BIOCHEMICAL MECHANISMS OF MACULAR CAROTENOID SYNTHESIS AND UPTAKE

by

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ABSTRACT

Fovea centralis is a depression located in the back of the primate retina. This region is crucial for sharp central vision. Age Related Macular Degeneration (AMD) is a debilitating disease that affects several million people in the world. In this disease, the retina and RPE surrounding the fovea undergoes degeneration, thereby compromising visual acuity. The fovea contains significant amounts of three carotenoids – lutein, zeaxanthin, and *meso*-zeaxanthin. Carotenoids are plant-derived pigment molecules. Vertebrates are unable to synthesize these compounds *de novo,* and have to obtain these through the diet. Carotenoids protect the foveal region from oxidative stress, light damage, and improves vision. Carotenoid supplementation is shown to alter the course of AMD.

Hundreds of carotenoids are present in nature, and the regular human diet consumes about 50 of these. Among these, only 15 are absorbed by the gut and present in the serum. However, only lutein, zeaxanthin, and *meso*-zeaxanthin are present in the retina. Retinal concentrations of these carotenoids are 5000 times greater than observed levels in the serum, suggesting a very specific transport mechanism into the retina. There are gaps in our knowledge of the mechanisms involved in carotenoid transport into the eye.

meso-Zeaxanthin is a retina-specific carotenoid that is rarely encountered in nature. No dietary sources of *meso*-zeaxanthin are identified. At the foveal pit, there are equal amounts of lutein, zeaxanthin, and *meso*-zeaxanthin. However, *meso*-zeaxanthin is absent in the peripheral retina. The mechanism by which *meso*-zeaxanthin is produced in the eye

and the physiological significance of its presence is not understood.

This dissertation has determined the biochemical mechanisms that underlie carotenoid production and uptake into the fovea.

To my wonderful husband, Shyam Gopinath.

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CHAPTER 1

INTRODUCTION

1.1 Basic Science of the Macular Carotenoids

Carotenoids are plant-derived pigment molecules that vertebrates cannot synthesize *de novo*. These molecules are responsible for bright colors in animals and plants. They are precursors to vitamin A and are necessary for retinoic acid signaling. Carotenoids also function as potent anti-oxidant molecules that are capable of blue light filtration, and these qualities of carotenoids enable them to prevent the progression of diseases such as age-related macular degeneration (AMD).

1.1.1 Carotenoid chemistry and stereo chemistry

Carotenoids can be broadly classified based on their hydrophobicity (Britton, 1995). Carotenes are hydrophobic with little to no solubility in water, whereas xanthophylls are moderately soluble in water because of the presence of one or more hydroxyl or carbonyl groups. Due to their limited polarity, carotenoids are often associated with the cell membrane or bound to proteins. Presence of conjugated double bonds in their hydrocarbon chain is responsible for the antioxidant properties of these molecules (Bernstein et al., 2016; Britton, 1995). The efficacy of quenching singlet oxygen depends on the number of conjugated double bonds (Bernstein et al., 2016; Woodall et al., 1997). For example – zeaxanthin is considered to be a better antioxidant than lutein because it has 11 conjugated double bonds, while lutein has 10. Some carotenoids when subject to cleavage can give rise to retinol (vitamin A). The most common carotenoids that can produce vitamin A are β-carotene, α-carotene, and β-cryptoxanthin. However, none of these carotenoids are found in significant amounts in the retina. Among the 700 carotenoids that are present in nature, only 15-30 carotenoids enter the blood. Two carotenoids, lutein and zeaxanthin and the metabolite of lutein,

meso-zeaxanthin, are present in the fovea. Collectively they are referred to as the macular pigment (Bone et al., 1993).

Macular carotenoids are characterized by the presence of hydroxyl groups at the 3 and 3’ end of the ionone rings. As shown in **Figure 1.1**, the hydroxyl group of lutein positioned at C-3’ is configured exactly opposite of that of zeaxanthin, while the C-3 and C-3′ hydroxyl groups in *meso*-zeaxanthin are positioned identically to lutein. The double-bond in lutein at the 4′, 5′ position is shifted to the 5′, 6′ position in zeaxanthin and *meso*-zeaxanthin (**Figure 1.1**). Similar conformations of *meso*-zeaxanthin and lutein suggests that lutein rather than zeaxanthin is the immediate precursor to *meso*-zeaxanthin because steric inversions at chiral centers are rare in nature (Bernstein et al., 2016; Bone et al., 1993). In fact, a simple shift of one double bond will produce *meso*-zeaxanthin from dietary lutein. Furthermore, production of *meso*-zeaxanthin supplements in industrial setting is carried out using lutein as the precursor in the presence of strong base at high temperature (Bernstein et al., 2016). *meso*-Zeaxanthin is an eye-specific carotenoid that is not in the human plasma and liver but is present in human macula, retina, and RPE/choroid (Khachik et al., 2002). Primates maintained on a xanthophyll-free diet but then given lutein supplements showed the presence of *meso*-zeaxanthin in their retina; the control animals that were provided no xanthophylls and the animals supplemented with zeaxanthin alone did not have *meso*-zeaxanthin in their retina (Johnson et al., 2005). In another study, Bhosale and coworkers fed deuterated lutein or zeaxanthin to female quails and reported the presence of labelled *meso*-zeaxanthin only in the retinas of birds fed with deuterated lutein (Bhosale et al., 2007). Both of the studies described above indicate that lutein is the major precursor of *meso*-zeaxanthin in the retina.

1.1.2 Foveal anatomy and macular carotenoids

The fovea centralis is a depression located in the middle of the macula of the primate retina. The ophthalmoscope invented by Hermann von Helmholtz in 1851 led to the discovery of the foveal pit in live humans (Nussbaum et al., 1981). Later studies showed that this region is responsible for the sharp central vision required for daily activities such as reading, driving, and recognizing faces. The central region of the human macula is free of rod receptors and is composed of tightly packed foveal cone cells. Unlike the rest of the macula, the foveal region lacks several retinal layers such as the inner nuclear layer, the inner plexiform layer, and the ganglion cell layer. Also, the basal lamina, the internal limiting membrane that separates the vitreous from the retina, is absent at the fovea. There is, however, a higher concentration of Müller glial cells in this area. The lack of cell layers, the tight organization of cone cells, and the absence of basal lamina are all thought to be adaptations to facilitate the passage of light (Yamada, 1969). Retina from non-human primates is considered an excellent non-human experimental alternative for high-resolution histological studies because it is often easier to obtain high-quality freshly fixed tissue. Monkey and human fovea share similarities; the center is free of rods and consist only of cones (Yamada, 1969).

The presence of yellow coloration in the foveal region resulted in the anatomical term *macula lutea* or yellow spot (Nussbaum et al., 1981). This color was later determined as a result of the fovea's high content of xanthophyll carotenoids. The carotenoids present in the macula lutea are lutein, zeaxanthin, and *meso*-zeaxanthin (Bone et al., 1993, 1997; Wald, 1948). Carotenoid concentrations were determined to range from 0.05 ng/mm^2 in the peripheral retina to 13 ng/mm^2 at the fovea (Bone et al.,

1993). Studies from our laboratory have identified various metabolites of lutein and zeaxanthin such as *meso*-zeaxanthin, 3′-epilutein, and 3-hydroxy β,ε-caroten-3′-one in the human retina, lens, and uveal tract (Bernstein et al., 2001). Trace amounts of carotenoid pigments were identified in the cornea and sclera, and the vitreous was completely free of carotenoids (Bernstein et al., 2001). The carotenoids are highly concentrated near the fovea, and their concentration decreases nearly 100-fold with increasing eccentricity (Snodderly et al., 1984). There is twice as much zeaxanthin and *meso*-zeaxanthin than lutein near the fovea; however, this relationship is reversed in the peripheral retina where zeaxanthin and *meso*-zeaxanthin levels are half as much as those of lutein (Bone et al., 1993, 1997). Foveal carotenoids are mainly present in the receptor axons as well as the Henle fiber layer (Bone and Landrum, 1984; Snodderly et al., 1984). In the central retina, equal concentrations of lutein, zeaxanthin, and *meso*-zeaxanthin are present; however, the ratio of *meso*-zeaxanthin to zeaxanthin decreases with the increased eccentricity to the fovea (Bone et al., 1993). Studies from our laboratory have identified and localized the carotenoid-binding proteins, glutathione S-transferase P1 (GSTP1) and steroidogenic acute regulatory domain protein 3 (StARD3), in the photoreceptors of the foveal region and Henle fiber layer (Bhosale et al., 2004; Li et al., 2011). These proteins facilitate the specific distribution and stability of carotenoids in the foveal region.

1.1.3 Functional properties

Carotenoids are excellent quenchers of singlet oxygen that react at the limits of diffusion without being consumed in the process (Foote et al., 1970). Reactive oxygen species (ROS) are either radicals such as hydroxyl radical or peroxyl radical, or they are reactive nonradical compounds such as singlet oxygen, peroxynitrite, or hydrogen

peroxide (Stahl and Sies, 2002). Singlet-state molecules rapidly form, and these molecules can then react with oxygen to produce ROS. These, in turn, can cause lipid peroxidation by attacking polyunsaturated fatty acids, resulting in DNA damage and protein and transmembrane glycoprotein oxidation (Winkler et al., 1999). Hydroxyl radical is the most reactive species (Woodall et al., 1997b) and, as a result, it immediately reacts with surrounding target molecules near its site of generation. Free radicals produced in the eye and other tissues are quenched by macular carotenoids, and in doing so, they can harmlessly release the energy as heat without undergoing any degradation (Krinsky, 1989).

The outer retina, especially membranes of the outer segments of the photoreceptors, has high concentrations of polyunsaturated fatty acids that are susceptible to photo-oxidation. Therefore, the risk of ROS production in the retina is higher than that of other tissues (Cai et al., 2000; Conn et al., 1991; Winkler et al., 1999). ROS are produced by absorption of UV and blue light by a photosensitizing compound or molecule (e.g., lipofuscin, protoporphyrin, or cytochrome).

Similar to their roles in plants, lutein, zeaxanthin, and *meso*-zeaxanthin act as protective antioxidants in the eye. These carotenoids undergo oxidation and a series of transformations to protect the macula (Khachik et al., 2002). Metabolites such as 3-hydroxy-β,ε-caroten-3′-one are produced as a result of direct oxidation of lutein in monkey retinas (Khachik et al., 1997a) and in human eye (Bernstein et al., 2001). Another compound, 3- methoxyzeaxanthin, identified in the macula of donor eyes was present only in older donors, indicating that methylation of carotenoids may occur in increased rates with age (Bhosale et al., 2007c).

Macular carotenoids are also capable of protecting the eye tissues from oxidative stress-induced damage. As an individual ages, the accumulation of lipofuscin, a fluorescent mixture consisting of lipid-protein complexes, in the RPE increases. (Bernstein et al., 2001; Bhosale et al., 2009; Boulton et al., 1990). Components of lipofuscin can damage the RPE, are toxic to mitochondria, and induce apoptosis of cultured RPE cells when exposed to blue light (Sparrow and Cai, 2001; Suter et al., 2000). When RPE cells are treated with lutein, this phototoxic effect is greatly decreased (Bian et al., 2012; Shaban and Richter, 2002). The presence of lutein and zeaxanthin has further been shown to reduce the amount of lipofuscin formed in cultured RPE cells and *in vivo* (Bhosale et al., 2009; Sundelin and Nilsson, 2001; Winkler et al., 1999).

1.1.4 Dietary sources

Carotenoids cannot be synthesized *in vivo* by vertebrates and invertebrates, and they therefore must be obtained from dietary consumption. Consumption of lutein- and zeaxanthin-rich green leafy vegetables and orange and yellow fruits and vegetables is beneficial to health and is associated with lower rates of various diseases such as cancer, cardiovascular disease, AMD, and cataract formation (Beatty et al., 1999; Krinsky et al., 2003; Landrum and Bone, 2001; Trumbo and Ellwood, 2006). Green leafy vegetables (kale, spinach, and broccoli) are abundant in lutein (Holden, 1999), while corn products are good sources of zeaxanthin (Perry et al., 2009). The carotenoid compositions of foods differ based on several factors such as species, cultivation, part of the plant, degree of maturity at harvest, and postharvest handling practices (Kimura and Rodriguez-Amaya, 1999; Rodriguez-Amaya, 2003). Therefore, selection and processing of samples under optimal conditions are important to retain most of carotenoids. The differences in lutein

and zeaxanthin levels among vegetables are often attributed to species variations (Azevedo-Meleiro and Rodriguez-Amaya, 2007; Ismail and Cheah, 2003; Rodriguez-Amaya, 2003).

Several dietary factors affect the bioavailability of carotenoids from vegetables and fruits (van Het Hof et al., 2000). Since carotenoids are hydrophobic, consumption of carotenoid-rich foods in the presence of oils or cholesterol may increase their uptake (Brown et al., 2004). In addition to vegetables, which are less bio-available, egg yolk (Goodrow et al., 2006; Kelly et al., 2014; Krinsky and Johnson, 2005) and fortified milk (Granado-Lorencio et al., 2010) are also good dietary and bioavailable sources of lutein and zeaxanthin. As expected, dietary intake of carotenoids differs widely between individuals, and epidemiological studies have outlined that among many different ethnicities and age-groups, lutein consumption is higher than that of zeaxanthin (Johnson et al., 2010).

meso-Zeaxanthin is an eye-specific carotenoid that is rarely found in the human diet. Outside of the vertebrate eye, this carotenoid has been detected in shrimp carapace, fish skin, and turtle fat (Maoka et al., 1986). A recent study has confirmed its presence in fish skin using more modern methods (Nolan et al., 2014; Thurnham et al., 2015). A significant amount of *meso*-zeaxanthin has been detected in commercially produced chicken eggs in Mexico where it is commonly added to the feed to achieve desirable coloration (Wang et al., 2007).

1.2 Uptake, Transport, and Metabolism of Carotenoids

1.2.1 Transport proteins

Due to the absence of carotenoid synthesis enzymes in the body, humans rely on carotenoid intake through diet. Most dietary carotenoids are consumed and embedded within a food matrix. Once they reach the gut, carotenoids are released from their matrix. The free carotenoids are then incorporated into micelles before their uptake by the intestinal mucosal cells. In the intestine, the carotenoids are subject to cleavage by BCO1 and/or BCO2 to give rise to vitamin A and other metabolites (Erdman et al., 1993). From the intestine, carotenoids and their metabolites are secreted into the lymphatic and portal circulations for transport to the liver. Here, xanthophyll carotenoids such as lutein and zeaxanthin are taken up by their transporters to be carried to the retina and other tissues via the circulatory system. In the human serum, water-soluble lipoproteins are responsible for carrying carotenoids, retinoids, vitamin E, and plasma lipids (Rigotti et al., 2003). Lipoproteins consist of an outer shell of polar proteins and phospholipids, and an inner core of neutral lipids. They are divided into six different groups: chylomicrons, chylomicron remnants, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins, and high-density lipoproteins (HDL) (Mahley et al., 1984). The smallest and densest of all plasma lipoproteins is HDL. This molecule plays a crucial role in cholesterol metabolism, especially in reverse cholesterol transport (Trigatti et al., 2000). Lutein and zeaxanthin are primarily associated with HDL in the blood stream, although the components of HDL that are responsible for binding carotenoids are not understood. In addition, studies in chickens have identified that lutein delivery into the retina is mediated by HDL (Connor et al., 2007).

A cell surface glycoprotein, scavenger receptor class B member 1 (SR-B1), binds HDL (Acton et al., 1996; Pagler et al., 2006). SR-BI is a member of the CD36 superfamily (Oquendo et al., 1989). Recent studies have identified that SR-B1 is involved in the uptake and transport of carotenoids into the human and fly retina. A study revealed that macular carotenoids are taken up by fully differentiated ARPE19 cells, an RPE cell line, in an SR-B1-dependent manner (During et al., 2008). In Drosophila, NinaD mutant flies lack scavenger receptor class B protein. As a result, these flies are incapable of transporting carotenoids into their retina, thereby rendering them blind (Kiefer et al., 2002).

CD36, a scavenger receptor family member of SR-BI, was shown to be well expressed in the primate neural retina (Tserentsoodol et al., 2006). In addition, Cameo2, a CD36 homolog in silkworms, is necessary for lutein uptake into the silk gland (Sakudoh et al., 2010). Recent studies have shown that genetic variants of CD36 are associated with serum lutein levels and macular pigment optical density (MPOD) in AMD patients (Borel et al., 2011), suggesting that CD36 is likely to be involved in the macular pigment (MP) uptake process. The role of third scavenger receptor family member, SR-B2, in carotenoid transport has been unexplored thus far.

1.2.2 Binding proteins

Specific accumulation of lutein, zeaxanthin, and *meso*-zeaxanthin in the outer plexiform layers of the human fovea is not very well understood. Studies from our laboratory identified glutathione S transferase P1 (GSTP1) as the zeaxanthin-binding protein from the total membrane proteins of the human macula (Bhosale et al., 2004). Immunohistochemistry analyses revealed that GSTP1 in the human and monkey retina

was abundant in the outer and inner plexiform layers of the fovea and in the photoreceptor inner segment ellipsoid region. Recombinant human GSTP1 bound macular zeaxanthins with high affinity as opposed to only low-affinity interactions with lutein. In addition, closely related proteins to GSTP1, GSTM1 and GSTA1, showed no preferential binding affinities for lutein or zeaxanthin, further confirming the specific nature of interactions between GSTP1 and the macular zeaxanthins.

Studies from our laboratory identified steroidogenic acute regulatory domain protein 3 (StARD3) as the lutein-binding protein (Li et al., 2011). Western blots of human macula demonstrated abundance of StARD3 here. Immunohistochemistry analysis of StARD3 revealed specific localization of this protein to all neurons of monkey macular retina and is especially present in foveal cone inner segments and axons. Surface plasmon resonance (SPR) of recombinant StARD3 showed high affinity interaction with lutein. Thus, macula-enriched proteins such as StARD3 and GSTP1 can account for specific localization of carotenoids in this region of the eye.

1.2.3 Cleavage proteins

There are two well-characterized carotenoid cleavage enzymes in vertebrates; BCO1 and BCO2. These proteins are present in human retina as well as RPE (Bhatti et al., 2003; Li et al., 2014; Lindqvist and Andersson, 2004; Lindqvist et al., 2005). Carotenoids are cleaved symmetrically by BCO1 at the 15-15′ carbon-carbon double bond. This is an essential step for generation of vitamin A, and such cleavage requires its substrates to have at least one nonsubstituted beta-ionone ring (dela Sena et al., 2013; Lindqvist and Andersson, 2002). Given the presence of hydroxyl groups at the ionone rings of both lutein and zeaxanthin, these carotenoids cannot be cleaved by BCO1.

BCO2, on the other hand, catalyzes eccentric cleavage of carotenes at 9′, 10′ carbon-carbon double-bonds, generating 10′-apo-β-carotenal (C27), β-ionone (C13), and C9 dialdehyde as three possible cleavage products (Krinsky et al., 1993; von Lintig et al., 2005). Studies from our laboratory have shown that, unlike ferret and mouse BCO2 that can cleave xanthophylls such as lutein and zeaxanthin (Mein et al., 2011), human BCO2 is a relatively inactive cleavage enzyme. This finding is considered to be the result of an unusual –GKAA- amino acid insertion near the substrate binding tunnel that appears to be unique to primates and whose insertion into the mouse enzyme leads to its inactivation (Li et al., 2014). Inactivity of retinal BCO2 can further explain the reason for the unique accumulation of lutein and zeaxanthin in primate retinas.

1.3 Overview of Chapters 2-4

Tserentsoodol *et al.* have shown that SRBs are present in the retina and RPE of primates (Tserentsoodol et al., 2006), but their immunohistochemistry (IHC) data do not support their Western blot data. For instance, in their IHC, SR-B1 is expressed in the primate retinal and RPE layers, but their western blot shows the expression of SR-B1 protein only in primate retina, not in the RPE. Similar inconsistencies were observed in the expression patterns of SR-B2 and CD36. Using ARPE19 cells, a human RPE cell line, previous work has identified the roles of SR-B1 and CD36 in carotenoid transport (During et al., 2008; Thomas and Harrison, 2016), but the third member of the SRB family, SR-B2 and its role in carotenoid transport have not been explored. *In the second chapter of this dissertation, I have identified the expression profile of SRBs in the primate eye as well as determined their differential ability to transport macular xanthophylls* **(Figure 1.2)**.

Abundant food sources contain lutein and zeaxanthin, but *meso*-zeaxanthin is rarely encountered in nature. There are no common dietary sources for this carotenoid (Bernstein et al., 2016). Apart from the retina and RPE, *meso*-zeaxanthin is not present elsewhere in the primate body. At the foveal center, the lutein:zeaxanthin:*meso*-zeaxanthin ratio is 1:1:1 (Snodderly et al., 1984). A few millimeters away, the concentration of *meso*-zeaxanthin drops dramatically, and the level of *meso*-zeaxanthin becomes nearly undetectable in the peripheral retina. Given its specific accumulation in the foveal pit, and its lack of presence in common dietary sources, it is hypothesized that *meso*-zeaxanthin may be produced as a result of chemical reaction in the retina and/or the retinal pigment epithelium (RPE) (Bernstein et al., 2016). Feeding studies in monkeys indicate that *meso*-zeaxanthin is present in the retinas of animals that were fed lutein supplements but not zeaxanthin supplements (Johnson et al., 2005), and supplementation experiments in quails conducted using deuterium-labelled lutein or zeaxanthin showed the presence of labelled *meso*-zeaxanthin in the eyes of birds that were fed with labelled lutein, not zeaxanthin (Bhosale et al., 2007). These studies indicate that lutein may be the precursor to *meso*-zeaxanthin in vertebrates. When subject to high temperature in the presence of a strong base, lutein readily converts to *meso*-zeaxanthin. However, this reaction has never been observed *in-vitro* under physiological conditions. Since lutein, zeaxanthin, and *meso*-zeaxanthin are structural isomers **(Figure 1.1),** it is hypothesized that an isomerase enzyme may be responsible for the conversion of lutein to *meso*-zeaxanthin. *In the third and fourth chapters, I have determined the biochemical mechanisms that underlie meso-zeaxanthin production* **(Figure 1.3).**

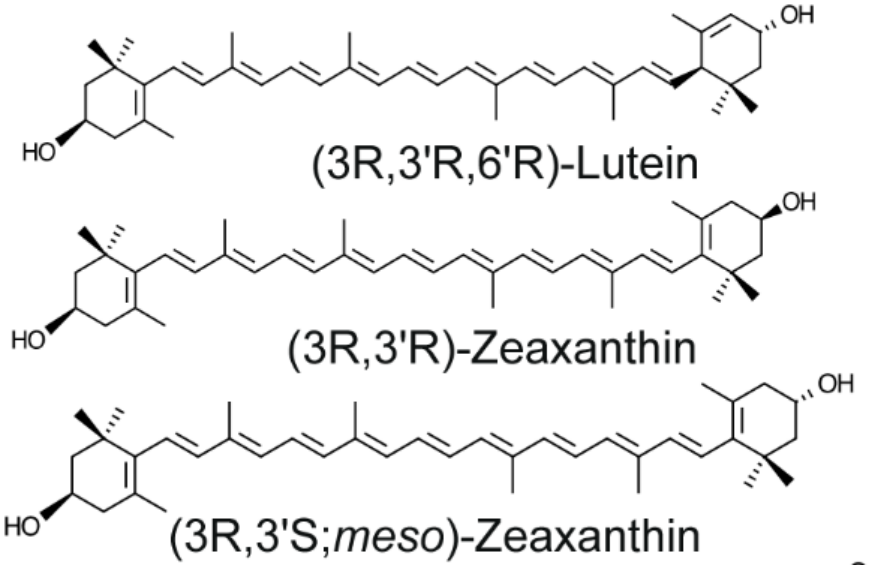

Figure 1.1: Structures of the macular xanthophylls. Lutein, zeaxanthin, and *meso*-zeaxanthin are the three carotenoids present in the primate macula. They are structural isomers with the same molecular formula $C_{40}H_{56}O_2$

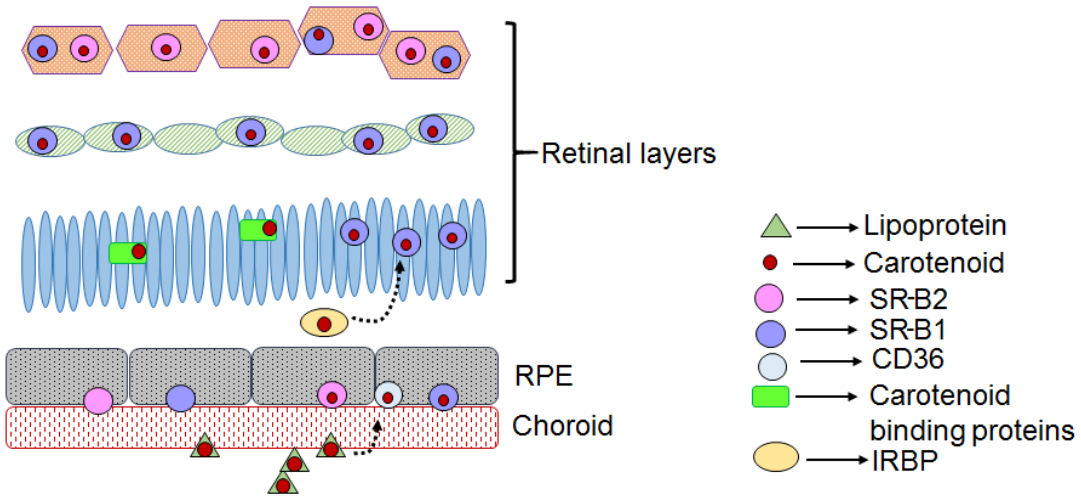

Figure 1.2: Schematic model outlining the major findings in Chapter 2.
Hypothesized transport pathway of carotenoids – Lipoprotein laden carotenoids enter the RPE from the choroidal circulation. SRB proteins (SR-B1, SR-B2 and CD36) present in the RPE and in the retina layers facilitate the movement of carotenoids into the different regions. In the present study, we determined that all three SRBs are capable of transporting macular carotenoids but not β-carotene. In addition, we identified that, while all three SRB proteins are expressed in the RPE, only SR-B1 is present in the outer nuclear layer of the retina. SR-B2 was the only SRB expressed in the outer nuclear layer of the retina. Transport proteins such as IRBP carry the carotenoids from the RPE to the retinal layers (Vachali et al., 2013). Carotenoids are retained in the retina and RPE by means of binding proteins such as GSTP1 and StARD3 (Bhosale et al., 2004; Li et al., 2011).

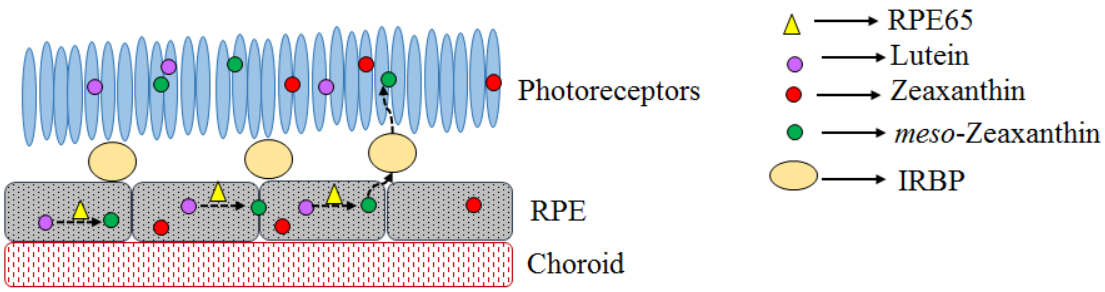

Figure 1.3: Schematic model outlining the major findings in Chapters 3 and 4. In Chapter 3, we discovered the presence of *meso*-zeaxanthin in a developmentally regulated manner in chicken embryonic RPE. In Chapter 4, we identified lutein to be the precursor of *meso*-zeaxanthin in chicken RPE. Furthermore, we discovered that RPE65 is the lutein to *meso*-zeaxanthin isomerase in vertebrates. The newly formed *meso*-zeaxanthin can be transported to the retinal layers by transporters such as IRBP (Vachali et al., 2013).

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CHAPTER 2

ALL THREE HUMAN SCAVENGER RECEPTOR PROTEINS CAN BIND AND TRANSPORT ALL THREE MACULAR XANTHOPHYLL CAROTENOIDS

Shyam R, Vachali P, Gorusupudi A, Nelson K, Bernstein P.S. All three human scavenger receptor class B proteins can bind and transport all three macular xanthophylls. (2017). Arch Biochem Biophys. 2017 Sep 22. pii: S0003-9861(17)30337-5. doi: 10.1016/j.abb.2017.09.013. [Epub ahead of print].

Credits

Paul Bernstein and I came up with the idea for this project. Preejith Vachali conducted Surface Plasmon Resonance, Aruna Gorusupudi performed HPLC analysis, Kelly Nelson dissected human tissues for this project. All other experiments were conducted by me. I wrote and Paul Bernstein edited this manuscript prior to submission. My committee members were instrumental in the organization of this manuscript.

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All three human scavenger receptor class B proteins can bind and transport all three macular xanthophyll carotenoids

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ABSTRACT

Carotenoids are plant pigment molecules that are potent antioxidants. Carotenoids cannot be synthesized *de novo*; therefore, their dietary intake and transport to various tissues are essential to harness their health benefits. Two of the three scavenger receptor class B (SRB) proteins, SR-B1 and CD36, have been implicated as carotenoid transporters in lower species and in various tissues of higher animals. The function of the third SRB protein, SR-B2, in carotenoid transport is unknown. Using surface plasmon resonance (SPR) analyses, we have determined that all three human SRB proteins are capable of binding the macular xanthophyll carotenoids; lutein, zeaxanthin, and *meso*-zeaxanthin. By over-expressing human SRB proteins in cells that do not endogenously express SRBs, we have determined that lutein uptake is enhanced in the presence of LDL and is mediated by SR-B1 and CD36. SR-B1, SR-B2, and CD36 were able to take up significant amounts of zeaxanthin as well as *meso*-zeaxanthin, and uptake was increased in the presence of HDL. Our analyses revealed no apparent differences in protein expression profiles of SRBs in central and peripheral regions of human donor tissues, indicating that carotenoid-binding proteins rather than transporters are likely to mediate selective accumulation of carotenoids into the macula.

1. Introduction

Carotenoids are plant-derived pigment molecules that vertebrates cannot synthesize *de-novo,* which must be obtained exclusively from the diet. These compounds are potent anti-oxidants and are the precursors to compounds such as retinoids that are crucial for normal physiological functions [1,2]. In 2002, Harrison and colleagues showed that an epithelial transporter, SR-B1, in the intestine is responsible for carotenoid absorption [3]. In that same year, NinaD, a class B scavenger receptor (SRB) in Drosophila that shares homology to vertebrate CD36 and SR-B1, was determined to be responsible for carotenoid transport into the eye [4]. These two studies brought attention to the role of scavenger receptor class B proteins as carotenoid transporters in the intestine. Since then, studies have examined the effects of SR-B1 and CD36 on the uptake of carotenoids. Over-expression of SR-B1 or CD36 was sufficient for the uptake of pro-vitamin A carotenoids [5]. SR-B1 was shown to preferentially take up macular xanthophylls over carotenes [6]. Enhanced uptake of lutein in LDL complexes and zeaxanthin in HDL complexes was facilitated by endogenously expressed SR-B1 [7].

The area in the primate body with the highest carotenoid concentration is the *macula lutea* of the retina [2,8]. This region is yellow in color due to the abundance of carotenoids. There are only three carotenoids present in the macula — lutein, zeaxanthin, and *meso*-zeaxanthin, along with lower levels of their oxidative metabolites (Fig. 1) [1]. These xanthophyll carotenoids are capable of filtering blue light from reaching the central retina, and they have strong antioxidant properties [9,10]. In addition, lutein and

Abbreviations: AMD, Age-related macular degeneration; CD36, Cluster determinant 36; GSTP, Glutathione *S*-transferase Pi isoform; IHC, immunohistochemistry; PVP, Polyvinylpyrrolidone; RPE, Retinal pigment epithelium; SR-B1, Scavenger receptor class B protein 1; SR-B2, Scavenger receptor class B protein 2; SRB, Scavenger receptor class B proteins; StARD3, steroidogenic acute regulatory domain 3; SPR, Surface plasmon resonance; THF, Tetrahydrofuran.

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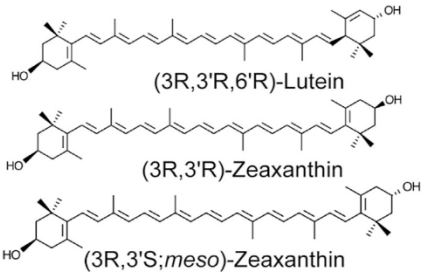

Fig. 1. Structure of macular xanthophylls. Lutein, zeaxanthin, and meso-zeaxanthin are the three carotenoids present in the primate macula. They are structural isomers with the same molecular formula $C_{40}H_{56}O_2$.

zeaxanthin supplements have been shown to increase macular pigment and alleviate the progression of age-related macular degeneration (AMD) [11], and supplements containing the non-dietary macular carotenoid *meso*-zeaxanthin have recently entered the market [12].

Several studies have revealed that SR-B1 and CD36 can function as carotenoid transporters into the retina. The role of the third SRB family member, SR-B2, in carotenoid transport is unknown. Given the ability of all SRBs to bind similar ligands and the presence of all three in the primate retina [13], we hypothesized that SR-B2 may also be involved in carotenoid transport in mammals. In the present study, we have characterized the binding affinities of SRBs to the three macular xanthophyll carotenoids and determined their roles as transport proteins.

2. Materials and methods

2.1. Human tissue isolation

Human eyes from donors (ages ranging from 59 to 81) from the Utah Lions Eye Bank were isolated within 24-h post-mortem. 6–8 mm punches of the macular retina, sub-macular RPE, peripheral retina, and peripheral RPE were obtained. The samples were stored in −80 °C in RNALater (Thermo Fisher Scientific, Waltham, MA). Tissue procurement and handling were in compliance with the Declaration of Helsinki.

2.2. Protein isolation and Western blot

For total protein isolation, cells and tissues were homogenized at 4 °C in RIPA buffer (25 mM NaCl, 0.5 mM EDTA, 25 mM Tris HCl (pH 7.2), 0.1% Tween 20) containing protease inhibitors. Cell debris was removed by centrifugation. BCA assay (Thermo Fisher Scientific) was carried out, and 20 μg of protein was resolved by 9% SDS-PAGE. Transfer was carried out to a 0.45 μm nitrocellulose membrane using a *trans*-blot SD semi-dry transfer cell (BioRad, Hercules, CA) at 25 V for 1 h. Membranes were subsequently washed in TBS with 0.01% Tween 20 and blocked using Odyssey blocking buffer (LICOR Biotechnology, Lincoln, NE) containing 0.01% Tween 20 for 1 h. Primary antibodies were diluted in the above blocking buffer, and the membranes were incubated overnight at 4 °C. The antibodies used and their dilutions were as follows−1:1000 dilution of rabbit monoclonal anti-SR-B1 (ab52629-Abcam, Cambridge, MA), 1:1000 dilution of goat polyclonal anti-SR-B2 (af1966-R&D Systems, Minneapolis, MN), 1:500 dilution of rabbit monoclonal anti-CD36 (14347, Cell Signaling Technology), 1:5000 dilution of mouse monoclonal *anti*-β-actin (8H10D10, Cell Signaling Technology), 1:2000 dilution of rabbit polyclonal anti-Na-K ATPase (3010, Cell Signaling Technology). Proteins were visualized using an Odyssey Image Analyzer (LICOR Biotechnology, Lincoln, NE) following incubation with IR dye conjugated secondary antibodies (LICOR Biotechnology) at 1:10000 dilutions for 1 h at room temperature. For CD36, we ran two gels with the same lysate (at the same concentration) simultaneously. One membrane was blotted for CD36, whereas the other one was blotted for Na-K ATPase.

2.3. Immunohistochemistry

Eyes from 4-6 year old Macaca mulatta monkeys were obtained after perfusion fixation with 10% paraformaldehyde for 15 min. These animals were provided by other University of Utah reserchers whose IACUC protocols did not utilize ocular tissues after sacrifice. The eyes were dissected, and 10 μm thick cryosections of the tissue were obtained. The sections were rinsed in 0.1 M PBS with 0.1% Triton X-100 (PBT) and blocked in 10% donkey serum in PBT for 1 h. Following this, primary antibody incubation was carried out overnight at 4 °C. The antibodies and their dilutions were as follows - 1:100 dilution of rabbit monoclonal SRB1 (ab52629-Abcam), 1:500 dilution of goat polyclonal *anti*-SRB2 (af1966-R&D Systems), and 1:100 dilution of rabbit monoclonal anti-CD36 (14347, Cell Signaling Technology). The sections were rinsed in PBT, and incubations using FITC- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were conducted at 1:1000 dilution at room temperature for 2 h. Control sections were incubated with only the corresponding secondary antibodies and not with primary antibodies. In order to quench autofluorescence, sections were treated with 0.1% Sudan black solution as previously described [14].

2.4. Cell culture and transient transfection

The ARPE-19 human RPE cell line and the HEK-293T human embryonic kidney cell line were purchased from ATCC (Rockville, MD). ARPE-19 cells were maintained in 1:1 Ham's F12 medium:-DMEM with 10% FBS and 1% penicillin streptomycin antibiotic mixture (Thermo Fisher Scientific). HEK-293Tcells were cultured in DMEM containing 10% FBS and 1% antibiotic mixture (Thermo Fisher Scientific). Both cell types were cultured in T-75 flasks until confluent. Cells were passaged using TrypLE Express (Thermo Fisher Scientific) and plated in 60 × 15 mm dishes for experiments. In order to ensure adequate differentiation, ARPE-19 cells were in culture for at least 6 weeks before experiments were conducted.

Over-expression was carried out by transient transfection of plasmids – pCMV-GFP SR-B1, pCMV-GFP SR-B2, and pCMV-GFP CD36 (Sino Biological Inc., Beijing, China). Cells were transfected with Jetprime reagent (Polyplus Transfection, Illkrich, France) following the manufacturer's instructions. Transfection efficiencies were determined to be between 80 and 90% for each plasmid. Experiments were conducted after 48 h of transfection.

2.5. Carotenoid delivery

Zeaxanthin was supplied by Zeavision (Chesterfield, MO), *meso*-zeaxanthin was from DSM Nutritional Products (Kaiseraugst, Switzerland), and lutein was provided by Kemin Health (Des Moines, IA). Carotenoid stocks were prepared in hexanes. 0.3% Tween 40 (Sigma-Aldrich) was added to appropriate volume of stocks, and the mixture was dried under nitrogen gas. For carotenoid delivery using HDL or LDL, either HDL (J64903) or LDL (J65039) (Alfa Aesar, Tewksbury, MA) were added to the dried stock solutions so that the final concentration of lipoproteins in the media were 10 μg/mL [7], and the mixture was vortexed overnight

at 4 °C in the dark. Serum-free medium was added, and the mixture was sonicated on ice for 1 h and then vortexed at room temperature for 30 min. The media were filtered using 0.2 μm filters, and extracted to validate the concentration of carotenoids.

Cells were washed with serum-free media, and the mixtures of media with carotenoids and lipoproteins were added to the cells to initiate carotenoid uptake. The dishes were placed into a 37 °C incubator for 30 min. The cells were subsequently washed with ice-cold 10 mM sodium taurocholate to remove any adsorbed carotenoids and then with 1X PBS. Cells were scraped into 1X PBS and centrifuged at 13000 rpm for 5 min. Excess liquid was removed, and carotenoid extraction was conducted.

2.6. Carotenoid extraction and HPLC analysis

Carotenoids were extracted following the addition of tetrahydrofuran (THF) containing 0.1% butylated hydroxytoluene (BHT). 1 mL of THF was added to the cell pellet from above, and this mixture was sonicated on ice (10 min) followed by vortexing for 5 min. The cell homogenates were centrifuged at maximum speed for 10 min, and the supernatant containing carotenoids was removed and dried under nitrogen gas. The above process was carried out three times to remove all carotenoid content in the cells. HPLC analyses were performed as previously described [15].

2.7. Surface plasmon resonance (SPR)

Recombinant proteins for CD36 (10752-H08H, Sino Biological Inc., Beijing, China), SR-B1 (11069-H08H, Sino Biological Inc., Beijing, China), and SR-B2 (BP001637-C532, Syd Labs, Natick, MA) were purchased. Proteins were immobilized onto hydroxyl gel modified sensor chips (Xantec, Dusseldorf, Germany) using standard amine coupling to obtain a density ranging from 6 to 12 kRU. Each of the three carotenoids (lutein, zeaxanthin, and *meso*-zeaxanthin) was dissolved in DMSO to obtain high concentration and then diluted to a final 5% DMSO concentration in running buffer. PBS with 0.05% Triton X-100 (for CD36 assay) or 1 mg/mL polyvinylpyrrolidone (PVP) (for SR-B1 and SR-B2 assays) was used as the running buffer. Carotenoid analytes' concentrations ranged from 10 to 30 μM. Each of the analytes was run in triplicate with a flow rate of 100–200 μL/min. All analyses were carried out at 25 °C using a SensiQ Pioneer optical biosensor (SensiQ Technologies Inc., Oklahoma City, OK). Data were collected at 10 Hz. SPR response data (sensorgrams) were zeroed at the beginning of each injection and double referenced. The responses were plotted against the analyte concentration and fit to a 1:1 (A + B = AB) binding model using Qdat analysis software (SensiQ Technologies) [16,17].

Table 1
Equilibrium dissociation constants (K_D) for carotenoid interactions with SRBs.

	Lutein	Zeaxanthin	*meso*-Zeaxanthin
SR-B1	2.04 ± 0.040 μM	1.60 ± 0.100 μM	3.10 ± 0.20 μM
SR-B2	2.51 ± 0.040 μM	1.53 ± 0.040 μM	3.90 ± 0.04 μM
CD36	0.73 ± 0.006 μM	0.92 ± 0.007 μM	1.66 ± 0.01 μM

2.8. Statistical analysis

Statistical analyses were conducted using Graphpad Prism software (La Jolla, CA). Values are listed as mean ± SEM. Results were analyzed using Student t-tests.

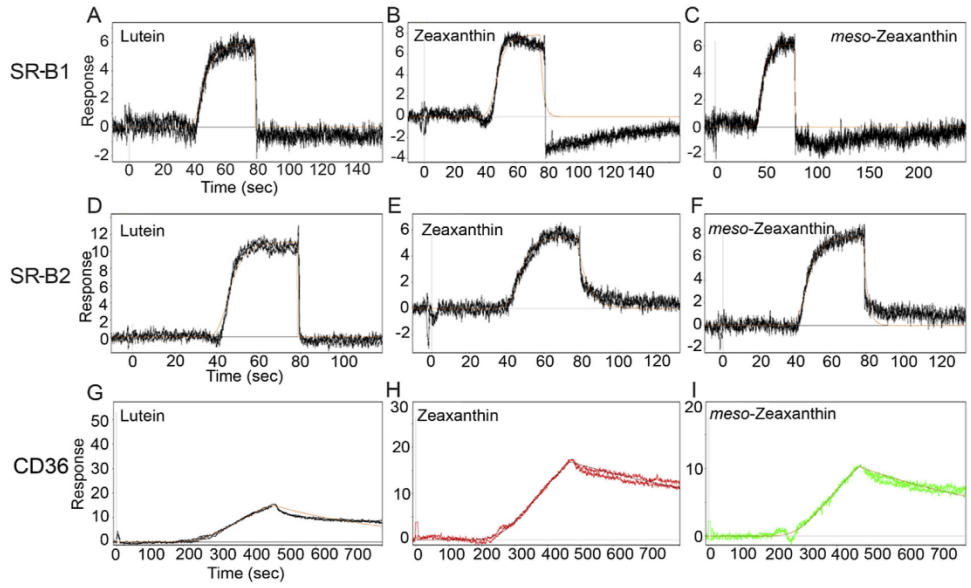

Fig. 2. Surface plasmon resonance sensorgrams obtained for the binding interactions of carotenoids with SRB proteins. CD36 binding affinities toward all three carotenoids were stronger than those of SR-B1 and SR-B2 as evidenced by the slower off-rate in the sensorgrams (G, H, and I). Solid red lines through the curve show model fit for the calculation of the affinities of interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

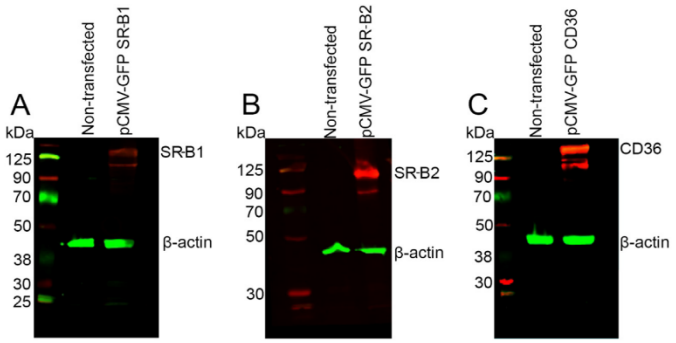

Fig. 3. Western blot showing the endogenous and over-expressed SRB proteins in HEK-293Tcells. Endogenous expression of SR-B1, SR-B2 and CD36 was absent in these cells. Upon over-expression, SRBs were detected. β-Actin was used as loading control.

3. Results and discussion

There are over 500 carotenoids present in nature. Humans and other primates consume about fifty of these, among which only three, lutein, zeaxanthin, and *meso*-zeaxanthin, are present in the macula. Such specificity in the accumulation of these three xanthophyll carotenoids into the eye suggests that this may be due to the function of specialized transport proteins and binding proteins [2,18,19]. Studies conducted using cell culture models as well as animal models have revealed that SR-B1 may be responsible for the transport of carotenoids from the intestine [3,4]. In addition, Sakudoh et al. showed that Cameo2, a CD36 orthologue, is responsible for selective accumulation of lutein in the cocoon [20,21]. Previous work has shown that SRB proteins, SR-B1 and CD36, can transport carotenoids into cells [3,4,20,21], but the role of SR-B2 in carotenoid transport has not been studied. In addition, studies on ARPE19 cells, an RPE cell line, have hypothesized that carotenoid uptake into these cells is mediated by SR-B1 [7]. In the current study, we chose to explore the roles of all three scavenger receptor proteins in carotenoid uptake.

In order to quantitatively assess the interactions between all three SRB proteins and carotenoids, we first determined the binding affinities of SRBs to macular xanthophylls on an SPR platform using human recombinant proteins of SR-B1, SR-B2, and CD36. Our results show that all three scavenger receptors bind macular xanthophylls with a binding affinity characteristic of transport proteins. As shown in Table 1 and Fig. 2, SR-B1 and SR-B2 showed comparable affinities with all three carotenoids (Fig. 2A–F). CD36 showed relatively stronger affinity toward macular xanthophylls, with K_D values in the sub-micromolar range. As seen in the sensorgrams (Fig. 2G, H, and I), this protein had a faster on-rate and slower off-rate with carotenoids. This resulted in a different kinetic profile of interaction than those of SR-B1 and SR-B2.

Previous studies from our laboratory on the interactions between binding proteins and carotenoids have determined K_D values in the sub-micromolar range. A low K_D is indicative of stronger interaction between the ligand and the protein. For instance, K_D values of StARD3 to lutein and GSTP1 to zeaxanthin were less than 0.6 μM [22,23]. However, binding affinities with carotenoids of a protein such as IRBP, a retinoid and carotenoid transporter that is present in the interphotoreceptor retinal space, was previously determined to be in the 1–2 μM range. Nonspecific binding

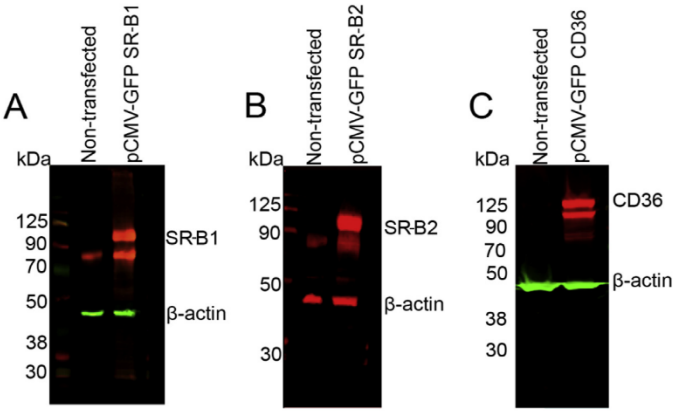

Fig. 4. Western blot showing the endogenous and over-expressed SRB proteins in ARPE-19 cells. Endogenous expression of SR-B1, SR-B2 was detected, but CD36 was absent in these cells. Upon over-expression, SRBs were detected. β-Actin was used as loading control.

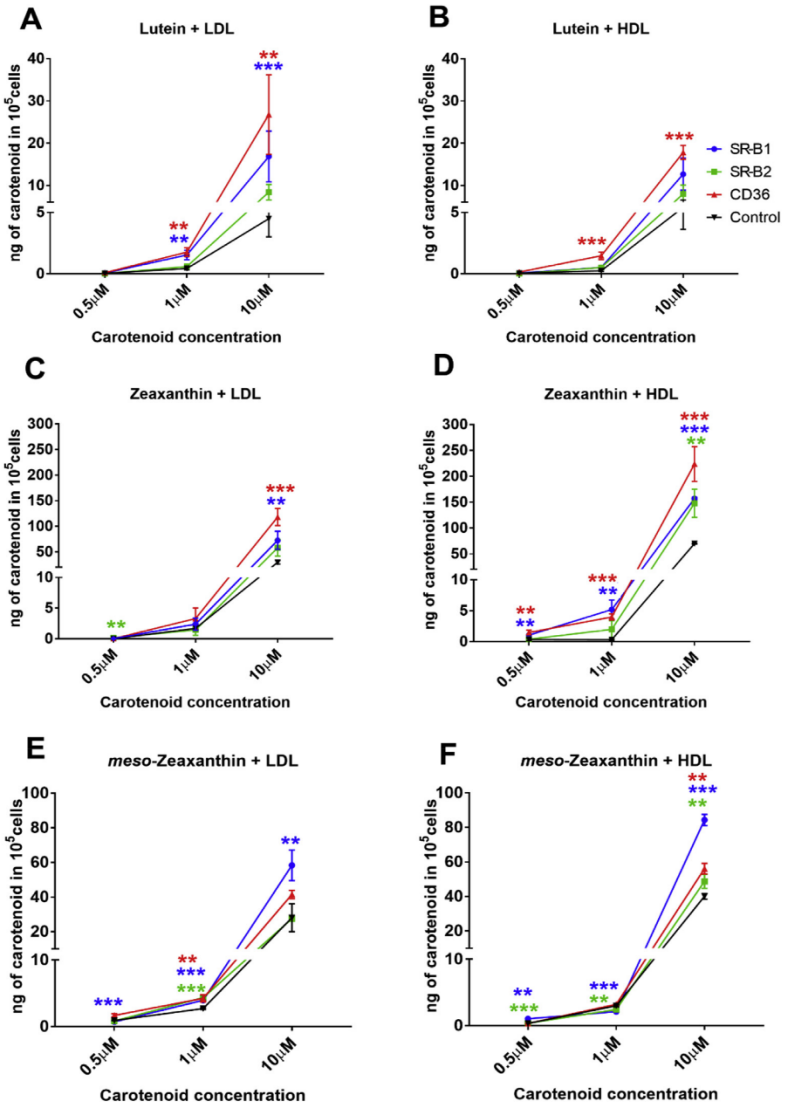

Fig. 5. – *Quantification of carotenoid uptake into HEK-293T cells over-expressing SRB proteins. Lutein uptake was increased in the presence of LDL, and zeaxanthin and meso-zeaxanthin uptake was higher in the presence of HDL. CD36 and SR-B1 were most efficient in the uptake of lutein and zeaxanthin, whereas SR-B2 showed significant uptake of zeaxanthin and meso-zeaxanthin.* Carotenoid uptake in non-transfected cells may be due to passive diffusion. Xanthophyll uptake into the control cells may be mediated by passive diffusion. It is also likely that lipoprotein laden carotenoids are endocytosed into the control cells. Non-specific uptake into the control cells may also be mediated by taurocholate ** - *p-value* < 0.05, ***-*p-value*<0.0005.

interactions result in K_D values that are higher than 10 μM. The K_D values observed for SRB proteins here are in the same range as we observed for IRBP, indicating that SRBs bind carotenoids with affinities characteristic of transport proteins.

In the next set of experiments, we determined whether the binding affinity of SRBs for macular xanthophylls resulted in their enhanced uptake in a cell culture system. We chose to study carotenoid uptake in HEK-293T cells since they are free of endogenous SRBs (Fig. 3), as opposed to the commonly used cell line, ARPE-19, which expresses both SR-B1 and SR-B2 endogenously (Fig. 4). By over-expressing each SRB protein, and by using empty vector transfected cells as controls for all experiments, we were able to compare and contrast the SRB-mediated as well as non-SRB-mediated uptake of carotenoids into HEK-293T cells. Physiological

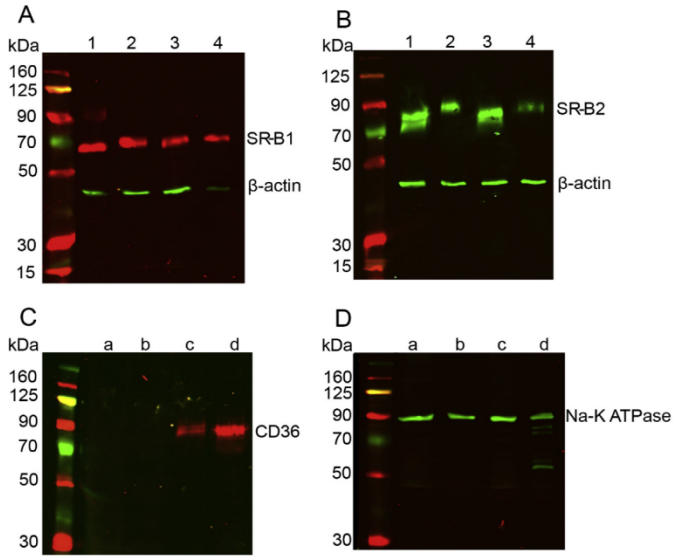

Fig. 6. Western blots conducted on human tissues. SR-B1 and SR-B2 proteins (A and B) were detected in total tissue lysates of human macular retina (lane 1), sub-macular RPE (lane 2), peripheral retina (lane 3), and peripheral RPE (lane 4). β-actin was used as loading control. Membrane protein isolation resulted in the detection of CD36 (C) only in the peripheral RPE (lane c) and sub-macular RPE (lane d). Retinal tissue (lanes a and b) did not contain detectable CD36. Na-K ATPase was used as the loading control for membrane proteins. Panel D shows the presence of Na-K ATPase in all four tissues. Experiments were conducted in tissues from three different donors of ages ranging from 50 to 70 years with similar results. Results from one representative experiment are presented.

levels of carotenoids in plasma are between 1 and 10 μM [24]; therefore, we tested the uptake using carotenoid concentrations ranging from 0.5 μM to 10 μM. Since carotenoids in circulation are present in mixed micelles of lipoproteins [25,26], we presented these molecules in LDL or HDL complexes to the cells.

For lutein, uptake was significantly increased in cells over-expressing CD36 and SR-B1. SR-B2 over-expressing cells were able to take up this carotenoid at levels higher than the control cells, but the difference lacked statistical significance. We also noticed that the transport of LDL complexes with lutein were higher than transport of HDL complexes with lutein (Fig. 5A and B).

Treatment of SRB over-expressing cells with zeaxanthin in HDL resulted in higher uptake than when this carotenoid was presented to the cells in LDL (Fig. 5C and D). Consistent with our observations for lutein, CD36 and SR-B1 over-expressing cells were able to take up significantly higher amounts of carotenoid than control cells. Interestingly, we noticed that the amount of zeaxanthin uptake into SR-B2 over-expressing cells was significant at higher concentrations of the carotenoid. In addition, the amount of zeaxanthin taken up by the cells was almost ten-fold higher than that of lutein.

With LDL as well as HDL, complexes of *meso*-zeaxanthin, SR-B1 over-expressing cells displayed the highest carotenoid uptake at all concentrations (Fig. 5E and F). *meso*-Zeaxanthin, similar to zeaxanthin, was taken up better by cells in the presence of HDL. Interestingly, the uptake of HDL complexes of *meso*-zeaxanthin was higher in all three SRB over-expressing cells. SR-B2 over-expressing cells showed increased uptake of *meso*-zeaxanthin, and this trend was evident at all three concentrations of the carotenoid. Control cells treated with zeaxanthin or *meso*-zeaxanthin took up higher amounts of carotenoid than with lutein treatment. This suggests an inherent preference of zeaxanthins over lutein by these cells that may be SR-B independent.

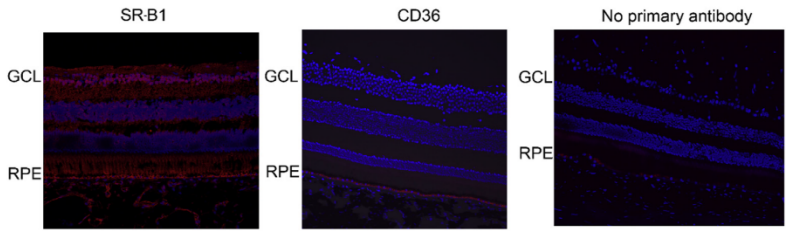

Fig. 7. Immunohistochemistry showing expression of SR-B1 and CD36 in macaque eye sections. SR-B1 expression (red) was detected throughout the RPE and retinal layers. CD36 expression (red) was limited to the RPE layer. Both CD36 and SR-B1 antibodies were rabbit polyclonal. "No primary antibody" control sections were incubated with the secondary antibody alone. Nuclei are stained using DAPI in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

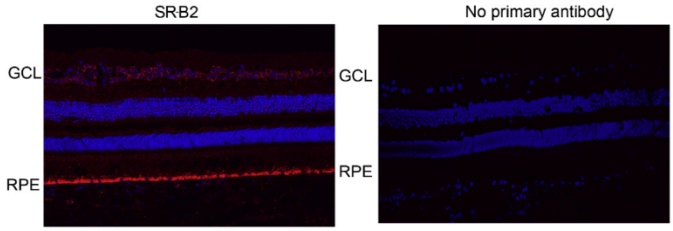

Fig. 8. SR-B2 staining in macaque eye sections. Protein expression was strong in the RPE layer and in the ganglion cell layer. Weak expression was detected throughout the retina. SR-B2 expression (red) was determined using a goat polyclonal antibody. "No primary antibody" control sections were incubated with only the secondary antibody. Nuclei are stained using DAPI in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Even though *meso*-zeaxanthin is not a common dietary component and is synthesized in the RPE enzymatically from lutein [28], supplements of this carotenoid are on the market [12]. It is hypothesized that consumption of such supplements may have positive effects on visual function [27], and protein mediated transport and deposition from the RPE to the retina would still be important. Identification that all three SRBs can function as *meso*-zeaxanthin transporters reveals a possible mechanism by which this carotenoid supplied by supplements may reach the retina.

With the understanding that SRB proteins can function as macular xanthophyll transporters, our next goal was to understand any differences in distribution of SRB proteins in the central and peripheral regions of human donor eye. We hypothesized that if SRB proteins are responsible for the selective accumulation of carotenoids in the macula, there will be differences in their protein expression profile in the macular and peripheral regions of the eye. Western blotting was conducted on human donor samples, and our data show the presence of SR-B1 and SR-B2 in peripheral retina, peripheral RPE, macular retina, and sub-macular RPE (Fig. 6A and B). These proteins were determined using total lysates of the above-mentioned tissues. CD36, however, could not be detected in the total lysate. Thus, we carried out membrane protein isolation followed by Western blot. In the membrane fraction, we observed the presence of CD36 in the sub-macular RPE and peripheral RPE but not in macular retina or peripheral retina. Detection of CD36 after membrane protein isolation but not in the total tissue lysate indicates its low abundance (Fig. 6C and D). We did not observe any differences in the expression patterns of SR-B1 and SR-B2 in the peripheral and macular regions of the eye. However, we noticed SR-B2 protein resolved at a slightly higher molecular weight in the RPE lysates. In addition, our data also revealed higher amounts of SR-B2 in the retina than in the RPE. Future studies may analyze the reasons behind the increased expression of this protein in the retina.

In order to determine the specific cell types in which these proteins are expressed, immunohistochemistry was conducted on sagittal sections of macaque eyes. SR-B1 was expressed throughout the retina and RPE (Fig. 7A). SR-B2, like SR-B1, was expressed throughout the retinal layers. Strong SR-B2 expression was observed in the RPE layer and ganglion cell layer of the retina (Fig. 8). Consistent with the Western blot data, CD36 expression was detected only in the RPE layer (Fig. 7B).

Tserentsoodol et al. have previously shown the presence of all three SRB proteins in primate eye tissues, but we noticed inconsistencies in their Western blots and IHC. For instance, their IHC shows the presence of SR-B1 and SR-B2 in the retinal layers and in the RPE of primates. In their Western blot, SR-B1 was visible in the monkey neural retina, but no bands at the right molecular weight were present in the RPE fraction. Similarly, no bands that correspond to SR-B2 were present in the monkey retina and RPE. Their IHC detected the presence of CD36 in various retinal layers and not in the RPE, but in their Western blots, CD36 was not detected in the primate retina or RPE [13]. In the present study, we were able to observe the presence of SR-B1 and SR-B2 throughout the retina and RPE, while CD36 was only in the RPE. Our Western blots are in agreement with the IHC findings.

In the present study, we have shown that all three SRBs are expressed in the primate macula as well as peripheral regions. Interestingly, we did not observe any changes in expression profiles of these proteins in the macula and peripheral retinal tissues. Our analyses suggest that SRB proteins, even though they are capable of

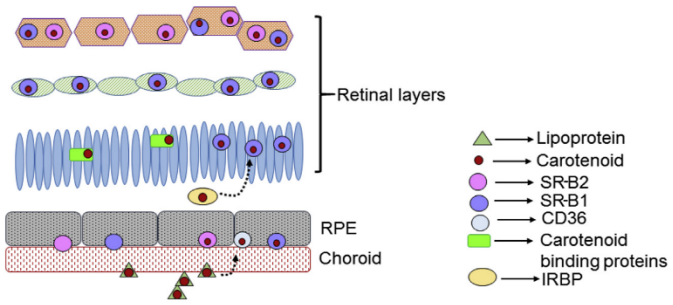

Fig. 9. Proposed model of carotenoid uptake into the macula. Carotenoids in lipoproteins reach the RPE through the choroidal circulation. Here, SRB proteins bind and transport the carotenoids. From the RPE, transport proteins such as IRBP may function to shuttle the carotenoids into the retina. Once in the retina, binding proteins retain the carotenoids. Low level of CD36 protein was detected only in the RPE and not in the retina.

transporting macular xanthophylls, may not be responsible for their selective accumulation in the macula. Consistent with previous work [7], we also found that LDL complexes of lutein and HDL complexes of zeaxanthin and *meso*-zeaxathin are taken up better by cells in culture. With SPR analyses, we have quantitatively determined the binding affinities of SRBs to macular carotenoids.

In conclusion, we have shown that all three human SRBs are capable of binding and transporting all three macular carotenoids. Previous studies from our laboratory have identified and characterized carotenoid binding proteins that have restricted expression in the macula and not in the peripheral regions. GSTP1 was determined to be a zeaxanthin binding protein. Its strong expression was detected in the inner and outer plexiform layers of primate macula [19]. Similarly, work in our lab characterized StARD3 as a lutein binding protein [22]. Expression of this protein was enhanced in the primate macula, specifically in the photoreceptor inner segments. The present study reveals the mechanism by which carotenoids in circulation reach the peripheral or macular retina (Fig. 9). The presence of higher levels of specific binding proteins in the macula is likely be responsible for the accumulation of carotenoids in this region.

Conflicts of interest

Authors declare no conflicts of interest.

Acknowledgements and grant support

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CHAPTER 3

DEVELOPMENTALLY REGULATED PRODUCTION OF *MESO*-ZEAXANTHIN IN CHICKEN RETINAL PIGMENT EPITHELIUM/CHOROID

Gorusupudi A, Shyam R, Li B, Vachali P, Subahni YK, Nelson K, Bernstein PS. Developmentally Regulated Production of *meso*-zeaxanthin in chicken retinal pgment epithelium/choroid and retina. (2016). Invest Ophthalmol Vis Sci. 57. 1853-61. With Permission from Investigative Ophthalmology and Visual Sciences.

Credits

Paul Bernstein and Binxing Li conceived the idea of this project. Aruna Gorusupudi and I performed all the experiments. Preejith Vachali helped optimize the experimental conditions. Kelly Nelson was in charge of the supplies including chicken eggs. Yumna Subhani helped with carotenoid extractions of brain samples. Paul Bernstein and I wrote the manuscript and answered all the reviewers' questions.

Biochemistry and Molecular Biology

Developmentally Regulated Production of *meso*-Zeaxanthin in Chicken Retinal Pigment Epithelium/Choroid and Retina

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AG and RS contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Purpose. *meso*-Zeaxanthin is a carotenoid that is rarely encountered in nature outside of the vertebrate eye. It is not a constituent of a normal human diet, yet this carotenoid comprises one-third of the primate macular pigment. In the current study, we undertook a systematic approach to biochemically characterize the production of *meso*-zeaxanthin in the vertebrate eye.

Methods. Fertilized White Leghorn chicken eggs were analyzed for the presence of carotenoids during development. Yolk, liver, brain, serum, retina, and RPE/choroid were isolated, and carotenoids were extracted. The samples were analyzed on C-30 or chiral HPLC columns to determine the carotenoid composition.

Results. Lutein and zeaxanthin were found in all studied nonocular tissues, but no *meso*-zeaxanthin was ever detected. Among the ocular tissues, the presence of *meso*-zeaxanthin was consistently observed starting at embryonic day 17 (E17) in the RPE/choroid, several days before its consistent detection in the retina. If RPE/choroid of an embryo was devoid of *meso*-zeaxanthin, the corresponding retina was always negative as well.

Conclusions. This is the first report of developmentally regulated synthesis of *meso*-zeaxanthin in a vertebrate system. Our observations suggest that the RPE/choroid is the primary site of *meso*-zeaxanthin synthesis. Identification of *meso*-zeaxanthin isomerase enzyme in the developing chicken embryo will facilitate our ability to determine the biochemical mechanisms responsible for production of this unique carotenoid in other higher vertebrates, such as humans.

Keywords: carotenoid, macular pigment, *meso*-zeaxanthin, lutein, chicken

Meso-Zeaxanthin is a major carotenoid component of the human fovea,[1,2] yet its physiological significance in ocular tissues remains elusive. This xanthophyll carotenoid is present only in ocular tissues, with peak concentrations at the fovea, but it is not detectable in the liver or in the serum,[3,4] strongly suggesting specific production of *meso*-zeaxanthin in the eye. Natural dietary sources of *meso*-zeaxanthin are limited to rarely eaten foods, such as turtle fat, fish skin, and shrimp shells.[5,6] Because these are not a significant part of a normal Western diet, the contribution of food intake toward *meso*-zeaxanthin is considered negligible. The biochemical process by which *meso*-zeaxanthin is produced in the retina is unknown. Feeding studies in Japanese quails and macaque monkeys have suggested that lutein is the precursor for *meso*-zeaxanthin.[7,8] *meso*-Zeaxanthin and lutein have identical stereochemistries at the 3 and 3′ positions and differ only in the position of one double bond (Fig. 1). Conversion of lutein to *meso*-zeaxanthin can be readily achieved under harsh industrial conditions of high temperature and strong base,[1] but this reaction has not yet been identified in vitro under physiological conditions.

Identification of the biochemical mechanisms of this isomerization reaction in an isolated biological system could provide insights into the function of *meso*-zeaxanthin in the ocular tissues in health and disease. Zeaxanthins are better antioxidants than lutein,[9] but lutein is much more abundant in dietary sources than zeaxanthin.[10] Therefore, the isomerization reaction to produce *meso*-zeaxanthin from lutein may be an effective way to improve the antioxidant properties of macular carotenoids in the fovea. Defects of *meso*-zeaxanthin production in the eye could lead to abnormal macular pigment levels and distributions, which has led various investigators to speculate that deficiencies of a putative *meso*-zeaxanthin isomerase could underlie macular disease, such as AMD or macular telangiectasia type II (MacTel), but to make such an argument, it is imperative to unequivocally show that such a process is enzymatically mediated. Here, we demonstrate that *meso*-zeaxanthin is produced in a developmentally regulated manner in the embryonic chicken eye and that it is likely that the *meso*-zeaxanthin isomerase enzyme is expressed in the RPE/choroid.

Methods

Incubation of Eggs, Staging, and Isolation of Tissues

Fertilized eggs from White Leghorn chickens were obtained from a local hatchery. Embryonic day 0 (E0) eggs were incubated at 37°C under 50% humidity in dark conditions.

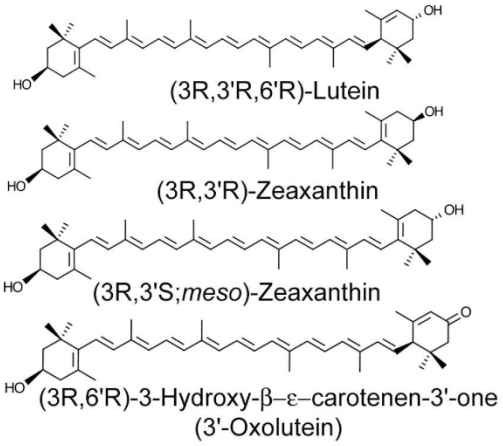

FIGURE 1. Structures of the macular carotenoids. The macular carotenoids are isomers with the same molecular formula: $C_{40}H_{56}O_2$. Lutein and *meso*-zeaxanthin differ in the position of the double bond in one of the ionone rings (4′–5′ vs. 5′–6′). Zeaxanthin and *meso*-zeaxanthin differ in the spatial arrangement of the 3′-OH group. 3′-Oxolutein is a xanthophyll carotenoid oxidation product with the molecular formula $C_{40}H_{54}O_2$.

Eggs were removed and processed at various time points during development. Embryos were weighed and staged according to Hamburger-Hamilton Staging. Yolk, brain, eyes, blood, and liver were isolated from the embryo. Dissection of ocular tissues was carried out in saline with direct visualization under a dissecting microscope; 0.25% trypsin in saline was used to facilitate separation of RPE/choroid from retina. After dissection, tissues were spun down briefly to remove any excess saline, and wet weight was measured.

Extraction and Saponification of Carotenoids

Yolk was homogenized on ice using three 30-second sonicator pulses; 0.5 mL homogenized yolk was removed and extracted using 1 mL tetrahydrofuran (THF) containing 0.1% butylated hydroxytoluene (BHT). Extractions were conducted by vortexing the samples for 2 minutes, followed by water bath sonication at 4°C for 10 minutes. Blood samples were centrifuged at 2000*g* at 4°C for 2 minutes to isolate serum from blood cells. Sera were extracted using THF (+0.1% BHT) after vigorous vortexing. Liver and brain tissues were homogenized following the addition of 0.5 mL 0.85% saline; 700 µL 1-mm silica beads were added to 0.25 mL of the homogenate along with 1 mL THF (+0.1% BHT). Extraction was carried out following three 30-second pulses in a Mini-Beadbeater (BioSpec Products, Tulsa, OK, USA). Samples were rested on ice for at least 1 minute following each 30-second pulse. Ocular tissues from each embryo were pooled,

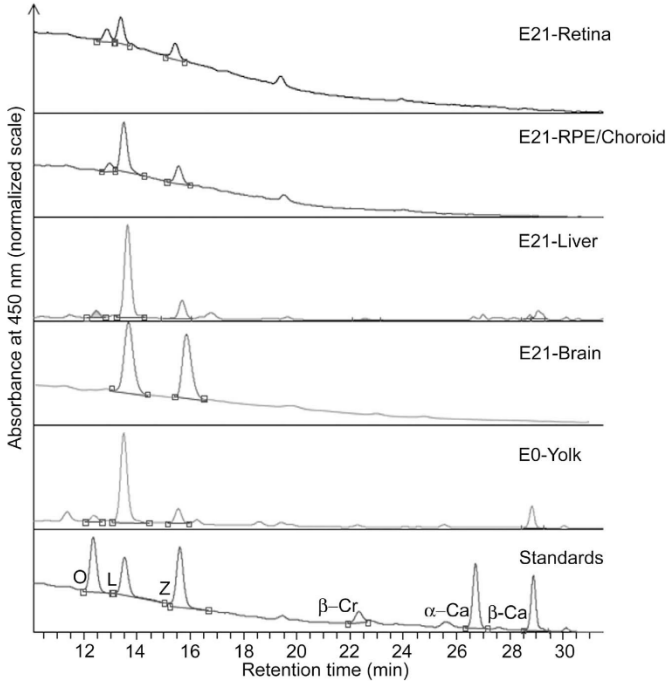

FIGURE 2. Complete carotenoid profile of chicken embryonic tissues obtained by HPLC separation using a C-30 column. Embryonic day 21 retina, RPE/choroid, and liver samples contained lutein and zeaxanthin along with trace amounts of 3′-oxolutein. Embryonic day 21 brain showed only the presence of lutein and zeaxanthin. Embryonic day 0 yolk had lutein, 3′-oxolutein, and zeaxanthin. Trace amounts of β-carotene were detected in the yolk and liver samples. The peaks observed at 19.5 minutes in E21 RPE/choroid and retina samples remain unidentified because their visible spectra are not characteristic of carotenoids. The presence of other carotenoids, such as astaxanthin, canthaxanthin, and β-cryptoxanthin,[7,17,18] were assessed in separate HPLC runs; none of these carotenoids that are occasionally added to chicken feed were present in our samples. O, 3′-oxolutein; L, lutein; Z, zeaxanthin; β-Cr, β-cryptoxanthin; α-Ca, α-carotene; β-Ca, β-carotene;.

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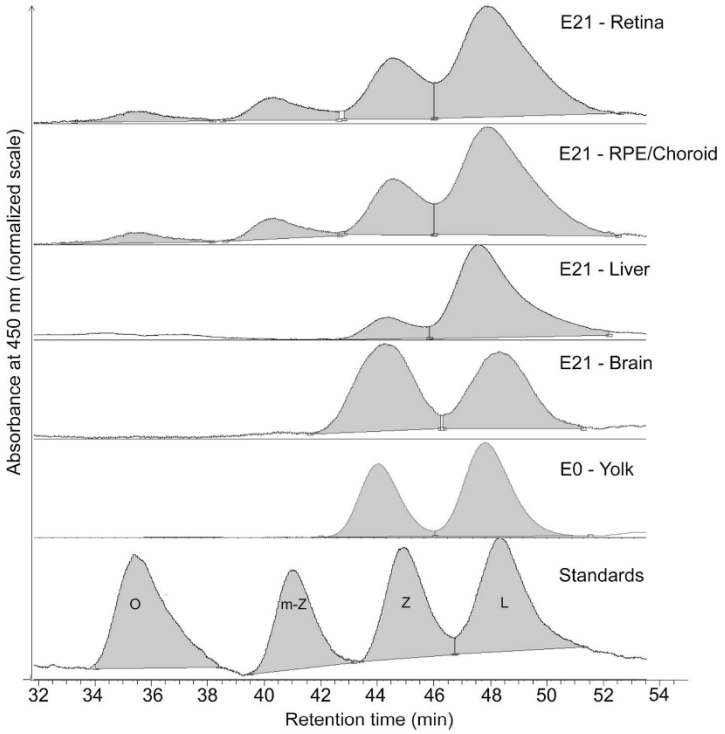

FIGURE 3. Complete carotenoid profile of chicken embryonic tissues obtained by HPLC separation using a chiral column. *meso*-Zeaxanthin was detected in E21 retina and RPE/choroid samples, but not in E21 liver, E21 brain, or E0 yolk samples. O, 3′-oxolutein; *m*-Z, *meso*-zeaxanthin; Z, zeaxanthin; L, lutein.

weighed, and extracted using 1 mL THF (+0.1% BHT), followed by bead beating as indicated above. Three extractions were conducted for each sample.

Extracts were combined and evaporated using a rotary evaporator under reduced pressure. All extracts, except sera, were subjected to saponification to hydrolyze esterified carotenoids and to break up fibrous tissues. Vertebrate serum contains only nonesterified carotenoids; therefore, saponification was not necessary for these samples.[3,4] Dried samples were treated with 0.1 mL methanol, 0.5 mL hexane, and 0.5 mL 20% KOH in methanol. The samples were saponified at 37°C for 45 minutes on a shaker. Following saponification, samples were washed with water, and 0.5 mL 90% hexane and 10% ethyl acetate was added to extract the cartenoids. The organic phase was removed following centrifugation at 2000*g* to remove any residual KOH and dried in a rotary evaporator under reduced pressure.

Preparation of Standards and HPLC Conditions

Two HPLC analysis methods were used. Method 1 was used for the separation of *meso*-zeaxanthin from zeaxanthin. In this method, a ChiralPak AD column (Daicel Technologies, West Chester, PA, USA) of 25 cm length × 4.6 mm internal diameter was used with a mobile phase that was composed of 95:5 (vol/vol) hexane:isopropanol. Method 2 was used to determine the complete carotenoid profile of samples. For this, a C-30 column (YMC Carotenoids, Allentown, PA, USA) of 25 cm length × 4.6 mm internal diameter was used. The mobile phase for the C-30 column was composed of a series of linear gradients of methanol:methyl tert-butyl ether (% methanol@-min: 99 @0; 90 @10; 70 @20; 0 @30; 99 @35; 99 @40). The dried samples were mixed in 200 µL HPLC mobile phase. Mobile phase flow rates of 0.7 mL per minute for method 1 and 1 mL per minute for method 2 were used to separate the carotenoids. Columns were maintained at room temperature with peak monitoring at 450 nm. The identities of the carotenoid peaks were confirmed by photo-diode array spectra, and by co-elution with authentic standards. Previously published extinction coefficients were used to calculate the concentrations of the standard carotenoid solutions. These solutions were injected in amounts ranging from 0.1 ng to 100 ng to plot standard curves. The carotenoid concentrations in the samples were determined from these standard curves.

RESULTS

Complete Carotenoid Profile of Chicken Embryos

To determine the range of carotenoids that may be present in the tissues of chicken embryos, we first analyzed the extracted carotenoids from liver, yolk, brain, RPE/choroid, and retina on a reverse-phase C-30 column. However, this column cannot be

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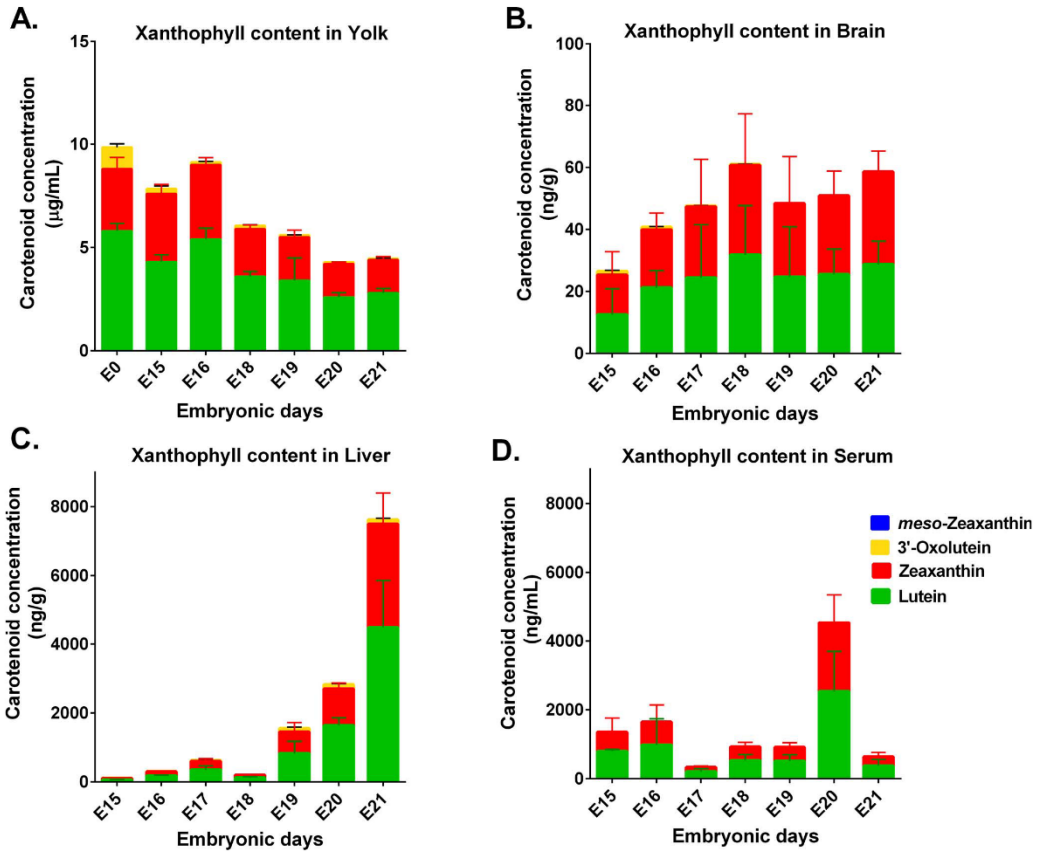

FIGURE 4. Xanthophyll carotenoid concentrations in chicken nonocular embryonic tissues. (**A**) In the yolk, the concentrations of lutein, zeaxanthin, and 3′-oxolutein decrease as the embryo ages. (**B**) Brain contained considerably lower amounts of these carotenoids, and their total concentrations increase with the development of the embryo. (**C**) As the embryo neared hatching, the liver accumulated very high concentrations of lutein, zeaxanthin, and trace amounts of 3′-oxolutein. (**D**) Serum carotenoids varied considerably in the embryo during development. *Error bars* represent SEM for five samples at each time point. Experiments were repeated three times, and similar results were obtained. *meso*-Zeaxanthin was never detectable in any of these nonocular tissues.

used for the detection of *meso*-zeaxanthin because dietary 3R,3′R-zeaxanthin and 3R,3′S-*meso*-zeaxanthin co-elute.

Analysis by C-30 column of E0 yolk samples showed the presence of only zeaxanthin, lutein, β-carotene, and 3′-oxolutein (Fig. 2). 3′-Oxolutein is a well-known oxidative product of dietary carotenoids.[11] Embryonic day 21 retina, RPE/choroid, and liver samples, when analyzed on the C-30 column, showed the presence of 3′-oxolutein, zeaxanthin, and lutein, but only liver had detectable amounts of β-carotene. Brain contained only lutein and zeaxanthin. We repeated all of these HPLC analyses on a chiral column that adequately separates zeaxanthin stereoisomers, and only ocular tissues had detectable *meso*-zeaxanthin (Fig. 3).

Carotenoid Profiles of Nonocular Tissues During Chicken Embryonic Development

Yolk contained significant amounts of lutein and zeaxanthin throughout embryonic development. At E0, 5.8 μg lutein and 3.3 μg zeaxanthin were present per milliliter of yolk. By E15, the yolk lutein concentration decreased to 4.3 μg/mL, whereas the zeaxanthin amount remained closer to E0 levels (Fig. 4A). Lutein concentrations decreased at a higher rate relative to zeaxanthin as the embryo neared the time of hatching. Decreasing amounts of 3′-oxolutein were detected in the egg yolk. At E0, the ratio of lutein to zeaxanthin in the egg yolk was close to 2.0, but between E15 and E21, the ratio had dropped to approximately 1.5 (Fig. 5A). No *meso*-zeaxanthin was observed in egg yolk at any time points.

In the brains of developing chicken embryos, we detected low amounts of lutein and zeaxanthin. As opposed to microgram levels of lutein and zeaxanthin that were observed in the yolk, brain contained only nanogram levels of these carotenoids (Fig. 4B). We observed an increasing trend in the concentration of lutein as well as zeaxanthin in the brain tissue with embryonic development. Only trace amounts of 3′-oxolutein were detected in the brain. In the time points under consideration, there were equal amounts of lutein and

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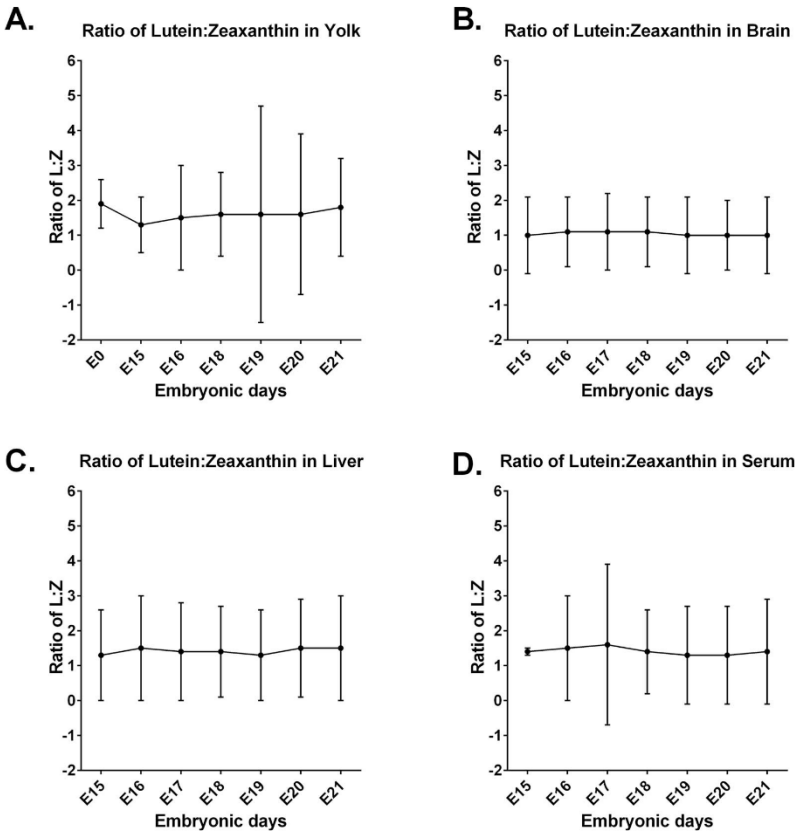

Figure 5. Ratios of lutein and zeaxanthin in nonocular tissues during chicken embryonic development. (**A**) Yolk contained twice as much lutein as zeaxanthin at E0; however, as the embryo ages, the ratio of lutein to zeaxanthin becomes closer to 1.5. (**B**) Brain samples contained almost equal amounts of lutein and zeaxanthin during development. (**C**, **D**) Liver and serum samples consistently had lutein:zeaxanthin ratios close to 1.5. *Error bars* represent SEM for five samples at each time point.

zeaxanthin in the brains (Fig. 5B). No *meso*-zeaxanthin was observed in brain samples at any time point.

Embryonic day 15 liver tissues contained 55.5 ng/g lutein and 43.2 ng/g zeaxanthin. By E21, the carotenoid concentrations were 4.5 µg/g and 3.0 µg/g, respectively (Fig. 4C). Small and increasing amounts of 3′-oxolutein were detected in the liver during development. Even though the carotenoid content in the liver of embryonic chicken increased 10^3-fold as the embryos neared hatching, the ratio of lutein to zeaxanthin remained close to 1.5 (Fig. 5C). No *meso*-zeaxanthin was observed in the liver samples.

Serum samples of chicken embryos showed considerable variation in lutein and zeaxanthin concentrations during development (Fig. 4D). It was challenging to remove blood samples from embryos without yolk contamination, which may be the reason for the fluctuation in lutein and zeaxanthin levels in our samples. Importantly, there was no *meso*-zeaxanthin present in the serum at any time points, and lutein:zeaxanthin ratios remained in the 1.3 to 1.6 range between E15 and E21 (Fig. 5D).

Ocular Tissues Show the Presence of *meso*-Zeaxanthin in a Developmentally Regulated Manner

In the RPE/choroid of chicken embryo, there was an increase in concentration of lutein as well as zeaxanthin with age. At E15, RPE/choroid samples contained 2.03 ng lutein and 4.03 ng zeaxanthin in a pair of eyes (Fig. 6A). By E21, the concentrations increased to 19.9 ng lutein and 18.6 ng zeaxanthin per pair of eyes. 3′-Oxolutein was first detected at E21 in the embryos. The ratio of lutein to zeaxanthin was 0.5 in E15 embryo, and it increased to 1.1 by E21 (Fig. 6C). Retinas of chicken embryos showed similar trends in carotenoid concentration. At E15, 0.84 ng lutein and 0.93 ng zeaxanthin per pair of eyes were observed (Fig. 6B). By E21, there were 8.51 ng lutein and 18.17 ng zeaxanthin per pair of eyes. Trace amounts of 3′-oxolutein were detected in the retina starting at E20. In the retina, the ratio of lutein to zeaxanthin was at 0.9 at E15 but decreased to 0.5 by E21 (Fig. 6C).

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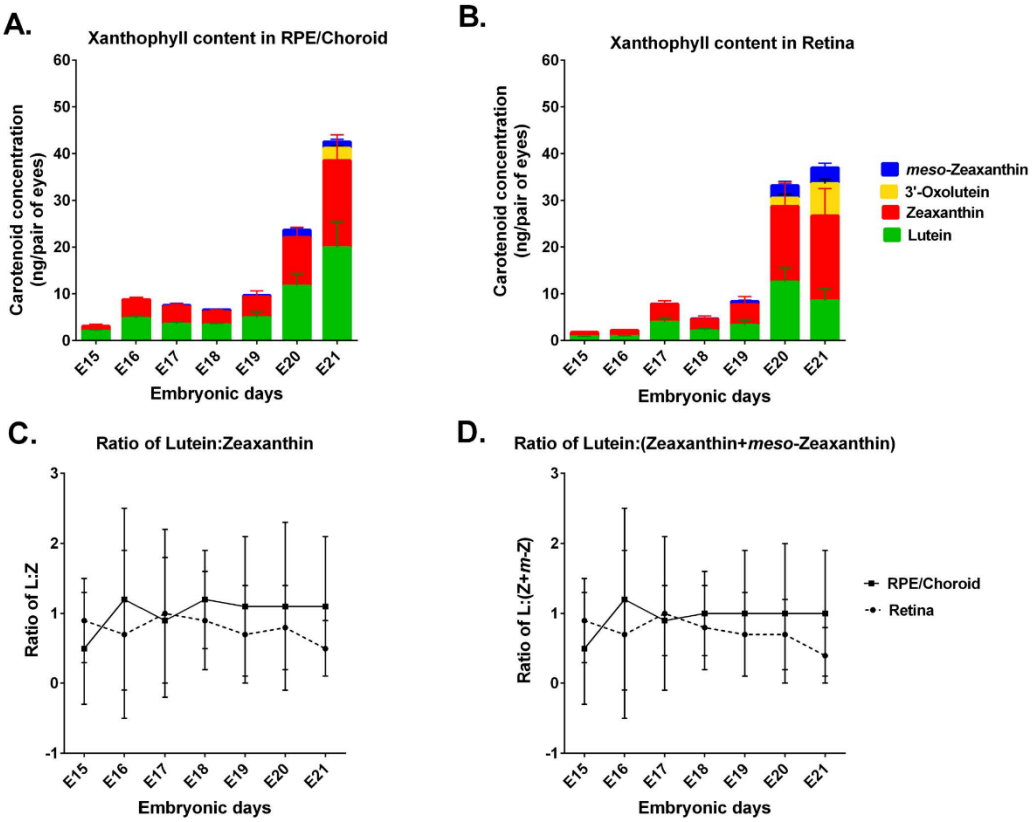

Figure 6. Xanthophyll carotenoid content in ocular tissues during chicken embryonic development. (**A**) Retinal pigment epithelium/choroid showed an increase in lutein and zeaxanthin as the embryo developed. *meso*-Zeaxanthin was first detectable in RPE/choroid at E17. As the embryo neared hatching, *meso*-zeaxanthin levels increased in the RPE/choroid. 3′-Oxolutein was first detected at E21 in the RPE/choroid. (**B**) Similar trends were observed in the retinas; however *meso*-zeaxanthin was first detectable in the majority of samples at E19. 3′-Oxolutein was first detected at E20. (**C**) Lutein:zeaxanthin ratios in the retina were less than 1. (**D**) Similar trends were observed in the ratios of lutein to zeaxanthin + *meso*-zeaxanthin. *Error bars* represent SEM for five samples at each time point. Experiments were repeated three times, and similar results were obtained.

Among the various tissues that were analyzed, retina and RPE/choroid were the only tissues that showed the presence of *meso*-zeaxanthin (Figs. 3, 6). Interestingly, we observed the presence of this carotenoid in a tissue-specific, developmentally regulated manner. In the RPE/choroid, we first detected *meso*-zeaxanthin at E17 (Fig. 7A). The concentration of this carotenoid steadily increased from 0.25 ng per pair of eyes at E17 to 1.34 ng per pair of eyes at E21 (Fig. 7A). In the retina, *meso*-zeaxanthin was detected in 80% of the analyzed samples at E19, and the concentration ranged from 0.63 ng per pair of eyes at E19 to 3.37 ng/pair of eyes at E21 (Fig. 7B). By E20 and E21, 100% of the analyzed retina and RPE/choroid samples contained *meso*-zeaxanthin (Fig. 7B). With the exception of just one E19 embryo, *meso*-zeaxanthin was consistently detected in the RPE/choroid starting at E17, several days before it was consistently detected in the retina (Figs. 6, 7). In addition, if RPE/choroid of an embryo was devoid of *meso*-zeaxanthin, the corresponding retina was always negative as well. Conversely, we never observed a case in which the retina was positive for *meso*-zeaxanthin when the corresponding RPE/choroid was negative.

Discussion

The macular pigment consists of three carotenoids: lutein, zeaxanthin, and *meso*-zeaxanthin. These carotenoids are highly concentrated at the fovea, and their concentration decreases toward the periphery of the retina.[1,2] In nonocular tissues and serum, the ratio of lutein:zeaxanthin:*meso*-zeaxanthin is approximately 4:1:0; however, in the peripheral retina it becomes 2:1:0, and in the fovea the ratio changes to 1:1:1.[2] The lack of *meso*-zeaxanthin in the serum has led to the hypothesis that this isomer may be formed as a result of metabolic transformations within the retina.[7,8] The macular carotenoids are isomers with a common $C_{40}H_{56}O_2$ composition. Dietary zeaxanthin and *meso*-zeaxanthin differ in the stereochemistry of just the hydroxyl group at the 3′ position, but biological interconversion reactions at a single chiral center rarely occur in nature.

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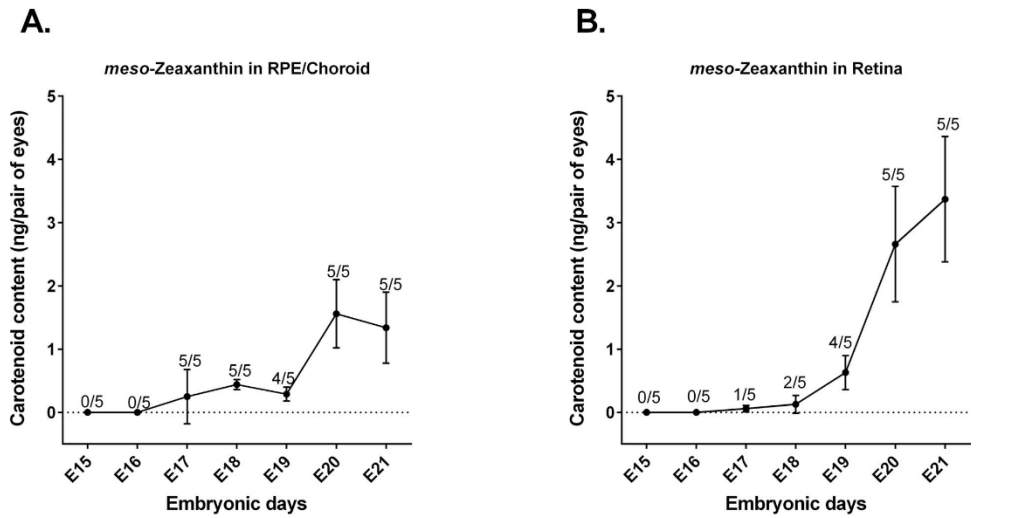

FIGURE 7. *meso*-Zeaxanthin content in RPE/choroid and retina of developing chicken embryos. (**A**, **B**) In the retinas, a few samples (1/5) contained detectable levels of *meso*-zeaxanthin at E17, whereas all (5/5) the RPE/choroid samples at E17 contained this carotenoid. As the embryo neared hatching, all the analyzed retinas and RPE/choroids contained *meso*-zeaxanthin. Even though most RPE samples were positive for *meso*-zeaxanthin earlier than the retina, the ultimate amounts of *meso*-zeaxanthin were higher in the retinas. Ratios indicated above the data points report the number of samples positive for *meso*-zeaxanthin in one representative experiment. *Error bars* represent SEM for five samples at each time point. Experiments were repeated three times with similar results.

In vivo studies conducted by Johnson and coworkers[8] indicated that lutein rather than zeaxanthin may be the precursor of *meso*-zeaxanthin in primate retina. In their study, carotenoid-deficient animals were maintained on lutein or zeaxanthin supplements. The animals that were fed with lutein showed the presence of *meso*-zeaxanthin in the retina. Control animals that were either maintained on a carotenoid-deficient diet or the ones fed with zeaxanthin alone did not have detectable *meso*-zeaxanthin in their retinas. This suggests a nondietary origin of *meso*-zeaxanthin in primates. Studies conducted in our laboratory using deuterium-labeled carotenoids produced similar results in Japanese quails.[7] These studies suggest that ocular-specific transformation of lutein to *meso*-zeaxanthin by an enzyme-mediated double-bond shift reaction is the source of the latter carotenoid.

Identification of the isomerase enzyme is complicated in primates and quails. This is because experiments in primates are difficult to execute and are expensive. Quail retina contains several rare carotenoids that may confound downstream enzymology assays. Therefore, in the present study, we chose to determine whether chicken embryos might be a better system to study the production of *meso*-zeaxanthin because they have a simpler ocular carotenoid profile, and their eggs are easily incubated in a laboratory setting.

The chicken has been a widely used model to study carotenoid transport and retention.[13,14] High-density lipoprotein (HDL)-deficient Wisconsin Hypoalpha Mutant chickens were used in studies in which the role of HDL-mediated transport of lutein into the retina was identified.[14] Work done by Connor et al.[14] showed that HDL deficiency led to decreased content of both lutein and zeaxanthin in the retina. In another study, it was determined that chickens maintained on a xanthophyll-free diet resulted in a decreased accumulation of lutein and zeaxanthin in tissues, consistent with a dietary origin of these carotenoids in birds.[13] Interestingly, it was shown that although plasma and other tissues of birds on a xanthophyll-free diet exhibited the loss of more than 90% of lutein and zeaxanthin, the retina selectively retained these carotenoids.[13] This is consistent with a profound physiological significance for lutein and zeaxanthin in the cone-rich retinas of birds.

Zeaxanthins are better antioxidants than lutein.[9] Because dietary sources typically contain higher concentrations of lutein,[10] it is not surprising that tissues have selective uptake/retention mechanisms optimized to maintain higher zeaxanthin concentrations. Our data show consistently lower lutein:zeaxanthin ratios in the retinas of developing embryos when compared with nonocular tissues (Figs. 5, 6).

Data presented by Wang and coworkers[13] documented the presence of *meso*-zeaxanthin in the retinas of 1-day-old chickens. The ocular concentration of this carotenoid was maintained in the birds fed with the xanthophyll-free diet, suggesting a potential physiological relevance. Consistent with their observations, our study shows the presence of *meso*-zeaxanthin in a developmentally regulated manner. In addition, our study reveals that *meso*-zeaxanthin is an ocular-specific carotenoid in the chicken embryos. More importantly, our data show that RPE/choroid is the likely site of *meso*-zeaxanthin production. At E17, we first observe the presence of *meso*-zeaxanthin in all of the RPE/choroid samples that were analyzed; however, only 20% of the retina samples showed the presence of this carotenoid. At E19, we identified *meso*-zeaxanthin in 80% of both retina and RPE/choroid samples. Whenever RPE/choroid was negative for *meso*-zeaxanthin, this carotenoid was absent in the retina as well, and we never had an instance in which an embryo's retina was positive for this carotenoid when its RPE/choroid was negative. Considering that we consistently observe the presence of *meso*-zeaxanthin in the RPE/choroid several days ahead of when it is present in retina and that we never

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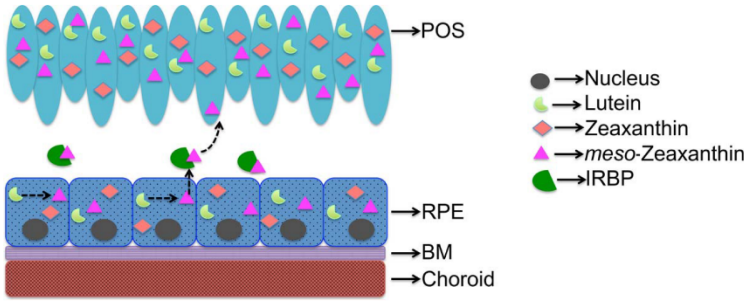

Figure 8. Model representing *meso*-zeaxanthin production in the RPE/choroid and uptake into the retina. Lutein is converted into *meso*-zeaxanthin in the RPE/choroid. The newly formed *meso*-zeaxanthin could be transported into the retina by transporter proteins such as IRBP.[16] Lutein and zeaxanthin would presumably use a similar transport pathway from the RPE to the retina via IRBP without metabolic transformations. *Dotted lines* indicate hypothesized pathway. POS, photoreceptor outer segment; BM, Bruch's membrane.

identified this carotenoid in the retina of an embryo when RPE/choroid was devoid of it, we can conclude that the RPE/choroid is the primary site of synthesis of *meso*-zeaxanthin. Because the eggs were kept in the dark during incubation, we can exclude the role of light in the production of *meso*-zeaxanthin. Previous studies have shown that in the RPE/choroid, carotenoids are nonesterified,[7] which provides higher accessibility of the carotenoids in this tissue to enzymes that may be responsible for the production of *meso*-zeaxanthin.

If the RPE/choroid is the primary site for *meso*-zeaxanthin production, then transporter proteins may be involved in carrying the newly formed *meso*-zeaxanthin from the RPE into the retina. A similar situation is observed for retinoid metabolism in which the visual chromophore 11-*cis*-retinal is enzymatically produced in the RPE and then transported to the photoreceptors.[15] Retinoids are closely associated to carotenoids, and retinoid metabolism involves coordination among choroid, RPE, and the photoreceptors mediated by transporter proteins.[15] Interphotoreceptor retinoid-binding protein (IRBP) is one such transport protein implicated in retinoid transport between RPE and retina.[15,16] It is an abundant transport protein in the interphotoreceptor matrix. Studies from our laboratory have shown that IRBP can bind macular carotenoids with moderate affinity, characteristic of a nonspecific transporter,[16] with equilibrium binding constants comparable to IRBP-retinoid interactions. We therefore hypothesize that IRBP may be involved in transporting *meso*-zeaxanthin from the RPE into the retina in chicken and human eyes as well (Fig. 8).

This is the first report of developmentally regulated synthesis of *meso*-zeaxanthin in a vertebrate model system. Our study has determined that the RPE/choroid is the primary site of *meso*-zeaxanthin synthesis. Because the carotenoids in the RPE are nonesterified, they are accessible to enzymes that may mediate the production of *meso*-zeaxanthin in this tissue. Future studies will determine candidate proteins that can function as a *meso*-zeaxanthin isomerase.

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CHAPTER 4

RPE65 HAS AN ADDITIONAL FUNCTION AS THE LUTEIN TO *MESO*-ZEAXANTHIN ISOMERASE IN THE VERTEBRATE EYE

Shyam R, Gorusupudi A, Nelson K, Horvath M.P, Bernstein P.S. RPE65 has an additional function as the lutein to *meso*-zeaxanthin isomerase in the vertebrate eye. (2017). Proc Natl Acad Sci U S A. 2017 Sep 5. pii: 201706332. doi: 10.1073/pnas.1706332114. [Epub ahead of print]. With permission from Proc Natl Acad Sci USA.

Credits

Paul Bernstein and I designed the research. I conducted all the experiments. Aruna Gorusupudi carried out the HPLC analysis, and Kelly Nelson was responsible for the collection and maintenance of chicken eggs. Martin Horvath conducted structural modelling. Paul Bernstein and I wrote the manuscript and answered all the reviewers' queries. Paul Bernstein and Martin Horvath edited the manuscript before submission.

RPE65 has an additional function as the lutein to *meso*-zeaxanthin isomerase in the vertebrate eye

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Carotenoids are plant-derived pigment molecules that vertebrates cannot synthesize de novo that protect the fovea of the primate retina from oxidative stress and light damage. *meso*-Zeaxanthin is an ocular-specific carotenoid for which there are no common dietary sources. It is one of the three major carotenoids present at the foveal center, but the mechanism by which it is produced in the eye is unknown. An isomerase enzyme is thought to be responsible for the transformation of lutein to *meso*-zeaxanthin by a double-bond shift mechanism, but its identity has been elusive. We previously found that *meso*-zeaxanthin is produced in a developmentally regulated manner in chicken embryonic retinal pigment epithelium (RPE)/choroid in the absence of light. In the present study, we show that RPE65, the isomerohydrolase enzyme of the vertebrate visual cycle that catalyzes the isomerization of all-*trans*-retinyl esters to 11-*cis*-retinol, is also the isomerase enzyme responsible for the production of *meso*-zeaxanthin in vertebrates. Its RNA is up-regulated 23-fold at the time of *meso*-zeaxanthin production during chicken eye development, and we present evidence that overexpression of either chicken or human RPE65 in cell culture leads to the production of *meso*-zeaxanthin from lutein. Pharmacologic inhibition of RPE65 function resulted in significant inhibition of *meso*-zeaxanthin biosynthesis during chicken eye development. Structural docking experiments revealed that the epsilon ring of lutein fits into the active site of RPE65 close to the nonheme iron center. This report describes a previously unrecognized additional activity of RPE65 in ocular carotenoid metabolism.

carotenoid | isomerase | retina | lutein | zeaxanthin

The rare carotenoid *meso*-zeaxanthin is present only in the eyes of higher vertebrates (1, 2). This is a unique phenomenon in nature, especially since vertebrates normally obtain carotenoids through their diet and are incapable of producing these molecules de novo (2, 3). *meso*-zeaxanthin is not commonly found in dietary sources; besides the eyes of vertebrates, this carotenoid is present in shrimp shells, turtle fat, and fish skin (2, 4, 5). Hundreds of carotenoids are present in nature, and even though primates consume more than 50 of them, *meso*-zeaxanthin is one of only three carotenoids present in the foveal center of the retina, the region responsible for sharp, central vision. Degeneration of the retina and retinal pigment epithelium (RPE) surrounding the fovea occurs in the disease state known as age-related macular degeneration (AMD). Carotenoid supplementation has been shown to be effective in curtailing the progression of this disease, because these molecules are capable of protecting the fovea from blue light damage and reactive oxygen species (2, 6). Despite the abundance of *meso*-zeaxanthin in the foveal center, its specific function relative to dietary lutein and zeaxanthin remains unknown.

Lutein, zeaxanthin, and *meso*-zeaxanthin are the three carotenoids present at the foveal center (2). These molecules share the same molecular formula, $C_{40}H_{56}O_2$ (Fig. 1*A*). Since lutein and zeaxanthin are abundant in a normal diet, it has long been hypothesized that an isomerase enzyme may be responsible for the metabolic transformations of either lutein or zeaxanthin to produce *meso*-zeaxanthin (2). In vivo studies from our laboratory using quail have shown that birds fed with deuterium-labeled lutein produce labeled *meso*-zeaxanthin, while labeled zeaxanthin feed did not have the same effect (7). Similar results have been observed in primates as well. When carotenoid-deficient monkeys were fed lutein, *meso*-zeaxanthin was present in their retinas, but no *meso*-zeaxanthin was detected when these animals were maintained on feed enriched with zeaxanthin (8). Both of these studies indicate that lutein undergoes metabolic transformations to form *meso*-zeaxanthin in vivo, but the biochemical mechanism by which this reaction occurs is unknown, although it is efficiently produced under harsh industrial conditions, such as high temperature and strong base (9). Conversion of dietary zeaxanthin to *meso*-zeaxanthin would require inversion of a chiral center at the 3′ position, a reaction rarely encountered in biological systems. In contrast, conversion of lutein to *meso*-zeaxanthin proceeds by the migration of just one double bond from the 4′-5′ position to the 5′-6′ position, a reaction that should be readily accomplished by coordinated acid-base catalysis as illustrated in Fig. 1*B*. Other mechanisms involving radical chemistry are also plausible.

In an effort to determine the biochemistry behind *meso*-zeaxanthin formation, our laboratory undertook studies in developing chicken embryos. In this isolated system, we determined that *meso*-zeaxanthin is produced in a developmentally regulated manner in the RPE/choroid of chicken embryos from the lutein and zeaxanthin naturally present in egg yolk (10). In a previous study, we detected the presence of *meso*-zeaxanthin in the RPE/choroid of E17 embryos, with increasing levels as the embryo neared hatching at E21 (10). Retinal detection of *meso*-zeaxanthin occurred only at E19, and all other tissues examined (brain, liver, serum, and yolk) were devoid of this carotenoid. Since the eggs were incubated in the dark, we could rule out the role of light in *meso*-zeaxanthin production. In the current study, we present evidence that RPE65, the isomerohydrolase enzyme of the vertebrate visual cycle responsible for the isomerization of

Significance

Carotenoids are plant-derived pigment molecules that cannot be synthesized de novo by higher organisms. These physiologically relevant compounds function as potent antioxidants and light screening compounds, and their supplementation has been shown to ameliorate the progression of such diseases as age-related macular degeneration. Hundreds of carotenoids are present in the plant world, but the primate macula contains only three: lutein, zeaxanthin, and *meso*-zeaxanthin. The presence of *meso*-zeaxanthin in the foveal region of primates is an unexplained phenomenon, given its lack of dietary sources. We show that RPE65 is responsible for the conversion of lutein to *meso*-zeaxanthin in vertebrates, a unique role for RPE65 in carotenoid metabolism beyond its well-known retinoid isomerohydrolase function in the vertebrate visual cycle.

Author contributions: R.S. and P.S.B. designed research; R.S., A.G., and K.N. performed research; R.S., A.G., M.P.H., and P.S.B. analyzed data; and R.S. and P.S.B. wrote the paper.

The authors declare no conflict of interest.

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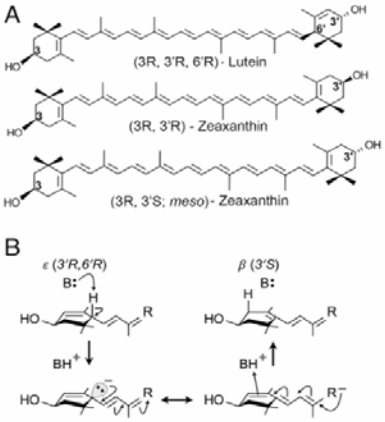

Fig. 1. Structures of macular xanthophylls (*A*) and simple mechanism of coordinated acid-base catalysis of lutein to *meso*-zeaxanthin (*B*). The first step involves base-catalyzed proton abstraction from the ε-ionone ring at position C6′. The resulting negative charge on the intermediate is expected to be resonance-stabilized (double-headed arrow). In the final step, BH+ acts as a source of proton for attachment to the ionone ring at the new position, C4′. The mechanism shown here illustrates how conversion might occur in vivo; alternate mechanisms involving radical chemistry (e.g., hydrogen atom transfer) are also possible.

all-*trans*-retinyl palmitate to 11-*cis*-retinol (11–14), is the lutein to *meso*-zeaxanthin isomerase.

Results

RPE65 Transcript and Protein Levels Are Significantly Up-Regulated in E21 Chicken RPE/Choroid. To identify the enzyme responsible for the production of *meso*-zeaxanthin in chicken RPE/choroid, we performed RNA sequencing to ascertain which transcripts of likely candidates were up-regulated. We used total RNA isolated from E16 RPE/choroid, a stage at which *meso*-zeaxanthin is not detectable in the RPE/choroid, and compared the expression profile of mRNA transcripts with those of E21 RPE/choroid, a stage at which substantial amounts of *meso*-zeaxanthin are present. Log2 FPKM values of at least three embryos from each stage were compared, and the relative abundance of gene transcripts normally involved in either carotenoid metabolism and transport (*GSTP1*, *STARD3*, *STARD1*, *BCO1*, *BCO2*, *SCARB1*, *SCARB2*, and *CD36*) or retinoid metabolism and transport (*RBP1*, *IRBP*, *CRALBP*, *RPE65*, *LRAT*, *CYP27C1*, *STRA6*, and *DES1*) are plotted in Fig. 2. Among the genes that we considered, *RPE65* was the most highly up-regulated between E16 and E21. Its transcript levels were 23-fold higher at E21 compared with at E16. GSTP1 and STARD3 are zeaxanthin- and lutein-binding proteins, respectively (15, 16), and their transcript levels did not show any significant increase between E16 and E21. BCO1 and BCO2 are carotenoid oxygenases (17–20), and both of their genes were slightly up-regulated at E21. STRA6 is a retinol transport protein (21), and DES1 is a vitamin A isomerase expressed in the Müller cells of the retina (22). While *STRA6* mRNA was up-regulated at E21, its magnitude of increase was lower than that observed for RPE65. *DES1* mRNA levels were not changed during development. SCARB1, SCARB2, and CD36 are carotenoid transport proteins in the eye (23). While *SCARB1* showed increased levels between E16 and E21, it is an unlikely candidate to catalyze the production of *meso*-zeaxanthin. LRAT, the acyl transferase enzyme required for visual pigment regeneration, was moderately up-regulated at E21 (12–14). CYP27C1, a protein known to convert vitamin A1 to vitamin A2 (24), showed a decrease in transcript abundance between E16 and E21. No significant differences in expression were observed for retinoid transporters, such as IRBP, CRALBP, and RBP1. From the RNA sequencing data, we concluded that among the relevant genes involved in the visual cycle and carotenoid metabolism, RPE65 is most likely to be responsible for *meso*-zeaxanthin production, especially since it is a relative of two carotenoid metabolic enzymes, BCO1 and BCO2.

We next determined whether the protein expression profile of RPE65 showed a similar trend as the mRNA levels. No RPE65 protein was detected in E16 chicken RPE/choroid, whereas strong expression was observed in E21 tissue (Fig. S1).

Overexpression of RPE65 Leads to the Production of *meso*-Zeaxanthin in HEK293T Cells. To determine whether RPE65 can catalyze the conversion of lutein to *meso*-zeaxanthin, we used the nonocular cell line HEK293T, which is derived from human embryonic kidney and does not express RPE65 or LRAT (12, 13). Overexpression of *pCDNA3.1-CRPE65* (chicken RPE65) resulted in strong expression of RPE65 at 48 h posttransfection that was sustained for another 4 d, while nontransfected cells had no

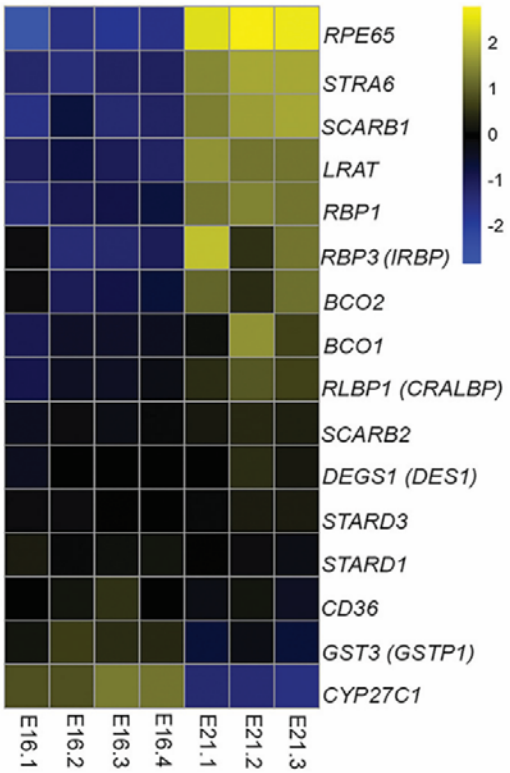

Fig. 2. Comparison of gene expression profiles in E16 and E21 chicken RPE/choroid. The FPKM value of each gene is compared with its expression across all samples to obtain the average expression. The ratio of gene expression in each sample is compared with the average, and the values are plotted on a log base2 scale. Positive values indicate above-average expression; negative values, below-average expression.

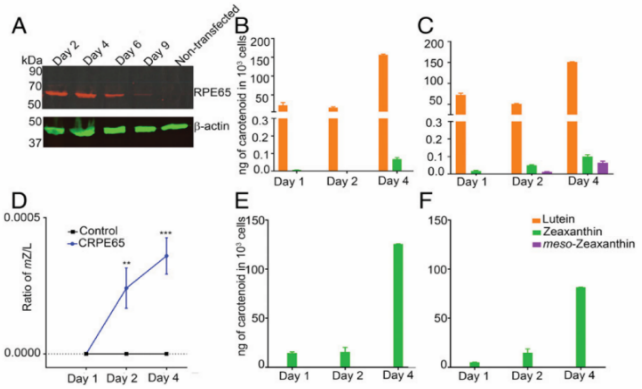

Fig. 3. CRPE65 overexpression followed by lutein treatment gives rise to *meso*-zeaxanthin in HEK293T cells. Nontransfected HEK293T cells do not express RPE65, but transient transfection results in expression of this gene for several days (*A*). Treatment of *CRPE65*-transfected cells with 4 μM lutein resulted in *meso*-zeaxanthin production (*C*), whereas the cells overexpressing control plasmid were devoid of *meso*-zeaxanthin (*B*). The ratio of *meso*-zeaxanthin to lutein shows an increase in the latter in a time-dependent manner (*D*). Treatment with 4 μM zeaxanthin in control plasmid-overexpressing cells (*E*) or CRPE65-overexpressing cells did not result in *meso*-zeaxanthin production (*F*). $n = 3$. Error bars represent SEM. $^{**}P < 0.005$; $^{***}P < 0.0005$.

RPE65 (Fig. 3*A*). We supplied HPLC-purified lutein with no detectable *meso*-zeaxanthin and <0.5% zeaxanthin (Fig. 4*B*) to HEK293T cells with no endogenous carotenoids (Fig. 4*C*). Treatment with 4 μM lutein for up to 4 d resulted in the production of progressively higher levels of *meso*-zeaxanthin in RPE65-overexpressing cells, while control cells transfected with *pCDNA3.1-GFP* did not show the presence of *meso*-zeaxanthin (Figs. 3 *B* and *C* and 4 *D* and *E*). The identity of biosynthesized *meso*-zeaxanthin was confirmed by comparing its retention time with that of authentic carotenoid standards (Fig. 4*A*) and by the observation of its characteristic tripeak spectrum with the highest peak at 450 nm using in-line photodiode array detection (Fig. 4*F* and Fig. S2). We observed small amounts of zeaxanthin in our experimental and control cells that increased in a time-dependent manner (Fig. 3 *B* and *C*). This is likely because our lutein stock, even though devoid of *meso*-zeaxanthin, contains ~0.5% zeaxanthin. To rule out zeaxanthin as the precursor to *meso*-zeaxanthin in CRPE65-overexpressing cells, we treated these cells with 4 μM isomerically pure zeaxanthin with no detectable lutein. Neither the control nor the experimental cells showed any detectable levels of *meso*-zeaxanthin or lutein (Fig. 3 *E* and *F*).

Previous studies have shown that CRPE65 is a better isomerohydrolase than its human counterpart (25). We conducted our next set of experiments to determine whether there were any differences between human RPE65 (HRPE65) and CRPE65 in *meso*-zeaxanthin isomerization (Fig. S3). We observed that

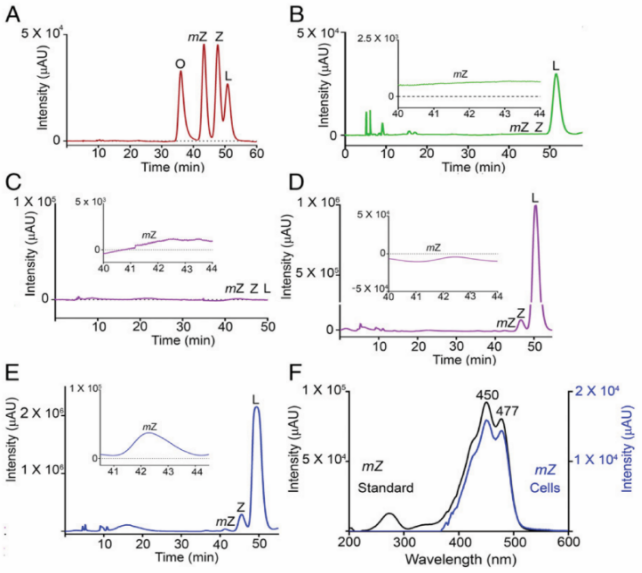

Fig. 4. Chromatograms showing the presence of carotenoids in HEK293T cells. (*A*) Retention times of authentic carotenoid standard mixture. (*B*) The HPLC-purified lutein used in our incubations did not contain any *meso*-zeaxanthin. (*C*) HEK293T cells were free of carotenoids. (*D* and *E*) Overexpression of pCDNA3.1-GFP followed by treatment with 4 μM lutein for 4 d did not result in *meso*-zeaxanthin production (*D*), whereas cells overexpressing CRPE65 when treated with 4 μM lutein for 2 d gave rise to detectable levels of *meso*-zeaxanthin (*E*). (*F*) Characteristic tripeak spectrum obtained for *meso*-zeaxanthin from authentic standard (black) and CRPE65-overexpressing cells (blue) (Fig. S2). (*Insets*) Zoom-in views (20×). L, lutein; mZ, *meso*-zeaxanthin; O, oxo-lutein; Z, zeaxanthin.

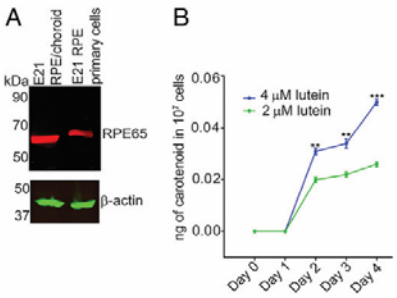

Fig. 5. Endogenous RPE65 in chicken RPE primary cells can catalyze the production of *meso*-zeaxanthin. (*A*) RPE primary cells from E21 chicken embryos retain RPE65 expression. (*B*) When treated with 2 μM lutein for 2 d, these cells produce *meso*-zeaxanthin, and higher levels of *meso*-zeaxanthin production are observed when cells are treated with 4 μM lutein. $n = 3$. Error bars represent SEM. $^{**}P < 0.005$; $^{***}P < 0.0005$.

HRPE65 transfection resulted in slightly lower *meso*-zeaxanthin production compared with CRPE65 at day 2; however, by day 4, both CRPE65- and HRPE65-overexpressing cells contained similar levels of *meso*-zeaxanthin (Fig. 3*C* and Fig. S3*C*). As with CRPE65, treatment of HRPE65-overexpressing cells with 4 μM zeaxanthin resulted in no detectable *meso*-zeaxanthin, and no *meso*-zeaxanthin was present in the control cells (Fig. S3 *E* and *F*). Comparison of the ratio of lutein to *meso*-zeaxanthin in CRPE65- and HRPE65-overexpressing cells revealed a conversion rate of <0.05% over a 4-d period (Fig. 3*D* and Fig. S3*D*).

E21 Chicken RPE Primary Cells Retain RPE65 Expression and Can Produce Meso-Zeaxanthin. In these experiments, we explored whether chicken RPE primary cultures from E21 embryos are capable of producing *meso*-zeaxanthin on lutein treatment. Unlike human primary RPE cells, these cells retain the expression of RPE65 even after five passages (26) (Fig. 5*A*). Treatment of 10^7 cells with 2 μM lutein for 2 d resulted in detectable levels of *meso*-zeaxanthin (Fig. 5*B*). Between days 2 and 4, we observed a progressive increase in *meso*-zeaxanthin levels. To determine whether a higher precursor concentration will result in increased production of *meso*-zeaxanthin, we treated these cells with 4 μM lutein. They produced significantly higher amounts of *meso*-zeaxanthin at days 2, 3, and 4 relative to the cells treated with 2 μM lutein (Fig. 5*B*).

Pharmacologic Inhibition of RPE65 in the Developing Chicken Embryo Decreases *meso*-Zeaxanthin Levels. We next examined whether pharmacologic inhibition of RPE65 activity could specifically inhibit *meso*-zeaxanthin production. Our previous studies have shown that *meso*-zeaxanthin is first present at detectable levels in the chicken RPE/choroid at E17. Therefore, we decided to introduce a competitive inhibitor of RPE65 activity into the yolk sac of chicken embryos at E17.

We used the pharmacologic inhibitor ACU-5200-HCl (ACU-5200) to knock down RPE65 function. ACU-5200 is an analog of emixustat, a highly specific RPE65 inhibitor that is currently undergoing clinical trials as a treatment for Stargardt disease (27–29) (Fig. 6*A*). ACU-5200 has been shown to inhibit production of 11-*cis*-retinoids in animals at very low oral doses (ED_{50} = 0.27 mg/kg in mice; proprietary data provided by Acucela Inc.). We injected various doses of ACU-5200 into the yolk sac of the developing embryo at E17 and then again at E19. The ACU-5200–injected embryos developed normally and had no obvious phenotypic abnormalities (Fig. 6*B*). Injection of two 2-mg doses (2 × 2 mg) of ACU-5200 resulted in significant down-regulation of RPE/choroid *meso*-zeaxanthin levels (Fig. 6*C*). Doubling this dose resulted in complete absence of *meso*-zeaxanthin in the injected embryos' RPE/choroid (Fig. 6*D*). The RPE/choroid lutein and zeaxanthin contents of the ACU-5200–injected embryos remained comparable to those of control embryos (Fig. 6 *C* and *D*). These results indicate that in an in vivo system, the function of RPE65 is necessary for the production of *meso*-zeaxanthin.

Structural Docking Experiments Reveal That the Epsilon Ring of Lutein Can Fit into the Active Site of RPE65. To determine whether lutein fits into the substrate tunnel of RPE65, we docked lutein molecules into a homology model for chicken RPE65. Fig. 7 shows a representative outcome that minimizes steric conflicts and maximizes hydrogen bonds. The ε-ionone ring of lutein rests on a ledge comprising the iron-coordinating histidine residues, and the β-ionone ring protrudes from an opening at the surface of the enzyme. The buried hydroxyl group is positioned in proximity with two hydrogen-bonding groups (indole amine of Trp-331 and carboxylate of Glu-417; Fig. S4). Steric complementarity is evident, with phenylalanine residues making a close approach on either side of the ε-ionone ring and also stacking with the polyene chain. The edge of the ionone ring containing atoms C4′, C5′, and C6′, which are involved in isomerization, was consistently found closer to the iron center and histidine residues. Docking outcomes with these atoms pointed away from the iron center were not observed among well-fitting outcomes, likely because in this orientation, the curvature observed for lutein molecules does not match the curvature of the substrate tunnel found in RPE65.

Discussion

RPE65 is an important enzyme in the visual cycle, responsible for the key all-*trans* to 11-*cis*-retinoid isomerization step of the visual cycle (11–13). Such diseases as Leber congenital amaurosis and retinitis pigmentosa arise from the loss of function of this gene (12, 13). Here we show that RPE65 is capable of carrying out an additional function in which it converts lutein to *meso*-zeaxanthin, an eye-specific carotenoid with no common dietary sources. Since *meso*-zeaxanthin is accumulated in high concentrations at the fovea of the retina, a region crucial for visual acuity, its hypothesized function is to protect the region from blue light damage and oxidative stress and to potentially enhance visual function. In support of this hypothesis, a previous study

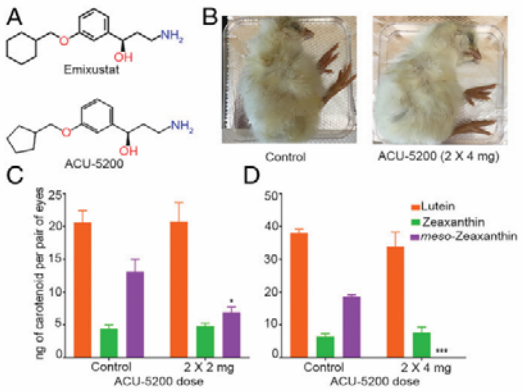

Fig. 6. ACU-5200 injection inhibits *meso*-zeaxanthin production in the RPE/choroid of developing chicken embryos. (*A*) ACU-5200 is an analog of emixustat. (*B*) The development of ACU-5200–injected embryos was comparable to that of control embryos. (*C* and *D*) No significant differences in lutein or zeaxanthin levels were observed in ACU-5200–injected embryos compared with corresponding control embryos. *meso*-zeaxanthin levels were either significantly down-regulated or completely absent following ACU-5200 injection. $n \geq 5$. Error bars represent SEM. $^{*}P < 0.05$; $^{***}P < 0.0005$.

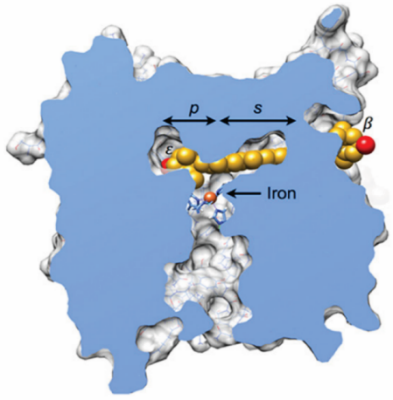

Fig. 7. Model of RPE65 complexed with lutein. A molecule of lutein (carbon, gold; oxygen red) is shown docked into a homology model of chicken RPE65. In this view, much of the protein structure has been cut away to reveal the substrate tunnel found in the interior. The palmitate-binding pocket (*p*) is above and to the left of the iron center, overlapping with the ε-ionone ring of lutein. The polyene chain extends through the presumed substrate-binding pocket (*s*), defined by the structure of the enzyme in complex with its competitive inhibitor emixustat (29). The β-ionone ring emerges at an opening found on the surface of the enzyme. A more detailed view of molecular interactions is provided in Fig. S4. Energy minimization was not used for these coarse-grid rigid-body docking experiments. The representative result shown here was selected from >33,000 trials because it has few steric clashes ($n = 9$; clash defined as interatomic distance less than 3 Å) and makes two hydrogen bonds. Steric conflicts would likely resolve on subtle adjustments of torsion angles in the lutein molecule and repositioning of sidechains. For comparison, lutein docked into the tunnel-like cavity of StARD3 with the same rigid-body protocol experiences 14 clashes (42), ligands palmitate and emixustat bound to RPE65 have two clashes, and lutein molecules found in structures of chlorophyll-binding proteins frequently have no clashes (42). This figure was prepared with UCSF Chimera (43, 44).

has shown that *meso*-zeaxanthin has stronger antioxidant properties than lutein and zeaxanthin (30), and another study has shown that oral supplementation with all three macular carotenoids can improve contrast sensitivity in normal individuals (31, 32). The process by which *meso*-zeaxanthin is produced in the eye has been a mystery. In the present study, we show in both in vitro and in vivo systems that RPE65 is the enzyme that catalyzes the conversion of lutein to *meso*-zeaxanthin.

After identifying RPE65 as a prime candidate for the *meso*-zeaxanthin isomerase in chicken RPE by RNA sequencing studies, we conducted overexpression experiments using both chicken and human *RPE65* plasmids in a nonocular cell culture system. Our studies in HEK293T cells show that RPE65 of both species is capable of producing *meso*-zeaxanthin from lutein, but not from zeaxanthin. HEK293T cells do not endogenously express LRAT, the acyl transferase enzyme essential to provide all-*trans* fatty acid ester retinoid substrates for RPE65 to catalyze their conversion into 11-*cis*-retinol (12, 13, 25). By overexpressing RPE65 in a system free of LRAT and treating these cells with HPLC-purified lutein, we were able to produce *meso*-zeaxanthin independent of LRAT's catalytic activity. The reaction is slow in cell culture, with no detectable product observed until several days after the addition of lutein. This is consistent with the relatively slow formation of *meso*-zeaxanthin during chicken eye development, which also takes several days (10).

Our structural modeling studies show that the epsilon ring of lutein can coordinate with the active site histidines and iron of RPE65 in a manner that could facilitate the double-bond shift reaction required to convert lutein to *meso*-zeaxanthin by a mechanism involving acid-base catalysis (Fig. 1*B*) or some other mechanism. We also found that an RPE65 inhibitor, ACU-5200, was able to specifically inhibit formation of *meso*-zeaxanthin during chicken eye development without affecting lutein or zeaxanthin uptake into the RPE/choroid. Its close analog, emixustat, is currently in clinical trials as a visual cycle inhibitor for various eye diseases (27–29). Our finding of RPE65's additional role in macular carotenoid metabolism suggests that it may be of interest to examine whether this compound detectably alters macular pigment levels or distributions in the participants in these clinical trials.

Mutations in human *RPE65* are quite rare and typically cause severe visual function deficits, and we suspect that individuals with deleterious mutations in *RPE65* may also have abnormalities in their macular pigment levels and distributions. Interestingly, SNPs in human *RPE65*, along with other carotenoid-associated genes, such as *GSTP1*, *BCO1*, and *SCARB1*, were identified as determinants of macular optical density in women participating in the CAREDS study (33).

The notion that RPE65 is the *meso*-zeaxanthin isomerase is appealing, since its carotenoid oxygenase family members BCO1 and BCO2 are known carotenoid cleavage enzymes. In fact, RPE65's alternate name is BCO3. These three proteins share significant sequence homology, and each plays a crucial role in vertebrate retinoid and carotenoid physiology. BCO1 cleaves β-carotene at the central 15, 15′ site to produce two molecules of retinal (18). This newly formed all-*trans*-retinal undergoes reduction and conversion into retinyl esters that are substrates for RPE65-mediated production of 11-*cis*-retinol; alternatively, retinal can be oxidized to retinoic acid, which is used for cell signaling and gene regulation. BCO2 cleaves a variety of xanthophyll carotenoid substrates at the 9′, 10′ double bond and is involved in the homeostasis of non-provitamin A carotenoids (17).

In other species, a single enzyme can perform the functions of BCO1, BCO2, and RPE65. Arthropods encode a single carotenoid cleavage enzyme, NinaB, that performs the functions of all three BCO family members (34). Carotenoid cleavage enzymes in lower organisms have a range of substrate specificities. ACO from cyanobacteria is capable of cleaving carotenoids of various lengths, ranging from C_{20} to C_{27}. This enzyme binds to substrates with either aldehydes or alcohols at their terminal ends distal to the ionone ring, and it also can accept apocarotenoids with or without 3-hydroxyl groups on the ionone rings (35–37). Therefore, it is not unprecedented that RPE65, whose known interactions until now have been only with retinoids, may interact with structurally similar molecules, such as carotenoids.

Our findings show that *meso*-zeaxanthin production from lutein occurs in the RPE, and that it is catalyzed by RPE65. The specific accumulation of this carotenoid in the fovea may be mediated by specific transporters as well as binding proteins. IRBP and class B scavenger receptor proteins are capable of shuttling carotenoids to the retinal layers from the RPE via the interphotoreceptor space (38, 39). GSTP1 is a known zeaxanthin-binding protein present in the primate RPE and foveal regions that binds *meso*-zeaxanthin with equally high affinity (15). It is plausible to hypothesize that newly formed *meso*-zeaxanthin from the RPE is shuttled into the subretinal space and then into the retinal layers by means of transport proteins, and that once in the retina, it may be held in place in the foveal region by specific binding proteins.

In the present study, we have described a novel function of RPE65 as the lutein to *meso*-zeaxanthin isomerase. We have shown that both chicken and human RPE65 are capable of converting lutein to *meso*-zeaxanthin. The reaction rate is slow, and *meso*-zeaxanthin isomerization is likely a secondary function of RPE65. The foveal presence of *meso*-zeaxanthin, especially given its lack of common dietary sources, has been a conundrum in the field of carotenoid biology. With the identification of RPE65 as the enzyme responsible for the production of

meso-zeaxanthin, future studies can further delineate the physiological role of this macula-specific carotenoid.

Materials and Methods

Total RNA Isolation and RNA Sequencing. Total RNA was isolated from E16 and E21 chicken embryos with the Qiagen RNeasy Kit. Intact poly(A) RNA was purified from total RNA samples (100–500 ng) with oligo(dT) magnetic beads, and stranded mRNA sequencing libraries were prepared as described using the Illumina TruSeq Stranded mRNA Library Preparation Kit. Details are provided in *SI Materials and Methods*.

Cell Culture and Transient Transfection. A primary cell culture of E21 chicken RPE was established, and HEK293T cells were transiently transfected with RPE65 or GFP for overexpression experiments, as described in *SI Materials and Methods*.

Carotenoid Treatment. HPLC-purified carotenoid stocks were prepared. Tween 40 was added to the dried stocks before the addition of medium. Cells were treated with the carotenoid-containing medium for 0, 1, 2, or 4 d. More details are provided in *SI Materials and Methods*.

Carotenoid Extraction and HPLC Analysis. Carotenoid extraction and chiral HPLC analyses were carried out as described previously (10) and in *SI Materials and Methods*.

Protein Isolation and Western Blot Analysis. Cell lysis, protein isolation, and Western blot analysis were done following standard protocols, as described in *SI Materials and Methods*.

Inhibitor Treatment of Chicken Embryos. E17 and the same chicken embryos at E19 were injected with appropriate concentrations of ACU-5200 (Acucela Inc.) diluted in Ringer's solution containing 1% penicillin-streptomycin (*SI Materials and Methods*).

Structural Modeling of Lutein Docking into RPE65. Lutein molecules were docked into a homology model of RPE65 obtained with *Phyre2* (40), by threading the amino acid sequence of CRPE65 into the structure of bovine RPE65 [Protein Data Bank (PDB) ID codes 4RSC and 3FSN] (29, 41). Additional details are provided in *SI Materials and Methods*.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Three isomeric carotenoids, lutein, zeaxanthin, and *meso*-zeaxanthin, are selectively accumulated within the retina in the macula region. Even though the concentration of these carotenoids in a normal diet ranges from 4:1:0 to 7:1:0, their levels at the macula are 1:1:1 (Bone et al., 1997). This suggests a selective transport of these carotenoids from the diet and retention into the macula. Even though studies have established the mechanism of carotenoid transport and retention into the primate retina (During et al., 2002, 2005; Voolstra et al., 2006), this process required further analysis. In the second chapter of this dissertation, we have expanded the understanding of the transport mechanism by which carotenoids reach the primate retina.

Carotenoid transport from the diet to various parts of the body has been well studied. Harrison and colleagues identified SRB family member, SCARB1, to be responsible for the intestinal uptake of carotenoids from the diet (During et al., 2002). Few studies have outlined the function of SRBs as carotenoid transporters into the retina (During et al., 2005; Thomas and Harrison, 2016). However, their transcript as well as protein expression profile in the macula, sub-macular regions, and peripheral regions of the eye were not known. In addition, the relative binding affinities of SRBs to lutein, zeaxanthin, and *meso*-zeaxanthin were also not studied. In the second chapter, we show that there are differences in the expression of SRBs within the eye (Shyam et al., 2017a). In addition, we show that SCARB2 undergoes posttranslational modifications in the RPE that may affect its function in this tissue. In addition, our study also shows that CD36 undergoes posttranscriptional modifications to limit its expression to the RPE layer. The nature of these modifications as well as their effects on the functions of these proteins need further evaluation. In our study, we identified that the amount of zeaxanthins taken

up by SRB over-expressing cells is significantly higher than that of lutein. This is interesting, especially since lutein and zeaxanthins are isomers. Such a difference in uptake implies that the SRBs may employ mechanisms for selective uptake of zeaxanthin and *meso*-zeaaxanthin over lutein. The domains of SRBs responsible for distinguishing carotenoid isomers can be identified in future studies. In addition, the physiological implications of selective uptake of zeaxanthins over lutein can also be determined in these analyses.

We focused on over-expressing scavenger receptors one at a time in a system that is free of their endogenous expression. Therefore, our studies did not investigate synergy between these receptors in carotenoid transport. Since all three SRBs are expressed in the primate RPE, and we hypothesize that the lipoprotein laden carotenoids enter the retina through the choroidal circulation via RPE, it will be useful to understand any crosstalk between the carotenoid receptors. A mixture of carotenoids in circulation is presented to these receptors, therefore the effects of a mixture of carotenoids on the transport mechanism also need further enquiry.

Of the three macular carotenoids, *meso*-zeaxanthin has no common dietary sources. However, this carotenoid is present at high concentration at the macula. Several studies in vertebrate models have shown that lutein undergoes metabolic transformations to produce *meso*-zeaxanthin. However, the biochemical mechanism behind this reaction was unknown. In the third and fourth chapter of this dissertation, we have identified the enzyme behind the conversion of lutein to *meso*-zeaxanthin. In the third chapter of this dissertation, we have determined that *meso*-zeaxanthin is produced in a developmentally regulated manner in the RPE in chicken embryos (Gorusupudi et al., 2016). With this

understanding, in the fourth chapter, we have elucidated the mechanism by which this process takes place in the chicken embryos (Shyam et al., 2017b). Here we have determined that RPE65, an RPE specific enzyme that is crucial for visual function, is responsible for the conversion of lutein to *meso*-zeaxanthin.

Identification of RPE65 as the *meso*-zeaxanthin isomerase in vertebrates has many implications. RPE65 is a well-studied enzyme which is responsible for the conversion of all-*trans* retinyl esters to 11-*cis* retinol (Jin et al., 2005; Moiseyev et al., 2005). Even though the substrates of RPE65, retinoids, are structurally similar to carotenoids, our report is the first to identify its interaction with carotenoids. Mutational analyses will reveal the nature of RPE65 residues that are responsible for the production of *meso*-zeaxanthin. Function of RPE65 independent of LRAT, the acyl transferase enzyme, was previously unknown. In our study, we show that lutein to *meso*-zeaxanthin isomerization is independent of LRAT activity. Whether the presence of LRAT would enhance the rate of conversion is yet to be explored.

Several supplement sources of *meso*-zeaxanthin are readily available; however, it is not known whether it is beneficial to bypass the ocular enzymatic metabolic pathway to supply it in a supplement form. The physiological implications of circumventing the slow conversion rate by providing supplements are also worth studying. Through the consumption of *meso*-zeaxanthin supplements, the subjects are introducing other regions in their body to this macula-specific carotenoid, which can have effects on their normal physiology. In addition, whether or not supplemented *meso*-zeaxanthin affect macular pigment levels also needs further exploration. A variety of health benefits are imparted to macular carotenoids. Their antioxidant as well as blue light filtering properties are

considered to be main reasons for their protective nature against AMD (Chew et al., 2014). Recent studies have also shown that in addition to their optical properties, macular pigment can affect the normal functions of neurons. Studies outline that carotenoids are capable of improving neuronal processing, neuronal communication through cell to cell channels, moderate the dynamic instability of microtubules (structural units of neurons), and protect against degradation of synaptic vesicle proteins (Bernstein et al., 2016). These studies show that in addition to their function in ameliorating the progression of AMD, carotenoids are important for the visual performance of normal subjects.

In this dissertation, I have outlined the elusive molecular mechanism behind the transformation of lutein into *meso*-zeaxanthin. In addition, I have expanded the understanding of the transport process by which carotenoids are deposited into the primate retina. This work can help future efforts in understanding the molecular mechanisms behind carotenoid metabolism in vertebrates.

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