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Review

Autophagy in the normal and diseased cornea

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ABSTRACT

The cornea and covering tear film are together the 'objective lens' of the eye through which 80% of light is refracted. Despite exposure to a physically harsh and at times infectious or toxic environment, transparency essential for sight is in most cases maintained. Such resiliency makes the avascular cornea a superb model for the exploration of autophagy in the regulation of homeostasis with relevancy to all organs. Nonetheless, missense mutations and inflammation respectively clog or apparently overwhelm autophagic flux to create dystrophies much like in neurodegenerative diseases or further exacerbate inflammation. Here there is opportunity to generate novel topical therapies towards the restoration of homeostasis with potential broad application.

1. Introduction

In myth and apparently in actual practice, the ancient Greeks took advantage of the health restorative properties of autophagy by exposure of the eye (Trompoukis and Kourkoutas, 2007) and body to epidermal growth factor (EGF) containing saliva (Angeletti et al., 1992) - likely from the nonvenomous snakes *Zamenis longissimus* (or 'Aesculapian snake' named after Asclepius, the god of medicine) and *Elaphe quatuorlineata*. The term 'autophagy' (or self-eating') was the inspiration of 1974 Nobel Laureate Christian de Duve in 1963 based on the morphological studies of others (De Duve, 1963; Klionsky, 2008), and on his earlier biochemical discovery of and invention of the term 'lysosome' (De Duve et al., 1955). Subsequent development of yeast mutants deficient in autophagy by 2016 Nobel Laureate Yoshinori Ohsumi (Takeshige et al., 1992), and soon after by Daniel Klionsky (Harding et al., 1995) together with other approaches (Liang et al., 1999) disclosed the main molecular machinery as a complex, constitutive (but stimulatable) process that diverts autophagosomal captured damaged proteins and organelles into the lysosomal system. Under normal circumstances, mitochondrial function and cellular homeostasis benefit (Fig. 1). This process is now more accurately referred to as 'macroautophagy' with 'microautophagy' pertaining to lysosomal capture of cargo via membrane protrusions. Different types of macroautophagy have been described, including at least seventeen different forms of cargo specific 'selective macroautophagy' such as mitochondrial selective 'mitophagy'. The first formal consideration of macroautophagy in the cornea was by Tom Ferguson's group (Leib et al., 2009). To date, there have been a number of reviews that focus on (Akira et al., 2010; Chai et al., 2016; Han et al., 2016; Karnati et al., 2016; Kempuraj and Mohan, 2022; Lavker et al., 2020; Martin et al., 2019; Martínez-Chacón et al., 2020; Peng et al., 2017) or touch on (Hsueh et al., 2022; Kaplan et al., 2021a; Kumar and Jurkunas, 2021; Laihia and Kaarniranta, 2020; Miyai, 2018; Mun et al., 2021; Shukla and Valyi-Nagy, 2022; Stepp and Menko, 2021; Stuard et al., 2020; Volatier et al., 2022) aspects of corneal macroautophagy. Here for the first time, we highlight methods employed and actual primary data. How autophagy is studied and interpreted is important, as detailed in the latest edition of 'Guidelines for the use and interpretation of assays for monitoring autophagy' (Klionsky et al., 2021).

2. Architecture

The multi-layered corneal architecture (Fig. 2) includes: (i) the stratified, highly innervated and tear-wettened epithelium (~51 μm thick) that is supported by a basement membrane known as Bowman's membrane (or 'layer'; ~16 μm), (ii) the ~160 μm thick connective tissue ('stroma') composed of parallel, hydrated, collagenous lamellae (Espana and Birk, 2020; Meek and Boote, 2009), and (iii) the simple squamous corneal endothelium adherent to another basement membrane known as Descemet's membrane (3–10 μm). Descemet's membrane adjoins the

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stroma. The endothelium is immersed in the aqueous humor.

The most commonly studied macroautophagy marker is the phosphatidylethanolamine modified inner membrane-associated microtubule associated protein 1 light chain 3 beta (MAP1LC3B-II or 'LC3B-II' or 'LC3-II'; Table 1). Western immunoblotting for LC3-II also detects precursor 'LC3-I', a cytoplasmic protein of slower mobility (Mizushima and Yoshimori, 2007). Not generally detected is the initial translation product 'LC3' that is rapidly processed to LC3-I by autophagy related 4B cysteine peptidase (ATG4B). To form LC3-II, LC3-I is first activated by the E1-like enzyme autophagy related 7 (ATG7) and then passed to E2-like autophagy related 3 (ATG3) for conjugation with phosphatidylethanolamine as LC3-II (Klionsky et al., 2021). LC3-II with or without family members (Shpilka et al., 2011), associates with macroautophagy machinery (Fig. 1) during formation of the nascent phagophore, a double membrane structure created for the capture of damaged proteins and organelles. The ensuing autophagosome subsequently fuses with the low pH and protease-rich lysosome or with early or late endosomes. The former creates an autolysosome (Fig. 1), and the latter an amphisome. The amphisome then fuses with a lysosome forming an autolysosome. Within autolysosomes, contents are subjected to degradation. The products of degradation then become available to cellular metabolic (Fig. 1) and synthetic machinery (Rabinowitz and White, 2010). For a complete list of macroautophagy machinery, see the BioGRID Autophagy Project (Oughtred et al., 2021).

Proper monitoring of macroautophagic and mitophagic (macroautophagy of mitochondria) 'flux' or flow is essential. Elevated immunodetection of LC3-II versus LC3-I in Western blots or enhanced cellular immunofluorescence is often offered as evidence. This can be misleading in the absence of data capturing the dynamic nature of autophagic flux. LC3-II accumulation can for example be the consequence of problems in autophagosome-lysosome or -late endosome fusion, post fusion degradation, potentially trafficking, or even utilization of LC3-II by non-autophagic processes (Klionsky et al., 2021). Moreover, accelerated autophagy (apparently with rapid turnover) can even present as less LC3-II (Wang et al., 2013), and for cellular immunofluorescence, no antibody can currently distinguish LC3-II from LC3-I. Proper detection of autophagic flux is detailed in 'Guidelines for the Use and Interpretation of Assays for Monitoring Autophagy (3rd Edition)' (Klionsky et al., 2021). One recommended approach is to transfect or transduce cells or employ mouse models with GFP- and mCherry- (or mRFP-) double tagged LC3B (or for mitophagy, replace LC3B with $FIS1_{101\text{-}152}$, a fragment of the outer mitochondrial protein 'fission, mitochondrial 1').

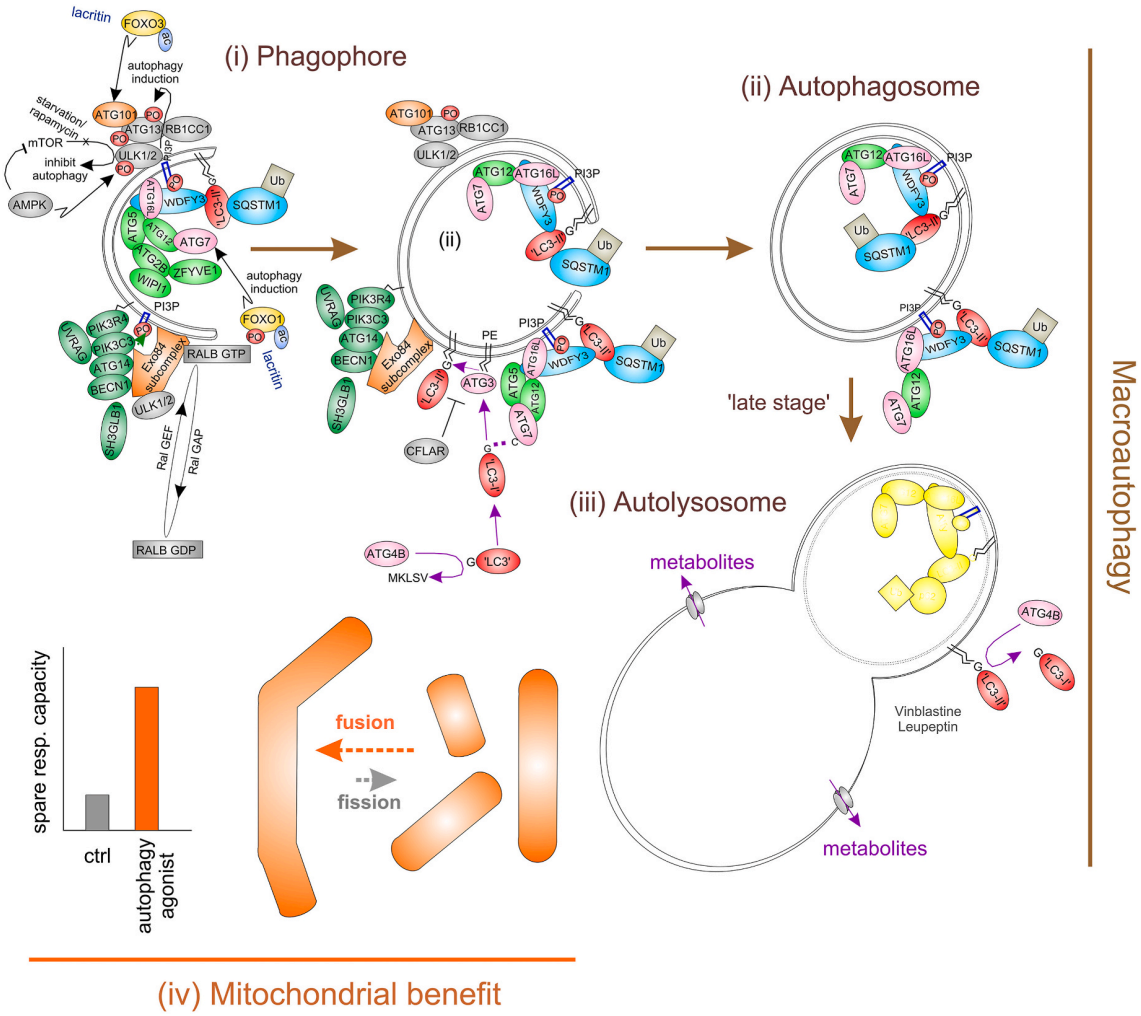

Fig. 1. Schematic diagram of macroautophagy beginning with formation of (i) the 'phagophore' that assembles around damaged organelles and proteins, and then fuses as (ii) the 'autophagosome'. Later fusion with endosome(s) or a lysosome creates (iii) the 'autolysosome'. Macroautophagy can benefit (iv) mitochondrial oxidative phosphorylation (including enhanced 'spare respiratory capacity') by elevating mitochondrial fusion (Wang et al., 2013). Basal macroautophagic flux is subject to inhibition or acceleration. Shown are mediators. Diagram was updated and modified from Experimental Eye Research Vol. 144/Authors: Karnati R, Talla V, Peterson K, Laurie GW/Title: 'Lacritin and other autophagy associated proteins in ocular surface health', Pages No. 4–13, 2016, with permission from Elsevier.

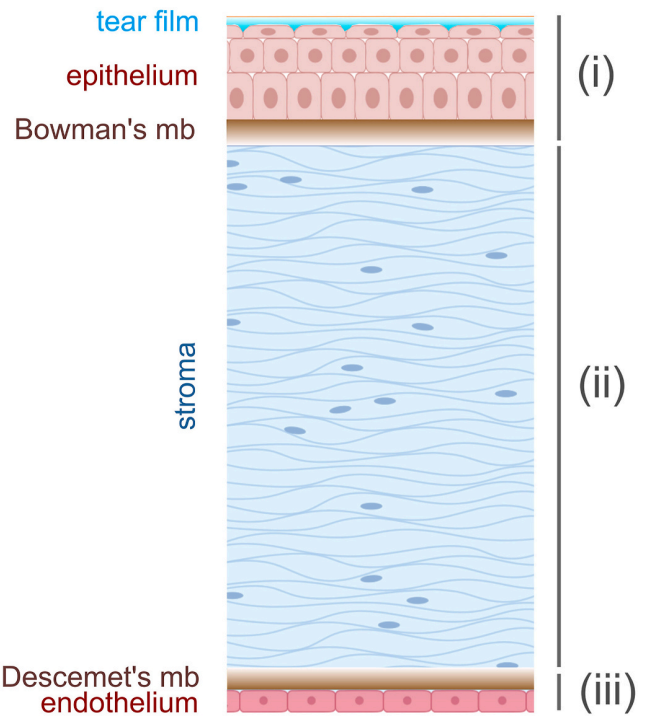

Fig. 2. Schematic depiction of the human central cornea with lipid (orange) covering the aqueous tear film (aqua) over outer cells of the stratified epithelium. The (i) epithelium is supported by Bowman's membrane (mb) and (ii) the underlying stroma. Associated with the deepest stromal layer is (iii) Descemet's membrane to which the endothelium is adherent. Created with BioRender.com (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dependent fluorescent detection of expressed LC3B (or $FIS1_{101\text{-}152}$) shifts from yellow (GFP plus mCherry) at the phagophore and autophagosome to only red as GFP (but not mCherry) is quenched in low pH autolysosomes. Also recommended is inclusion of macroautophagic inhibitors, although with the reminder that all pharmacologic inhibitors can have additional targets dependent on dose and duration of treatment (Klionsky et al., 2021). Eight (McWilliams et al., 2019; Miao et al., 2019; Shetty et al., 2017; Shyam et al., 2021; Stuard et al., 2022; Tang et al., 2022; Wang et al., 2021, 2013) of 89 primary research articles on corneal macroautophagy to date have employed either of these tandem constructs. An excellent example is the study of adult and E16.5 corneas of transgenic mCherry-GFP-MAP1LC3B and mCherry-GFP-$FIS1_{101\text{-}152}$ mice - as respectively macroautophagy and mitophagy reporter mice (McWilliams et al., 2019). For reporter imaging, mice were first cleared of blood cells by intracardial perfusion with PBS, followed by tissue fixation in buffered formaldehyde and cryosectioning. Scattered mCherry punctae indicative respectively of macroautophagy or mitophagy were detected in epithelial, stromal and endothelial cells. The level of macroautophagy exceeded that of mitophagy, and E16.5 levels of each slightly exceeded adult levels. Mitochondria were concentrated in the outer two-thirds of the corneal epithelium, as previously noted by others (McWilliams et al., 2019). Further evidence of macroautophagy in the epithelium, stroma and endothelium are discussed below.

3. Corneal epithelium

3.1. Regeneration

Regeneration of the corneal epithelium is regulated by the *microRNA-103/107* (*miR-103/107*) expressing (Peng et al., 2015) limbal stem cell compartment (Cotsarelis et al., 1989; Schermer et al., 1986) located at the corneal/conjunctival junction. Here, transit-amplifying cells arise. Migration of transit-amplifying cells from the limbus to the base of the peripheral and central cornea regenerates the epithelium (Lehrer et al., 1998). Exploration of the mechanism revealed that *miR-103/107* is essential for promoting late stage macroautophagy necessary for maintenance of proliferative capacity. Late stage macroautophagy is the maturation and fusion of autophagosomes with lysosomes or endosomes (Mehrpour et al., 2010). Studies were performed in a limbal-derived epithelial stem cell line with and without antagomirs, siRNA's and the late stage autophagic inhibitor bafilomycin together with MAP1LC3B ('LC3B') immunostaining, as well as in GFP-LC3B transgenic mice (Park et al., 2016). Bafilomycin A1 is a specific inhibitor of the vacuolar type H (+)-ATPase that thereby blocks lysosomal acidification (Yoshimori et al., 1991) and inhibits autophagosome - lysosome fusion (Klionsky et al., 2021). By single cell RNA-seq, beclin $1^{+/-}$ (BECN1) heterozygous mice deficient in macroautophagy displayed a 50% reduction in limbal stem and transit-amplifying cells, in keeping with a substantial deficit of mRNA's linked to cell replication (Kaplan et al., 2019). RNA-seq is a powerful technique for comprehensively documenting all transcribed RNA's (Nagalakshmi et al., 2008). BECN1 (Fig. 1) regulates phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3; Fig. 1) (Panaretou et al., 1997) necessary for the promotion of autophagy (Liang et al., 1999). In the central and peripheral cornea but not limbus, late stage macroautophagy and ciliogenesis are regulated by the EPH receptor A2 (EPHA2) (Kaplan et al., 2021b) of the ephrin receptor subfamily whose expression is negatively modulated by ligand ephrin A1. This arrangement contributes to the compartmentalization of the limbus (Kaplan et al., 2018). Evidence is based largely on siRNA and bafilomycin manipulation of a human corneal epithelial cell line with LC3B, SQSTM1 and PLD1 immunoblotting, together with use of GFP-LC3 transgenic and EPHA2 knockout mice (Kaplan et al., 2021b). EPHA2 interacts broadly with macroautophagy mediators including autophagy related 12 (ATG12) and BECN1 (Kaplan et al., 2021b), several cyclin dependent kinases, integrins, and the cell junctional protein cadherin 1 (CDH1) and syndecan-1 (SDC1). LC3A, LC3B and ATG7 mRNA have been detected in human limbus explant cultures at levels exceeding that of the conjunctiva (Dhamodaran et al., 2015).

3.2. Pathology

3.2.1. Ocular surface disease

Dry eye is the most common eye disease affecting 3–8% of the world's population, rising to 12% in those greater than 50 years old (Dana et al., 2019). An unbiased biochemical screen for endogenous tear agonists identified *lacritin* (LACRT) (Sanghi et al., 2001), a novel tear protein that promotes basal tearing (Samudre et al., 2011; Vijmasi et al., 2014; Wang et al., 2015) including release of the tear proteins lactoferrin (LTF) and lipocalin (LCN1) (Fujii et al., 2013) and exosomes (Lee et al., 2020). *Lacritin* was later found by proteomics to be selectively deficient in aqueous deficient, combined aqueous deficient/meibomian gland disease, contact lens-related and both primary and secondary Sjögren's Syndrome dry eye (Georgiev et al., 2021; Karnati et al., 2013; Willcox et al., 2017). *Lacritin* targets cell surface syndecan-1 (SDC1) via a heparanase-dependent 'off/on switch' mechanism (Ma et al., 2006; Zhang et al., 2013) complexed with a pertussis toxin sensitive (Wang et al., 2006) G-protein coupled receptor. SDC1 is a heparan sulfate proteoglycan. Under conditions of stress, *lacritin* rapidly stimulates macroautophagy in a calcineurin- (PPP3CC), but not rapamycin-dependent manner mediated by cytoplasmic forkhead box O1 (FOXO1) and forkhead box O3 (FOXO3). Calcineurin is a phosphatase; rapamycin inhibits mTOR complex 1 (mTORC1) to promote autophagy; and FOXO1 and FOXO3 are transcription factors that undergo translocation between the cytoplasm and nucleus. *Lacritin* does so by promoting the phosphorylation FOXO1 that together with stress-dependent acetylation can then ligate ATG7 (Fig. 1), and through

Table 1
Demonstrated corneal autophagic stimulators, inhibitors, mediators, miR's and pathologies.

Demonstrated corneal autophagic[a]					
Corneal layer	Stimulator	Inhibitor	Mediator	miR	Pathology
Tear film	EGF LACRT	IGFBP3			ocular surface disease
Epithelium	*A. fumigatus* blue or UV light calcitriol EGF EPHA2 *HSV-1* LACRT *P. aeruginosa* (pyoverdin or ExoU) particulate matter < 2.5 μm rapamycin *S. marcescens* (prodigiosin or ShlA) S100A4 shRNA cigarette smoke stress (dessic, inflamm., hyperosm., oxidative) trehalose	3-MA bafilomycin chloroquine high glucose IGFBP3 melatonin stress (oxidative) trehalose *HSV-1* (ICP34.5) *RV* *P. aerug.* (ExoS) *S. marces.* (prodigiosin) wortmanin	ATG4B ATG5 ATG7 ATG12 ATG14 ATG16L1 ATG101 BECN1 BNIP3 CGAS DDIT4 FOXO1 FOXO3 HMOX1 'LC3-II' PIK3C3 PINK1 PRKN MFN2 SIRT3 SQSTM1 WDFY3	34c 103/107 129–5p 181a 205-3p	diabetes keratitis keratoconus ocular surface disease wounding GCD2 LCD
Stroma	melatonin rapamycin	3-MA bafilomycin	ATG5 BECN1 'LC3-II' SQSTM1		GCD2 LCD SCD
Endothelium	lithium carbonate menadione	bafilomycin	ATG5 ATG12 'LC3-II' PINK1 PRKN SQSTM1		CHED FECD

[a] References are provided in the text.

lacritin-dependent acetylation of FOXO3 for binding of autophagy related 101 (ATG101; Fig. 1) associated with the phagophore assembly site (Wang et al., 2013). Stress was created by dry eye inflammatory cytokines interferon-γ (IFNG) and tumor necrosis factor (TNF) or by overexpression of huntingtin mutant Htt103Q-mCFP, as monitored in a human corneal epithelial cell line transfected with mCherry-GFP-MAP1LC3B (Wang et al., 2013). Red puncta elevated 10 min after the addition of *lacritin* progressively decreased over 24 h - a benefit negated by the late stage macroautophagy inhibitor vinblastine and absent in cells treated with the inactive *lacritin* truncation mutant C-25. Vinblastine depolarizes microtubules to block autophagosome - lysosome or -endosome fusion (Klionsky et al., 2021). Transiently elevated autophagy leads to restoration of oxidative phosphorylation and spare respiratory activity via mitochondrial fusion (Wang et al., 2013), and is presumed to contribute to the diminishment of lissamine corneal staining in Aire$^{-/-}$ dry eye mouse mice treated topically with *lacritin* (Vijmasi et al., 2014). Spare respiratory activity was assessed using 'mitochondrial stress tests' that measure cellular oxygen consumption after the progressive introduction of selective mitochondrial inhibitors. Similar results were observed with nineteen amino acid synthetic peptide *N-94/C-6* (*Lacripep*) from *lacritin's* C-terminus, as replicated in a 204 patient Primary Sjögren's Syndrome phase I/II trial (NCT03226444) for which further dose optimization is necessary (Tauber et al., 2022).

There are various examples of corneal epithelial cell macroautophagy in the context of H_2O_2 oxidative, desiccating or hyperosmolar stress - each of which trigger epithelial inflammation. Oxidative stress is an apparent macroautophagic inhibitor in a human corneal cell line (Yin et al., 2018) and in mouse primary corneal epithelial cells (Zhou et al., 2016). Yet, in an immortalized murine limbal cell line it stimulates macroautophagy (Zhou et al., 2016), as monitored by LC3 and sequestosome 1 (SQSTM1 or p62; Fig. 1) immunoblotting with or without macroautophagy agonist rapamycin (Yin et al., 2018). Dessicating stress of a human corneal epithelial cell line by media removal and 10 min of air drying promoted macroautophagy as indicated by LC3B-II immunoblotting with or without autophagic inhibitor chloroquine (Shivakumar et al., 2018). Chloroquine appears to block autophagosome-lysosome fusion (Mauthe et al., 2018), rather than stably increasing lysosomal pH as was previously thought (Homewood et al., 1972). Dessicating stress of mouse eyes or hyperosmolar stress (500 mOsM) of a human corneal epithelial cell line and of primary human corneal epithelial cells substantially enhanced macroautophagy as demonstrated by bafilomycin-augmentable LC3B-II immunoblotting. Surprising accumulation of SQSTM1 rather than autophagic degradation was interpreted as evidence of faulty late stage macroautophagy that appeared to be corrected by siRNA knockdown of DNA damage inducible transcript 4 (DDIT4) (Wang et al., 2019). DDIT4 is required for the stress-dependent induction of reactive oxygen species, and is an mTORC1 independent regulator of macroautophagy (Qiao et al., 2015). A similar benefit was achieved by introduction of *N-acetylcysteine*, a scavenger of reactive oxygen species (Wang et al., 2019).

A number of authors have sought reagents that might modulate macroautophagy to alleviate pathology under conditions of dessicating or hyperosmolar stress. Examples to date include *calcitriol*, the corticosteroid *fluormetholone*, *melatonin*, *rapamycin* and *trehalose*.

- *Calcitriol* is a form of vitamin D3 with current clinical application in support of blood calcium levels and with a long history of autophagic stimulation in breast cancer and other cells (Høyer-Hansen et al., 2005). *Calcitriol* stimulates macroautophagy in a hyperosmolar stressed human corneal epithelial cell line over an apparent bell-shaped dose response (N. Lyu et al., 2020) inversely analogous to *trehalose's* U-shaped dose response (Liu et al., 2020b). This significantly promotes viability in a bafilomycin-inhibitable manner that is erased after siRNA knockdown of vitamin D receptor mRNA. A rat dry eye model generated by topical treatment with pro-apoptotic ophthalmic preservative benzalkonium chloride (BAC) also appears to benefit from *calcitriol*-stimulated macroautophagy of the corneal epithelium, as indicated by LC3B-II immunoblotting (N. Lyu et al., 2020).
- *Fluormetholone*, a corticosteroid approved by the FDA for 'treatment of steroid responsive inflammatory condition of the palpebral and bulbar conjunctiva, cornea and anterior segment of the eye' was topically applied four times daily for one month to 40 patients suffering from Primary Sjögren's Syndrome dry eye. Immunoblotting of tears using an anti-LC3B antibody revealed substantially less LC3-II after treatment and little LC3-II in normal and non-Sjögren's Syndrome dry eye tears. Whether immunoblotting of tears accurately reflects the state of ocular surface health is uncertain although tear LC3-II presumably derives from epithelial sloughing. The authors inaccurately attributed anti-LC3B immunostaining of conjunctival impression cytologies and conjunctival RT-PCR to LC3-II. Western immunoblotting is required to distinguish LC3-II (lipidated; autophagosome-associated) from precursor LC3-1 (non-lipidated; cytoplasmic; Fig. 1) (Karnati et al., 2016). Nonetheless, fluorescein corneal staining was less and tears collected without topical anesthesia were greater (Byun et al., 2017). Topical *fluormetholone* increases the risk of developing glaucoma and cataracts beyond two weeks of use.
- The pineal gland hormone *melatonin* has long been associated with macroautophagy. *Melatonin* enhanced the viability of a human corneal epithelial cell line subjected to oxidative stress. It did so by partially suppressing excessive macroautophagy as suggested by LC3B-II immunoblotting and double tagged mCherry-GFP-LC3 fluorescence, a benefit lost after siRNA knockdown of heme oxygenase 1 (HMOX1). HMOX1 overexpression promotes oxidative stress and stress-dependent autophagy (Song et al., 2012). Eyes of mice subjected to ocular dessicating stress benefited from intraperitoneal injection of *melatonin* as indicated by decreased corneal fluorescein staining, and increased tearing and goblet cell number (Wang et al., 2021).
- *Rapamycin* restored the viability of a human corneal epithelial cell line (Yin et al., 2018) and slightly improved the viability of primary human corneal epithelial cells subjected to oxidative stress (Liu et al., 2020a). When topically applied twice daily to eyes of elderly mice between 16.5 and 20.5 months old, some benefit was detected including a modest increase in goblet cells on the ocular surface and of $CD4^{+}$/Foxp3+ T regulatory cells in both lacrimal glands and draining lymph nodes, and a reduction in some transcripts of lacrimal gland inflammatory cytokines (Trujillo-Vargas et al., 2020).
- Appropriate dosing of *trehalose*, a glucose dissacharide, slightly increased the viability of a human corneal epithelial cell line subjected to dessicating stress. In LC3B-II immunoblotting, *trehalose* appeared to impair macroautophagy (Panigrahi et al., 2019), in keeping with substantial evidence in primary cortical neurons and a neuroblastoma cell line that *trehalose* may be an effective inhibitor of autophagic flux (Yoon et al., 2017). Yet, Liu et al., 2020b) provide evidence for *trehalose* as an autophagic stimulator in the context of osmotic stress of primary corneal epithelial cells using LC3B, ATG5, ATG7, BECN1 and SQSTM1 immunoblotting together with the early stage macroautophagy inhibitor *3-methyladenine* (3-MA). 3-MA inhibits the class III phosphatidylinositol 3-kinase complex of catalytic subunit PIK3C3 and regulatory subunit PIK3R4 in an autophagy inducing complex with BECN1 and other proteins (Fig. 1). Nuclear transcription factor EB (TFEB) is elevated in the presence *of trehalose* and proposed to be the mediator (Liu et al., 2020b). TFEB is a key transcriptional regulator of both lysosomal biogenesis and macroautophagy (Settembre et al., 2011).

3.2.2. Diabetes

Culturing the TKE2 cell line developed from mouse limbal epithelium in high glucose media decreased levels of LC3B-II, sirtuin 3 (SIRT3), FOXO3, and parkin RBR E3 ubiquitin ligase (PRKN) in Western blots. SIRT3 is a mitochondrial class III deacetylase (Schwer et al., 2002) described as both a positive and (occasionally) a negative regulator of macroautophagy. PRKN phosphorylated by PTEN induced kinase 1 (PINK1) contributes to the ubiquitination of proteins on depolarized mitochondria to enhance mitophagy (Pickles et al., 2018) and thereby counter inflammation. PRKN and PINK1 mutations are linked to Parkinson disease (Sliter et al., 2018). Transducing high glucose incubated cells with SIRT3 adenovirus restored levels of FOXO3 and 'wound healing' in cellular scratch culture, and promoted the overexpression of PRKN (Hu et al., 2019b). Similarly, corneas of SIRT3 adenoviral injected conjunctiva of $Ins2^{Akita/+}$ type 1 diabetic mice displayed healing of algerbrush created wounds at a level approximating that of the $Ins2^{+/+}$ normal mouse (Hu et al., 2019b).

The trigeminal ganglion receives sensory nerves from the ocular surface. Trigeminal ganglia neuronal explant culture from mice made diabetic via prior injection of pancreatic β-cell toxin streptozotocin, and ganglia from normal mice incubated in high glucose, displayed some reduced immunoblotting of LC3B-II (Hu et al., 2019a; 2019b) that was not apparent in follow up immunoblotting (Hu et al., 2020). Also reduced was ATG4B (Hu et al., 2019a) and autophagy related 5 (ATG5) (Hu et al., 2020). ATG4B cleaves cytoplasmic LC3-I at its C-terminal arginine to expose glycine (Satoo et al., 2009) thereby enabling ATG7 catalyzed attachment of phosphatidylethanolamine (PE) to form LC3-II. Through PE, LC3-II associates with the autophagosome membrane (Fig. 1). ATG5 in a complex with ATG12 and tectonin beta-propeller repeat containing 1 (TECPR1) together trigger the fusion of autophagosomes with lysosomes (Chen et al., 2012). Both ganglia also displayed elevated levels of *miR-34c* (Hu et al., 2019a) and *miR-181a* (Hu et al., 2020). *miR-34c* is thought to be associated with oxidative stress (Pavlides et al., 2010), and *miR-181a* inhibits macroautophagy by reducing levels of ATG5 (Tekirdag et al., 2013). Subconjunctival injection with antagomirs of *miR-34c* (Hu et al., 2019a) or *miR-181a* (Hu et al., 2020) prior to Algerbrush corneal wounding of normal or diabetic mice restored LC3B-II, ATG4B and ATG5 immunoblotting and healing.

Insulin like growth factor binding protein 3 (*IGFBP3*) is detectable in normal human tears by ELISA and, at much lower levels in type 2 diabetic tears (Wu et al., 2012). *IGFBP3* suppressed basal macroautophagy and mitophagy in primary and immortalized human corneal epithelial cells (Stuard et al., 2022). This is in keeping with siRNA IGFBP3 depletion in which cells transfected with mCherry-GFP-MAP1LC3B displayed more red puncta suggestive of increased macroautophagic flux that shifted to yellow puncta (slowed or blocked flux) when the same cells were treated with recombinant *IFGBP3* (Stuard et al., 2022).

3.2.3. Keratoconus

Keratoconus is a bulging, noninflammatory aberration of the cornea with progressive thinning that deleteriously affects visual acuity, and is currently addressed by corneal transplantation and corneal crosslinking (Iqbal et al., 2013). Oxidative stress is thought to contribute. Significantly decreased LC3A/B-II immunoblotting was observed in the periphery of clinical grade I and III epithelial samples, and of SQSTM1 in grade II peripheral epithelium (Shetty et al., 2017). Grading is based in part on: the radius of the cornea, the level of myopia and/or astigmatism and scarring or perforation. In 20–30% of patients, the superficial and basal corneal epithelial layers may contain unusual iron deposits.

Transferrin (*TF*) and *lactotransferrin* (*LTF*) are important in ferric iron binding, transport and homeostasis. Both are downregulated in the tear (López-López et al., 2021) and corneal (Chaerkady et al., 2013) keratoconus proteome. Excessive cellular iron is associated with lipid peroxidation and cell death by ferroptosis.

3.2.4. Pathogen antagonism or agonism

Ocular pathogens, including *Aspergillus fumigatus* (C.-Y. Han et al., 2021; Li et al., 2019; Li et al., 2020), *Fusarium solani* (Lin et al., 2021), *Herpes simplex virus type 1* (*HSV-1*) (Hsu et al., 2016; Jiang et al., 2015; Leib et al., 2009; Parker et al., 2015; Petrovski et al., 2014; Yakoub and Shukla, 2015), *rubella virus* (*RV*) (Pásztor et al., 2014), *Pseudomonas aeruginosa* (Angus et al., 2010; Hardy et al., 2022; Karthikeyan et al., 2013; Kroken et al., 2022; Rao et al., 2021; Tam et al., 2007) and *Serratia marcescens* (Brothers et al., 2018; Di Venanzio et al., 2014), affect corneal epithelial and stromal macroautophagy in different ways, most commonly by interfering with host autophagic machinery. Differentiation of *Acanthamoeba castellanii* trophozoites into chemical resistant cysts is via macroautophagy and can be inhibited by early stage autophagic inhibitor *3-methyladenine* and mid-stage inhibitor *chloroquine*, but not by LY294002 or *bafilomycin A* and only slightly by *wortmannin* (Moon et al., 2015). LY294002 (Vlahos et al., 1994) and *wortmannin* (Arcaro and Wymann, 1993), like *3-methyladenine*, inhibit the class III phosphatidylinositol 3-kinase complex of catalytic subunit PIK3C3 and regulatory subunit PIK3R4 in an autophagy inducing complex with BECN1 and other proteins (Fig. 1). Adhesion of trophozioites to silicon hydrogel contact lenses is inhibited by *3-methyladenine* and *chloroquine* (Lee et al., 2017). This is important because most cases of *Acanthamoeba* keratitis occur in contact lens wearers.

Macroautophagy as a consequence of ocular *Aspergillus fumigatus* infection was compared in C57BL/6 versus BALB/c mice with corneal LC3-II and beclin-1 immunoblotting greater (and SQSTM1 less) in the former, as was keratitis (C.-Y. Li et al., 2019). Cells from infected human corneas displayed a similar immunoblotting pattern. Treatment of infected mouse eyes with *3-methyladenine* or *chloroquine* increased while *rapamycin* decreased the level of keratitis, in keeping with respective immunoblotting of C-X-C motif chemokine ligand 1 (CXCL-1) (Li et al., 2020), a neutrophil chemoattractant. In a human corneal epithelial cell line and in eyes of infected C57BL/6 mice, *Aspergillus fumigatus*-dependent macroautophagy was mediated in part by the cyclic GMP-AMP synthase (CGAS), a stimulator of the interferon response cGAMP interactor 1 (STING1) signaling pathway. CGAS inhibitor *RU.521* and CGAS siRNA knockdown partially abrogated *Aspergillus fumigatus* infection-dependent macroautophagy and corneal fluorescein staining, and enhanced detection of CGAS and of STING phosphorylated on serine 366 (Han et al., 2021). STING1 serine 366 phosphorylation suggested activation of the CGAS-STING1 pathway. Accumulation of LC3B immunoblotting in the presence of *chloroquine* was evidence of macroautophagic flux (Han et al., 2021). In a *Fusarium solani* mouse keratitis model, *miR-129-5p* antagomir increased LC3B-II, BECN1 and autophagy related 14 (ATG14) immunoblotting and stroma cell viability (Lin et al., 2021). mir-129–5p appears to reduce BECN1 expression by targeting its 3′UTR (Xiao et al., 2015). Taken together, these results pointed to macroautophagy as an innate immune response that, when accelerated, enhanced fungal clearance leading to improved corneal health.

HSV-1 surface glycoproteins gB, gC and gD ligate heparan sulfate chains of cell surface proteoglycans to trigger membrane fusion, entry (Shukla et al., 1999) and STING1-dependent (Parker et al., 2015) macroautophagy. gD does so by preferentially binding 3-O sulfated heparan sulfate (Shukla et al., 1999). Viral protein ICP34.5 then antagonizes macroautophagy through ligation of host cell BECN1 (Orvedahl et al., 2007) necessary for successful *HSV-1* replication 5–7 days post-infection of scarified cornea. Removal of the BECN1 binding domain in ICP34.5 abrogated this response (Leib et al., 2009). *HSV-1* proteins *ICP0, Us3* and *Us11* antagonize macroautophagy through targeting of other host cell proteins as listed below. None have apparently been examined in the cornea to date.

- *ICP0* - Downregulation of SQSTM1 and optineurin (OPTN) (Waisner and Kalamvoki, 2019). OPTN is a TANK binding kinase 1 (TBK1) enhanceable autophagy receptor important in selectively coupling ubiquitinated proteins to LC3. Mutated forms are causative for adult-onset primary open-angle glaucoma (Rezaie et al., 2002).
- *Us3* - Phosphorylation of both BECN1 and unc-51 like macroautophagy activating kinase 1 (ULK1) (Rubio and Mohr, 2019). ULK1 (Kuroyanagi et al., 1998) is a serine/threonine kinase that complexes with RB1CC, ATG13 and ATG101 as an initial step in the initiation of autophagy (Fig. 1).
- *Us11* - Ligation of eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2 or PKR) (Lussignol et al., 2013). EIF2AK2 ligation of signal transducer and activator of transcription 3 (STAT3) regulates fatty acid-induced autophagy (Niso-Santano et al., 2013).
- *Us11* - Ligation of tripartite motif containing 23 (TRIM23) (Liu et al., 2019). TRIM23 is a GTPase that promotes the activation of TBK1 in a complex with SQSTM1. TRIM23 is important in virus selective autophagy (Sparrer et al., 2017).

Rubella virus also impedes macroautophagy, as studied by LC3B-II immunoblotting with and without *bafilomycin* in lysates of an infected human corneal epithelial cell line with fibroblastic characteristics. Inclusion of *rapamycin* significantly reduced viral titer and apoptosis (Petrovski et al., 2014).

Pseudomonas aeruginosa exoenzyme effector ExoU and iron siderophore pyoverdin respectively stimulate mitophagy, whereas exoenzyme effector ExoS inhibits macroautophagy. Both ExoU and ExoS are injected into host cells (Hardy et al., 2022; Kroken et al., 2022; Rao et al., 2021) via the *P. aeruginosa* type III secretion system. ExoU is a potent ubiquitin-activated phospholipase A2, that inserts into mitochondrial membranes to promote oxidative stress and mitophagy in neutrophils, macrophages and epithelia (Hardy et al., 2022; Tam et al., 2007) thereby enabling later colonization of scarified mouse cornea (Tam et al., 2007). In host cells ExoS′ C-terminal ADP-ribosylation domain partially inhibits PIK3C3 (Fig. 1) necessary for the initiation of macroautophagy as part of the ATG14-BECN1 complex (Rao et al., 2021). This thereby promotes survival of *P. aeruginosa* within the membrane replicative niche of host corneal epithelial cells (Angus et al., 2010). ExoS was reported to be substantially more prevalent than ExoU in *P. aeruginosa* keratitis isolates (Karthikeyan et al., 2013). *P. aeruginosa* secretes pyoverdin into the extracellular environment where, upon uptake into host cells, it promotes oxidative stress and mitochondrial damage by capturing mitochondrial iron. Damaged mitochondria trigger mitophagy thereby conferring host resistance to *P. aeruginosa* (Kirienko et al., 2015).

Filter sterilized overnight growth media from *S. marcescens* and from various Gram-negative and MRSA Gram-positive species triggered macroautophagy in a human corneal epithelial cell line transfected with LC3B-GFP, as optimized using *rapamycin*, *ammonium chloride*, *3-methyladenine* and *bafilomycin*. *Ammonium chloride* elevates lysosomal and endosomal luminal pH to minimize degradation of autophagosomal contents, thereby inhibiting macroautophagic flux. Several Gram-positive bacterial growth media were inactive (Brothers et al., 2018). *S. marcescens* mutant screening pointed to macroautophagy activating roles of the proapoptotic red pigment *prodigiosin* and the pore forming toxin *ShlA*. *Prodigiosin* has been previously described as both an agonist (Lin et al., 2017) and antagonist (Zhao et al., 2020) of macroautophagy, and *ShlA* as an agonist (Di Venanzio et al., 2014).

3.2.5. Environmental/particulate matter initiated

Macroautophagy in human cell lines was triggered by cigarette smoke extract (Miao et al., 2019; Otsu et al., 2021), particulate matter less than 2.5 μm in diameter (D. Kashiwagi and Iizuka, 2020; Lyu et al., 2020), and by environmental blue (Li et al., 2021) and ultraviolet (UV)

light (Fu et al., 2020). Cigarette smoke extract also promoted corneal epithelial cell hypertrophy and loss of viability (Miao et al., 2019) associated with loss of transepithelial resistance and reduced mRNA expression of mucins 1, 4 and 16 (MUC1, 4 and 16) that form the glycocalyx of the apical corneal surface. Cell hypertrophy and loss of viability, MUC1, 4 and 16 mRNA's was preventable by co-treatment with *cysteamine* (Miao et al., 2019) or *N-acetylcysteine* (Otsu et al., 2021) indicative of cigarette smoke induced lipid peroxidation (Otsu et al., 2021).

SQSTM1 binds multiple macroautophagy-associated proteins including LC3B, autophagy related 16 like 1 (ATG16L1), ubiquitins (UBB, UBC, UBD) and ubiquitinated proteins. ATG16L1 couples with the ATG12 - ATG5 complex to regulate LC3 lipidation (Fujita et al., 2008). Ubiquitinated proteins were more abundant in SQSTM1 pulldowns from cigarette extract treated cells versus controls. This is in keeping with the perinuclear colocalization of SQSTM1 with ubiquitinated proteins and ubiquitinated proteins with LC3B, together with reactive oxygen species detectable by dichloro-dihydro-fluorescein diacetate. *Cysteamine* treatment erased this protein complex (Miao et al., 2019). Immunoblotting for LC3B-II, SQSTM1 and ferritin together with immunofluorescent colocalization of ferritin, SQSTM1 and lysotracker suggested that faulty macroautophagy led to accumulation of a ferritin proteolytic fragment and subsequent lipid peroxidation coupled with a form of cell death known as ferroptosis (as noted above). Lysotracker is a fluorescent probe with an affinity for the luminal acidic compartment of lysosomes and other acidic organelles. Ferroptosis inhibitor *Fer-1* partially restored viability and no ferritin proteolytic fragment was evident after *chloroquine* or *bafilomycin* treatment (Otsu et al., 2021). *Cysteamine* and *N-acetylcysteine* are both commercially available as eye drops respectively for pediatric or adult cystinosis, or for ocular discomfort. A 38 person randomized, controlled, double blind trial for the treatment of dry eye using the fellow eye for vehicle control resulted in 24 h of enhanced tear film thickness after a single dose, but not after repeated doses, and no significant further benefit (Schmidl et al., 2017).

Exposure of rat eyes and a human corneal epithelial cell line to particulate matter less than 2.5 μm in diameter upregulated expression of serpin family B member 2 (SERPINB2 or PAI-2), LC3B-II and BECN1 - an effect reversed by SERPINB2 shRNA knockdown (D. Lyu et al., 2020), although no inhibitor of macroautophagy was used in this study. Rat eyes were treated four times daily with particulate matter both in PBS and in a hyaluronate gel. This promoted a substantial decline in tear volume, and an increase in fluorescein corneal staining (D. Lyu et al., 2020). SERPINB2 is thought to play a role in inflammation (Brauze, 2021).

Exposing wild type and nucleotide binding oligomerization domain containing 2 (NOD2) knockout mice to 410 nm blue light for 10 days promoted enhanced LC3B-II, SQSTM1 and ATG16L1 immunoblotting of the cornea and conjunctiva, as well as corneal fluorescein staining and evidence of oxidative stress (Li et al., 2021). NOD2 is involved in adaptive and innate immunity in the intestine (Kobayashi et al., 2005). With the exception of LC3B-II, both ATG16L1 and SQSTM1 immunoblotting were somewhat less in mice lacking NOD2 (Li et al., 2021), suggesting involvement of NOD2 in the inflammatory and autophagic response, much like in the murine dessicating stress model of dry eye (Y. Li et al., 2019). The latter model involves prolonged drying of the ocular surface by treating mice with scopolamine hydrobromide to decrease parasympathetic stimulation of tearing while being housed in a dry environment with continuous fan exposure. Three hours of 250–350 nm UVB exposure of a human corneal epithelial cell line suppressed SQSTM1 but enhanced immunoblotting for LC3-II, ATG5 and BECN1 in an *miR-205-3p* inhibitable manner in part via toll like receptor 4 (TLR4) (Fu et al., 2020). Elevated *miR-205-3p* is associated with cancer (Jiang et al., 2013).

3.2.6. Wounding

Macroautophagy and corneal diabetic wound healing were noted above. Scrutiny of corneal wound models suggest the importance of macroautophagy in corneal repair. *S100 calcium binding protein A4 (S100A4)* is a calcium binding protein associated with fibrosis. ShRNA suppression of S100A4 in rabbits enhanced healing from corneal alkali burns coincident with apparently elevated macroautophagy *in vivo* (Wang et al., 2020). Augmented autophagy was implied by some slightly or modestly increased LC3B-II, BECN1 and ATG4B immunoblotting but without validating flux analyses. Enhanced LC3B-II, BECN1 and ATG4B immunoblotting was more apparent in alkali treated and shRNA transduced rabbit corneal stromal cells *in vitro*. Enhancement coincided with suppression of phosphorylated MTOR. Overexpression of S100A4 diminished detectable LC3B-II, BECN1 and ATG4B (Wang et al., 2020).

Epidermal growth factor (EGF) appeared to benefit human corneal epithelial cell culture scratch healing. Healing was characterized in part by enhanced LC3-II immunoblotting and cellular staining by a mitochondrial affinity dye (Huo et al., 2020) with fluorescence optimum at pH 4 achievable upon lysosomal fusion with autophagosome-captured mitochondria (Iwashita et al., '17). That EGF signals through beneficial ROS (D'Autréaux and Toledano, 2007) was suggested by partial or complete negation of benefit (Huo et al., 2020) by ROS suppressors *Mdivi-1* (Bordt et al., 2017) or *N-acetylcysteine*. *Clotrimazole*, often used as an inhibitor of transient receptor potential cation channel subfamily M member 2 (TRPM2), was also inhibitory in keeping with EGF enhanced TRPM2 expression (Huo et al., 2020).

4. Corneal stroma

4.1. Dystrophies

'Epithelial-Stromal TGFBI' corneal dystrophies comprise a progressive, degenerative disorder affecting the stroma and Bowman's membrane of the eye (Chao-Shern et al., 2019). Underlying this disorder are over 54 missense mutations of transforming growth factor beta induced (TGFBI; Human Gene Mutation Database), including those at R124C, R124H, R124L, P501T, R555W, R555Q (Online Medelian Inheritance in Man). TGFBI (Skonier et al., 1992) is a secreted extracellular matrix protein of the corneal epithelium, stroma and endothelium (Choi et al., 2019) as respectively the 21st, 2nd and top most abundant protein per mass spectrometry (Dyrlund et al., 2012). It features an integrin binding 'RGD' sequence and an interactome inclusive of collagens 1 (COL1A1, A2), 2 (COL2A1) and 4 (COL4A1, A2, A3, A4), as well as fibronectin 1 (FN1) and matrix metallopeptidase 9 (MMP9). Age-dependent, intracellular and extracellular progressive accumulation of mutated TGFBI as large aggregates promotes cell stress and eventual death (Choi et al., 2012) linked to impairment of corneal transparency and defective macroautophagy (Choi et al., 2019; Klionsky et al., 2021). Other mutational risk factors include that of carbohydrate sulfotransferase 6 (CHST6; Macular ['spot'] Corneal Dystrophy (Zheng et al., 2020)) and UbiA prenyltransferase domain containing 1 (UBIAD1; Schnyder Corneal Dystrophy [lipid accumulation]). Nonamyloidogenic and amyloidogenic corneal dystrophies have been described. Granular Corneal Dystrophy is nonamyloidogenic, whereas *Lattice Corneal Dystrophy* is amyloidogenic (Lakshminarayanan et al., 2015), that is extracellular deposits in the former are amorphous, whereas in the latter they are assembled as 'amyloid fibrils'.

4.1.1. Granular corneal dystrophy (GCD; Epithelial-Stromal TGFBI)

Primary corneal fibroblasts isolated from granular corneal dystrophy type 2 (GCD2) patients displayed delayed cellular transit of TGFBI R124H as compared to wild type TGFBI after treatment with cycloheximide. Accumulated TGFBI R124H was partially depleted following treatment with *rapamycin* in a *3-methyladenine* inhibitable manner, and mitochondrial profiles were short - in keeping with poor metabolic activity (Choi et al., 2012). Also, autophagic agonist *melatonin* reduced levels of accumulated TGFBI R124H by inhibiting MTOR to promote a modest increase in autophagic flux as monitored with and without

bafilomycin in a *melatonin* receptor antagonist (*luzindole*) non-inhibitable manner (Choi et al., 2013). The *Ki* of *luzindole* with 2-[^{125}I]iodomelatonin is 7.3 ± 2 nM for the melatonin receptor 1B (MTNR1B) (Dubocovich et al., 1998). The authors concluded that the activity does not directly involve this receptor (Choi et al., 2013). Combining *melatonin* (100 μM) with *rapamycin* (0.1 μM) further enhanced LC3B-II immunoblotting and MTOR inhibition (Choi et al., 2013). The mechanism was explored by adenoviral overexpression of TGFBI R124H or use of TGFBI R124H homogeneous corneal fibroblasts from GCD2 patients (Choi et al., 2012). Overexpressed TGFBI R124H substantially enhanced LC3B-II immunoblotting as compared to the overexpressed wild type TGFBI control and promoted the degradation of regulatory associated protein of MTOR complex 1 (RPTOR). Degradation of RPTOR was blocked by the proteosome inhibitor MG132 (Choi et al., 2014). Interestingly, fibroblasts isolated from GCD2 patients displayed a deficiency of active lysosomal cysteine proteases cathepsin B, K and L (respectively CTSB, CTSK and CTSL) but not CTSD downstream of insufficient TFEB. Overexpression of either CTSL or TFEB helped restore cellular homeostasis by degradation of TGFBI R124H (Choi et al., 2020).

4.1.2. Lattice Corneal Dystrophy (LCD; Epithelial-Stromal TGFBI)

Stromal TGFBI L558P deposits in Lattice Corneal Dystrophy (LCD) are commonly encircled by macrophages (Han et al., 2019). Isolated macrophages were equally capable of internalizing either recombinant TGFBI L558P or wild type TGFBI whether derived from individuals with LCD or from normal individuals. However, TGFBI L558P degradation, macrophage activation and macroautophagy were faulty, as shown by LC3B versus SQSTM1 immunoblotting with or without macroautophagic inhibitors (Han et al., 2019).

4.1.3. Schnyder Corneal Dystrophy (SCD; stromal)

UBIAD1 is a membrane-associated UbiA prenyltransferase responsible for mediating the synthesis of coenzyme Q10 and the vitamin K2 isoform MK-4 (Shearer and Okano, 2018). Accumulation of missense mutants of UBIAD1 is associated with Schnyder Corneal Dystrophy (SCD) (Orr et al., 2007). Study of SV-589 human fibroblasts lacking UBIAD1 with and without wild type or SCD mutant UBIAD1 N102S (or nineteen other SCD UBIAD1 mutants) revealed that all mutants lacked substantial enzymatic activity (Jun et al., 2020). *Compactin* and more so *bafliomycin* enhanced immunoblotting of wild type UBIAD1 and LC3-II. *Compactin* (also known as *mevastatin*) is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme-A reductase, an enzyme required for the biosynthesis of UBIAD1 side chain substrate geranylgeranyl diphosphate (Hirota et al., 2015) and of cholesterol. Geranylgeranyl diphosphate induces the Golgi transport of UBIAD1 and synthesis of MK-4 (Jun et al., 2020). Some, or substantially, more accumulation of UBIAD1 N102S over wild type UBIAD1 was apparent, as was also the case following siRNA depletion of macroautophagy mediators ATG7, ATG12, ATG16L1 and autophagy and beclin regulator 1 (AMBRA1). AMBRA1 is required for BECN1 dependent autophagy (Fimia et al., 2007). In immunofluorescent images wild type UBIAD1 colocalized for the most part with Golgi marker GOLGA2 (GM-130), but following *bafilomycin* treatment, wild type UBIAD1 instead colocalized with LC3 (likely LC3-II) (Jun et al., 2020). In contrast, UBIAD1 N102S displayed a distribution consistent with localization to the endoplasmic reticulum that was largely unaffected by *bafilomycin* treatment and did not colocalize with LC3 (likely LC3-II). Thus, whereas wild type UBIAD1 is transported to the Golgi and then into the macroautophagy pathway, SCD mutant UBIAD1 N102S (and others that are incapable of recognizing geranylgeranyl diphosphate) instead accumulate in the endoplasmic reticulum (Jun et al., 2020).

5. Corneal endothelium

Corneal endothelial cells line the inner surface of the cornea in apposition to the aqueous humor as a simple squamous sheet on Descemet's membrane. Here it pumps excess water from the stroma made available by hydrophilic glycosaminoglycans whose content is most elevated in the stromal layers adjacent to Descemet's membrane (Castoro et al., 1988). Stromal edema from inadequate pumping separates collagenous lamellae to deleteriously affect the refractive index and transparency (Maurice, 1957). It also accelerates G1-arrested endothelial cell loss (Joyce et al., 1996). Starvation induced macroautophagy in cultured primary corneal endothelial cells is coupled with loss of gap junction protein alpha 1 (GJA1, also known as connexin 43) (D'hondt et al., 2016) necessary for gap junction function. The toxic herbicide *paraquat* increased levels of LC3-II and SQSTM1 and promoted apoptosis of a corneal endothelial cell line. Co-treatment with *ascorbic acid* abrogated apoptosis, and restored LC3-II and SQSTM1 immunoblotting to control levels (Hsueh et al., 2020). Although ascorbic acid was claimed to alleviate 'autophagic flux blockage', no formal flux analysis was performed. Others have tested the antibiotic *moxifloxacin* on a human corneal endothelial cell line (Park et al., 2021). Moxifloxacin enhanced LC3A/BII immunoblotting and LC3B immunofluorescence in a dose-dependent manner that was further elevated by treatment with *chloroquine*. This is indicative of enhanced autophagic flux since *chloroquine* blocks autophagosome-lysosome fusion (Klionsky et al., 2021). *Moxifloxacin* also triggered the production of reactive oxygen species and apoptosis (Park et al., 2021).

5.1. Dystrophies

Much like stromal dystrophies, single missense mutations underlie most endothelial corneal dystrophies: 'Congenital Hereditary Endothelial Dystrophy' (CHED), 'Fuchs Endothelial Corneal Dystrophy' (FECD), 'Posterior Polymorphous Corneal Dystrophy' (PPCD), and 'X-Linked Endothelial Corneal Dystrophy' (XECD). To date, macroautophagy has been explored in CHED and FECD.

5.1.1. Congenital Hereditary Endothelial Dystrophy (CHED)

Missense/nonsense mutation of solute carrier family 4 member 11 (SLC4A11) is causative for CHED. Over 44 SLC4A11 mutations have been recorded, in keeping with SLC4A11's key role in corneal endothelial water transport (Loganathan et al., 2016). In its absence in SLC4A11 null mice corneal stromal edema is observed (Vilas et al., 2013). Also causative are at least one duplication mutation and three regulatory substitutions in ovo like zinc finger 2 (OVOL2) that trigger aberrant OVOL2 corneal endothelial expression (Davidson et al., 2016). OVOL2 represses MYC proto-oncogene, bHLH transcription factor (MYC) and notch receptor 1 (NOTCH1) in keratinocytes (Wells et al., 2009). It also is a transcriptional repressor of the zinc finger E-box binding homeobox 1 (ZEB1) promoter (Roca et al., 2013). Mutationally altered ZEB1 is associated with PPCD (Davidson et al., 2016).

Immortalized mouse corneal endothelial cells from SLC4A11 null mice displayed increased LC3B-II immunoblotting in the presence of *bafilomycin* and decreased MTOR phosphorylation (Shyam et al., 2021). Elevated GFP in cells transfected with GFP-LC3-RFP-LC3ΔG and accumulation of SQSTM1 indicated a late block of macroautophagy in keeping with lack of CTSB and the multisubunit vacuolar ATPase in both the cell line and corneal endothelial tissue. This was associated with a deficiency of CTSL activity, and of nuclear (but not cytoplasmic) TFEB. Correction was for the most part achieved by *glutamine* depletion or by addition of mitochondrial antioxidant *MitoQ* in both *in vitro* and *in vivo* (Shyam et al., 2021).

5.1.2. Fuchs Endothelial Corneal Dystrophy (FECD)

Missense/nonsense mutations causative for FECD include those in collagen type VIII alpha 2 chain (COL8A2; 5 mutations), lipoxygenase homology PLAT domains 1 (LOXHD1; 16 mutations), AGBL carboxypeptidase 1 (AGBL1; 2 mutations) and collagen type XVII alpha 1 chain (COL17A1; 1 mutation). FECD is also associated with a splicing mutation and two repeat variation mutations of transcription factor 4 (TCF4).

One group (Loganathan et al., 2016) noted involvement of SLC4A11, although not apparently listed at present in the Human Gene Mutation Database. COL8A2 resides in Descemet's membrane (Levy et al., 1996). LOXHD1 is best known as a stereociliary protein in hair cells of the ear (Grillet et al., 2009) but is expressed at very low levels in the corneal endothelium (Riazuddin et al., 2012). AGBL1 removes glutamyl groups from polyglutamylated proteins (Riazuddin et al., 2013). COL17A1 is a transmembrane protein (McGrath et al., 1995) expressed by corneal endothelial cells. SLC4A11 is a proton transporter (Zhang et al., 2015). $COL8A2^{Q455K/Q455K}$ (Jun et al., 2012) and $COL8A2^{L450W/L450W}$ (Meng et al., 2013) transgenic mice mirror human FECD. Corneal endothelial cell density and morphological defects observed in two month old $COL8A2^{Q455K/Q455K}$ mice were partially rescued by feeding *lithium carbonate*-enriched chow for seven months to stimulate macroautophagy. Evidence of the latter was suggested by slightly increased detection of ATG5-ATG12 conjugate, although SQSTM1 mRNA was elevated (Kim et al., 2013). ATG5-ATG12 conjugate immunoblotting was also slightly less in $COL8A2^{L450W/L450W}$ corneal endothelium, but LC3A/B-II was not detected (Jun et al., 2012). CRISPR/Cas9 may be a future treatment approach. Decreased expression of $COL8A2^{Q455K/Q455K}$ protein in mutant mice by CRISPR/Cas9 gene editing partially reduced endothelial cell loss (Uehara et al., 2021).

To explore why mutations in TCF4 may be causative (Hwang et al., 2020), primary human corneal endothelial cells were subjected to TCF4 siRNA knockdown or to overexpression with a transcriptional activator peptide coupled with dead Cas9. With knockdown, LC3-II and caspase 9 immunoblotting were greater and viability less (Hwang et al., 2020), but autophagic flux was not addressed. FECD cell lines displayed less MitoTracker Green FM detectable mitochondrial mass than normal corneal endothelial cells, as do normal cells treated with *carbonyl cyanide m-chlorophenyl hydrazone (CCCP)* (Benischke et al., 2017). *CCCP* is a known uncoupler of oxidative phosphorylation, as well as a *bafilomycin* blockable inducer of mitophagy. LC3A/B-II immunoblotting was elevated in most FECD corneal endothelial specimens and somewhat more in a human FECD cell line as compared to normal corneal endothelial cells. *Bafilomycin* and *CCCP*, both alone and together, increased LC3A/B-II immunoblotting or LC3 immunofluorescence, the latter on cell lines and on *ex vivo* normal and FECD corneal specimens. In keeping with reduced mitochondrial mass, mitofusin 2 (MFN2) involved in mitochondrial fusion was deficient in a FECD specimen and in FECD cell lines compared to a normal specimen and normal cell line, a deficit increased by FECD cell line exposure to *CCCP*. Partial rescue was achieved by combined *CCCP* and *bafilomycin* treatment that at 20 h increased the colocalization of MFN2 and LC3 (Benischke et al., 2017). Evidence for deficiency of dyamin 1 like (DNM1L, also known as Drp 1), although a regulator of mitochondrial fission, suggested involvement of mitophagy (Miyai et al., 2019). Introduction of the ROS generator *menadione* to normal human corneal endothelial cells mimicked FEDC-triggered mitochondrial fragmentation and mitophagy. This was indicated by: (i) LC3A/B-II immunoblotting of a mitochondrial-enriched fraction that exceeded the cytsolic fraction, (ii) time-dependent reduction in DNM1L, PRKN, and PINK1, and (iii) increased phosphorylation of PRKN. After treatment instead with *CCCP*, PRKN decreased but neither DNM1L nor PINK1 were apparently affected. Inclusion of the proteosome inhibitor *epoxomicin* (Meng et al., 1999), but not *bafilomycin*, in FECD cells blocked *CCCP*-dependent loss of PRKN, whereas *bafilomycin* blocked the loss of PRKN in normal cells treated with *menadione* - the latter in keeping with involvement of mitophagy (Miyai et al., 2019).

6. Conclusions

Macroautophagic mechanisms are powerful and essential guardians of corneal homeostasis - a homeostasis that is remarkably resilient, likely in keeping with the cornea's exposure to the external environment. Nonetheless, macroautophagic problems arise affecting the visual acuity of many patients. In the corneal epithelium, macroautophagic problems are manifested in ocular surface disease, keratoconus, microbial and viral infection, and reaction to cigarette smoke and other environmental particulate matter. Additionally, corneal dystrophies prevail when macroautophagic machinery is overwhelmed by cellular or stromal accumulation of aggregated mutated proteins or by deficiency of lysosomal enzymes. Such problems offer a rich opportunity to further explore the fundamental cell biology of macroautophagy - both known and unknown, and to pursue unbiased screens for natural transient stimulators of corneal macroautophagy that might have disease benefits. This approach offers an opportunity for success that may be tissue and autophagy selective, and well-tolerated yet has been rarely applied. Candidate testing could also be productive but has been limited to date and with mixed results. Accordingly, with the apparent exception of *lacritin* (fruit of an unbiased screen), drugging corneal macroautophagy has received remarkably little attention. No ocular hits arise from 'Autophagy' and 'Cornea' keyword search of ClinicalTrials.gov, despite the excellent accessibility of the cornea for drug delivery - and the investment community's expressed interest in 'disruptive technologies'. Here there is opportunity to generate novel topical therapies towards the restoration of homeostasis with potential broad application.

Declaration of competing interest

None for KLD-T, MSG, JR, FN. GWL is cofounder and CSO of TearSolutions, Inc. that has provided an unrestricted gift to UVA's Department of Cell Biology for use by GWL's lab. GWL is also an inventor on several issued and pending patents owned by UVA.

Data availability

No data was used for the research described in the article.

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