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GENETIC INSIGHTS INTO THE ROLE OF PAX6 IN OCULAR DEVELOPMENT

submitted by NAIF SAMI SANNAN in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in EXPERIMENTAL MEDICINE

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The fovea is a small retinal indentation packed with specialized cone photoreceptors. Despite its key-role in central vision, little is known about foveal pathologies and development at the cellular and molecular levels. Therefore, no treatment is yet available for vision loss resulting from underdeveloped-fovea (foveal hypoplasia (FH)).

First, I used aniridia as a disease model to better understand FH at the cellular and molecular levels. Thirty-three aniridia subjects from British Columbia underwent a thorough ophthalmic examination with in-vivo imaging of foveal structure. Molecular investigations include sequencing of PAX6, candidate genes, in addition to 11p chromosomal analysis. In those in whom imaging was possible, FH was seen in the majority (80%) of cases. Best corrected visual acuities in the cohort ranged from normal vision to no light-perception. Molecular genetic defects involving PAX6 were identified in 30 participants, including 4 novel PAX6 mutations and 4 novel chromosome 11p deletions inclusive of PAX6 or its regulatory region.

Then as a proof-of-principle, we employed the SMaRT (spliceosome-mediated RNA trans-splicing) method to rescue Pax6 defects in homozygous-mutant mouse embryonic-fibroblasts and then in-vivo in a naturally occurring Pax6 mouse model. We showed that by using SMaRT technology we were able to rescue Pax6 expression in-vitro and in-vivo, paving the way for potential future therapies for FH.

Finally, we tested the feasibility of using Anolis carolinensis (green anole lizard) as a novel foveated model. With its complete published genome, bioinformatic analysis revealed that 85% of human candidate FH genes had an orthologous gene or DNA sequence in the anole. Eyes were collected at various stages of prehatching development for histological analysis,
immunofluorescence, and apoptosis analysis. We demonstrated that embryonic foveal development in green anoles resembles human foveal development during infancy. Additionally, at embryonic stage (ES) 14 Pax6 was localized across the entire retina. However, at ES17 Pax6 expression in the ganglion cells of the central retina was markedly reduced. These findings provide the first insight into foveal morphogenesis in the green anole and suggest that it could be an ideal model for perturbing the molecular signals driving foveal development, thus informing on human foveal development and disease.
LAY SUMMARY

As light enters the eye it is focused on the fovea, a small indentation at the back of the eye essential for sharpness of central vision. As you read this, you are using your foveal vision to navigate between these letters, and your brain is busy processing the foveal visual information. There is an untreated disease called foveal hypoplasia (FH) which affects central vision from birth. To find a treatment for FH, we need to better understand foveal structure, which currently is poorly understood in the literature. Therefore, I’ve conducted the following experiments: 1) studied FH in patients, 2) tried to repair the *Pax6* gene mutation in a cell line and in a *Pax6* mutant mouse, as this gene is implicated in FH, and 3) studied foveal development in a novel animal model (green anole lizard). These studies showed that all patients with a *PAX6* gene mutation had variable grades of FH. We rescued the *Pax6* defects in mutant cell line and in mutant mouse model. Finally, we characterized foveal development in green anoles and found there were similarities to human foveal development, suggesting this model could be used to study human foveal disease.
PREFACE

Work presented hereafter was conducted in the Department of Ophthalmology and Visual Sciences in the Eye Care Centre, Vancouver General Hospital Campus, University of British Columbia. The research reported here was approved by the UBC Office of Research Services in accordance with the general guidelines set by the Declaration of Helsinki, the Association for Research in Vision and Ophthalmology, and the Canadian Council on Animal Care (protocols# H11-01618, A14-0029, and A14-0062). Animal ethics, practical animal care, and biosafety certifications are: #5462-12, RBH-85-12, RA-33-13, 2014-iYtAe.

This thesis covers the following themes: Chapter 1 introduces the current understanding of fovea development and structure and the known molecular genetic data. Chapter 2 presents the genotype and phenotype of patients with Aniridia that have been ascertained from British Columbia with focus on foveal hypoplasia (FH). This study has been published in the Canadian Journal of Ophthalmology (Sannan et al., 2017) with the following contributing authors: Cheryl Gregory-Evans, Christopher J. Lyons, Anna M. Lehman, Sylvie Langlois, Simon Warner, Helena Zakrzewski, Kevin Gregory-Evans. I was involved in study design, genotyping, phenotyping, data interpretation, and manuscript writing. Chapter 3 delves deeper into the genetic defects found in Chapter 2 and is complemented with other genetic defects found in the literature to understand their possible role in FH. Data in this chapter is not yet published. Chapter 4 describes the utilization of RNA trans-splicing to rescue Pax6 mutations, which is implicated in foveal abnormalities in mutant cell line and in a naturally occurring mutant mouse model. Data generated in this chapter is being prepared for submission to the Journal of Experimental Eye Research. Chapter 5 describes the characterization of the Anolis carolinensis
lizard as a novel animal model to better understand the fovea at the cellular and molecular level. This study has been published in the Experimental Eye Research journal (Sannan et al., 2018) with the following contributing authors: Xianghong Shan, Kevin Gregory-Evans, Kenro Kusumi, and Cheryl Gregory-Evans. I was involved in study design, data collection, interpretation, and manuscript writing. **Chapter 6** offers a discussion of the major findings, significance, and future directions.
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<th>Full Form</th>
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<tbody>
<tr>
<td>ASO</td>
<td>Antisense oligo</td>
</tr>
<tr>
<td>BCVA</td>
<td>Best-corrected visual acuity</td>
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<tr>
<td>BD</td>
<td>Binding domain</td>
</tr>
<tr>
<td>BP</td>
<td>Branching point</td>
</tr>
<tr>
<td>CC</td>
<td>Connecting cilium</td>
</tr>
<tr>
<td>CF</td>
<td>Central fovea</td>
</tr>
<tr>
<td>Chor</td>
<td>Choroid</td>
</tr>
<tr>
<td>CP</td>
<td>Conus papillaris</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DsRed2</td>
<td>Discosoma red fluorescent protein</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinogram</td>
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<tr>
<td>ES</td>
<td>Embryonic stage</td>
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<tr>
<td>FAZ</td>
<td>Foveal avascular zone</td>
</tr>
<tr>
<td>FH</td>
<td>Foveal hypoplasia</td>
</tr>
<tr>
<td>fH</td>
<td>Fibers of Henle</td>
</tr>
<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>Inner plexiform layer</td>
</tr>
<tr>
<td>IS</td>
<td>Inner segment</td>
</tr>
<tr>
<td>ISL</td>
<td>Inner segment lengthening</td>
</tr>
<tr>
<td>logMar</td>
<td>Logarithm of the minimum angle of resolution</td>
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LOVD  Leiden open variation database
MLPA  Multiplex ligation dependent probe amplification
mRNA  Messenger ribonucleic acid
NFL   Nerve fiber layer
NGS   Next generation sequencing
OCT   Optical coherence tomography
ON    Optic nerve
OLM   Outer limiting membrane
OMIM  Online Mendelian inheritance in man
ONL   Outer nuclear layer
OPL   Outer plexiform layer
OS    Outer-segment
OSL   Outer segment lengthening
PAX   Paired box
PAX6  Paired box-6 gene
PBST  PBS + 0.1% Tween 20
PCR   Polymerase chain reaction
PEDF  Pigment epithelium derive factor
PPT   Poly pyrimidine tract
pre-mRNA Pre-messenger ribonucleic acid
PTMs  Pre-messenger ribonucleic acid trans-splicing molecules
RNA   Ribonucleic acid
RPE   Retinal pigment epithelium
<table>
<thead>
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<th>Description</th>
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<tr>
<td>S/M/L</td>
<td>Short (blue)/medium (green)/long (red) wavelengths</td>
</tr>
<tr>
<td>SD-OCT</td>
<td>Spectral domain-optical coherence tomography</td>
</tr>
<tr>
<td>SLC38A8</td>
<td>Solute Carrier Family 38 Member 8 gene</td>
</tr>
<tr>
<td>SMaRT</td>
<td>Spliceosome-mediated RNA <em>trans</em>-splicing</td>
</tr>
<tr>
<td>SS</td>
<td>Splice site</td>
</tr>
<tr>
<td>TF</td>
<td>Temporal fovea</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>WAGR</td>
<td>Wilms tumour, aniridia, genitourinary malformations, and mental retardation syndrome</td>
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This work could not be done without the presence of many brilliant minds. Here I present only few. First and foremost, I acknowledge Dr. Cheryl Gregory-Evans (my research supervisor) for her guidance and presence during my PhD journey. Also, I would like to acknowledge my Ph.D. supervisory committee, Drs. Christopher Lyons, Daniel Goldowitz, and Joy Richman for their valuable recommendations throughout this work. I acknowledge Xianghong Shan (lab manager), Anat Yanai (research associate), Ishaq Viringipurampeer (research associate), Xia Wang (post-doctoral fellow), Vahitha Nizamudheen (PhD candidate), Jessica Morrice (PhD candidate), and Aisha Soliman (MSc candidate) for their valuable discussions and technical assistance. Also, I acknowledge Laura Hall from UBC Department of Ophthalmology for retinal imaging of patients and Ingrid Barta from the Department of Pathology at UBC for her assistance with processing of histological specimens.

I owe particular thanks to all patients and their families who participated in this study. Their contribution is substantial in producing this thesis, which hopefully will push the field a step forward in the right direction.

I also acknowledge King Saud bin Abdul Aziz University for Health Sciences, the Saudi Cultural Bureau in Ottawa, and Sharon Stewart trust for financially supporting this research.
DEDICATION

To my dear parents (Sami and Nisreen), to my soulmate (Lamees), and to my three wildcard-kids (Omar, Ayah, and Ahilla). No words can express my gratitude...
Chapter 1: INTRODUCTION

1.1 Opening remarks

Vision is a primary source of our perception and it relies on healthy eyes, healthy visual pathways, and healthy visual processing areas in the brain (Purves, 2012). Although more than 80% of all vision impairments can be prevented or cured, visual impairments are considered a major global burden. According to World Health Organization, it is estimated that there are about 253 million people living with vision impairment of which 36 million are legally blind. Whereas 19 million children are afflicted worldwide, about 1.4 million live with blindness and require access to vision rehabilitation services for coping with the disability. In general terms, vision function can be classified into four major groups: normal vision, moderate impairments, severe impairments, and blindness (WHO, 2017).

A poorly understood ocular condition affecting the fovea, a specialized retinal structure located at the centre of gaze, is among those untreatable debilitating abnormalities. This condition is clinically known as foveal hypoplasia (FH) or less commonly as macular hypoplasia (online Mendelian inheritance in man (OMIM # 136520 and 609218). In this condition the fovea is significantly underdeveloped, and patients could have vision impairment from early childhood ranging from moderate vision impairment to full blindness. The work presented in this thesis focuses on foveal development, its importance to normal vision, the structural abnormalities, a possible treatment strategy targeting the paired box-6 gene (PAX6) which is implicated in FH, and evaluation of A. carolinensis (green anole lizard) as a possible model to study FH.
1.2 Overview of the eye

Anatomically, the eye is a fluid filled sphere composed of three layers of tissue: fibrous tunic (sclera and cornea); vascular tunic (iris, ciliary body, and choroid); and retina (neural and pigmented layers). Different eye structures contribute in forming a clear image representing the outside world. In brief, arrays of light enter the eye through the cornea, a clear and curved membrane covering the front of the eye, which bends and focuses the light rays before it passes through a central opening in the iris (i.e. the pupil) toward the lens. The pupil constricts and dilates to control the amount of incoming light through the coordinated actions of pupillary sphincter and dilator muscles. The lens, a biconvex structure, focuses the light further by the action of the ciliary muscle. Focused light rays then travel through the vitreous which is a jelly-like transparent substance filling the inside of the eye. Lastly, focused light rays hit the retina (Standring, 2016).

1.3 Retina overview

The initial step in seeing begins in the retina. It is where incoming light rays are sensed, translated, and transmitted via the optic nerve to the visual cortex of the brain for processing and ultimately, the perception of the visual scene. It is an essential and non-replaceable ocular structure, susceptible to a broad spectrum of abnormalities (Standring, 2016, Margalit and Sadda, 2003). The retina is considered as a peripheral part of the central nervous system because it originates from the neural tube and has similar types of functional elements to those found in the brain and spinal cord (Purves, 2012). During early embryogenesis, the diencephalon forms an out-pocketing on both lateral sides of the developing neural tube known as optic vesicles. These primordial optic vesicles then invaginate (~4.5 fetal weeks) to form the optic cups with the inner
side of the cup becoming the neural retina and the outermost layer forming the retinal pigment epithelium (RPE) (Mann, 1964). At this stage, the developing retina has a massive expansion in the number of the dividing cells. Cell birth starts at the central retina and then spreads toward the periphery in a wave like pattern. By the seventh fetal week, the first axon leaves the eye through the optic nerve to connect to the visual processing areas in the brain. By the fifth fetal month, most retinal neural connections are established (Mann, 1964, Kolb et al., 1995).

The neurosensory retina is a thin, delicate membrane that coats the inner surface of the eyeball. It has three main classes of neurons forming a complex circuitry: sensory neurons (rods and cones), intermediate neurons (horizontal, bipolar, and amacrine), and output neurons (ganglion cells). In addition there are auxiliary glial cells (Müller cells, astrocytes, and microglia) which serve several structural and functional benefits. Perikarya of these neurons and their axons are organized in seven major layers stacked on top of the RPE layer (Figure 1.1). These layers are, from outer to inner retina: 1) photoreceptor outer and inner segments (OS/IS), carrying the light-sensitive pigments and cellular organelles, respectively; 2) outer nuclear layer (ONL), which contains rods and cones nuclei; 3) outer plexiform layer (OPL), where axons of photoreceptors meet the dendrites of intermediate neurons; 4) inner nuclear layer (INL), which carries the intermediate cell nuclei; 5) inner plexiform layer (IPL), containing axons of intermediate neurons synapsing with dendrites of ganglion cells; 6) ganglion cell layer (GCL), carrying ganglion cell nuclei; and 7) nerve fiber layer (NFL), where ganglion cell axons leave the eye forming the optic nerve (ON), a visual link to the brain. These layers are nourished either by the innervated blood capillaries (NFL to OPL) or by the choriocapillaries network (ONL and RPE) (Kolb et al., 1995).
Therefore, in this structurally complexed retina, incoming light rays are first filtered by passing through the inner and outer retinal layers and blood vessels before it reach photoreceptor outer segment where it get converted to neuronal impulses that travel in the opposite direction to intermediate neurons, to ganglion cells, to the ON, and then to the visual cortex for signal interpretation and image formation and interpretation.

Figure 1.1 Overview of the eye and the retina
Cartoon representation showing cross sectional view of the human eye (left) and the retina (right). Slightly modified version from OpenStax.org (under a creative commons attribution license 3.0).

Retinal functions require photoreceptors. These photoreceptors are known as rods (long and slender) and cones (short and tapered), and they have the following components: 1) a synaptic terminal connected to a bipolar and horizontal cells; 2) a cell body where the nucleus reside; 3) a mitochondria-rich IS; 4) a connecting cillum (CC), connecting the IS with; 5) the OS, which is composed of several hundred lammellar-shaped discs carrying different light sensitive molecules known as opsin.
Opsins are the light sensitive pigments involved in the phototransduction cascade, i.e. converting light stimuli into electrical impulses. Each photoreceptor type reacts to a different wavelength of light depending on the type of opsin it holds. Rods carry rhodopsin photopigment and mediate perception of shades of grey and light-dark contrast, and therefore are active in low light conditions. Cones, on the other hand, are active in well-lit conditions and carry one of three different cone opsins. Cone pigments are maximally sensitive to either short wavelengths (S) of light (peak at 420nm and sensitive to blue light), medium wavelengths (M) of light (peak at 530nm and sensitive to green light), or long wavelengths (L) of light (peak at 560nm and sensitive to red light). These photopigments are the basis of our trichromatic visual color perception (Bowmaker and Dartnall, 1980, Kolb et al., 1995).

1.4 The macula lutea

The retina is a circular disk-shaped structure of about 30-40 mm in diameter. The central retina is the most central ~5mm in diameter zone while the rest is known as the peripheral retina. The retina is generally thicker at the centre compared to periphery due to the increased packing of cones and their associated intermediate and output neurons (Kolb et al., 1995).

The central retina is also known as macula lutea (hereafter; macula) which is an ill-defined circular region temporal to the optic disc, outlined by the superior and inferior temporal branches of the vascular arcades (Figure 1.2). A yellowish pigmented region on the otherwise pale retina is due to the presence of different carotenoids (Nguyen, 2010, Rodieck and Nirenberg, 1973). Anatomically, the macula can be divided into three main concentric zones: perifovea; parafovea; and most centrally the fovea (Figure 1.2) (Wandell, 1995, Penfold and Provis, 2005).

Abnormalities in the perifovea and parafovea account for vision loss in the central 20 and 8
degrees of the visual field, respectively. These two peripheral zones have no sharp boundaries and mainly differ in the number of stacked nuclei (i.e. cell rows) at different retinal layers. For instance, perifovea represents the outer macular zone and has one row of cells in the GCL and more rods than cones in the ONL (approximate ratio 21:1). Whereas the inner zone, parafovea, is recognized by the presence of 4 cell-deep GCL (closer to perifovea) which doubles close to the foveal zone. Also, the parafovea has reduced rods to cone ratio at the ONL (approximate ratio 8:1) and a thick OPL (Penfold and Provis, 2005, Yuodelis and Hendrickson, 1986).

Figure 1.2 Fundus photograph of right eye
The macula is a pigmented region located temporal to the optic disc and can be divided into three main concentric zones (perifovea, parafovea, and fovea) with a central pit (foveola). The approximate diameter and the subtending visual field are stated between brackets for each zone. The central retinal vessels are seen branching from the optic disc. Note that the darkly pigmented region located between 3 and 4 o’clock side of the image (circled, nasal to optic disc) is known as choroidal nevus (i.e. eye freckle) and is different from macular pigment (original figure).
1.5 The fovea

The fovea, also known as fovea centralis, is a retinal structure essential for central vision accounting for the central 5.2° of the visual field. It is the inner macular zone (about 1.5-1.85mm in diameter) and can be further divided into: foveola, foveal slope, and periphery (Standring, 2016). The foveola is the most central region (0.25-0.35mm in diameter) representing the actual pit. It is the thinnest region in the retina (about 130 μm) and forms after lateral extrusion of inner retinal layers starting at late pregnancy within the foveal avascular zone (FAZ). The mature foveola is characterized by the presence of a peak density of M and L cones (40,000 cones or 200,000/mm²), tightly packed and with maximally elongated IS and OS, Müller cell processes, and the absence of rods and S-cones. The foveola is normally avascular, therefore, M and L cones rely on the underlying choroid for their oxygen and nutrients needs. Whereas the foveal slope contains the highest number of ganglion cells (about 35,000 cells/mm²), the peripheral retina has about 5,000 ganglion cells/mm². This high density of ganglion cells in the slope correspond to the high density of cones in the foveola. Then the foveal region gradually transitions to a fully layered retina at the peripheral rim margin (Curcio and Allen, 1990, Fulton, 2000, Penfold and Provis, 2005).

1.5.1 Benefits of having a fovea, current understandings and theories

The fovea lies on the central visual axis of the eye, so that light rays passing perpendicular through the centre of the lens will fall on it. Therefore, binocular foveal vision is achieved when both eyes converge to fixate the incoming light on the bilateral foveas. This region of highly specialized cells and unique retinal structure is believed responsible for the sharpest central color vision. Therefore, activities such as reading, driving, and seeing faces are
heavily dependent on healthy foveas (Rayner and Bertera, 1979). For example, when reading a line of text, the letters are projected on the reader’s fovea and the acuity drops as the stimulus is projected farther from the foveola. This provides the highest visual acuity covering an approximately 2° wide area centred at the point of fixation (i.e. at the foveola). Therefore, brief fixational pauses followed by rapid saccadic eye movements constantly bring word characters into foveal vision. Furthermore, it is known that reading can be impaired when experimentally blocking the foveal vision or due to natural foveal pathologies.

Few hypotheses have been proposed regarding the benefits of having a fovea. For example, we know that cell nuclei, their axons, and the innervated blood vessels have an inherent ability to scatter light at different degrees. Therefore, the presence of all of these structures on top of the buried and inverted photoreceptor OS predicts less effective signal transfer in the retina. This is in contrast to the foveola, where the absence of inner retinal layers and vasculature reduces the attenuation of the incoming arrays of light. In other words, the photoreceptors are more accessible to incident light rays (Weale, 1966). In addition, in 1937 Walls demonstrated that the concave structure of the foveal-pit is able to scatter light rays over a wider area of photoreceptors and therefore may act as an image magnifier (Walls, 1937).

Furthermore, photoreceptors (the main functional unit in the retina) are scattered differently across the retina. Rods outnumber cones throughout the retina, but are absent in the foveola. S-cones are also absent in the central 100μm foveola-region. Foveal M and L cones are densely packed with thinner and longer IS/OS than those in other retinal region. Such distribution of photoreceptors across the retina infers an important function that benefits human vision which is mainly cone-dependent (Bowmaker and Dartnall, 1980, Kolb et al., 1995, Bumsted and Hendrickson, 1999, Provis et al., 2013).
At birth, the fovea is relatively immature but reaches maturity around 4 years after birth and continues to develop further through IS/OS elongation (Hendrickson and Yuodelis, 1984, Vajzovic et al., 2012). This may correspond to the time-frame where 20/20 vision is achieved in children (4-9 years of age) (McDonald et al., 1985, Stephens, 1995, Almoqbel F et al., 2018). Other contributing postnatal morphological changes in the brain may also have an equal or higher role in infant vision development, such as the increase in the visual cortex synapses as well as the myelination of the visual pathway (Sireteanu et al., 1994). Interestingly, approximately 25% of the total ganglion cells are in the foveal region with each foveal cone synapsed to three ganglion cells via three bipolar cells (0-1.8 mm eccentricity, known as the midget circuit). This ratio reduces to one ganglion cell/cone at 3-4mm away from the foveal centre. The midget circuit was found in human (Sjostrand et al., 1994) and in monkey (Schein, 1988) foveas. In other words, this suggests that foveal information employs a large portion of the visual cortex. It is estimated that the central 5° of the visual field, where the fovea is located, employs up to 40% of the activity in the primary visual cortex. This may aid in visual resolution and cortical signal magnification (Sjostrand et al., 1994, Ahmad et al., 2003).

Another interesting macular feature is the presence of macular pigments. These pigments are either of entirely dietary origin (lutein and zeaxanthin) or formed in the retina from lutein (meso-zeaxanthin). These pigments are positioned in the photoreceptors with the highest concentration found in the axon layer (fibers of Henle (FoH)) at the foveola and are believed to protect the eye from oxidative stress. These pigments can absorb the high energy waves of blue and ultraviolet light, i.e. short wavelength filter, additional to that provided by the lens (Nguyen, 2010, Rodieck and Nirenberg, 1973).
In summary, the foveal pit captures the incoming light and refracts it over a wider region of cone photoreceptors. In addition packed foveal cones are able to process more visual input. This has allowed us to see better and to resolve fine spatial details and colors. Interestingly, German engineers designed a fovea-inspired photovoltaic system and showed an enhanced light absorption ability (Shalev et al., 2015), providing further evidence that a fovea structure has a benefit.

1.5.2 Foveal imaging

The ophthalmoscope is routinely used to look at the back of the eye (i.e. retinal fundus) through the pupil, allowing assessment of the presence or absence of the macular pigmentation and foveal reflex, representing the actual pit, and is somewhat a crude assessment tool. However, eye enucleation, processing, and imaging was the only method with the highest resolution available to look at normal and pathological foveal architectures. A relatively recent in vivo non-invasive technique has allowed clinicians to thoroughly investigate normal and pathological foveas in more depth with comparable resolution to histological specimens. Nowadays, optical coherence tomography (OCT) has an important role in the management of retinal conditions, and particularly foveal abnormalities. OCT is a continuously and rapidly evolving technique, mainly in depth and resolution. It employs the natural inherent reflectance property of the retinal layers and processes the reflected light waves to generate retinal cross sections in clinical as well as in research settings (Kashani et al., 2017).
1.5.3 Foveal variations

Several studies have reported a racial and sex-based variations in foveal pit morphology between healthy individuals (Song et al., 2010, Kashani et al., 2010, Sull et al., 2010, Grover et al., 2009, Kelty et al., 2008, Asefzadeh et al., 2007, Wagner-Schuman et al., 2011). These variations were in pit depth, pit diameter, and foveal retinal thickness. For example, Africans and African Americans display reduced foveal retinal thickness, and deeper and broader foveal pits than Caucasians. Women have reduced central subfield thickness when compared to men. These slight measurement differences are still considered as normal vision.

Progressive minor structural changes in foveal architecture were anticipated as a biomarker for early detection of Parkinson’s, Alzheimer’s, and other neurological disorders. For example, features such as reduced foveal pit thickness and width were positively correlated with Parkinson’s severity and duration. Whereas, reduced foveal NFL thickness was reported in Alzheimer’s and in patients with mild cognitive impairment. Another study found a strong correlation between the reduction in macular NFL and GCL with Huntington’s progression (Ding et al., 2014, Pilat et al., 2016, Slotnick et al., 2015, Salobrar-Garcia et al., 2015, Gulmez Sevim et al., 2018). For these studies, only patients with normal or near normal visual acuity (logMar 0.3) were included.

1.5.4 Foveal hypoplasia

FH is a congenital structural abnormality. It is characterized by the complete or partial bilateral absence of the foveal pit due to the persistence of inner retinal layers resulting in a flat retina. It results in visual abnormalities from early infancy and is often associated with the absence of foveal pigmentation, absence of a FAZ, and nystagmus (McTrusty et al., 2013, Perez
et al., 2014, Oliver et al., 1987, Azuma and Nishina, 1996). FH is rarely described as an isolated entity, rather, it is more commonly reported with other diseases such as achromatopsia, Aland eye disease, albinism, Amish microcephaly, aniridia, anterior segment dysgenesis, Chediak-Higashi, Hermansky Pudlak syndrome, incontinentia pigmenti, microphthalmia, Pierson syndrome, Stickler syndrome, Pelger-Huët anomaly, and retinopathy of prematurity (Gregory-Evans and Gregory-Evans, 2011, Shah et al., 2016, Matsushita et al., 2017, Park and Lee, 2017).

Advancement in OCT imaging (Figure 1.3) has led to the development of a FH grading scheme (Figure 1.4) (Thomas et al., 2011). Briefly, the fovea is graded from 1 to 4 based on the presence or absence of three structural landmarks: 1) the pit; 2) cone packing; and 3) cone outer segment lengthening (OSL). Accordingly, grade 1 is characterized by the incomplete extrusion of inner retinal layers leading to a shallower foveal pit. Grade 2 is identified by the complete absence of the foveal pit leading to a flat retina. Grade 3 is when the foveal cones are not fully developed as evident with shorter OS. Finally, grade 4 is characterized by the absence of ONL widening which represents the loss of cone migration to the foveal region (Figure 1.4).

Figure 1.3 Advancement in foveal OCT-imaging
A representation of older-lower resolution (left image; (Recchia et al., 2002)) versus newer-higher resolution (right image; in-house image) images of foveal structure. Note that the retinal layers are more prominent on the right image with more foveal details compared to the left image. CC, connecting cilium; ELM, external limiting membrane; fH, fibers of Henle; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS/OS, inner-segment/outer-segment; ISL, inner segment lengthening; NFL, nerve fiber layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; OSL, outer segment lengthening; RPE, retinal pigment epithelium.
The normal fovea (Figure 1.3, right) has a deep pit, thick ONL, cone ISL, and cone OSL. Whereas in grade 1 (upper left), the pit is shallower due to the presence of residual inner retinal layers. Grade 2 (upper right) the fovea is flat with ONL thickened, ISL, and OSL. Grade 3 (lower left) the only foveal feature present is the ONL thickening. Grade 4 (lower right) is when all foveal landmarks are lost. Yellow numbers indicate the presence of different features: 1) the pit, 2) ONL thickening, 3) ISL and, 4) OSL.

1.5.5 Genetics of foveal hypoplasia

To date, cases of FH have been linked to just two genes, SLC38A (solute carrier family 38 member 8) (OMIM# 609218) and PAX6 (OMIM# 136520).

1.5.5.1 The SLC38A8 gene

SLC38A8 is located on chromosome 16q23 and was recently linked to FH with or without other ocular abnormalities. The gene encodes a putative sodium-dependent neutral amino acid transporter with eleven transmembrane domains, an extracellular N-terminus and an intracellular C-terminal tail. The protein is expressed in the entire retina with higher expression in the IPL,
OPL, and photoreceptor layer. The mode of inheritance in patients with SLC38A8 mutations is autosomal recessive with either homozygous or compound heterozygous mutations (Perez et al., 2014). To date, there are 35 pathogenic variants identified in SLC38A8 and these are deposited in the national center for biotechnology information - clinical variants database: (ClinVar database: http://www.ncbi.nlm.nih.gov/clinvar).

1.5.5.2 The PAX6 gene

A better understood gene, PAX6 is part of a family of nine genes (PAX: paired box) coding for tissue specific transcription factors that orchestrate complex processes during early development. The PAX family is characterized by the presence of two DNA binding domains, a paired domain and a partial or a complete homeodomain (Blake and Ziman, 2014).

PAX6 (chromosome 11p13) is essential for the developing brain, eye, olfactory system, spinal cord, and pancreas. It is one of few master regulatory genes that work at the top of genetic networks to direct cell type specification and differentiation “i.e. molecular switch” by interacting with other genes with the appropriate cis-regulatory sequence. It controls the expression of a wide range of molecules, including transcription factors, signaling molecules, hormones, and structural proteins. It has been implicated in different key biological processes such as cell proliferation, migration, and differentiation in diverse neuronal and non-neuronal tissues. PAX6 spans about 28kb with 14 coding exons. It has two different 5’-promoters (P0 and P1) and one internal promoter (Pα), and therefore able to produce different spliced mRNAs including the canonical PAX6 and the PAX6-5a. The canonical PAX6 has four functional domains; two DNA binding domains (paired and homeodomains) which are connected by a

Tracking PAX6 expression during human eye development has revealed the following: at 6 weeks of gestation, PAX6 expression was seen in the surface ectoderm, lens vesicle, inner and outer layers of optic cup, and optic stalk. Whereas at 8 to 22 weeks of gestation, expression was located at the corneal epithelium, conjunctiva, lens, and non-pigmented ciliary epithelia. Notably, it is expressed in the entire developing retina between 8 to 10 weeks of gestation and in the GCL and INL after 21 weeks of gestation (Nishina et al., 1999).

To date, hundreds of pathogenic mutations have been identified across the whole PAX6 gene and these have been deposited in the Human PAX6 allelic variant database (LOVD database: http://lsdb.hgu.mrc.ac.uk/home.php?select_db=PAX6). About two-thirds of PAX6 variants are transmitted through germline mutations and the rest are de-novo mutations (ClinVar database). PAX6 haploinsufficiency leads to a broad spectrum of dominantly inherited eye abnormalities including ocular coloboma, optic nerve coloboma, optic nerve hypoplasia, morning glory disc anomaly, anterior segment dysgenesis, cataract with late onset corneal dystrophy, aniridia, keratitis, and FH (OMIM# 607108). Other recently identified non ocular phenotypes was also linked to PAX6 gene including in sensory, neural, cognitive, and pancreatic diseases (Bobilev et al., 2016). For instance, a smaller pineal gland, lower melatonin secretion, and sleep disturbance was reported in several patients with PAX6 heterozygous mutations (Hanish et al., 2016).

Pax6 is highly conserved among vertebrates and invertebrates and is labelled as a master control gene for eye morphogenesis. Interestingly, different Pax6 genes from different animal phyla are capable of inducing ectopic eye formation (Gehring and Ikeo, 1999). In 1995, Halder
and co-workers induced ectopic eye on different parts of the fruit fly (*Drosophila melanogaster*) including the wings, legs, and antennae by specifically overexpressing the *Drosophila eyeless* gene (*Pax6* homologue) and the mouse *Pax6* gene. Mammals have a single *Pax6* gene whilst *Drosophila* and other holometabolous insects have two *Pax6* paralogs known as the eyeless (*ey*) and twin of eyeless (*toy*). Another study was able to generate an ectopic eye structure by injecting the *Drosophila ey* and *toy* mRNA into *Xenopus* embryos at the two-cell-stage embryos (Onuma et al., 2002).

A number of vertebrate models have been used to study the functions of the *Pax6* gene. In a mouse model, *Pax6* spontaneous or induced mutations give rise to ocular abnormalities similar to those seen in aniridic patients. For instance, *Pax6*<sup>sey/+</sup> mice carry a spontaneous mutation in one allele of the *Pax6* gene where guanine is changed to thymine, which may changes the normal codon for glycine at position 194 to a stop codon, resulting in nonsense mediated decay (Figure 1.5). These mutant mice have smaller eyes with poor ocular morphology including: a smaller lens that is incompletely separated from the overlying cornea, opaque and hypertrophic cornea, morphologically abnormal retina, and a smaller and less myelinated optic nerve. Remarkably, homozygotes *Pax6*<sup>sey/sey</sup> can reach full term but lack both eyes with poorly developed nasal cavities; therefore, they die soon after birth as they cannot breathe while suckling (Roberts, 1967, Hill et al., 1991).

Zebrafish has two *Pax6* homologs, *Pax6.1* and *Pax6.2*. Twelve amino acids substitutions in a highly conserved region are associated with the *Pax6.2* gene. Both Pax 6.1 and 6.2 are similarly expressed in the eye and the central nervous system (Nornes et al., 1998). In zebrafish, knocking down either of the homolog with morpholinos results in a smaller eye as well as other central nervous structures with early lethality (Coutinho et al., 2011).
In *Xenopus tropicalis*, knocking out *Pax6* also generated an aniridia-like-phenotype in the *Pax6* partial state and lethality in *Pax6* null state (Nakayama et al., 2015). Unfortunately, neither mouse, *Xenopus* nor zebrafish possess a fovea, thus these models are not suitable to look at the foveal development and genetic manipulation. They are however useful for studying *Pax6* function during eye development.

**Figure 1.5 *Pax6*<sup>Sey/+</sup> mouse model genotype and ocular phenotype**

The mouse *Pax6* gene encodes 12 exons with translation starting at exon 3. In this mouse model (*Pax6*<sup>Sey/+</sup>) the mutation is located at exon 7, changing the amino acid glycine (GGA) to a stop codon (TGA). This results in a truncated protein with 193 amino acids instead of the full length 423 amino acids. Ocular phenotypes include small eye and corneal opacity. UTR: untranslated region.

*Pax6* is involved in the development of different tissues. It interacts with various transcription factors and genes to regulate different cellular processes through its two DNA binding domains and the potent transcription activation domain in the C terminus. *Pax6* can act as a transcriptional activator (MITF RPE-promoter element; Baumer et al., 2003) and repressor (several β-Crystallin genes and L-MAF; Duncan et al., 1998, Duncan et al., 2004, Reza et al., 2002). It has an interactive role in different signaling pathways. For example, it is a downstream target of the *Wnt/β-catenin* pathway to facilitate self-renewal and neurogenesis during development (Gan et al., 2014). Hedgehog signaling is also influenced by the loss of *Pax6* allele.
leading to corneal phenotype (Kucerova et al., 2012). Tgfβ2, Bmp4, and Foxc1 are direct downstream targets of Pax6 in the developing iris and ciliary body (Wang et al., 2017).

It is also regulated by other factors such as HIPK2, Pax2 and Six3 (Kim et al., 2006, Schwarz et al., 2000, Goudreau et al., 2002, Ashery-Padan et al., 2000). Additionally, Smad3 can represses autoregulation of the Pax6 P1 promoter (Grocott et al., 2007). While shuttling of Pax6 between nucleus and cytoplasm is influenced by TGFβ and SPARC via the TGFβ/Smad signaling pathway.

1.5.6 Comparative studies on foveated animals

The fovea is one of three different types of specialized retinal topographies seen in different vertebrates. Linked to animal lifestyle, these topographies are the visual streak, area centralis, and fovea centralis (Hughes, 1977). Comparative histology suggests that those species living in thick forests and can’t see the horizon tend to have an area centralis, whereas those living in open plains tends to have a visual streak. The visual streak is a horizontal band with high density of ganglion cells in the naso-temporal axis of the retina and is found in animals such as the rabbit and eastern chipmunk (Provis, 1979, Hughes, 1971, Wakakuwa et al., 1985). The area centralis tends to have both ganglion cells and photoreceptors concentrated in the temporal retina in an area aligned with the central visual field. The area centralis is found in predatory animals such as cats (Stone, 1978, Hughes, 1975). Interestingly, a combination of both visual streak and area centralis is found in animals living in habitats where sight is obscured by vegetation such as in nocturnal, insectivorous, and fruit eating species including different Australian marsupial species, the ox, horses, and pigs (Tancred, 1981, Kassab and Sugita, 2000).
In the latter category, some species have one feature more than the other as found in strepsirrhine primates (lemurs, lorises, galagos) where more area centralis is present than visual streak.

The fovea centralis, as explained earlier, is a more developed retinal topography with two main features, a pit lacking the inner neuronal layers with increasingly packed cones. It is a characteristic feature in all primates except in the nocturnal owl monkey (Penfold and Provis, 2005). It is also present in other species such as in some teleost fishes (e.g. Girella species), birds, and reptiles. Furthermore, diurnal raptors such as hawks, eagles, and falcons and some lizard species are bi-foveate (i.e. having two foveas). In these bi-foveated animals, the central fovea is deep and employed in the lateral monocular vision whereas the temporal fovea is employed in the frontal field of view (i.e. for binocular vision). Furthermore, foveated birds and fishes usually have smaller pit diameter with steeper walls (convexiclivate). For example, pigeons, ostriches, and northern blue jay birds have shallower pit than human and presumably poorer acuity. This is in contrast to the wedge-tailed eagle that have better visual acuity than primates due to higher cones density and steep structure of the fovea. In addition, many vertebrates’ foveas contain residual INL and GCL across the foveola (Woollard, 1927, Rohen and Castenholz, 1967, Wolin and Massopust, 1970, Dkhissi-Benyahya et al., 2001).

1.5.7 Fovea development

Most of our current understanding originates from a limited number of human and monkey fetal and postnatal tissues (Hendrickson 1992). In general, primate fovea is formed by migration and maturation of various cell types during development. In general, the foveal pit formation and peak cone migration occurs between late pregnancy up until early postnatal life.

In humans, foveal cells become post mitotic by the 10th fetal week. By the 11th fetal week
rod photoreceptors are already absent from the foveal centre and present at the foveal edge. At this stage, foveal cones are short and cuboidal. Later, during the 22 fetal week, the incipient foveal region is defined by the accumulation of ganglion cells which results in a hump at the retinal surface. Then the actual pit invaginates by the 25th fetal week in the avascular region due to peripheral migration of inner retinal layers including the ganglion cells. After birth, cone photoreceptors migrate in high-numbers toward the foveola. And lastly, elongation and thinning of cone IS and OS continues, so at about 15 months after birth it matches the peripheral cone characteristics, and are 4 times longer by 13 years after birth (Figure 1.6). Interestingly though, foveolar cones continue to thin and elongate throughout life as shown in one study where they compared early-staged fovea with a 72 years of age fovea (Hendrickson et al., 2012, Penfold and Provis, 2005).

In comparison, tracking foveal development in closely related Macaca species (nemestrina, fascicularis, and mulatta) revealed a large number of ganglion cells accumulating at the incipient foveal region, temporal to the optic disc by mid-gestation (74/~168 fetal days) (Bumsted and Hendrickson 1999). The first foveal depression was seen approximately 50 days before birth, again due to peripheral displacement of ganglion, amacrine, horizontal, Müller, and bipolar cells. During this time frame, cones are migrating into the foveal region. Although the actual foveal depression is formed shortly after birth, cones are constantly migrating toward the foveal region up until three months after birth where cone basal axon are elongated, IS are thinned to half of those seen at birth, and IS/OS are increased in numbers to fourfold (Bumsted and Hendrickson 1999). A pure-cone area decreases in size with increase in age, reaching 200μm in diameter by 8 weeks after birth and remaining unchanged into adulthood (Hendrickson and Kupfer, 1976). A recent longitudinal study looked at postnatal fovea maturation in Macaca
mulatta using OCT (Patel et al., 2017) and showed the following interesting findings. Most morphological changes occurred from 120 days post-gestation and continued up until 1 year after birth. Between 0-18 months of age, the mean retinal thickness at the foveola increased by 21.4% with corresponding decrease in the pit depth to 20.3%.

Tracking retinal vascularization is another interesting topic that is related to foveal development. Blood vessels migrate and proliferate emerging from the optic disk in response to increase in oxygen demands of the developing retinal cells (Madan and Penn, 2003). Retinal vascularization beings at 14-16 weeks post-conception. The developing foveal region was shown to express anti-proliferative and anti-angiogenic factors which prevent capillary growth within the foveola during the formation of the FAZ (Figure 1.7). Thus, the foveola is normally avascular throughout development as it is in adult life. Consequently, abnormal foveal vascularization is not due to failed vascular regression, but rather due to vascular growth. The FAZ is about 500 μm in diameter at 35 weeks gestation and 300-350 μm at 40 weeks of gestation (Provis and Hendrickson, 2008, Provis et al., 2013). The FAZ is a roundish, well-defined area surrounded by the perifoveal capillary plexus. Several studies suggested that the absence of the FAZ leads to absence of a foveal pit, hence reduced visual acuity (Mietz et al., 1992, Azuma and Nishina, 1996, Meyer et al., 2002, McGuire et al., 2003, Meyer et al., 2003). Furthermore, abnormal foveal structure in 24 preterm children born at 23-27 weeks post-conception has been reported (Yanni et al., 2012). These children showed shallower and less steep foveal pits with decreased FAZ diameter.
At 22 fetal weeks, a hump is formed due to accumulation of ganglion cells at the incipient foveal region. At 28 fetal weeks, the inner retinal layers are slightly displaced from the centre forming the initial foveal pit. During development, the pit deepens as more cells migrate from the centre to the periphery leaving only ONL as seen in 15 and 45 months after birth cartoons. Cones (red, green, and blue circles) migrate in high numbers as shown in 45 months after birth, which also represents the blue cone free zone. Cones start to form their IS/OS prenatally, which fully elongate and thin postnatally. The choroid is also thickened at this region to meet the demands of thickened ONL. Chor, Choroidal vasculature; fH, Fibers of Henle; GCL, Ganglion cell layer; INL, Inner nuclear layer; ONL, Outer nuclear layer; OS, Outer segment; RPE, Retinal pigment epithelium. Used with permission from (Gregory-Evans and Gregory-Evans, 2011).
1.6 Research goals and overall significance

Surprisingly, little is known about the cellular and molecular signaling pathways that regulate tissue remodeling to form a fovea. This is mainly due to limited access to developmentally-staged fetal human tissue, in addition to absence of a foveal structure in most routinely used animal models. Better understanding of the fovea may assist in finding a cure for FH from early infancy, otherwise destined for poor vision. Therefore, in this dissertation I set out the following aims: 1) to understand the cellular and molecular basis of FH in affected patients (covered in Chapter 2 and 3), 2) to test the potential benefits of correcting the foveal gene (PAX6) from early development (covered in Chapter 4), and lastly 3) to characterize a novel reptile model to better understand the fovea at the cellular and molecular levels (covered in Chapter 5).
Chapter 2: CORRELATION OF NOVEL PAX6 GENE ABNORMALITIES IN ANIRIDIA AND CLINICAL PRESENTATION

2.1 Chapter goals

FH (OMIM 136520) is rarely diagnosed in isolation. It is often presented with other ocular diseases such as aniridia. Although it is considered as a rare disease, in fact, FH is often reported in aniridia patients (Nelson et al. 1984). In addition, aniridia patients across British Columbia are continually referred to the Eye Care Centre (Vancouver General Hospital) where this research is conducted for proper management of the disease. Therefore, we employed aniridia as a disease model to investigate the cellular and molecular changes in foveal abnormalities.

This work is divided between two chapters each with different goals. In this chapter (Chapter 2) more clinically oriented data is presented investigating the general genotype and phenotype of 33 aniridia patients with a focus on FH. While in the following chapter (Chapter 3), the goal was to characterize the molecular defects presented in chapter 2 in conjunction with similar molecular-foveal work found in the literature. Defining the link between aniridia and FH at the genetic level is essential knowledge for better understanding of foveal abnormalities in general.

2.2 Overview

Aniridia (OMIM 106210) a congenital, bilateral, panocular condition (Nelson et al., 1984). About two-thirds of cases are inherited in an autosomal dominant manner. The rest is
sporadic with no previous family history of the disease (Valenzuela and Cline, 2004). Population based studies in the USA and Europe have estimated the prevalence of the condition to be 1:64 000 to 1:96 000 (Nelson et al., 1984, Eden et al., 2008). Iris hypoplasia comprises minor iris anomalies (pigmentary defects, stromal defects, coloboma), remnant iris root tissue, or complete absence of the iris (total aniridia) (Nelson et al., 1984, Ivanov et al., 1995, Hingorani et al., 2012, Kokotas and Petersen, 2010). Other common ocular manifestations include nystagmus, cataract, glaucoma, corneal opacification, and FH (Hingorani et al., 2009, Lee and Colby, 2013, Gupta et al., 1998, Park and Oh, 2013). Systemic abnormalities have also been observed, including interhemispheric brain anomalies, olfactory defects, obesity, and diabetes (Sisodiya et al., 2001, Peter et al., 2013, Netland et al., 2011). Aniridia may also coexist with various systemic syndromes, such as Gillespie syndrome (partial aniridia, cerebellar-ataxia, and mental retardation) (Glaser et al., 1994), and WAGR syndrome (Wilms tumour, aniridia, genitourinary malformations, and mental retardation) (Riccardi et al., 1978).

Aniridia is caused by heterozygous mutations in the PAX6 gene (Jordan et al., 1992), its regulatory elements, or, more rarely, by chromosomal defects (Ton et al., 1991, Fantes et al., 1995, Lauderdale et al., 2000, Kleinjan et al., 2001) leading to haploinsufficiency of the PAX6 transcription factor. In 8% – 20% of cases, no defects in PAX6 have been identified (Traboulsi et al., 2008, Bobilev et al., 2016, Ansari et al., 2016). Variable expressivity in phenotype has been well documented and does not appear to correlate well with genotype. In addition to its role in eye development, PAX6 is also important in brain, olfactory, and pancreas development, which may provide a rationale for the condition’s coexistence with various systemic syndromes (Hingorani et al., 2012, Hittner et al., 1980).
The literature investigating the FH associated with aniridia remains scarce. Foveal development begins during midgestation and continues in postnatal life, reaching full cellular maturity by approximately 4 years of age (Yuodelis and Hendrickson, 1986). FH describes a foveal pit or depression that is partially or completely absent (Gregory-Evans and Gregory-Evans, 2011). Despite the implications of FH on vision impairment, the process by which arrested development of the fovea occurs remains unknown. Spectral domain optical coherence tomography (SD-OCT) has improved evaluation of FH (Recchia et al., 2002, Thomas et al., 2011). An SD-OCT-based grading system of FH has been proposed (Thomas et al., 2011), which suggests a relationship between the gradation of FH and visual acuity. However, the gradation of FH in most studies using this tool has been limited to that seen in albinism (Harvey et al., 2006, Chong et al., 2009). We undertook an assessment of the ocular clinical presentation and genotype of individuals with aniridia living in British Columbia to study the association of aniridia, grade of FH, and genotype.

2.3 Materials and methods

Participants with aniridia were prospectively enrolled into a clinical and genetic study. Seven other affected family members were subsequently recruited, totaling 33 participants who were examined. Informed written consent for clinical investigations was obtained from those enrolled, in accordance with the University of British Columbia Clinical Research Ethics Board and the tenets of the Declaration of Helsinki.
2.3.1 Clinical investigations and imaging

Full ophthalmic examinations were performed. Posterior segment SD-OCT imaging was undertaken *in vivo* using a Spectralis SD-OCT platform (Heidelberg Engineering, Carlsbad, Calif.). In those with an absent foveal pit, scans of the posterior pole were centred approximately 3 mm directly temporal from the edge of the optic nerve head. Two ophthalmologists (Kevin Gregory-Evans and Christopher J Lyons) with expertise in evaluation of posterior segment SD-OCT imaging independently graded FH as per the grading system previously described (Thomas et al., 2011). Briefly, images were graded on a scale of 0 to 4, where 0 is normal foveal architecture, grade 1 shows a shallow foveal pit, grade 2 features no foveal pit, grade 3 has no foveal pit plus loss of photoreceptor outer segment thickening at the fovea, and grade 4 is a complete absence of differentiation at the fovea.

2.3.2 PAX6 sequencing analysis

Genomic DNA was extracted either from buccal swabs (DNA Isolation Kit, Isohelix, Harrietsham, UK) or from 10 mL EDTA blood samples using a Nucleon BACC Genomic DNA Extraction Kit (GE Healthcare, Baie d’Urfe, Que.). *PAX6* sequencing was performed using primers (Table A.1; Appendix A) as previously described (Love et al., 1998, Robinson et al., 2008).

2.3.3 Multiplex ligation-dependent probe amplification

A commercially available P219 multiplex ligation-dependent probe amplification (MLPA) protocol (MCR Holland, Amsterdam, Netherlands) was used to detect chromosomal duplications or deletions in the *PAX6* genomic region when sequencing of *PAX6* failed to detect
abnormalities as previously described (Redeker et al., 2008). Each MLPA analysis included 3 control DNA samples from healthy individuals.

2.3.4 Quantitative polymerase chain reaction

Deletions detected with MLPA were confirmed using a quantitative real-time polymerase chain reaction (PCR) assay with predesigned TaqMan probes to target multiple loci of the suspected deleted region (Table A.2; Appendix A). Reactions were analysed on a ViiA7 real-time PCR system (Applied Biosystems), and results were assessed using CopyCaller software v2.1 (Life Technologies).

2.3.5 Cytogenetic analysis

In one family, standard fluorescence in situ hybridization was carried out on cultured lymphocytes using bacterial artificial chromosome probes: RP11-133E13, RP11-915F11, and CTD-2643K156. PCR-mediated amplification of microsatellite markers in the WAGR region were analyzed by gel electrophoresis as follows: tel-D11S914, PAX6, WT1, D11S4101, D11S672, D11S1301, and D11S907-cen. In a second family, chromosomal microarray analysis using the CytoScan HD array platform was used to identify the extent of a deletion on chromosome 11. Chromosome Analysis Suite version 1.15.1 (Affymetrix, Santa Clara, Calif.) was used in the data analysis.
2.3.6 Candidate gene and single nucleotide polymorphisms sequencing in PAX6-negative subjects

Gene-specific PCR-amplified products were generated and screened by direct sequencing using primers and conditions, as previously described, or were redesigned in-house (Table A.3; Appendix A) using the Primer3 Program (http://bioinfo.ut.ee/primer3-0.4.0/). These candidates have previously been associated with aniridia (FOXC1, PITX2) (Weisschuh et al., 2006), anterior segment dysgenesis (BMP4) (Mangino et al., 1999), or isolated FH (SCL38A8) (Poulter et al., 2013) or were previously found to be downstream targets of PAX6 in the iris (BMP4, TGFβ2) (Wang et al., 2017b).

2.4 Results

2.4.1 Subject characteristics and clinical examination

Thirty-three participants of various ethnic backgrounds living in British Columbia with a diagnosis of aniridia were recruited over a 2-year period (Table 2.1). The average age of the cohort was 25 years, and 50% of the cohort was male. There was a family history of disease in 56% of cases. Nystagmus was found in 19 participants (58%), and keratopathy was seen in 31 eyes (41% of eyes). This keratopathy was a peripheral corneal neovascular pannus in all 29 eyes. In addition, mild stromal opacities were seen in 7 of these 29 eyes. A further 4 of the 29 exhibited advanced or severe stromal scarring (patients A3, A16, A27, and A33; Table 2.1); all consequently underwent keratoplasty (using Boston Keratoprosthesis in the cases of A16 and A27). Total absence of iris tissue was noted in 42 eyes (64%). In those with partial loss of iris tissue (Figure 2.1), a remnant of iris root tissue was seen in 16 eyes (24%), iris coloboma was evident in 6 eyes (9%), and milder iris stroma defects only were seen in 2 eyes (3%). Cataract or
intraocular lens *in situ* was evident in 48 eyes (72%), and a history of glaucoma was elicited in 48%. No cases with optic nerve anomalies (e.g., hypoplasia, coloboma) were seen. Glaucomatous optic nerve changes, however, were present in all eyes with a history of glaucoma (48%).
Table 2.1 Patient characteristics and clinical findings

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
<th>BCVA L/R</th>
<th>Nystagmus</th>
<th>Keratopathy</th>
<th>Iris defect</th>
<th>Cataract</th>
<th>Glaucoma</th>
<th>FH grade</th>
<th>Eyes with FH L/R</th>
<th>PAX6 genetic abnormality</th>
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<td>A1</td>
<td>F</td>
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<td>-</td>
<td>Remnant/aniridia</td>
<td>+/-</td>
<td>+/-</td>
<td>4/4</td>
<td>Bilateral FH</td>
<td>Ex9 ELP4: Ex4 DCDC1</td>
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<tr>
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<td>F</td>
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<td>Aniridia/aniridia</td>
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<td>+/-</td>
<td>1/1</td>
<td>Bilateral FH</td>
<td>Ex9 ELP4: Ex4 DCDC1</td>
<td></td>
</tr>
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<td>+/-</td>
<td>Scar/normal</td>
<td>No FH</td>
<td>Not found</td>
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<tr>
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<td>+/-</td>
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<tr>
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<td>+/-</td>
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<td>+/-</td>
<td>Normal/Normal</td>
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<td></td>
</tr>
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<td>+/-</td>
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<td>+/-</td>
<td>4/4</td>
<td>Bilateral FH</td>
<td>PAX6 del - D11S914</td>
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<td>+/-</td>
<td>4/4</td>
<td>Bilateral FH</td>
<td>Ex4 PAX6: Ex9 ELP4</td>
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<tr>
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<td>Bilateral FH</td>
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<td>+/-</td>
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<td>+/-</td>
<td>No FH</td>
<td>c.112delC; p.Arg38Glyfs*16</td>
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<td>80</td>
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<td>Normal/Normal</td>
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<td>8</td>
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<td>-</td>
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<td>+/-</td>
<td>+/-</td>
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<td>c.112delC; p.Arg38Glyfs*16</td>
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<td>Scar/scar</td>
<td>No FH</td>
<td>Ex9 ELP4: PAX6</td>
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<td>+/-</td>
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<td>c.53G4T; p.Gly18Val</td>
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<td>Bilateral FH</td>
<td>c.1058C4A; p.Ser353*</td>
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<td>Remnant/remnant</td>
<td>+/-</td>
<td>+/-</td>
<td>4/4</td>
<td>Bilateral FH</td>
<td>Ex6 PAX6: Ex4 DCDC1</td>
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<tr>
<td>No.</td>
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<td>Age</td>
<td>BCVA LogMAR L/R</td>
<td>Nystagmus</td>
<td>Keratopathy</td>
<td>Iris defect</td>
<td>Cataract</td>
<td>Glaucoma</td>
<td>FH grade</td>
<td>Eyes with FH L/R</td>
<td>PAX6 genetic abnormality</td>
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</tr>
<tr>
<td>A20</td>
<td>F</td>
<td>33</td>
<td>1.0/1.3</td>
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<td>+/-</td>
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<td>4/4</td>
<td>Bilateral FH</td>
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<td>aniridia/aniridia</td>
<td>+/-</td>
<td>+/-</td>
<td>4/1/4</td>
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<td>c.40_41delGT; p.Val14Leufs*41</td>
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<td>coloboma/remnant</td>
<td>+/-</td>
<td>+/-</td>
<td>normal/scar</td>
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<tr>
<td>A24</td>
<td>M</td>
<td>4/12</td>
<td>FL/FL</td>
<td>+</td>
<td>+/-</td>
<td>remnant/remnant</td>
<td>-/-</td>
<td>+/-</td>
<td>+/-</td>
<td>3/3</td>
<td>11p14.2 - p12 del</td>
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<tr>
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<td>M</td>
<td>48</td>
<td>HM/HM</td>
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<td>aniridia/aniridia</td>
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<td>c.718C4T; p.Arg240*</td>
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<td>aniridia/aniridia</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>3/3</td>
<td>c.781C4T; p.Arg261*</td>
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<td>aniridia/aniridia</td>
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<td>+/-</td>
<td>3/3</td>
<td>c.781C4T; p.Arg261*</td>
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<td>A28</td>
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<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>c.781C4T; p.Arg261*</td>
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<tr>
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<td>3/4</td>
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<td>+/-</td>
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<td>+/-</td>
<td>aniridia/aniridia</td>
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<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>c. 961_962insGTTT; p.Ser321Cysfs*34</td>
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</table>

Grayed entries indicate novel mutations identified in this study. BCVA, best-corrected visual acuity; NR, not recorded; - unable to obtain imaging; FL, follows light; CF, counting fingers; HM, hand motion; LP, light perception; NLP, no light perception.
Figure 2.1 Iris phenotype variability observed in the study cohort

(A) Iris phenotype observed in patient A5 (p.Ser65Profs*14). (B) Remnant iris observed in patient A6 (no PAX6-related mutation identified). (C) Ring iris defect observed in patient A13 (p.Pro399Pro). (D) Remnant iris observed in patient A14 (Ex9 ELP4 - Ex4 DCDC1 deletion).

2.4.2 Assessment of foveal hypoplasia

SD-OCT imaging was obtained in 56 eyes. Anterior segment disease, which limited the view of the posterior pole; young age of the participant; or severe nystagmus precluded imaging in others. SD-OCT imaging revealed that FH was present in 45 eyes (80%). In the other 11 eyes, 6 fovea were considered normal, and scar tissue (unrelated to aniridia) was recorded in the remainder. In this latter group, macular scarring was attributed to previous retinal vein occlusions (A3 and A23), bilateral retinal detachments (A16), and wet macular degeneration (A29). Most eyes with FH were observed to exhibit severe hypoplasia (grade 4, 28 of 44 eyes; Figure 2.2 and Figure 2.3). Agreement between raters for gradation of FH was good (κ = 0.60).
Figure 2.2 Posterior segment spectral domain-optical coherence tomography (SD-OCT) imaging of patients with novel PAX6 mutations

(A) SD-OCT imaging right eye of patient A5 (p.Ser65Profs*14) demonstrating significant FH (grade 4). (B) SD-OCT imaging right eye of patient A17 (p.Gly18Val) demonstrating significant FH (grade 4). White arrow denotes thickening of the outer nuclear layer. (C) SD-OCT imaging of the right eye of patient A29 (p.Met337Argfs*28) demonstrating significant FH (grade 4).

Figure 2.3 FH phenotypic variability observed in the study cohort

Each bar denotes the number of eyes observed with each grade of FH. Two staff ophthalmologists with expertise in evaluation of posterior SD-OCT imaging independently graded observed FH as per the grading system previously described. Anterior segment disease, which limited the view of the posterior pole, or age of the patient precluded the imaging of 10 eyes.
2.4.3 Molecular genetic analysis

PAX6 genetic defects were identified in 91% (30 of 33) of subjects within the cohort (Table 2.1). Three novel PAX6 sequence mutations were identified (Gly18Val; Ser65Profs*14; Met337Argfs*18), one novel insertion (Ser321Cysfs*34) and one synonymous PAX6 sequence change (Pro399Pro). MLPA analysis uncovered 4 different deletions in 9 participants that were either within or downstream of the PAX6 genomic region Figure 2.4 (Redeker et al., 2008, D'Elia et al., 2007). This included 2 parent/sibling families with novel deletions (A9 and A10; A19 and A20). Cytogenetic analysis yielded novel positive results in a further 2 unrelated individuals (A8; A24) (Table 2.1, Figure 2.4). A total of 6 families were enrolled, allowing comparison of phenotypes among family members with the same PAX6 mutation. A1 and A2 were mother and daughter with a DCDC1-ELP4 deletion. Interestingly, the daughter (A2) had mild FH only (grade 1) whereas the mother had severe FH (grade 4). There were no other differences on clinical examination. This same deletion was also seen in 2 other unrelated individuals (A14 and A15), in whom quite variable grading of FH again was seen (grade 1 and grade 3, respectively). In all 4, complete aniridia was seen without keratopathy or glaucoma (despite 2 of the 4 being over 30 years of age). That no cataract was visible in A14 is probably attributable to her young age. In another deletion family (A11 and A12), significant differences in clinical appearance were again seen both in the anterior segment and posterior segment (milder iris abnormalities and milder FH in older family member A11). Contrastingly, in the other 3 families (A19:A20, A26:A27: A28, and A31:A32) clinical features among family members were either nearly identical or could be attributed to age differences. No PAX6 abnormality could be identified in 3 unrelated participants (A3, A6, and A23). In addition, a screen for molecular abnormalities in genes with a functional relationship to PAX6 (PITX2, FOXC1, and SCL38A8) (Weisschuh et al.,
also failed to identify genetic mutations. It was noticeable that in each case, milder iris defects were seen in comparison to PAX6-mutant aniridia cases and that none of the 3 exhibited FH. A Val152Ala polymorphism in BMP4 (Mangino et al., 1999) was identified in A3, A6, and A23; however, this polymorphism was also seen in 80% of the rest of the cohort and is a known non-pathogenic variant. We also took this opportunity to screen PITX2, FOXC1, and SCL38A8 to see whether sequence variants might explain the extreme phenotypic heterogeneity seen in FH in aniridia. A heterozygous p.Val64Asp substitution in SLC38A8 was identified in A2, who had partial FH (grade 1) with good visual acuity (0.4 logMAR right eye, 0.1 logMAR left eye). The participant’s affected mother (A1) had a normal SLC38A8 genotype and significant FH with poorer vision (0.4 logMAR right eye; hand motion left eye). Paternal DNA was not available for testing so it could not be determined if the Val64Asp mutation was a spontaneous mutation or of paternal origin. We could not identify sequence variants in SLC38A8 in the other participant in the study (A14) with grade 1 FH who coincidentally had the same PAX6 deletion as A2 (Ex9 ELP4-Ex4 DCDC1). All participants within the cohort were identified as having the alternate major G alleles and not the minor effect alleles for both SLC38A8 single nucleotide polymorphisms.
Figure 2.4 Chromosome deletions in patients without PAX6 point mutations identified in this study

The gene organization of chromosome 11p is represented (not to scale) with horizontal arrows denoting the direction of gene transcription. DRR (vertical arrows) is the DNA regulatory region containing hypersensitivity sites. D11S914 denotes microsatellite marker. Gray bars indicate the extent of deletion in each patient as detected by multiplex ligation-dependent probe amplification analysis. White bars indicate deletions in patients as determined by cytogenetics analysis. Patient ID number is denoted inside each bar.
2.5 Discussion and conclusion

With an incidence of aniridia of 1:64,000–1:96,000, the 33 cases presented here represent approximately 46%–69% of the estimated cases in British Columbia (population 4.6 million), so they are reasonably representative of aniridia cases across the province. One recent Mexican study (Villarroel et al., 2008) identified PAX6 genetic defects in as little as 30% of cases, whereas in Saudi Arabian (Khan et al., 2011) and Korean studies (Park et al., 2012) PAX6 genetic abnormalities were reported in as many as 88% of cases. In this study PAX6 genetic abnormalities were seen in the majority of cases (91%), higher than in previous reports. I identified 4 novel PAX6 mutations (1 missense, 2 nonsense, and 1 insertion) and 4 novel chromosomal deletions inclusive of PAX6 or a known PAX6 regulatory region.

One structural feature detected in posterior segment SD-OCT imaging used to grade FH is outer segment (OS) lengthening (Thomas et al., 2011). In that study, no cases were reported in which a foveal pit was present without OS lengthening (OSL), suggesting these 2 features are mutually coincident. However, in this study we observed a partial foveal pit, but no OS lengthening in one participant (A2), and accumulation of photoreceptors was present in the region of the expected foveal pit in 2 others (A11, A17). These observations suggest that OS lengthening and foveal pit excavation are 2 distinct events. This is supported by the known timeline of foveal development (Yuodelis and Hendrickson, 1986, Gregory-Evans and Gregory-Evans, 2011): Foveal pit excavation begins at 28 weeks gestation and is complete by birth, whereas outer segments of cone photoreceptors start to form at 36 weeks, but do not reach their full length until approximately 3-4 years of age. The mechanism by which PAX6 regulates various phases of foveal pit formation remains to be determined, but multiple target genes are
likely to be involved in the different phases. It might also be expected that foveal development would be arrested earlier in gestation in those with significant FH.

A broad range of best-corrected visual acuity was found in this study. This is a feature of many ocular syndromes in which acuity may be reduced due to a number of ocular abnormalities: corneal scarring, nystagmus; amblyopia, cataract, advanced glaucoma, and FH are common causes of reduced acuity in aniridia patients. In such a multifactorial condition, it can be challenging to apportion percentages of vision loss to each specific abnormality. In the context of FH, though, it would be expected that the proportion of vision loss that can be ascribed to this abnormality will be relatively small in the context of significant corneal scarring, for example. Furthermore, previous studies have suggested that advanced stages of FH can be associated with relatively good vision (Marmor et al., 2008).

Six independent families were enrolled, and although none were large families and despite previous reports on single, individual families (Hingorani et al., 2009, Davis and Cowell, 1993, Gregory-Evans et al., 2011), this study is one of the few to concurrently study intrafamilial variation in a number of families. Of particular note was the marked variation in FH seen with the ELP4-DCDC1 deletion (family A1/A2 and unrelated individuals A14 and A15). This was seen in the context of total aniridia, but otherwise mild anterior segment changes were observed in each subject. This has led to the hypothesis that the factors (either genetic or environmental) causing variation in the posterior segment are not an influence in anterior segment pathogenesis.

Of particular note were the 3 unrelated cases in which no PAX6 abnormality could be identified (A3, A6, and A23), although an extensive study was not undertaken of PAX6 3’-cis regulatory regions, some of whom can map hundreds of kilobases from the PAX6 gene locus (Kleinjan et al., 2001, Kawano et al., 2007, Chen et al., 2013, Kammandel et al., 1999, McBride
et al., 2011). Therefore, it was noted that these cases without detectable \textit{PAX6} mutations differed clinically from others in this study in that iris anomalies were milder, and none exhibited fovea hypoplasia. It should be noted, however, that some \textit{PAX6} mutant aniridia cases were seen with partial aniridia (A11 and A18) and normal foveal architecture (A13), although not with both.

Previous reports have suggested that non-\textit{PAX6} aniridia-like phenotypes are associated with \textit{FOXC1} and \textit{PITX2} (Weisschuh et al., 2006). However, in this screen abnormalities of these genes or in the functionally related \textit{SCL38A8} gene were not identified. A review of these past cases noted that none have reported classical ocular aniridia (complete absence of iris tissue, juvenile onset glaucoma, epithelial keratopathy, cataract, and frequent \textit{FH}). A \textit{FOXC1} mutation was associated with partial aniridia, aniridia with corneal disease (Peter’s anomaly), or aniridia with congenital glaucoma (not normally present in classic aniridia). Furthermore, \textit{FH} was not reported in these cases (Ansari et al., 2016, Khan et al., 2008, Ito et al., 2009, Arcot Sadagopan et al., 2015). Similarly, in the 2 \textit{PITX2} mutation patients, the phenotypic descriptions were limited with no report on foveal architecture (Traboulsi et al., 2008, Perveen et al., 2000). It is striking to note that in this study all aniridia cases with \textit{PAX6} mutation had abnormal foveal architecture. This calls into doubt the existence of aniridia without \textit{PAX6} mutation and suggests that these other cases might more correctly be labelled as anterior segment dysgenesis, in which the aniridia is only a physical sign of a lack of iris tissue and not indicative of the genetic syndrome of aniridia. This genetic syndrome of aniridia could therefore be defined as a condition with a \textit{PAX6} defect, anterior segment dysgenesis, and \textit{FH} (with additional high risk of keratopathy, cataract, glaucoma, and systemic defects). Whereas the less severe iris hypoplasia with no other clinical presentation and normal \textit{PAX6} are better described as anterior segment
dysgenesis. A larger survey of aniridia patients’ needs to be undertaken to verify this observation.

Although PAX6 genetic abnormalities were first identified more than 2 decades ago, only now is the value of their identification coming to the fore. Screening for 11p13 chromosomal deletions is now routine in patients diagnosed with aniridia because it identifies those at risk for Wilms tumor, prompting regular kidney ultrasound surveillance. Sequencing of the PAX6 gene is often limited, however, because it is seen as having a lesser impact on medical management. This may be about to change as new concepts in therapeutics emerge. It was recently reported a small-molecule, nonsense-suppression therapy in the Pax6Sey mouse model of aniridia (Gregory-Evans et al., 2014) that has now progressed to a clinical trial (ClinicalTrials.gov identifier: NCT02647359). Approximately 40% of those diagnosed with aniridia are identified as having PAX6 nonsense mutations (Tzoulaki et al., 2005); this makes this therapy potentially useful to a significant proportion of individuals with the disease. Such mutation-based therapeutic options mandate more widespread molecular genetic testing and new natural history studies to more closely examine the clinical variability seen in this and other studies.
Chapter 3: ASSESSMENT OF PAX6 ALLELES IN PATIENTS WITH FH

3.1 Chapter goals

In Chapter 2, all PAX6-positive patients showed abnormal foveal architecture. Interestingly, similar PAX6 defects resulted in variable foveal grades between related and unrelated individuals. Therefore, this chapter further characterizes the molecular findings previously presented in Chapter 2 with the following specific goals: 1) to classify PAX6 mutations from our cohort and other molecular-foveal published materials, 2) to correlate those mutations with foveal health, whenever possible.

Work in this chapter may aid in thorough and effective genetic counselling of patients since molecular knowledge is vital for diagnosis, management, and treatment of different clinically overlapping diseases. The end goal of this chapter is to understand the phenotypic consequences of different types of PAX6 mutations.

3.2 Methods

3.2.1 Revisiting molecular findings in Chapter 2

http://promoter.bx.psu.edu/hi-c/) was used to look for potential long-range interaction between PAX6 promoter and different loci in the deleted region.

3.2.2 Literature search for PAX6-related FH

LOVD database was searched for patients with PAX6 results and a report on foveal health. Then each referenced article was visited and information regarding the genotype and foveal grade were extrapolated. If no specific grade was given for FH, we used the term grade 1 or greater. One major limitation of this approach is that we may have missed a few studies that are not deposited in the LOVD database or in the ClinVar database.

3.3 Results

3.3.1 A glimpse into PAX6 mutations in the study cohort from Chapter 2

Characterization of PAX6 mutations has displayed the following features: 37% of mutations identified were in the paired domain, 32% in the homeodomain, 21% in the proline-serine-threonine rich domain, and 10% in the glycine-rich linker region (Figure 3.1). About 90% of mutations identified resulted in a premature stop codon and were spread across all the major domains. Additionally, a missense mutation was detected in the paired domain and a silent variant in the proline-serine-threonine rich domain. In addition, five novel mutations were identified. Among those was a glycine to valine substitution at position 18 of the amino acid sequence, which is a highly conserved amino acid in macaque, mouse, chicken, frogs, zebrafish, Drosophila, and C. elegans species. Besides the clinical phenotype of the patient carrying this mutation (A17; Table 2.1), this mutation is predicted to have a deleterious effect on the PAX6 protein (Table B.1; Appendix B). The other three novel mutations were frame-shifting deletions
or insertions (A5, A29, and A33; Table 2.1) resulting in a premature stop codon and probably a degradable mutant transcript (nonsense mediated decay) (Table B.2; Appendix B). A proline to proline variant at position 399 located at the proline-serine-threonine-rich domain was also identified in one patient with normal foveal architecture and bilateral partial loss of iris tissue (A13; Table 2.1). This might suggest a positive correlation between PAX6 and foveal morphogenesis. However, it does not explain the partial iris phenotype. In relation, some studies have demonstrated that such mutations, i.e. silent variant, might affect protein synthesis, folding, and function (Chevance et al., 2014, Bali and Bebok, 2015). Two potential hypotheses stemming from this finding is that unlike retina, the iris is more sensitive to slight PAX6 dosage changes. Alternatively, this particular silent variant is detrimental for PAX6 interactions with other genes involved in iris formation, but not with genes involved in retinal morphogenesis. However, it is also possible that the iris phenotype is due to other unknown genetic defect(s) or due to exposure to different environmental factors (e.g. infectious, chemical, or physical) during normal iris development (Nelson et al., 1984).

Gross chromosomal deletions were identified in eleven subjects (Figure 2.4) and this information was compared to the human genome using the UCSC genome browser and the BLAST program, for better visualization of different endogenous genomic components (Figure 3.2). Whole PAX6 was deleted in three patients (A8, A16, and A24) and partially deleted in four patients (A9, A10, A19, and A20). In addition to PAX6 complete or partial deletion, a region known as SIMO element (a remote cis-acting regulatory element, OMIM# 617141, (Ton et al., 1991, Fantes et al., 1995, Bhatia et al., 2013)) was also deleted in these patients (Figure 3.2). The largest deletion encompasses 17mb (A24; Table 2.1) and comprises 55 OMIM genes including PAX6. This patient had partial iris loss, keratopathy, cataract, and glaucoma with
no information regarding non-ocular conditions. In this patient retinal imaging was not possible due to the presence of severe anterior segment abnormalities.

In two unrelated families (A1:A2, A14:A15; Table 2.1), a 250kb deletion downstream of PAX6 was found (Ex9 ELP4 - Ex4 DCDC1). This deletion encompasses 4 OMIM genes (DCDC1, DNAJC24, IMMP1L, and ELP4), 5 long-range PAX6 promoter interacting loci (Figure 3.3), and two VISTA enhancers elements. Enhancer element_863 is located within the IMMP1L gene and is expressed in the midbrain of the developing mouse embryo, thus, might have no ocular function. Contrastingly, element_565 is located within the ELP4 gene and is expressed in the midbrain and the forebrain of the developing mouse embryos, suggesting a potential role in ocular development, i.e. the retina originates from the diencephalon of the developing forebrain. However, it is not yet been demonstrated if element_565 drives Pax6 expression in the forebrain (Duan et al., 2013) and more specifically in the developing eye. Interestingly, gene synten analysis of this region also revealed a conservation pattern across human (foveated), mouse (afoveated), and green anole (foveated) (Figure 3.4). It is also possible that the deletion of the 4 OMIM genes be the cause directly rather than the loss of an enhancer elements.
Human PAX6 is segmented into three major domains and yields 422 amino acids (Ensembl transcript id: ENST00000638914.1). Most mutations identified in the study cohort are in the paired and homeodomains. Novel mutations are in red font. Written in parentheses beside the mutation are the number of times this mutation was found in our cohort.

Top black bars represent the deleted region in each patient (Figure 2.4). PAX6 is partially or totally deleted in several patients. SIMO element is also deleted in those patients with partial or total PAX6 deletion. (Chromosome 11 (hg19) deletion coordinates; A1:31329311-31671729, A2:31329311-31671729, A8:31363277-31907189, A9:31671656-31828466, A10:31671656-31828466, A14:31329311-31671729, A15:31329311-31671729, A16:31671656-31907189, A19:31329311-31824382, A20:31329311-31824382, A24:26043425-43443424).
Figure 3.3 Long-range interactions between PAX6 promoter and other genomic regions in erythroblasts

Five interactions were found between PAX6 promoter and different loci in the PAX6 downstream region. The deletion found in the two families is shown by the red bar. The deleted region encompasses five PAX6-interactions and two enhancers. The deleted enhancers are expressed in midbrain (element 863) and in midbrain and forebrain (element 565). PAX6 promoter (red line) is at chr11:31826234-31834881 (hg19). PAX6 promoter-interactions coordinates (hg19) from left arch to right: 31388885-31389367, 31397081-31399258, 31428086-31430642, 31524868-31525183, and 31530655-31532402. Enhancer data was extrapolated from VISTA enhancer browser. Element 863 is located intragenic to ELP4 (hg19 coordinates: chr11:31502035-31503157). Element 565 coordinates are located intragenic to IMMP1L (hg19 coordinates: chr11:31622822-31624118).
Figure 3.4 Interspecies comparison of genes synten and sequence conservation

Mouse and green anole display similar gene synteny to humans’ chr11:30830369-32105755 (hg19). Interestingly, the foveated green anole shows the ddc1 gene, which absent in the mouse region. Human VISTA enhancer element_863 (green rectangle) and its orthologues are located intragenic within the IMMP1L gene in both human (chr11:31502035-31503157, hg19) and mouse (chr2:105753493-105754643, ncbi37) and extragenic in green anole (chr1:60410148-60418627, AnoCar2). Element_565 (brown rectangle) is located within the ELP4 gene in human (chr11:31685357-31686884, hg19), mouse (chr2:105618687-105620196, ncbi37), and green anole (chr1:60595894-60597067, AnoCar2). Genes and enhancer elements are not to scale.

3.3.2 PAX6 and fovea: database mining

This analysis was strengthened by combining the data from this study with data gathered from the LOVD database, which results in 122 unique variants with a report on foveal architecture (Figure 3.5). These foveal-graded variants are distributed across the gene. Fifty-nine (48%) variants are located in the paired domain, 27 (22%) in the proline-serine-threonine rich region, 20 (16%) in the linker region, 15 (12%) in the homeodomain, and 1 (<1%) in the initiation exon. Approximately, 41% of mutations are frameshift, 31% nonsense, 24% missense, and the remainders are in frame insertion/deletion, silent, and c-terminal run-on mutations.

Not surprisingly, since most studies were conducted before the OCT era, detailed descriptive data on foveal architecture is limited. Here we found that only 26/122 PAX6 variants were reported with OCT images or specific foveal grade. The rest (96/122) were examined with an ophthalmoscope, which only can reveal the presence or absence of the foveal pit. In this case,
we termed the absence of foveal reflex as grade one or more (grade 1 or >) (Figure 3.5). The majority of PAX6 mutations (~93%) showed variable degrees of FH. The remainder resulted in either normal foveal architecture (three mutations: Lys86Serfs*35, Gln205*, and Pro282Alafs*86; (Azuma et al., 2003, Perez-Solorzano et al., 2017)) or in both normal and abnormal fovea. The latter includes three nonsense mutations: Arg103*, Gln180*, Arg203* (Kondo-Saitoh et al., 2000, Godavova et al., 2014, Park et al., 2012, Perez-Solorzano et al., 2017), one missense mutation: Gln47Arg (Perez-Solorzano et al., 2017, Vasilyeva et al., 2017), polyphen-2 score = 0.036 (benign)) which is also registered in the ClinVar SNP database as a normal variant, and one in-frame deletion: Lys28del (Wang et al., 2012, MutationTaster = disease causing).
Figure 3.5 Distribution of PAX6 variants

122 variants are dispersed across the PAX6 major domains. 1 mutation in the initiation codon, 59 in the paired domain, 20 in the glycine-rich linker region, 15 in the homeodomain, and 27 in the proline-serine-threonine rich domain. Mutation types are organized in the following order from top to bottom: premature stop, missense, and others. The latter includes in-frame insertion/deletion, silent, and run on mutations. The study cohort mutations are indicated with three asterisks. aa: amino acids.
3.4 Discussion

PAX6 displays DNA-binding complexity because of its multiple DNA-binding domains. It has two major DNA binding domains (paired and homeodomains). In this patient cohort, the majority of variants were in the paired domain. Interestingly combining our PAX6 data with data extrapolated from the database also resulted in more mutations in the paired domain. One relevant use of this observation is for PAX6 testing facilities as they may reduce the cost and time of genetic testing by sequencing the paired domain prior to other domains.

Furthermore, the paired domain carries two functionally independent DNA binding subdomains (PAI and RED). These bind to a 20-base pair DNA sequence known as P6CON (i.e. PAX6 consensus binding site, (ANNTTCACGC(A/T)T(G/C)ANT(G/T)(A/C)N(T/C))). The 5’-half of the paired domain (PAI) recognize 5’-P6CON and the 3’-half (RED) recognize 3’-P6CON. Interestingly, P6CON can also be recognized by other PAX genes including: Pax -1, -2, -5, and -8 (Epstein et al., 1994, Manuel et al., 2015). Higher foveal grades were associated with the 5’ end of the paired domain (PAI). However, since we have a limited number of accurately labeled FH, it is not possible to fully support this observation.

Of particular interest are those reports with PAX6 mutations and a normal fovea. One variant (Gln47Arg) is predicted benign by the PolyPhen2 server while other variants (7 in total) are predicted as disease-causing and yet still show a normal fovea. The mechanism by which PAX6 regulates various phases of foveal pit formation remains to be determined, but multiple genes are likely involved in different phases of foveal development.

A literature search for FH associated with the recessively inherited gene (SLC38A8) has revealed 7 different mutations in multiple patient families: p.(Ile32Ser); p.(Met34Arg); p.(Val236Asp); P.(Ser336Afs*15); p.(Gln200*)/p.(Ala282del); p.(Glu233Lys);
p.(Leu344Cysfs*7); and p.(Gly412Arg)/large deletion. These mutations resulted in grade 4 FH in most cases (about 4/7 families), or were ungraded FH (Poulter et al., 2013, Perez et al., 2014). Therefore, I sequenced the SLC38A8 gene in the study cohort of patients with PAX6-negative FH (Chapter 2), but no mutations were identified. This may suggest that FH has other unidentified causative genes in addition to PAX6 and SLC38A8.

In this study several tissue-specific enhancers were identified upstream, intronic, and more-distally-downstream of PAX6 (up to 165k away from P0 promoter). Interestingly, several microRNAs have been shown to manipulate Pax6 expression level, for example miR-450b-5p targets the 3’-UTR and inhibits Pax6 expression in the corneal epithelium (Shaham et al., 2012). Therefore, we bioinformatically analyzed the deleted region in several patients with no PAX6 related abnormalities and found two possible interacting loci downstream of PAX6. However, these do not correspond with the miR-binding site that has been previously described.

In conclusion, predictive genetic testing for FH is challenging due to the variable expressivity of fovea related genes (PAX6 and SLC38A8). In addition, there are reports of PAX6- and SLC38A8-negative FH, which may suggest the involvement of other genes in the disease etiology.
Chapter 4: RNA TRANS-SPICING: A GENE THERAPY APPROACH TO CORRECT PAX6 MUTATIONS

4.1 Chapter goals

As shown previously in Chapter 2, all aniridic patients with PAX6 mutations presented with abnormal foveal architecture. This suggests that PAX6 is an essential gene for foveal development in addition to its vital role in eye, nose, pancreas, and brain morphogenesis. Because currently there are no treatment options for foveal deficit and by knowing that the fovea develops postnatally for a few years after birth, then, correcting PAX6 defects at an early stage of development might have a beneficial implication on further foveal maturation. Therefore, in this chapter I propose RNA trans-splicing as a novel method to correct the transcription factor PAX6 defects.

4.2 Trans-splicing overview

To express a gene, DNA is first transcribed to pre-messenger RNA (pre-mRNA) which later processed into mRNA. This mRNA is then translated into a chain of amino acids to serve a specific function. For pre-mRNA to mature into mRNA, there are three crucial steps: 1) 5’ end capping, where a guanine is added to the terminal base of the transcript via the guanylyl-transferase, shortly after transcription initiation which is vital for mRNA stability; 2) splicing of intronic sequences which will be explained in the following paragraph and; 3) polyadenylation of the 3’ end which controls mRNA stability and influences translation.
Splicing is an intermediate process, subject to very strict regulations, and is responsible for creating diversity in gene expression. It is a process by which introns are excised out of the pre-mRNA, while coding exons are joined. There are two forms of splicing, the predominant \textit{cis}-splicing and the less common \textit{trans}-splicing.

\textit{Trans}-splicing is a natural form of RNA splicing, where different pre-mRNA transcripts are joined to form a single chimeric mature mRNA (Mansfield et al., 2004, Lei et al., 2016). It was first described in trypanosomes (Murphy et al., 1986, Sutton and Boothroyd, 1986), then in mammals (Caudevilla et al., 1998), and later in humans (Li et al., 1999, Takahara et al., 2000, Finta and Zaphiropoulos, 2002, Flouriot et al., 2002, Wu et al., 2014). In mammals, it was observed in different physiological and pathological processes such as in cancer (Li et al., 2014).

\subsection{Spliceosome-mediated RNA \textit{trans}-splicing (SMaRT)}

The principle of mRNA reprogramming has been applied successfully in RNA-based therapy in different models of human genetic diseases (Berger et al., 2016). The SMaRT approach has the potential to correct genetic defects at the pre-mRNA level with rationally designed constructs known as PTMs (pre-mRNA \textit{trans}-splicing molecules). Often a single PTM is introduced and typically consists of: 1) binding domain (BD), a complementary sequence to guide the construct to the genomic target; 2) splicing essential elements including a branching point (BP), a poly-pyrimidine-tract (PPT) and, a splice site (SS); and 3) a segment or full-length wildtype cDNA sequence lacking the initiation codon. By shuffling these elements, one can replace the 3’, 5’, or specific internal exons of the targeted gene. One of the main advantages of this technique is the use of endogenous regulatory machinery; therefore, it will not alter the targeted-gene expression level. Therefore, in designing the PTM it is important to maximize
*trans*-splicing level and to reduce the endogenous *cis*-splicing (Reviewed in (Mansfield et al., 2004)). Several *in-vitro* (Liu et al., 2005, Rodriguez-Martin et al., 2009, Tockner et al., 2016, Zayed et al., 2007, Rindt et al., 2012) and *in-vivo* studies (Chao et al., 2003, Coady and Lorson, 2010, Lorain et al., 2013, Berger et al., 2015) have employed this approach to rescue different genes.

Here, I employed this endogenously regulated process to correct *Pax6* mutations in a mutant cell line and in a *Pax6* mutant mouse model by delivering a construct carrying the 3’-segment of the mouse *Pax6* wildtype cDNA. This construct is guided by complementary base pairing to an intronic sequence in the host’s *Pax6* gene. I designed several constructs and tested their ability to rescue, at the protein level, a stop codon mutation in a *Pax6* homozygous mutant cell line (*Pax6*<sup>Sey/Sey</sup> mouse embryonic fibroblasts (MEFs<sup>Sey</sup>)). The most efficient PTM was then delivered by intraocular injection into the naturally occurring *Pax6*<sup>Sey/+</sup> mouse model (study overview: Figure 4.1).

![Figure 4.1 RNA trans-splicing study overview](image)

(1) PTMs were made by amplifying different regions of genomic DNA and RNA from wildtype mouse and ligated together in a mammalian expression vector using NEBuilder HiFi assembly cloning system. (2) To test their ability in making Pax6, each PTM was transfected into MEFs<sup>Sey</sup> using lipofectamine 3000®. A construct with full length cDNA was used as a transfection control. (3) The PTM with highest efficiency was then intraocularly injected into *Pax6*<sup>Sey/+</sup> mouse model.
4.3 Materials and methods

4.3.1 Engineering the 3’-PTM-plasmid constructs

The mouse *Pax6* gene is composed of 12 exons with the ATG codon located in exon 3 (Transcript ID: ENSMUST00000111082.7). DNA and RNA were isolated from wild type mouse tissue (brain) using the DNeasy and RNeasy extraction systems (Qiagen) following the manufacturer's protocols. RNA was then converted into cDNA using Quantitect reverse transcription protocol (Qiagen). Briefly, different DNA fragments were amplified from the wildtype DNA and cDNA with overlapping sequences and ligated together into an expression vector driven by a CMV promoter (pIRES2-DsRed2, Clontech) using the NEBuilder HiFi DNA Assembly system (New England BioLabs). PTMs were then sequenced by the Sanger method with primers binding the vector backbone and different loci in the construct. More details about the PTMs and their sequences are included in the appendix (Figure C.1, Tables C.1-C.6; Appendix C). Six different PTMs were created to replace the full length *Pax6* cDNA except exon 3 where the initiation codon is located. Each PTM was composed of: 1) ~150 bases binding to complementary sequence in intron 3; 2) a pre-defined splicing domain with a BP, a polypyrimidine tract, and the 3’ splice site and; 3) wildtype cDNA devoid of the initiation codon. Two of these constructs were made with the 3’ untranslated region (Figure 4.2). Furthermore, an antisense oligonucleotide (ASO) was added to the reaction to bind to the intron3-exon4 boundary, which may prevent cis-splicing and increase the chance of trans-splicing (Figure 4.2).

If trans-splicing occurs, cells should express both Pax6 and a *Discosoma* red fluorescent protein (DsRed2) from one transcript which makes it possible to track trans-spliced *Pax6*. In other words, the presence of the gene (*Pax6*) is synonymous with the presence of red tag (DsRed2).
Each PTM binding target is boxed in red or green in the above sequence. All PTMs have the same essential splicing elements (underlined in the above intronic sequence). A short antisense oligo was created to span the intron-exon4 boundary as indicated above. PTMs with higher efficiencies are marked with an asterisk. BD, binding domain; BP, branch point; PPT, poly-pyrimidine tract; SS, splice site; UTR, untranslated region.

4.3.2 In-vitro PTMs selection

4.3.2.1 Cell transfection

MEFs\textsuperscript{Sey} cell line was generated by our collaborator Dr. Elizabeth Simpson (UBC). In a six-well plate, MEFs\textsuperscript{Sey} cells were transiently co-transfected with a single PTM and an ASO using Lipofectamine 3000 (Thermofisher Scientific) by following the manufacturer’s protocol. Cells were grown in high glucose-containing Dulbecco’s modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1% L-glutamine (Invitrogen), and 1% non-essential amino acids (Invitrogen). In this pilot study, transfection efficiency was not measured.
4.3.2.2 Western blotting

Three days post-transfection, cells were scraped from the incubation plate on ice and transferred to ice-cold lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 1% SDS, 1X Protease Inhibitor Cocktail (Roche)). Cells were then freeze-thawed three times and sonicated (30 secs on:15 sec off) for 5 minutes at 4°C. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay). Fifty micrograms of protein was loaded per lane, separated on a 10% SDS-polyacrylamide gel and then transferred by electrophoresis to Immobilon-FL membrane (Millipore). The membrane was pre-blocked with 3% bovine serum albumin for 1 h at room temperature and then incubated with a 1:500 dilution of a polyclonal antibody against the C-terminus region of the Pax6 protein (901301, Biolegend) overnight at 4°C. After several washes with PBS + 0.1% Tween 20 (PBST), the membrane was incubated with the secondary antibody IRDye800 goat anti-rabbit (61113200, Rockland Immunochemicals). The membrane was next blocked with 5% non-fat milk for 1 h at room temperature and then incubated with anti β-actin antibody at a dilution of 1:3,000 in PBST for 1 h at room temperature. After several washes in PBST, the membrane was incubated with the secondary antibody IRDye680 goat anti-mouse (610144002, Rockland Immunochemicals). Protein bands were visualized using a Li-COR Odyssey detector.

4.3.2.3 Immunolabelling

MEFs_{Sey} cells were plated on sterile coverslips placed inside a six well plate. Three days post-transfection, cells were washed with PBS, fixed for 15 minutes in 4% PFA, blocked for 1 h at room temperature with blocking buffer (2% normal goat serum, 0.1% Triton X-100 in PBS), then incubated overnight in a humidified chamber with a 1:500 dilution of anti-Pax6 antibody
(901301, Biolegend) in blocking buffer. The next day, cells were washed extensively in PBST then incubated with a 1:200 dilution of AlexaFluor® 488 goat anti-rabbit IgG (A-11008, Thermofisher Scientific) in PBST for 1 h at room temperature. Nuclei were counter-stained with DAPI (4′, 6-diamidino-2-phenylindole) for 5 minutes at room temperature and then was washed with PBS for 5 minutes. Slides were then mounted with a coverslip using fluoromount-G (Southern Biotech). Immunofluorescent images were acquired using a Zeiss 510 laser scanning confocal microscope.

4.3.3 In-vivo assessment of candidate PTMs

4.3.3.1 PTM-complex formation and injection

The Pax6 Ser/+ mouse at postnatal day 14 (P14) was used, as this is the time of eye opening, by following standard laboratory procedures in accordance with the Association of Research in Vision and Ophthalmology and the guidelines of the University of British Columbia Animal Care Committee and Veterinary Service.

Injecting naked plasmid DNA is the simplest approach, but it is generally not effective because it can be easily detected and degraded by endogenous nucleases, in addition to limited uptake, thus lower gene expression level would be expected. Therefore, for in-vivo PTM delivery, PTM was complexed with a polymer, 1-2 h before injection. Briefly, in a 25µl reaction, 25µg of PTM-plasmid construct was mixed with in-vivo JetPEI® at N/P=8 (i.e. the number of nitrogen residues of in-vivo jetPEI per DNA phosphate) (in-vivo JetPEI®, Polyplus) following manufacture’s protocol. To anesthetize the animals, we used intraperitoneal injection of 100µl/10g of anesthetic cocktail (0.17ml ketamine hydrochloride (100mg/ml, Vetalar) + 0.5ml xylazine (20mg/ml, Bayer) + 4.33ml normal saline). Once the animal was completely sedated, by
testing the pinch-toe reflex, 2µl (2µg) of the PTM-complex was administered either to the anterior chamber or to the sub-retinal space of the eye. To reverse the anesthesia, on the other side of the intraperitoneal cavity, 1mg/kg of atipamezole hydrochloride (Zoetis) was injected. All reagents were freshly prepared prior injection.

4.3.3.2 Eye collection and immunolabelling

Three days post-injection, eyes were enucleated, washed briefly in PBS and then fixed overnight in 4% paraformaldehyde at 4°C. The next day, eyes were incubated overnight in PBS at 4°C, and then transferred to 30% sucrose on the third day for cryopreservation. Eyes were then embedded in polyfreeze medium (Polysciences). Sagittal cryo-sections (10 µm) were incubated with an antibody against the Pax6 c-terminus (ab5790, Abcam) and an anti-DsRed2 antibody (632496, Clontech) following a standard immunolabelling protocol (Wang et al., 2017a). Secondary antibodies used were 1:200 AlexaFluor® 488 goat anti-rabbit IgG (A-11008, Invitrogen) and 1:200 AlexaFluor® 594 goat anti-mouse IgG (A-11005, Invitrogen).

4.3.3.3 Statistics

Analyses were done with GraphPad Prism 5.0. For parameter comparisons between groups, an unpaired two-tailed Student's t-test was performed. P-values of <0.05 were considered significant. Results are reported as mean ± SEM.
4.4 Results

4.4.1 Trans-splicing corrects mutant Pax6 in-vitro

Finding a cell line that doesn’t express Pax6 was challenging as most common cell lines do express Pax6 at different levels such as the wildtype MEFs (Dong et al., 2008). Therefore, we used a homozygous mutant cell line (MEFs\textsuperscript{Sey}) lacking the typical Pax6 expression profile when tested with an antibody against the c-terminal region of the Pax6 protein (Figure 4.3 A.i & B, western blot). This cell line was used to test the efficiency of six different PTMs. Transfecting these cells with full length Pax6 cDNA, as an experimental control, leads to Pax6 expression (Figure 4.3 A.ii) and was used as a reference for PTM efficiency scoring (Figure 4.3 B, bar graph). Treating the cells with PTM1 and PTM2 (Figure 4.3 A.iii & A.iv, respectively) resulted in 80-70\% of expression compared to cells transfected with full length cDNA (Figure 4.3 B, bar graph). Statistical significance was determined using student $t$-test on the four different treatment groups. The addition of ASO did not improve the splicing efficiency (Figure 4.3 B). The other four PTMs tested didn’t result in detectable Pax6 expression.
Figure 4.3 Rescue of Pax6 expression in MEF<sup>Sey</sup> cell line

(Panel A) no Pax6 was detected in the untransfected MEFs<sup>Sey</sup> cell line (i). Pax6 and DsRed2 expression was detected in MEFs<sup>Sey</sup> cells transfected with wildtype full length cDNA (ii) and with two different PTM constructs (iii and iv). Arrows in the iv row indicates either a non-transfected cell or absence of trans-splicing. (Panel B) 50µg of protein were loaded. Western blot (top) and the corresponding densitometric data (bottom) are presented. No Pax6 was detected in MEFs<sup>Sey</sup>. Rescue of Pax6 protein was seen when transfected with full length cDNA and with different constructs. No significant increase in trans-splicing was seen with antisense oligonucleotides. Pax6 antibody used is against the c-terminus. Scale bars: 100µm. Data plotted as mean ± SEM (n = 3), and statistical significance was determined using the Student t-test, *P < 0.01, **P < 0.001.

4.4.2 Tracking PTM1 expression in-vivo

From previous experiments, PTM1 appeared to be more effective (Figure 4.3 B, bar graph) in trans-splicing and therefore was tested in-vivo. Sub-retinal and anterior chamber injections into Pax6<sup>Sey/+</sup> mice resulted in noticeable Pax6-DsRed co-expression as shown in Figure 4.4 & Figure 4.5 respectively. The construct that was injected in the narrow anterior chamber of the Pax6<sup>Sey/+</sup> mouse resulted in mosaic Pax6-DsRed expression in the ciliary body, although we did not use ciliary body specific markers. Whereas sub-retinal injection resulted in mosaic expression in the ONL and RPE (n=3). Unlike the homozygous Pax6 null cells (MEFs<sup>Sey</sup>), the Pax6<sup>Sey/+</sup> mouse model is a heterozygous mutant; therefore, they have lower Pax6 expression compared to wildtype (about 50% less).
Figure 4.4 Three days post-subretinal injection of PTM1-plasmid-complex into Pax6<sup>Sey</sup> eye
Subretinal injection led to Pax6-DsRed expression in the ONL and RPE as seen in the merged panel. Yellowish color in the merged figure represents co-labelling of both Pax6 and DsRed. Insets on the left image represents the typical retina (R) structure of both mutant (upper) and wildtype (lower) mouse at P14. Note the typical distorted retinal structure in the mutant model. Representative confocal image (n=3) from two independent experiments. Arrows in the Pax6 panel represents the endogenous Pax6 expression whereas exogenous expression is outlined.

Figure 4.5 Three days post-anterior chamber injection PTM1-plasmid-complex
Pax6-DsRed expression was noted in the iris region. Insets in the left merged image show the fused cornea (C) and lens (L) in the mutant mouse model (upper) compared to wildtype (lower). CB, ciliary body; R, retina. Representative confocal image (n=3) from two independent experiments. Arrows in the Pax6 panel represents the endogenous Pax6 expression whereas exogenous expression is outlined.
4.5 Discussion and conclusion

The human fovea is about 2mm in diameter and 131μm in depth (Tick et al., 2011) thus restorative micro surgical intervention at this scale is not yet available neither are any other forms of pharmacological approach. Therefore, correcting the underlying genetic defect has the potential to reverse or prevent disease progression at early stages of development. One option for PAX6 mutations causing haploinsufficiency would be gene augmentation therapy. Although this has been done in Pax6 mice (Schedl et al., 1996) one of the critical issues is that the regulation of Pax6 expression is complex, especially with long-range regulatory elements. For example, too much Pax6 dosage can make the pathology worse (Manuel et al., 2008). Recently, gene therapy vectors have been developed to produce restricted Pax6 gene expression which could be particularly advantageous for dosage-sensitive genes like Pax6 (Hickmott et al., 2016).

To attempt to mitigate the complex regulatory issues associated with correct Pax6 expression I therefore decided to test an RNA-based approached to overcome this problem. In this approach, the cell’s own RNA regulatory machinery is used to avoid over-expression of Pax6. There are several different approaches to correct a gene defect at the RNA (ribonucleic acid) level such as RNA interference (gene silencing), nonsense-mediated mRNA decay inhibition (produces a truncated protein that may be functional), nonsense suppression (full length protein production), and RNA trans-splicing (joining different RNAs together). RNA repair is a new avenue for gene therapy and a potential approach to treat FH for many reasons such as: 1) the fovea continues to develop postnatally up to 4 years of age (Hendrickson, 1992) giving a window of opportunity to treat foveal defects; 2) the genetic cause is known in most patients; 3) PAX6 cDNA is a small enough sequence to easily fit into an expression vector for therapy; 4) a small increase in the protein level may have therapeutic benefits (Gregory-Evans et
al., 2014); 5) availability of different *Pax6* mutant animal models for testing therapies and; 6) the eye has immune privilege and is easily accessible for delivery of therapy. Two related *Pax6* isoforms (*Pax6* and *Pax6*+5a) show different expression profiles (Pinson et al., 2005). Azuma et al., 2005 have suggested that the *Pax6*+5a isoform is important for foveal development. So in future experiment we may need to simultaneously introduce a combination of both the canonical and the 5a *Pax6*-isoforms to treat foveal defects. Also, we do not know yet the long-term consequences of manipulating the *Pax6*:*Pax6*+5a ratio.

In this proof-of-principle study, we have targeted the canonical *Pax6* where most mutations reside. The overarching goal was to restore normal *Pax6* expression in a single reaction with one molecular tool using the SMaRT approach. In a homozygous mutant *Pax6* cell line, we have demonstrated that PTM1 produced the highest *Pax6* expression level when compared to the other five constructs. These constructs differ in the intronic binding site as well as the addition of 3’-UTR. UTRs are known to play an important roles in gene expression including stabilizing the mRNA during transport out of the nucleus (Mignone et al., 2002). Therefore, PTM1 was designed to contain the full-length mouse 3’-UTR to potentially aid in construct stability. However, no *trans*-splicing event was seen with PTM3 which also carried the 3’-UTR. PTM1 and 3 have different binding sites which may have assisted in the superior functioning of PTM1 at restoring the *Pax6* expression level. Interestingly, the binding domain of the PTMs with the highest *trans*-splicing efficiency was when it was near the 5’-splice donor site, while those PTMs with binding region further away from the splice donor site did not show similar *trans*-splicing efficiency. Binding specificity is needed to bring the construct in close proximity with the endogenous gene for successful *trans*-splicing. Additionally, unlike in other
studies (Mansfield et al., 2003, Lorain et al., 2013), the inclusion of intronic sequence in the replacement cDNA (PTM6) did not result in noticeable Pax6 rescue.

To estimate trans-splicing efficiency, we have compared cells transfected with PTM to those transfected with full length canonical cDNA. Further experiments are needed to compare the expression levels to those in wildtype MEFs. This will help in better estimating the percentage of the overall expression rescue.

For in-vivo work, plasmid-based PTM delivery offers several advantages, they do, however, have a number of drawbacks. It is known that transgene expression is commonly lower in plasmid vector-based system compared to viruses. In addition, in rapidly dividing cells, most plasmids are not passed to daughter cells leading to gradual loss of expression and, therefore, it is considered ineffective in tissues with high mitotic index. In addition, transfected cells or the expressed protein may elicit an immune response. Furthermore, microbial DNA sequences are prone to methylation by the host cell leading to inhibition of transcription (Morrissey et al., 2013). However, studies have shown that plasmid-DNA is stable for up to two years after intramuscular injections in mice with long term transgene expression. This may be due to the nondividing nature of the adult muscle cells (Armengol et al., 2004), which could be similar to the eye. Furthermore, the immune-privileged status of the eye makes it an ideal organ for testing different therapeutic approaches including the plasmid based system.

Gene delivery using non-viral vectors offers potential advantages over viral system. However, transfection efficiency is generally lower compared to viral-based delivery vectors. Yet, several studies have shown effective gene delivery to different tissues including different ocular tissues using the polyethylenimine cationic carrier approach (i.e. in-vivo JetPEI system) (Liao and Yau, 2007, Zhang et al., 2012, Yuan et al., 2013).
In addition to a retinal abnormality, \(Pax6^{Stv/+}\) mice do have other ocular phenotypes including corneal opacity, iris hypoplasia and ciliary body defects. Therefore, we attempted to deliver the PTM1 mixture to the iris/ciliary body by injecting it into the anterior chamber and aiming to see increase in Pax6 expression. In addition Pax6 might be able to penetrate the cornea and possibly reduce corneal opacities. No improvement was noticed one month post-injection in the cornea nor in the iris length/attachment to cornea and ciliary body structure which may be due to the: 1) short follow up time; 2) insufficient trans-splicing rate to restore normal corneal Pax6 profile; 3) inability of the Pax6 to penetrate the corneal tissue; or 4) limited spatial delivery.

Finally, the fovea is the least matured area of the retina at birth, accounting for the poor visual acuity characteristic of human infants. Since the fovea is still developing postnatally this may provide a window of opportunity to remodel the abnormal foveal architecture through pharmacological approaches in young patients with FH who are otherwise destined for poor vision.

To date, more than 1000 variants across \(PAX6\) are reported in human. Since only 5% of these mutations occur in the first four exons, the 3’ replacement approach could potentially rescue 95% of the known \(PAX6\) mutations, by providing the full length cDNA consisting of the four first native exons and the remainder from the PTM construct.
Chapter 5: *ANOLIS CAROLINENSIS* A MODEL TO UNDERSTAND
MOLECULAR AND CELLULAR BASIS OF FOVEAL DEVELOPMENT

5.1 Chapter goals

The fovea is an anatomical specialization of the central retina containing closely packed cone-photoreceptors providing an area of high acuity vision in humans and primates. Despite its key role in the clarity of vision, little is known about the molecular and cellular basis of foveal development, due to the absence of a foveal structure in commonly used laboratory animal models. Of the amniotes the retina in birds of prey and some reptiles do exhibit a typical foveal structure, but they have not been studied in the context of foveal development due to lack of availability of embryonic tissue, lack of captive breeding programs, and limited genomic information. However, the genome for the diurnal bifoveate reptile species *Anolis carolinensis* (green anole) was recently published and it is possible to collect embryos from this species in captivity. Here, we tested the feasibility of using the anole as a model to study foveal development.

5.2 Overview

Humans and most primates are equipped with a specialized retinal area essential for high acuity vision known as the fovea (from the Latin meaning pit). The foveal architecture is thought to be crucial for activities that require high visual acuity, refined color vision, and contrast sensitivity (Provis et al., 1998). Anatomically, the fovea is an avascular depression with high cone photoreceptor density located at the centre of the macula region of the retina. At the foveal
centre (i.e. foveola), the inner retinal layers are peripherally displaced. During retinogenesis, a shallow foveal pit appears in the third trimester gestation in both monkeys (*Macaca nemestrina*) and human and fully develops within a few weeks or years after birth, respectively (Hendrickson, 1992).

Most of our current histological and basic molecular understanding of fovea originates from a few human studies and from non-human primates (Borwein et al., 1980, Hendrickson, 1992, Provis et al., 1998, Springer et al., 2011). As a disease model non-human primates suffer from the limited number of off-spring, slow time course of development, they are costly to maintain and difficult to manipulate genetically. However, the use of non-invasive OCT in the clinical setting has been used to great advantage in the diagnosis of foveal abnormalities and has been employed to track postnatal foveal development in both humans and non-human primates (Dubis et al., 2012, Lee et al., 2015, Patel et al., 2017). However, to deepen our understanding of pre-natal foveal development and the molecular and cellular drivers of the process a model organism is required.

High visual acuity is critical to the survival of many vertebrate species that are predators and capture live prey primarily through visual guidance. However, routinely used laboratory animal models such as the mouse (Huberman and Niell, 2011), rats (Meier and Reinagel, 2013), pigs (Beauchemin, 1974), dogs (Beltran et al., 2014), cats (Rapaport and Stone, 1984), and zebrafish (Schmitt and Dowling, 1999) do not have a fovea, rather they have an area of high cone photoreceptor density (area centralis) without a typical foveal pit. Animal species with a foveal pit include: raptors, pigeons, blue jay birds, ostriches, albatross (O’Day, 1940, Mitkus et al., 2017), seahorses, pipefishes (Walls, 1942, Hard, 1972, Collin and Collin, 1999, Boire et al.,
2001), and some reptiles (Walls, 1942, Roell, 2001). In each of these species the foveal pit differs in pit depth, pit width, and cellular composition.

*Anolis carolinensis*, also known as the green anole, is an arboreal reptile commonly used in comparative developmental biology. Green anoles are found largely in southeastern United States and in the Caribbean Islands. They have two foveal pits, a central convex-sided (convexiclivate) pit and a shallow temporal pit (Makaretz and Levine, 1980). The green anole genome is sequenced (Genome assembly: AnoCar2.0) (Alfoldi et al., 2011), their eggs are laid very early in development prior to foveal development, foveal histogenesis proceeds rapidly after laying (22-27 days of incubation) (Sanger et al., 2008), are inexpensive to maintain, and this animal is abundant and not endangered species. To determine the validity of this model system for studying foveal development, we tracked foveal morphogenesis, examined the spatiotemporal localization of cone-opsin and Pax6, and investigated the role of programmed cell death in foveal pit formation. In addition we used bioinformatics to interrogate the recently published anole genome for genes that were homologous to 98 human genes associated with FH defects.

### 5.3 Materials and methods

#### 5.3.1 Tissue collection

This study was approved by the Animal Care Committee at the University of British Columbia in accordance with the guidelines established by the Canadian Council on Animal Care. Adult female anoles were purchased from the Pet Boutique, North Vancouver, BC, Canada, that were procured and imported from Charles Sullivan Co., Nashville, TN, USA. Animals were maintained on a 14:10 light:dark cycle with an ambient temperature of 28°C. Humidity was kept at about 70% to maintain reproductive activity. A diet of live crickets (*Acheta*
domesticus), that had been dusted with a 2:1 mix of calcium powder:vitamin supplement (Rep-Ca Research Laboratories, Los Gatos, CA, USA), was provided every 2 days. In addition twice weekly live mealworms (Tenebrio molitor) were provided (2 per lizard). A detailed description of housing requirements and husbandry conditions has been standardized (Lovern et al., 2004). Nesting boxes were checked daily for newly laid embryos. The embryos were collected at different time points and staged according to the nineteen stages of embryonic development (Sanger et al., 2008). Briefly, embryos that were 0-15 days post-laying were removed from their eggs, decapitated, and immediately placed into 4% (w/v) paraformaldehyde fixative (Electron Microscopy Sciences, Hatfield, PA). Embryos that were beyond 15 days post-laying were euthanized by injecting 1% sodium bicarbonate-buffered MS222 (Sigma-Aldrich, Oakville, ON, Canada), pH 7.2, into the intracoelomic cavity followed by removal from the egg, decapitated, and immediately placed into 4% (w/v) paraformaldehyde. Juveniles and adults were first anesthetized by inhalation of isoflurane prior to decapitation and fixation.

5.3.2 Histology, immunolabelling, and TUNEL assay

For images of the adult retina in situ, the anterior segment was removed and then photographed using a Leica MZ16F stereomicroscope. For histology, fixed heads from embryos at ES7, ES10, and ES12 were submerged in decalcifying solution-lite (D0818-1L, Sigma Aldrich, Oakville, ON, Canada) for 3 h with mild agitation prior to embedding in paraffin wax. Dorso-ventral sections that were 5 µm thick were stained with hematoxylin and eosin. Fixed eyes from later staged embryos at ES14, ES16, ES17, hatchlings, and adults were embedded in wax and sectioned in a naso-temporal orientation. All sections were examined carefully for early signs of foveal development such as GCL widening and early pit formation. Images were
photographed by an Aperio ScanScope digital scanning system. Image measurements were done using ImageScope viewing software. For immunofluorescence staining, fixed ocular tissues were cryoprotected in 30% w/v sucrose overnight at 4°C, infiltrated with optimal cutting temperature compound (Tissue-Tek; Torrance, CA) and snap-frozen on dry ice. Ten micron thick cryosections were incubated overnight at 4°C in a humidified chamber with an anti-Pax6 antibody (1:500 ab5790, Abcam, Toronto, ON, Canada) or an anti-opsin antibody (1:250 ab5405, Millipore, Toronto, ON, Canada), followed by a 1 h incubation at room temperature with an AlexaFluor® 488 goat anti-rabbit IgG secondary antibody (1:200 A-11008, ThermoFisher Scientific, Waltham, MA, USA). Sections were protected with antifade mounting medium with DAPI (Vector laboratories, Burlingame, CA, USA). Apoptotic cells in cryosections were labeled with an ApopTag® Fluorescein In Situ Apoptosis Detection Kit (S7111, Millipore, Toronto, ON, Canada) following the manufacturer’s protocol. Immunofluorescent images were captured using a Zeiss 510 laser scanning confocal microscope.

5.3.3 Bioinformatic analysis of foveal and macular genes

The literature was reviewed for candidate genes that could be potentially involved in foveal and macular development and homeostasis. A list of 98 candidate genes was compiled (Bernstein et al., 1996, Bernstein et al., 1995, Sharon et al., 2002, Rickman et al., 2006, Kozulin et al., 2009, Gregory-Evans and Gregory-Evans, 2011, Poulter et al., 2013). These genes were either mutated in patients with FH (PAX6 and SLC38A8), mutated in other ocular diseases usually accompanied with FH (e.g. TYR and OCA2), or differentially expressed at foveal and macular regions when tested in fetal and postnatal donor eyes, or had comparative data from embryonic chick. The Ensembl Genome Browser was used to find orthologues in the...
A. carolinensis genome and determined the degree of protein homology with human sequences (available at: https://grch37.ensembl.org/index.html).

5.4 Results

5.4.1 Adult retina

To observe the major retinal landmarks of the green anole retina, the anterior segment was removed and images taken of the retina in situ. The location of the central fovea (CF), temporal fovea (TF), conus papillaris (CP) and optic nerve (ON) in an adult eye (3 months of age) are shown in Figure 5.1A. The CP is highly vascularized and the pigmented optic nerve projects into the vitreous chamber (Figure 5.1B & Figure D.1; Appendix D). These two features are commonly found in birds and reptiles (Brach, 1976). The TF is located at the far superior-temporal region, exhibits a very shallow pit and is thought to mediate binocular vision (Fite and Lister, 1981). The CF is located more centrally along the typical optic axis and is slightly shifted to inferior temporal quadrant of the vertical and horizontal meridians.
Figure 5.1 A. carolinensis eye structures

(A) Fundus view of the A. carolinensis eye with the anterior segment removed. The central area of the retina is demarcated by a darker circular line indicated with two arrowheads in the superior right and left quadrants. CF, central fovea; TF, temporal fovea; ON, optic nerve head; CP, conus papillaris; L, lens. Scale bar = 1 mm. (B) Retina section demonstrating the CP emanating from the optic nerve head. Scale bar = 1 mm. *Insets show higher magnification of the vascularized structure of the CP. Scale bar = 50µm.

Histology was used to analyze the retinal structure at 3 months of age when the retina is fully developed (Figure 5.2A). The retina is thickest at approximately 500 µm away from the foveal pit (Figure 5.2B). At this position, the ganglion cell layer (GCL) is about 5 nuclei deep, the inner nuclear layer (INL) has about 20 rows of stacked nuclei, and the outer nuclear layer (ONL) has about 6 rows of photoreceptor cell nuclei (Figure 5.2C). The overall thickness of the retina is approximately 425 µm at this position. Retinal thickness declines toward the peripheral retina and measures approximately 121 µm at a position 4 mm away from the foveal pit. In this location, the GCL is one cell thick, the INL has 2 rows of cell nuclei and the ONL contains single row of cell nuclei. Quantitation of retinal layer thickness and number of rows of cell nuclei are presented in Figure 5.2E. The CF has steep sides that is convexiclivate in shape.
The retina is about 150 μm thick at the foveola, which is acellular, with a thick nerve fiber layer (NFL) that is visibly-packed with elongated photoreceptor inner (IS) and outer segments (OS). The pigmented microvilli emanating from the apical surface of the RPE are elongated at the foveola. A circular line in wholemount retina (Figure 5.1A, arrowheads) correlates with the change in cellular density observed in the retina (Figure 5.2A, asterisk).

**Figure 5.2 Histological analysis of the adult *A. carolinensis* eye at 30 days of age**

(A) Cross-section through the eye showing the central foveal pit (arrow). L, lens; *, corresponds to the circular line demarcating the central retina indicated in Fig. 1. Scale bar = 1 mm. (B) Retinal thickness measurements were taken every 500μm away from the foveola. The size of each bar is annotated in μm. Thickness was measured from inner boundary of the nerve fiber layer to the basal membrane of the pigment epithelium. Scale bar = 1 mm. (C) Higher magnification of foveal pit and the retina at 500μm and 4000μm away from the foveal pit. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 50 μm. (D) Higher magnification of the foveal pit. NFL, nerve fiber layer; IPL, inner plexiform layer; OPL, outer plexiform layer; IS, inner segment; CC, connecting cilium; OS, outer segment; RPE, retinal pigment epithelium. Each retinal layer is demarcated by thin black lines. Size bar = 100μm. (E) Quantification of retinal layer thickness (left graphs) and rows of nuclei of each nuclear layer (right graphs) across the retina. Red dots indicate the foveal centre. Note that the increase in NFL thickness at the asterisk-labelled site indicates the proximity to the ON.
The TF is located in the superior periphery of the retina, is shallow in depth and different in structure compared to the CF (Figure 5.3A). At ES17 the centre of the TF is packed with 3-4 rows of photoreceptor nuclei compared to the lateral edges of the fovea where only a single row of photoreceptors is present (Figure 5.3B). Similarly, in the adult TF there is an accumulation of photoreceptor nuclei (4-5 rows) in the ONL at the centre of the fovea (Figure 5.3B). In addition there is a reduced thickness of the INL comprising 6-7 rows of nuclei, whereas there are 10-11 rows more laterally. There is no change in the thickness of the GCL at the TF.

Figure 5.3 Histological analysis of the temporal fovea in the A. carolinensis eye
(A) Sagittal sections through an ES17 eye (left) and an adult eye (right) showing the location of the temporal fovea, which is positioned in the superior retina (dotted black circles). CP, conus papillaris; L, lens. Scale bar = 1 mm. * area magnified in upper panel of (B); ** area magnified in lower panel of (B). (B) Higher magnification of the ES17 fovea* and the adult fovea **. Scale bar = 100 µm. The centre of the ES17 temporal fovea (inset, single yellow asterisk) is packed with 3-4 rows of photoreceptor nuclei compared to a single row of photoreceptors at the periphery (inset, double yellow asterisks). Scale bar = 25 µm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.
In unstained frozen sections through the central foveal retina, a yellowish pigmentation was observed in the adult photoreceptors (Figure 5.4A), which is reminiscent to lutein macular pigmentation in human retina (Trieschmann et al., 2008). Interestingly, the temporal fovea did not have the same pigmentation (Figure 5.4B) suggesting it did not need antioxidant protection. It was very clear from an unfixed posterior segment eyecup that there was a large area of yellow pigmentation that radiated beyond the central foveal pit (Figure 5.4C).

Figure 5.4 Yellow pigment in the foveal region of an adult anole
(A) Extent of pigment in the central foveas shown by white arrowheads in an unstained cryosection of the retina. Scale bar = 300µm. Inset: higher magnification of the area containing yellow pigment in the photoreceptors. Scale bar = 50µm. (B) Yellow pigment is absent from the temporal foveal region of an adult anole. Scale bar = 300µm. (C) En face view of an unfixed posterior eyecup from an adult anole showing a large region of yellow pigmentation. Dotted red line denoted the foveal pit; white dotted line denotes the extent of the yellow pigmentation. PE, pigment epithelium. Scale bar = 300µm.
5.4.2 Temporal development of the central fovea

To determine the developmental stage when the central foveal pit begins to form we examined the retinal histology at 4 time points. At ES7 there is no evidence of foveal pit formation (Figure 5.5Ai-ii) and at the expected position of the presumptive foveal region, the GCL layer is 6 nuclei thick, 15 nuclei thick at the INL, and 1 cell nucleus at the ONL (Figure 5.5Bi). At ES10, there is an increase in GCL (19 nuclei thick), the inner plexiform layer (IPL) thickness is greater than at ES7 (Figure 5.5Aiii-iv), and there is still a single row of photoreceptor nuclei present (Figure 5.5Bii). At ES14, GCL thickness is reduced to 6 nuclei (Figure 5.5Av-vi) and the INL layer shows two morphologically distinct nuclear zones; the inner zone nearest the IPL contains the amacrine cell bodies (~522 nuclei/500 µm) and the outer zone nearer to the OPL contains horizontal and bipolar cell bodies (~895 nuclei/500 µm). At this stage, the single row of photoreceptors starts to develop inner segments (Figure 5.5Biii). The foveal pit starts to invaginate at ES17 (~5 days prior to hatching) with a reduction of ganglion cells in the foveal centre (3 nuclei deep) and an increase in ONL thickness up to 4-6 cells in depth (Figure 5.5Avii-viii). At this position, IS/OS are elongated as shown in (Figure 5.5Biv). At the region of foveal invagination, the two nuclear zones of the INL (15 nuclei in depth combined) become less distinct and thinner (9 nuclei deep). At the hatchling stage (Figure 5.5Aix-x), the CF is very prominent and relatively mature, although not fully formed when compared to adult fovea (Figure 5.5Axi-xii).
Figure 5.5 Remodeling of the *A. carolinensis* retina during development from ES7 to adult
(A) Histological sections through the whole eye (upper panels; size bar = 600 µm, except in panel (11) and higher magnification of the corresponding incipient foveal region (lower panels; size bar = 100 µm). White brackets in panels 6 & 8 denote 2 INL zones of different nuclear densities. The nerve fiber layer (NFL) appears very thick when sections are taken close to the optic nerve in this species. The conus papillaris (CP) is denoted by black arrow in panel 9. ON, optic nerve; FF, future fovea. (B) Higher magnification of the ONL showing an increase on ONL thickness from a single row of cone nuclei at ES7 to up to 4 layers of nuclei at ES17. Note the start of elongation of IS at ES14 (arrow). White dotted lines denote the boundary of the ONL and IS/OS. Outline of nuclei boundaries (in white) in panels 1-3 are denoted for clarity. Scale bar = 20 µm. (C) M/L opsin immunolabelling of the foveal region at different developmental stages. M/L opsin intensity increases with development from labeling of the whole photoreceptor cell/perinuclear region of photoreceptors to the OS/IS at hatchling and adult stages. Yellow arrows in ES17 shows expression of M/L opsin in elongating IS. White brackets indicate cell boundaries. Scale bar = 50 µm. Days of incubation: ES7, 4-7 days; ES10, 8-11 days; ES14, 15-18 days; ES17, 19-22 days.
5.4.3 Protein localization during fovea remodeling

To follow photoreceptor maturation we examined M/L opsin immunolabelling as retinal development progressed (Figure 5.5C). From ES7 to ES14 M/L opsin antibody labelled the whole cell body/perinuclear region of the photoreceptor as no inner segments were present at these stages. At ES17 M/L opsin is detected in the elongating IS. At hatchling and adult stages, there is strong labeling of M/L opsin in IS/OS region.

Since PAX6 mutations are associated with FH in humans we assessed Pax6 localization in the developing fovea. At ES14 Pax6 labelling was detected across the whole retina and was more prominent in the GCL and in the less dense nuclear zone of the INL (Figure 5.6A), where the amacrine cells are located and known to express Pax6 protein (Hitchcock et al., 1996). At ES17 where the foveal pit is invaginating (Figure 5.6B), there appears to be a reduction of Pax6 labelling in the GCL at the foveal region when compared to the peripheral retina (Figure 5.6C,D). We did not observe any changes to Pax6 expression in the GCL in the temporal foveal region.
Figure 5.6 Expression of Pax6 protein in the developing *A. carolinensis* retina

(A) Pax6 is expressed in nuclei of the GCL and the nuclei of the inner zone of the INL at ES14. (B) Pax6 labelling in the foveal region compared to more peripheral retina at ES17. Boxed region (*) shown at higher magnification in panel (C). Boxed region (**) shown at higher magnification in (D). Scale bar = 500 µm in all panels.

5.4.4 *TUNEL* staining in the developing anole retina

As the retina remolds during development histogenic cell death occurs to remove excess cells (Young, 1984). Therefore we hypothesized that cell death could contribute to foveal pit invagination. At stage ES7, *TUNEL*-positive cells were more prominent in the INL compared to the GCL (Figure 5.7A,E). At ES12, *TUNEL*-positive cells were more widely distributed across the retina in the INL and GCL (Figure 5.7B,E), whereas fewer *TUNEL*-positive cells were observed in ES16 retina and only in the INL (Figure 5.7C). No *TUNEL* positive cells were seen
in the foveal region of ES17 stage embryos (Figure 5.7D), suggesting that cell death did not contribute to foveal invagination.

![Figure 5.7 TUNEL staining at different developmental stages in A. carolinensis retina](image)

(A) Whole eye at ES7 (upper panel). Size bar = 500 µm. *, incipient foveal area magnified in lower panel (scale bar = 100 µm). TUNEL-positive cells in green; DAPI counterstain. (B) Whole eye at ES12 (upper) and magnified foveal area (lower). (C) Whole eye at ES16 (upper) and with magnified foveal area (lower). (D) High magnification of invaginating foveal retina at ES17. Arrow indicates the start of a foveal pit. Scale bar = 50 µm. (E) Quantitation of TUNEL-positive cells in the foveal region (500 µm width) compared to the whole retina.

5.4.5 **Comparison of human and green anole foveal gene sequences**

Database mining revealed 84 of the 98 human foveal candidate genes (86%) examined had orthologous protein sequence matches in the green anole genome. Protein homology varied from 5 to 100% reflecting a broad range of sequence matches. For instance, 76-100% protein homology was found in 42 of the genes, 51-75% homology in 29 of the genes, 26-50% homology in 12 of the genes, and below 25% homology in just 1 gene. Fourteen of the examined candidate genes (15%) have no orthologous sequence in green anoles (Table 5.1).
### Table 5.1 Ninety-eight candidate human proteins examined for the presence of an *A. carolinensis* orthologue

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### Relevant embryonic chick data (da Silva and Cepko, 2017)

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*Percentage of the orthologues protein sequence matching the human protein sequence is noted for each gene. NP, anole orthologue not present. Coverage = percentage of amino acids covered by the alignment.

### 5.5 Discussion and conclusion

Very little is known about the molecular and biochemical cues that drive foveal development in humans as a suitable model system has not been available. In this study we set out to determine whether the green anole species *A. carolinensis* would be a useful model in which to study foveal development. The green anole breeding season occurs from April through July and so many research laboratories purchase them during this time frame from commercial suppliers in the USA (~$8/lizard). They have nineteen in-ovo developmental stages (ES1-19) and 1 egg is laid every 1-2 weeks at about ES4 and they hatch between 22 and 27 days after laying (Sanger et al., 2008). This window of hatching varies due to factors including ambient temperature of the soil, humidly and animal stress. A total of 8-12 eggs per female are laid per season, therefore the number of anoles to be obtained from specific experiments can be estimated. Some laboratories have breeding colonies however, we have found that after the keeping the anoles over the winter their reproductive capacity is reduced in the following season.
The green anole has two foveas, a central deep convexiclivate fovea and a much shallower, temporal fovea. In this study, we focused mainly on the central fovea for the following reasons: it has a more mature and deeper pit similar to the human fovea; it has a higher cellular density than the temporal fovea; and it is employed in central vision. In addition to its role in central vision and based on modeling estimates, the deep central fovea of reptiles and birds may be used for image magnification and maintenance of accurate fixation of moving objects (Pumphrey, 1948, Fite and Lister, 1981).

Using histological analysis we found that at the incipient central foveal region the GCL layer began to thicken at ES10 (8-11 days post-laying) approximately halfway through development in the anole, which is consistent with human GCL thickening at 20-22 weeks of gestation prior to pit formation (Hendrickson et al., 2012). Foveal indentation first appeared at ES17 (2-4 days prior to hatching), whereas in humans foveal pit formation began earlier at 25 weeks gestation (mid-gestation). At the hatchling stage, the foveal pit is deep, the cone IS/OS are significantly elongated, and there are no photoreceptor nuclei present. In humans at birth the pit is relatively shallow, there is a single layer of photoreceptor nuclei and the OS/IS have not yet elongated. In both the lizard and humans the foveal pit matures after birth. In the anole the foveal pit is fully mature by 3 months of age, whereas in humans the fovea is not mature until 15 months of age (Hendrickson et al., 2012). The main structural difference between the human and anole mature fovea is that the shallow human foveal pit overlies an accumulation of cone photoreceptor nuclei in the ONL, whereas in the anole there are no nuclei, just a thick nerve fiber layer and a substantial population of cone inner and outer segments. The inner and outer segments are very dense and cause an increased thickness of the RPE to accommodate this high density of cone outer segments (Figure 5.2D).
An interesting observation that we found was the presence of a very prominent yellow pigment in the central foveal region, that was absent from the temporal fovea. In human retina it is thought that oxidative damage from blue light is mitigated through antioxidative mechanisms including the presence of carotenoids such as lutein and zeaxanthin (Bone et al., 1985). These macular pigments not only absorb blue light but also act as free radical scavengers (Khachik et al., 1997). Furthermore, these pigments might act as an optical filter to counteract chromatic aberrations and light scattering (Wooten and Hammond, 2002). Perhaps the shallower temporal fovea does not require such protection as there may be less incident light in the far superior retinal position (Williams et al., 2004). In humans and primates the yellow pigments are most highly concentrated in an area radiating out approximately 200-400 μm from the fovea centre, and extending out to about 1 mm at a lower concentration (Snodderly et al., 1984). It would be interesting to determine in future studies the composition of the yellow pigment in the anole retina in comparison to human data.

It is likely that the earlier foveal maturity in young anoles and the deep fovea pit would be required evolutionarily due to the prey-capture nature of their survival.

Several studies have shown that apoptosis is required for vertebrates’ retinal development. For instance, retinal apoptotic activity peaked at pre-hatching/birth in zebrafish, *Xenopus*, chicken, and in humans whereas it peaks post-birth in rats and mice. In general, it spreads in a center-periphery wave-like pattern affecting the GCL first, then the INL, and Lastly ONL (Vecino et al., 2004). In human retina, foveal pit development occurs through a thinning of the GCL and INL due to an outward displacement of the inner retinal layers (Diazaraya and Provis, 1992) rather than through a cell death mechanism (Georges et al., 1999). In the developing anole foveal pit at ES17 there is also a reduction of nuclei in the GCL and INL, which is coincident with an
increase in photoreceptor nuclei in the ONL. We also found that there were no TUNEL-positive cells at this stage, confirming that apoptosis is not a significant factor in foveal pit morphogenesis. However, at ES7 and ES12 there were many TUNEL-positive cells in the INL at the region of the incipient fovea. This has also been observed in the human incipient fovea at 15 weeks gestation and this was attributed to a wave of bipolar cell death (Georges et al., 1999). There are also TUNEL-positive cells in the GCL and this is related to development of GCL topography (Provis et al., 1983). Thus, the cell death features seen in the anole retina prior to pit formation is similar to that of human retina.

PAX6 is a transcription factor essential for eye development and in aniridia patients with haploinsufficient PAX6 gene mutations about 70% of patients have FH (lack of a foveal pit), suggesting correct levels of PAX6 expression are required for normal foveal pit development. Therefore, we investigated Pax6 protein expression profiles during retinogenesis at the foveal region. Pax6 was expressed across the whole retina in early developmental stages and at ES14 was more prominent in the inner zone of the INL, where Pax6-positive amacrine cells are known to reside in other species (Hitchcock et al., 1996), and at high levels in the GCL. This expression in the GCL is consistent with the requirement for Pax6 to activate the ATOH7/Math5 transcription factor essential for ganglion cell specification and differentiation (Matter-Sadzinski et al., 2005). At ES17, an area of reduced Pax6 protein expression in the foveal region was observed, especially in the ganglion cells, the opposite of what we would have predicted if Pax6 loss-of-function was directly involved in defective foveal pit formation. This observation implies that Pax6 activity is required prior to foveal pit formation. Thus, the underlying cause of FH due to PAX6 haploinsufficiency remains to be determined. One avenue of investigation might be to examine the downstream targets of Pax6 and ATOH7/Math5 (e.g., BRN3 gene family; (Pan et
al., 2008)) and other retinal ganglion cell markers (e.g., Islet-1, γ-Synuclein; (Surgucheva et al., 2008)) during foveal pit development. This would now be possible in the anole model.

Based on our observations we conclude that similarities in anole and human foveal morphogenesis are apparent. Specifically both species have: i) thickening of the GCL layer at the incipient foveal region occurring mid-gestation; ii) TUNEL-positive cells in the GCL and INL occurs prior to pit formation; iii) foveal pit formation that is not associated with apoptotic mechanisms; iv) foveal pit formation occurring before birth/hatching; v) they have a foveal/central retinal region with yellowish pigmentation; vi) retinal foveal maturation occurs after birth in both species; vii) the fovea have an avascular rod-free zone; viii) 84 out 98 human genes associated with the macular/foveal regions of the human retina have homologues in the anole genome. However, there are some differences between species: ix) the foveal pit starts to invaginate at about 25 weeks mid-gestation in humans whereas it is nearer hatching in green anoles; x) at hatching in anoles the density of foveal cones has increased and they already have elongated IS/OS expressing M/L opsin, whereas at birth in humans there is only a single row of cones photoreceptors without IS/OS present. The cones in humans migrate to the foveal region after birth; xi) the mature fovea in anoles at 3 months of age has a thick nerve fiber layer and no photoreceptor nuclei, whereas the human fovea at 15 months of age has an accumulation of cone photoreceptor nuclei. These observations suggest that the green anole could be used to define the mechanistic pathways leading to normal foveal development, especially at the early stages of morphogenesis such as specification of the incipient fovea and foveal pit formation.

There is still ongoing debate about the significance of the foveal pit for normal visual function in humans. For example, in patients with FH several reports positively correlate visual acuity to foveal ONL thickness and not the actual pit formation (Marmor et al., 2008, Kirchner et
al., 2017). Future experiments on this foveated animal model could improve our understanding of foveal development and its clinical significance. For example, knockdown of foveal candidate genes and examining the effect on foveal development would be informative. Although the thick soft-shelled eggs has hampered in-ovo manipulation of embryos in the past, a few pioneering works have reported in-ovo gene delivery or ex-ovo culture with snake, lizard and turtle embryos (Nomura et al., 2015, Yamashita et al., 2017). SMaRT technology (presented in Chapter 4) has the potential to rescue most Pax6 mutations by using a single construct which may be superior to other gene-editing technologies. Yet, the consequence of Pax6 mutations in green anoles remains an unstudied area of research. In addition there have been several reports showing that is it now possible to use CRISPR/Cas9 technology (Mali et al., 2013) in a variety of animals and non-model organisms (Shrock and Güell 2017, Mendoza and Trinh, 2018) suggesting that this technology could be applied to the developing green anole. Another application might be to consider long-term treatment of female anoles with phenylthiourea to see if this affects foveal development, since most patients with oculocutaneous albinism have variable degrees of foveal hypoplasia (Wilk et al., 2014). In addition it will also be interesting to utilize the accessible eyes of the post-hatching anole as a model for postnatal changes in the foveal maturation.
Chapter 6: DISCUSSION

6.1 Overview

The fovea is an excavated retinal area with specialized cone photoreceptors located in the central retina. Human visual activities that employ central vision such as seeing faces, reading, writing, driving, etc. are affected in patients with severe FH. These patients are destined for poor vision due to the unavailability of treatment options. It is unfortunate that such important structure is not very well understood in the literature which may be attributed to the lack of foveal architecture in all animal models currently used in ocular research. We have investigated the human foveal pathologies in aniridia patients, possible treatment option targeting the Pax6 gene through RNA trans-splicing and presenting a novel foveated animal model (green anole). This study is a preliminary study which paves the way for a variety of future research experiments.

6.2 PAX6 and ocular diseases

Observing family members with isolated FH and PAX6 mutation (Azuma and Nishina, 1996) has raised the question about the role of PAX6 gene in foveal pathologies and foveal development. FH is commonly associated with other ocular conditions such as oculocutaneous albinism (TYR, TYRPI1 genes) and aniridia syndrome (PAX6 gene). Both diseases (albinism and aniridia) differ in the underlying pathophysiological mechanisms. TYR (Tyrosinase) is required by melanocytes to produces melanin, therefore albino patients tend to have light skin and hair coloration with FH (OMIM# 606933). PAX6 on the other hand, is a nuclear transcription factor and expressed in eye, central nervous system, and pancreas and its mutations manifest mainly in
aniridic phenotype (i.e. loss of iris tissue). There is an interesting link between PAX6 and TYR which may help in explaining the reason why FH is present in both aniridia and albinism. PAX6 is a complex protein that interacts with the promoter region of other genes to drive their transcription. A study was published on the role of murine Pax6 in melanogenesis (i.e. melanin synthesis) which showed that Pax6 synergized with MITF (melanogenesis associated transcription factor) to activate genes involved in RPE pigment biogenesis. In addition, Pax6 was required for the expression of key melanogenic genes including Tyr through activation of MITF (Raviv et al., 2014). Related to this, RPE as well as GCL secrete an anti-angiogenic factor called pigment epithelium derive factor (PEDF). This factor prevents retinal vasculature from invading the foveal region during early development, thus has a role in FAZ formation, where the initial foveal depression forms (Provis et al., 2013). In addition, PEDF serves as a neurotrophic and neuroprotective agent (OMIM# 172860). Therefore, we propose that the RPE may have a contributing role in fovea development and pathologies.

Furthermore, PAX6 is a complex gene leading to phenotypic heterogeneity in affected patients. PAX6 haploinsufficiency is linked to nine ocular conditions registered in the OMIM database (OMIM# 607108). These diseases affect multiple different eye structures derived from different embryological germ layers: ectoderm (lens and cornea), mesoderm (iris), and neuroectoderm (retina and optic nerve). Phenotypic heterogeneity is well documented between PAX6 patients. Although some earlier work tried to correlate missense mutations to weaker disease phenotype (Hanson et al., 1999), no precise correlation could be made between mutation type and phenotype.

In Chapter 2 and 3, the genetic and foveal architecture in 33 cases of aniridia was studied. PAX6 defects was identified in 30 participants (91%), including 4 novel PAX6 mutations
(Gly18Val; Ser65Profs*14; Met337Argfs*18; Ser321Cysfs*34) and 4 novel chromosome 11p deletions inclusive of PAX6 or a known PAX6 regulatory region. Interestingly, the number of PAX6 mutations causing aniridia continues to increase suggesting it is sensitive to gene mutation. Furthermore, we showed that in those patients in whom SD-OCT was possible, FH was seen in the majority of aniridia cases (80%). The cohort best corrected visual acuity (BCVA) ranged from 0.0 logMAR to no light perception. Also, within a family, SD-OCT has revealed that partial FH correlated with better visual acuity, suggesting that the degree of FH at birth, but not that of the parent, may be a useful predictor of future visual outcome. However, vision loss in aniridia is multifactorial, thus it is harder to predict on likely visual acuity from foveal architecture. Even with evaluation of each factor contributing to acuity, there would be insufficient power in a cohort of this size for a multifactorial non-parametric comparison of clinical condition and visual acuity. We also showed that all patients with FH show abnormally variable foveal architectures. Variable foveal architecture despite nearly identical anterior segment disease in 4 participants with an Ex9 ELP4-Ex4 DCDC1 deletion suggested that molecular cues causing variation in disease in the posterior segment differ from those at play in the anterior segment.

Since foveal development extends for a few years after birth, in Chapter 4 I hypothesized that correcting PAX6 defects would improve foveal maturation outcome. In this proof-of-principle experiment, the Pax6 defect in both in-vitro and in-vivo mouse model was rescued. Although the mouse model doesn’t exhibit a fovea, this work set the foundation for similar work on a foveated animal model in the near future. In theory the SMaRT approach has the ability to correct the majority of PAX6 defects. Therefore, it has the potential to treat aniridia, FH, and other PAX6-related conditions. In order to treat FH, we need a therapy which is able to: 1) displace residual inner retinal layers to form the foveal pit; 2) assist in cone packing; 3) stimulate
IS & OS lengthening, and less importantly 4) help in forming the FAZ and the; 5) displacement of rod & S-cones from the foveal centre. To achieve this we may need more than one molecule. Hence, our approach of solely targeting Pax6 might not be sufficient to completely restore foveal landmarks. However, our approach of Pax6 treatment might only be effective in patients with the following criteria: 1) having foveal hypoplasia; 2) with PAX6 point mutation; and 3) being under 4 years of age when the fovea ceases remodeling.

To date, over 100 different genomes can be browsed in the Ensembl genomic database. Most of these genomes belong to animals with no foveal structure, and very few have documented their retinal morphology. Those reported in the Ensembl database with a fovea include primates, zebra-finch, ducks, turkeys, elephants, and green anoles however, most have not been studied in the context of foveal development. Therefore, in Chapter 5, we tested the feasibility of using the green anole as a model to study foveal development. Eyes were collected at various stages of development for histological analysis, immunofluorescence, and apoptosis. Similarities to human foveal development include: a peak of ganglion cell density at the incipient central foveal region with a single row of cone photoreceptor nuclei; the foveal pit forms before hatching; post-hatching there is an increase in cone density and lengthening of inner and outer segments; a yellowish pigment was seen in the adult foveal region; and bioinformatic analysis revealed that 85% of human candidate FH genes had an orthologous gene or DNA sequence in the green anole. A few differences to human foveal development include: foveal pit invagination occurs mid-gestation in humans but near hatching on anoles; at hatching cone density and elongated IS/OS are well advanced in the anole compared to humans; the mature fovea in anoles at 3 months of age has a thick nerve fiber layer and no photoreceptor nuclei, whereas the human fovea at 15 months of age has an accumulation of cone photoreceptor nuclei.
Overall these findings provide the first insight into foveal morphogenesis in the green anole and suggest that it could be a very useful model for investigating the molecular signals driving foveal development, and thus inform on human foveal development and disease. One aspect of a good model system is that it should be amenable to genetic manipulation, or at least have a phenotype similar to the human disease that could be introduced by a physical or chemical means. Although this model requirement has yet to be reached in anoles it seems likely that new technologies will help to drive forward this need, enabling therapeutic strategies to be tested.

6.3 Future work

Next generation sequencing (NGS) including exome sequencing can be a very useful tool to look at the genetic makeup of patient families with isolated or syndromic foveal diseases. This may assist in a clearer understanding of the role of several candidate genes as well as others in FH. However, this requires a large patient base because the many base changes that are identified by NGS need to be verified in other patients, and segregation of potential mutations in families is crucial. Furthermore, significantly more funding will be required to carry out such studies on patients and their carefully nominated affected and unaffected family members.

The recent advances in programmable genome-editing technology, CRISPR/Cas9, has allowed the manipulation of gene expression at the DNA level (rather than RNA level, although this is now possible). Several studies have shown correction of point mutations using this technology in animal models of retinitis pigmentosa (Wu et al., 2016, Bakondi et al., 2016). Therefore, knocking out \( Pax6 \) and \( Tyr \) genes in green anole through a CRISPR/Cas9 approach might precisely identify their role in foveal development.
Over the past two decades many studies have identified ocular disease genes, have investigated the disease pathogenesis, have attempted therapies in animal models and a few clinical trials have been instigated. Despite these initial functional tests we are still far away from developing approved therapies. The first gene therapy treatment for Leber congenital amaurosis (RPE65 defect) was recently approved by the FDA (Luxturna®; FDA News Release Dec 19, 2017). It is expected that the current trials for other retinal diseases such as choroideremia and Stargardt’s disease will soon follow (MacLaren et al., 2014, Parker et al., 2016). There is an ongoing trial for aniridia (NCT02647359; ClinicalTrials.Gov) that is using nonsense suppression (Ataluren®) to overwrite premature stop codons in PAX6 mRNA to increase the amount of PAX6 protein. This was based on research carried out in the PAX6<sup>Sey/+</sup> mouse model that tested the efficacy of a small molecule drug (Ataluren®) using a systemic approach and a topical delivery method (Gregory-Evans et al., 2014, Wang et al., 2017). It will be interesting to see if any of the very young children in this clinical trial had any benefits to their foveal development.

### 6.4 Structure-function relationships between foveal morphology and visual acuity

A few studies have reported an underdeveloped fovea (fovea plana) in a number of healthy individuals with normal to near normal visual acuity (Marmor et al., 2008, Noval et al., 2014, McTrusty et al., 2013). These foveas lack pit curvature, but they have cone packing and cone maturation (i.e. grade 3 FH or less). Such extreme variation in morphology raises the question about the importance of the actual pit. However, since optics are very complicated, a major caveat of these studies which may be overlooked is correlating BCVA with foveal structure. BCVA is the best vision one could achieve with corrective glasses or lenses. Visual correction may mask the real impact of flat fovea. In theory, Walls (1937) suggested that the
foveal pit may act as an image magnifier due to its concave nature, thus being able to refract incoming light rays over a wider area of photoreceptors. This optical advantage may also be achieved, if lost through a flat fovea, using corrective glasses and lenses.

A very recent report again contradicts what we know about foveal development due to the new technique of OCT-angiography which allows high-resolution imaging of the retinal vasculature (Yokoyama et al., 2018). They showed several patients with 20/20 vision or better, with a foveal pit, and the presence of outer retina that was packed with mature cone photoreceptors. However, the FAZ was absent. It was previously believed that the FAZ was a requirement so that light could enter the foveal pit was able to function unimpeded by the vasculature. Also, Balaratnasingam et al. 2016 have shown that abnormal foveal vascularization affects visual acuity. However, it could also be argued that these patients have a grade 1 FH and that although they have 20/20 vision that their eyes are not normal. A longitudinal study will be required to further investigate if visual acuity changes occur over time. In addition, a large survey of normal eyes with OCT imaging is required to better understand variation in foveal morphology.

### 6.5 Closing remarks

With limited government annual research budgets, pharmaceutical-funded university research and philanthropic donations are becoming more prominent in supporting research projects, particularly those that involve common diseases such as cancer and heart disease that affect many people. On a national/international scale this might be considered as good resource allocation. However, on the other side of the spectrum, rare diseases are affecting millions of people worldwide, diseases such as FH and aniridia. Research in these orphan diseases requires
almost as much funding as common diseases, however, they are often ignored by pharmaceutical companies because there are relatively few patients with a specific disease and thus a perceived lack of investment return. Therefore, studies in orphan diseases are often heavily dependent on the generous donations by the affected families. These families are hoping to see a cure or improved care for their specific diseases. This is particularly poignant as this study was in part covered by a generous gift from an aniridia family and I am fully cognizant of their commitment to funding research into their disease and the benefit it may have to others. Furthermore, rare diseases offer a chance to understand gene function in the human context. Also PAX6 has functions in other organs and may be dysregulated in cancer (Xia et al., 2015). Therefore understanding more about PAX6 will be important for many other diseases.


Kirchner, I. D., Waldman, C. W. and Sunness, J. S. (2017) 'A series of five patients with foveal hypoplasia demonstrating good visual acuity', Retinal Cases and Brief Reports, Publish Ahead of Print.

and transgenic analysis redefine the functional domain of PAX6', *Human Molecular Genetics*, 10(19), pp. 2049-2059.


recombinant adeno-associated virus partially restores cystic fibrosis transmembrane conductance regulator function to polarized human cystic fibrosis airway epithelial cells', *Human Gene Therapy*, 16(9), pp. 1116-1123.


peripapillary segmentation in early alzheimer's disease patients', *Biomed Research International*.


APPENDICES

Appendix A Supplementary data for Chapter 2

Table A.1 Primers used to amplify and sequence PAX6 gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
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<td>GCTGGGCGTGGAATTAAGGA</td>
<td>CTTTGATTGGGGACAGCAG</td>
</tr>
<tr>
<td>2</td>
<td>TTATCTCTCAGCTTGCAAC</td>
<td>GGAGACCTGCTGAAATTTG</td>
</tr>
<tr>
<td>3</td>
<td>GCTGGTGAACCACAGCTTGA</td>
<td>CTGTTTGTGGGTTCGAGCC</td>
</tr>
<tr>
<td>4</td>
<td>TTGGGAGTTGAGGCTTACTC</td>
<td>TCTGGGCTTTTGGGACTTC</td>
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<tr>
<td>5</td>
<td>CTCTTTCTCTCCTCTCTC</td>
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<td>5a</td>
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<td>6</td>
<td>CTCTACAGTTGACTCTG</td>
<td>AGGAGAGAGCATTGGGCTTA</td>
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<td>9</td>
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<td>10&amp;11</td>
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<td>GCCACTCTCCTCTCCTCTC</td>
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<td>12</td>
<td>GCTGTGGAATGTGTTCTCTCA</td>
<td>TAAACACGCTCCCTCTCAT</td>
</tr>
<tr>
<td>13</td>
<td>CATGTCTGTITCTCAAAAGGG</td>
<td>CCGATACGTGAATTAAACAC</td>
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Primers marked with an (*) were previously reported (Love et al., 1998)

Table A.2 TaqMan assays for qRT-PCR to confirm MPLA results

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<th>TaqMan® Assay ID</th>
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<th>Transcript Accession</th>
<th>Location in gene or transcript</th>
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<td>Hs04389405_cn</td>
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<td>Hs02310190_cn</td>
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<td>Within exon 13</td>
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<td>Hs06287561_cn</td>
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<td>Hs03792128_cn</td>
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Table A.3 Primers for PCR/Sequencing of candidate genes and SNPs

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<td><strong>SCL38A8 RS7200988</strong></td>
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## Appendix B Supplementary data for Chapter 3

### Table B.1 Pathogenicity scores of novel PAX6 glycine to valine substitution

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<th>MutationTaster</th>
<th>SIFT</th>
<th>MutPred</th>
<th>Blosum62</th>
<th>PROVEN</th>
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<tr>
<td>Glycine18Valine</td>
<td>Probably damaging (score 1.000)</td>
<td>Disease causing (prediction probability 0.999)</td>
<td>Damaging (score 0)</td>
<td>Deleterious (score 0.866)</td>
<td>Damaging (score -3)</td>
<td>Deleterious (score -7.641)</td>
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<table>
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<td>GMQLGGFVNYORPLDSPRQKIV</td>
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<td>not conserved</td>
<td>18</td>
<td>GMQLGGFVNYORPLDSPRQKIV</td>
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<td>Muscidae</td>
<td>no homologs</td>
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### Table B.2 Pathogenicity predictions of the novel PAX6 frameshifting mutations

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<th>Mutation</th>
<th>PolyPhen2</th>
<th>Summary</th>
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</table>
| Ser65Profs*14  | Disease causing (prediction probability 1) | • Nonsense mediated decay  
• Amino acid sequence changed  
• Frameshift  
• Protein features (might be) affected  
• splice site changes |
| Ser321Cysfs*34 |                 |                                              |
| Met337Argfs*28 |                 |                                              |
Appendix C Supplementary data for Chapter 4

Figure C.1 PTM-plasmid design

All PTMs shares the following features: 1) a binding domain complementary to genomic target; 2) sequences essential for splice machinery including branch point site, poly-pyrimidine tract, and 3’ acceptor site sequence; and 3) full length cDNA devoid of the initiation codon. BD, binding domain; BP, branch point; PPT, polypyrimidine tract; UTR, untranslated region.
Table C.1 PTM1 sequence

PTM1 sequence:  
TAATCTTAAAGGAAATTATGGAGGTAAACGCTAGATCAACGATCAATGCTGGTATCGGCACGGCTACTGGTTTATACCGTATTACCGGCATGCATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGGCTCGCAGCCAACGTCGGGGCGGCAGGCCCTGCCATAGCCTCAGGTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATAAAAAGACAGAATAAAACGCACGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCACCGAGACCCCATTGGGGCCAATACGCCCGCGTTTCTTCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCTGCATACGCTTGATCCGGCTACCTG

Keys:
CMV promoter, BD, Splicing elements, cDNA lacking initiation exon (exons 4-12), Intron 4, 3UTR, IRES2, DsRed2, Pires-dsred2 backbone
Table C.2 PTM2 sequence

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| Keys: | CMV promoter, BD, Splicing elements, cDNA lacking initiation exon (exons 4-12), Introns 4, IRES2, DsRed2, Pires-dsred2 backbone |
Table C.3 PTM3 sequence

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| | | CCATTGACGTCAATGGGTGGAGTATTTACGG |
| | | TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCG |
| | | PTM3: |
| | | BD, Splicing elements, eDNA lacking initiation exon (exons 4-12), Intron |
| | | 4, 3'UTR, IRES2, DsRed2, Pires-dsred2 backbone |

**Keys:**

- **CMV promoter**
- **BD**
- **Splicing elements**
- **eDNA lacking initiation exon (exons 4-12)**
- **Intron**
### Table C.4 PTM4 sequence

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<th>Pires</th>
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#### PTM4 sequence

CCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCATGCAT

#### Keys:

- **CMV promoter**, **BD**, **Splicing elements**, **cDNA lacking initiation exon (exons 4-12)**, **Intron**
- **IRES2**, **DsRed2**, **Pires-dsred2 backbone**
Table C.5 PTM5 sequence

**CMV promoter, BD, Splicing elements, eDNA lacking initiation exon (exons 4-12), Intron**

4, IRES2, DsRed2, Pires-dsred2 backbone
Table C.6 PTM6 sequence

| PTM6 sequence | IRES2, DsRed2, Pires, Splicing elements, cDNA lacking initiation exon (exons 4-12), Intron |

Keys:

- **CMV promoter**, BD, Splicing elements, cDNA lacking initiation exon (exons 4-12), Intron
Appendix D Supplementary data for Chapter 5

Figure D.1 Development of conus papillaris
At ES17 (left), the conus papillaris evolves from the retina and appears as an extension of the optic nerve (ON) in the adult’s stage (right). Insets: higher magnification of the structure tip (*) and base (**). Scale bars: 100 μm (ES17), 1mm (adult), and 50 μm (adult’s insets).