferation in non-proliferating cells (Ratajczak et al., 2006). Exosomes have also been engineered to deliver small molecules, as has been demonstrated for curcumin delivery to activated myeloid cells (Sun et al., 2010). Together, these studies demonstrate that exosomes can function as delivery vehicles for a variety of therapeutic cargos.

A number of studies have also shown that exosomes can target specific cells types and tissues to deliver their cargos. One successful strategy used DCs engineered to express a modified exosomal protein, LAMP2b fused to a peptide from the rabies viral glycoprotein. These exosomes were loaded with siRNA targeting GAPDH and administered intravenously to mice. These engineered exosomes specifically knocked down GAPDH in neurons and microglia in the brain (Alvarez-Erviti et al., 2011). Similar strategies of using modified exosomal proteins to target specific cell subtypes and deliver cargo have also been published (Ohno et al., 2013; Tian et al., 2014). Finally, a recent cancer study examining organ specific metastasis found that α/β integrin expression patterns on the exosomes resulted in organ specific uptake (Hoshino et al., 2015). This suggest that exosome-based therapies could be designed to target specific tissues in the eye once injected locally and supports the possibility that exosomal therapies targeting eye tissues could be administered intravenously, significantly reducing the cost of treating patients.

Exosome-based therapies have a number of potential applications in the eye. As already discussed, neovascularization underlies a number of eye diseases including neovascular AMD, diabetic retinopathy, macular edema, neovascular glaucoma and corneal neovascularization (Neely and Gardner, 1998). A number of groups have demonstrated anti-angiogenic properties of exosomes. For example, exosomes from retinal astrocytes have anti-angiogenic components that were able to suppress vessel leakage and inhibit choroidal neovascularization in a mouse laser CNV model (Hajrasouliha et al., 2013). Exosomes containing the membrane-bound Notch ligand Dll4 suppress vascular sprouting, a fundamental part of angiogenesis (Sharghi-Namini et al., 2014). Anti-VEGF therapies are effective in many of these ocular neovascularization diseases and exosomes derived from mesenchymal stem cells can suppress angiogenesis by down regulating the expression of VEGF, partly due to the microRNA miR-16 (Lee et al., 2013).

Inflammation and fibrosis in the retina, leading to macular degeneration, and in the cornea, leading to dry eye disease are hypothesized to be mediated by activation of immune cells (Cousins et al., 2004; Ishikawa et al., 2016; Pflugfelder, 2004). The immunomodulatory effects of exosomes may be used to address these pathologies. For example, mesenchymal stem cell-derived exosomes possess anti-inflammatory properties that may be applicable to inflammatory eye diseases (Blazquez et al., 2014; Zhang et al., 2014). As mentioned previously, RPE cells are thought to use exosomes to modulate local immune responses by killing monocytes (Knickelbein et al., 2016). Exosomes also appear to deliver anti-inflammatory drugs to microglial cells to suppress neuroinflammation (Zhuang et al., 2011) or αB-crystallin to the neural retina, which could act as neuroprotection to photoreceptors (Sreekumar et al., 2010). Exosomes may also be able to facilitate neural repair. For example, MiR-133b containing exosomes transferred this microRNA to astrocytes and neurons in rats resulting in changes to gene expression that led to neurite remodeling and recovery from stroke (Xin et al., 2013). These neuroprotective effects of exosomes from mesenchymal cells have recently been shown useful in supporting retinal ganglion cells in a model of glaucoma (Mead and Tomarev, 2017). Finally, exosomes can induce proliferation in a number cell types (Deregibus et al., 2007; Jeong et al., 2014; Raimondo et al., 2015). Proliferation of TM cells can restore IOP homeostasis in animal models of glaucoma (Zhu et al., 2016).

We speculate that future studies will determine the minimal essential components of exosomes that mediate the anti-angiogenic, anti-inflammatory, neuroprotective and proliferative effects mentioned above. In combination with targeted delivery methods, engineered exosomes will likely be a viable therapy for the treatment of numerous eye diseases. Further, the heterogeneous nature of many eye diseases means biomarkers will help guide the design of exosomal therapies to provide a personalized, highly effective treatment as outlined in Fig. 6.

9. Conclusions and future directions

The etiology of a number of eye diseases involve activation of immune cells, inflammation, degeneration of neurons, neovascularization and fibrosis (Cousins et al., 2004; Hernandez et al., 1990; Howell et al., 2013; Ishikawa et al., 2016; Neely and Gardner, 1998; Pflugfelder, 2004; Tektas and Lutjen-Drecoll, 2009; Vranka et al., 2015). As discussed in this review, exosomes are likely mediating some, if not all of these effects. More importantly, the use of exosomes has been experimentally shown to predict or combat these processes. While the eye field is significantly trailing other fields in exosome research (Fig. 1, inset), the framework created by these fields will allow for rapid acceleration of exosome research in the eye.

To aid in this endeavor, in Fig. 7 we have indicated a select subset of the published exosome and small EV studies in different parts of the eye. In addition, in supplementary table S3 we have listed all eye-related exosome studies to date (excluding review articles) with brief descriptions of the exosome tissue/cell origin, isolation methods, analysis methods, and main findings in each study. Using exosomes as biomarkers or therapeutic vehicles hold the potential to lead to better, personalized treatments for patients with eye diseases, as outlined in Fig. 6. This summary emphasizes the immense research opportunities that exist to understand the physiological role and clinical potential of exosomes in ocular health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MVE	multivesicular endosome
EVs	extracellular vesicles
DCs	dendritic cells

RPE	retinal pigmented epithelium
CNV	choroidal neovascularization
FasL	Fas ligand
ECM	extracellular matrix
LC	lamina cribrosa
TM	trabecular meshwork
MMPs	matrix metalloproteinases
AMD	age-related macular degeneration
ApoE	apolipoprotein E
PCP	protein correlation profiling
AH	aqueous humor
PEG	polyethylene glycol
VEGF	vascular endothelial growth factor
PEDF	pigment epithelium-derived factor
fn	fibronectin
dex	dexamethasone

Glossary

Apoptotic bodies or blebs
Large portions (1–5 microns) of cells undergoing apoptosis which are released into the extracellular milieu by blebbing from the plasma membrane

Ectosome
Sometimes used to indicate neutrophil- or monocyte-derived MVs specifically, sometimes used to indicate MVs from any cell type

Endosome
Membrane-bound compartment inside eukaryotic cells. It is a compartment of the endocytic membrane transport pathway originating from the trans-Golgi membrane

ESCRT
Endosomal sorting complexes required for transport (ESCRT). Are made up of cytosolic protein complexes ESCRT-0 through –III

Exosome
Smallest subset of EVs. Of endolysosomal origin and released to extracellular milieu upon fusion of MVE with plasma membrane

Extracellular vesicle (EV)
Any lipid bilayer vesicle released from the cell in a controlled manner in an approximate size range of 30–1,000 nm. Does not include apoptotic blebs/bodies

Invadopodia
Newer, broader term replacing the terms invados ome, podosome and PILS

Invadosome, podosome, PILS
Any of several actin-rich adhesion structures. Podosome or invadopodia-like structures (PILS)

Lipoprotein particle
HDL, LDL, VLDL & Chylomicrons. Particles containing lipids, cholesterol and apolipoproteins

Lysosome
Organelle with an acidic interior containing a large range of digestive enzymes used primarily for digestion and removal of excess or damaged organelles, proteins, and engulfed viruses or bacteria

Multivesicular endosome (MVE)
ESCRT complexes generate exosomes by a controlled inward budding process from the endosome membraneresulting in an MVE

Microparticle, microvesicle (MP, MV)
EVs budding directly from the plasma membrane into the extracellular milieu. Approximate size range 150–1,000 nm

Proteasome
A multisubunit enzyme complex that plays a central role in the degradation of proteins that have been tagged by ubiquitin

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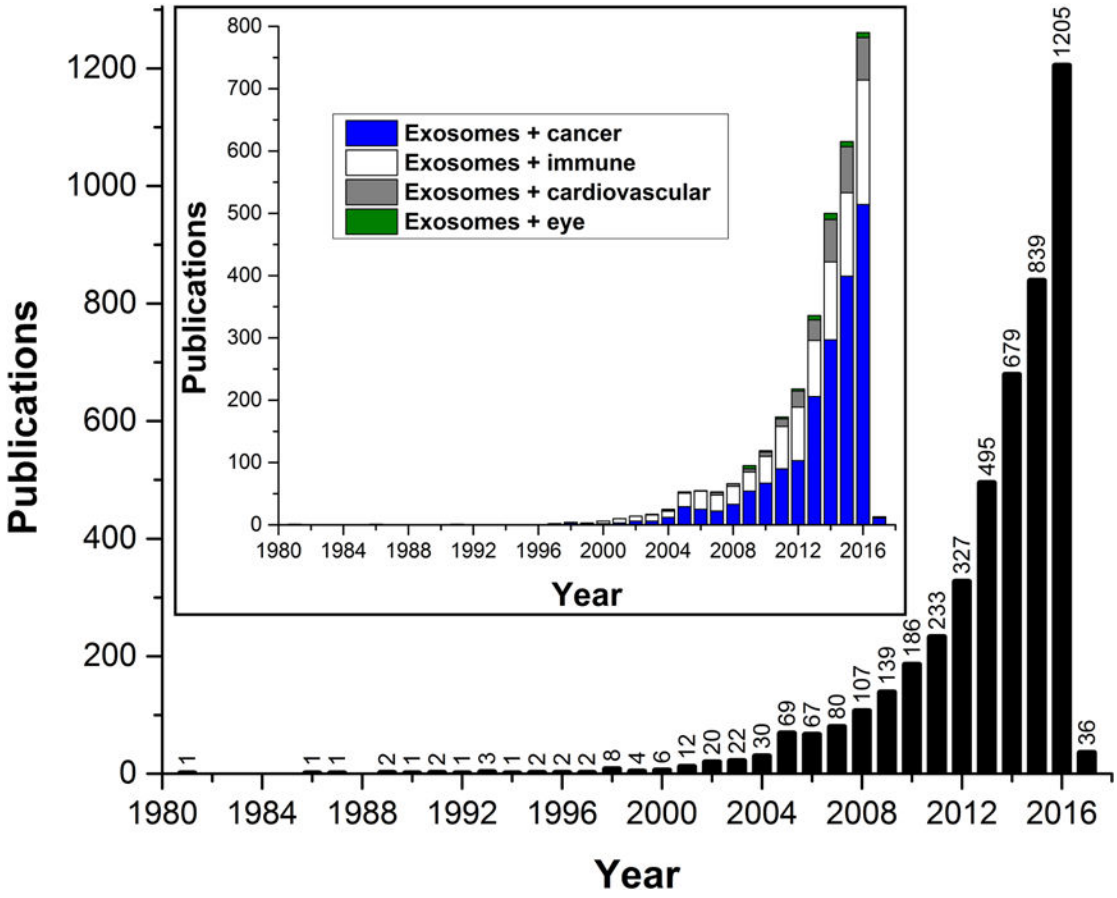

Figure 1. Publications with the keyword "exosomes" in the NCBI PubMed database as of December 2016

Note the exponential growth in the number of published articles, in particular in the last decade. (Inset) Publications with the indicated search terms in the NCBI PubMed database show a significant increase of exosome publications in the cancer (blue), immune (white), and more recently cardiovascular (gray) research fields; while the eye research field (green) is trailing far behind with only 8 articles in each of the last two years.

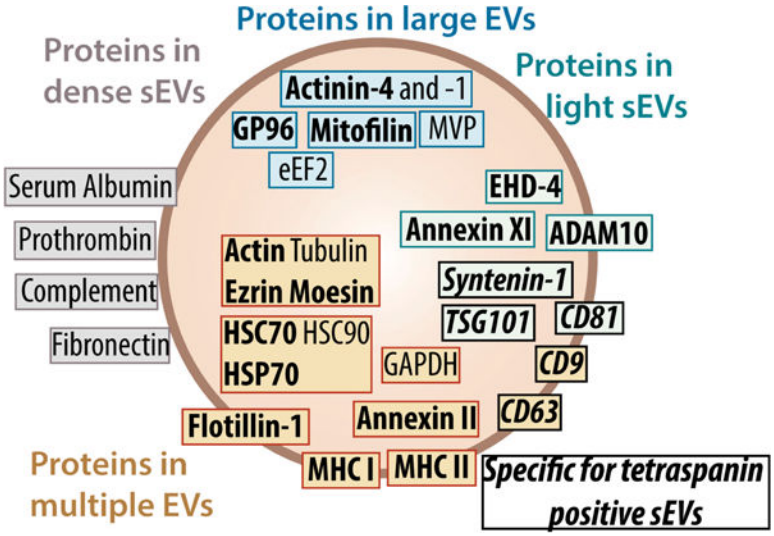

Figure 2. Extensive subfractionation of EV preparations identified highly enriched and/or specific markers for different subsets of EVs

Exosomes make up a subset of tetraspanin positive small EVs (sEVs), here identified by Syntenin-1, TSG101 and CD81. Figure from (Kowal et al., 2016); with permission and copyright PNAS. Permission Pending.

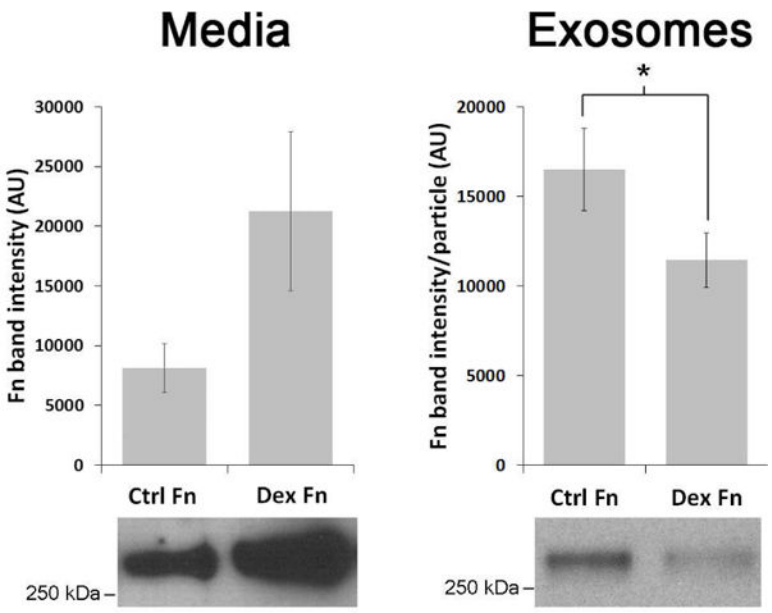

Figure 3. The corticosteroid, dexamethasone (Dex) treatment of human trabecular meshwork explants increases fibronectin secretion but decreases exosome affinity for fibronectin (Fn) surface

Western blot analysis of exosome-depleted conditioned media and exosomes isolated from conditioned media atop cultured human trabecular meshwork explants in the presence or absence of 100 nM dexamethasone for 72 hrs. Fibronectin content in exosome preparations was normalized to particle number using nanoparticle tracking analysis and quantified via densitometry (n=6) (Dismuke et al., 2016). Permission pending.

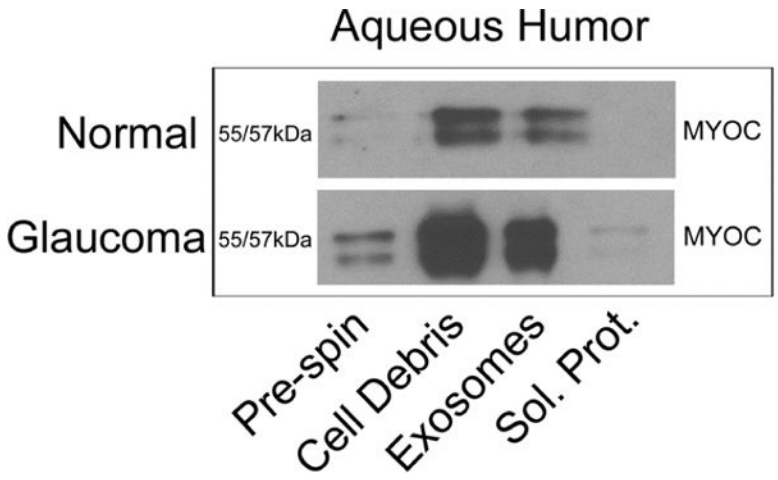

Figure 4. Comparison of myocilin-associated exosomes in fresh aqueous humor samples from normal and glaucoma patients undergoing surgery

Shown are representative Western blots of volume-equivalent samples taken from aqueous humor subjected to differential centrifugation steps. Pre-spin shows whole aqueous humor; cell debris shows resuspended pellet of whole aqueous humor subjected to 10,000 *g*; supernatant from pre-spin was subjected to centrifugation at 100,000 *g* giving exosomes in pellet and soluble proteins (sol. Prot.) in supernatant (Perkumas et al., 2007). Permission pending.

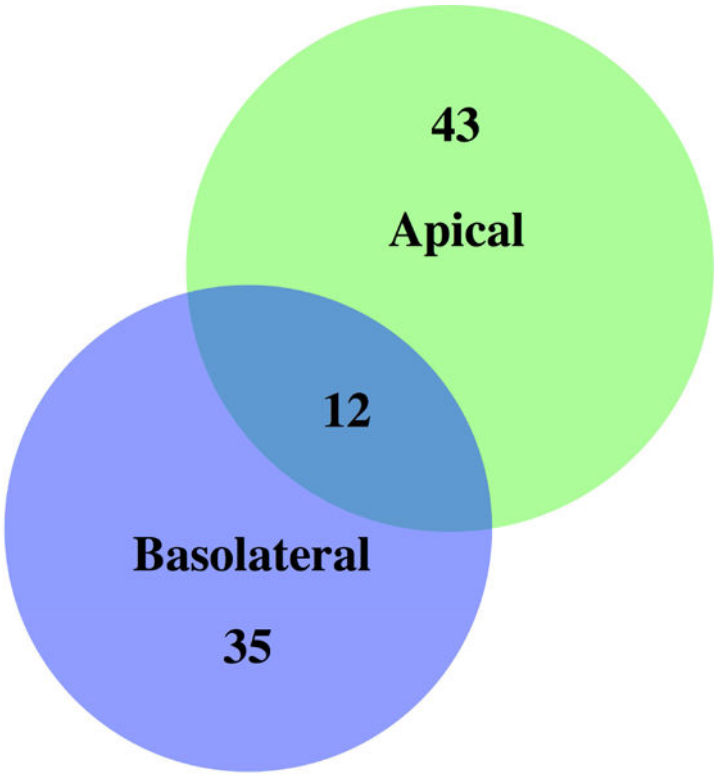

Figure 5. Exosomal proteins highly co-enriched with the exosome-specific marker Syntenin-1, differ markedly between apically and basolaterally released exosomes in a polarized RPE cell culture model

Note that in these highly stringent exosome-specific proteome datasets, only 12 proteins are released in exosomes from both sides, emphasizing the exceptional directionality in EV release from polarized RPE monolayers (Klingeborn et al., 2017). Permission pending.

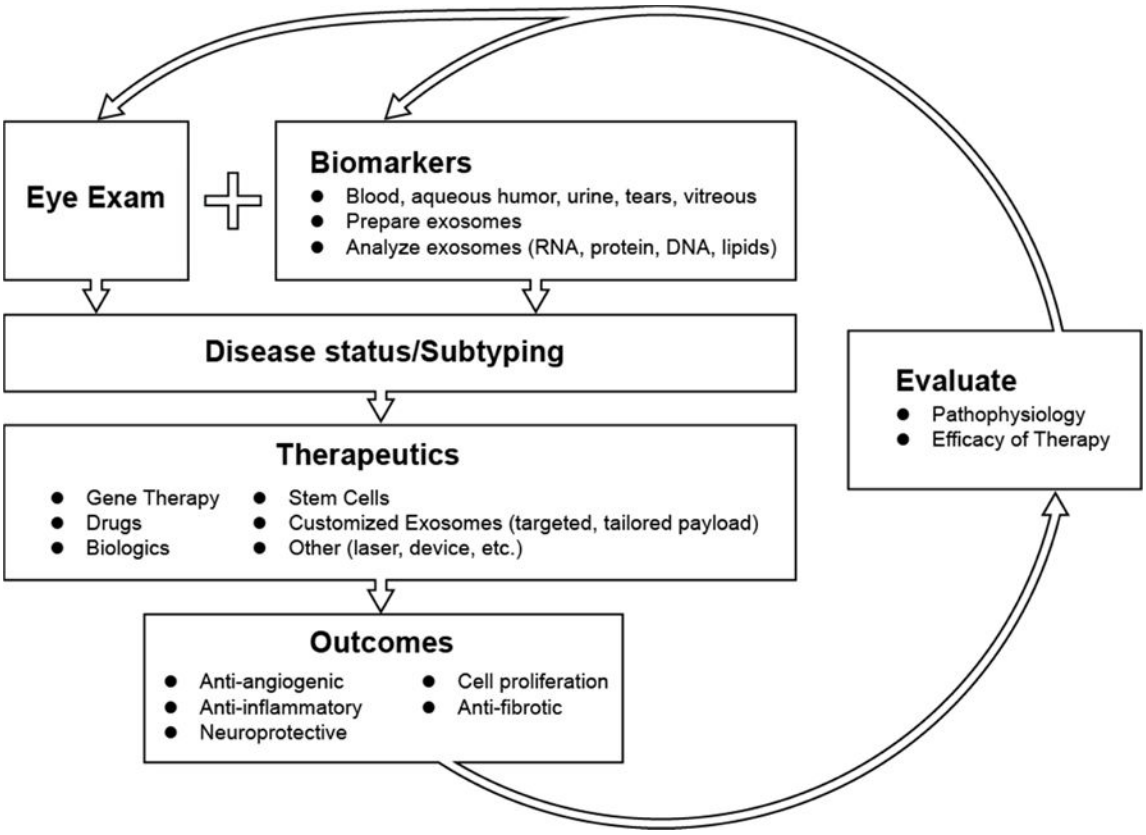

Figure 6.

Flow chart outlining an exosomal theragnostic approach to personalized treatment involving development and delivery of therapeutic, followed by diagnostic and prognostic evaluations.

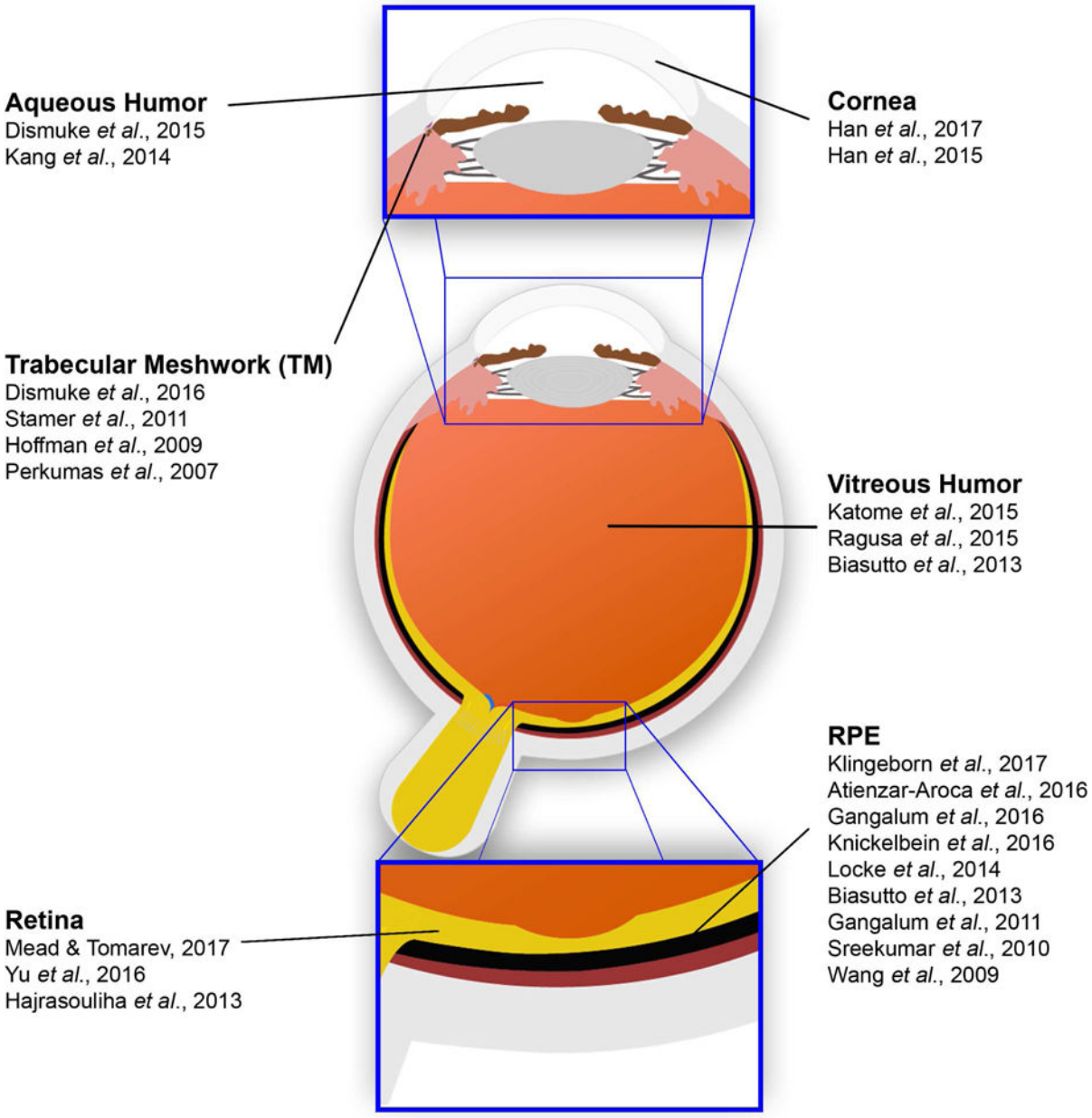

Figure 7. Select exosome studies in the eye

Exosome and small EV research in the eye has to date been limited. Exosome studies in different parts of the eye are indicated. Corresponding citations are shown in the reference list at the end of the article. TM, trabecular meshwork; RPE, retinal pigmented epithelium.

Table 1

A summary of the most relevant characteristics of common exosome and small EV isolation methods.

Method	Yield	Exo purity	Ease of use	Cost	Biological activity*	Preferred analysis†
DUC	++	++	++	Low	++	EM, NTA, WB
PEG	+++	+	+++	Low - High	+	2nd isolation method required
DGUC	+	+++	+	Low	++	EM, WB, NTA, MS, NGS, LA
IAC	++	++/+++	+/++	Medium to High	+	WB, MS, NGS, LA
SEC	+/++	++	++	Medium	+++	EM, NTA, WB, MS, NGS, LA

*Biological activity is impacted by different isolation methods by altering exosome integrity. Examples of biological activity includes exosome binding and uptake, enzyme activity, and other protein and nucleic acid-induced activity measured in target cells.

†Analyses that the resulting EV preparation is most suited/optimal for based on purity and physical integrity of the exosomes and small EVs. DUC, differential ultracentrifugation; PEG, polyethylene glycol precipitation; DGUC, density gradient ultracentrifugation; IAC, immunoaffinity capture; SEC, size exclusion chromatography; EM, electron microscopy; NTA, nanoparticle tracking analysis; WB, western blotting; MS, mass spectrometry; NGS, next-generation sequencing; LA, lipidomic analysis.