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Clusterin in the eye: an old dog with new tricks at the ocular surface

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Abstract

The multifunctional protein clusterin (CLU) was first described in 1983 as a secreted glycoprotein present in ram rete testis fluid that enhanced aggregation ('clustering') of a variety of cells in vitro. It was also independently discovered in a number of other systems. By the early 1990s, CLU was known under many names and its expression had been demonstrated throughout the body, including in the eye. Its homeostatic activities in proteostasis, cytoprotection, and anti-inflammation have been well documented, however its roles in health and disease are still not well understood. CLU is prominent at fluid-tissue interfaces, and in 1996 it was demonstrated to be the most highly expressed transcript in the human cornea, the protein product being localized to the apical layers of the mucosal epithelia of the cornea and conjunctiva. CLU protein is also present in human tears. Using a preclinical mouse model for desiccating stress that mimics human dry eye disease, the authors recently demonstrated that CLU prevents and ameliorates ocular surface barrier disruption by a remarkable sealing mechanism dependent on attainment of a critical all-or-none concentration in the tears. When the CLU level drops below the critical all-or-none threshold, the barrier becomes vulnerable to desiccating stress. CLU binds selectively to the ocular surface subjected to desiccating stress in vivo, and in vitro to LGALS3 (galectin-3), a key barrier component. Positioned in this way, CLU not only physically seals the ocular surface barrier, but it also protects the barrier cells and prevents further damage to barrier structure. CLU depletion from the ocular surface epithelia is seen in a variety of inflammatory conditions in humans and mice that lead to squamous metaplasia and a keratinized epithelium. This suggests that CLU might have a specific role in maintaining mucosal epithelial differentiation, an idea that can now be tested using the mouse model for desiccating stress. Most excitingly, the new findings suggest that CLU could serve as a novel biotherapeutic for dry eye disease.

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Clusterin in the Eye: An Old Dog with New Tricks at the Ocular Surface

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Abstract

The multifunctional protein clusterin (CLU) was first described in 1983 as a secreted glycoprotein present in ram rete testis fluid that enhanced aggregation ('clustering') of a variety of cells *in vitro*. It was also independently discovered in a number of other systems. By the early 1990s, CLU was known under many names and its expression had been demonstrated throughout the body, including in the eye. Its homeostatic activities in proteostasis, cytoprotection, and anti-inflammation have been well documented, however its roles in health and disease are still not well understood. CLU is prominent at fluid-tissue interfaces, and in 1996 it was demonstrated to be the most highly expressed transcript in the human cornea, the protein product being localized to the apical layers of the mucosal epithelia of the cornea and conjunctiva. CLU protein is also present in human tears. Using a preclinical mouse model for desiccating stress that mimics human dry eye disease, the authors recently demonstrated that CLU prevents and ameliorates ocular surface barrier disruption by a remarkable sealing mechanism dependent on attainment of a critical all-or-none concentration in the tears. When the CLU level drops below the critical all-or-none threshold, the barrier becomes vulnerable to desiccating stress. CLU binds selectively to the ocular surface subjected to desiccating stress *in vivo*, and *in vitro* to LGALS3 (galectin-3), a key barrier component. Positioned in this way, CLU not only physically seals the ocular surface barrier, but it also protects the barrier cells and prevents further damage to barrier structure. CLU depletion from the ocular surface epithelia is seen in a variety of inflammatory conditions in humans and mice that lead to squamous metaplasia and a keratinized epithelium. This suggests that CLU might have a specific role in maintaining mucosal epithelial differentiation, an idea that can now be tested using the mouse model for desiccating stress. Most excitingly, the new findings suggest that CLU could serve as a novel biotherapeutic for dry eye disease.

Highlights

- The multifunctional protein clusterin, first described in 1983, is expressed throughout the body, including in the eye. Its homeostatic activities in proteostasis, cytoprotection, and anti-inflammation have been well documented, however its roles in health and disease are still not well understood.
- CLU is especially prominent at fluid-tissue interfaces. CLU was demonstrated to be the most highly expressed transcript in the human cornea, the protein being localized to the apical layers of the mucosal epithelium. CLU protein is also present in human tears.
- Using a preclinical mouse model for desiccating stress that mimics human dry eye disease, the authors recently demonstrated that CLU prevents and ameliorates ocular surface barrier disruption by a remarkable sealing mechanism dependent on attainment of a critical all-or-none concentration in the tears.
- CLU depletion from the ocular surface epithelia is seen in inflammatory diseases that lead to squamous metaplasia, suggesting that CLU might have a specific role in maintaining mucosal epithelial differentiation. Because new mouse models have been developed, this idea can now be tested.
- The new findings suggest that CLU could serve as a novel biotherapeutic for dry eye.

Key Words

Clusterin, Chaperone, Ocular Surface, Cornea, Proteostasis, Cytoprotection, Inflammation, Dry Eye, Epithelial Barrier, Mucosal Epithelium, Squamous Metaplasia, Biotherapeutic

Abbreviations

HUGO nomenclature is used in this article for genes and their protein products. Non-standard abbreviations: FDA: U.S. Food & Drug Administration; FECD: Fuchs' Endothelial Corneal

Dystrophy; HDL: high-density lipoprotein; PXG: pseudoexfoliation glaucoma; RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

1. Introduction

The multi-functional protein clusterin was first described in 1983 as a secreted glycoprotein present in ram rete testis fluid that enhanced aggregation ('clustering') of a variety of cells *in vitro* [1, 2]. The protein was subsequently re-identified in a number of other studies and was given different names based on the activity investigated. Clusterin is identical to serum protein 40,40 (SP-40,40) found in the SC5b-complex of complement and in immune deposits in glomerulonephritis [3, 4]. It is also the same as Apolipoprotein J (ApoJ), a protein associated with high-density lipoprotein and very high-density lipoprotein in human serum [5, 6], as well as sulfated glycoprotein-2 (SGP-2), the major secreted product of rat Sertoli cells [4], and the protein translated from testosterone-repressed prostate message-2 (TRPM-2), which is upregulated in the regressing rat ventral prostate [7]. Participants in the inaugural International Workshop on Clusterin held in Cambridge, England in 1992 agreed to the name clusterin, acknowledging the original reports of its identification [8]. The HUGO nomenclature committee has given clusterin the designation "CLU".

CLU is nearly ubiquitously expressed in tissues, and is constitutively present in most biological fluids [9]. The first publication on CLU in the eye was in 1992, describing elevated CLU expression in the degenerative disorder, retinitis pigmentosa [10]. CLU expression in various parts of the eye was subsequently documented in developmental studies in rats [11] and mice [12], including in the lens, cornea and ciliary body, and CLU protein was demonstrated in the aqueous and vitreous of the mature human eye [12]. A number of studies at that time investigated a role for CLU in retinal degenerative disease. In 1996, a DNA sequencing study was published highlighting CLU as the most highly expressed gene in the adult human corneal epithelium [13], sparking interest in examining the role of CLU at the ocular surface, as discussed below. The most recent study of expression demonstrated CLU mRNA in adult

human and monkey eyes localized to the lens, cornea, limbus, sclera, orbital muscle, ciliary body, retina, and retinal pigment epithelium /choroid, as well as to retinal pigment epithelial cells in culture [14].

When we began to write this article, we performed a search of PubMed using the term “clusterin”, and turned up more than 2,000 articles. Despite all this research, new knowledge continues to emerge. We refer the reader to the numerous excellent review and perspective articles on CLU, a selection of which are listed here [8, 15-22]. The current article provides a brief overview of the history and current knowledge on CLU. It then offers an updated review and perspective on the physiologic role of CLU in the eye, including some new insight from our group on its role at the ocular surface [23, 24].

2. Gene and Protein Structure

In humans, a single *CLU* gene of nine exons is located on chromosome 8. The sequence is highly conserved across species, showing 70–80% identity at the amino acid level amongst mammals [20]. Transcription results in an mRNA of ~2-kb, from which is produced a primary polypeptide chain of 449 amino acids. Figure 1 is a schematic of the CLU molecule based on information deduced from sequence analysis and biochemical studies. An N-terminal signal peptide of 22 amino acids is removed in the endoplasmic reticulum to produce a protein with a predicted mass of ~50 kDa. Subsequently, CLU is proteolytically cleaved to form two anti-parallel polypeptide chains of similar size connected at a central core by 5 disulfide bonds. Six predicted N-linked glycosylation sites clustered around the disulfide-bonded core were confirmed by mass spectroscopy [25]. This results in a secreted glycoprotein with an apparent mass of 75–80 kDa by SDS-PAGE, although the actual mass is approximately 58–63 kDa, which is 17–27% carbohydrate by weight. Other N-terminally truncated clusterin isoforms have

been proposed, including one thought to localize to the nucleus (e.g., [26, 27]), however unequivocal identification of any of these in cells has yet to be achieved.

Sequence analysis of the CLU mRNA predicts that the glycosylated, disulfide-bonded core of the encoded protein is flanked by five amphipathic α-helices [28]. The result is a four armed molecule with regions of native disorder, resulting in a dynamic, molten globule-like structure with the capacity to bind a variety of different molecules [28]. This includes hydrophobic regions exposed on denatured proteins, important for CLU function as a chaperone [28, 29]. CLU also binds a number of specific proteins, including the SC5b-9 complex of complement and immunoglobulins [8]. There have been no crystal structure determinations for CLU, and only limited analyses by mass spectrometry [25, 30] and nuclear magnetic resonance [31].

3. Biochemical Activities and Roles in Health and Disease

3.1. Complement Inhibition

Characterized as SP-40,40, CLU was identified in glomerular immune deposits as part of the membrane attack complex of complement [3]. Purified CLU was shown to inhibit C5b-6-initiated hemolysis in a dose-dependent manner [32] by binding to complement component SC5b-9 [33]. The idea that CLU is a physiological inhibitor of complement-mediated cytolysis was tested using erythrocytes and cells stably transfected with a membrane-anchored form of CLU as targets for complement-mediated cytolysis [34]. CLU gave dose-dependent protection of antibody-coated sheep erythrocytes against complement-mediated lysis by diluted normal human serum, however extrapolation to undiluted serum showed that a CLU concentration at least two orders of magnitude greater than its physiological concentration would be needed to confer protection in the circulation [34]. Once deposited in tissues however, the effective

concentration of CLU may be much higher. The physiologic significance of complement inhibition by CLU remains to be established.

3.2. Lipid Transport

Characterized as apolipoprotein J [35, 36], CLU was found to exist in human plasma, associated with high-density lipoproteins (HDL), and specifically with subclasses of HDL that also contain APOA1 (apolipoprotein A1) and CETP (cholesteryl ester transfer protein) activity. CLU is also associated with HDL in cerebrospinal fluid [37]. The major physiological role postulated for HDL is to mediate reverse cholesterol transport, a process in which excess cholesterol is removed from peripheral cells and returned to the liver for eventual excretion as bile acids [38, 39]. Like APOA1, CLU was found to promote cholesterol efflux from cells *in vitro* [40], although it remains to be shown whether this is an important role for CLU *in vivo*. More recent studies suggest that CLU is important for stabilizing APOA1, PON1 (paroxonase 1) and other proteins in the HDL (see discussion of chaperone function below), thus maintaining their anti-atherogenic properties [41, 42]. CLU is also a component of low density lipoproteins [43].

CLU protein is not found in the normal aorta, but it is distributed in the intima and media of aortas with diffuse, intimal thickening or atherosclerotic lesions [44]. CLU expression is upregulated after vascular injury and appears to prevent endothelial cell activation and limit the proinflammatory response in atherosclerosis [45]. Apolipoprotein mimetic peptides, designed around the sequence of the amphipathic helices, dramatically reduce atherosclerosis in animal models and may provide therapeutic value in a variety of human vascular inflammatory conditions [46]. An orally-delivered amphipathic helix peptide based on CLU reduced atherosclerosis in APOE-null mice [47].

3.3. Anti-Apoptosis and Cell Survival

A notable property of CLU is its induction during programmed cell death in a variety of different tissues. Testosterone-repressed prostate message-2 [7] and sulfated glycoprotein-2 mRNA [48] were independently cloned from the prostate undergoing involution following castration by two different groups. Sequence analysis showed they were identical to one another and to CLU. Later studies identified CLU induction in many other organ systems undergoing massive apoptosis (e.g., [49]), leading to the general idea that CLU might play a causative role in programmed cell death. However, this concept was ultimately reversed by the finding that overexpression of CLU conferred resistance to TNFA-induced apoptosis in human prostate cancer cell cultures [50]. Conversely, CLU knockdown resulted in a significant reduction of cellular growth and higher rates of spontaneous apoptosis [51]. These experiments mimic natural changes in CLU levels; expression is low in most unstressed cells, but is stimulated by different stressful conditions and agents [52, 53].

Secreted CLU may protect cells from undergoing apoptosis in several ways. First, extracellular CLU is cytoprotective in its role as a molecular chaperone, as discussed above. CLU can also protect directly against apoptosis. In one mechanism described, this begins by binding to cell surface receptors of the low-density lipoprotein family such as LRP2 (megalin) [54], LPR8, or VLDLR [55]. Binding of CLU to LRP2 induces activation of AKT, promoting cell survival [55]. It should be noted that, while a large number of studies have reported that CLU confers protection against apoptosis, some studies report the opposite [56]. The molecular basis of this apparent conflict remains to be resolved.

The anti-apoptotic activity of CLU has been well studied in connection with resistance to chemotherapeutics in cancer [17, 57-59]. Custirsen (OGX-011/TV-1011; OncoGeneX Pharmaceuticals, Inc., Bothell, WA, USA) is a second-generation antisense oligonucleotide that

reduces the production of secreted CLU [60]. Custirsen was developed to bind CLU mRNA and reduce CLU protein expression as a strategy for treatment resistance in various cancer types. A 2'-O-methoxyethyl (2'-MOE) modification enhances binding to the target mRNA and resistance to nucleolytic degradation, thus prolonging tissue half-life and reducing dose frequency when compared with the first-generation antisense oligonucleotide. The drug is currently under investigation in patients with solid tumors treated by chemotherapy [60, 61].

3.4. Chaperone Activity and Proteinase Inhibition

Proteostasis describes the maintenance of the individual proteins of the proteome in the conformation, concentration, and location required for their correct function. Chaperones are involved in controlling the movement of intractably misfolded proteins toward the intracellular degradation machinery and some are also involved in refolding misfolded proteins. Most of the current information on the function of chaperones relates to those found inside cells, with the superfamily of heat shock and related proteins being a well-known example. CLU was the first of the extracellular chaperones to be identified [9]. It was characterized as a potent small heat shock protein-like chaperone that inhibits stress-induced amorphous protein aggregation and the fibrillar aggregation of many amyloidogenic proteins and peptides. CLU forms high-molecular-weight ‘solubilized’ complexes with heat- or reduction-stressed proteins, inhibiting their precipitation [62]. CLU can stabilize stressed proteins but, like small heat shock proteins, cannot catalyze protein refolding. However, on a molar basis, CLU is considerably more potent than small heat shock proteins at inhibiting stress-induced protein precipitation [63]. The structural elements responsible for the chaperone activity of CLU are not yet known, but the ability to bind to misfolded proteins is thought to relate to surface hydrophobicity, which is enhanced by acidic pH (49). The chaperone activity of CLU is ATP independent and, in the case of amorphously aggregating clients, results in the formation of soluble, high molecular mass complexes ≥40,000 kDa (57).

The physiologic significance of CLU's chaperone function is demonstrated by the observation that immunodepletion from human blood plasma renders plasma proteins susceptible to stress-induced precipitation [64]. CLU knockout mice have increased tissue damage after heat shock [65], myosin-induced autoimmune myocarditis [66], or post-ischemic brain injury [67]. Aging CLU knockout mice develop protein deposits in the kidney and glomerular neuropathy, which directly implicates CLU in the clearance of misfolded proteins [68].

Many age-related, inherited, systemic and neurological disorders are characterized by the deposition of highly structured protein aggregates known as amyloid or amyloid-like fibrils. This includes Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Creutzfeldt-Jakob disease, Down's syndrome and atherosclerosis [69]. Aggregates can be located intra- or extracellularly, exerting pathogenic effects by organ disruption or cytotoxicity. Intracellular amyloid aggregates are found co-localized with components of the intracellular protein quality control system [70]. In a striking parallel, all disease-associated insoluble extracellular protein deposits tested, including those characterized as amyloid, co-localize with CLU [71]. Evidence has been presented that, when present at low concentrations, CLU incorporates into amyloid deposits, perhaps in an aborted attempt to fulfill its role as an extracellular chaperone. However, if CLU attains a critical concentration threshold, it potently inhibits amyloid formation and provides substantial cytoprotection [71].

Based on these and other findings, it has been proposed that CLU forms part of an extracellular protein quality control system that helps to maintain proteostasis [72]. The CLU gene has been identified as an important risk locus for Alzheimer's disease. Functional analyses suggest reduced secretion of the CLU protein as the mode of action for three CLU coding mutations [73]. CLU concentration in cerebrospinal fluid is low compared to other bodily fluids, suggesting

protective activity could be easily overwhelmed and that supplementation might be of therapeutic value in Alzheimer's disease [73].

Chaperoning is one way to maintain proteostasis; another way is by inhibition of proteolysis. Two recently published studies made the unexpected new finding that CLU is a potent inhibitor of Matrix Metalloproteinase (MMP) activity.

In the first study [74], a Madin-Darby canine kidney (MDCK) cell line was created, stably expressing a soluble form of MMP25, a neutrophil-specific enzyme that normally is tethered to the cell surface via a covalent glycosylphosphatidylinositol link. When the resulting soluble MMP25 was isolated, it was found to be in complex with CLU. Soluble MMP25 was enzymatically inactive in the complex. Moreover, the activity of purified soluble MMP25 was inhibited by addition of CLU. This activity was specific, as CLU had no effect on MMP2 or soluble MMP14.

In the second study [23], a yeast-two hybrid screen, using MMP9 as bait, identified CLU. CLU was found to bind very strongly to the truncated form of MMP-9 lacking the pro-domain, with an affinity constant of 2.63 nmol/L. CLU had an even higher affinity for pro-MMP9 than this activated form of MMP9. CLU inhibited the enzymatic activity of MMP9, comparing quite favorably to inhibition by the synthetic small molecule inhibitor SB-3CT. In this study, CLU also was found to inhibit enzymatic activity of MMP2, as well as MMP3, and to a lesser extent, MMP7. Physiologic relevance was demonstrated by showing that CLU inhibited MMP9-mediated dissolution of tight junctions in human epithelial cell cultures.

The mechanism of CLU inhibition of MMP activity remains to be investigated. Intriguingly, another extracellular chaperone, A2M (α-2-macroglobulin)[75], is also a broad-spectrum

proteinase inhibitor, with well-known action against MMPs [76]. It might be speculated that proteinase inhibition is part of an anti-inflammatory suite of activities shared by at least some of the extracellular chaperones.

4. CLU in the Eye

4.1. Retinal Degeneration

The neurodegenerative disease retinitis pigmentosa (RP) served as a model for investigating CLU's role in apoptosis [10]. CLU mRNA was localized to the retinal pigment epithelium cells, photoreceptor inner segments, inner nuclear layer, and ganglion cell layer of normal retina. Differential hybridization screening of a retinal cDNA library revealed an increase in CLU expression in diseased retina. A subsequent study localized CLU expression to apoptotic photoreceptors in RP [77]. An increase in CLU expression was also seen in light-induced retinal damage in rats [78]. Improper photoreceptor development in the vitiligo mutant mouse was accompanied by increased expression of CLU mRNA in the retinal pigment epithelium [79]. In the retinal degeneration slow mutant mouse, over-expression of CLU co-localized with apoptotic nuclei [80, 81]. The pattern of apoptotic nuclear labeling was examined in a rat model of light-induced retinal degeneration. In control retinal sections, CLU expression decreased in photoreceptors and retinal pigment epithelium cells, which progressively degenerated, and increased in the preserved inner nuclear layer, in proportion to the duration of light exposure in both cyclic light- and dark-reared animals [82]. These studies linked CLU to apoptosis, but did not establish whether its role was causal or protective.

To address the question of CLU's role in apoptosis directly, transgenic mice were generated in which a rat CLU transgene was expressed in photoreceptor cells under the transcriptional control of the human IRBP (interphotoreceptor binding protein) promoter. A reduction in

apoptotic staining in the transgenic retinas was observed from birth to postnatal day 15. These results suggested that CLU is not causally involved in photoreceptor cell death, but appeared instead to be cytoprotective [83], as discussed above for other tissues and cancers. CLU expressed by retinal Muller cells was shown to be assembled into lipoprotein particles [84]. A recent study provided evidence that CLU protein protects retinal pigment epithelial cells against oxidative stress [85].

4.2. Eye Diseases of Protein and Lipid Deposition

4.2.1. Age-related macular degeneration – This disease is characterized in its early stages by the presence of “drusen”, i.e., extracellular deposits that accumulate between the basal surface of the retinal pigment epithelium and Bruch's membrane, an extracellular matrix complex that separates the neural retina from the capillary network in the choroid. Drusen are regarded as hallmarks of underlying degeneration. They are comprised of carbohydrates, zinc, and proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease, and include CLU and other apolipoproteins, as well as complement components [86-93]. DNA sequence variants in several complement proteins found in drusen are associated with increased disease risk [94-97], but no variants in CLU have yet been associated with disease.

4.2.2. Pseudoexfoliation glaucoma – Pseudoexfoliation syndrome is a systemic condition with eye manifestations. Pseudoexfoliation material, when deposited on various structures of the anterior segment, causes pseudoexfoliation glaucoma (PXG), the most common cause of secondary open-angle glaucoma worldwide [98]. CLU is a component of pseudoexfoliation deposits [99-101], and a deficiency of CLU has been suggested as a factor in accumulation of deposits [102], which appears to lead to complement activation [103]. Variants of LOXL1, an enzyme involved in cross-linking elastin fibers, are highly associated with PXG in most

populations. Two SNPs in LOXL1 confer a higher than 99% population attributable risk for PXG in the Nordic population, however, they carry different risks in other populations. Common CLU variants may contribute to modest PXG risk but larger datasets are required to confirm these findings [104].

4.2.3. Corneal dystrophies – This is a group of inherited disorders characterized by deposition of insoluble protein material in the form of extracellular deposits or intracellular cysts. The deposits are localized to various layers of the cornea depending on the gene involved and its specific mutation, and they affect corneal transparency and visual acuity. CLU has been found co-localized in deposits of two types of superficial and stromal corneal dystrophies: the TGFBI-linked corneal dystrophies [105, 106] and the lattice type I corneal dystrophy linked to mutations in the gene for TACSTD2 (Tumor-Associated Calcium Signal Transducer 2) [107]. In addition, CLU is markedly elevated in Fuchs' Endothelial Corneal Dystrophy (FECD), the most common cause of corneal endothelial dysfunction [108-110]. The disease is characterized by accumulation of extracellular collagenous deposits called "guttae" posterior to Descemet's membrane, the specialized extracellular matrix that backs the corneal endothelium [111]. Early-onset FECD has been linked genetically to a mutation in the COL8A2 (α2 chain of collagen VIII) gene encoding a component of Descemet's membrane [112]. Polymorphisms in the CLU gene have been associated with late-onset FECD [113, 114]. CLU expression was demonstrated in human corneal endothelium by both PCR and immunohistochemistry [115] and CLU has a protective effect against oxidative stress-induced cell death in these cells [116].

4.3. Proliferative Disorders

Two studies suggest that CLU promotes proliferative disorders in the eye. Pterygium, also known as "surfer's eye" or "farmer's eye", is a benign growth of the conjunctiva associated with exposure to sunlight. CLU is one of the more highly expressed genes in pterygium [117]. CLU is

also highly expressed in retinoblastoma, a childhood cancer that begins in the retina [118]. As with other cancers, apoptosis of retinoblastoma cell death due to treatment with cisplatin was prevented by co-treatment with, or over-expression of CLU. Targeting CLU in both of these lesions using antisense agents could provide therapeutic value.

4.4. Stem Cell Expansion and Transplantation

For many years, corneal epithelial stem cells isolated from the limbal niche located between cornea and sclera have been used for ocular surface reconstruction. Originally these cells were isolated and expanded on feeder layers of mouse 3T3 fibroblasts [119]. In a recent study, CLU was overexpressed in 3T3 cells by transfection of a vector encoding full-length CLU. The colony forming efficiency of corneal limbal epithelial stem cells was significantly enhanced by growth on the CLU transfected cell feeder layer. Expression of transfected CLU stimulated production of the growth-promoting cytokine, hepatocyte growth factor, by the feeder cells [120].

Another way to isolate stem cells is by identifying those that exclude the DNA-binding dye Hoechst 33342 by fluorescence-activated cell sorting, i.e., the “side population”. Side population cells isolated from mouse lacrimal and salivary glands were transplanted into the glands of mice made hypo-functional by irradiation. The secretions from both glands in the recipient mice were restored within 2 months of transplantation, although the transplanted cells did not appear to expand. Side population cells isolated from salivary glands of CLU knockout mice had no therapeutic potential, whereas lentiviral transduction of CLU restored function. CLU directly inhibited oxidative stress and oxidative stress-induced cell damage in these cells [121].

4.5. Retinal Vascular Barrier Function

Breakdown of the blood-retinal barrier occurs following retinal ischemia. CLU expression increased when human retinal endothelial cells were exposed to oxygen-glucose deprivation, whereas tight junction proteins OCLN and ZO1 markedly decreased. Tight junction proteins were restored by CLU treatment [122]. CLU also effectively inhibited vascular endothelial growth factor-induced hyperpermeability in advanced glycation end product-treated human retinal microvascular endothelial cells and in the retinas of mice with streptozotocin-induced diabetes [123]. Again, the antipermeability activity of CLU was related to the restoration of tight junction proteins. Thus CLU may have therapeutic potential in the treatment of diabetic blood retinal barrier breakdown.

5. CLU at the Ocular Surface

5.1. Ocular Surface Barrier Function in Dry Eye Disease

In an early study to characterize CLU, *In situ* hybridization analysis was performed in mouse embryos and adult tissues. This revealed a striking level of expression in epithelial and secretory cells from a broad range of tissues that form the cellular interface with fluid compartments, as well as several non-epithelial secretory cell types that line fluid compartments, including synovial lining cells and ovarian granulosa cells [124]. The results suggested that localized CLU synthesis serves a general role in protection of secretory, mucosal, and other barrier cells from the extracellular environment.

The ocular surface barrier is comprised of such mucosal epithelia. The molecular structure has been described in several publications (e.g., [125]). Membrane-associated mucins emanating from the microplicae (finger-like membrane folds) on the apical layer of epithelial cells, project into the tear film [126]. Their glycan groups bind multiple oligomers of the network-forming

galectin, LGALS3 (galectin-3), creating the transcellular barrier; tight junctions composed of OCLN (occludin), ZO1 (zonula occludens-1), and other molecules, seal the space between adjacent cells, creating the paracellular barrier. The barriers are functionally linked via the cytoskeleton [127]. Barrier disruption is assessed clinically by measuring intracellular uptake of water-soluble dyes such as rose-bengal, lissamine green or fluorescein [128, 129]. The normal ocular surface exhibits low, variable levels of dye uptake, which occurs in a distinctive punctate pattern, possibly reflecting cellular desquamation and shedding of mucin ectodomains [129-131]. Higher levels of dye uptake in the same punctate pattern are associated with dry eye syndrome [129, 132, 133].

Dry eye syndrome is a common affliction that affects 5% to 34% of all people globally, and prevalence increases with age [134]. The disease is caused by inadequate hydration and lubrication of the ocular surface, which can be brought on by a variety of factors. Symptoms include pain, burning, itching, redness, sensitivity to light and other discomfort. If left untreated, severe cases may result in vision loss due to corneal scarring. In all forms of dry eye, reduced tear flow and/or increased evaporation leads to tear hyperosmolarity. This initiates the vicious circle of dry eye pathology in a final common pathway. Hyperosmolarity induces inflammatory cascade activation [135-137], increases apoptosis [138-140], and stimulates expression and activity of MMPs [141, 142], causing ocular surface barrier disruption [143, 144]. In severe cases, dry eye also leads to squamous metaplasia involving ocular surface epithelial cell transdifferentiation from a wet mucosal phenotype to a keratinized skin-like phenotype [145].

As noted above, a DNA sequencing study published in 1996 identified CLU as the most highly expressed gene in the human corneal epithelium [13]. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) demonstrated CLU mRNA in both corneal and conjunctival epithelial cells of the ocular surface [13, 146, 147]. In situ hybridization revealed CLU mRNA in all layers

of the corneal epithelium, but most prominently in the basal cells. In contrast, immunohistochemical analysis revealed positive immunostaining for CLU protein only in the apical cell layers of the ocular surface epithelia, suggesting translational regulation [13, 146, 147]. CLU mRNA is also abundant in the human lacrimal glands [148, 149], meibomian glands [149] and accessory lacrimal glands of Wolfring [150] and mass spectrometric analyses have demonstrated CLU protein in human tears [151-164]. This localization pattern is consistent with the notion of a protective role for CLU at the fluid-tissue interface of the ocular surface epithelia.

Use of mice for experimental disease models affords the opportunity to take a genetic approach to identify causal factors through gene knockout technology. One of the first mouse models for dry eye applied an air-draft plus scopolamine protocol to create desiccating conditions at the ocular surface [165]. In this procedure, desiccating stress is created through the use of blowers to cause tear film evaporation. Pharmacologic inhibition of tear secretion with the anti-cholinergic agent scopolamine is used to further decrease tear production and clearance. A simple fluorometric assay was developed to quantify fluorescein dye uptake, representing an advantage over grading scales used to evaluate fluorescein uptake as a measure of ocular surface barrier disruption in humans.

A search for possible causal mediators of barrier disruption identified an increase in MMP9 protein in the tears and at the ocular surface subjected to desiccating stress [166]. Elevated gelatinolytic activity was detected within the ocular surface epithelia by in situ zymography [132]. MMP9 levels in human tears correlated with dry eye signs [167]. As noted above, MMP9 is a marker of inflammatory cascade activation. To test for MMP9 causality, we took a genetic approach using the then new MMP9 knockout mouse. It was found that loss of MMP9 activity completely protects ocular surface barrier function against desiccating stress [144]. Importantly, topical addition of MMP9 protein to the ocular surface of MMP9 knockout mice “rescued” the dry

eye phenotype. In other words, barrier disruption due to desiccating stress returned when MMP9 was added, meaning that MMP9 activity is necessary for barrier disruption.

How does MMP9 compromise barrier function? Various proteinases, including MMP9, catalyze cleavage of transcellular barrier components, including LGALS3 [168-170] and MUC16 (a membrane-associated mucin) [171], as well as paracellular barrier components, including ZO1 [172] and OCLN [173]. We found that barrier disruption under conditions of desiccating stress was associated with an increase in the cleaved form of OCLN, as well as a loss of OCLN at cell-cell borders [144]. Loss of MMP9 activity in MMP9 knockout mice protected against this [144]. MMP9 proteolysis also controls activity of cytokines, thus modulating leukocyte migration and inflammation [174]. Knockout mouse studies performed in our lab demonstrated that MMP9 modulates ocular surface activity of inflammatory signaling pathways by its effects on interleukin-1 isoforms and transforming growth factor-β isoforms [175]. MMP9 might also cause any of the barrier protein cleavages indirectly, for example by cleaving and activating other proteinases [176]. Thus, MMP9 is likely to have a cascading action in disruption of the ocular surface barrier subjected to desiccating stress.

An early study using *in situ* hybridization [12] demonstrated CLU mRNA in the ocular surface epithelia of mouse embryos, but the adult conjunctival epithelium appeared negative. However, RT-PCR demonstrated CLU mRNA in mouse epithelial cells cultured from adult corneas [147]. Moreover, in a gene expression microarray analysis of normal and healing mouse corneal epithelium, CLU was identified as one of the more highly expressed genes, upregulated 1.8-fold in the repairing epithelium [177]. Most recently, analysis of corneal sections from mice revealed immunoreactive CLU protein within the apical layers of the ocular surface epithelia in the same location as seen in humans, and RT-PCR demonstrated the presence of CLU mRNA [23]. A more recent study demonstrated immunoreactive CLU protein in cells of the mouse lacrimal

gland [121]. CLU was most recently identified and quantified in mouse tears by enzyme-linked immunosorbent assay [24]. These studies support the mouse as a valid model for study of CLU's role at the ocular surface.

We hypothesized that the desiccating stress of dry eye might overwhelm the protective capacity of CLU at the ocular surface. If this was the case, treatment with CLU topically might restore protection. In a recently published study, we used the mouse air-draft-plus-scopolamine model described above to test this idea [24]. In a series of experiments, we applied the desiccating stress protocol treated topically with CLU, and quantified the effects on the ocular surface barrier by measuring fluorescein dye uptake. CLU formulated in PBS, topically applied to the ocular surface, 4 times/day, at the same time as the desiccating stress protocol was applied completely protected the ocular surface against desiccating stress. This effect occurs via a striking all-or-none response over a very precise threshold range of 0.6-1 ug/mL. Strikingly, the same dose of CLU also ameliorated pre-existing ocular surface barrier disruption due to desiccating stress.

Since CLU was so effective at ameliorating pre-existing barrier disruption, we wondered whether it might have a direct sealing effect. Figure 2, taken from our recent paper [24], shows representative results of these experiments. When CLU was applied only a single time, and when fluorescein uptake was assayed within 15 minutes before repair could occur, pre-existing barrier disruption was completely ameliorated. The effect lasted for at least 2 hours, but was gone within 16 hours. These results indicate that CLU acts to seal the ocular surface barrier against fluorescein uptake. The all-or-none threshold range was higher in this case – 3-6 ug/mL – for reasons not yet understood.

We next tested the capacity of CLU to protect the barrier against physical damage. We showed for the first time that LGALS3 is cleaved at the mouse ocular surface subjected to desiccating stress. LGALS3 cleavage products are found at the ocular surface and in tears of dry eye patients [178], providing evidence that similar mechanisms are operative in human dry eye. We also found that topical CLU treatment protects OCLN in the tight junctions of the paracellular barrier *in vivo*, as previously shown in a cell culture model *in vitro* [23]. These results demonstrate that CLU maintains protein structure at the ocular surface subjected to desiccating stress.

We also demonstrated that topical CLU is cytoprotective, preventing the increase in apoptosis that occurs at the ocular surface subjected to desiccating stress [24]. As discussed above, cytoprotection by CLU has been well studied in connection with resistance to chemotherapeutics in cancer, but this the first time CLU has been demonstrated to be anti-apoptotic at the ocular surface subjected to desiccating stress, and the first time CLU delivered topically has been shown to provide this beneficial effect.

The observation that CLU seals the ocular surface against fluorescein uptake immediately after being applied, and that sealing is maintained for at least two hours, strongly suggested that CLU must bind at the ocular surface and we showed that this is indeed the case [24]. Importantly, CLU binding was found to be selective for the ocular surface subjected to desiccating stress (as compared to the unstressed ocular surface). This suggested that CLU binds specifically to disrupted areas of the barrier. CLU was identified as an interacting candidate in a recent proteomics screen for potential LGALS3 interacting proteins from human prostasomes [179]. We validated this finding for the first time, showing that CLU applied to an LGALS3-Sepharose affinity column bound to the beads, but was eluted with the counter-receptor β-lactose. This

suggests that CLU interacts with the carbohydrate-binding domain of LGALS3. Further studies will be important to understand the selectivity mechanisms.

Since LGALS3 is present at the normal ocular surface, how could it provide for selectivity? We suggest this could involve proteolysis. All galectins have a C-terminal carbohydrate recognition domain, but LGALS3 is unique in also possessing an N-terminal extension with a repeating motif that enables multimer formation [180]. This gives it the capacity to forms networks that bridge membrane-associated mucin ectodomains to organize the ocular surface barrier. Bridging of membrane-associated mucins by LGALS3 has been shown as essential for exclusion of the clinical dye rose-bengal [181]. MMPs and other proteinases can cleave the multimerization domain from the body of LGALS3, reducing self-association [168-170]; truncated LGALS3 interferes with network formation and rose-bengal exclusion [182]. As reported above, LGALS3 cleavage increases at the mouse ocular surface subjected to desiccating stress, and that CLU protects against cleavage. Cleavage of LGALS3 at the ocular surface subjected to desiccating stress would disrupt interactions with other LGALS3 molecules as well as membrane-associated mucins, freeing it for interaction with CLU. Proteolysis of other molecules might similarly provide for selectivity of CLU binding. These ideas remain to be tested.

CLU binding at the ocular surface must also relate to the observed all-or-none sealing effect. All-or-none responses are seen in many biological processes [183-185] and often involve the assembly of multimeric complexes at a critical concentration [186]. Significantly, LGALS3 binding to counter-receptors is of higher affinity after removal of the multimerization domain [170]. Thus when the critical threshold is attained at the ocular surface that has been subjected to desiccating stress and subsequent proteolysis, CLU might intercalate into the LGALS3-membrane-associated mucin network [24].

The available data suggest that CLU might act as a “spot weld” to bind to and seal the ocular surface. An artist’s conception of sealing is depicted in Figure 3. CLU binding to the ocular surface and sealing might involve the amphipathic helices, which could mediate interaction with proteins at the ocular surface denatured by proteolysis, as well as with the plasma membrane [24]. All exchangeable apolipoproteins, including APOA1 and APOE, bind lipids via their amphipathic helix domains, and can insert into lipid bilayers [187].

As shown in Table 1, the concentration of CLU varies greatly among human bodily fluids. CLU has been identified in basal and reflex tear proteomics profiles of normal human subjects [156-164], dry eye subjects [151, 152, 155], and subjects with pterygium, Sjögren’s syndrome, diabetes, diabetic retinopathy, and multiple sclerosis [151-154, 164], however the actual concentration of CLU in tears has never been measured. In our recently published paper, we report the first ever measurement of tear CLU concentration [24]. We determined that the basal CLU tear concentration in 6 week old female C57BL/6 mice is ~5-6 ug/mL. Significantly, this fits within the range of the all-or-none CLU threshold that we observed for sealing (3-6 ug/mL).

In vitro, CLU potently inhibits amyloid formation characteristic of many genetic diseases of protein deposition. This provides substantial cytoprotection, but depends on achieving a critical molar ratio of CLU to substrate [71]. As noted above, CLU concentration in cerebrospinal fluid is low, suggesting the levels could be easily overwhelmed in disease and that CLU supplementation might be of therapeutic value in Alzheimer’s [73]. Considering the low level of CLU in mouse tears, the same argument might be made for dry eye. Perhaps of significance, increased CLU in the saliva has been suggested as a biomarker for Sjögren’s syndrome [188]. This increase likely represents a protective stress response. It remains to be learned whether the increase extends to the tears.

This vulnerability idea might be tested using CLU knockout mice, but first we had to demonstrate that their ocular surface was anatomically normal. We performed extensive characterization and found no differences from wild type mice [24]. Next we had to demonstrate that reduction in genetic dosage resulted in a corresponding reduction in the tear concentration of CLU. Heterozygous CLU knockout mice had half the CLU tear concentration – 2.5 ug/mL – as expected for half the gene dosage [24]. Importantly, this half dose is beneath the critical threshold for sealing (3-6 ug/mL). Having established these criteria, we tested the vulnerability hypothesis. We found that the ocular surface barrier of heterozygous CLU knockout mice was ~3-fold more sensitive to desiccating stress than wild type mice [24]. This supported the hypothesis that the level of CLU in tears is limiting, and that a reduction can create vulnerability for barrier disruption.

In summary, topical application of CLU prevents and ameliorates ocular surface barrier disruption due to desiccating stress by a remarkable sealing mechanism dependent on a critical all-or-none concentration. When the endogenous CLU level drops below the critical all-or-none threshold, the barrier becomes vulnerable to desiccating stress. CLU binds selectively to the ocular surface subjected to desiccating stress *in vivo*, and *in vitro* to the network-forming galectin, LGALS3, a key barrier component. Positioned in this way, CLU not only seals the disrupted barrier, but it also prevents further structural damage due to proteolysis. These are fundamentally new observations about CLU functionality. Further studies to investigate the mechanisms of binding and selectivity for the disrupted ocular surface barrier, as well as the factors leading to ocular surface vulnerability will be very important.

5.2. Is CLU a Regulator of Mucosal Differentiation?

Depletion of intracellular CLU from the ocular surface epithelial is seen in a variety of inflammatory conditions in humans (e.g., [189, 190]), including such ocular surface disorders as

Stevens-Johnson syndrome and cicatricial pemphigoid [191, 192]. These latter diseases result in a deficiency of mucins in the tears, leading to evaporation of the aqueous layer of the tears resulting in a severe form of dry eye. Immunohistochemical analysis of tissues obtained from such eyes revealed that CLU depletion from the apical epithelial cells correlated strikingly with expression of markers of squamous metaplasia [193]. At the time it was suggested that CLU might be essential for protecting against inflammatory and desiccating stress, maintaining ocular surface barrier function and mucosal differentiation [194, 195]. However the experimental mouse models needed to test this idea had not yet been developed.

Squamous metaplasia occurs in experimental mouse dry eye models [196], including the model used in our lab [197]. Analysis of corneal sections from mice maintained under ambient conditions revealed immunoreactive CLU protein within the sub-apical epithelial cells, in a pattern very similar to that seen in human corneas. When eyes were subjected to the desiccating stress protocol, CLU immunostaining was diminished while MMP9 immunostaining was enhanced. CLU expression in the ocular surface epithelia, quantified by both RT-PCR and western blotting, showed a ~30% reduction at the mouse ocular surface under desiccating stress, a reduction similar in size to what we observed in tears [23]. Like the previous study, this is consistent with a possible regulatory role for CLU in squamous metaplasia. It was further found that treatment of cultured human corneal epithelial cells with inflammatory mediators resulted in a strikingly down regulation of CLU, while expression of MMP9 was enhanced [23]. This suggests that inflammation could make the ocular surface vulnerable to squamous metaplasia by depleting CLU in the ocular surface epithelia.

Several studies have suggested that CLU may have direct effects on inflammation through inhibition of NF-κB activity, suggesting a possible mechanism for squamous metaplasia (e.g., [198, 199]). However, loss of intracellular CLU also might simply serve as a marker of squamous

metaplasia. With the new dry eye model, the availability of CLU knockout mice, and the ability to "replace" CLU in tears by topical treatment, this idea can now be investigated.

5.3. CLU as a Possible Biotherapeutic for Dry Eye

There is a major unmet need for new therapeutics to prevent or treat dry eye. Restasis (cyclosporine A), an immunosuppressant drug widely used in organ transplantation to prevent rejection, is currently the only prescription medicine available, however health care providers report a high failure rate [200]. The U.S. Food & Drug Administration (FDA) approved Restasis in 2002. Since then, criteria for approval have become more stringent, and 15 companies have unsuccessfully attempted to secure FDA approval for a dry eye drug [201]. The most recent was Shire with rejection of Lifitegrast, a small molecule integrin α4 antagonist with anti-inflammatory activity, in October 2015. Likely there are numerous factors contributing to this failure, but one may be the existence of multiple types of dry eye. Each form not only exhibits variable severity, but also responds differently to upstream interventions. At present it is not usually possible to accurately distinguish one form from another, making it impossible to design clinical trials towards a single type. Most efforts for drug development in the dry eye arena have been devoted to targeting of inflammation (like Restasis), tear production, tear film movement and tear chemistry, i.e., factors located upstream in the cascade of events leading to dry eye and ocular surface barrier disruption. Therapeutics targeting common downstream effects in the vicious circle of dry eye may provide an advantage towards meeting FDA approval.

Currently there is a new focus on biologics in the pharmaceutical industry, the goal to address the high attrition rate in preclinical and clinical trials ascribed to toxicity, insufficient efficacy, or inadequate selectivity of small molecules [202]. In this regard, the natural proteins of the tears may offer much opportunity [160]. Proteins that have been considered include LCN1 (lipocalin), a multifunctional protein that serves as the predominant lipid carrier in human tears and which is

critical to functions involving lipids in protection of the ocular surface [203-205]. Another example is LACRT (lacritin), a glycoprotein discovered in an unbiased screen for novel factors that stimulate tear secretion [206, 207]. LACRT has prosecretory activity in the lacrimal gland and mitogenic activity at the corneal epithelium. In the Aire knockout mouse model of dry eye (considered similar to human Sjögren's syndrome), topical LACRT restores pilocarpine-induced tearing and largely eliminates lissamine green staining [208]. A third example is PRG4, a mucin-like secreted glycoprotein localized to the ocular surface, where it functions as a boundary lubricant [209]. PRG4, also called lubricin, may have clinical utility as a topical treatment for dry eye, or as a contact lens biomaterial coating to promote more comfortable wear [210].

The natural tear protein CLU could be an ideal therapeutic to treat dry eye. As discussed in this article, CLU exhibits a variety of homeostatic activities that enable it to protect cells and tissues under conditions of stress and we now know that topical CLU delivers several of these benefits to the ocular surface subjected to desiccating stress in the preclinical mouse model [211]. Most novel and exciting, CLU directly seals the disrupted ocular surface barrier [211]. This means that CLU can target both upstream effects leading to dry eye, as well as dry eye's final common pathway.

FDA approval of pharmacotherapies for dry eye has typically required a statistically significant superiority of the drug to its vehicle in both a sign (usually fluorescein uptake) and a symptom. Consistent amelioration of fluorescein uptake has been a difficult endpoint for many investigational new drugs to meet [212, 213]. If the all-or-none response seen in mice holds in humans, the "all" part would be an important advantage. CLU's proven ability to seal the ocular surface barriers and inhibit apoptosis, accompanied by reduced inflammation and proteostasis, may not only improve the signs of dry eye (dye uptake), but could also quiet symptoms, e.g., irritation, dryness, gritty feeling and burning. Human studies are the best way to determine

whether CLU can improve such symptoms, making patients “feel better”.

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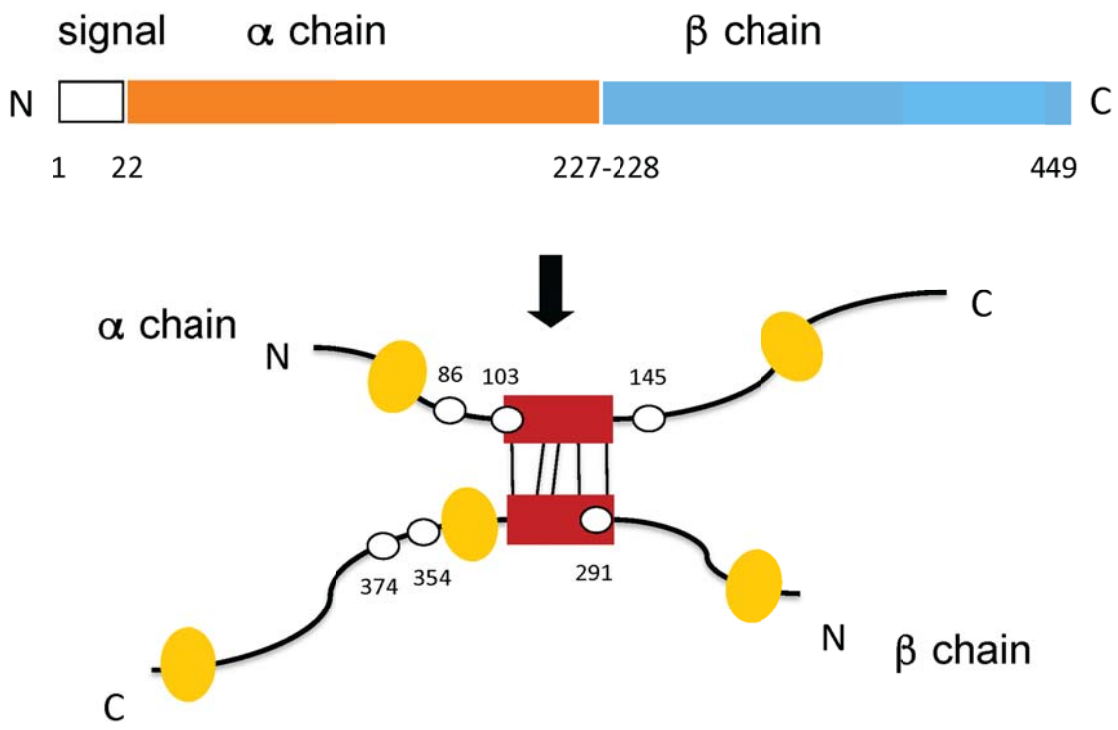
Tables

Table 1. Concentration of CLU in Bodily Fluids

Human seminal plasma		
	250-500 ug/mL	[214]
	438±235 ug/ml	[215]
Human serum		
	35-105 ug/ml	[3]
	111±50 ug/ml	[215]
	340 ug/mL	[216]
	325±100.3 ug/ml	[115]
	101±42 ug/ml	[217]
	52.8±0.8 ug/ml (Japanese men) 49.3±0.5 ug/ml (Japanese women)	[218]
Human plasma		
	72 ug/ml	[3]
	50-100 ug/ml	[214]
Human cerebrospinal fluid		
	1.6-3.6 ug/ml	[219, 220]
Human aqueous humor		
	0.8 ± 0.5 ug/ml	[115]
C57BL/6J mouse basal tears		
	5.2 ug/mL	[24]
C57BL/6J mouse dry eye tears		
	3.6 ug/mL	[24]

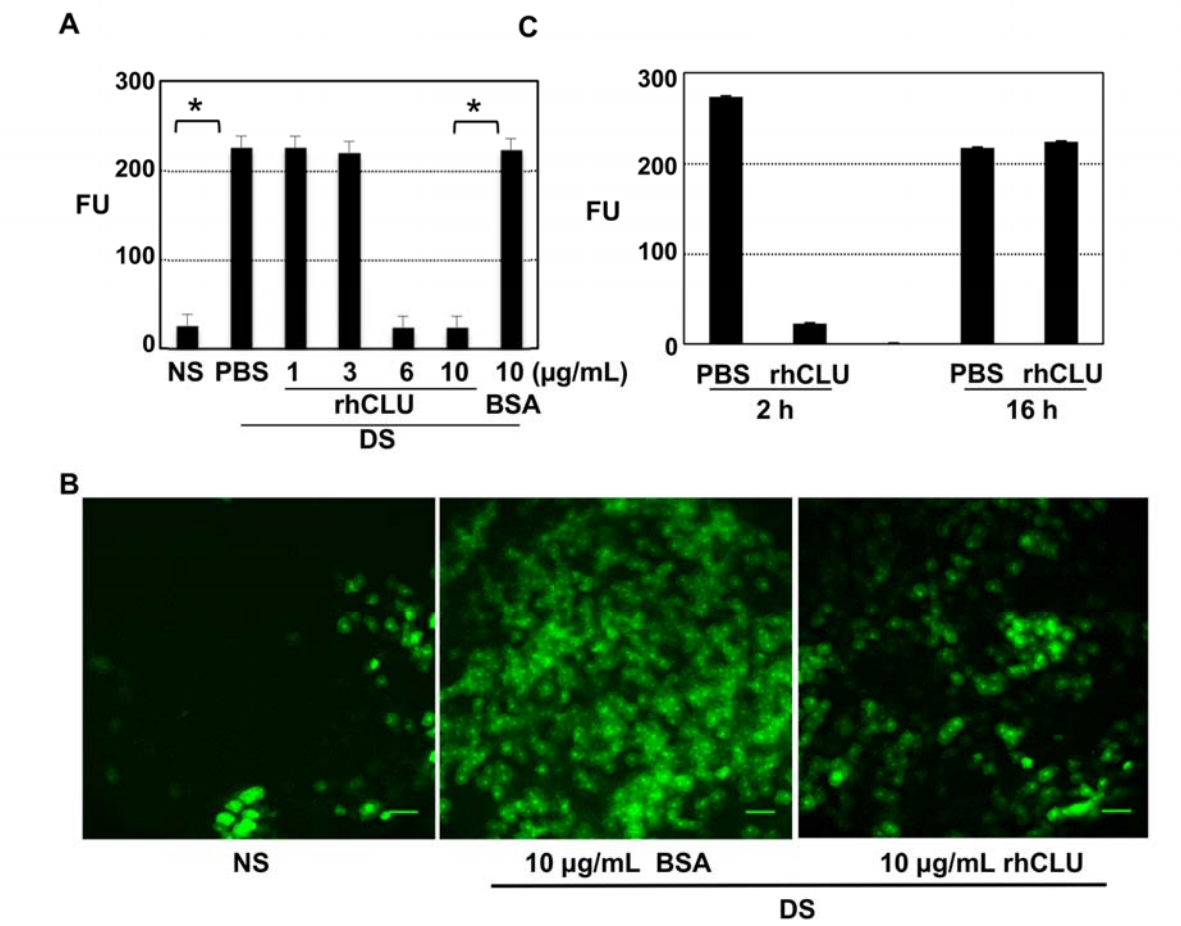
Figure Captions

Figure 1. Predicted human CLU structure



Schematic adapted from [8, 20, 28]. The 22-mer secretory signal peptide is proteolytically cleaved from the 449 amino acid precursor polypeptide chain and subsequently the chain is cleaved again between residues Arg227-Ser228 to generate an α-chain and a β-chain. These are assembled in anti-parallel fashion to generate a heterodimeric molecule in which the cysteine-rich centers (red boxes) are linked by five disulfide bridges (black lines) and flanked by five predicted amphipathic α-helices (yellow ovals). The six sites for N-linked glycosylation are indicated (white spots). Amino acid numbering for the N- and C-termini, the cleavage sites, and the sites for N-linked glycosylation are indicated, as in [25].

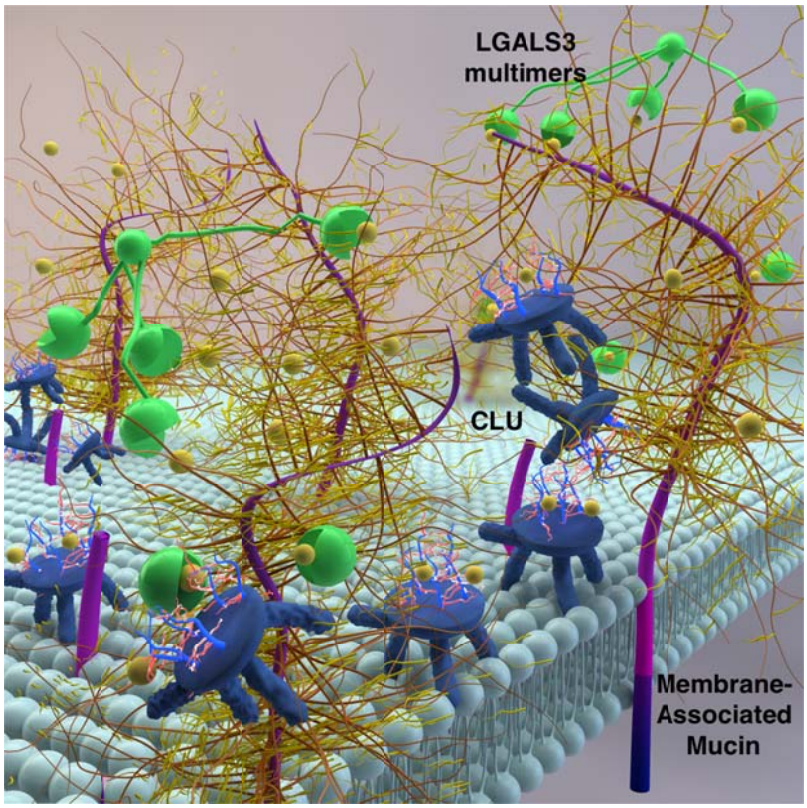
Figure 2. Topical CLU directly seals the ocular surface barrier disrupted by desiccating stress.



The standard desiccating stress protocol was applied for 5-days to create ocular surface disruption. Non-stressed (NS mice) housed under normal ambient conditions served as the baseline control. Eyes with desiccating stress were then treated topically, a single time, with 1 µL of CLU formulated in PBS, 1 µL of BSA formulated in PBS for comparison, or 1 µL of PBS control. Barrier disruption was assayed by measuring corneal epithelial uptake of fluorescein (FU = Fluorescence Units at 521 nm). Values are expressed as the mean ± SD. (A) Eyes were treated a single time with recombinant human CLU (rhCLU) at 1, 3, 6 or 10 µg/mL, BSA at 10 ug/mL, or PBS. Fifteen minutes later, the fluorescein uptake test was performed, before there was time for barrier repair to occur. *P<0.0001 (n = 4). (B) Images of central cornea from the experiment shown in (A), obtained using laser scanning confocal microscopy at 10X magnification. One representative image out of two independent experiments is shown. Scale bar = 100 µm. (C) Eyes were treated a single time with rhCLU at 10 µg/mL (right eyes) or PBS (left eyes). Then the mice were kept further for 2 h or 16 h while continuing with the same desiccating stress protocol. The fluorescein uptake test was performed following the indicated time period to assess the time length of treatment effect. *p<0.0001 (n = 4).

From: Clusterin Seals the Ocular Surface Barrier in Mouse Dry Eye. Bauskar A et al. PLoS one. 2015. 10(9) doi: 10.137/journal.pone.0138958, CC-BY; used with permission from the publisher.

Figure 3. Conceptual model depicting CLU binding to areas of barrier disruption at the ocular surface subjected to desiccating stress.



Membrane-Associated Mucins (fuscia, dark blue and gold), LGALS3 (green) and CLU (dark blue with blue and coral "antlers") are shown interacting with one another, and with the lipid bilayer of the apical epithelial cells (light blue), in this artist's conception of the ocular surface. Membrane-Associated Mucins are depicted as long, flexible rods (fuscia) traversing the lipid bilayer of the apical epithelial cells of the ocular surface, with their intracellular domains projecting into the cytoplasm (blue). The carbohydrate chains (gold) linked to the extracellular domains are extensively branched. Following exposure to desiccating stress, membrane-associated mucins may be proteolytically cleaved, leaving membrane-embedded protein "stubs" (fuscia).
LGALS3 molecules (green) are shown with the C-terminal carbohydrate-binding domain appearing as a "mouth" linked to the N-terminal multimerization domain by a long thread. Some of these LGALS3 molecules are depicted as self-associating via their multimerization domains, a requirement for network formation and exclusion of clinical dyes. In other cases, the multimerization domain is drawn as proteolytically cleaved, leaving only the carbohydrate-binding domain.
CLU molecules (blue) are schematically modeled after a milking stool. The "seat" of the stool represents the disulfide-bonded region of the polypeptide chains decorated by carbohydrate

chains (blue and coral) emanating from six attachment sites. The three legs of the stool represent the C-terminal and N-terminal portions of the molecule containing the amphipathic helices. The “arm” of the stool is the C-terminal portion lacking an amphipathic helix. Galactose moieties on both the mucin and CLU carbohydrate chains are depicted as small “marbles” (yellow). The carbohydrate-binding domains (“mouths”) of LGALS3 molecules are shown binding to (“eating”) the yellow globes. CLU molecules are shown in various interactions 1) self-associating, 2) binding to the lipid bilayer, and 3) associating with proteolyzed mucin “stubs”. In the foreground, the proteolytically cleaved carbohydrate-binding domain of an LGALS3 molecule is shown binding to a marble on a carbohydrate chain of a CLU molecule. This drawing aims to illustrate the idea that all-or-none sealing of the ocular surface barrier disrupted by desiccating stress occurs when the concentration of CLU molecules is high enough to compete effectively with mucins for binding to LGALS3 molecules.

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