In vitro effects of Insulin and VEGF on the Choroidal and Scleral Components of Eye Growth

By

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Abstract

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Emmetropization occurs in most vertebrates to regulate the axial length of the growing eyes, so that the focal plane of the eye can match its ocular length, and a clear image of a distant object can fall on the retina. It has been well established that vision can control eye growth (change in the ocular length). When the image of the distant object is not on the retina, change in eye growth occurs to correct this visual error, so that the position of the retina can be moved and the image of a distant object can fall on the retina again. Visual error can be induced by using optical lenses, or by depriving the eye of form vision (form deprivation), or by allowing the eye to recover from form deprivation. The eye will compensate the induced error by changing the choroidal thickness and rate of ocular elongation. This compensation can occur without any connection to the brain, which suggests that eye growth can be regulated by local retinal signals. A signal cascade is presumed to be present at the posterior part of the eye, where retina produces the signal to act on the retinal pigment epithelium (RPE), and RPE produces another signal to affect choroid and/or sclera. It is also possible that the signal that regulates the choroid is different from the signal that regulates the sclera. Many molecules have been suggested to be

involved in eye growth. Among them, insulin and vascular endothelial growth factor (VEGF) are potential regulatory signals. Insulin injected into the eye can decrease choroidal thickening caused by positive lenses and increase ocular elongation as well as scleral glycosaminoglycan (GAG) synthesis, an indicator of ocular elongation *in vitro*. VEGF mRNA expression in the RPE increases when eye growth is enhanced. In this thesis, we used a new experimental system to study the *in vitro* effect of insulin and VEGF on the choroidal and scleral components of eye growth. Eye-cups with vitreous and retina removed were prepared. The RPE and choroid of the eye-cups can be removed separately. Therefore, the effect of how the RPE and choroid mediate the effect of insulin and VEGF to affect the choroidal and scleral components (indicated by scleral GAG synthesis *in vitro*) of eye growth can be studied. We found that *in vitro* as *in vivo*, insulin can reduce choroidal thickening and increase scleral GAG synthesis. Our findings also suggest that insulin can cause the RPE to produce secondary signaling molecules that thin the choroid. Furthermore, we found that VEGF can reduce choroidal thickening transiently and increase scleral GAG synthesis in the eye-cups with choroid and sclera. We suggest that both insulin and VEGF act on the choroid to affect scleral GAG synthesis. We also suggest that insulin might cause the RPE to produce VEGF to thin the choroid, and VEGF might be one of the initial signals that cause choroidal thinning in eye growth. We suggest future experiments to explore further this relationship between insulin and VEGF in guiding eye growth.

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Table of Contents

List of Figures and Table

Chapter 1 General introduction

Emmetropization occurs in most vertebrates postnatally to regulate the axial length of the growing eye. The function of emmetropization is to match the position of the retina with the focal plane of the eye so that images of distant objects can be focused on the photoreceptor layer of the eye and can be seen clearly by the organism. In this thesis, the word eye-growth refers to the growth of the axial length of the eye. It has been well established that vision can control eye growth and many potential molecules have been suggested to affect this process. In our study, we used *in vitro* eye-cups, a new experimental system, to study the effect of two molecules, insulin and VEGF, on the choroidal and scleral components of eye growth. In the general introduction, we first introduce vision (1.1) and emmetropization (1.2) as well as how different visual factors can affect emmetropization. Then we discuss what biological molecules have been indicated in the signal cascades (1.3) that control eye growth, and the linkage between the choroidal and the scleral components of it. At the end of this chapter, we will introduce the general method in our experimental system (1.4) in studying the effect of insulin and vascular endothelial growth factor (VEGF) in eye growth.

1.1 Vision

1.1.1 Light pathway

When we look at an object, light reflected from it enters our eyes. The reflected light travels through and is focused by the cornea, which has three quarters of the eye's focusing power, then by the lens, which is a quarter of eye's focusing power. Ultimately, the reflected light of the focused object falls on the photoreceptors of the retina which converts light into electrical and ultimately neuronal signals that are sent to the higher visual processing center in the brain.

1.1.2 Ocular structures and their functions

Inside the eye and behind the cornea is the anterior chamber filled with watery liquid called aqueous humor. The size of the aperture of the eye, pupil, was adjusted by the iris that limits the amount of light passing through the eye. When light is dim, iris relaxes and allows more light entering the eye. The depth of focus, which is the distance that everything is in focus, increases with decrease in the pupil size. Behind the iris is the lens, which is held between the zonules of Zinn. Even though it has less focusing power than the cornea, power of the lens is adjustable and is controlled by the ciliary muscles. Contraction of the ciliary muscles relaxes the zonules of Zinn and leads to the lens increasing its curvature and consequently its focusing power. This process is called accommodation and it occurs when we focus on a near object. On the contrary, when we focus on a far object, ciliary muscles contract the zonules leading to the lens decreasing its curvature and power. Behind the lens is the vitreous humor, a gelatinous substance which fills the main cavity of

the eye. Vitreous humor keeps the eye in shape and maintains the retina position at the back of the eye. Retina is the light sensitive layer where visual processing starts.

1.1.3 Vision and visual processing in vertebrates

The structure and organization of the eye are fundamentally alike across vertebrates, including fish, birds and mammals. In the retinal level, there are six principal cell types in vertebrates: photoreceptors, projection neurons (retinal ganaglion cells), three types of interneurons (horizontal, bipolar, and amacrine cells), and glila cells (muller glia). The retinal ganglion cells send axons to more than ten areas of the brain. In most vertebrates, such as birds and fishes, the main target is the optic tectum, also called superior colliculus in mammals. In higher mammals, such as cats and primates, the dorsal lateral geniculate nucleus (LGN) in the thalamus is the major target. The lateral geniculate nucleus in primates comprises six cellular laminae which are innervated by three major groups of retinal ganglion cells. The optic tectum is highly laminated in lower vertebrates, where it functions as a higher visual processing center.

1.1.3.1 Birds versus primates

Despite the overall similarity, there are several main characteristics in avian eyes that are different from primates. First, avian eyes are a lot larger relative to the brain than primates. Their eyes occupy more than half of the cranial volume, whereas eyes in human, for example, only take up about 5%. The large eye size allows birds to obtain larger images projected on the retina. Furthermore, avian eyes have also

higher density of photoreceptors and more types of cones than primate eyes. In addition to the choroidal blood supply, retinal blood vessels in the avian eye are concentrated in a single structure, known as the pecten, as opposed to being embedded throughout the retina as in the human eye. This offers less visual interference and provides greater sensitivity to motions. Furthermore, both birds and primates have highly differentiated visual brains that are similar in their basic structures. The general visual processing is also similar, where visual information from the retina is conveyed to higher brain regions though two routes: the collothalamic pathway and the lemnothalamic pathway. Collothalamic pathway starts from the retina to the midbrain, then to the thalamus and telencephalon (cerebrum). Lemnothalamic pathway is a more direct route, in which information goes from the retina to the thalamus, then to the telencephalon. In primates, the primary route for visual processing is the lemnothalamic pathway; whereas avian collothalamic is hypertrophic and the roles of lemnothalamic pathway are seemingly minor but recent research on this pathway is still emerging.

1.2 Emmetropization

Most organs have a homeostatic control that regulates their size/shapes during and after development. For example, when some of the lobes of the livers in rat are removed, remaining liver tissue will restore to its original mass within days (Michalopoulos and DeFrances, 1997). In eyes, there is an additional challenge that the focal length of the eye must match its ocular length so that the image of a distant object can fall on the retina, not in front of or behind it, resulting in a clear image

(emmetropia, Fig 1.1). In all studied species, ocular length at birth or hatching is either too long or too short for its focal length. An interesting phenomenon is that whether the eye is too long or too short at birth, it can become emmetropic early in life. This process is called emmetropization.

Emmetropization occurs in most vertebrates postnatally to regulate the axial length of the growing eye. Refractive errors are caused by the error in emmetropization. Myopia is a condition when the eye grows longer than its optical focal point, and thus the image of a distant object falls in front of the retina (Fig 1.1). Hyperopia occurs when the image of a distant object falls behind the retina (Fig 1.1). Animal studies done over decades show that visual conditions can affect eye growth. When a positive or negative lens is put in front of an eye, the position of the retina can be moved as a result of changing the choroidal thickness and rate of ocular elongation (Fig 1.2). A positive lens, which puts the image in front of the photoreceptors creating a myopic defocus, inhibits ocular elongation and increases choroidal thickness. Because of these changes, the retina is moved forward to match the image plane. A negative lens, which puts the image behind the photoreceptors creating a hyperopic defocus, increases ocular elongation and decreases choroidal thickness. Furthermore, choroidal thickness changes in the opposite direction to the change in scleral extracellular matrix, which is indicated by scleral glycosaminoglycan (GAG) synthesis (will be discussed later in section 1.3.5.1 of this chapter). In these lens-compensation experiments, when choroidal thickness

increases, scleral GAG synthesis decreases or vice versa. In the following paragraphs, we discuss how different visual factors can affect emmetropization.

1.2.1 Role of accommodation

Accommodation was thought to be an important factor guiding emmetropization, because atropine, a muscarinic antagonist that paralyzes accommodation, can successfully prevent myopia development in humans. Also, there is a high correlation of near work with myopia. This may indicate that accommodation in near work may affect eye growth. However, findings in chicks showed that accommodation does not prevent lens compensation. First, lesion of Edinger Westphal nucleus, ciliary nerve section or optic-nerve section does not prevent eyes from compensating for myopic or hyperopic defocus (Schaeffel et al., 1990; Troilo et al., 1987; Wildsoet, 2003; Wildsoet and Wallman, 1995; Wildsoet and Pettigrew, 1988). Second, the muscarinic antagonist pirenzepine that does not block accommodation, can inhibit myopia development in chicks (Cottriall and McBrien, 1996). Third, chicks can still compensate for hyperopic and myopic defocus, with or without cycloplegia (applications of drugs which block accommodation) (Diether and Schaeffel, 1997).

1.2.2 Vision controls eye-growth

Studies done over decades showed that vision can control eye-growth. The first evidence showed the role of vision in eye growth came from studies in monkey and tree shrew. When lids of the animals were surgically fused, they developed myopia

by enhancing ocular elongation (Sherman et al., 1977; Wiesel and Raviola, 1977). Also, this lid-fusion myopia could not be induced if monkeys were raised in the dark (Raviola and Wiesel, 1978). The role of vision in eye growth was further confirmed when the animals were deprived of form vision, by putting translucent occluders in front of the eyes, the eyes elongated much more than the normal eyes (Wallman et al., 1978). Later on, as mentioned previously before, it was found that ocular elongation can be driven or inhibited by wearing negative and positive lenses (Schaeffel et al., 1988).

1.2.3 Sign of blur vs. amount of blur

Both the position of the image, whether it is in front of or behind the retina (signs of blur), and the difference in the amount of blur induced by positive and negative lenses have been suggested to guide lens compensation. A positive lens, which puts the image of a distant object in front of the retina, creates myopic defocus; whereas a negative lens, which puts the image behind the retina as mentioned previously, creates a hyperopic defocus. Since the eye is not a perfect optical system, longitudinal chromatic aberration and astigmatism induced by positive and negative lenses are different. By discerning these different types of defocus or different signs of blur induced by different lens-wears, the eye might be able to tell if it should increase or decrease its elongation and change its choroidal thickness. Alternatively, the eye might grow by measuring the amount of blur. If an animal looks mostly at near objects, its eye accommodates by changing the optical power of the crystalline lens which brings the image of a near object from behind the retina to be on the

retina. Since accommodation is not perfect, and sometimes the increased optical power is still insufficient to put the image on the retina, a positive lens will help bring the image of a close object on the retina. Whereas a negative lens will increase this blur by moving the images further behind the retina. Thus, if vision is primarily at near, the eye can also discern a positive lens from negative lens by the amount of blur.

Recent findings favor that the eye can discern the sign of blur induced by spectacle lenses. When the amount of blur is increased by mixed astigmatic lenses or by a diffuser on top of a positive or negative lens, the eyes do not become more myopic as the amount of blur increases (McLean and Wallman, 2003; Park et al., 2003). Instead, these eyes compensate for the signs of the blur induced by the spherical lenses in both directions (McLean and Wallman, 2003; Park et al., 2003). Furthermore, when chicks are confined to the center of a cylinder in which the only thing they can see is the wall of the cylinder which is beyond their far point, neither positive nor negative lenses can put the image of the wall on the retina. Their eyes still compensate in both directions for the positive and negative lenses.

Another theory hypothesizes that the eye might not be able to discern the sign of blur, but instead, it grows by trial and error. Like focusing an image under the microscope, the eye just grows in one way first, and if it results in blurred images, it grows in the opposite way. However, chick eyes can compensate positive and negative lenses by just a brief episode of lens-wear as short as minutes (Zhu et al.,

2005). Ten minutes of positive lens-wear followed by an hour in darkness can cause a significant thickening in the choroid and a decrease in ocular length (Zhu et al., 2005). An hour of negative lens-wear can cause a significant change in the opposite direction (Zhu et al., 2005). This indicates that the eye knows which way to grow instead of doing it by trial and error.

1.2.4 Longitudinal chromatic aberration

The findings indicating that the eye can discern the sign of blurs raise question of what error signal can guide the eye to grow in the right direction. Since the optics of the eyes are not perfect, optical aberrations might provide the signed error signal. There are different aberrations in the eyes, and recent findings suggest that longitudinal chromatic aberration alone can tell the eye which way to grow.

In longitudinal chromatic aberration, short-wavelength (blue) light is focused more strongly than long-wavelength (red) light. If a black/white edge is in focus, the blue light will be focused in front of the photoreceptors, while the red would be focused behind the photoreceptors. Therefore, if the blue aspect of the image were sharper than the red aspect, it would indicate that the eye was defocused in the hyperopic direction (image focused behind the photoreceptors), whereas if the red aspect were sharper, it would indicate myopic defocus (image focused in front of the photoreceptors). When chicks are exposed to images that use chromatic contrast simulating hyperopic and myopic defocus, the eyes grow in the corresponding directions (Rucker and Wallman, 2009).

1.2.5 Temporal factors in lens compensation

In daily lives, our eyes face different signs of blur which are fluctuating constantly, depending on one's accommodative states, fixation point and surrounding environment. For example, when you are reading this paper, the image of the text will presumably fall on your retina. But the image outside the paper, such as the image of the object on your desk, which is further away than this paper from you, will fall in front of the photoreceptor (be myopically defocused). The image of anything between the thesis and your eyes, such as the frame of your glasses in your peripheral field, or a pen you are holding to mark this thesis, will fall behind the retina. Your eyes change their focus all the time. When you are just looking around, the images on your retinas are changing their positions. Your eyes need to integrate and compensate these different kinds of defocus with time.

Studies on the temporal components of lens compensation in chicks showed that the compensation for repeated episodes of defocus does not add linearly. It was found that frequent, brief episodes of defocus, with darkness between lens-wearing episodes, are more potent than infrequent, long episodes with the same amount of lens-wearing time (Winawer and Wallman, 2002). However, if the duration is less than 2 minutes, no matter how frequent that defocus is given, the eye will not compensate for it.

When myopic (created by positive lens) and hyperopic defocus (created by negative lens) is given alternately, the compensation that results is in the positive-lens direction. The effect of positive lens is so much more potent than negative lens that only four episodes of either 2 minutes or 15 minutes of positive lens-wear can cancel negative lens-wear for the rest of the day (Zhu et al., 2003).

Also, inhibition of ocular elongation caused by the positive lens can last much longer than the stimulation of ocular elongation caused by the negative lens. In a study of how quickly the signals for positive and negative lenses decline, chicks were put on positive or negative lens-wear for half an hour before they were put in the dark. Inhibition of ocular elongation induced by positive lenses sustained 24 hours; whereas stimulation of ocular elongation induced by negative lenses weakened within an hour (Zhu and Wallman, 2009b).

We will summarize what we have discussed about emmetropization in the following: (1) emmetropization occurs in most vertebrate to match the focal length of the eye to its ocular length; and error in it can result in myopia or hyperopia; (2) the eye can discern the sign of blur (whether the image of the distant object is in front or behind the retina) and can grow corresponding mainly by changing its choroidal thickness and ocular elongation which can also be indicated by changes in scleral extracellular matrix; (3) longitudinal chromatic aberration alone can tell the eye which way to grow; (3) error signals from hyperopic (blurred image in front of the retina) and myopic (blurred image behind the retina) defocus do not add linearly, and myopic defocus

induced by the positive lens is more potent than hyperopic defocus induced by the negative lens. Next, we will discuss what retinal signals may be used to signal the choroid and scleral to change in the corresponding direction in eye growth.

1.3 Signal cascades in regulating eye growth

1.3.1 Local retinal signals controlling ocular elongation

Eye growth can be regulated by local retinal signals. Form-deprivation or lens-wear in either the nasal or temporal part of the retina changes the ocular elongation rate of that part only (Diether and Schaeffel, 1997; Wallman et al., 1987). Furthermore, as mentioned previously, disconnection of the eyes from the brain by optic-nerve section cannot inhibit accelerating or decelerating eye growth induced by form-deprivation, recovery from form-deprivation or lens-wear (Troilo et al., 1987; Wildsoet, 2003; Wildsoet and Wallman, 1995; Wildsoet and Pettigrew, 1988). Even though the brain can still affect emmetropization, as optic nerve section alters the set-point of the compensation for lens-wear (Wildsoet, 2003), and monkeys that are surgically induced to become amblyopic develop hyperopia (Kiorpes and Wallman, 1995), without the connection to the brain, the eye still knows which way to grow. Therefore, the retina is not only able to discern the blur (whether the image of the distant object is in front or behind the retina), but it is also able to change its position by causing changes in the choroid (thinning or thickening) and the growth of the posterior sclera. More than a sensory organ as it was previously thought, the retina is a sensorimotor apparatus.

Since the two main components change in eye growth are the choroidal thickness and ocular length, which involves changes in scleral extracellular matrix mainly at the posterior part of the eye, the signal cascade is presumed to be present at the posterior part of the eye. The retina triggers the initial signal to regulate eye growth. However, any molecule secreted by the retina will be less likely to act on the choroid and sclera by itself because of the tight junctions in the retinal pigment epithelium (RPE) and the vasculature of the choroid which may wash away any signal (Wallman and Winawer, 2004). Thus, a signal cascade should be present.

Moreover, if there is only one signal pathway regulating first the choroid and then the sclera, both responses should be affected in a similar extent when the stimuli are weakened. However, when brief episodes of lens-wear are given infrequently, only the choroidal response of the positive lens or the scleral response of the negative lens is inhibited (Wallman and Winawer, 2004). Also, when a weak diffuser is put over a positive lens or when light intensity is lowered in positive lens-wear, only the choroidal response is inhibited (Park et al., 2003; Roberts et al., 2003). These previous findings suggested that choroidal and scleral responses are not suppressed in a similar extent, and therefore they might be regulated by two different pathways (Wallman and Winawer, 2004). Recent findings have favored the inhibition of eye growth involve the same pathway to thicken the choroids and decrease ocular elongation, as the inhibition of ocular growth is always preceded by a thickened choroid (refer to section 1.3.6.2 for details).

In the following section, we will first discuss what signals can be produced by the retina to initiate the process. Then we will discuss the roles of other structures in the posterior part of the eye in the signal cascade of eye growth.

1.3.2 Retinal signals

As mentioned before, the retina does not only sense light, it can also discern the sign of blur and transduce the error signal to initiate the signal cascade that regulates eye growth. The retinal structure is highly heterogeneous. It is indicated that the amacrine cells in the retina are responsible for inducing signals in eye growth, because they can release neurotransmitter and neural modulators. Furthermore, several biochemical markers, like glucagon, dopamine, retinoic acid binding protein and others, have been suggested to be related to eye growth and they were found in the amacrine cells (Ashby and Feldkaemper, 2010). We will discuss five of them below.

Glucagon & Insulin

Glucagon was once recognized to be the most promising player in the signal cascade of eye growth. First, the expression of the immediate early gene ZENK increases and decreases bidirectionally only in the glucagonergic amacrine cells (Bitzer and Schaeffel, 2002; Fischer et al., 1999). Its expression increases in visual conditions suppressing eye growth and decreases in visual conditions enhancing eye growth (Bitzer and Schaeffel, 2002; Fischer et al., 1999). When chicks are confined to the center of a drum, so that only one viewing distance is available (only

the wall of the drum is seen), the level of ZENK still responds to the signs of imposed defocus (Bitzer and Schaeffel, 2002). Second, glucagon or glucagon agonist, [Lys(17,18),Glu(21)]-glucagon-NH(2)], which is five times higher in binding affinity with the glucagon receptor than glucagon, prevents myopia caused by form deprivation (Vessey et al., 2005b) or negative lens-wear (Feldkaemper and Schaeffel, 2002); whereas glucagon antagonists can inhibit hyperopia development (Feldkaemper and Schaeffel, 2002; Zhu et al., 2001). Third, glucagon content in the suprachoroidal fluid changes bidirectionally with positive and negative lens-wear (Zhu et al., 2006). Retinal glucagon content decreases after negative-lens treatment and choroidal glucagon content increases after positive-lens treatment (Feldkaemper and Schaeffel, 2002). Fourth, accelerating eye growth stimulates the proliferation of progenitor neurons in the avian retinal margin, and injections of glucagon or GLP-1 suppress the progenitor proliferation (Fischer, 2005).

There are some ambiguous findings on glucagon which cannot be explained. First, glucagon mRNA expression changes in both lens-wearing eye and contralateral control eye (Buck et al., 2004). It is also unknown why there is a transient increase in glucagon receptor mRNA expression in both negative or positive lens-wearing eyes (Buck et al., 2004). Second, how glucagon acts on the eye to affect ocular growth is unknown. Pre-administration of glucagon antagonist, des-His(1),des- Phe(6),Glu(9)]-glucagon-NH(2) followed by glucagon do not prevent the choroid from thickening (Vessey et al., 2005a). It indicates that glucagon might act on something else other than its own receptors. It is known that glucagon receptor mRNA expression is

higher in the RPE than retina or choroid and it has been suggested that glucagon acts on the RPE (Buck et al., 2004), whether via its own receptors or not, to affect eye growth.

In the eye as in the liver, insulin has effects opposite to those of glucagon: injected insulin reduces choroidal thickening and increases ocular elongation, countering the effect of positive lenses. Even in normal eyes and eyes wearing negative lenses, insulin injected into the eyes can further increase ocular elongation (findings on the insulin effect in eye growth will be discussed in detailed in chapter two). Therefore, glucagon and insulin may be output signals from the retina that decode the sign of defocus and modulate eye growth.

Retinoic acid (RA)

Retinoic acid (RA) is a molecule previously shown to exhibit bidirectional changes with positive and negative lenses in chicks. Positive lens-wear decreases retinal RA and increases choroidal RA (Mertz and Wallman, 2000) and four episodes of 15 minutes lens-wear can cause these opposite changes (Richiert et al., 2004). Ten minutes of positive lens-wear can induce a detectable change in retinal RA six hours later. When scleral punches are cultured with a physiological concentration of RA, scleral GAG synthesis decreases (Mertz and Wallman, 2000). It is suggested that a higher level of choroidal RA induced by positive-lens wear inhibits scleral GAG synthesis (Mertz and Wallman, 2000). Furthermore retinal mRNA levels of a RA synthetic enzyme, aldehyde dehydrogenase-2 (AHD2) and retinal mRNA level of a

receptor of RA, RAR-β , are differentially affected by lens-wear (Bitzer et al., 2000). However, retinal RA might not be a signal to regulate eye growth because the bidirectional changes in retinal and choroidal RA can be uncoupled, possibly because the retina does not produce much retinoic acid (Bitzer et al., 2000). Exogenous RA cannot inhibit choroidal RA, indicating retina RA cannot affect choroidal RA (Mertz and Wallman, 2000). Therefore, retinal RA can be an indicator of other retinal functions besides, or other than a signal initiating eye growth.

Dopamine

Studies have been done to investigate the role of dopamine in eye growth because retinal dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) (Iuvone et al., 1989; Pendrak et al., 1997; Stone et al., 1989), as well as tyrosine hydroxylase activity (Iuvone et al., 1989), decrease in form-deprived eyes. Also, the DOPAC/dopamine ratio, which indicates the metabolism of dopamine, and the level of DOPAC increase in chick eyes recovering from form-deprivation (Pendrak et al., 1997). Moreover, a dopamine agonist, apomorphine, has been shown to inhibit both form-deprivation myopia (Rohrer et al., 1993; Schmid and Wildsoet, 2004; Stone et al., 1989) and lens-induced myopia (Schmid and Wildsoet, 2004). It can also enhance the lens-induced hyperopia (Schmid and Wildsoet, 2004).

There are still a lot of unanswered questions regarding the role of dopamine. If dopamine can regulate eye growth by responding to either myopic or hyperopic defocus, its level should be higher in one type of compensation but lower in the other.

Bidirectional changes in the level of dopamine or its metabolite in positive versus negative lens-compensations should be found. This was found in one study (Guo et al., 1995) but not in the others (Bartmann et al., 1994; Schaeffel et al., 1995). It is also puzzling that when dopamine antagonist, haloperidol, is injected alone to the form-deprived eye, it does not increase ocular elongation. On the contrary, it inhibits ocular growth (Stone et al., 1989). Unless where these drugs act on the eye is known, it is hard to explain their effects. We only know that dopamine receptors are found in the RPE and they are suggested to interact with the retinal dopamine to affect choroid and/or sclera [review (Witkovsky, 2004)]. Recent findings have shown that when apomorphine and quinpirole (D2 agonist) were injected into chick eyes wearing negative lenses, it caused a transient choroidal thickening before ocular growth was inhibited (Nickla and Totonelly, 2011). Therefore, dopamine may act on the upstream signaling on initiating transient choroidal thickening which is associated with inhibition of ocular elongation (section 1.3.6.2), possibly by acting on the D2-receptor (Nickla and Totonelly, 2011).

Muscarinic agents

Muscarinic receptors have been studied for a very long time because an antagonist, atropine, has been used to inhibit myopia development. The whole muscarinic receptor family has 5 subtypes, M1 to M5. Atropine is a non-specific muscarinic antagonist. It has strong side effects of causing pupil dilation and paralyzing accommodation. Even after decades of study, how atropine acts on the eye to stop myopia is still unknown. Its possible action in the retina was excluded, as eyes with

damaged cholinergic amacrine cells can still compensate for form-deprivation myopia and recover from it (Fischer et al., 1998). Also, the form-deprivation myopia induced after the destruction of the cholinergic amacrine cells can be prevented by the application of atropine (Fischer et al., 1998). An M1 specific antagonist, pirenzepine, has been found to inhibit myopia without affecting accommodation in tree shrews and chicks (Cottriall and McBrien, 1996; Truong et al., 2002). Recently, it was found that pirenzepine can inhibit human myopia with mild effects on accommodation (Bartlett et al., 2003). However, same as atropine, it is still not known where this antagonist acts on the eye. Even though the binding site for the ligand of the M1 receptors were found in choroid and retina in chicks (Vessey et al., 2002), the M1 mRNA expression cannot be found in chick ocular or brain tissue (Yin et al., 2004). Pirenzepine is suggested to act on something other than its receptor to inhibit myopia. A recent unpublished study has shown that muscarinic agonists, oxotremorine, carbachol and arecaidine can thin choroid *in vivo* and *in vitro* without the retina (Nickla et al., 2013); and pirenzepine can cause transient choroidal thickening *in vivo*, and *in vitro* without the retina (Nickla et al., 2013). Therefore, it is suggested that muscarinic agents do not act on the retina and they can directly act on the choroid (Nickla et al., 2013). It is still uncertain whether pirenzepine can act on sclera directly or not. Its application decreases the sulfate incorporation of the cultured scleral chondrocytes (Lind et al., 1998). However, a study in scleral chondrocyte binding with muscarinic ligands failed to show a saturation curve, which indicates no specific binding (Vessey et al., 2002). As some Asian countries, like Singapore (Chua et al., 2006) and Taiwan (Lee et al., 2006), still use atropine and

pirenzepine to treat myopia, it is crucial to find out how it acts on the eye and what side effects it might cause.

1.3.3 Retinal pigment epithelium (RPE)

Lying between the retina and the sclera is a single layer of pigmented cells called the retinal pigment epithelium (RPE) and the choroid. The apical side of the RPE faces the retina and its basolateral side faces the Bruch's membrane, which separates the RPE from the choroid. The tight junctions in the RPE form a fluid and ionic barrier. The polarized distribution of ion transporters in the RPE provides transepithelial transport of nutrients and ions between photoreceptors and choriocapillaries [review (Strauss, 2005)].

Functions of RPE include improving vision by absorbing scattered light not focused by the lens, transporting nutrients from the choroids to the retinas, removing metabolic end products from the retinas to the choroids, phagocytosis of the outer segment of the photoreceptors, stabilizing ion compositions in the subretinal area, and reisomerizing retinal after phototransduction as well as producing various growth factors, such as insulin like growth factor-1, vascular endothelial growth factor (VEGF), transforming growth factor-ß, fibroblast growth factors to maintain the retina and choroid at a healthy state [review (Strauss, 2005)].

Compared with the other ocular tissue in the posterior part of the eye, the role of RPE in eye growth has been studied the least. Previously, RPE was found to

enlarge passively during eye growth, as its number of cells remains the same when ocular elongation increases (Lin et al., 1993). Because RPE acts a barrier between the retina and the choroid and RPE is highly polarized, its role in eye growth is thought to facilitate the change in choroidal thickness which will be discussed in section 1.3.4. As mentioned in the beginning of section 1.3 in the signal cascade of eye growth, any molecule produced by the retina will be less likely to act on the choroid and sclera directly because of the tight junctions in the RPE and the vasculature of the choroid which may wash away any signals (Wallman and Winawer, 2004). Thus, upon receiving signal from the retina, RPE is presumed to produce another signal to affect choroid/sclera.

1.3.4 Choroid

Choroid is a vascular layer between the RPE and the sclera. It comprised of capillaries, connective tissues, fibroblasts and melanocytes. In the transition zone between choroid and sclera, which is also called suprachoroid, there are endothelium lined spaces which empty into veins. These spaces are called lacunae and they are part of the lymphatic system. Functions of choroid include absorption of scattered light and heat. The changes in choroidal thickness with lens compensation and recovery from form-deprivation have been well demonstrated in chicks (Wallman et al., 1995). Similar, but smaller, compensatory changes in choroidal thickness have been found in tree shrews (Siegwart and Norton, 1998), marmosets (Troilo et al., 2000), rhesus macaques (Hung et al., 2000), guinea pigs (Howlett and McFadden, 2006, 2009), and even in humans (Read et al., 2012).

The mechanism of choroidal thickening is still unknown. Major theories based on the increase in fluid entry into the choroid. One theory suggested that the transepithelial transport of Cl^- across the RPE, would accompany water transport from the subretinal space of retina to the choroids, as a source to thicken choroids in positive-lens compensation or recovering from form-deprivation (Crewther et al., 2008; Rymer and Wildsoet, 2005; Zhang et al., 2011). Others theories involves the expansion of lacunae, membrane bounded spaces found in the choroid lymphatic system. When choroid was expanded, it was found that these lacunae expanded as well. Possible suggested mechanism are [review (Nickla and Wallman, 2010)]: (a) changes in the synthesis of large osmotically active proteoglycans, which attract water molecules; (b) changes in choroidal vascular permeability, which allows leaking of protein molecules and passive flow of fluid to fill in the lacunae; (c) choroids have a lot of non-vascular smooth muscle cells, which are also called myofibroblasts, and they were suggested to control the size of the lacunae by contraction (squeezing fluid out) and relaxation (allowing fluid entering by passive fluid entry). It is unknown if there is local regulation in the lacunae membrane. For example, whether there are ion transporters on the membrane, to regulate ion influx and outflux and the subsequent inflow or outflow of fluid that can change the size of the lacunae.

1.3.5 Sclera

Sclera is the outermost layer of the eye containing collagen and connective tissues that is comprised of extracellular matrix (ECM) and matrix secreting fibroblasts.

Extracellular matrix of the sclera is altered in eye growth

The changes in the ocular length were shown to be caused by the changes in the extracellular matrix in the sclera which is anatomically different in chicks from that in mammals. In mammals, there is only one scleral layer. In chicks, there are two layers: The cartilaginous layer, and the fibrous layer that is similar to the mammalian sclera. When a chick eye elongates more than usual, the cartilaginous layer gets thicker and the fibrous layer gets thinner (Gottlieb et al., 1990; Marzani and Wallman, 1997). The thinning in the fibrous layer in chicks is similar to the thinning of sclera in mammals in accelerating eye growth. These changes are associated with the changes in the scleral extracellular matrix which is made of proteoglycan, collagen, fibroblasts (in the fibrous layer) and chondrocytes (in the cartilaginous layer). Proteoglycan is made of a protein core with at least one glycosaminoglycan (GAG) residue. It is shown that when eye-growth accelerates, scleral GAG synthesis, which can be studied by measuring radiolabeled sulphate incorporation in the sclera, increases in the cartilaginous layer and decreases in the fibrous layer of avian sclera (Marzani and Wallman, 1997). When eye growth is inhibited, the scleral GAG synthesis decreases in the cartilaginous layer and increases in the fibrous layer (Marzani and Wallman, 1997). Aggrecan in the cartilaginous layer has a lot more GAG chains than decorin found in the fibrous layer. Therefore, even though the GAG synthesis of the cartilaginous layer and the fibrous layer always change in opposite directions, when scleral GAG synthesis is examined without the two layers

being separated, the overall change in GAG synthesis is similar to the change found in the cartilaginous layer. Thus, enhancement of ocular elongation (caused by wearing diffusers or negative lenses) and inhibition of ocular elongation (caused by removal of diffusers or wearing positive lenses) have been associated with increased (Nickla et al., 1997; Rada and Matthews, 1994; Rada et al., 1992; Rada et al., 1991) and decreased (Nickla et al., 1997; Rada et al., 1992) scleral GAG synthesis respectively. The change in GAG synthesis in the mammalian sclera, at least in tree shrew, is similar to the fibrous layer of the avian sclera with response to accelerating and decelerating eye growth (McBrien et al., 2000). Furthermore, when proteoglycan synthesis is inhibited by beta-xylosides, the ocular growth associated with diffusers is inhibited (Rada et al., 2002). Also, there are diurnal rhythmic changes in both scleral GAG synthesis and ocular elongation, and they share similar patterns: Both being higher in the morning and lower at night (Nickla et al., 1999). Fourth, when ocular elongation is not possible to detect only after a few hours of visual manipulation, scleral GAG synthesis can still respond in the corresponding direction. For example, when the effect of chromatic aberration on eye-growth was studied in chick eyes, eyes were treated with different chromatic stimulus simulating myopic and hyperopic defocus for only a few hours. Since changes in ocular elongation were not possible to detect after a few hours of treatment, scleral GAG synthesis instead was studied and it responded in the corresponding directions in simulations of myopic and hyperopic defocus (Rucker and Wallman, 2009).

Active growth and passive stretch of sclera

Both active growth and passive stretch of the sclera have been suggested to cause the change in the scleral matrix leading to ocular elongation. Evidence supporting the active growth theory is: (1) dry weight of the sclera of the deprived eyes increases faster than that of the non-deprived eyes (Christensen and Wallman, 1991); (2) DNA synthesis (Christensen and Wallman, 1991), GAG synthesis (Christensen and Wallman, 1991), protein synthesis (Christensen and Wallman, 1991) and mitotic activity (Christensen and Wallman, 1991; Seko et al., 1994) of sclera increase in form deprivation in chicks. The theory of passive scleral stretch came from the findings on (1) marked thinning of the posterior sclera in myopic human eyes [review (Rada et al., 2006)]; and (2) decrease in intraocular pressure in embryonic chick eye prevents normal eye growth (Neath et al., 1991). However, recent findings do not favor the stretch theory. On one hand, the thinning sclera found in human study always came from donor eyes after death when myopia had already developed for decades. The thinning was suggested to be the consequence rather than the cause of myopia [review (Rada et al., 2006)]. In fact, no significant difference in scleral thickness is found in myopic or emmetropic children (Schmid et al., 2003). On top of that, a correlation between IOP and eye growth has never been found in chick (Schmid et al., 2000) or human (Edwards and Brown, 1996). A higher IOP is reported only after but not before myopia is developed (Edwards and Brown, 1996). Thus, the change in scleral matrix is likely to be an active process.

Vascular endothelial growth factor (VEGF)

In addition to its prominent role in vascularization and angiogenesis, it has been shown that VEGF is involved in choroidal development and maintenance (Saint-Geniez et al., 2009; Saint-Geniez et al., 2006). In addition, when ocular growth is enhanced, RPE cells expand which was attributable to the passive stretch in ocular growth (Lin et al., 1993). Previous *in vitro* studies showed that when RPE was stretched in a pulse-like manner, VEGF and its mRNA expression increased (Seko et al., 1999). Furthermore, VEGF mRNA expression in the chick RPE can be upregulated after either 48 hours or 2 weeks of minus lens-wear [Nguyen ARVO abstract 09]. The role of VEGF has never been studied thoroughly. Whether VEGF can affect the choroidal and scleral components has never been studied, and we will discuss this topic in detail in Chapter 3.

Transforming growth factor β (TGFβ)

Transforming growth factor (TGFβ) has been suggested to play a role in eye growth but findings have been conflicting. Its concentration was found to be higher in the retina, RPE and choroid as well as the sclera of the form-deprived eyes in one study (Seko et al., 1995) but not in another (Honda et al., 1996). It can prevent the inhibition effect of fibroblast growth factor (FGF) in form deprivation (Seko et al., 1995) and promote the growth of scleral chondrocytes and fibroblasts in a dose-dependent manner (Rohrer and Stell, 1994). However, it cannot affect form deprivation myopia or normal eye growth (Rohrer and Stell, 1994). A change in the ratio of its different isoforms is found in myopia progression (Jobling et al., 2004). TGF-β 1 and TGF-β 2 were suggested to help laying down new synthesized matrix

in a disorganized order and inhibit MMP activity; whereas, TGF-β 3 does the opposite and promotes matrix organization. It was suggested that coordinated changes in expression levels of all three isoforms are needed to control scleral matrix remodeling in myopia (Jobling et al., 2004). TGF-β 2 mRNA expression was also found to be higher in the cartilaginous layer of the positive-lens wearing eyes than that of the control eyes (Schippert et al., 2006). Since TGF β could be found in all the tissues (retina, RPE, choroid and sclera) in the posterior eye, it is hard to study how it involves in the pathway by doing *in vivo* experiment.

1.3.6 Choroid can regulate scleral growth.

Choroid has been suggested and shown to regulate ocular elongation. Apart from the tight link between choroidal thickness and ocular elongation as mentioned before, emerging evidence has been showing that change in ocular elongation or scleral GAG synthesis, is always linked to or preceded by the change in choroidal thickness.

1.3.6.1 Choroid can regulate scleral GAG synthesis

Choroid can regulate scleral GAG synthesis. When choroid from eyes that are no longer elongating are co-cultured with scleral punches from normal eyes, scleral GAG synthesis of the scleral punches decreases; whereas choroids from elongating eyes increase scleral GAG of normal scleral punches (Marzani and Wallman, 1997).

1.3.6.2 Thickened choroid always precedes inhibition of ocular elongation

The shift in diurnal rhythms of ocular length and choroidal thickness under different visual manipulations has shown that thickened choroid always precedes inhibition of ocular elongation. In young chicks and marmosets, ocular length and choroidal thickness fluctuate diurnally, and these diurnal rhythms are approximately out of phase (Nickla et al., 1998). In chicks, ocular length is longest at noon when the choroid is thinnest; in midnight, the ocular length is shortest but the choroid is thickest. When ocular elongation is inhibited, however, these two rhythms will shift into exactly in phase; in contrast, when ocular elongation is enhanced, these two rhythms will shift to be exactly out of phase. Thus, the thickest choroid will be found with the longest ocular length a day before ocular elongation is inhibited (Nickla, 2006). Similar phase shift is also found in marmosets (Nickla et al., 2002).

Transient choroidal thickening always precedes the inhibition of ocular growth. As mentioned before, myopia can be induced by putting an occluder in front of an eye. If the occluder is removed, or when the eye is allowed for unrestricted vision for 2 hours a day while the rest of the day with the occluder on, the eye will recover from myopia. A transient increase choroidal thickening was found in both cases which precedes the inhibition of ocular elongation (Nickla, 2007). Similar transient choroidal thickening can be found when eyes wearing negative lenses are injected with muscarinic antagonists (Nickla, et al. 2013) or with dopamine agonists (Nickla et al., 2010a) that inhibit ocular growth. When transient choroidal thickening was inhibited in eyes recovering from form deprivation by injected nitric oxide synthase inhibitor l-NAME or spiperone, a dopamine D2 antagonist, the eyes did not recover

from form-deprivation (Nickla and Totonelly, 2011; Nickla et al., 2006). Thus, choroidal thickening is suggested to inhibit ocular growth either by (1) simply providing a mechanical barrier, so that molecules maintaining ocular elongation cannot reach the sclera; or (2) producing some molecules that inhibit eye-growth (Nickla 2002).

We have discussed different ocular structures and their functions as well as different potential molecules involve in the signal cascade of eye growth. However, it is still unknown how visual signals received by the retina can induce the retinal production of these molecules. Studies on eye growth were previously mostly focused on the retina, second on the sclera and recently have been more on the choroids [review: (Nickla and Wallman, 2010)]. The RPE, however, has been mostly neglected. To decipher the signal cascade of eye growth, roles of different structures at the posterior part of the eyes on the effect of different molecules should be studied.

In recent years, our laboratory has been working on an *in vitro* system to study signaling molecules in the posterior part of the eye (Nickla et al., 2010b; Nickla et al., 2013; Sheng et al., 2008, 2009; Zhu and Wallman, 2005). There are several advantages of using *in vitro* eye-cups. First, by successively removing RPE and choroid, the effect of the molecule on different ocular layers can be explored, which would be impossible to do *in vivo*. Second, recent findings have shown that visual treatments of one eye can cause changes of ocular parameters (Rucker et al., 2009) or the protein expression (Bitzer and Schaeffel, 2002) of the fellow untreated eyes,

indicating interaction between paired eyes *in vivo*. In our eye-cups, the effect of the studied molecule on the choroid and sclera is not affected by interactions between the eyes. Third, because we can successively remove RPE and choroid, the concentration of the molecule required to produce an effect in each layer can be known. If the molecule is injected into the eye, it might be metabolized in the vitreous, and the concentration reaching the RPE, choroid or sclera would be unknown.

In the following section, we will introduce the general method of our eye-cup system, and it will be repeated (except 1.4.4) in the method section of the insulin and VEGF chapter.

1.4 General methods

1.4 .1 Animals

White Leghorn chicks (*Gallus gallus domesticus*) were hatched from eggs supplied from Cornell University (Cornell K-strain; Ithaca, NY). The chicks were housed in heated brooders on a 14:10 light-dark cycle. Food and water were supplied *ad libitum*. One-week-old chicks were used in all experiments. Care and use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

1.4.2. Eye-cup dissection and tissue culture

We made eye-cups consisting of either RPE, choroid, and sclera (RCS eye-cups), just choroid and sclera (CS eye-cups), or just sclera (scleral eye-cups) and used them according to different experimental paradigms.

Chicks were sacrificed by intracardiac injection of pentobarbital sodium (Beuthanasi-D Special; Schering-Plough Animal Health Corp) and were kept postmortem at room temperature for different intervals of time depending on whether the RPE was to be removed or not. Eyes were hemisected along the equator perpendicular to the optical axis. Vitreous, retina and pectin were removed. For RCS eye-cup preparation, in which the RPE was retained, we waited 10 minutes postmortem prior to enucleating the eyes. At the 10 minute postmortem time point, we found that the RPE was firmly attached to the choroid, facilitating removal of the retina while leaving the choroid in place. Any pair of RCS eye-cups in which either eye retained less than 80% of the RPE was discarded. Scleral and CS eye-cups, in which the RPE was not retained, were prepared from eyes enucleated 20-30 minutes postmortem, as at this time point we found that the RPE was firmly attached to the retina, and both were removed concurrently. Any residual RPE left on the choroid was gently removed by a brush. Any pair of scleral or CS eye-cups in which either eye-cup retained more than 10% of the RPE was discarded. Individual eye-cups were put into wells of a 24-well tissue culture plate on ice. Each well contained 3 mL of CO_2-independent medium (Gibco; Invitrogen), which maintained the physiological pH of the medium during dissection and during the first measurement of choroidal thickness. After the first measurement, eye-cups were transferred to wells containing

3 mL of L-15 medium with or without the studied molecules according to the experimental paradigms, and cultured at 37^{o}C with 5% CO_2.

1.4.3 Choroidal thickness measurement in eye-cups

Choroidal thickness in eye-cups was measured by A-scan ultrasonography (Fig 1.3). For ease of alignment, eye-cups were centered on top of nylon nuts (10.8 mm width x 8.4 mm height; Small Parts Inc.) with the scleral side down. The ultrasound transducer with an attached, water-filled conical standoff was aligned onto the posterior pole (Fig 1.3A). Choroidal thickness was measured with a 30 MHz polymer transducer (Panametrics Model 176599) linked to a computer interface with a Sonix 8100 A/D board, allowing sampling of the output signal at 100 MHz (Nickla, 1996). With good alignment, peaks of reflections from the RPE/choroid and scleral surfaces are illustrated in Fig 1.3B For measurement of each eye-cup, two alignments were made, with 5 traces saved per alignment.

1.4.4 Correlation of *in vivo* and *in vitro* ultrasound measures

To examine the correlation between *in vivo* and *in vitro* ultrasound measurements, we created a range of choroidal thicknesses in the chick eyes. First we placed translucent diffusers over one eye of 5-day-old birds for 5 days. The other eye of the same birds was covered with diffusers for 3 days, and then uncovered for two days. On the 5th day of treatment, choroidal thickness was measured *in vivo*. The treated birds were sacrificed as previously described. Eyes were then enucleated, and RCS

eye-cups or CS eye-cups were prepared. Choroidal thickness in eye-cups was then measured again approximately 2 hours after the corresponding *in vivo* measurement.

Choroidal thickness of eye-cups was well correlated with the *in vivo* measurement made 2 hours earlier on the same eye (RCS eye-cups: r^2=0.88, p<0.0001; CS eye-cups: r^2=0.43, p<0.01; SD for repeated measurements for RCS eye-cups vs. CS eye-cups: 10 μm vs. 17 μm, Fig 1.4). In addition, most data points fell above the line of identity indicating that choroids in eye-cups were thicker than choroid *in vivo*.

1.4.5 Scleral GAG measurement

After incubation, 7 mm punches of sclera were taken from the posterior pole of each eye-cup and cultured in L-15 medium containing $Na_2{}^{35}SO_4$ (4 μL/mL) for 2 hours. Punches were then digested in proteinase K (in 10 mM EDTA, 0.1 M sodium phosphate, pH 6.5, protease type XXVIII, Sigma) overnight at 57^{o}C. Newly synthesized ^{35}S labeled GAGs were precipitated by adding chondroitin sulphate (1mg/mL in distilled water, Sigma) and 0.5% of cetylpyridinium chloride (Sigma) at 37^{o}C. Precipitated GAGs were filtered with Whatman filters (GF/A; Fischer Scientific), which were then washed repeatedly and dried overnight at room temperature. Radioactivity in the filters was measured by liquid scintillation counter in 20 mL of scintillation fluid (Ultima Gold F, Perkin Elmer).

Chapter 2 *In vitro* studies of insulin

Abstract

Purpose: In chick eyes, exogenous insulin prevents the choroidal thickening caused by wearing positive lenses and increases ocular elongation and scleral GAG synthesis, both indicators of eye growth. Using *in vitro* eye-cups, a novel experimental system, we examined the role of the RPE and insulin on choroidal thickness and scleral GAG synthesis. Specifically, we asked whether insulin causes the release of diffusible factors from the RPE that affect the choroid.

Method: Experiment 1: Effect of insulin on choroidal thickness and scleral GAG synthesis. Eye-cups consisting of RPE, choroid, and sclera (RCS), choroid and sclera (CS), or just sclera, were prepared from pairs of eyes. One eye-cup was cultured in 0.037, 0.37, 3.7 or 37 μM insulin dissolved in L-15 medium, and its pair was cultured in plain medium. Choroidal thickness in eye-cups was measured by A-scan ultrasonography before and after 20 hours of incubation. Sulfate incorporation into GAGs (scleral GAG synthesis), was measured after 44 hours of incubation. Experiment 2: Effect of RPE and insulin on choroidal thickness. Pairs of CS eye-cups were cultured with vs. without RPE transplanted from donor eyes, in the presence, or absence of 37 μM insulin. Choroidal thickness was measured before and after 20 hours of incubation. Experiment 3: Effect of RPE conditioned medium in the presence or absence of 37 μM insulin on the choroidal thickness of CS eye-cups was studied. RPE was cultured with (experimental conditioned medium) or without 37 μM insulin (control conditioned medium) for 20 hours. Experimental and control conditioned medium were collected separately, and an equal volume of medium

containing a concentration of 37 μM insulin was added to both experimental and control medium. Pairs of CS eye-cups were cultured in conditioned medium (experimental vs. control). Choroidal thickness was measured before and after 20 hours of incubation.

Results: Experiment 1: Choroids cultured with insulin were significantly thinner than those cultured without insulin, but only if the RPE was present. This effect was dose-dependent and strongest at 37 μM. Insulin increased scleral GAG synthesis in both RCS and CS eye-cups, having a greater effect in the CS eye-cups. Insulin had no effect on scleral GAG synthesis in scleral eye-cups. Experiment 2: Choroids of CS eye-cups cultured with transplanted RPE plus insulin were significantly thinner than choroids of eye-cups cultured with insulin but without the RPE. The thinning effect was similar to that seen in eye-cups with intact RPE (RCS) in Experiment 1. Experiment 3: CS eye-cups cultured with experimental conditioned medium had thinner choroids than their pairs cultured with control conditioned medium.

Conclusion: *In vitro*, as *in vivo*, insulin prevents choroidal thickening and increases scleral GAG synthesis. Our findings suggest that Insulin might cause the RPE to synthesize diffusible molecules that inhibit choroidal thickening. Insulin might also act on the choroid to affect scleral GAG synthesis.

2.1 Introduction

Animal studies in eye growth have shown that the eyes compensate for defocus and respond to blur by changing choroidal thickness and the rate of ocular elongation. In chicks, negative lenses, which make the eyes hyperopic by shifting the focal plane behind the retina, decrease choroidal thickness (Wallman et al., 1995) and enhance ocular elongation (Diether and Schaeffel, 1997; Schaeffel et al., 1988; Wallman et al., 1995). A similar effect is seen with translucent diffusers (Wallman et al., 1987; Wallman et al., 1978), which deprive the eyes of form-vision (form deprivation). Conversely, when positive lenses are worn (Diether and Schaeffel, 1997; Schaeffel et al., 1988), which make the eyes myopic by shifting the focal plane in front of the retina, the choroid thickens, and the rate of ocular elongation decreases (Wallman et al., 1995). A similar effect is seen when translucent diffusers are removed (Wallman et al., 1995). Similar, but smaller, compensatory changes in choroidal thickness have been found in tree shrews (Siegwart and Norton, 1998), marmosets (Troilo et al., 2000), rhesus macaques (Hung et al., 2000), guinea pigs (Howlett and McFadden, 2006, 2009), and even in humans (Read et al., 2012). Changes in the rate of ocular elongation have been associated with corresponding changes in the scleral extracellular matrix indicated by scleral GAG synthesis (a measure of scleral proteoglycan synthesis). In chicks, the increase in ocular elongation that is seen with negative-lenses or translucent diffusers has been associated with increased scleral GAG synthesis (Nickla et al., 1997; Rada and Matthews, 1994; Rada et al., 1992; Rada et al., 1991); while the decrease in ocular elongation that is seen with positive

lenses or removal of translucent diffusers has been associated with decreased scleral GAG synthesis (Nickla et al., 1997; Rada et al., 1992).

Evidence suggests that the control of eye growth is retinal in origin. In chicks, optic nerve section cannot inhibit accelerating eye growth induced by form-deprivation or accelerating or decelerating eye growth induced by negative or positive lens-wear (Troilo et al., 1987; Wildsoet and Wallman, 1995). Also, the eye can control its growth locally: Form deprivation or lens-wear in either the nasal or temporal part of the retina changes the ocular elongation rate in that area (Diether and Schaeffel, 1997; Wallman et al., 1987). Local control of eye growth supports the hypothesis that the retina itself generates signals to regulate eye growth.

Lying between the retina and the sclera is the choroid and a single layer of pigmented cells called the RPE. The apical side of the RPE faces the retina, and its basolateral side faces the Bruch's membrane, which separates the RPE from the choroid. The tight junctions in the RPE form a fluid and ionic barrier. The retina might initiate a signal cascade that acts on the choroid and sclera directly. Alternatively, the signal from the retina might act indirectly and cause the RPE to produce another signal that acts on the choroid and directly or indirectly on the sclera. Ultimately, the signal cascade causes changes in the choroidal thickness and the rate of ocular elongation.

Insulin is well known to lower glucose and to enhance cell growth and cell proliferation (Taniguchi et al., 2006). Insulin can act on both insulin receptor and IGF-1 receptor. When bound by insulin, insulin receptor phosphorylates the insulin receptor substrates, which act as docking sites for effecting different signaling cascades. The most well-known two cascade pathways are: (1) The phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway, which is responsible for most of the metabolic actions of insulin including glucose metabolism, and (2) Ras-mitogen-activated protein kinase (MAPK) pathway, which co-ordinates with the PI3K pathway to control cell growth and differentiation (Taniguchi et al., 2006). In glucose metabolism, the ultimate goal of insulin is to translocate glucose transporter 4 to the plasma membrane.

Insulin is not present or synthesized in the pancreas only. Its presence has been shown in the central nervous system (Boyd et al., 1985), the photoreceptors (de la Rosa et al., 1994), retinal glial cells (Das et al., 1984), and RPE (Waldbillig et al., 1991). It was also shown that chick sclera could bind insulin and IGF-1, and the binding affinity of them on the sclera is regulated during development (Waldbillig et al., 1990).

Insulin receptor monomer which composed of one α and one β subunit bridged by an intrinsic disulfide bond can homodimerize with another insulin receptor monomer or heterodimerize with an IGF-1 receptor monomer, becoming an insulin/IGF-1 hybrid receptor [review: (Hernandez-Sanchez et al., 2006)]. It is known that insulin

can act both on insulin receptors and IGF-1 receptors as well as the insulin/IGF hybrid receptors (Chiu and Cline, 2010; Hernandez-Sanchez et al., 2005). Proinsulin, an insulin precursor, stimulates the development of the neuroretina in chicks, and its action is mediated by the regulation of insulin/IGF-1 hybrid receptor (Hernandez-Sanchez et al., 2006). Even though this hybrid receptor decreases during retinal development, it is still present in postnatal tissues of other mammalian tissues (Bailyes et al., 1997; Hernandez-Sanchez et al., 2006). Furthermore, both insulin and IGF-1 can increase the activities of chondrocytes (Bohme et al., 1992; Torres et al., 2003), which might lead to increased scleral GAG synthesis resulting in ocular elongation.

Recent studies have shown that when insulin is injected into normal chick eyes, it enhances ocular growth (Feldkaemper et al., 2009; Zhu and Wallman, 2009a) and scleral GAG synthesis (Zhu and Wallman, 2009a). When insulin is injected into eyes wearing positive lenses, it inhibits choroidal thickening (Zhu and Wallman, 2009a), disinhibits ocular growth (Feldkaemper et al., 2009; Zhu and Wallman, 2009a) and increases scleral GAG synthesis (Zhu and Wallman, 2009a). Injected insulin can even make the eyes over-compensate for negative lenses (Feldkaemper et al., 2009) by further increasing ocular length. Furthermore, in glucagonergic amacrine cells, the expression of transcription factor ZENK, which increases in positive lens-wear and decreases in negative lens-wear (Bitzer and Schaeffel, 2002; Fischer et al., 1999), is also decreased by injected insulin (Feldkaemper et al., 2009).

In the present study, we used *in vitro* eye-cups, a novel experimental system, to examine the role of the RPE in mediating the effect of insulin on the choroid and sclera, using changes in scleral GAG synthesis as an indicator of eye-growth. Even though the natural ligand in the ocular tissues will possibly be IGF-1 or IGF-2, the use of insulin provides an economical way to study how the insulin family members can affect eye growth.

2.2 Materials And Methods

The following sections before the experimental design are the same as those in the general method and VEGF chapters.

2.2.1 Animals

White Leghorn chicks (*Gallus gallus domesticus*) were hatched from eggs supplied from Cornell University (Cornell K-strain; Ithaca, NY). The chicks were housed in heated brooders on a 14:10 light-dark cycle. Food and water were supplied ad libitum. One-week-old chicks were used in all experiments. Care and use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2.2 Eye-cup dissection and tissue culture

We made eye-cups consisting of either RPE, choroid, and sclera (RCS eye-cups), just choroid and sclera (CS eye-cups), or just sclera (scleral eye-cups) and used them according to different experimental paradigms.

Chicks were sacrificed by intracardiac injection of pentobarbital sodium (Beuthanasi-D Special; Schering-Plough Animal Health Corp) and were kept postmortem at room temperature for different intervals of time depending on whether the RPE was to be removed or not. Eyes were hemisected along the equator perpendicular to the optical axis. Vitreous, retina and pectin were removed. For RCS eye-cup preparation, in which the RPE was retained, we waited 10 minutes postmortem prior to enucleating the eyes. At the 10 minute postmortem time point, we found that the RPE was firmly attached to the choroid, facilitating removal of the retina while leaving the choroid in place. Any pair of RCS eye-cups in which either retained less than 80% of the RPE was discarded. Scleral and CS eye-cups, in which the RPE was not retained, were prepared from eyes enucleated 20-30 minutes postmortem, as at this time point we found that the RPE was firmly attached to the retina, and both were removed concurrently. Any residual RPE left on the choroid was gently removed by a brush. Any pair of scleral or CS eye-cups in which either eye-cup retained more than 10% of the RPE was discarded. Individual eye-cups were put into wells of a 24-well tissue culture plate on ice. Each well contained 3 mL of CO_2-independent medium (Gibco; Invitrogen), which maintained the physiological pH of the medium during dissection and during the first measurement of choroidal thickness. After the first measurement, eye-cups were transferred to wells containing 3 mL of L-15 medium with or without insulin according to the experimental paradigms, and cultured at 37^{o}C with 5% CO_2 for 20 hours.

2.2.3 Choroidal thickness measurement in eye-cups

Choroidal thickness in eye-cups was measured by A-scan ultrasonography (Fig 1.3). For ease of alignment, eye-cups were centered on top of nylon nuts (10.8 mm width x 8.4 mm height; Small Parts Inc.) with the scleral side down. The ultrasound transducer with an attached, water-filled conical standoff was aligned onto the posterior pole (Fig 1.3A). Choroidal thickness was measured with a 30 MHz polymer transducer (Panametrics Model 176599) linked to a computer interface with a Sonix 8100 A/D board, allowing sampling of the output signal at 100 MHz (Nickla, 1996). With good alignment, peaks of reflections from the RPE/choroid and scleral surfaces are illustrated in Fig 1.3B. For measurement of each eye-cup, two alignments were made, with 5 traces saved per alignment.

2.2.4 Scleral GAG measurement

After incubation, 7 mm punches of sclera were taken from the posterior pole of each eye-cup and cultured in L-15 medium containing $Na_2{}^{35}SO_4$ (4 µL/mL) for 2 hours. Punches were then digested in proteinase K (in 10 mM EDTA, 0.1 M sodium phosphate, pH 6.5, protease type XXVIII, Sigma) overnight at 57^{o}C. Newly synthesized ^{35}S labeled GAGs were precipitated by adding chondroitin sulphate (1mg/mL in distilled water, Sigma) and 0.5% of cetylpyridinium chloride (Sigma) at 37^{o}C. Precipitated GAGs were filtered with Whatman filters (GF/A; Fischer Scientific), which were then washed repeatedly and dried overnight at room temperature. Radioactivity in the filters was measured by liquid scintillation counter in 20 mL of scintillation fluid (Ultima Gold F, Perkin Elmer).

2.2.5 Experimental Design:

2.2.5.1 Experiment 1: Effects of insulin on choroidal thickness and scleral GAG synthesis

After the initial ultrasound measurement, paired RCS, CS and scleral eye-cups from the same animals were cultured with or without bovine insulin (Sigma) at one of the following concentrations: 0.037 µM, 0.37 µM, 3.7 µM, or 37 µM in L-15 medium (Millipore). Choroidal thickness was measured in the RCS and CS eye-cups after 20 hours of incubation at 37 ^{o}C with 5 % CO_2. Since our preliminary findings did not show that insulin could affect scleral GAG synthesis after 20 hours of incubation (data not shown), medium with or without insulin was renewed and eye-cups were cultured for another 24 hours. Scleral GAG synthesis was thus measured after a total of 44 hours of incubation. The number of chicks used at each concentration of insulin in RCS and CS eye-cups is shown in Figure 2.1 and 2.2.

2.2.5.2 Experiment 2: Effect of insulin plus transplanted RPE on choroidal thickness

Previous experiments identified that the effect of insulin on choroidal thickness was attenuated by the absence of the RPE. To rule out that this effect was due to inadvertent damage to the choroid during RPE removal, we assessed the viability of the choroidal tissue by culturing CS eye-cups with RPE from donor eyes. To harvest the intact donor RPE, RCS eye-cups were prepared as previously described and cultured in 1mM EDTA in PBS at 37 ºC for 2 hours (adapted from (Rizzolo, 1991)).

The RPE was then removed in one piece using forceps and subsequently transferred by a dropper to recipient CS eye-cup pairs prepared from another set of birds. The CS eye-cups pairs were divided into three groups. In the first group, all CS eye-cups were cultured in the presence of 37 μM of insulin (n=7 pairs), with vs. without RPE from one donor eye in pairs. In a second group, all CS eye-cups were cultured in the presence of 37 μM of insulin (n=7 pairs), with or without RPE from two donor eyes in pairs. In the third group (n=10 pairs), all eye-cups were cultured in L-15 without insulin, with or without RPE from one donor eyes in pairs. Therefore, the experimental eye-cups of each pair from all three groups were cultured with RPE from one or two donor eyes, whereas the paired control eye-cup received no RPE. Choroidal thickness was measured after 20 hours of incubation for all three groups.

2.2.5.3 Experiment 3: Effect of RPE-conditioned medium

To examine the role of the RPE in the mediation of choroidal thickness via diffusible factors, conditioned medium was made from RPE cultured with or without insulin (Bovine insulin from Akron). To increase the concentration of the hypothetical molecules produced by the RPE in the presence of insulin, RPEs of two donor eyes from two birds (one from each bird) were cultured in the same well filled with 37 μM of insulin in 3 mL L-15 medium (experimental conditioned medium); RPEs from the other eyes of the same two birds were cultured in the paired well without insulin in 3 mL L-15 medium (control conditioned medium). A total of 24 RPEs from 12 birds were used and cultured in 6 pairs of wells. Experimental and control conditioned medium were pooled separately after 20 hours of incubation.

To exclude the possibility that insulin thins choroids directly in the CS eye-cups, rather than by producing a molecular cascade involving the RPE, a critical procedure in this experiment was to add equal volume of 37 μM of insulin to both experimental and control conditioned medium separately. Therefore, the experimental conditioned medium still contained 37 μM insulin (in addition to the hypothetical signaling molecules produced by the RPE in the presence of insulin during the first 20 hours of incubation), but the control conditioned medium contained 18.5 μM insulin. Our findings in Experiment 1 revealed that a two-fold difference in the concentration of insulin did not cause a difference in the relative change in the choroids (change in the choroidal thickness cultured with insulin minus the change in the choroidal thickness cultured without insulin) of the CS eye-cups (Fig 2.1B). Paired CS eye-cups prepared from another set of birds (n=12) were each then cultured in 3 mL experimental and control conditioned medium respectively. Choroidal thickness was measured before and after 20 hours of incubation with conditioned medium. Our hypothesis was that a significant difference in choroidal thickening after incubation between the paired eyes would indicate that signaling molecules contained in conditioned medium were acting on the choroid.

2.2.6 Statistics

All data are means (±SEM) unless otherwise specified. We increased the sensitivity of statistical tests by comparing relative changes in choroidal thickness (the difference of changes in thickness between paired eyes (ΔX-ΔN)) at different

concentrations of insulin using one-way analysis of variance (ANOVA) with LSD post hoc test. The ratio of scleral GAG synthesis was calculated by dividing the values of radioactivity from the experimental eye-cups by the values from the paired control eye-cups. To avoid the asymmetry of averaging ratios (e.g., a ratio of 5 having more weight than the inverse ratio of 0.2 and making one treatment appear more effective than another), each ratio was taken to logarithm of base 10 and the mean of the calculated ratio (mean log ratio) for each concentration of insulin was converted back by taking its anti-logarithm. Effects of insulin on the scleral GAG synthesis was analyzed by comparing the logarithm of the count from the experimental eye-cups with that of the paired control eye-cups by paired Student *t*-test. One-way or two-way ANOVA was performed and was followed by LSD post hoc test. Data were also analyzed by mixed factorial ANOVA in which presence of the RPE and concentrations of insulin were the between-group variables, and paired eye from the same birds was the within-group variable; this was followed by a Holm – Bonferroni post hoc test.

Data points falling more than 3 standard deviations from the average of all data in the same kind of eye-cups were omitted. By this criterion, one out of 63 pairs of choroidal thickness data points for CS eye-cups, and one out of 46 pairs of scleral GAG synthesis data points for scleral eye-cups were omitted.

2.3 Results

2.3.1. Experiment 1: Effects of insulin on choroidal thickness and scleral GAG synthesis in eye-cups

Choroids cultured with insulin were significantly thinner than those cultured without insulin, but only if the RPE was present (Fig 2.1A). Choroidal thickness of all RCS eye-cups cultured without insulin increased by 117 μm on average after 20 hours of incubation. This choroidal thickening was significantly reduced in RCS eye-cups cultured with insulin (ΔX vs. ΔN: 37 μM of insulin: 54 μm vs. 118 μm, $p<0.0001$, two-tailed paired Student's *t*-test; 3.7 μM of insulin: 78 μm vs. 110 μm, $p<0.01$, mixed factorial ANOVA, $p<0.001$, Fig 2.1A). Moreover, we found that the effect of insulin on the relative change in choroids (ΔX -ΔN) of the RCS eye-cups was dose-dependent ($p<0.01$, one-way ANOVA, $p<0.0001$, mixed factorial ANOVA), with the strongest effect at 37 μM ($p< 0.01$, LSD post hoc test, Fig 2.1A).

Presence of the RPE significantly affected choroidal thickening whether insulin was present or not. Without insulin, choroids of RCS eye-cups thickened more than the choroids of CS eye-cups (all control RCS eye-cups vs. all control CS eye-cups: 117 μm vs. 80 μm, $p<0.001$, two-tailed two-sample equal variance Student's *t*-test, Fig 2.1C, mixed factorial ANOVA, interaction between RPE & concentration, $p<0.0001$). Thus, although the presence of the RPE by itself increased choroidal thickening, the RPE together with insulin reduced choroidal thickening. Presence of the RPE also significantly affected insulin dose-response on the relative change of the choroids (ΔX -ΔN, $p<0.001$, two-way ANOVA).

In CS eye-cups without the RPE, the effect of insulin was not statistically significant at any concentrations by paired-eye comparison (two-tailed paired Student's *t*-test, Fig 2.1B). However, at the higher two concentrations (3.7 and 37 μM), choroids cultured with insulin seemed to thicken less than their paired control choroids; whereas at the lower two concentrations (0.37 and 0.037 μM), choroids cultured with insulin seemed to thicken more than their paired choroids. Therefore, when the relative changes in the choroidal thickness ($\Delta X - \Delta N$) of these CS eye-cups at different doses were analyzed, they were significantly different ($p < 0.05$, one-way ANOVA, Fig 2.1B), even though insulin did not cause any significant difference in these choroids between the paired eyes.

Insulin increased scleral GAG synthesis in eye-cups with choroid and sclera, whether the RPE was present or not (Fig. 2.2); indeed, the effect of insulin was greatest if the choroid was present and the RPE was absent as in CS eye-cups (two-way ANOVA, $p<0.05$, mixed factorial ANOVA, interaction between RPE and concentration, $p<0.001$). In the CS eye-cups, although the highest dose was ineffective (37 μM), 0.37 and 3.7 μM of insulin increased the scleral GAG synthesis 2-3 fold relative to the fellow eye ($p<0.05$, two-tailed paired Student's *t*-test, Fig 2.2B) and the effect at these doses was 2-3 fold stronger than that at 37 μM ($p<0.01$, one-way ANOVA, $p<0.05$, LSD post hoc test,). In contrast, when the RPE was left on the choroid, insulin significantly increased scleral GAG synthesis (1.5-2 fold), but only at the highest concentration (37μM, $p<0.05$, two-tailed paired Student's *t*-test, Fig 2.2A). Insulin had no effect on GAG synthesis of isolated sclera (Fig. 2.2C).

2.3.2 Experiment 2: Roles of RPE in the insulin effect on the choroid

We found that insulin can reduce choroidal thickening in the presence of transplanted RPE. When paired CS eye-cups were cultured with 37 μM insulin, the choroid with RPE transplanted from one donor eye ended up 50 μm thinner than the fellow choroid without the RPE (ΔX vs. ΔN, 45 μm vs. 95 μm; $p<0.05$, two-tailed paired Student's *t*-test, Fig 2.3A). Furthermore, in the presence of insulin, choroids with RPE transplanted from two donors ended up 95 μm thinner than their paired fellow choroid without the RPE (-5μm vs. 90μm: $p<0.05$, Fig 2.3B). The relative difference between transplanted RPE from one and two donors was not significant (two-tailed 2 sample equal variance Student's *t*-test). Those eye-cups that received RPE from two donor eyes showed less thickening than CS eye-cups in Experiment 1, even though the effect was not significant (-5 μm vs. 53 μm; $p=0.06$, two-tailed two-sample equal variance Student's *t*-test, Fig 2.3B).

The presence of insulin was necessary for the donor RPE to affect choroidal thickness. Without insulin, transplanted RPE did not affect choroidal thickness in recipient CS eye-cups compared with their paired CS eye-cups without the RPE (Fig 2.3C: ΔX vs. ΔN**,** 126 μm vs. 109 μm). That is, similar to eye-cups without insulin in Experiment 1, choroids thickened after 20 hours of incubation (RCS eye-cups with RPE intact in Experiment 1 vs. CS eye-cups with transplanted RPE in Experiment 2, 117 μm vs. 126 μm, $p>0.05$ two-tailed 2 sample equal variance Student's *t*-test). With insulin, transplanted RPE from a single donor caused similar choroidal

thickening in CS eye-cups to that in RCS eye-cups with their RPE intact, as was seen in Experiment 1 (45 μm vs. 54 μm, $p>0.05$, two-tailed 2 sample equal variance Student's *t*-test, Fig 2.3A).

An additional two-way ANOVA (with the amount of RPE and the concentration of insulin as factors) followed by a post-hoc LSD test was done to further assess if the amount of the transplanted RPE and the concentration of insulin could significantly affect the relative change in the choroidal thickness across eye-cups [i.e., the change in choroidal thickness in the eye-cups cultured with transplanted RPE minus the change in choroidal thickness of the paired eye-cups without RPE (ΔX-ΔN), also indicated by the bars in Fig 2.3]. Similar to what we found with the Student's *t*-test presented in section 2.3.2, the 2-way ANOVA revealed that the amount of RPE did not significantly affect the relative change in choroidal thickness ($p=0.2$). It also showed that concentration of insulin significantly affected the relative change in choroidal thickness ($p<0.05$).

2.3.3. Experiment 3: Effect of insulin on RPE-conditioned medium

Choroids of CS eye-cups cultured with experimental conditioned medium thickened significantly less than their controls cultured with control conditioned medium (mean change in choroidal thickness: 126 μm vs. 209 μm, $p< 0.05$, two-tailed paired Student's *t*-test, Fig 2.4). Thus, even without the RPE, experimental conditioned

medium containing the signaling molecules (presumably produced by the RPE in the presence of insulin) inhibited choroidal thickening in eye-cups.

2.4 Discussion

Our findings suggest that in response to insulin, RPE produces secondary signaling molecules that reduce the natural choroidal thickening *in vitro*. Furthermore, insulin indirectly increases scleral GAG synthesis by acting on the choroid.

2.4.1 Choroid thickens *in vitro*

Our findings suggest that insulin can reduce choroidal thickening (or cause relative thinning of the choroids) by acting on the RPE. During incubation, all choroids in eye-cups thickened. Choroidal expansion has been associated with the expansion of lacunae, spaces within the choroidal lymphatic system (Junghans et al., 1999). Increased fluid transfer into the lacunae is found during choroidal expansion when eyes are recovering from form-deprivation (Junghans et al., 1999). In the *in vitro* environment, the absence of intra-ocular pressure and the oncotic pressure, caused by the high plasma concentration in the choroid, draw fluid from the tissue culture medium into the lacunae and can therefore ultimately thicken the choroids during incubation.

2.4.2 The role of the RPE in mediating the thinning effect of insulin

Insulin reduced the natural choroidal thickening in a dose-dependent manner, but only when the RPE was present. When CS eye-cups were cultured with transplanted

RPE from donor eyes, the relative thinning effect of insulin on the choroids was recovered. In the presence of insulin, transplanted RPE caused the choroids in CS eye-cups to respond similarly to the choroids in RCS eye-cups as in Experiment 1, with (Fig 2.3A).

Because insulin did not cause significant changes in CS eye-cups in Experiment 1, one might argue that the choroids in the CS eye-cups might have been damaged during dissection when we removed the RPE or perhaps the longer postmortem time of preparing CS eye-cups than that of the RCS eye-cups before we enucleated the eyes might make the choroids less viable. In Experiment 2, choroids in CS eye-cups cultured with insulin and transplanted RPE from one or two donor eyes thickened significantly less than those of their paired CS eye-cups cultured with insulin but without transplanted RPE. Therefore, choroids in CS eye-cups were not damaged, or at least not damaged to an extent that can limit the thinning effect of insulin. Also, the natural physical binding of RPE and choroid was unnecessary for the relative thinning effect of insulin in eye-cups. We further found that medium conditioned by the RPE in the presence of insulin reduced choroidal thickening in CS eye-cups. The findings suggest that insulin might cause the RPE to produce secondary signals that reduce choroidal thickening.

We found that choroids in CS eye-cups seemed to thicken less consistently than choroids in RCS eye-cups. Choroids of CS eye-cups cultured in medium conditioned by the RPE (Experiment 3) seemed to thicken more than those in CS eye-cups from

Experiments 1 or 2, with 37 μM insulin. However, comparing choroids of CS eye-cups in Experiment 3 with those in Experiment 1 or 2 might not be appropriate because medium in Experiment 3 was conditioned by the RPE with 37 μM insulin for 20 hours before equal amounts of 37 μM insulin was added to it. It is not clear how this difference can affect the natural choroidal thickening *in vitro*. Batch differences among birds might also play a role, as Experiment 3 was performed several years after Experiments 1 and 2. Furthermore, choroids in CS eye-cups in Experiment 2 seemed to thicken more, even though not significantly so, than the choroids of CS eye-cups in Experiment 1 (both cultured with 37 μM insulin without the RPE; denoted by open square and circle in Fig 2.3B respectively). However, when CS eye-cups were cultured with RPE transplanted from two donor eyes, choroidal thickening was reduced further, almost significantly more ($p=0.06$) than those CS eye-cups in Experiment 1 (Fig 2.3B).

To study if the inconsistent change in the control choroids was perhaps caused by other variables the way we ran the experiments, Factorial ANOVA was done to study the effect of the following factors on all the control choroids cultured without insulin: (1) use of antibiotics, (2) date of experiment, (3) initial choroidal thickness, (4) different kinds of eye-cups (RCS eye-cups vs. CS eye-cups: i.e. the presence of the RPE), (5) different concentrations of insulin (see if there were any block effects even though the paired eye-cups were randomized). Factorial ANOVA showed that only the initial choroidal thickness and the presence of the RPE (i.e. types of eye-cups) have a significant effect on the choroidal change in the control eye-cups without

insulin. The effect of the RPE on the choroidal thickness is consistent with what we found by doing 2-sample equal variance Student *t*-test as it is shown in Figure 2.1C: Without insulin, choroids of RCS eye-cups thickened more than choroids of the CS eye-cups (indicated by the diamond and the hexagon respectively in Figure 2.1C; we will come back to discuss the role of the RPE in maintaining choroidal thickness *in vitro* later in section 2.4.6). We also looked into the initial choroidal thickness of the CS eye-cups at different concentrations. The mean initial choroidal thickness in each group was similar (data not shown), even though the changes in choroids after 20 hours of each group were different. Therefore, it did not explain the inconsistency in choroids of CS eye-cups.

2.4.3 Can paired comparison of choroids diminish the effect of insulin in CS eye-cups?

Since we did paired comparisons of choroids of the same animals, one might argue that the thinning effect of insulin would be diminished if there were variation among control eyes in choroidal thickening. For example, in Figure 2.1B at 37 μM insulin, the mean change of the choroids in CS eye-cups without insulin was only 63 μm; this might indicate something different or aberrant with these eyes. We therefore selected a subset of these cases whose mean choroidal thickening was more similar to other CS cases by removing data points of paired eye-cups in which the control choroids thickened less than 50 μm. In doing this, the mean change of the control choroids increases to 89 μm (Table 1) which is close to the other control groups in Figure 2.1B. However, the mean change of choroids cultured with insulin also

increases to 65 μm. Paired *t*-tests showed that insulin cannot significantly reduce choroidal thickening in these eye-cups. We then selected another subset whose mean choroidal thickening was similar to that of the RCS eye-cups by removing data points of the control choroids that thickened less than 60 μm, and made the mean thickness change of control choroids of CS eye-cups increased to 107 μm; however, it also increased the mean thickness change of paired choroids cultured with insulin to 84 μm (Table 1). Therefore, even if the comparison is restricted to the CS pairs having control choroids that thickened similarly to those choroids in RCS eye-cups, insulin did not significantly reduce choroidal thickening. It also suggest that changes in choroids in eyes of the same animals are more similar than eyes from different animals. By doing paired-eye comparison, it increases the sensitivity of the statistical test to the effect of insulin in the choroids.

Even though the baseline of the CS eye-cups were not as consistent as the RCS eye-cups, we always ran experiments with paired eyes of the same birds. With this paired-eye comparison, the relative thinning effect (or the effect of reducing choroidal thickening) of insulin related to RPE was consistently found in all three experiments. Since reduced choroidal thickening in CS eye-cups resulted from both transplanted RPE in the presence of insulin, and medium conditioned by the RPE in the presence of insulin, our findings suggest that insulin causes the RPE to produce secondary signaling molecules that inhibit choroidal thickening.

2.4.4 Insulin effect on scleral GAG synthesis

Our findings suggest that insulin affects scleral GAG synthesis by acting on the choroid. Insulin increased scleral GAG synthesis in eye-cups with choroid whether the RPE was present or not. In fact, we found that the effect of insulin was greatest when the choroid was present and the RPE was absent in the CS eye-cups. Furthermore, because eye-cups were put in the medium with insulin, one can argue that the sclerae of CS eye-cups were also exposed to insulin, and our system cannot exclude the direct effect of insulin on the sclera. However, without the RPE and choroid, we found that insulin cannot affect scleral GAG synthesis in scleral eye-cups; thus, insulin did not have any direct effect on the sclera. Therefore, our results suggest that insulin might cause the choroid to produce signaling molecules that increase scleral GAG synthesis.

2.4.5 Insulin might increase scleral GAG synthesis by acting on the choroid

Our findings suggest the same notion similar to earlier findings showing that the choroid can regulate scleral GAG synthesis (Marzani and Wallman, 1997). When choroids of eyes recovering from myopia caused by form-deprivation are co-cultured with scleral punches of normal eyes, scleral GAG synthesis decreases-whereas choroids of myopic eyes increase scleral GAG synthesis (Marzani and Wallman, 1997). Recent findings have shown that mRNA level of IGF-1 receptor increases in the choroid of elongating eyes (Penha et al., 2011). Since insulin can act on both insulin and IGF-1 receptors, and the concentrations of insulin we used were above

physiological range, it is possible that insulin might act on IGF-1 receptors on the choroid to enhance scleral GAG synthesis.

2.4.6 The role of the RPE in maintaining choroidal thickness

After 20 hours of incubation, choroidal changes are more consistent when the RPE is present, whether insulin is present or not. RPE is well known for its role in maintaining the physiology of the overlying retina, stabilizing ion compositions in the sub-retinal area and maintaining the health of retina and choroid by producing various growth factors [review (Strauss, 2005)]. The polarized distribution of ion transporters in the RPE provides transepithelial transport of nutrients and ions between photoreceptors and choriocapillaries [review (Strauss, 2005)]. Thus, the RPE might maintain choroidal thickness by controlling transepithelial transport of certain ions, such as Cl^- or by producing other growth factors that lead to stabilization of ion composition in the choroid. In fact, when chick eyes are recovering from form-deprivation, it was found that retina, RPE, and choroid contained more sodium and chloride ions, and the accumulation of these ions resulted in edema in these ocular layers (Liang et al., 2004).

2.4.7 Potential signaling molecules produced by the RPE to inhibit choroidal thickening

RPE can produce and secrete many growth factors, and VEGF might be one of the signaling molecules that is produced by the RPE in the presence of insulin to inhibit choroidal thickening. VEGF is well known for its role in vasculogenesis and

angiogenesis (Ferrara and Gerber, 2001). In studies of diabetic retinopathy, insulin can increase VEGF mRNA expression in the RPE (Lu et al., 1999). It would be interesting to study if VEGF is one of the signals produced by the RPE in the presence of insulin. It would also be interesting to study if VEGF can affect choroidal and scleral components in growth, which will be discussed in chapter 3.

IGF-1 and IGF-2 can possibly be the natural ligands in regulating eye growth. As mentioned before, insulin can bind to insulin receptor, IGF receptor and the insulin/IGF hybrid receptor [review: (Hernandez-Sanchez et al., 2006; Taniguchi et al., 2006)]. Similar to its effect on scleral GAG synthesis, the insulin concentrations that lead to a significant choroidal response in our findings are higher than the physiological concentration. Therefore, it is likely that insulin induces its effects on the choroid through the IGF receptors. Because of that, IGF might be the natural ligand for regulating the choroidal and scleral components in eye growth. In fact, recent findings have shown that lens treatment did not alter insulin mRNA level in the retina, RPE or choroids, but IGF-1 mRNA level was lower in the RPE of eyes treated with positive lenses compared with eyes treated with negative lenses (Penha et al., 2011). In addition, insulin and IGF-1 receptor mRNA levels were significantly lower in the RPE with positive lens-wear (Penha et al., 2011).

2.4.8 Why might insulin have different effects *in vivo* versus *in vitro?*

Since we found such a strong effect of insulin on the choroid in eye-cups made from normal eyes without lens treatment, it is curious that when insulin was injected into

normal chick eyes, it did not affect choroidal thickness but only increased ocular elongation (Zhu and Wallman, 2009a). One explanation is that choroids of live birds do not thicken over time as *in vitro*. In fact, when eyes wearing positive-lenses, which cause choroidal thickening, are injected with insulin, insulin does prevent choroids from thickening in those birds (Zhu and Wallman, 2009a). Since injected insulin can increase ocular growth and scleral GAG synthesis in normal eyes *in vivo* when the RPE is present (Feldkaemper et al., 2009; Zhu and Wallman, 2009a), it is also curious why the effect of insulin on scleral GAG synthesis is less when the RPE is present with the choroid in eye-cups. Furthermore, it is surprising that insulin does not affect isolated sclerae *in vitro*. This negative finding is unexpected as insulin family member, IGF-1, is known to increase proteoglycan synthesis (Bohme et al., 1992; Torres et al., 2003).

One possible explanation for the differences between *in vivo* and in vitro findings is that our *in vitro* findings are the effect of insulin relative to without insulin. In our *in vitro* conditions, the physiological environment of the tissues was not the same as they were *in vivo. In vivo*, other endogenous biological molecules may have synergistic or additive effects with insulin, causing greater effects on scleral GAG synthesis than we found under the *in vitro* conditions. Therefore, insulin might have an effect on sclera *in vivo* when other growth factor(s) is/are also present. It is also possible that the significant difference we found did not result from how the tissues respond to insulin specifically, but rather the tissues respond differently when insulin was absent. For example, perhaps the choroid should not be thickened *in vitro*, and

insulin reduced this choroidal thickening because the tissue culture medium was too deprived of nutrients or biological molecules. The presence of insulin changed the physiological condition closer to that *in vivo*. Therefore, insulin did not induce the changes, but rather, without insulin the tissues did not respond as they were *in vivo*. However, our findings are similar to the *in vivo* findings that *in vitro* as *in vivo*, insulin can reduce choroidal thickening and increase scleral GAG synthesis (Zhu and Wallman, 2009a). While we retain the possibility of differences in the *in vivo* and *in vitro* conditions might contribute to some of the effect we found, the possibility that all the significant effects we found for insulin is solely due to the abnormal condition in our system would be rather unlikely.

2.5 Conclusion

Our findings suggest that insulin causes the RPE to produce secondary signals that reduce the natural choroidal thickening in eye-cups, and that insulin acts on the choroid to increase scleral GAG synthesis.

Chapter 3 *In vitro* studies of VEGF

Abstract

Purpose: VEGF causes vascularization of the retina and choroids in mammals, among other effects [review: (Ferrara 2009)]. Its mRNA expression in the RPE increases when eye growth is enhanced. Insulin or IGF-1 can induce VEGF mRNA expression in the RPE (Lu et al. 1999; Weng et al. 2009), and our findings suggest that insulin causes the RPE to produce secondary signals that thin the choroid *in vitro* (Sheng at al., ARVO abstract 2008, 2009). VEGF may therefore be one of the signals that causes choroidal thinning. We studied whether VEGF165 (V165), a major isoform of VEGF, can affect ocular parameters *in vivo*, and if it can affect choroidal thickness and scleral GAG synthesis *in vitro*.

Methods: Experiment 1. One-week-old chicks were injected with 1 µL of 100 µg/mL of V165 in one eye intravitreally, the contralateral eye was injected with 1 µL of PBS. Intravitreal injection was done every day for three days. Ocular parameters were measured before injection for the first three days and the fourth day without injection. Experiment 2. Eyes from 1-week-old chicks were hemisected with retinas removed to make eye-cups with either RPE/choroid/sclera (RCS eye-cups), choroid/sclera (CS eye-cups) or just sclera (S eye-cups) in pairs from individual birds. One eye-cup of each pair was cultured with V165 (1,10 or 100 ng/mL) in L-15 medium, and the fellow eye-cup was cultured in L-15 alone. Choroidal thickness of some eye-cups cultured with 100 ng/mL was measured before and after 1, 2, and 20 hours of incubation. The rest were measured after 1 hour and 20 hours of incubation. Scleral GAG synthesis was measured after 20 hours or 44 hours of incubation.

Results: Experiment 1. Injected V165 at 666 ng/mL did not affect the change in choroidal thickness, ocular length or any other measured ocular parameters over the three days period. Experiment 2. In CS, but not RCS, eye-cups, one hour of incubation with V165 significantly reduced the natural choroidal expansion seen *in vitro* (i.e., caused choroids to be thinner than those of control eyes) at 10 and 100 ng/mL (ΔX-ΔN [mean±SEM]: both -43 µm), but not at 1ng/mL. This thinning effect at 100 ng/mL was not sustained after 2 and 20 hours of incubation. The effect of V165 on CS and RCS eye-cups was not significantly different (two-way ANOVA). Furthermore, after 44 hours (but not after 20 hours), V165 significantly increased scleral GAG synthesis 1.4-fold in CS eye-cups at 100 ng/mL but not in RCS eye-cups or S eye-cups at any concentrations.

Conclusions: The direct transient thinning effect of VEGF on the choroid suggests that VEGF may be one of the signals produced and released by the RPE in the presence of insulin to initiate choroidal thinning. The weak effect of VEGF in eye-cups containing the RPE might be due to the RPE acting as a barrier or interfering with the effect of exogeneous VEGF. The stimulating effect of 100 ng/mL of VEGF on scleral GAG synthesis in CS eye-cups but not in RCS or scleral eye-cups suggest that VEGF increase scleral GAG synthesis by acting on the choroid.

3.1 Introduction:

As mentioned earlier in the general introduction and the insulin chapters, emmetropization that occurs in most vertebrates postnatally has the function of matching the position of the photoreceptor of the retina with the focal plane of the eye so that images of distant objects are focused on the photoreceptor. In myopia, the eye grows longer than its focal length, and thus the image of a distant object falls in front of the retina. In hyperopia, the eye is shorter than the focal length, and the image of a distant object falls behind the retina. Studies done over decades showed that visual conditions can affect eye growth. A positive lens, which puts the image in front of the photoreceptors creating a myopic defocus, inhibits ocular elongation and increases choroidal thickness (Wallman et al., 1995). A negative lens, which puts the image behind the photoreceptors, creating a hyperopic defocus, increases ocular elongation and decreases choroidal thickness. Because of these changes, a clear image is again focused on the photoreceptor layer. In the conditions mentioned above, choroidal thickness always changes in opposite direction from the rate of ocular elongation. Whenever choroidal thickness increases, the rate of ocular elongation decreases and vice versa. How the choroid changes its thickness in ocular growth is still unknown. In avian choroids, there are spaces called lacunae, which are part of the drainage of the lymphatic system. These lacunae were found expanded when choroid thickened in visual conditions suppressing eye-growth and shrunk when choroid thinned in visual condition enhancing eye-growth (De Stefano and Mugnaini, 1997; Junghans et al., 1999). Therefore, it has been suggested that the choroid changes its thickness through changing the volume/size of the lacunae (Wallman, 1995).

Changes in ocular elongation are associated with changes in scleral extracellular matrix (ECM), which can be indicated by the rate of synthesis of glycosaminoglycan (GAG) chain of proteoglycan, a major component in the scleral ECM. Because these GAG chains are sulfated, scleral proteoglycan synthesis, or more precisely, scleral GAG synthesis, can be studied by measuring radiolabeled sulphate incorporation in the sclera. Findings in chicks have shown that enhancement of ocular elongation (caused by wearing diffusers or negative lenses) and its inhibition (caused by removal of diffusers or wearing positive lenses) have been associated with increased (Nickla et al., 1997; Rada and Matthews, 1994; Rada et al., 1992; Rada et al., 1991) and decreased (Nickla et al., 1997; Rada et al., 1992) scleral GAG synthesis, respectively.

A signal cascade is thought to be present in regulating eye growth (Wallman and Winawer, 2004) in which retina can produce signaling molecules that act on the RPE; and the RPE can produce other signaling molecules that act on choroid and/or sclera. In the insulin chapter, we suggested that insulin causes the RPE to produce secondary signaling molecules that inhibit the natural choroidal expansion *in vitro*; and insulin acts on the choroid to increase scleral GAG synthesis (Chapter 2 of this thesis). These *in vitro* findings are consistent with recent *In vivo* findings that insulin can reduce choroidal expansion (Zhu and Wallman, 2009a) and increase rate of ocular elongation (Feldkaemper et al., 2009; Zhu and Wallman, 2009a) as well as scleral GAG synthesis (Zhu and Wallman, 2009a). Previous studies have also

shown that insulin (Lu et al., 1999) or IGF-1 (Lu et al., 1999; Seigel et al., 2006; Weng et al., 2009) can cause the RPE to produce vascular endothelial growth factor (VEGF), which affects the vasculature of the choroid. It is not known if VEGF can affect choroidal thickness or enhance ocular growth like the effect of insulin. Therefore, in this chapter, we studied if VEGF can affect any ocular parameters *in vivo* as well as the choroidal thickness and scleral GAG synthesis in eye-cups.

VEGF is a member of a big family, consisting of placenta growth factor (PIGF), VEGF-A (also denoted as VEGF, since it was the first molecule found in the family), VEGF-B, VEGF-C and VEGF-D and the parapox-virus-encoded VEGF-E (Ferrara, 2009). VEGF-A is the most studied one in the family, and it is well known for its roles in vasculogenesis and angiogenesis. Since the discovery of VEGF-A inhibition suppressing tumor growth (Kim et al., 1993), many drugs targeting to inhibit VEGF-A have been developed for cancer treatment (Ferrara, 2010b). VEGF-A is also indicated in the development of nervous system in vertebrate (Eichmann and Simons, 2012; Thomas and Eichmann, 2013), and its role in the nervous system is still emerging. There are at least 8 isoforms of VEGF-A found in human, produced by alternate splicing of exons. Among them, VEGF-165 is the most abundantly found isoform [review: (Ferrara, 2010a)]. Another VEGF family member, VEGF-B was suggested to regulate VEGF-A by binding to neurophilin-1. Recently, VEGF-B was suggested to be a neuron survival factor as it suppresses apoptosis and pathological vascular angiogenesis (Li et al., 2009). VEGF-C and VEGF-D have been found to

induce lymphangiogenesis [Review: (Alitalo and Detmar, 2012; Makinen et al., 2001)], and their roles in development and treatment of cancer are still growing.

VEGF receptors are receptor tyrosine kinases, known as VEGF receptor-1, -2, and -3 (VEGF-R1, -R2 and -R3). There are also co-receptors that lack VEGF-induced catalytic function, such as heparin sulphate proteoglycans and neuropillins (Olsson et al., 2006). Even though VEGF receptors are structurally highly related, they display functional differences [review:(Olsson et al., 2006) (Shibuya and Claesson-Welsh, 2006)]. Specifically, VEGFR1, also known as fms-like tyrosine kinase 1 (Flt-1), can bind PlGF, VEGF-A and VEGF-B. VEGF-A can also bind to neuropilin-1 and neuropillin-2, which act as co-receptors for VEGF-A. VEGF-R2, also known as KDR in human or Flk-1 in mouse, can bind VEGF-A and proteolytically processed VEGF-C and VEGF-D [review: (Koch et al., 2011)]. Activation of VEGFR1 and VEGFR2 leads to induction of angiogenesis and vasculogenesis, and it can also lead to regulation of vascular permeability, macrophage functions and placenta functions [review: (Shibuya, 2006)]. VEGFR3, also known as Flk-4, can bind VEGF-C and VEGF-D. Activation of VEGF-R3 leads to embryonic angiogenesis and lymphangiogenesis (Alitalo and Detmar, 2012).

In the eye, VEGF is responsible for the vascularization and angiogenesis of retinal vessels and choroids in embryogenesis as well as in the neovascularization in several ocular diseases (Ferrara et al., 2007; Miller et al., 2013). It has been shown that VEGF-R2 is present in mouse retinal progenitor cells and therefore, VEGF can

also be responsible for neurogenesis as well as vasculogenesis of the retina (Yang and Cepko, 1996). In the conventional view of VEGF production in the eye, VEGF is produced in the RPE under hypoxia condition via HIF-dependent transcriptional activation (Blaauwgeers et al., 1999; Safran and Kaelin, 2003). VEGF is then produced from the basolateral side of the RPE to act on the choroids and that sequentially leads to choroidal neovascularization (Miller et al., 2013; Schlingemann, 2004). Drugs like Avastin, VEGF trap, Ranibuzumab have been developed and studied for treatment of various kinds of ocular diseases [review: (Kaiser, 2006)]

Recently, it has shown that VEGF is involved in the choroidal development and maintenance (Saint-Geniez et al., 2009; Saint-Geniez et al., 2006). VEGF 165, one of the major isoforms of VEGF-A, is found in the RPE of the adult mice (Saint-Geniez et al., 2006). VEGF-A, VEGF-C and VEGF-D are also found in human RPE (Ikeda et al., 2006). Their common receptors, VEGF receptor-2 is found in mice (Saint-Geniez et al., 2006), bovine (Saint-Geniez et al., 2006) and human choroid (Blaauwgeers et al., 1999); whereas, receptor of VEGF-C and VEGF-D, VEGF-R3, was found in human choroid (Blaauwgeers et al., 1999). A preliminary studies have very recently shown that VEGF-A, VEGF-C and VEGF-D and their receptors VEGF-R2 and VEGF-R3 are present in the avascular retina, RPE and choroid in chicks (Feldkaemper et al., 2013).

The effect of VEGF on the choroidal and scleral components in eye growth has never been studied. VEGF-A and VEGF-C can increase the permeability of blood

vessels (Murohara et al., 1998; Witzenbichler et al., 1998) that might allow plasma to leave the choroid. The possible subsequent decline in the oncotic pressures might decrease fluid transfer to the choroid and ultimately reduce choroidal thickness.

In this chapter, we will discuss the effect of VEGF 165 (V165), the most abundant isoform of VEGF-A, in the choroidal and scleral components in eye-cups. Some of the findings of this study were published in an abstract (Sheng et al., 2012).

3.2 Methods

The following sections before statistics (section 3.2.5) are similar to those in the general methods and insulin chapters.

3.2.1 Animals

White Leghorn chicks were hatched in our laboratory from eggs supplied from Cornell University (Cornell K-strain; Ithaca, NY). The chicks were raised and housed in heated brooders on a 14:10 light-dark cycle. One-week-old chicks were used in all experiments. The number of birds used in each experiment was stated on the figures in the result section. Care and use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

3.2.2 Eye-cup dissection and tissue culture

Chicks were sacrificed by intracardiac injection of pentobarbital sodium (Beuthanasi-D Special; Schering-Plough Animal Health Corp) and were kept postmortem at room temperature until eyes were hemisected along the equatorial axis perpendicular to

the optical axis of the eye with vitreous, retina, and pecten removed. To make RCS eye-cups (eye-cups consisting of RPE/Choroid/sclera): eyes were enucleated from birds 10 minutes postmortem, as the RPE was found to be attached to the choroid more than to the retina after this interval. Retina was removed and separated from the RPE. Any pairs of eye-cups with one of the pairs possessing less than 80% of RPE left on the choroid were discarded. To make CS (eye-cups consisting of choroid/sclera) or scleral eye-cups: eyes were enucleated after 20-30 minutes postmortem. After this interval, the RPE was more attached to the retina than to the choroid. In CS eye-cups, retina and RPE were removed together from the choroid. Any RPE left on the choroid, usually on the periphery, was brushed off. Any pairs of eye-cups with one of the pairs possessing more than 10% of RPE on the choroid were discarded. To make scleral eye-cups retina with the RPE were removed together, and choroid was then brushed off from the sclera. Each eye-cup was put into a well of a 24-well tissue culture plate on ice, and each well was filled with 3mL CO2-independent medium (Gibco; Invitrogen), which maintained the physiological pH of the eye-cups during dissection and first measurement of choroidal thickness before incubation. After the first measurement of choroidal thickness, the medium was changed to L-15 medium (Millipore). The plates were incubated at 37^{o}C with 5% CO_2.

3.2.3 Choroidal thickness measurement

Choroidal thickness was measured by A-scan ultrasound. To make alignment easier and more precise, each eye-cup prepared was put on top of a nylon nut of 10.8mm

width and 8.4mm height (Small Parts Inc: HNN-1420), with the scleral side pointing down and facing the nut. The posterior pole of the eye-cup was centered with the center of the nut and aligned with the ultrasound transducer. Choroidal thickness was measured by high frequency A-scan ultrasound with a 30 MHz polymer transducer (Panametrics Model 176599) linked to a computer interface with a Sonix 8100 A/D board, which allowed sampling of the output signal at 100 MHz (Nickla, 1996). The velocity of sound used to covert time to distances was 1534 meters per second for choroid (Wallman and Adams, 1987). A cone of plexiglass, which was filled with distilled water and sealed with a wrapping film (Parafilm) was attached to the transducer. The transducer was mounted on a micromanipulator and was aligned with each eye-cup in the well by moving the transducer in three axes. After the first five tracings were taken and saved, the transducer was moved up, and the eye-cup was re-centered before another five tracings were taken.

3.2.4 Scleral GAG synthesis

After incubation, scleral punches of 7mm in the posterior eye-cups were cultured in L-15 medium containing $Na_2{}^{35}SO_4$ for 2 hours. The scleral punches were then digested with proteinase K (protease type XXVIII, Sigma, in 10mM EDTA, 0.1M sodium phosphate, pH 6.5) overnight at 57^{o} C. Newly synthesized labeled GAGs were precipitated by adding chondroitin sulphate (Sigma; 1mg/ml in distilled water) and 0.5% of cetylpyridinium chloride (CPC) at 37^{o}C. Precipitated GAGs were filtered with Whatman filters (GF/A; Fischer scientific), which were then washed repeatedly and dried overnight at room temperature. Radioactivity in the filters was measured

by liquid scintillation counter in 20mL of scintillation fluid (Ultima Gold F, Perkin Elmer).

3.2.5 Statistics

All data are means (±SEM) unless otherwise specified. Data were analyzed in four ways. (1) Choroidal change between the eyes/eye-cups treated with V165 and their paired controls was compared by repeated measures ANOVA over the studied period. (2) At each time point, change between the paired eyes/eye-cups was compared by two-tailed paired student's *t* test. (3) Data were analyzed by mixed factorial ANOVA in which presence of the RPE and concentrations of VEGF were the between-group variable, and paired eye from the same birds was the within-group variable; this was followed by a Holm – Bonferroni post hoc test. (4) We increased the sensitivity of statistical tests by comparing relative changes in choroidal thickness (the difference of changes in thickness between paired eyes (ΔX-ΔN)) at different concentrations of V165 using one-way analysis of variance (ANOVA) with LSD post hoc test. The ratio of scleral GAG synthesis was calculated by dividing the values of radioactivity from the experimental eye-cups by the values from the paired control eye-cups. To avoid the asymmetry of averaging ratios (e.g., a ratio of 5 having more weight than the inverse ratio of 0.2 and making one treatment appear more effective than another), each ratio was taken to logarithm of base 10 and the mean of the calculated ratio (mean log ratio) for each concentration of V165 was converted back by taking its anti-logarithm. Effect of V165 on the scleral GAG synthesis was analyzed by comparing the logarithm of the count from the

experimental eye-cups with that of the paired control eye-cups by paired Student *t*-test. One-way or two-way ANOVA was performed and was followed by LSD post hoc test.

3.2.6 Experimental design

3.2.6.1 Experiment 1: Effect of injected V165 on choroidal thickness and ocular elongation

To study if injected V165, a major isoform of VEGF, can affect choroidal thickness and ocular elongation *in vivo*, paired eyes of individual animals were invitreally injected with 1 µL of recombinant human V165 (Peprotech) (at 100 µg/mL) versus 1 µL PBS (n=5), once a day for three days. Assuming the volume of the vitreous to be 150µL, the concentration of V165 used was 666 ng/mL in the vitreous. Ocular parameters were measured by ultrasonography before injection every day for the first three days and on the fourth day.

3.2.6.2 Experiment 2: Dose response of V165 on choroidal thickness and scleral GAG synthesis in eye-cups.

To study if V165 can directly affect the choroidal thickness and/or scleral GAG synthesis in eye-cups, eye-cups with RPE/choroid/sclera (RCS eye-cups), with choroids and scleras (CS eye-cups) and scleral eye-cups were made in pairs from individual birds. One eye-cup of the pairs was cultured in L-15 medium with V165 at various concentrations (1ng/mL, 10 ng/mL and 100 ng/mL) and the fellow eye-cups of the pairs were cultured in L-15 medium without V165. The concentrations we

used to study the effect of V165 were based on the optimal concentration of VEGF for its angiogenic effect found *in vitro* (Pepper et al., 1992).

Choroidal thickness of RCS eye-cups and CS eye-cups cultured with 100ng/mL of VEGF was measured before and after 1, 2, and 20 hours of incubation. Choroids of eye-cups cultured with 1 and 10 ng/mL of VEGF were measured after 1 hour of incubation. Our preliminary findings showed that V165 did not affect scleral GAG synthesis after 20 hours of incubation (data not shown). Therefore, in some of the eye-cups (RCS, CS and scleral), medium was renewed after 20 hours and eye-cups were cultured for another 24 hours before scleral GAG synthesis was studied at the end.

3.3 Results:

3.3.1 Experiment 1: Effect of injected 666ng/mL of V165 on choroidal thickness and ocular length *in vivo*

Injected V165 at 666 ng/mL had no apparent effect on any axial parameters of any ocular structures. There was no significant difference in the changes of choroidal thickness or the ocular length between the eyes injected with V165 (X) and the contralateral control eyes injected with PBS (N).

Over a four day period, mean change in choroidal thickness of eyes injected with V165 vs. the contralateral control eye injected with PBS (ΔX vs. ΔN) were: after 1 day: 32 μm vs.16 μm; after 2 days, 13 μm vs. 40 μm; after 3 days, 9 μm vs. 38 μm (p>

0.05, two-tailed paired Student *t*- test, Fig 3.1A). The mean change in ocular length (ΔX vs. ΔN) after 1 day, 2 days and 3 days were: 71 μm vs. 67 μm; 100 μm vs. 147 μm; 199 μm vs. 202 μm; p> 0.05, two-tailed paired Student *t*- test, Fig 3.1B). There were no significant differences in the anterior chamber depth, lens thickness or vitreous chamber depth, either (data not shown).

3.3.2 Experiment 2: Dose responses of V165 on choroidal thickness and scleral GAG synthesis in eye-cups

Without the RPE, V165 thinned choroid in eye-cups transiently and increased scleral GAG synthesis after 44 hours of incubation. Choroids of both RCS eye-cups and CS eye-cups without V165 expanded over time. When change in choroidal thickness was analyzed by repeated measure ANOVA, time did not cause any significant difference in the change of choroids in RCS or CS eye-cups. However, when the changes of the choroids between the paired eyes were analyzed by paired t-test, 100 ng/mL of V165 reduced this natural expansion, in other words, caused relatively thinner choroids in CS eye-cups compared with the paired eye-cups cultured without V165 after 1 hour of incubation (Fig 3.2B). The mean change in choroidal thickness in eye-cups cultured with 100 ng/mL of V165 (ΔX) versus without V165 (ΔN) were: -3 vs. 40 μm, p<0.05; relative difference between the paired eye-cups, ΔX - ΔN: -43 μm, paired 2-tailed Student *t*- test; Fig 3.2B). But the effect was not significant after 2 (ΔX vs. ΔN: 25 μm vs. 45 μm; ΔX - ΔN, -20 μm) or 20 hours of incubation (ΔX vs. ΔN: 69 μm vs. 82 μm, ΔX - ΔN, -13 μm, Fig 3.2B).

In the presence of the RPE, 100ng/mL of V165 did not cause any significant difference in the choroidal thickness in RCS eye-cups (ΔX vs. ΔN at 1 hour, 2 hours and 20 hours were: -4 μm vs. 17 μm; 34 μm vs. 29 μm; 88 μm vs. 86 μm, Fig 3.2A). The relative difference of the change between the paired eye-cups, ΔX - ΔN at 1 hour, 2 hours and 20 hours were: -22 μm, 5 μm, 2 μm, Fig 3.2A).

Dose-responses of V165 in CS eye-cups and RCS eye-cups on the choroid were further studied after 1 hour of incubation (Fig 3.3). V165 significantly thinned choroid in CS eye-cups at 10 and 100 ng/mL (Δ X vs. Δ N at 10 and 100 ng/mL (mean): -15 vs. 28 μ m, -3 vs. 40 μ m; relative difference (Δ X – Δ N) at 10 and 100 ng/mL: -43 μ m at both concentrations, Fig 3.3B), but not at 1ng/mL (Δ X vs. Δ N; 7 vs. 23 μ m; Δ X – Δ N, -16). However, ANOVA and mixed factorial ANOVA showed that the effect of V165 on the choroidal thickness was not significantly affected by different concentrations. With the presence of the RPE in RCS eye-cups, V165 at 1, 10, or 100 ng/mL did not cause any significant effect on the choroids after an hour of incubation (mean change in choroidal thickness, Δ X vs. Δ N at 1, 10 and 100 ng/mL: 22 μ m vs. 13 μ m, 12 μ m vs. 26 μ m, -5 μ m vs. 17 μ m). However, the dose response of V165 on the choroids of RCS eye-cups was similar to the dose response of V165 in CS eye-cups (relative difference between paired RCS eye-cups (Δ X – Δ N) at 1, 10 and 100 ng/mL: 9 μ m, -14 μ m, -22 μ m, Fig 3.3A).

The presence of the RPE had a significant effect on the scleral GAG synthesis in eye-cups ($p<0.05$, mixed factorial ANOVA). Without the RPE, V165 increased

scleral GAG synthesis in CS eye-cups, but not in scleral eye-cups or RCS eye-cups. After 44 hours of incubation, V165 increased scleral GAG synthesis 1.4 fold in CS eye-cups at 100 ng/mL (Fig 3.4) ($p<0.05$, paired 2-tailed Student *t*- test). The dose-responses of V165 at different concentrations in CS eye-cups were not significantly different from each other. After 20 hours of incubation, V165 did not affect scleral GAG synthesis in S eye-cups, RCS eye-cups or CS eye-cups as mentioned before (data not shown).

3.4 Discussion

Our findings indicate that without the RPE, V165 can directly thin the choroids transiently and increase scleral GAG synthesis in CS eye-cups. We will discuss the effect of VEGF on the choroid and scleral ECM in CS eye-cups first, before we discuss its lack of effect when the RPE is present *in vivo* and in RCS eye-cups.

3.4.1 V165 reduces choroidal thickening transiently in CS eye-cups

V165 can transiently thin choroids significantly in CS eye-cups only after 1 hour of incubation. Therefore, VEGF might be one of the first short-term signals that initiate choroidal thinning in negative-lens compensation. In fact, at 100 ng/mL, the transient thinning in the choroids caused by VEGF in eye-cups was accompanied by the corresponding increase in scleral GAG synthesis in the CS eye-cups, which will be discussed later in detail. The presence of VEGF receptors on the choroid have been shown in mice and human (Saint-Geniez and D'Amore, 2004; Saint-Geniez et al., 2009), and very recently in chicks as well (Feldkaemper et al., 2013). Therefore, it is

possible that VEGF produced by the RPE acts on the choroid to initiate choroidal thinning.

3.4.2 V165 might increase scleral GAG synthesis by acting on the choroid

Our findings suggest that V165 might increase scleral GAG synthesis indirectly by acting on the choroids. 100 ng/mL of V165 increases scleral GAG synthesis in CS eye-cups after 44 hours of incubation but not after 20 hours of incubation. Since V165 does not directly increase scleral GAG synthesis in scleral eye-cups, and it takes 44 hours for V165 to increase scleral GAG synthesis, we suggest that V165 causes the choroid to produce secondary signals that increase scleral GAG synthesis. This finding suggests the same rationale of the recent findings that choroid is involved in regulating eye-growth as mentioned in the general introduction chapter (1.3.6.1). Furthermore, recent findings have also shown that when VEGF expression in the RPE is specifically knocked out in VEGF $^{rpe-/-}$ mice, it results in absence of choroids (Marneros et al., 2005). On top of that, eyes of the treated mice were strikingly small compared with the control mice. These findings suggest that VEGF from the RPE might initiate ocular elongation by acting on the choroid.

3.4.3 Does V165 affect choroids in RCS eye-cups?

Our findings in RCS eye-cups is a bit ambiguous as VEGF did not affect choroidal thickness significantly when the RPE was left on the choroid, but its trend of thinning is similar to CS eye-cups with RPE removed, whether it was in the time-course study (Fig 3.2) or in the dose-response (Fig 3.3). One possibility is that RPE either acts as

a mechanical barrier that prevents exogenous VEGF in the tissue culture medium, or possibly *in vivo*, RPE prevents VEGF in the vitreous from reaching the choroid. It might also be possible that RPE in the eye-cups interferes with the effect of VEGF on the choroid and sclera. In the eye, VEGF is responsible for the vascularization and angiogenesis as well as neovascularization of retina and choroid in several ocular diseases (Ferrara et al., 2007) as mentioned before. To prevent neovascularization in the retina and choroid under normal conditions, the RPE, in addition to being a mechanical barrier to VEGF, might be actively involved in lowering the concentration of VEGF in the subretinal space. In fact, when cultured RPE was mounted in a chamber where medium bathing the apical side of the RPE (facing the subretinal space *in vivo*) was separated from medium bathing the basal side (facing the choroid) of it, a significant fraction of VEGF added to the apical side was endocytosed and degraded (Peng et al., 2010). This finding implicates the possibility that RPE might have a mechanism that actively limits the amount of VEGF in the subretinal space to prevent retinal neovascularization and diffusion of VEGF to the choroid. It has been found that other ocular tissues like cornea, in order to maintain its avasculature, can secrete soluble VEGFR-1 to prevent VEGF from building new blood vessels (Ambati et al., 2006). Thus, the presence of the RPE in RCS eye-cups and *in vivo* might limit VEGF action with the choroid and weaken its effect on choroidal thickness and scleral extracellular matrix. Even though the RPE might interfere with exogeneous VEGF in the tissue culture medium or in the vitreous *in vivo*, in normal physiological conditions, endogeneous VEGF produced by the RPE (Saint-Geniez et al., 2006) can diffuse from the basal side of the RPE and

act on the choroid. In our *in vitro* studies, small amount of VEGF might still be able to pass through the RPE via the edge of the eye-cup (where it was hemisected) and affect the choroid. Therefore, even though the effect is weak to detect, the time-course or the dose-response of V165 of the RCS eye-cups still shares similar pattern to CS eye-cups. Whether RPE can interfere with VEGF or not, different from insulin, VEGF can thin the choroid directly, even though the effect is transient.

3.4.4 Differences in *in vivo* and *in vitro* findings

Our *in vitro* findings have shown that VEGF thins choroid transiently and increases scleral GAG synthesis, but the corresponding choroidal and ocular elongation cannot be found or demonstrated by our *in vivo* method. Our *in vitro* findings show that the effect of V165 on the choroid is transient and is not sustained after the first hour of incubation. Since we only measured ocular parameters in our *in vivo* experiment once a day, at least 24 hours after the previous injection, and we only tested one concentration, our negative findings in the *in vivo* experiment might be due to the possibility that we did not use the right dose and measure at the right time point to see the transient effect. It would be rather difficult, however, to estimate the right time point to detect any transient changes in choroid after each injection. VEGF can reach its target site much slower *in vivo* than *in vitro*, and part of it might be metabolized before it reaches the choroid. It would also be rather difficult to detect any transient subtle changes *in vivo* as the eye would have just been insulted by injection shortly before. Even though we tried to maximize the amount of V165 that can reach its target site on the choroid by using a concentration which was over six

times higher than the highest concentration we used in eye-cups (after taking the volume of vitreous, the concentration we used was 666 ng/mL), we did not find any effect in either the choroid or the ocular length. As discussed before, RPE can also interfere with the effect of exogeneous VEGF. Therefore, we did not pursue to further study the dose-response of VEGF on the choroid and ocular elongation *in vivo*.

3.4.5 V165 is possibly a stimulating agent in eye growth

Our findings are consistent with previous notion that VEGF is involved in enhancing eye-growth. When ocular growth is enhanced, RPE cells expand which was attributable to the passive stretch in ocular growth (Lin et al., 1993). Previous *in vitro* studies showed that when RPE was exposed to mechanical stress, VEGF and its mRNA expression increased (Seko et al., 1999). Furthermore, a preliminary study has shown that VEGF mRNA expression in the chick RPE can be upregulated after 48 hours and 2 weeks of minus lens-wear (Nguyen and Wildsoet, 2009). Since we found that VEGF has a transient effect on choroidal thinning and it enhances scleral GAG synthesis through the choroid after 44 hours of incubation, our findings suggest that VEGF can be one of the signals in initiating choroidal thinning and a long- term signal in enhancing ocular elongation.

3.5 Conclusion

VEGF can directly thin the choroid transiently and enhance scleral GAG synthesis in CS eye-cups with choroid/sclera but without the RPE. When the RPE is present,

whether it is *in vivo* or *in vitro*, VEGF does not affect choroidal thickness, ocular elongation or scleral GAG synthesis. RPE might have some mechanisms to interfere with the effect of exogeneous VEGF in the tissue culture medium or in the vitreous *in vivo*. Our findings are consistent with previous findings that VEGF is involved in enhancing ocular growth.

Chapter 4 General discussion

4.1 Signal cascade in eye growth

Our findings on the role of insulin and VEGF suggest that a signal cascade is present in the posterior part of the eye. The different effects of insulin and VEGF on the choroid and scleral GAG synthesis on different kinds of eye-cups (RCS, CS and scleral eye-cups) suggest that RPE and choroid can mediate the effect of these molecules in eye growth. We will first discuss the role of the RPE, before we discuss the role of the choroid in regulating eye growth.

4.1.1 Role of RPE in eye growth

Our findings suggest that RPE can produce secondary signaling molecules to affect choroidal thickness and scleral GAG synthesis; and it is also possible that RPE can actively prevent molecules from acting on the choroid and sclera.

RPE has been suggested to facilitate choroidal thickening by transepithelial transport of Cl^- from the subretinal space to the choroid (Rymer and Wildsoet, 2005). The passive flow of fluid acts as a source to fill in and expand the choroidal lacunae, and this may occur in positive-lens compensation or in eyes recovering from form-deprivation (Crewther et al., 2008; Rymer and Wildsoet, 2005; Zhang et al., 2011). Our *in vitro* findings, however, have shown that with or without insulin, transplanted RPE removed from donor eyes, where the RPE is no longer attached to the choroids, can still cause choroidal thickening in CS eye-cups similar to RCS eye-cups (in

which RPE is naturally attached to the choroid). Therefore, at least *in vitro*, our findings have suggested that it would be less likely choroidal thickening or its inhibition is solely regulated by the transepithelial ion transport across the RPE. One of the major functions of RPE is to produce growth factors to maintain the health of retina and choroids [review (Strauss, 2005)]. Because we found that reduced choroidal thickening in CS eye-cups resulted from both transplanted RPE in the presence of insulin, and medium conditioned by the RPE in the presence of insulin. Therefore, we suggest that insulin causes the RPE to produce secondary signaling molecules that inhibit choroidal thickening. At 37 μM of insulin, it is interesting to see that insulin can increase scleral GAG synthesis if only the RPE is present. When the RPE is removed, in CS eye-cups, insulin has no effect which is very different from its strong stimulating effect at 10 (3.7 μM) to a 100 (0.37 μM) times lower concentrations in the CS eye-cups. Perhaps at 37 μM, insulin concentration is so high that it activates other growth factors on the RPE that stimulate scleral GAG synthesis. Without the RPE but with the choroid, 37 μM insulin might cause the choroid to produce other growth factors that counter the stimulating effect induced by insulin.

Furthermore, we found that VEGF can thin choroids transiently only in CS eye-cups without the RPE, but not in RCS eye-cups or when VEGF is intravitreally injected *in vivo* (even though we only tried one dose). Therefore, as discussed in the VEGF chapter, we suggest the possibility that RPE might actively lower the concentration

of VEGF in the tissue culture medium and in the vitreous *in vivo* and ultimately prevent VEGF from reaching the choroids/sclera.

4.1.2 Role of choroid in eye growth

Our findings are consistent with the notion that choroids can regulate ocular growth. As previously mentioned (general introduction), when choroids from stop elongating eyes are co-cultured with scleral punches from normal eyes, scleral GAG synthesis of the scleral punches decreases; whereas choroids from elongating eyes increase scleral GAG of normal scleral punches (Marzani and Wallman, 1997). It indicates that choroids from elongating or stop elongating eyes can produce signaling molecules that stimulate or inhibit scleral GAG synthesis respectively. Since both insulin and VEGF can stimulate scleral GAG synthesis in CS eye-cups but they have no direct stimulating effect on scleral eye-cups, we suggest that VEGF causes the choroid to produce other molecules that increase scleral GAG synthesis.

4.1.3.Different pathways in regulating choroidal and scleral components

In the general introduction chapter, we have mentioned that the choroidal and the scleral components of stimulating eye growth were thought to be regulated by two different pathways rather than the same pathway (section 1.3.1).

Based on our findings, we suggest that there can be different pathways regulating the choroidal and scleral components, at least, when eye growth is enhanced *in vitro*. Our findings have suggested that insulin acts on the RPE to produce secondary

molecules that thin the choroids, and both insulin and VEGF act on the choroids to increase scleral GAG synthesis. Therefore, the pathways regulating the choroids and sclerae can be different. A recent unpublished study has also shown that several muscarinic agonists can thin the choroid when they are injected into the eyes *in vivo* or when they are used to culture eye-cup *in vitro*, but they do not affect ocular elongation (Nickla et al. 2013). These findings suggest that when ocular growth is enhanced, the pathway that thins the choroid is different from the one that increases ocular elongation.

4.2 The linkage between insulin and VEGF

As mentioned in the chapters on insulin and VEGF, previous studies have shown that insulin (Lu et al., 1999) or IGF-1 (Lu et al., 1999; Seigel et al., 2006; Weng et al., 2009) can cause the RPE to produce VEGF which affects the vasculature of the choroid. We found that VEGF reduces choroidal thickening within an hour of incubation, it is possible that VEGF is one of the initial signaling molecules produced by the RPE in the presence of insulin. In mice and very recently in chicks (Feldkaemper et al., 2013), VEGF receptor 2 (VEGF-R2), which binds VEGF, has been found in the choroids. It is possible that insulin can cause the RPE to produce VEGF that acts on its receptor to initiate choroidal thinning in CS eye-cups.

We tried to study this relationship by culturing paired CS eye-cups with antibodies that block VEGF-R2 (anti-VEGFR2 antibodies) versus the isotype control antibodies. We then cultured these treated CS eye-cups with the medium conditioned by the

RPE in the presence of insulin as in Experiment 3 in the insulin chapter. Our hypothesis is that if insulin can cause the RPE to produce VEGF in the conditioned medium, when paired CS eye-cups are both cultured with this conditioned medium, the CS eye-cups that are previously treated with anti-VEGFR2 antibodies would have thicker choroids than their paired eye-cups that are treated with the isotype control. Indeed, that is what we found (see Appendix I for detailed experimental implementation), and the effect was transient (Fig a in Appendix I).

One possible explanation for the transient effect is that VEGF can start binding to receptors other than VEGF-R2 on the choroid. In fact, VEGF can bind to VEGF-R1, VEGF-R2, neuropillin -1 and neuropillin-2. Another possible explanation is that, rather than a long term signal for choroidal thinning, VEGF is just one of the initial signals in reducing choroidal thickening as indicated by its transient effect in thinning the choroids. Because we did not knock out VEGF completely, we cannot exclude either possibility. Future studies can be done on culturing insulin-RPE conditioned medium with Bevacizumab or VEGF trap, which can neutralize VEGF in the medium if any. The treated medium will then be used to culture CS eye-cups to study if the relative thinning effect of the insulin-RPE conditioned medium on the choroid can be inhibited. A control experiment should also be done with the medium conditioned by the RPE without the insulin and see if the inhibitor or the anti-VEGFR2 antibodies still have the same effect on the choroid to confirm the findings.

4.3 Other future studies

Future studies can be done on studying the effect of IGF-1 or IGF-2 on the choroidal thickening in eye-cups. Since the effective concentrations of insulin in reducing choroidal thickening are outside the physiological range, it is possible that the thinning effect is caused by IGF-1 or IGF-2 receptor, in addition to insulin receptor. It will also be interesting to explore the signaling pathway of insulin/IGF in reducing choroidal thickness *in vitro*. In the insulin chapter, we mentioned that the most well-known two cascade pathways for insulin are: (1) The phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway, and (2) Ras-mitogen-activated protein kinase (MAPK) pathway [review: (Taniguchi et al., 2006)]. We can prepare medium conditioned by the RPE in the presence of insulin with the inhibitor that specifically blocks either the PI3K-AKT pathway or MARPK pathway, and study how the relative thinning effect of this conditioned medium can be affected.

As we discussed before, insulin and VEGF might increase scleral GAG synthesis indirectly by acting on the choroid, we can study if insulin or VEGF causes the choroid to produce some secondary signaling molecules by preparing medium conditioned by the choroid in the presence of insulin or VEGF. This conditioned medium can then be used to culture scleral eye-cups. It will also be interesting to see what signaling cascade insulin or VEGF uses to cause an effect on scleral GAG synthesis.

4.4 Limitations of our study

Finally, the findings in this thesis have been based on the effect of the drugs, whether it was insulin or VEGF, relative to without the drug on the posterior ocular tissues *in vitro*. The effect we found can be different when the drugs that are injected into the eyes *in vivo*. As we have discussed in the insulin chapter, the presence of other endogenous biological molecules can have some additive or synergistic effect with the drugs which account for the differences between the *in vitro* and *in vivo* effect.

After all, our findings can only provide some insights on the signal cascade involving the RPE, choroid and sclera. When we look at a bigger picture of eye growth and step out of the studied area of this thesis, how the retina transduces the visual signal into the production of biological molecules that regulate eye growth is still unknown. As mentioned in the general introduction chapter, we have only known that the eye can know which way to grow by discerning the sign of blur at the local retinal level (section 1.2). Longitudinal chromatic aberration alone can tell the eye which way to grow (Rucker and Wallman, 2009) and positive lens (or myopic) defocus is more potent than negative lens (or hyperopic) defocus. In terms of local retinal processing, we still do not know how the retina computes the signal to discern whether blue aspect of an image is more focused than the red aspect of the image, nor do we know how the retina can compute the temporal factors in all the defocus we encounter over time. Hopefully, in the near future, new methodologies can be developed to study these areas.

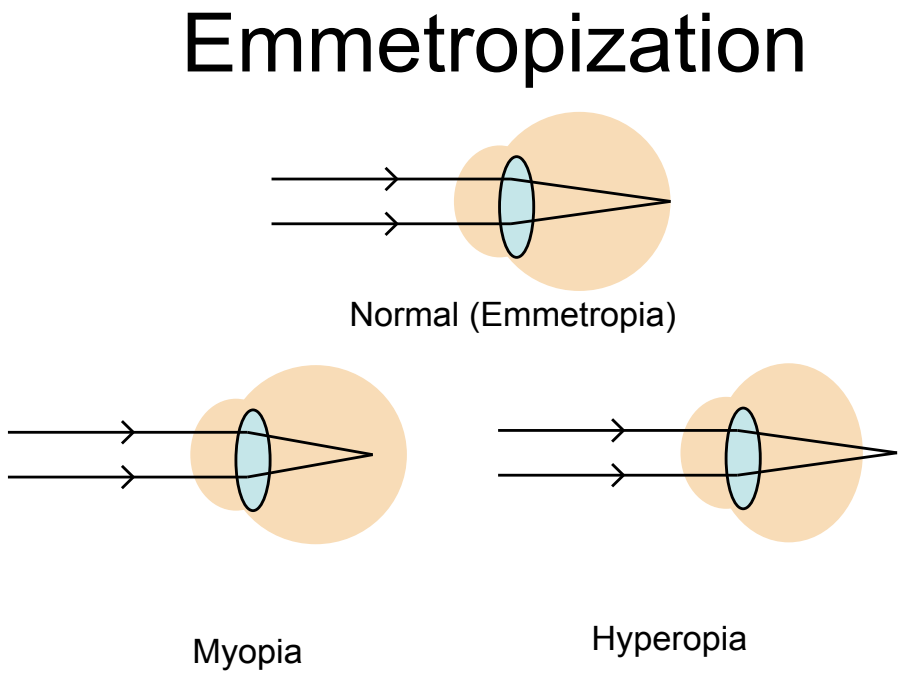

Figure 1.1 Emmetropization is a process that occurs in most vertebrates. It occurs postnatally to match the position of the retina with the focal plane of the eye, so that images of a distant object can be focused on the retina (emmetropia). Errors in emmetropization can result in myopia (ocular length is longer than the focal length of the eye; image of a distant object falls in front of the retina), or hyperopia (ocular length is shorter than the focal length; image of a distant object falls behind the retina).

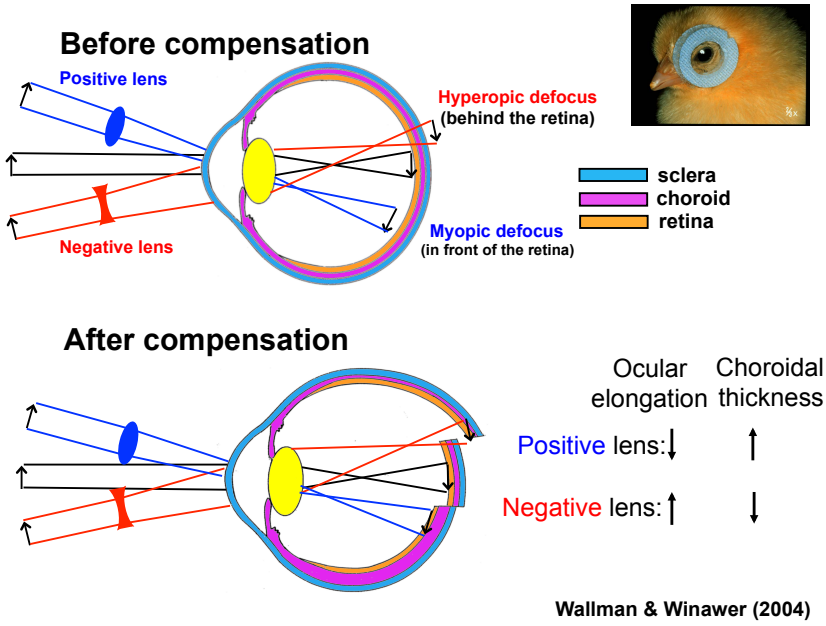

Figure 1.2 Choroidal and scleral components of eye growth. Positive lens creates a myopic defocus (image of distant object falls in front of the retina), which induces choroidal thickening and inhibition of ocular elongation. Negative lens creates a hyperopic defocus (image of distant object falls behind the retina), which induces choroidal thinning and enhances ocular elongation. The changes in the choroidal thickness and rate of ocular elongation move the position of the retina, so that images of distant objects will fall on the retina.

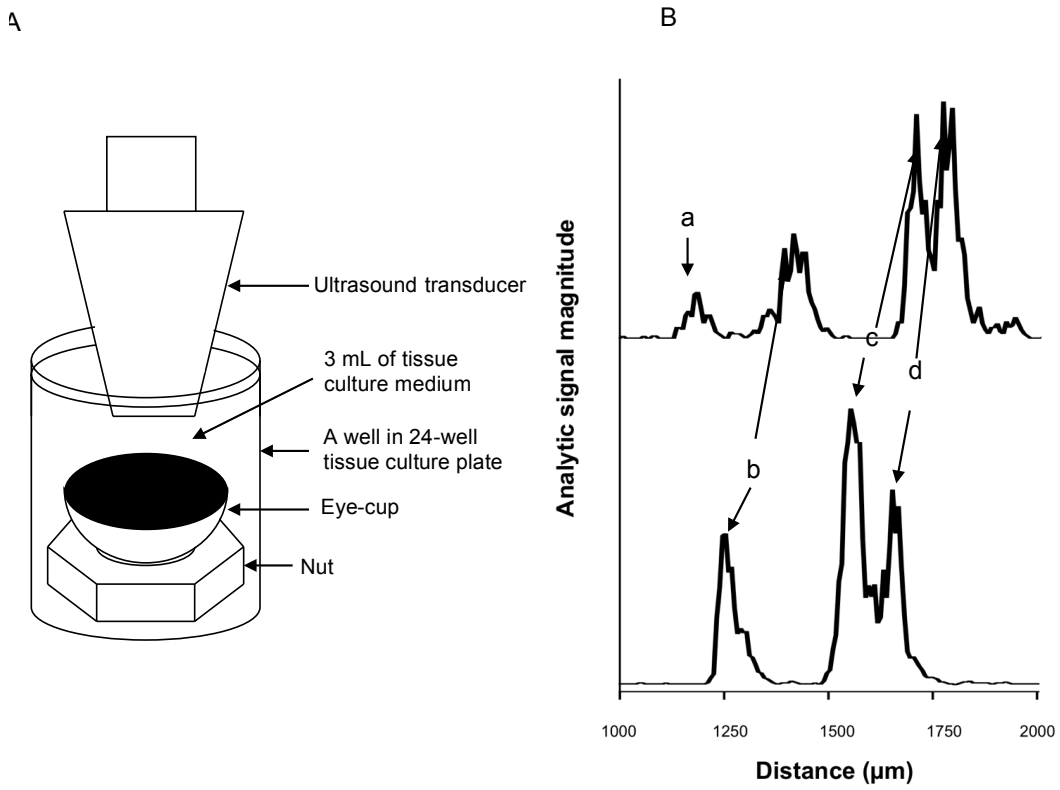

Figure 1.3 A-scan ultrasound biometry & traces. (A) Schematic diagram of an eye-cup in tissue culture and choroidal thickness measurement. (B) Ultrasound traces from *in vivo* (upper graph) and *in vitro* (with retina removed, lower graph) measurement of the same eye: (a) the anterior retinal surface, (b) the anterior choroidal surface, (c & d) the anterior and posterior sclera. Choroids can be easily distinguished from the ultrasound traces.

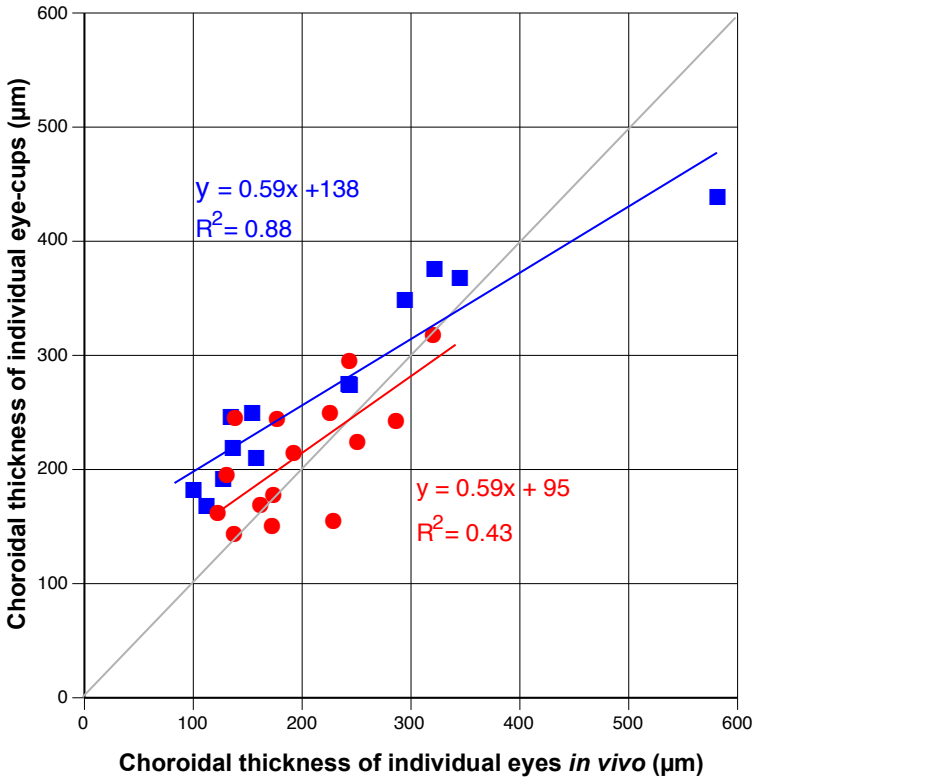

Figure 1.4 Correlation of *in vitro* and *in vivo* measurement of choroidal thickness (RCS eye-cups, n=12; CS eye-cups, n=15). Choroid thickness of eye-cups was positively correlated with the *in vivo* measurement made 2 hours earlier (RCS eye-cups r^2=0.88, p<0.0001; CS eye-cups, r^2=0.43, p<0.001)

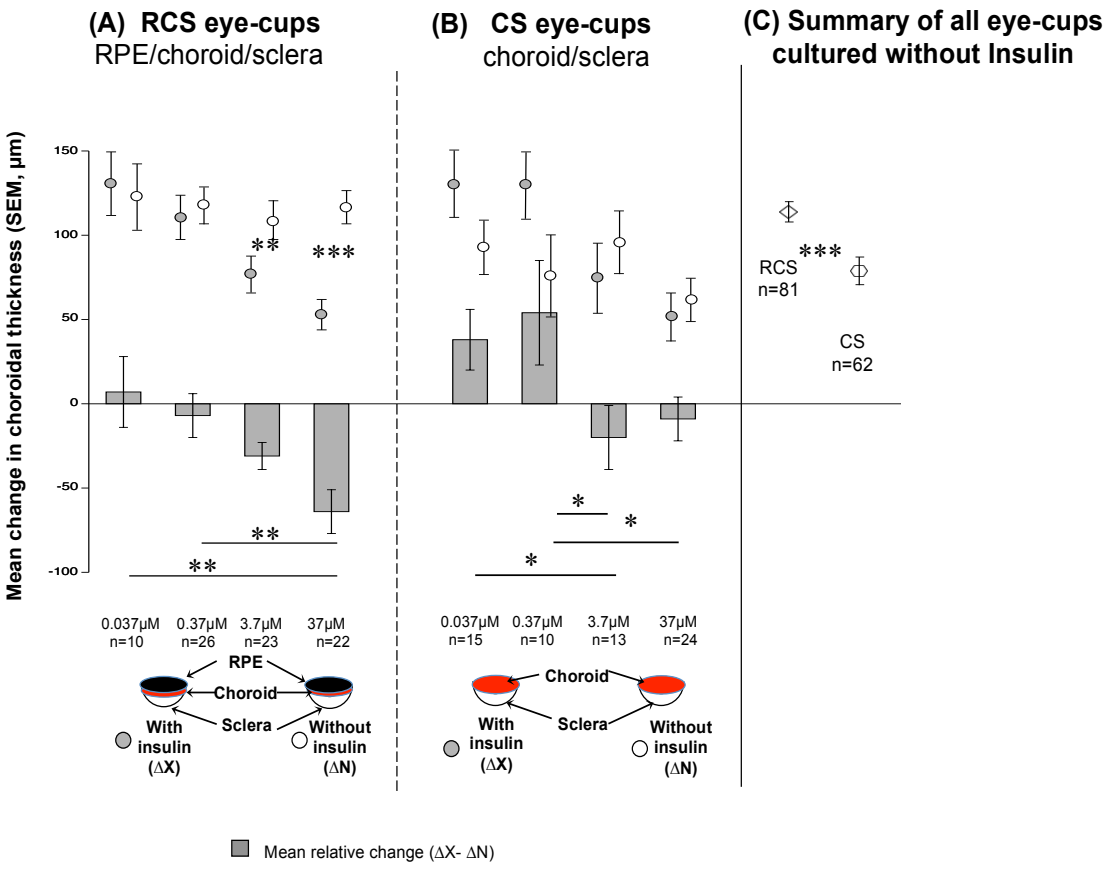

Figure 2.1 Effect of insulin on choroidal thickness in eye-cups. (A) RCS and (B) CS eye-cups after 20 hours of incubation, showing effect of insulin on choroidal thickness. (C) Summary of all eye-cups cultured without insulin. *p<0.05; **p<0.01; ***p<0.001

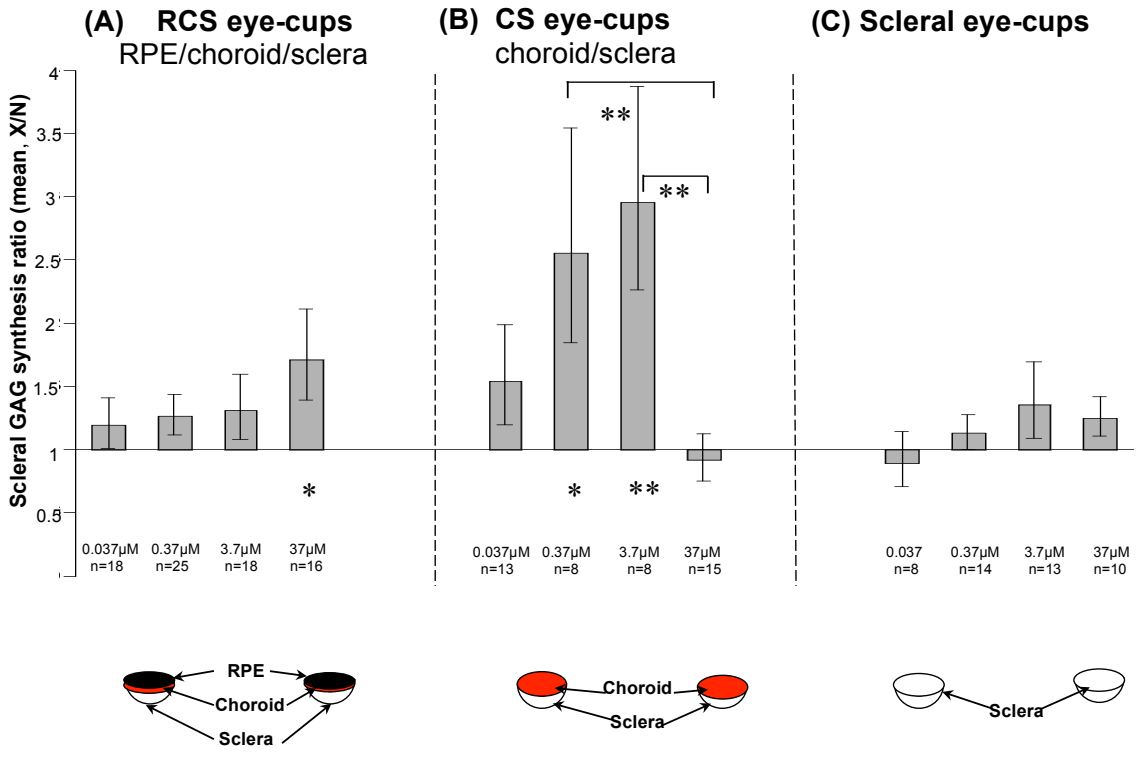

Figure 2.2 Effect of insulin on scleral GAG synthesis in (A) RCS, (B) CS, or (C) scleral eye-cups. Insulin increased scleral GAG synthesis in RCS and CS eye-cups. Asterisks below the bars show significant differences between the logarithms of the count from the eye-cups cultured with insulin and that of the paired eye-cups cultured without insulin. Asterisks below the lines and above the bars show significant differences in the log ratios between different doses of insulin in CS eye-cups. * $p<0.05$; ** $p<0.01$

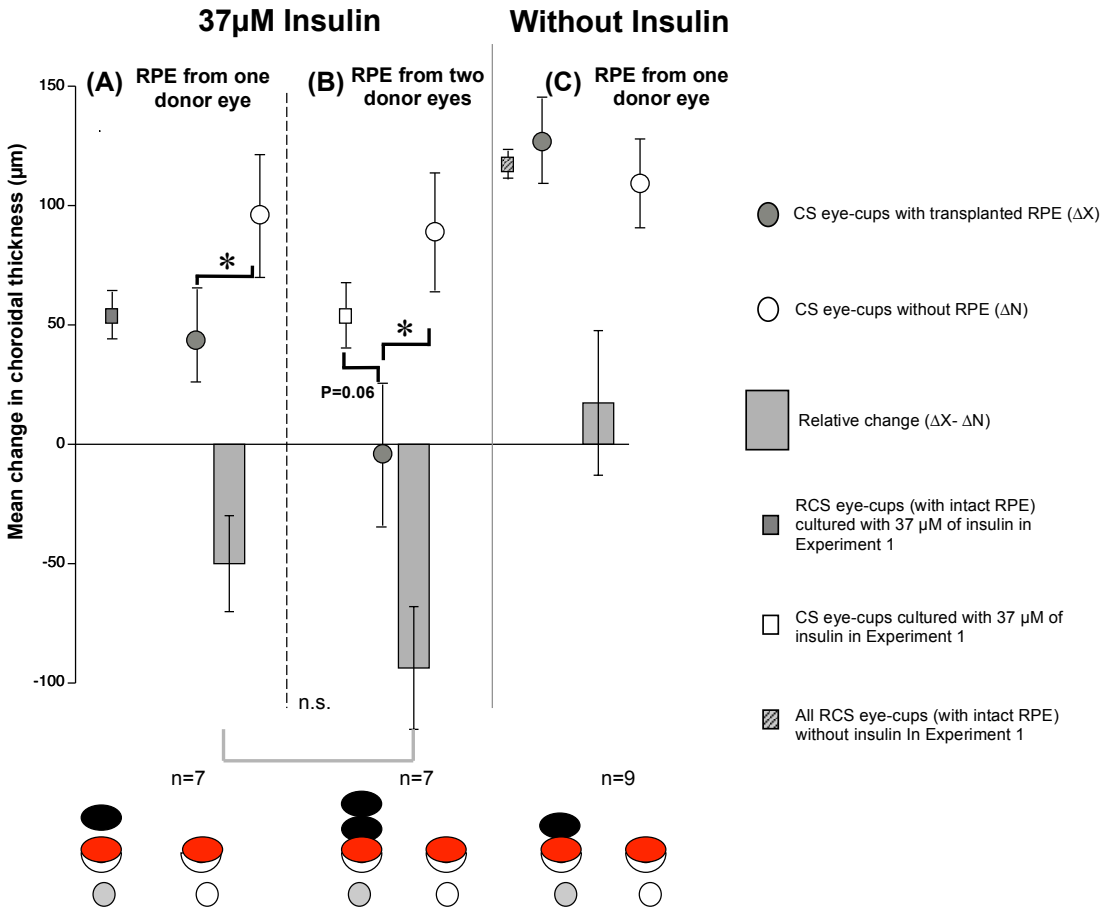

Figure 2.3 Effect of transplanted RPE on the thinning effect of insulin on choroidal thickness of CS eye-cups. (A) CS eye-cups cultured with or without transplanted RPE from one donor eye in pairs and with 37 μM of insulin. (B) CS eye-cups cultured with or without transplanted RPE from two donor eyes and with 37 μM of insulin. (C) CS eye-cups cultured with or without transplanted RPE in pairs but without insulin. * $p<0.05$; n.s. non-significant.

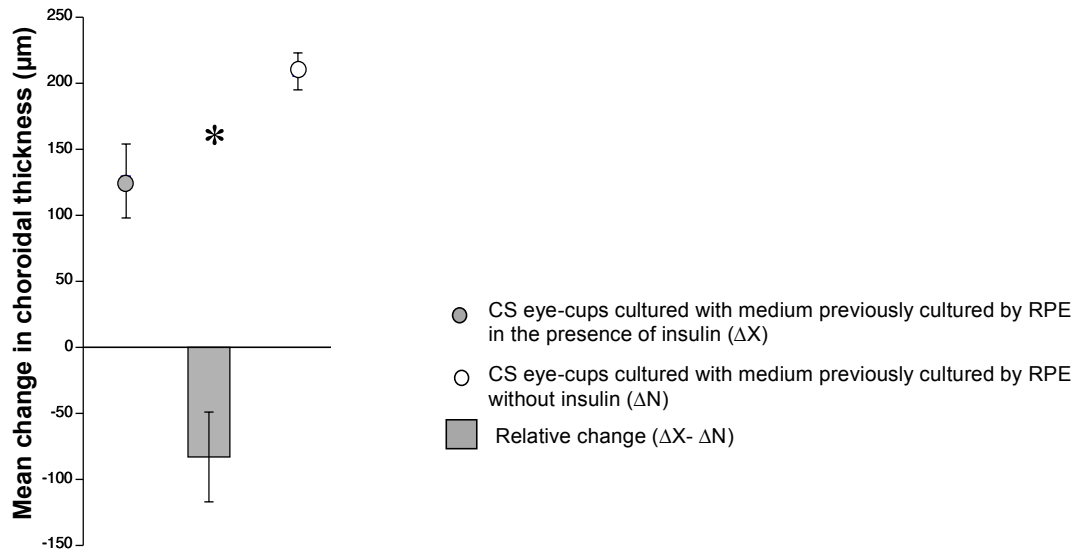

Figure 2.4 Effect of conditioned medium on the choroidal thickness of CS eye-cups. Medium were conditioned by RPE in the presence of 37 µM of insulin (X) or without (N) for 20 hours. They were then diluted with equal volume of 37 µM of insulin before they were used to culture paired CS eye-cups for 20 hours (n=12). *p<0.05

Criteria to remove pairs of eye-cups	Sample size	Change in choroidal thickness in eye-cups cultured with insulin (mean±SEM, µm)	Change in choroidal thickness in eye-cups cultured without insulin (mean±SEM, µm)	p value of paired 2-tailed student's t-test
0	24	53±13	63±13	0.50
With control choroids thickened less than 50µm	18	65±17	89±9	0.12
With control choroids thickened less than 60 µm	12	84±19	107±10	0.19

Table 1: Removal from the sample of control choroids that did not thicken much does not increase the effect of insulin on CS eye-cups.

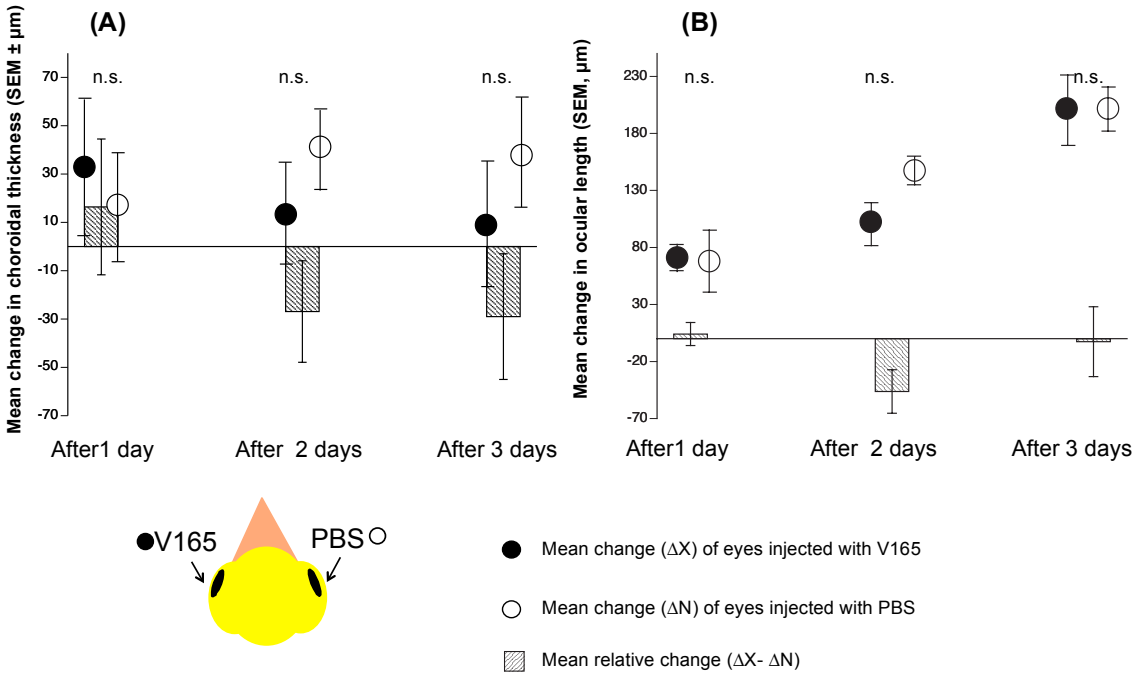

Figure 3.1. Effect of injected V165 on (A) the choroidal thickness and (B) ocular length of the eyes. Paired eyes from individual birds (n=5) were intravitreally injected with V165 (100 µg/mL in 1 µL) versus PBS once a day for three days. Ocular parameters were measured by ultrasonography for the first three days before injection and the fourth day. No significant difference was found between the paired eyes as shown. n.s. non-significant.

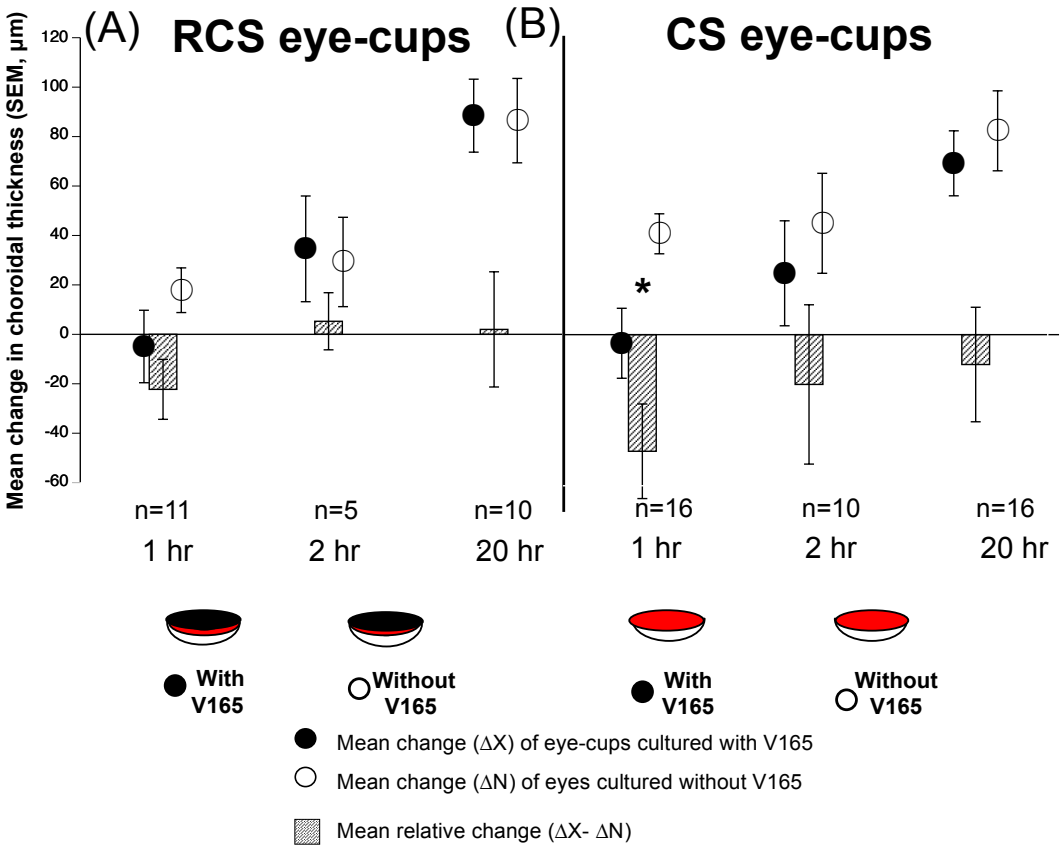

Figure 3.2 Effect of 100ng/mL of VEGF on the choroidal thickness in (A) RCS and (B) CS eye-cups over 20 hours of incubation. VEGF decreased choroidal thickening significantly in CS eye-cups after 1 hour of incubation, but the effect was transient.

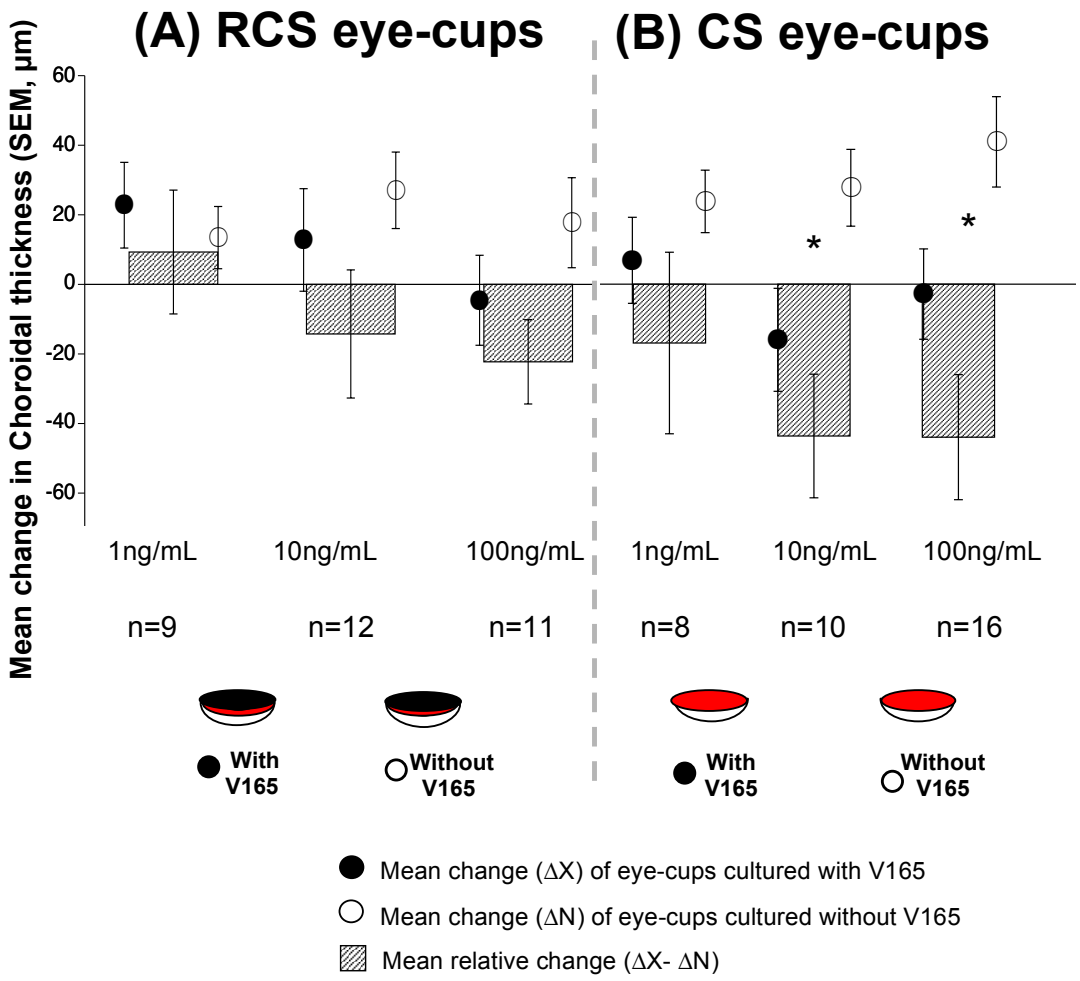

Figure 3.3. Dose responses of VEGF on the choroids in eye-cups after 1 hour of incubation. Dose response of V165 on the choroids of (A) RCS and (B) CS eye-cups. V165 reduced choroidal thinning in CS eye-cups at 10 and 100 ng/mL. Thinning effect of V165 in RCS eye-cups was not significant. *p<0.05

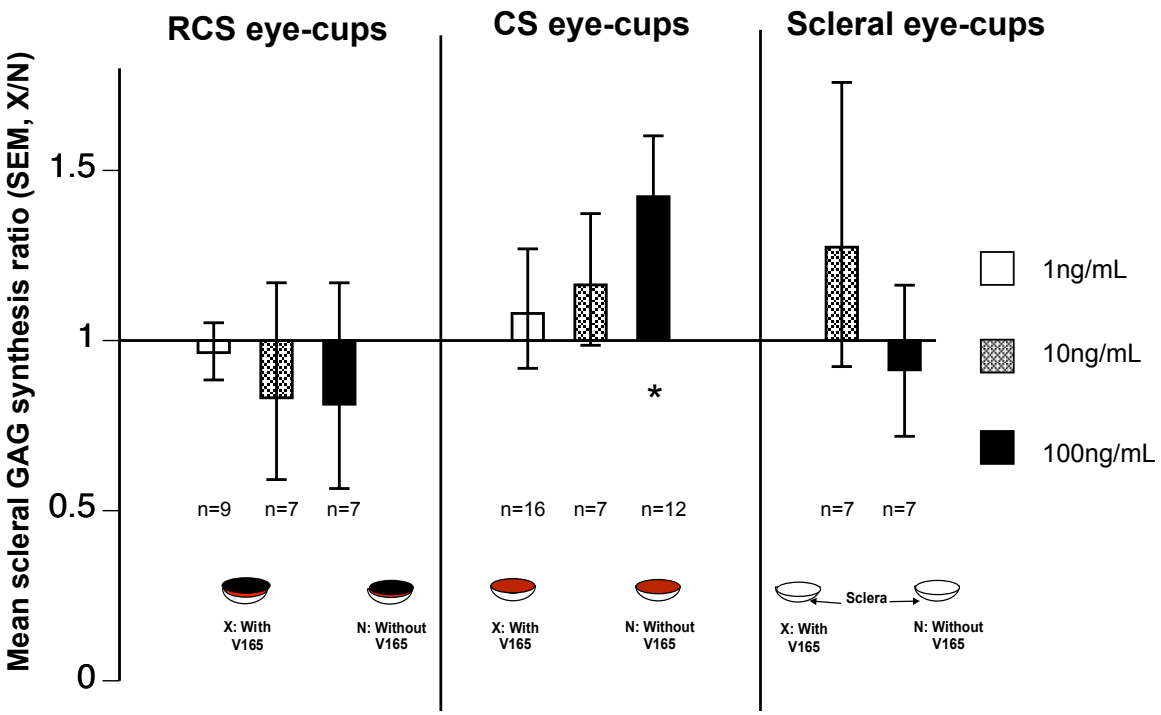

Figure 3.4 Dose responses of VEGF on scleral GAG synthesis in eye-cups after 44 hours of incubation. Effect of VEGF on RCS, CS and scleral eye-cups. V165 increases scleral GAG synthesis in CS eye-cups at 100 ng/mL but not in RCS or scleral eye-cups. *P<0.05

Appendix I

Effect of anti-VEGFR2 on the thinning effect of RPE-conditioned medium

To study if VEGF is one of the molecules produced by the RPE in the presence of insulin that thins the choroids in CS eye-cups, effect of ant-VEGFR2 antibodies on the thinning effect of conditioned medium was studied. RPEs of two donor eyes were cultured in a well filled with 3mL L-15 medium (Sigma) with 37μM of insulin. A total of 36 RPEs from 18 birds were used and cultured in 18 wells. After 20 hours of incubation, medium from all the wells was pooled together. CS eye-cups were made and cultured with anti-VEGFR2 antibodies (2 μg/mL, mab 3572, R&D Systems R&D systems) vs. its isotype controls (mouse IgG1, 2 μg/mL, R&D systems) for 2 hours in pairs (n=17). The CS eye-cups were then cultured with the conditioned medium for 20 hours in pairs. Because we knew that VEGF has a fast but transient response on the choroid, choroidal measurements were done after 1, 3 and 20 hours of incubation with conditioned medium. Data were analyzed by (1) repeated measures ANOVA, then followed by a post-hoc LSD test; (2) two-tailed paired student's *t* test for the paired eye-cups at 1, 3 and 20 hours of incubation. A repeated measures ANOVA showed that both time and the pre-treatment of anti-VEGFR2 had a significant effect on the change in choroidal thickness (time, $p<0.05$; treatment of anti-VEGFR2, $p<0.05$). At 1 and 3 hours of incubation, choroids pre-treated with anti-VEGFR2 thickened significantly more than their paired controls (at 1 hour, $p<0.05$; at 3 hours, $p<0.01$, LSD post-hoc test, two-tailed paired student's *t* test Fig a p. 103). The effect was still there after 3 hours of incubation but disappeared after 20 hours of incubation

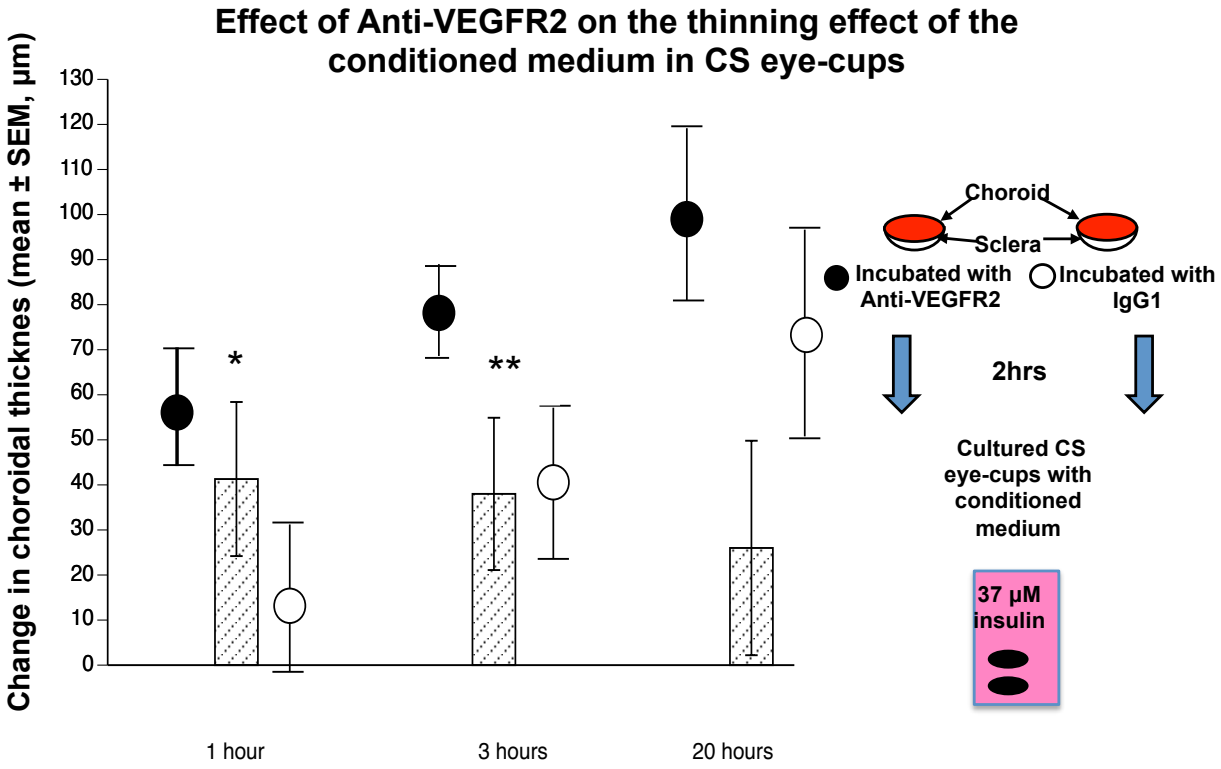

Figure a Effects of neutralizing antibodies of VEGF-R2 (anti-VEGFR2) on the insulin-RPE conditioned medium on choroids. Choroids of CS eye-cups pretreated with anti-VEGFR2 antibodies had significantly thicker choroids than their paired eye-cups pretreated with the antibody isotype control, after 1 and 3 hours, but not after 20 hours of incubation (n=17, * $p < 0.05$, **$p<0.01$).

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