REVIEW ARTICLE

Proline metabolism and transport in retinal health and disease

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Abstract

The retina is one of the most energy-demanding tissues in the human body. Photoreceptors in the outer retina rely on nutrient support from the neighboring retinal pigment epithelium (RPE), a monolayer of epithelial cells that separate the retina and choroidal blood supply. RPE dysfunction or cell death can result in photoreceptor degeneration, leading to blindness in retinal degenerative diseases including some inherited retinal degenerations and age-related macular degeneration (AMD). In addition to having ready access to rich nutrients from blood, the RPE is also supplied with lactate from adjacent photoreceptors. Moreover, RPE can phagocytose lipid-rich outer segments for degradation and recycling on a daily basis. Recent studies show RPE cells prefer proline as a major metabolic substrate, and they are highly enriched for the proline transporter, SLC6A20. In contrast, dysfunctional or poorly differentiated RPE fails to utilize proline. RPE uses proline to fuel mitochondrial metabolism, synthesize amino acids, build the extracellular matrix, fight against oxidative stress, and sustain differentiation. Remarkably, the neural retina rarely imports proline directly, but it uptakes and utilizes intermediates and amino acids derived from proline catabolism in the RPE. Mutations of genes in proline metabolism are associated with retinal degenerative diseases, and proline supplementation is reported to improve RPE-initiated vision loss. This review will cover proline metabolism in RPE and highlight the importance of proline transport and utilization in maintaining retinal metabolism and health.

Keywords Proline · Metabolism · Transport · Retina · Retinal pigment epithelium · Retinal disease

Abbreviations

RPE	Retinal pigment epithelium
PRODH	Proline dehydrogenase
SLC	Solute carrier family
OAT	Ornithine aminotransferase
P5C	Pyrroline-5-carboxylate
TCA cycle	Tricarboxylic acid cycle
TGF-β	Transforming growth factor-beta
EMT	Epithelial-to-mesenchymal transition
AMD	Age-related macular degeneration
PVR	Proliferative vitreoretinopathy

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Introduction

Vertebrate retinas are light-sensitive neuronal tissues with large metabolic demands for phototransduction, maintenance of ion gradients, and biosynthesis of daily-shed photoreceptor outer segments (Hurley et al. 2015). Due to the absence of a direct blood supply, photoreceptors must absorb nutrients, including glucose, amino acids, fatty acids, ketone bodies and vitamins through the retinal pigment epithelium (RPE) (Du et al. 2013; Hurley et al. 2015; Li et al. 2020). The RPE consists of a monolayer of polarized epithelial cells that transports metabolites between the choroidal blood supply and the outer retina. Similar to tumor cells, retinas have robust aerobic glycolysis (the Warburg effect) and produce large amounts of lactate from glucose. In turn, RPE utilizes lactate from the Warburg effect as a fuel to preserve glucose for the retina (Kanow et al. 2017). RPE phagocytizes ~ 10% of outer segments shed from photoreceptors each day and

processes them into fatty acids and ketone bodies as metabolic substrates for the retina (Adijanto et al. 2014; Reyes-Reveles et al. 2017). RPE could also recycle succinate into malate to feed the retina (Bisbach et al. 2020). This synergistic effect is crucial for photoreceptor survival, as metabolic dysfunction or degeneration of RPE can result in photoreceptor degeneration, leading to blindness in retinal degenerative diseases, including inherited retinal degenerations and age-related macular degeneration (AMD) (Ferrington et al. 2020; Lefevere et al. 2017; Zhao et al. 2011a). Likewise, separation of the neural retina from RPE in retinal detachment causes rapid photoreceptor degeneration (Lo et al. 2011). Of note, recent studies demonstrate that RPE prefers to use proline as a metabolic substrate, and dietary proline protects retinas from degeneration induced by the oxidative damage in the RPE (Chao et al. 2017; Yam et al. 2019). Mutations of genes involved in proline metabolism have previously been shown to cause inherited retinal degeneration (O'Donnell et al. 1978; Wolthuis et al. 2014). Furthermore, the proline transporter, SLC6A20, is highly enriched in the RPE and is associated with macular thickness (Bennis et al. 2015; Gao et al. 2019; Strunnikova et al. 2010). This review discusses proline transport and metabolism in RPE and how proline can affect retinal metabolism, retinal health, and retinal degenerative diseases.

## Proline catabolism, synthesis and transport in RPE

The metabolic fate of proline

The RPE is capable of using proline as a major metabolic substrate. Typical RPE culture medium contains 0.447 mM proline, which is almost completely consumed within 24 h in primary human RPE culture (Chao et al. 2017; Yam et al. 2019). Infusion of ^{13}C proline in vivo also shows that RPE actively consumes this substrate (Yam et al. 2019). Why does RPE need so much free proline? The fate of proline utilization includes the synthesis into proline-rich proteins and catabolism into ornithine and glutamate for the urea cycle and mitochondrial tricarboxylic acid (TCA) cycle (Fig. 1).

Proline makes up to 25% of collagen, the most abundant protein in the human body (Phang et al. 2010). Collagen is a major component of the extracellular matrix (ECM), which is very dynamic in its turnover to interact with cytokines and growth factors in response to cellular environmental changes. RPE cells attach to a collagen-rich, five-layered ECM structure called Bruch's membrane (BrM), a molecular sieve for small molecule exchange between RPE and choroid blood circulation (Murali et al. 2020). RPE is critical for the composition, stability and thickness of BrM in healthy and diseased

conditions (Campochiaro et al. 1986). ^{14}C-proline tracing showed that cultured RPE synthesizes and secretes collagen in a time-dependent manner, peaking between 60 and 108 days (Li et al. 1984). Proline analogs such as *cis*-hydroxyproline and azetidine carboxylic acid can incorporate into collagen polypeptides, destabilizing the collagen structure, inhibiting collagen synthesis and accelerating collagen degradation (Tan et al. 1983; Uitto et al. 1984). *Cis*-hydroxyproline inhibits RPE proliferation and collagen synthesis, while simultaneous addition of proline to the culture blocks these inhibitory effects (Yoo et al. 1997). The thickness of ECM underneath the RPE cell layer can reach 30 μM after 360 days of culture (Kigasawa et al. 1998). About half of proline in collagen peptides is post-translationally modified into 4-hydroxyproline or 3-hydroxyproline by prolyl-4-hydroxylase or prolyl-3-hydroxylase (Wu et al. 2011). This hydroxylation is important for increasing the stability of the collagen triple helix structure. Aside from the 28 classic collagens, many other proteins contain collagen-like triple helix domains, including complement 1q (C1q), adiponectin, ficolins, and macrophage receptors (Ricard-Blum 2011). These proteins are critical in immune recognition and anti-inflammation, both of which are involved in the pathogenesis of AMD (Cao et al. 2015; Nita et al. 2014a; Tan et al. 2020).

Proline is catabolized through flavin-dependent proline dehydrogenase (PRODH) in the mitochondrial matrix into pyrroline-5-carboxylate (P5C) (Fig. 1). PRODH can donate electrons directly to ubiquinone through FADH$_2$. P5C is a key intermediate in proline metabolism, as it connects with other amino acids and also serves as a precursor for proline synthesis. P5C produces glutamate through a NAD-dependent P5C dehydrogenase (P5CDH), encoded by *ALDH4A1* gene in the mitochondria. Glutamate, an important neurotransmitter in the retinal neurons, is also a precursor for glutamine, gamma-aminobutyric acid (GABA) and mitochondrial TCA cycle intermediates (Li et al. 2020). Glutamate can convert into αKG to enter the TCA cycle through either glutamate dehydrogenase or transaminases such as aspartate transaminase (AST), alanine transaminase (ALT), and phosphoserine aminotransferase (PSAT). RPE relies on aminotransferases rather than glutamate dehydrogenase for this process (Xu et al. 2020). Proline stimulates the production of glutamate, aspartate, alanine, serine, αKG, and other TCA cycle intermediates in human RPE culture, whereas the inhibition of PRODH substantially decreases these metabolites (Chao et al. 2017). Similar to cancer cells, RPE cells can use reductive carboxylation that produces mitochondrial citrate directly from αKG through NADP-dependent isocitrate dehydrogenase 2 (IDH2). This reductive carboxylation allows for the synthesis of citrate without acetyl-CoA and the export of NADPH into the cytosol to confer resistance to oxidative damage (Du et al. 2016). Tracing with ^{13}C proline
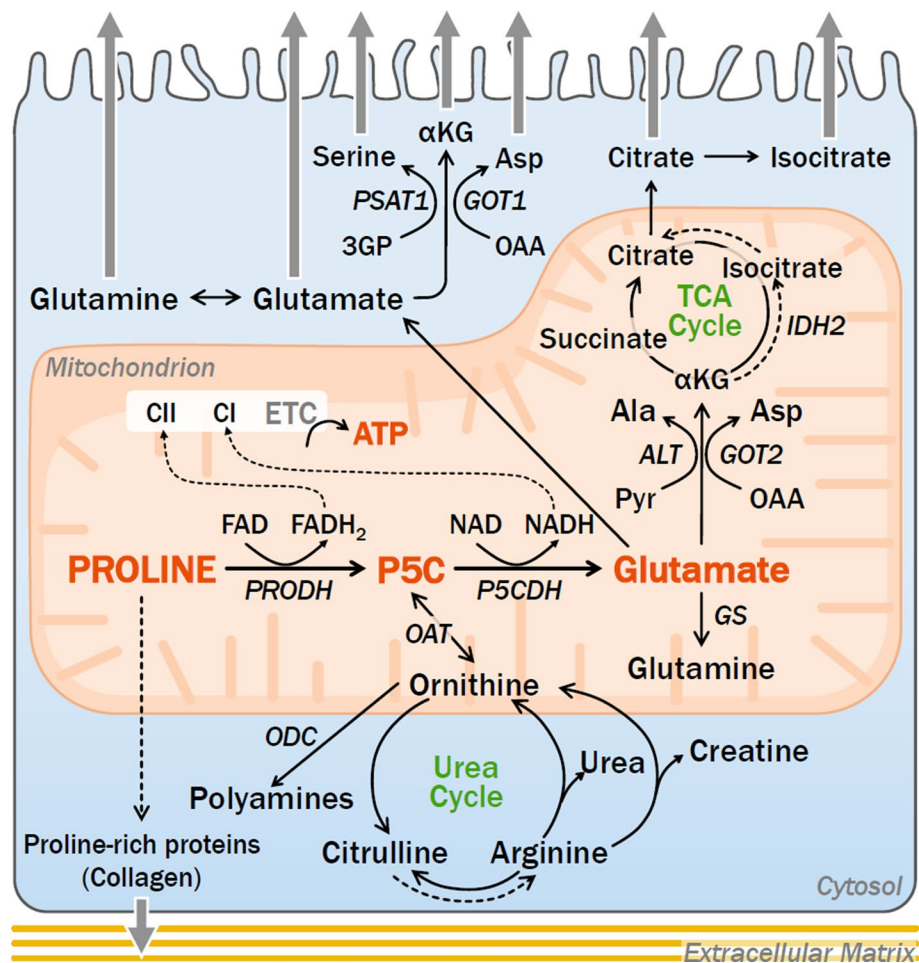
Fig. 1 The metabolic fate of proline. Proline is catabolized into P5C, a key intermediate which serves as a precursor for glutamate, glutamine and ornithine. FADH$_2$ and NADH from the catabolism fuel the electron transport chain to generate ATP. Glutamate can be transaminated into αKG, producing alanine, aspartate and serine. RPE is highly efficient in reductive carboxylation, generating mitochondrial citrate directly from αKG through IDH2. Substrates including glutamine, glutamate, serine, αKG, aspartate, citrate and isocitrate are exported to the apical photoreceptors. P5C is reversibly converted into ornithine depending on the availability of precursors. Ornithine enters the urea cycle to produce arginine, citrulline, and creatine. Ornithine is also a precursor for polyamines. Additionally, proline is incorporated into proteins, especially proline-rich proteins such as collagen to form the RPE extracellular matrix. *3PG* 3-phosphoglycerate, *αKG* alpha-ketoglutarate, *Ala* alanine, *ALT* alanine transaminase, *Asp* aspartate, *AST* aspartate transaminase (GOT1 & GOT2 isozymes), *GS* glutamine synthetase, *IDH2* isocitrate dehydrogenase 2, *OAA* oxaloacetate, *OAT* ornithine aminotransferase, *ODC* ornithine decarboxylase, *P5C* pyrroline-5-carboxylate, *P5CDH* P5C dehydrogenase, *PRODH* proline dehydrogenase, *PSAT* phosphoserine aminotransferase, *Pyr* pyruvate

in RPE shows that proline can efficiently be catabolized through this reductive carboxylation pathway (Chao et al. 2017).

P5C can also reversibly convert into ornithine through ornithine aminotransferase (OAT), depending on the availability of P5C or ornithine. Ornithine is a critical intermediate in the urea cycle and is closely involved in the metabolism of arginine, citrulline, creatine and polyamines (Wu et al. 2005) (Fig. 1). In neonates, OAT is an important source for arginine. OAT whole-body knockout mice die within 2 days of birth with symptoms of ornithine deficiency but survive with arginine administration (Wang et al. 1995). Human

neonates with OAT deficiency have low concentrations of ornithine and citrulline but high concentrations of proline in their plasma (de Sain-van der Velden et al. 2012). In cultured human fetal RPE, ^{13}C proline labels about half of the pool of ornithine within one hour (Chao et al. 2017), confirming that OAT is active and that ornithine turns over rapidly in the RPE.
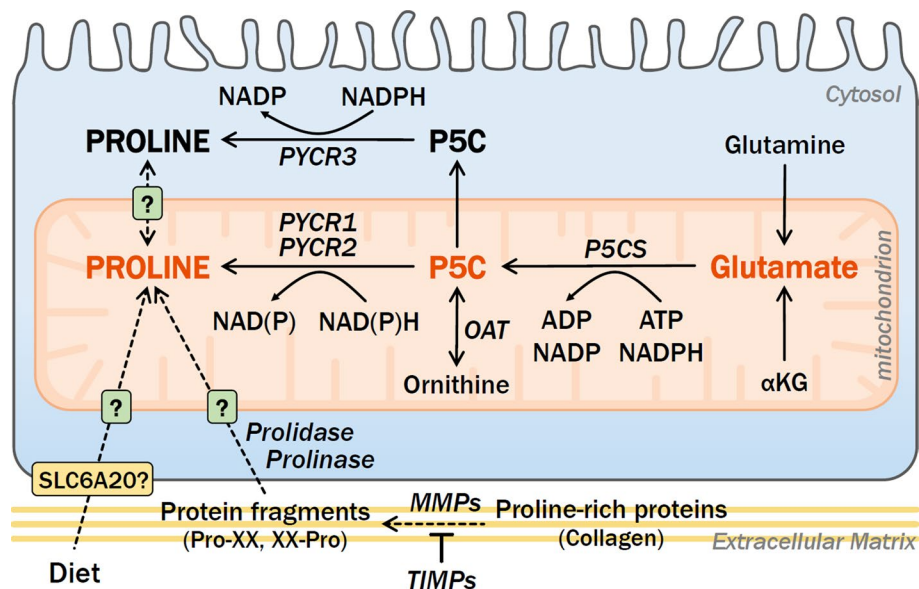
Sources of free proline

In addition to dietary intake, the major sources of proline in mammalian tissues are from de novo synthesis and

degradation of proline-enriched proteins such as collagen. Glutamate, glutamine, ornithine and arginine are precursors for proline synthesis, but the pathways can be cell- and species-specific (Fig. 2). P5C is the common intermediate in proline synthesis. Glutamate and glutamine can produce P5C through P5C synthase (P5CS), encoded by the *ALDH18A1* gene. P5CS is an ATP- and NADPH-dependent mitochondrial enzyme. Patients with mutations of *ALDH18A1* have hypoprolinemia and retinal degeneration (Baumgartner et al. 2000, 2005; Wolthuis et al. 2014). Fibroblasts from these patients are deficient in their ability to convert glutamate into proline for protein synthesis, demonstrating that proline synthesis from glutamate is critical for normal proline metabolism. Ornithine and arginine can convert into P5C through the reverse reaction of OAT. The direction towards proline synthesis is dominant in adults, which is the opposite of neonates (de Sain-van der Velden et al. 2012; Wang et al. 1995). The abnormalities of OAT mutations in patients are limited to gyrate atrophy in the eye (Mitsubuchi et al. 2008), suggesting that there might be a special need for proline metabolism in the RPE and retina.

Proline synthesis from P5C needs NAD(P)H-dependent P5C reductase (PYCR). PYCR has three known isoforms using both NADH and NADPH. PYCR1 and 2 are located in the mitochondria and prefer NADH as the co-factor, while PYCR3 is located in the cytosol and prefers NADPH (Fig. 2). The expression of PYCR isoforms is cell-specific, and different isoforms may contribute differently to proline synthesis. In Lu1205 cells, knockdown of PYCR1 and PYCR2 reduce the ratio of proline to glutamate, while knockdown of PYCR3 decreases the ratio of proline to ornithine (De Ingeniis et al. 2012). It is postulated that PYCR3 and PRODH can work together to shuttle proline between mitochondria and cytosol, also called the proline cycle. This cycle can transfer electrons from NADPH into the mitochondria and stimulate flux of the pentose phosphate pathway (PPP) (Phang et al. 2010). Mutations of *PYCRs* have similar clinical features to *ALDH18A1* but without visual defects (Wolthuis et al. 2014). Although, the knockout of PYCR1 in zebrafish shows disrupted RPE and retinal degeneration (Liang et al. 2019).

Proline incorporated proteins are another important source of free proline. Proline-rich collagen can serve as a reservoir to store proline. During stress or nutrient-deprivation, collagen degrades into proline to fuel energy metabolism (Olivares et al. 2017). Collagen can be cleaved into peptides by proteases such as matrix metalloproteinases (MMPs), which are inhibited by specific endogenous tissue inhibitors of MMPs (TIMPs). MMPs and TIMPs are critical regulators of ECM turnover and remodeling. Glucose

Fig. 2 Sources of proline in RPE. Dietary proline is taken up by RPE cells likely through SLC6A20 transporter. Collagen and other proline-rich proteins are degraded via MMPs, and proline-containing fragments are further degraded into free proline by prolidase and prolinase enzymes. TIMPs are endogenous inhibitors of MMPs, whose mutation results in excess degradation of ECM. Proline is also generated via de novo synthesis from glutamate and ornithine substrates. Glutamate is converted into P5C intermediate through P5CS, which is then reduced to proline by PYCRs. Ornithine and arginine can also convert into proline via reverse reaction of OAT. Biosynthesis of proline is energetically expensive, requiring 2 NADPH and 1 ATP from glutamate or glutamine pathway, and 1 NAD(P)H from arginine or ornithine pathway. The genetic identity of transporters responsible for proline transport between cytosol and mitochondria is still unknown. *αKG* alpha-ketoglutarate, *MMP* matrix metalloproteinases, *OAT* ornithine aminotransferase, *P5C* pyrroline-5-carboxylate, *P5CS* P5C synthase, *PYCR1,2,3* P5C reductase (isoforms 1,2,3), *TIMP* tissue inhibitors of MMP

stress, hypoxia and inflammation can activate MMPs to degrade collagen (Pandhare et al. 2009; Phang et al. 2010). Mutations of many ECM genes including MMPs and TIMPs are associated with retinal degeneration (Anand-Apte et al. 2019; Garcia-Onrubia et al. 2020); however, how the abnormal ECM turnover impairs proline supply and metabolism in RPE remains unknown.

Cells can take up MMP-hydrolyzed collagen fragments through the urokinase receptor associated protein (uPARAP/Endo 180), and further degrade them into tripeptides, dipeptides and free amino acids by cathepsins and peptidases (Curino et al. 2005; Phang et al. 2010). The unique pyrrolidine ring in proline induces conformational constraints on the peptide bond to protect the iminopeptides from hydrolysis by most peptidases (Cunningham and O'Connor 1997). Prolidase and prolinase are the only known enzymes that are capable of hydrolyzing proline-containing dipeptides, also called iminodipeptides, into free proline. Prolidase such as peptidase D (PEPD) specifically hydrolyzes C-terminal proline, while prolinase such as dipeptidyl peptidase IV (DPP4) and its family, specifically cleaves off N-terminal proline

(Misiura and Miltyk 2020; Waumans et al. 2015). In addition to collagen, many bioactive peptides contain proline to protect them from unexpected degradation. Prolidase and prolinase can degrade proline in these peptides to modulate the immune response, cell growth and neural development (Dunaevsky et al. 2020; Misiura and Miltyk 2020). Mutations of *PEPD* gene can cause prolidase deficiency, a rare autosomal recessive disorder with severe skin lesions, immunodeficiency and mental retardation (Kitchener and Grunden 2012). Some *PEPD* deficiency patients also have ocular symptoms including amblyopia, keratitis, optic atrophy, and chorioretinal atrophy (Ogata et al. 1981).

Proline transport and transporters

Proline requires specific transporters for its cellular import and export to maintain proline homeostasis. Twelve transporters are capable of transporting proline (Table 1). SLC6A20 is the only proline transporter that is highly enriched in the RPE/choroid (Takanaga et al. 2005b). Multiple independent findings demonstrate that *SLC6A20* or the

Table 1 List of proline transporters in humans

Gene	Synonym	Substrates*	Tissue specificity	Localization	Ions**	References
SLC6A7	PROT	Pro	Brain	Membrane	Na$^+$, Cl$^-$	Shafqat et al. (1995)
SLC6A15	B0AT2	Leu, Val, Ile, Met, Pro	Brain	Nucleus, vesicles	Na$^+$	Takanaga et al. (2005a)
SLC6A17	NTT4	Leu, Met, Pro, Cys, Ala, Gln, Ser, His, Gly	Brain	Vesicles	Na$^+$, Cl$^-$	Hagglund et al. (2013); Zaia and Reimer (2009)
SLC6A19	B0AT1	Leu, Met, Ile, Val, Asn, Phe, Ala, Ser, Thr, Gly, Pro	Intestine	Membrane	Na$^+$	Broer (2006)
SLC6A20 Slc6a20a (mouse)	SIT1	Pro, betaine, 4-OH-Pro	RPE/choroid, intestine, kidney	Membrane	Na$^+$, Cl$^-$	Broer et al. (2009); Kowalczuk et al. (2005)
SLC36A1	PAT1	Ala, Gly, GABA, taurine, Pro, 4-OH-Pro	Brain, intestine	Membrane, lysosome, nucleus	H$^+$	Jensen et al. (2014); Schroder et al. (2007)
SLC36A2	PAT2	Ala, Gly, Pro, 4-OH-Pro, scarosine	Kidney, muscle	Membrane	H$^+$	Kennedy et al. (2005)
SLC36A4	PAT4	Trp, Pro	Ubiquitous	Membrane, cytosol, Golgi	Unknown	Pillai and Meredith (2011)
SLC38A1	SNAT1	Ala, Ser, Gln, Asn, His, Cys, Met, Gly, Thr, Pro, Tyr, Val	Ubiquitous	Membrane	Na$^+$	Albers et al. (2001)
SLC38A2	SNAT2	Ala, Met, Asn, Gln, Ser, Pro, Gly, Thr, Leu, Phe	Ubiquitous	Membrane, vesicles	Na$^+$	Hatanaka et al. (2000)
SLC38A4	SNAT4	His, Arg, Ala, Asn, Lys, Gly, Gln, Ser, Pro, Leu, Phe	Liver	Membrane	Na$^+$	Hatanaka et al. (2001)
SLC1A4	ASCT1	Cys, Ala, Ser, Thr, Pro, 4-OH-Pro	Brain	Membrane	Na$^+$	Pinilla-Tenas et al. (2003)

4-OH-Pro 4-hydroxyproline, *Ala* alanine, *Arg* arginine, *Asn* asparagine, *Cys* cysteine, *GABA* gamma-aminobutyric, *Gln* glutamine, *Gly* glycine, *His* histidine, *Ile* isoleucine, *Leu* leucine, *Lys* lysine, *Met* methionine acid, *Phe* phenylalanine, *Pro* proline, *Ser* serine, *Thr* threonine, *Trp* tryptophan, *Tyr* tyrosine, *Val* valine

*Substrates are listed in the order of affinity from high to low. **Ions co-transported with the substrates

mouse homologue *Slc6a20a* is a highly conserved gene. By comparing the expression of mature human RPE cells with 53 human tissues, Liu et al. found that SLC6A20 is an RPE-specific gene (Liu et al. 2019). By comparing the expression of genes from native and cultured RPE with 78 different tissues, Sheldon Miller and colleagues selected 154 "RPE signature genes" including *SLC6A20* that were expressed tenfold higher in the RPE than any other tissues (Strunnikova et al. 2010). Similarly, Liao et al. profiled global gene expression of stem-cell-derived RPE cells, native and cultured human fetal RPE cells. They identified a set of 87 RPE signature genes in which *SLC6A20* is one of them (Liao et al. 2010). Meanwhile, Arthur Bergen and colleagues profiled native human RPE and native C57 mouse RPE, and found *SLC6A20* or *Slc6a20a* as one of the 22 signature genes shared by both human and mouse RPE (Bennis et al. 2015). Interestingly, proline consumption increases during RPE differentiation (Yam et al. 2019), and *SLC6A20* transcripts are substantially upregulated during RPE maturation, whereas other proline transporters are either not detected or unchanged (Radeke et al. 2015). These reports strongly suggest that SLC6A20 is responsible for mediating the robust proline consumption in differentiated RPE.

SLC6A20 is Na^+ and Cl^- dependent. Two Na^+ and one Cl^- molecule are co-transported together with each proline molecule (Broer et al. 2009). Human *SLC6A20* is a unique gene with two transcript variants. Compared to variant 1, variant 2 lacks an alternate in-frame exon, resulting in a shorter protein. Mouse *SLC6A20* has two homologous genes, *Slc6a20a* and *Slc6a20b*, located next to each other along the gene locus. *Slc6a20a* has 92% homology as human *SLC6A20* and is functionally active in transporting proline (Kowalczuk et al. 2005). Slc6a20b has a longer N-terminus with 81% identity to human SLC6A20 but does not transport proline or other amino acids; hence its function still remains unknown (Kowalczuk et al. 2005).

In addition to RPE, SLC6A20 is also found in epithelial cells of the intestine, kidney and lung. SLC6A20 genetic polymorphisms are associated with Hirschsprung's disease, iminoglycinuria, degenerative macular diseases and severe Covid-19 with respiratory failure (Ellinghaus et al. 2020; Gao et al. 2018; Kim et al. 2014; Lee et al. 2016; Xie et al. 2019). Hirschsprung's disease is a congenital and heterogeneous disorder characterized by missing nerves in the colon. The availability of proline may be important for neuronal cell development. Iminoglycinuria, a rare inherited disorder associated with multiple genetic mutations of glycine and proline transporters, results in poor amino acid absorption in the kidney and excess urinary excretion of proline, hydroxyproline and glycine. In humans, the macula is responsible for central, high acuity vision. Macular degenerative diseases such as AMD can result in structural changes to reduce macular thickness. A genome-wide association study (GWAS) of 68,423 participants identifies 139 loci associated with macular thickness, and *SLC6A20* is one of the four most significant loci (Gao et al. 2018). Furthermore, SLC6A20 expression is downregulated in retinas from human donors with AMD (Ratnapriya et al. 2019). Interestingly, a GWAS study of severe Covid-19 patients with respiratory failure identifies *SLC6A20* as one of six associated genes (Ellinghaus et al. 2020). SLC6A20 can functionally interact with angiotensin-converting enzyme 2 (ACE2), the receptor for Covid-19 spike glycoprotein in the intestine and lung (Camargo et al. 2020; Singer et al. 2012; Wang et al. 2020). The expression of ACE2 in the RPE is very low, but the overexpression of ACE2 shows protection against RPE cell death and diabetic retinopathy (Dominguez et al. 2016; Fu et al. 2017). It is unclear whether this protective mechanism is associated with SLC6A20 function. Despite many genetic association studies, the significance of SLC6A20 in physiology and disease remains largely unknown.

In addition to plasma transporters, proline also needs to be transported across the mitochondrial membrane to be oxidized by PRODH and P5CDH, located in the mitochondrial matrix. An early study of isolated rat liver mitochondria showed that proline does not travel through mitochondria by free diffusion but instead by an unidentified energy-dependent transporter (Meyer 1977). Using ^{14}C proline and spectroscopic measurements in isolated rat kidney mitochondria, Atlante et al. characterized two mitochondrial proline transporters: proline uniporter and proline/glutamate antiporter (Atlante et al. 1994). A mitochondrial proline transporter was also characterized in tsetse fly flight muscle mitochondria, which primarily use proline for energy (Njagi et al. 1992). Although the biochemical properties of mitochondrial proline transporters have been extensively characterized, the genetic identities of these transporters a still unknown.

Proline metabolism in retinal health

RPE uses proline as a metabolic fuel to nourish the neural retina

Proline is an energy substrate in many organisms including bacteria, plants, and animals (McDonald et al. 2018). Some species such as the tsetse fly and Colorado potato beetle use proline as the primary fuel to power their flight. Interestingly, the utilization of proline in their muscles stimulates lipolysis of the fat body to synthesize more proline (Arrese and Soulages 2010). Proline can serve as an "alternative fuel" to generate oxaloacetate to enhance the oxidation of acetyl-CoA through TCA cycle (McDonald et al. 2018). Both human and mouse RPE prefer to use proline to fuel their mitochondrial TCA cycle (Chao et al. 2017; Yam et al.

2019). The addition of proline doubles the maximum O_2 consumption compared to glucose alone in human RPE (Yam et al. 2019). This is consistent with reports in other species that proline can elicit maximal mitochondrial respiration (McDonald et al. 2018). However, the neural retina rarely uses proline directly for its mitochondrial metabolism (Yam et al. 2019). Its robust production of lactate and succinate may repress the activity of PRODH, and the hypoxic microenvironment can downregulate complex IV (Bisbach et al. 2020; Hancock et al. 2016; Kowaloff et al. 1977). These factors may make proline catabolism unfavorable in the neural retina. Nevertheless, the neural retina uses proline indirectly from the RPE, which exports proline-derived intermediates such as citrate, glutamate and aspartate towards the neural retina (Fig. 3).

What is the advantage of using proline as a metabolic substrate, especially in the RPE? First, proline has the highest solubility among the 20 amino acids, making it easy to store and transport (Bowden et al. 2018). It is 15-fold more soluble in aqueous solutions than glutamine, glutamate or branched chain amino acids. Second, proline synthesis is energetically expensive. Proline biosynthesis from glutamate or glutamine requires 2 NADPH and 1 ATP, while proline biosynthesis from arginine or ornithine requires 1 NAD(P)H (Fig. 2). NADPH is critical for energy metabolism and cellular antioxidant defense (Bradshaw 2019). RPE lives in a highly oxidative environment, demanding efficient NADPH supply to combat oxidative stress (Datta et al. 2017; Du et al. 2016). The uptake of proline can not only spare NADPH, but also result in the production of NADPH and glutathione (Fig. 4). Third, proline efficiently replenishes TCA cycle intermediates, despite the abundance of other substrates including glucose, lactate and fatty acids. Conventionally, these substrates enter the TCA cycle as acetyl-CoA which
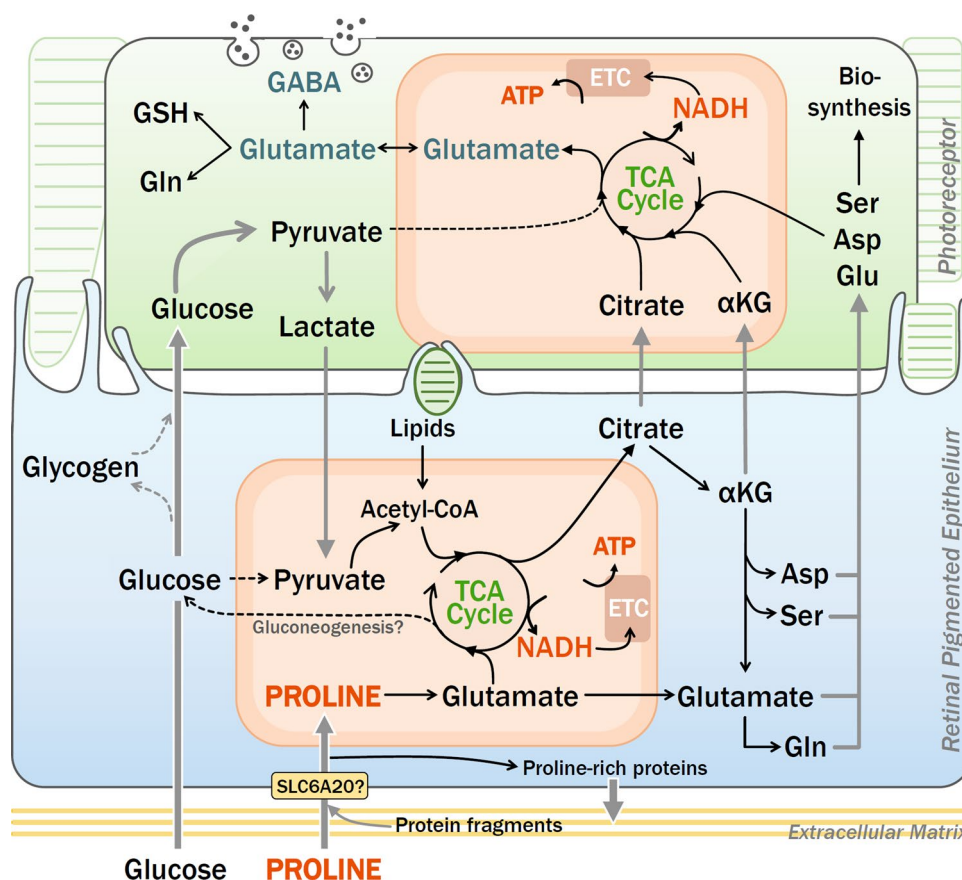
Fig. 3 RPE uses proline to fuel metabolism in both RPE and the retina. Blood glucose enters the RPE to be stored as small amounts of glycogen, and used minimally in the RPE mitochondrial metabolism. Most glucose is transported into the retina, which undergoes robust aerobic glycolysis to produce massive amounts of lactate. The exported lactate can be utilized by RPE as a fuel to preserve glucose for the retina. RPE also phagocytoses shed photoreceptor outer segments and degrades lipids to be used in the TCA cycle. Proline in the RPE serves as both a carbon source to replenish TCA cycle interme- diates, and as a nitrogen source to generate amino acids including glutamate and aspartate. These intermediates are exported in large amounts to fuel the TCA cycle in the retina, and support biosynthesis of lipids to replenish outer segments. Glutamate in the retina is an important neurotransmitter, and also the precursor for GABA, glutamine and GSH. *αKG* alpha-ketoglutarate, *Asp* aspartate, *GABA* gamma-aminobutyric acid, *Gln* glutamine, *Glu* glutamate, *GSH* glutathione, *Ser* serine
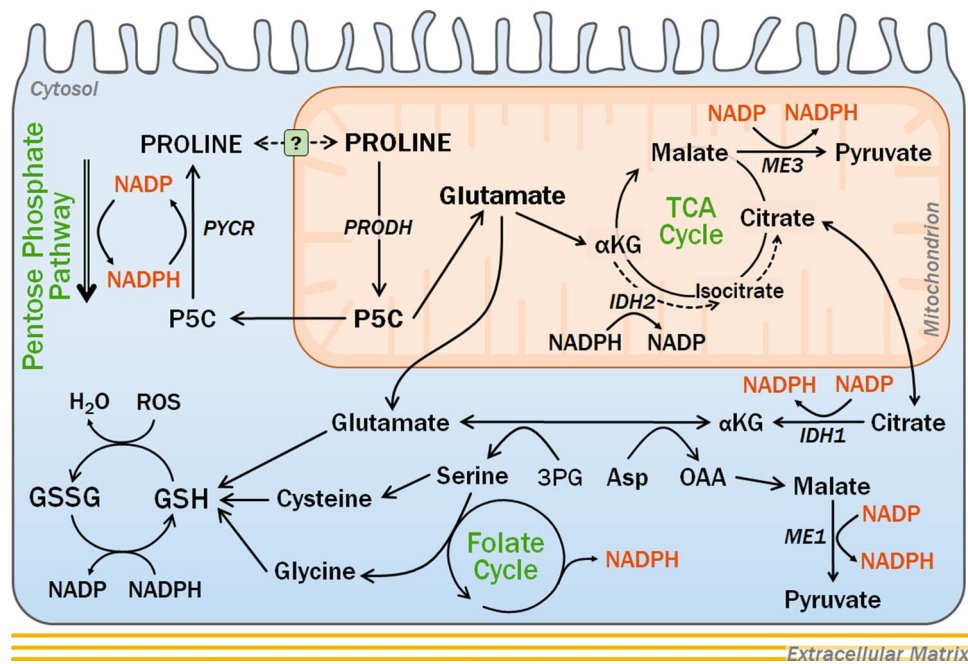
Fig. 4 Proline generates GSH and NADPH to counter oxidative stress. Proline can shuttle between the mitochondria and cytosol to form the proline cycle, which transfers electrons from NADPH into the mitochondria and stimulate flux of the pentose phosphate pathway. Proline catabolism can stimulate NADPH formation by driving malic enzymes, folate cycle and reductive carboxylation through IDH1/2. Glutamate, cysteine and glycine forms the tripeptide GSH, which is used by antioxidant enzymes to scavenge reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). Oxidized glutathione (GSSG) is then reduced back to GSH by NADPH-dependent glutathione reductase, thereby consuming NADPH. *3PG* 3-phosphoglycerate, *αKG* alpha-ketoglutarate, *Asp* aspartate, *IDH* isocitrate dehydrogenase (isoforms 1,2), *GSH* glutathione, *GSSG* oxidized GSH, *ME* malic enzyme (isoforms 1,3), *OAA* oxaloacetate, *P5C* pyrroline-5-carboxylate, *ROS* reactive oxygen species

is mostly oxidized as CO_2 and H_2O, and produces intermediates that leave the cycle (mitochondrial efflux) to support key biosynthetic pathways including synthesize of amino acids, fatty acids, or other nutrients (Fig. 3). To sustain the TCA cycle in the presence of fast influx and efflux, four or five carbon molecules are required to replenish the cycle, a process called anaplerosis. Stable isotope tracing studies show that proline-derived TCA cycle intermediates are exported to support retinal mitochondrial metabolism (Chao et al. 2017; Yam et al. 2019). Fourth, proline is an important nitrogen source. Our recent studies show that both human and mouse retinal explants preferentially uptake glutamate and aspartate, whereas proline could generate and release these amino acids to be used by the retina (Chao et al. 2017; Li et al. 2020). Glutamine is a common precursor for glutamate and aspartate; however, glutamine catabolism can also produce toxic free ammonia. Interestingly, when proline and other nutrients are available, RPE exports rather than consumes glutamine (Li et al. 2020). In rat hepatocytes, proline produces glutamate and aspartate much faster than glutamine (Baquet et al. 1991). Finally, proline can increase the availability of glucose. Photoreceptors in the outer retina rely on glucose for its energy metabolism, which has to be transported across RPE (Kanow et al. 2017). The block of glucose transport or excessive glucose utilization in the RPE is sufficient to cause photoreceptor degeneration (Kurihara et al. 2016; Swarup et al. 2019). Lactate utilization in the RPE could similarly spare glucose for the photoreceptors (Kanow et al. 2017). Additionally, proline is also a known glucogenic amino acid and is used in supplements to maintain blood glucose during exercise (Nogusa et al. 2014). It was shown to potently stimulate glycogen synthesis in hepatocytes (Baquet et al. 1991; Bode et al. 1992). Glycogen is rapidly accumulated in cultured RPE after refeeding the media (Senanayake et al. 2006). Preferential proline uptake by the RPE may contribute to glycogenesis to support the high metabolic demand for glucose by photoreceptors.

Proline generates glutathione and NADPH to protect against oxidative stress

Glutathione (GSH) coupling with NADPH is the primary antioxidant system in mammalian cells (Forman et al. 2009). GSH is a tripeptide synthesized from glycine, glutamate and cysteine. Its regeneration from oxidized GSH (GSSG) requires NADPH. Inhibition of PRODH in human RPE substantially diminishes the amount of intracellular GSH and its precursors (Yam et al. 2019), indicating that proline is a

critical component for GSH biosynthesis, likely by increasing the availability of glutamate, glycine and serine (a precursor for cysteine) in the RPE (Fig. 4). Consistently, proline has been shown to confer strong protection against oxidative damage in RPE and other cells in vitro (Krishnan et al. 2008; Natarajan et al. 2012). Multiple pathways contribute to NADPH production including the PPP, NADP-dependent IDH1/2, NADP-dependent malic enzyme, and serine-driven folate pathways (Fan et al. 2014) (Fig. 4). Key enzymes in these NADPH production pathways are highly enriched in the RPE compared to the neural retina in both humans and mice (Li et al. 2020). PPP is the classic pathway for NADPH production. However, a recent NADPH tracing study shows that serine-driven NADPH is comparable to PPP in cancer cells (Fan et al. 2014). We have reported that reductive carboxylation through IDH1/2 is highly active in human RPE cells, conferring RPE protection against oxidative damage (Du et al. 2016). Proline can activate PPP and NADP-dependent malic enzymes, increase the flux of reductive carboxylation through IDH1/2, and stimulate serine metabolism in folate pathways (Allmann et al. 2013; Chao et al. 2017; Hagedorn and Phang 1986). Consequentially, dietary proline improves visual function in an RPE-specific oxidative damage mouse model (Yam et al. 2019). These protections may be attributed to the enhancement of the GSH and NADPH system through proline catabolism.

Proline is specifically used in differentiated RPE

Terminally-differentiated RPE is pigmented to reduce light damage in the tissue, contains tight junctions to form a blood-retinal barrier, and polarized to bidirectionally transport nutrients and waste products (Lakkaraju et al. 2020). Human RPE cells in culture typically take 4–6 weeks to adopt a polarized epithelial morphology. Interestingly, proline is the only nutrient whose consumption increases with RPE differentiation (Yam et al. 2019). Poorly-differentiated RPE cells including ARPE-19 cells and hRPE-1 cells, consume almost no proline as a nutrient (Yam et al. 2019). Consistently, the expression of SLC6A20 and PRODH are upregulated in RPE differentiation, whereas their expression is downregulated in de-differentiated RPE cells (Radeke et al. 2015). The dedifferentiation of RPE into a non-polarized fibroblast-like phenotype is commonly referred to as epithelial-to-mesenchymal transition (EMT). RPE with EMT can lose its normal function, contributing to retinal degenerative diseases such as AMD and proliferative vitreoretinopathy (PVR) (Zhou et al. 2020). Co-treatment with TGF-β and TNF-α accelerates EMT in adult human RPE cells, which downregulates the transcripts of both SLC6A20 and PRODH more than 400 fold (Boles et al. 2020). The downregulation of PRODH by TGF-β also occurs in renal and airway epithelial cells undergoing EMT (Brennan et al.

2012; Tian et al. 2015). An important feature in RPE differentiation is a reprogramming of metabolic dependency from glycolysis to mitochondrial oxidative phosphorylation (OXPHOS) (Agathocleous and Harris 2013; Zheng et al. 2016). OXPHOS genes and mitochondrial mass significantly increase during RPE differentiation (Iacovelli et al. 2016). Proline may efficiently fuel mitochondrial metabolism to meet the increased metabolic demand in differentiation. Either inhibition of mitochondrial OXPHOS or augmentation of glycolysis in RPE can result in dedifferentiation and loss of its epithelial properties (Adijanto and Philp 2014; Kurihara et al. 2016; Rosales et al. 2019; Zhao et al. 2011a). Inhibition of mitochondrial OXPHOS, particularly of complex III, blocks proline uptake in RPE, suggesting that the demand for proline catabolism is the driving force for the high proline utilization in differentiated RPE (Zhang et al. 2021).

Proline metabolism in retinal diseases

Mutations of genes involved in proline metabolism are associated with inherited retinal degenerations. A multitude of metabolomics studies on human plasma and vitreous samples repeatedly show that proline is among the most significantly altered metabolite in retinal degenerative diseases including AMD, proliferative vitreoretinopathy (PVR), diabetic retinopathy, and glaucoma (Table 2). We will focus on retinal diseases that are highly relevant to RPE dysfunction or degeneration in this review.

Proline metabolism in inherited retinal degeneration

Inherited retinal degenerations consist of a diverse group of retinal diseases characterized by progressive vision loss due to genetic mutations (Duncan et al. 2018). Inborn errors or genetic deficiency of several enzymes in proline synthesis result in inherited retinal degenerations. Patients with *ALDH18A1* (the gene encoding P5CS) mutations have retinal degeneration cutis laxa (loose skin), and fat pads (Wolthuis et al. 2014). Zebrafish carrying the *pycr1* gene deficiency show behavioral abnormalities, damaged RPE and retinal degeneration (Liang et al. 2019). Mutation of prolidase causes proline deficiency and chorioretinal atrophy in patients (Ogata et al. 1981). OAT deficiency causes gyrate atrophy, characterized by progressive and lobular loss of RPE/choroid and accumulation of ornithine in the plasma (Lodato et al. 1981; O'Donnell et al. 1978). Attempts to correct ornithine accumulation by dietary reduction of its precursor, arginine, show efficacy in halting the progression of retinal degeneration (Kaiser-Kupfer et al. 1991; Wang et al. 2000). The OAT deficiency causes over tenfold

Table 2 Proline levels in retinal diseases

Diseases	Species	Comparison (n)	Samples	Fasting	Value* (FC/μM)	References
AMD	Human	AMD (314) Control (82)	Plasma	Yes	0.76 (FC)	Lains et al. (2019)
	Human	Early/intermediate AMD (72) Control (72)	Serum	No	AMD: 263.6 Control: 254.4	Kersten et al. (2019)
	Human	Exudative-AMD (40) Control (40)	Plasma	Yes	AMD: 191.2 Control: 165.4	Chao de la Barca et al. (2020)
	Human	Wet AMD (26) Control (20)	Aqueous humor	N/A	0.04 (FC)	Han et al. (2020)
DR	Human	PDR (20) Control (31)	Vitreous	N/A	3.3 ~ 5.7 (FC)	Paris et al. (2016)
	Human	PDR (21) Diabetic control (21)	Plasma	Yes	0.52 (FC)	Zhu et al. (2019)
	Human	PDR (9) Control (8)	Vitreous	N/A	PDR: 25.2 Control: 6.6	Haines et al. (2018)
	Human	PDR (28) Control with macular hole (22)	Vitreous	N/A	2.1 (FC)	Wang et al. (2020)
	Human	DR (174) Control (143)	Serum	N/A	1.13 ~ 1.50 (FC)	Yun et al. (2020)
	Mouse	OIR model (4) Control (5)	Whole eye	N/A	5.0 (FC)	Paris et al. (2016)
Glaucoma	Human	POAG (36) Control with cataract (27)	Plasma	Yes	POAG: 211.7 Control: 173.5	Leruez et al. (2018)
	Human	PCG (45) Control with ARC (10)	Aqueous humor	N/A	5.27 (FC)	Chen et al. (2019)
	Human	PCG (45) Control with CC (10)	Aqueous humor	N/A	5.68 (FC)	Chen et al. (2019)
	Human	POAG (26) Control with cataract (26)	Aqueous humor	Yes	POAG: 29.0 Control: 29.6	Buisset et al. (2019)

*Value represents fold change (FC) of metabolite levels over control patients or absolute concentrations in μM. *AMD* age-related macular degeneration, *DR* diabetic retinopathy, *PDR* proliferative DR, *OIR* oxygen-induced-retinopathy, *POAG* primary open-angle glaucoma, *PCG* primary congenital glaucoma, *ARC* age-related cataracts, *CC* congenital cataracts

accumulation of ornithine in the plasma, which in vitro has been shown to inhibit P5CS (Hu et al. 1999). Excessive proline with normal ornithine in the urine is correlated with atypical gyrate atrophy in patients, suggesting that proline is deficient in the RPE (Hayasaka et al. 1982; Saito et al. 1981). Oral supplementation of ornithine significantly increases plasma proline in healthy controls but not in patients with gyrate atrophy. Deficiencies of OAT in both humans and mice support the idea that OAT proceeds in the direction of proline synthesis from ornithine in the adults but the opposite direction in neonates (de Sain-van der Velden et al. 2012; Wang et al. 1995). Inhibition of OAT in conjunction with ornithine administration could induce cytotoxicity in both bovine and human RPE, whereas supplementation with proline prevents this ornithine-induced cytotoxicity (Ando et al. 2000; Ueda et al. 1998). Still, there are few data on how these genetic mutations influence tissue proline levels and proline utilization in the RPE.

Proline is essential for the biosynthesis of collagen and the maintenance of ECM structure and composition (Karna et al. 2020; Van de Water and Galinovic-Schwartz 1986; Yoo et al. 1997). Mutations in the genes coding for ECM components are associated with retinal degeneration and choroidal neovascularization (Table 3). Mutations in the collagen 2A1, 9A1 and 11A1 result in Stickler syndrome, where affected individuals have ear, nose, throat and ophthalmologic abnormalities (Boysen et al. 2020; Kaarniranta et al. 2006; Nikopoulos et al. 2011), underscoring the importance of collagen structure on pathophysiology. Furthermore, mutations in ECM modulatory protein EFEMP1 lead to macular degeneration with subretinal deposits (Stone et al. 1999). TIMP-1 and -3 are secreted by RPE cells for regulation of MMP activity. TIMP-3 has the broadest inhibition spectrum and is tightly bound to the BrM in the human retina (Fariss et al. 1997). Mutations of the *TIMP3* gene result in Sorsby fundus dystrophy (SFD), a rare autosomal dominant inherited retinal degeneration that shares several similar clinical features with AMD including sub-RPE deposits, geography atrophy, and choroidal neovascularization (Anand-Apte et al. 2019; Weber et al. 1994). Mutant TIMP-3 was later found

Table 3 Genetic mutations in ECM components that causes retinal pathology

Gene	Genetic disease	Retinal features	Species	References
COL2A1	Stickler syndrome, type I	Membraneous vitreous Retinal detachment Paravascular pigmented lattice degeneration	Human Mouse	Ballo et al. (1998); Richards et al. (2000); Go et al. (2003); Kaarniranta et al. (2006)
	Epiphyseal Dysplasia, Multiple, with Myopia and Conductive Deafness	Asteroid hyalosis Retinal thinning	Human	Beighton et al. (1978)
COL9A1	Stickler syndrome, type IV	Chorioretinal degeneration Retinal detachment	Human	Van Camp et al. (2006); Nikopoulos et al. (2011)
COL11A1	Stickler syndrome, type II Marshall syndrome	Beaded vitreous Retinal detachment Paravascular pigmented lattice degeneration	Human	Annunen et al. (1999); Richards et al. (1996); Boysen et al. (2020)
C1QTNF5	Macular dystrophy, late onset	Macular degeneration Chorioretinal atrophy Choroidal neovascularization	Human Mouse	Hayward et al. (2003); Ayyagari et al. (2005); Borooah et al. (2009); Shu et al. (2011); Chavali et al. (2011)
TIMP3	Sorsby fundus dystrophy	Subretinal neovascularization Central macular lesion Chorioretinal atrophy Retinal pigment epithelial atrophy Geographic atrophy	Human Mouse	Weber et al. (1994); Langton et al. (2000); Gliem et al. (2015); Weber et al. (2002)
EFEMP1	Doyne honeycomb retinal degeneration (Malattia Leventinese)	Radial drusen Geographic atrophy Abnormal retinal pigmentation Choroidal neovascularization	Human Mouse	Stone et al. (1999); Kermani et al. (1999); Tarttelin et al. (2001); Fu et al. (2007)

to be secreted at elevated levels by RPE cells, albeit having decreased inhibition on MMPs (Engel et al. 2021, bioRxiv). Consequentially, iPSC-derived RPE cells from SFD patients have increased intracellular levels of 4-hydroxyproline, indicating enhanced ECM degradation. Degradation of ECM not only releases bound growth factors that can induce a response from RPE cells, MMP cleavage also produces short bioactive fragments called matrikines (Patel and Snelgrove 2018). Tripeptide Pro-Gly-Pro was demonstrated in other tissues to have the capacity to regulate processes including proliferation and migration (Ma et al. 2011), angiogenesis (Monboisse et al. 2014) and chemotaxis (Karsdal et al. 2015). How matrikines affect RPE metabolism and the outer retinal environment has not yet been investigated.

Proline metabolism in AMD

AMD, the leading cause of irreversible central blindness in the elderly population, is characterized by drusen deposits, geographic atrophy, and choroidal neovascularization. The causes of AMD have been attributed to a multitude of environmental and genetic factors, with aging being the major risk (Datta et al. 2017; Handa et al. 2019). RPE dysfunction due to impaired mitochondrial metabolism, oxidative stress, aging and inflammation, is thought to underlie the pathogenesis of AMD (Ferrington et al. 2020). Inhibition of mitochondrial metabolism, specifically in RPE, is sufficient to induce AMD-like retinal degeneration in mice (Kurihara

et al. 2016; Rosales et al. 2019; Zhao et al. 2011a). RPE cells from AMD donors have impaired mitochondrial metabolism (Ferrington et al. 2017; Golestaneh et al. 2016). Patients with an A3243G point mutation in mitochondrial DNA have RPE atrophy, subretinal deposits, and maculopathy (Daruich et al. 2014; Fung et al. 2013; Smith et al. 1999). Altered mitochondrial metabolism is a metabolic signature of aging RPE and retina (Wang et al. 2018). The inhibition of mitochondrial metabolism in human RPE culture can block proline utilization and markedly reduce the secretion of intermediates and amino acids to support the neural retina (Zhang et al. 2021). In aging mice, plasma proline levels are reduced (Seo et al. 2016). Multiple metabolomics studies identified significant changes in proline in AMD patients (Hou et al. 2020) (Table 2). A recent targeted-metabolomics study also found proline to be one of six significantly changed metabolites among the 188 metabolites analyzed in the plasma of AMD patients (Chao de la Barca et al. 2020). Additionally, *SLC6A20* is one of the most significant new loci linked to AMD from GWAS (Gao et al. 2019), while SLC6A20 expression is reported to be downregulated in retinas from AMD donors (Ratnapriya et al. 2019). Likewise, a transcriptome database shows that P5CS is significantly downregulated in RPE from both dry and wet AMD donors (Newman et al. 2012).

Oxidative damage is another major cause of RPE dysfunction in AMD (Cai et al. 2000). Under conditions of oxidative stress, cellular GSH concentrations are markedly

reduced due to a combination of enhanced degradation and decreased synthesis (Wu et al. 2004). Supplementation of GSH precursors protects RPE from oxidative damage (Kularatne et al. 2020; Terluk et al. 2019). The deficiency of *Nrf2*, a transcription factor in the GSH system, results in drusen-like deposits similar to AMD in mice. (Zhao et al. 2011b). We have found that inhibition of reductive carboxylation disrupts the redox balance and increases the sensitivity of cultured RPE cells to oxidative damage. Supporting reductive carboxylation protects RPE cell viability from oxidative stress (Du et al. 2016). Proline is sufficient to increase reductive carboxylation, promote GSH synthesis and regeneration, and protect RPE against oxidative damage.

ECM remodeling resulting from RPE dysfunction plays an important role in AMD pathogenesis. The collagen-rich BrM can be three-fold thicker with reduced elasticity in AMD (Nita et al. 2014b) as a result of reduced solubility and increased cross-linking with specific biomolecules (Eamegdool et al. 2020; Nita et al. 2014b). Basal laminar deposits and drusen are hallmarks of aging and early AMD (Bhutto and Lutty 2012; Fernandez-Godino et al. 2016, 2018). ECM proteins including collagen, MMPs and TIMP3, are key components within these deposits, and ECM dysregulation may play a role in the AMD-like macular degeneration seen in patients with *TIMP3 and EFEMP1* mutations (Anand-Apte et al. 2019; Hulleman 2016) (Table 3). Autoradiographic studies of aged primate and human retina with ^{3}H proline show that the rate of ECM turnover is much slower in the regions with drusen and basal deposits (Hirata and Feeney–Burns 1992). Local ECM degradation supplies proline to tissues or cells, which is especially important under conditions of oxidative stress (Pandhare et al. 2009). It remains to be determined whether proline utilization is impaired in AMD.

Proline metabolism in Proliferative Vitreoretinopathy (PVR)

PVR is one of the most common and severe complications following the treatment of rhegmatogenous retinal detachment, resulting in poor visual outcomes (Idrees et al. 2019). PVR is characterized by the formation of scar-like fibrocellular membranes in the vitreous cavity and surfaces of the retina. These fibrocellular membranes are composed of excessive ECM and RPE cells that have undergone EMT, which can contract to result in retinal folds, re-detachment, and vision loss (Hiscott et al. 1999; Idrees et al. 2019). The dedifferentiation of RPE cells into fibroblast-like cells through EMT due to exposure to growth factors and cytokines is pivotal in the pathogenesis of PVR. These de-differentiated cells migrate to retinal surfaces, producing collagen (mostly type I), MMPs, fibronectin and TIMPs to rebuild the ECM (Greene et al. 2017; Hiscott et al. 1999). Treatment of human RPE with growth factors or

cytokines could induce EMT and substantially increases type I collagen synthesis (Boles et al. 2020; Itoh et al. 2007; Jing et al. 2019). The proline analog, *cis*-hydroxyproline, inhibits collagen synthesis, attachment and migration of bovine RPE cells in dose- and time-dependent manner (Yoo et al. 1997). In a rabbit PVR model, *cis*-hydroxyproline significantly reduces the rate of retinal detachment (Radtke et al. 1986; Yasukawa et al. 2002). Epithelial cells including RPE that has undergone EMT can suppress the expression of PRODH (Boles et al. 2020; Brennan et al. 2012; Tian et al. 2015). These studies suggest that RPE in PVR may have altered proline metabolism, shifting from proline catabolism to collagen synthesis.

Conclusion and perspectives

Proline transport and metabolism emerge as important regulators in retinal physiology and diseases through modulating mitochondrial metabolism, ROS protection, ECM remodeling and cell differentiation. However, except for transcriptomics data, there is a lack of information on the expression and localization of proline transporters and key enzymes in proline metabolism in normal and diseased retinas. Furthermore, there is scarce data on proline levels in the RPE and neural retinas in retinal disease models. Performing loss-of-function experiments specifically in RPE for genes in proline metabolism will provide important insights on the roles of proline in retinal health and diseases. These genetically deficient models will also be useful tools to investigate metabolic communications between RPE and the neural retinas. SLC6A20 and PRODH are downregulated in RPE with EMT and AMD, which could make them potential therapeutic targets for PVR and AMD.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article reviews published studies and does not require either the approval of animal use or human consent.

Informed consent No informed consent is required for this study.

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