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Lens Glutathione Homeostasis: Discrepancies and gaps in knowledge standing in the way of novel therapeutic approaches

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Abstract

Cataract is the major cause of blindness worldwide. The WHO has estimated around 20 million people have bilateral blindness from cataract, and that number is expected to reach 50 million in 2050. The cataract surgery is currently the main treatment approach, though often associated with complications, such as Posterior Capsule Opacification (PCO)-also known as secondary cataract. The lens is an avascular ocular structure equipped with an unusually high level of glutathione (GSH), which plays a vital role in maintaining lens transparency by regulating lenticular redox state. The lens epithelium and outer cortex are thought to be responsible for providing the majority of lens GSH via GSH *de novo* synthesis, assisted by a continuous supply of constituent amino acids from the aqueous humor, as well as extracellular GSH recycling from the gamma-glutamyl cycle. However, when *de novo* synthesis is impaired, in the presence of low GSH levels, as in the aging human lens, compensatory mechanisms exist, suggesting that the lens is able to uptake GSH from the surrounding ocular tissues. However, these uptake mechanisms, and the GSH source and its origin, are largely unknown. The lens nucleus does not have the ability to synthesize its own GSH and fully relies on transport from the outer cortex by yet unknown mechanisms. Understanding how aging reduces GSH levels, particularly in the lens nucleus, how it is associated with age-related nuclear cataract (ARNC), and how the lens compensates for GSH loss via external uptake should be a major research priority. The intent of this review, which is dedicated to the memory of David C. Beebe, is to summarize our current understanding of lens GSH homeostasis and highlight discrepancies and gaps in knowledge that stand in the way of pharmacologically minimizing the impact of declining GSH content in the prevention of age-related cataract.

Introduction

The lens has evolved as an anaerobic biological system with millimolar concentrations of glutathione (GSH). The critical role of GSH in maintaining lens redox status and

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transparency is well recognized and has been, over the years, the subject of several excellent reviews(Giblin, 2000; Lou, 2000; Reddy, 1990; Truscott, 2005). While one could argue that nothing new was to be expected concerning the protective role of GSH in the lens, our interest in GSH homeostasis in the lens was rekindled with the unexpected finding that lenticular GSH levels were not completely suppressed in the LEGSKO mouse in spite of complete absence of γ-glutamylcysteine ligase(Fan et al., 2012). This issue, which is the subject of intense investigation in our laboratory, is closely linked to lenticular ascorbate metabolism and cataractogenesis and the work of David Beebe who has pioneered the importance of the vitreous as a source of oxidative stress to the lens(Beebe et al., 2014; Holekamp et al., 2005; Li et al., 2013b; Shui et al., 2009). These paradigm shifting studies inspired us to study lens biology in connection not only to the aqueous humor but also to the vitreous humor. Additionally, Dr. Beebe's pioneer studies provide the mechanistic framework for a potential therapeutic treatment of high risk (>90%) and rapid (within two years) nuclear cataract formation after vitrectomy surgery(Petrash, 2013).

In order to provide a complete coverage of lens GSH homeostasis, we have to discuss the lenticular GSH dynamics from the perspective of both protein conjugated GSH and free GSH/oxidized GSH (GSSG). Since several excellent reviews have covered the protein glutathionylation(Lou, 2000, 2003; Lou and Dickerson, 1992; Lou et al., 1990; Lou et al., 1995), we will mainly focus on the roles of latter. Below we review the established mechanisms and pathways that are involved in lens GSH homeostasis. We also provide a brief summary of recent progress regarding lens nucleus GSH homeostasis, as well as the impact of aging on lens GSH homeostasis, since age-related nuclear cataract (ARNC) is often believed to be, in part, associated with declining nuclear GSH levels in the aging human lens(Giblin, 2000).

GSH de novo synthesis and its constituent amino acids transport

Intracellular GSH is synthesized by two ATP-dependent enzymes: γ-glutamylcysteine ligase (GCL) and glutathione synthase (GS) to produce γ-glutamylcysteine and GSH, respectively. The mammalian GCL is a heterodimer enzyme consisting of a 73-kDa catalytic subunit, Gclc, and a 28-kDa modulatory subunit, Gclm. The catalytic subunit, Gclc has the enzymatic activity and is regulated via a GSH feedback inhibition mechanism(Richman and Meister, 1975). Gclm has no enzymatic activity, but heterodimer formation of Gclm and Gclc significantly decreases the Km value for glutamate and increases the Ki value for the feedback inhibition by GSH(Chen et al., 2005).

Like other tissue systems, the lens has a functional GSH *de novo* synthesis machinery, which mostly lies in the epithelial and cortical layers, while mature fibers cells sit in inner layers of the lens that have lost cell organelles such as nucleus and mitochondria(Bassnett and Beebe, 1992). However, due to the avascularity of the lens, for GHS biosynthesis to take place, the constituent amino acids, glutamic acid, glycine and cysteine have to be transported to the epithelial and outer cortical fibers cells. Pioneering work from Reddy et al. has demonstrated that these amino acids are delivered to the lens from the plasma via the aqueous humor and the lens epithelium, from which they are delivered to the rest of the lenticular system(Reddy, 1973, 1979) (Fig.1). For GSH synthesis, the Km value of GCL for cysteine is ~0.15 mM,

while that for glutamate is ~1.7 mM, and that of GS for glycine is ~0.8 mM(McBean, 2012). In order for GSH biosynthesis to take place, the required intracellular concentration of these amino acids is thought to be close to their Km value. However, various studies suggest species-specific results regarding these amino acids levels in the lens epithelium and outer cortex. For example, Lim et al.(Lim et al., 2007) find that the concentration of the three amino acids in the rat lens cortex is lower than that required for the GCL and GS Km value of its proper constituent amino acids. In contrast, other studies in human, rabbit and bovine lens demonstrate much higher values than these required for the Km(Barber, 1968; Kern and Ho, 1973; Reddy, 1973). It has to be pointed out that these studies measured the total GSH from the homogenate of the lens tissue, and that this does not exclude the possibility that intracellular amino acid level might be much higher to fulfill the needs of GSH *de novo* synthesis. Nevertheless, similar to other body systems, such as the central nervous system (CNS)(Aoyama et al., 2012), cysteine level is relatively lower than glutamic acid and glycine. It is, therefore, the rate-limiting substance in lenticular *de novo* GSH synthesis and an adequate cysteine supply is essential wherever GSH *de novo* synthesis is taking place.

1. Cysteine/cystine transport—From the above considerations, it is clear that cellular cysteine and GSH synthesis are tightly linked. Multiple mechanisms have been postulated in terms of intracellular cysteine homeostasis in studies of the central nervous system (CNS) or hepatocytes(Lu, 1999; McBean and Flynn, 2001). The sodium independent cystine/glutamate exchanger (Xc^-) has been shown to take up cystine into cells, which is subsequently reduced into cysteine for GSH synthesis(Lewerenz et al., 2013; Lim and Donaldson, 2011). In one set of studies, the Xc^- exchanger was found present in the entire rat lens, predominantly in the cytoplasm in the outer cortex cells, while it was more membranous in the inner cortex region(Lim et al., 2005). In the human lens, Xc^- is present in the entire lens region at a young age, but no immunoreactivity is found in the central lens region of aged human lenses(Lim et al., 2013). In contrast, in other studies, Xc^- was reported to be predominately present in the membranes of outer cortex cells, and no detection was observed in the nuclear region of the dog lens(Lall et al., 2008). These studies provide evidence for the presence of Xc^-, but whether this exchanger is important for lens cysteine homeostasis is still not very clear. Several findings point to quite different research directions. In a vascular eye perfusion study in guinea pigs, radiolabeled cysteine, cystine and methionine were injected through the common carotid artery(Mackic et al., 1997). In this study, cysteine, but not cystine, was readily taken up by the lens epithelial and cortical fiber layers, while infused cystine failed to incorporate into GSH synthesis. Other evidence in support of cysteine rather than cystine uptake is that high levels of free cysteine, but not cystine, are found in human(Barber, 1968), monkey(Gaasterland et al., 1979) and calf(Kern and Ho, 1973) aqueous humor, though different results was reported in the aqueous humor of guinea pigs(Mackic et al., 1997). Furthermore, recent reports(Martis, 2015) indicate has no impact on lens GSH level based on tests with Xc^- knockout mice.

On the other hand, earlier studies indicate that over 90% of cystine transport is actually processed by sodium-dependent high affinity glutamate transporters (X_{AG}-), based on rat brain tissue uptake experiments(Flynn and McBean, 2000) and a cultured astrocytes study(Bender and Norenberg, 2000). Also, cysteine was found to be able to inhibit X_{AG}-

facilitated transport(McBean and Flynn, 2001). Altogether, five subtypes of high-affinity glutamate transporters (excitatory amino acid transporters 1–5 (EAAT1–5)) have been identified in mammalian tissues(Aoyama and Nakaki, 2013; Bridges and Esslinger, 2005). More recent studies demonstrate that EAAT3, also named “excitatory amino acid carrier 1” (EAAC1), is more functional in cysteine transport than the control of extracellular glutamate levels(Aoyama and Nakaki, 2013; Holmseth et al., 2012). The brain cysteine and GSH levels are significantly reduced in EAAC1-deficent mice, and this can be attenuated by the treatment with N-acetylcysteine (NAC), whose uptake proceeds via different uptake mechanisms than cysteine/cystine(Aoyama et al., 2006). All five types of EAATs are found present in rat lens based on RT-PCR and western-blot analysis(Lim et al., 2005). We therefore speculate that lenticular cysteine transport from aqueous humor occurs, most likely via a sodium-dependent transporter system, such as EAAC1, and that high cysteine transport activity occurs at the lens equator region, as reported by Truscott’s group(Sweeney et al., 2003).

The metabolic pathway transsulfuration can also supply cysteine from methionine via the transmethylation pathway(McBean, 2012). The transsulfuration pathway is also present in the lenticular system(Persa et al., 2004). Cystathionine-beta-synthase (CBS), one of the enzymes utilizing methionine to produce cysteine via transmethylation is elevated in human lens nucleus but decreased in the epithelial layer with aging. CBS expression can also be stimulated with oxidative stress, such as H_2O_2 in lens epithelial cell culture(Persa et al., 2004). In addition, the betaine-homocysteine S-methyltransferase 1(BHMT1), a remethylation enzyme that converts homocysteine to methionine, is found to be down-regulated in aged human lens nuclei(Zhou et al., 2015). This may explain the findings that the free cysteine levels are elevated in the lens nucleus under oxidative stress(Giblin et al., 1995; Lou, 2000). However, in a vascular eye perfusion study in guinea pigs(Mackic et al., 1997), methionine failed to produce cysteine and incorporate into GSH, suggesting minimal contribution of methionine in circulation to lenticular cysteine homeostasis(Mackic et al., 1997). Apparently, more study is needed to clarify whether the transsulfuration pathway plays a significant role in lens cysteine and GSH homeostasis.

2. Glutamate and glycine transporter—As mentioned above, high affinity sodium-dependent glutamate transporters have been located in rat lenses(Lim et al., 2005), though no report exists so far about their expression level in the human lens. We anticipate that these transporters will also be present in human lenses. Extensive studies and reviews have addressed their roles in the CNS(Divito and Underhill, 2014; Vandenberg and Ryan, 2013). On the other hand, it is well established that cellular glutamate originates from glutamine, which is readily taken up by the lens via a transport mechanism that is hundreds of times more efficient than that of glutamate. Glutamine was shown to convert into glutamate and be incorporated into GSH synthesis in *ex vivo* calf and rat lens culture systems(Kern and Ho, 1973). However, no *in vivo* confirmation of this *in vitro* finding was reported. Since the anterior, but not posterior, part of the lens is equipped with a monolayer of epithelial cells(Beebe, 2008), it is anticipated that the anterior will be more selective and specific for amino acid transport than the posterior of the lens. In that regard, it is our point of view that *ex vivo* lens culture systems using total immersion are convenient, but not suitable for lens

transporter studies because lens anterior and posterior surfaces have completely different structures. More work is needed to test whether glutamine uptake and conversion to glutamate plays a significant role in lens GSH homeostasis. At least one neutral amino acid transporter, ASCT2, has been found present in rat lenses(Lim et al., 2006), and free glutamine measured in calf aqueous humor is more than double that of glutamate(Kern and Ho, 1973).

Intracellular glycine homeostasis, like other neurotransmitters, is mainly regulated by the high affinity sodium-dependent transporters GLYT1 and GLYT2, which belong to the solute transporter family (SLC6)(Chen et al., 2004). Both glycine transporters have been identified in rat lenses(Lim et al., 2007; Lim et al., 2006) (Fig.1).

3. Glutathione metabolism/γ-glutamyl cycle—Gamma-glutamyl transpeptidase (GGT), also known as gamma-glutamyl transferase, a glycoprotein, is localized at the cell surface and anchored to the cell membrane via a single N-terminus transmembrane domain. It cleaves only the extracellular GSH, oxidized GSH (GSSG), as well as glutathione S-conjugates(Ikeda et al., 1995; Wickham et al., 2012), therefore providing the cells with the amino acids necessary for intracellular GSH synthesis(Hanigan, 2014). GGT was reported present in the lens, ciliary body and cornea over 40 years ago in two independent studies(Reddy and Unakar, 1973; Ross et al., 1973) (Fig.1). GGT activity enables the cells to maintain their intracellular GSH levels, thus coping with reactive oxygen species (ROS) attack. Both GGT knockout and mutant mice develop cataract in a very short time period after birth(Chevez-Barrios et al., 2000; Yamada et al., 2013). GGT deficient mice were found to have severe cysteine deficiency (~20% of WT plasma cysteine level)(Lieberman et al., 1996). Interestingly, despite high plasma GSH level in GGT deficient mice, eye and lens GSH levels are markedly reduced (~5% of WT)(Chevez-Barrios et al., 2000). The drastic reduction of lens GSH content in GGT KO mice cannot simply be explained by impaired GSH *de novo* synthesis due to cysteine deficiency. The lens Gclc conditional knockout mouse (LEGSKO mouse) recently created by our group(Fan et al., 2012) is able to maintain ~50% GSH level (1–2mM) relative to wild type (WT) mice on the C57 black background (unpublished), while levels were lower in the FVB/B6 hybrid strain (Fan et al., 2012). We believe that both low cysteine and GSH supply to the lens are necessary for drastic GSH reduction. In other words, lenses lacking efficient *de novo* GSH synthesis may take up either GSH or GSSG from surrounding ocular structures, i.e. the aqueous humor or the vitreous humor. We will discuss this aspect in the following section. Needless to say, the gamma-glutamyl cycle is an important amino acid recycling mechanism for maintenance of lens GSH homeostasis, particularly in the metabolically active regions of the lens.

Lens GSH/GSSG uptake

An *in situ* vascular eye perfusion study in guinea pig has come to the very surprising conclusion that the *de novo* GSH synthesis from circulating and aqueous sulfur amino acids, such as cysteine, cystine or methionine can be only a minor source of the millimolar concentration of GSH in the epithelium(Mackic et al., 1997). A $t_{1/2}$ of 5480 hours was estimated for lens epithelium GSH to be replaced entirely if solely based on circulating sulfur amino acids, the limiting amino acids in intracellular GSH biosynthesis. This

obviously is not the case based on human lens GSH turn over studies, whose $t_{1/2}$ is around 85.4 hours(Rathbun and Murray, 1991). Studies indicate that GSH can be taken up into *ex vivo*-cultured lenses, such as rabbit and bovine lenses(Hockwin et al., 1985; Reddy, 1973). Again, studies carried out utilizing *in situ* vascular eye perfusion with [S^{35}-cysteine] GSH and/or [H^3-glycine] GSH in guinea pigs have demonstrated that the lens is capable of taking up circulating GSH(Mackic et al., 1996; Zlokovic et al., 1994). The rate for lens epithelium GSH uptake from aqueous humor is more than three times than the rate of aqueous humor uptake from plasma within only 10 minutes of vascular eye perfusion, and only simple diffusion is observed when GSH enter aqueous humor from plasma(Mackic et al., 1996; Zlokovic et al., 1994). Based on this study, a $t_{1/2}$ of 85.4 hours is estimated if endogenous epithelial GSH has to be replaced entirely by plasma-derived GSH(Mackic et al., 1996), which is similar to the $t_{1/2}$ estimated in human lens (90 hr)(Rathbun and Murray, 1991). By perfusing with an equal ratio of [S^{35}-cysteine] GSH /[H^3-glycine] GSH, then determining the ratio in plasma, aqueous humor and lens epithelium, this study demonstrates that lens GSH uptake from aqueous humor is not due to the gamma-glutamyl cycle(Zlokovic et al., 1994). However, this study only tested the amino acids and GSH levels in aqueous and lens without measurements in vitreous humor. The secretion from both ciliary body and retina could also boost vitreous humor GSH or amino acids level in a significant manner. The GSH or amino acids uptake from posterior of the lens can then contribute to lens GSH or amino acid level, but these amino acids stay in lens posterior are not able to incorporated into GSH biosynthesis due to mature fiber cells that lack translational machinery. Overall, it will interfere the estimation of $t_{1/2}$ if only based on the calculation from aqueous humor and entire lens. Furthermore, the LEGSKO mouse(Fan et al., 2012), which lacks GSH biosynthesis, maintains around 50% of its lenticular GSH content relative to WT in the C57BL6 strain (unpublished). This indicates that the uptake from surrounding ocular tissue can only make up approximately half of lens GSH content. Stewat-DeHaan et al(Stewart-DeHaan et al., 1999) performed an *in vivo* transport study in rats by intraperitoneal injection of either H^3 or S^{35} labeled GSH, then measured the plasma and lens GSH accumulation. Over a 4 hour period, the lens can accumulate 12.3% of its total GSH from injected GSH, though other radiolabeled GSH species appear as fractionated GSH based on HPLC analysis. GSH fractionation would be expected, since the majority of GSH will be metabolized in the liver following i.p. injection. Nevertheless, ~12% of intact GSH accumulated in the lens indicating a remarkable uptake force by the lens.

Taken together, these studies support existence of compensatory mechanisms, i.e. somehow taking it up from circulating GSH, also plays an important role in lenticular GSH homeostasis. Thus, the LEGSKO mouse model of GSH depletion provides a useful tool for studying the mechanisms of weakened lenticular GSH homeostasis in old age. By measuring the differential in GSH level between aqueous humor to plasma, and lens epithelium to aqueous, respectively, Zlokovic et al(Zlokovic et al., 1994) predicted that a high affinity sodium-dependent GSH transporter might be present in lens epithelium, since very low levels (<50μM) of GSH are present in aqueous humor, particular in rodents. In order for the lens to take up GSH from the aqueous, it must pump across a GSH concentration gradient, and only an energy-dependent carrier-like transport system can fulfill such a task. However, whether a molecular GSH influx transporter exists in mammals is a still a debated

topic(Bachhawat et al., 2013; Gukasyan et al., 2007; Kaplowitz et al., 1996), despite several studies supporting the existence of a GSH transporter in the lenticular system(Kannan et al., 1996a; Kannan et al., 1996b; Li et al., 2010). Additionally, a high affinity GSH transporter (Hgt1) that has been identified in baker's yeast (*Saccharomyces cerevisiae*)(Bourbouloux et al., 2000). However, the Kannan studies(Kannan et al., 1996b; Kannan et al., 1995) were dismissed as a cloning artifact(Li et al., 1997), leaving Li's studies(Li et al., 2010) as the sole evidence in support of a specific GSH transporter in the lens. There have also been several studies, particularly in corneal GSH homeostasis, which suggest that, GSSG, but not GSH, is a better substrate for uptake in ocular tissues and then reduction back to GSH(Nakamura et al., 1994; Veltman et al., 2004). Whether this mechanism significantly contributes to the lens GSH pool requires further clarification.

1. Aqueous humor as the potential source of GSH

Aqueous humor is a clear fluid that fills and helps form the anterior and posterior chamber of the eye (Fig.1). The aqueous humor serves as "ocular blood" to provide nutrition, remove excretory products from metabolism and contribute to homeostatic regulation of these avascular structures, i.e. the lens and cornea(Goel et al., 2010). Aqueous humor is secreted by the ciliary epithelium lining the ciliary processes and first enters the posterior chamber, then reaches to the anterior aqueous humor chamber after passing through the capillary wall, stroma and epithelial bilayer(Goel et al., 2010). The aqueous humor is a dynamic system, and therefore helps to supply nutrition and remove metabolic waste in a continuous fashion.

Much effort has been focused on GSH supply from the aqueous humor, as mentioned above. Plasma GSH can indeed reach the aqueous humor by either vascular eye perfusion(Mackic et al., 1996) or *in vivo* GSH intraperitoneal injection(Stewart-DeHaan et al., 1999). A large aqueous humor GSH concentration (~1.5mM) can also be achieved by giving large, pharmaceutical doses of GSH (20mM) via perfusion, suggesting the importance of this channel in maintaining lens GSH homeostasis (Mackic et al., 1996). GSH/GSSG efflux transporter, such as the multidrug resistance-associated protein family, have been found expressed in ciliary body in human as well as in rat(Li et al., 2013a; Pelis et al., 2009). However, more study is needed to decipher the exact role that aqueous humor plays in maintaining lens GSH homeostasis besides supplying constituent amino acids for GSH biosynthesis, arguably due to low level of GSH in the aqueous humor.

2. The vitreous as a potential source of GSH

The vitreous humor accounts for up to 80% of the volume of the eyeball. It is an optically transparent gel formed by fine fibers and small molecular solutes that attach to lens and retina. However, the vitreous humor has functions beyond just being a space filling gel. Soluble extracellular proteins and small molecule solutes present in the vitreous are anticipated to play a vital role in maintaining homeostasis of adjacent ocular tissues, i.e. the lens and retina(Berman, 1991; Lund-Andersen, 2003; Monteiro et al., 2015). Outstanding work from Dr. Beebe's group suggestes another important functional mechanisms of vitreous humor, namely the regulation of molecular oxygen levels(Barton et al., 2007; Beebe et al., 1986; Filas et al., 2013; Harocopos et al., 2004; Holekamp et al., 2008; Shui et al., 2009). The gel-structure and high ascorbic acid concentration in vitreous humor is believed

to continuously prevent oxygen from reaching the lens, thus maintaining the lens under anaerobic conditions.

GSH is one of many small molecule solutes in vitreous humor, and it works to restore dehydroascorbic acid (DHA) into ascorbic acid in the so-called ascorbic-GSH cycle(May et al., 2001). Additional studies conclude that the vitreous humor is indeed in a more dynamic state than previously thought(Lumi et al., 2015; Monteiro et al., 2015). The soluble proteins and small molecular solutes are constantly transported in and out of the vitreous humor. Therefore, we speculate that vitreous GSH may also help regulate adjacent ocular tissue glutathione homeostasis. The vitreous humor GSH can come either from ciliary body secretion(Li et al., 2013a; Pelis et al., 2009) or from retinal metabolism, i.e. via retinal-pigmented epithelial (RPE) cells(Garcia et al., 2011; Lu et al., 1995). In addition, the vitreous humor is a nonhomogeneous tissue that displays a graded density at different anatomical locations, which have been subdivided into three main anatomical regions: the vitreous core, vitreous base, and vitreous cortex. Though, no regional GSH concentrations have been reported to our knowledge, one would expect these to vary; being e.g. much higher at the vitreous base if ciliary body is the actual source of GSH secretory tissue, or in vitreous cortex if the retina is the main provider of vitreous GSH. The total vitreous GSH varies according to species as recently observed in our laboratory (unpublished data) and reported by others(Nozal et al., 1997; Sulochana et al., 1999). Rabbit, rat, guinea pig and mouse are reported to have high GSH concentration (>250 μM) in their vitreous humor, while bovine and human have much lower GSH content (<50 μM). One reasonable explanation is that rodent lens is relative larger and rounder than human lens, and, as such, occupies much of the eye space, which leaves a very small vitreous humor volume. In rodent, the GSH obtained from its appropriate ocular tissue can be quickly mixed in the vitreous humor and the total GSH level will be high. In contrast, bovine and human have much larger vitreous humor volumes, and even if high GSH concentration is maintained at the vitreous base or cortex, the vitreous core may have very low GSH levels, and the total GSH concentration is still extremely low. In terms of the possibility of lens GSH uptake approaching from the vitreous humor, the GSH content at the vitreous base is apparently more important than other anatomical regions. The gel-like vitreous structure and large volume in human rules out the possibility for the vitreous cortex to serve as a source for lens GSH homeostasis. The measurement of GSH content in different regions of the vitreous humor in different species will shed much needed light on deciphering the GSH vitreous humor dynamics, and thus provide insight into the relationship between lens and vitreous GSH exchange, and the question of the extent to which the lens might be the GSH supplier for adjacent ocular tissues, as suggested by Umapathy et al.(Umapathy, 2014).

Lens nucleus GSH homeostasis

From the inner core to the periphery, the lens nucleus is constituted by an embryonic nucleus, a fetal nucleus, and an adult nucleus formed by differentiated fiber cells. Each of these can be delicately peeled off the human lens using simple tweezers(Beebe, 2008). These mature fibers cells have no capacity for protein or small molecule synthesis, including that of GSH. However, the lens nucleus still contains substantial level of GSH for maintenance of lens nucleus redox status(Giblin, 2000; Truscott, 2005). Obviously, lens

nucleus GSH content is reliant on transport mechanisms from the outer cortex. Similar to the lens epithelium and outer cortex, whether a GSH transporter is responsible for such tasks has not yet been determined. Currently, the well-recognized concept is that of concentration gradient diffusion into the nucleus via gap junctions, which connect the mature fiber cells(Beebe et al., 2011). More recently, Slavi N. et al found a significant GSH reduction in lens nucleus in Cx46 (connexin 46) knockout mouse(Slavi et al., 2014). Cx46 and Cx50 (connexin 50) are two gap junction channel proteins expressed in the lens. The remarkable reduction of lens nucleus GSH in Cx46 KO mouse suggests the importance of gap junctions in lens nucleus GSH homeostasis. However, only a low degree of GSH permeation is found in single channels formed by either Cx46 or Cx50. Whether gap junctions are playing a major role in maintaining lens nucleus GSH homeostasis is thus still unclear. In addition, rapid nuclear cataract formation in Cx46 KO mice (Xia et al., 2006) further complicate the content of lens nucleus GSH. In a nutshell, much more work is needed in order to better understand the mechanisms regulating lens nucleus GSH homeostasis and its role in age-related nuclear cataract formation. It has been proposed that approximately 1 mM is the threshold GSH concentration for human lenses for avoiding rapid cataract formation(Giblin, 2000). The LEGSKO mouse in the FVB/B6 hybrid background has significant decreases in lens nucleus GSH, contributing to the development of nuclear cataract at four months of age(Fan et al., 2012). An alternative hypothesis is that the lens “microcirculation” might be responsible for supplying and removing metabolites in the lens. However, such a hypothesis, as suggested by Beebe et al.(Beebe et al., 2011), is not a suitable mechanism, at least for GSH circulation in the lens. One argument is that the water, flowing from center to lens periphery, will block the GSH diffusion.

Aging impact on lens GSH homeostasis

The GSH level declines with age in the lens, particularly in the lens nucleus, and this is widely believed to be a major mechanism for age-related nuclear cataract formation(Giblin, 2000; Lou, 2003; Truscott, 2005). Multiple causes have been proposed for this decrease:

1. The age-related loss of synthetic enzymes for *de novo* GSH synthesis is considered one of the major mechanisms. GCL, the GSH *de novo* synthesis rate limiting enzyme, has a 16-fold decrease in activity over a 83-year time frame(Rathbun, 1984). Interestingly, such drastic activity loss is not unequivocally associated with lens GSH content, which is only reduced around 2–3 fold over several decades in normal lenses without cataract(Lou, 2003). This finding further suggests a compensatory mechanism is in place for maintenance of lens GSH homeostasis, which is most likely linked to uptake from surrounding ocular structures, as already argued above. The mechanisms of this loss of function in GSH biosynthesis enzyme is unknown and probably due to a combination of stochastic damage by oxidation and glycation(Beebe et al., 2010; Nagaraj et al., 2012) and other factors, as described in several reviews(Knight, 2000; Liu et al., 2004).
2. Concerning the source of extra-lenticular GSH or its constituent amino acids, the aging ciliary body might have a down-regulation of its amino acid, GSH and

GSSG secretory function to the aqueous humor or vitreous humor. Studies have found that the stroma of the aging ciliary body processes becomes collagenized, and that the processes become less vascularized(Folberg, 1996; Grossniklaus et al., 2013), therefore slowing down secretory functions. Similarly, aging also has a major impact on the retina. The retinal pigment epithelial (RPE) cell numbers are found decreasing at continuous rate from the 20s to 90s in human(Gao and Hollyfield, 1992), and this will have a remarkable impact on GSH efflux if, indeed, RPE cells are responsible for vitreous humor GSH homeostasis.

3. Several studies have found elevated lens stiffness and barrier formation with age(Beebe et al., 2011; Heys et al., 2004; Heys and Truscott, 2008; Sweeney and Truscott, 1998; Truscott, 2000), and a decline in GSH diffusion into the inner layer of the lens. Nuclear cataract is just an extreme example of lens hardening and impaired GSH diffusion, where barely detectable GSH is found in the cataract region, while millimolar levels of GSH are still available in the outer cortex(Pau et al., 1990). More work is needed to understand the molecular mechanisms responsible for the lens metabolic barrier formation during aging.

4. GSH usually works as a key partner with glutathione peroxidase (GPx), glutathione reductase (GR) and glutaredoxin to form a “GSH antioxidant network”, and GSH can be regenerated from this network system(Lou, 2003). However, during aging of the lens, many of the enzymes involved in this network are impaired(Zhu et al., 2010), including the thiol repair system(Wei et al., 2015). This is also reflected by the elevated protein-S-S-glutathione (PSSG) mixed disulfides in aged human lens which have been very well documented in the past(Lou, 2000, 2003; Lou and Dickerson, 1992; Lou et al., 1990; Lou et al., 1995). All of these changes are strongly associated with increased crystallin oxidation and crosslinking(Fan et al., 2006; Fan et al., 2009; Wang et al., 2014) and may ultimately hamper GSH regeneration.

Concluding remarks and perspectives

The lens is an avascular ocular tissue equipped with an unusually high level of GSH. The high concentration of GSH works with its antioxidant partners to keep the lens redox system in check and maintain lens transparency for several decades in humans. In addition to GSH *de novo* synthesis that only occurs in the epithelium and outer cortex, the lens is believed to also take up large portions of GSH from surrounding ocular tissues, i.e. the aqueous humor and the vitreous humor. However, more studies are needed to address the following questions: First, urgent study is needed to understand the mechanisms by which the lens manages to take up GSH from the circulation. If it is bycarrier-based mechanisms, most likely at the anterior pole of the lens, then what is the identity of the transporter? If it is by passive diffusion, most likely at posterior pole of the lens, then where are the sources and the origin of the GSH? Second, what is the contribution of lens GSH uptake vs. *de novo* synthesis in young and aged human lenses? We speculate that GSH uptake from surrounding ocular tissue will take a more important role in aged human lenses due to impaired *de novo* synthesis,. In that case, we need to understand what kinds of genes are adapted with aging.

Third, we need to understand the role of the vitreous humor in lens GSH homeostasis including its dynamics and which ocular tissue is the main source of vitreous GSH. Fourth, more work is needed to understand the transport mechanisms for maintenance of lens nucleus GSH homeostasis, and verify whether gap junctions are indeed responsible for lens nucleus GSH homeostasis. Are other mechanisms rather than gap junctions or those that work together with gap junction playing a role in lens nucleus GSH homeostasis? Lastly, since GSSG is unable to permeate through gap junction channels(Slavi et al., 2014), the obvious question is how does the lens eliminate GSSG if it is beyond the lens regeneration capacity? We believe that addressing these critical questions is urgently needed to understand the age-related decline in lens GSH homeostasis, and open the door to novel therapeutic approaches for the prevention or delay of age-related nuclear cataract. Dr. Beebe has been the pioneer in many research areas of the lens field, such as his recent work on the oxygen regulatory role by the vitreous humor. This has provided us with the new concept that we should place the lens in the context of the eye rather than as an independent island when we study its many biological phenomena.

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Highlights

- Glutathione is playing vital role in lens biology.
- The lens GSH homeostasis is maintained via biosynthesis and transport.
- The aqueous humor and gamma-glutamyl cycle continuously supplies constituent amino acids for GSH synthesis.
- Aqueous and vitreous humor are providing glutathione for transporting into lens.
- The mechanisms of lens GSH transport and nucleus GSH homeostasis are unknown.

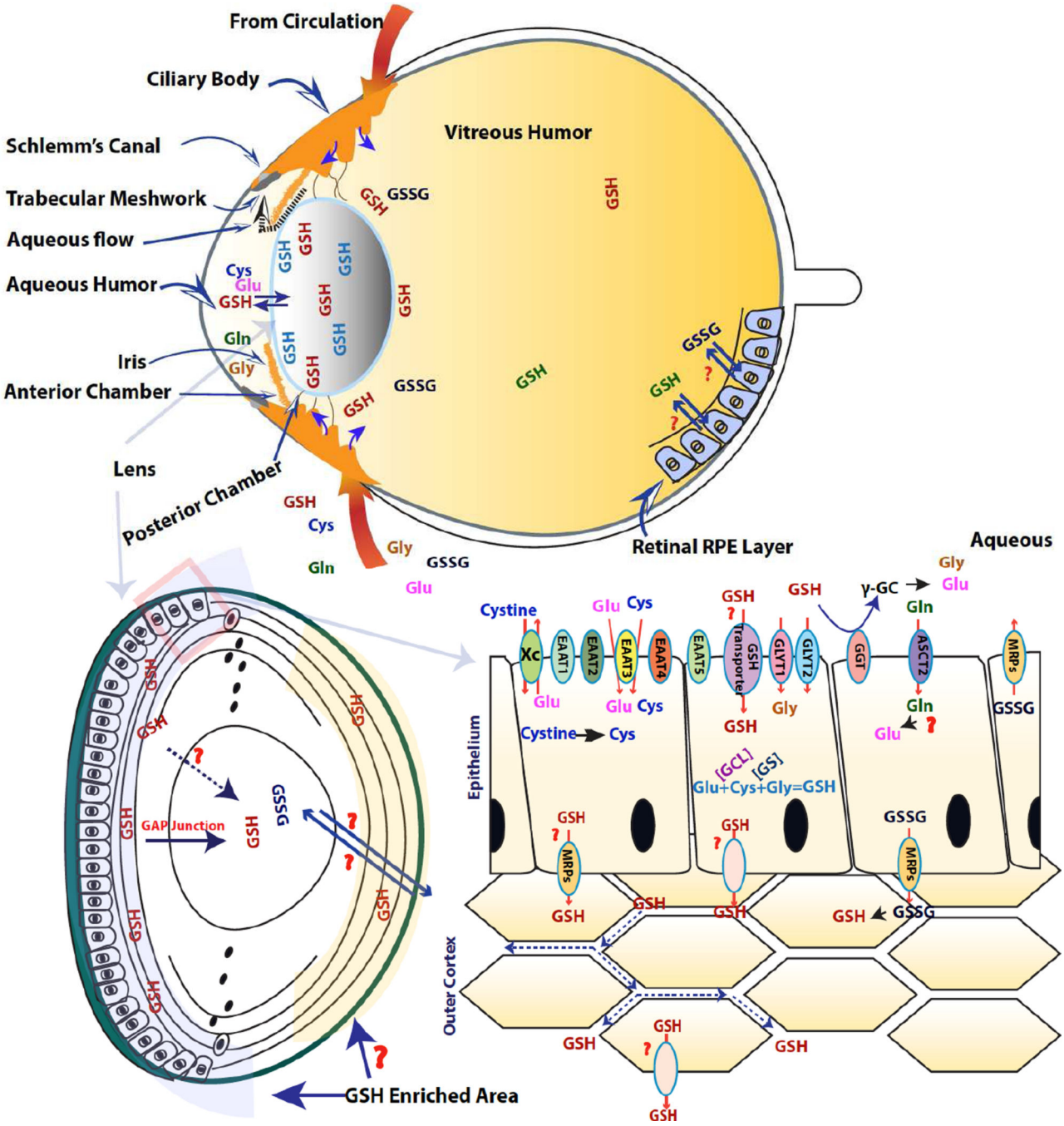

Figure 1. Lens glutathione homeostasis

(Top panel): Illustration of the lens and its conjunction ocular tissues. The ciliary body with its vascular circulation system continuously produces the aqueous humor that contains both the constituent amino acids for GSH synthesis and GSH/GSSG. It first enters the posterior chamber, then reaches to the anterior aqueous humor and then drains out via trabecular meshwork and Shclemm's canal. Both ciliary body and retina also produce molecular solute into the vitreous humor to maintain vitreous humor in a more dynamic, and this may also include GSH and GSSG. The lens will then utilize the amino acids, GSH or GSSG supply to

maintain its GSH homeostasis via *de novo* synthesis and uptake, respectively. The pathway with a question marker indicates that it is currently unknown.

(Lower left panel): The illustration of lens structure. The anterior of the lens is assembled with monolayer of epithelial cells, and their continuously proliferation and differentiation produce lens fiber cells to form outer and inner cortical fiber layer. The lens nucleus is assembled with the embryonic nucleus and the fetal nucleus. The lens epithelial cells and outer cortical fiber cells have the ability to synthesis GSH, while lens nuclear GSH is completely reliant on transport from outer cortex. The outer cortex has been found possess high concentration of GSH than lens nucleus, though whether this is also true in posterior outer cortex is still unclear.

(Lower right panel): Illustration of lens epithelial and outer fiber layer. The epithelial cells are responsible for transporting the constituent amino acids from aqueous humor for GSH synthesis, which is taking place in both epithelial and outer fiber cell layer. The lens epithelial cells may also take up GSH or GSSG from aqueous humor. The high GSH concentration gradient between lens and aqueous humor implicates it might be a carried based transporting mechanisms, though the identity of the transporter is still unknown. The lens fiber cells are enriched with gap junctions, and it has been suggested that gap junction might playing a role in lens nuclear GSH homeostasis.