THE ROLE OF CD24 IN
AGE RELATED CATARACTS

by

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ABSTRACT

Age related cataract (ARC) is a leading cause of visual disability. While the environmental determinates of the disease are known, genetics also plays a role, particularly in younger patients. However, the genetic variants that predispose a patient to cataract are not well defined. We have used a combination of bioinformatics and RNA sequencing to define the fiber cell transcriptome in order to predict genes likely to play a role in ARC. We identified a novel gene, CD24, a membrane-associated cell adhesion protein expressed at high levels in immune system and cancer tissues, as a potential mediator of ARC.

In accordance with the bioinformatics tool iSyTE, immunostaining of wildtype lenses with CD24 confirmed that it is expressed from the onset of fiber cell differentiation until adulthood. For the first time, I have shown that CD24 was preferentially expressed in lens fibers, and not in epithelial cells, in mice. Also, CD24 protein might be a clinically relevant candidate in ophthalmology as it is expressed in both the epithelial and fiber cells of human lens tissue.

Morphological analysis using bright field microscopy and refractive analysis using a 200-mesh electron microscopy grid, revealed that CD24 null mouse lenses develop normally, however they exhibit a refractive discontinuity at 4-5 months of age followed by cataract development by 1 year, attenuating their ability to refract light. Ultra-structure analysis using scanning electron microscopy revealed that the fiber cells are disorganized from 6 months onward in CD24 null lenses as opposed to wildtype
lenses. Cross sectional staining of CD24 and wheat germ agglutinin (WGA) in wildtype lenses identified that both of them are present more in shorter side of the lens fibers than the broader sides. WGA staining is reduced in CD24 null lens fibers and not in epithelial cells, suggesting that CD24 might be a predominant glycoprotein in the lens fibers. Further studies of the molecular pathways that CD24 participates in to regulate these phenotypes will shed more light on the prevention of age related cataracts.
Chapter 1

INTRODUCTION

1.1 Human Eye vs. Digital Camera

The design of a camera must be inspired by the human eye. Incoming light is primarily focused by the cornea, the clear front surface of the eye which refracts nearly two-thirds of the incoming light (Hung 2001), acting like a camera lens. The iris functions analogous to the diaphragm which controls the amount of light reaching the retina by correspondingly altering the size of the pupil which can be compared to the aperture. The ocular lens is located behind the iris (Bassnett et al. 2011) which further focuses the light and automatically adjusts focus for near and far objects like an autofocus camera lens.

The focused light then reaches the retina, the light sensitive inner lining situated at the rear of the eye which functions like an electronic image sensor, converting optical images into electric signals. These signals are transmitted to the visual cortex by the optic nerve (Selhorst and Chen 2009) which helps us visualize the final image.
1.2 The Lens – Structure and Function

The crystalline lens is a transparent, avascular tissue that helps to focus light onto the retina (Bazan 1988). The lens is encapsulated by a specialized, uninterrupted basement membrane referred to as the lens capsule, which sequesters the lens from other ocular tissues, and protects it from infection (Danysh and Duncan 2009).

The lens is composed of two types of cells- a monolayer of cuboidal lens epithelial cells (LECs) on its anterior surface, and concentric layers of elongated fiber cells. The
primary lens fibers, which are formed early in development, becomes the nucleus of the adult lens. Secondary fiber cells are formed by the continuous and regulated differentiation of epithelial cells at the equatorial zone. These cells elongate beneath the apical surface of the epithelium, and the posterior capsule, until their basal ends meet at the anterior and posterior sutures. These fiber cells are essential in maintaining the transparency of the lens and must degrade their nuclei and most other organelles, resulting in an organelle free zone (Piatigorsky 1981, Bassnett and Mataic 1997, Bassnett, Wilmarth et al. 2009). The refractive surfaces of the lens are bathed by aqueous humor in the anterior and vitreous humor in the posterior, both of which are rich in growth and regulatory factors. The aqueous humor regulates epithelial growth and maintenance while the vitreous humor supports and mediates fiber cell growth and differentiation. (Yamamoto 1976, Lovicu and McAvoy 2005, Bassnett et al. 2009). The lens is connected to the adjacent ciliary body by the zonules of Zinn (Bassnett et al. 2011).
1.2.1 Lens Development

Eye development begins with the closure of the neural tube and the head ectoderm acquiring competence to become lens and corneal epithelium (Chow and Lang 2001, Donner et al. 2006). The developing forebrain evaginates to form optic vesicle (Figure 1.3: A) and lens morphogenesis begins when the head ectoderm and the optic vesicle becomes closely associated to form lens placode (Figure 1.3: B) (McAvoy and Chamberlain et al. 1999, Lovicu and McAvoy 2005, Gunhaga 2011).

Figure 1.2 The anatomy of the ocular lens. Image adapted from Danysh and Duncan 2009.
Figure 1.3  Lens development. Image adopted from Lovicu and McAvoy 2005.

The invagination of lens placode and the optic vesicle results in the formation of the lens pit and optic cup (Figure 1.3: C), which later develops into retina. The lens pit detaches from the surface ectoderm to form lens vesicle (Figure 1.3: D) which contains precursors of the cells that will give rise to the adult lens. It is sequestered from the other ocular tissues by a surrounding basement membrane which thickens to form the lens capsule (Chow and Lang 2001, Lovicu and Robinson 2004, Lovicu et al. 2011). After the closure of the lens vesicle, all the vesicle cells are developmentally equivalent (Lovicu and Robinson 2004). Thereafter, the posteriorly situated vesicle cells exit the
cell cycle and elongate to form primary lens fibers (Figure 1.3: E) while the anterior cells remain as a monolayer, resulting in the establishment of lens epithelial cells. The apical ends of the primary fiber cells contact the apical surface of the anterior lens epithelium (Figure 1.3: F), resulting in the distinctive polarity of the lens which is maintained throughout life (Lovicu and Robinson 2004, Lovicu et al. 2011).

The lens increases in size as the epithelial cells proliferate. As the anterior epithelium proliferates, the adjacent epithelial cells migrate to the lens equator into a region referred as the transitional zone, where they withdraw from the cell cycle, differentiate, and elongate into secondary lens fibers (Lovicu and Robinson 2004, Shi et al. 2015). Many molecules regulate these processes (Duncan et al. 2000, Yoshida et al. 2001, Faber et al. 2002, Jia et al. 2007, Boswell and Musil 2015, Audette et al. 2016).

1.2.2 Lens – Transparency and Refractive index

The refractive index of the lens varies from 1.380 near the surface to 1.409 in the center, which is significantly higher than that of the surrounding media (n=1.336), thus facilitating the focus of light (Bassnett et al. 2011). The high refractive index in the center can be attributed to high concentration of crystallin proteins (>450 mg/ml) in the cytoplasm of the lens fibers (Duncan et al. 2004, Bassnett et al. 2011). As light scatter in protein solutions is directly proportional to the protein concentration, it is superficially paradoxical for the lens to be transparent. Despite the remarkable concentration of crystallins and other proteins, the short range interactions between them eliminate the light scatter by destructive interference (Bassnett et al. 2011). In addition, the intracellular desmosomes, tight junctions, adherens junctions, and gap
junctions help minimize the dimensions of the extracellular space facilitating focusing (Bassnett et al. 2011).


1.3 Cataracts

Cataract is defined as the opacification of the lens that obstructs vision which accounts for more than half of global blindness (Truscott and Friedrich, 2016). In the United States, more than 24.4 million people were affected by cataract in 2010, which is projected to increase to 50 million by 2050 (NEI). Several factors such as oxidative stress (Thiagarajan and Manikandan 2013), metabolic dysfunction, loss of ion/water balance (Donaldson et al. 2009), genetic defects/gene mutations (Hejtmancik 2008, Wang et al. 2011), aging (Hejtmancik and Kantorow 2004) and other non-genetic defects including certain diseases such as diabetes, personal behaviors such as smoking and alcoholism, as well as environmental factors such as exposure to UV radiation
(McCarty and Taylor 2002) influence cataract formation (Hejtmancik and Kantorow, 2004).

Figure 1.4  Cataracts. (A) A hypermature age related cortico-nuclear cataract with a brunescent nucleus (B) An acute sudden onset cortical cataract in a person with juvenile diabetes. Image adapted from http://www.nei.nih.gov/photo/cataract

Cataracts can be classified based on the onset of the anomaly. Congenital or infantile cataract occurs within the first year of life, while the juvenile cataract occurs within the first decade of life. Presenile cataracts are characterized by the onset of the condition before the age of 45, while age related or senile cataracts occur in the later stages (typically above 50 years of age) of life.
1.4 Congenital Cataracts

Congenital cataracts account for roughly 10% of childhood blindness (Gilbert et al. 1993, Foster et al. 1997, Gilbert and Foster 2001, Muhit and Gilbert 2003). Between 8.3 and 25 percent of congenital cataracts are known to result from gene mutations, however the number might be underestimated as 50-60% of cases are reckoned as idiopathic (Francois J 1982, Merin S 1991, Haargaard et al. 2004, Hejtmancik 2008, Yi et al 2011, Shiels and Hejtmancik 2013, Shiels and Hejtmancik 2015, Ma et al. 2016). About 72 per 100,000 children are affected by congenital cataracts, and this number spikes up to 136 per 100,000 in less-developed countries (Shiels and Hejtmancik 2013). Most of the known inherited forms of cataract are autosomal dominant, but can be X-linked or autosomal recessive, as well as syndromic or non-syndromic (Hejtmancik et al. 2001, Shiels and Hejtmancik 2013).
Figure 1.5  Morphological classification of congenital cataracts proposed by Merin. (A) Slit lamp view of a dense anterior polar cataract (B) Reflex view of posterior subcapsular cataract (C) Dense nuclear cataract (D) Punctate nuclear cataract (E) Reflex view of a lamellar pulverulent cataract with a cortical rider in the upper right (F) Sutural cataract with a pulverulent nuclear lamellar component. Image adopted from Hejtmancik 2008.

1.5 Genetics of Congenital Cataracts

About 45 genetic loci and 38 specific genes have been shown to influence cataract, though the numbers of known loci are still increasing. Among the mutant genes known, almost half are in the crystallins, about one-sixth in connexins, one-tenth in various transcription factors, and the rest bear mutations in several important genes including, but not limited to, heat shock transcription factor-4 (HSTF-4), beaded filament structural protein-2 (BFSP-2), and aquaporin-0 (AQP0, MIP) (Hejtmancik 2007, Shiels and Hejtmancik 2013, Shiels and Hejtmancik 2015).

1.5.1 Lens Crystallins

Mutations in the αA-crystallin gene has been reported to cause both autosomal dominant and recessive cataracts. For instance, while a chain termination mutation converting the tryptophan codon at position 9 into a termination codon results in autosomal recessive cataracts (Pras et al. 2000), while autosomal dominant cataracts result from non-conservative missense mutations altering a neutral or hydrophobic amino acid to arginine in αA-crystallin (Litt et al. 1998, Mackay et al. 2003, Graw et al.

1.5.2 Membrane Proteins

A missense mutation -E134G in Aquaporin-0, which is the most abundant integral membrane protein in the lens, causes non-progressive congenital lamellar and sutural cataract while another mutation replacing threonine by arginine results in a severe opacity which progresses with age (Park and Saier 1996, Francis et al. 2000). Mutation in LIM2, which is found at the protein junctions of lens membrane and binds calmodulin, cause presenile cataracts (Pras et al. 2002). The major lens-preferred gap junctions in the lens are comprised of connexins 46 (cx46) and 50 (cx50). Mutations in both cx46 (N63S) and cx50 (P88S) genes result in autosomal dominant nuclear and zonular pulverulent cataracts (Hejtmancik 2008).

1.5.3 Beaded Filament Proteins

Beaded Filaments are lens-specific intermediate filaments which are made up of BFSP1 (also known as filensin or CP115) and BFSP2 (also known as phakinin or CP49) (Sandilands et al. 1995, Blankenship et al. 2001). These proteins interact with each other as well as α-crystallin to form the beaded filament structure. Absence of exon 6 from BFSP1 results in developmental cataracts which are autosomal recessive, while a non-conservative missense mutation in exon 4 of BFSP2 results in congenital and juvenile
cataracts with autosomal dominant inheritance. (Conley et al. 2000, Ramachandran et al. 2007).

1.5.4 Growth and Transcription Factors

Mutations in HSF4, a member of the heat-shock transcription factor family, engender both autosomal dominant and recessive cataracts. However, the dominant cataracts manifest in early childhood, whereas the recessive cataracts are congenital (Bu et al. 2002, Forshew et al. 2005, Smaoui et al. 2004). Mutations in FOXE3 (Semina et al. 2001), EYA1 (Azuma et al. 2000), CHX10 (Plotnikova et al. 2007), MAF (Jamieson et al. 2002, Vanita et al. 2006), and PAX6 (Graw et al. 2006, Bremond-Gignac et al. 2010, Chograni et al. 2014) result in congenital cataracts. In addition, mutations in several other genes have been shown to cause a variety of cataracts (Shiels and Hejtmancik 2015).

1.6 Age Related Cataracts (ARC)

Age related cataracts are defined as an acquired opacity of the lens with aging. In spite of improvements in treatment, ARC is still the leading cause of blindness worldwide, affecting more than 80% of the blind people who are above 50 years of age (Jefferis et al. 2010, Zetterberg 2015, Truscott and Friedrich 2016). Several extrinsic factors such as smoking, ultraviolet radiation, injuries, obesity, diabetes, alcoholism, and aging can trigger the formation of age related cataracts (Hejtmancik and Kantorow 2004), but genetics can also play a role.
13.1.1 Aging and Cataracts

Many changes in elastic properties (Glasser and Campbell 1998), total ion content (Duncan et al. 1989), light scatter, and refractive properties (Moffat et al. 2002) take place in the lens with aging. Lens membranes contains a unique composition of lipids such as cholesterol, plasmalogen and dihydrosphingomyelin (Byrdwell and Borchman 1997), whose alteration upon aging will have functional consequences with respect to membrane fluidity, and hence binding of proteins to membranes (Cobb and Petrash 2002). Also, several post translational modifications (PTMs) upon aging lead to
cataractogenesis (Truscott 2005). Increasing oxidative stress during aging results in the thiolation of lens proteins by GSSG (Glutathione disulfide) which increases disulfide formation in crystallins, resulting in cataract formation (Takemoto 1996, Lou 2003). UV radiation catalyzes the formation of reactive oxygen species (ROS), which fosters oxidative stress, leading to the disruption of lens fiber cells leading to cataract (Wang, Lofgren et al. 2010).

1.6.2 Post-Translational Modifications with Aging

Several sites of cleavage in crystallins and many other lens proteins have been reported (Lampi et al. 1998, Shih et al. 1998). For example, truncation of MIP26/AQP0 may alter its functional properties and may compromise cell to cell communication (Kistler et al. 1995, Ball et al. 2003). Other modifications in the amino acids such as isomerization of aspartate residues (Masters et al. 1977) and deamidation of glutamine and asparagine (Lampi et al. 1998) can result in protein denaturation and hence alter protein-protein interactions. Additive modification such as methylation (Lapko et al. 2002), acetylation (Lin et al. 1998) and carbamylation (Lapko et al. 2001) also influence age related cataract formation.

1.6.3 Genetic Factors and Age Related Cataracts

Studies on congenital cataracts have shown that genetic alterations can cause cataract pathology. Recent meta-analysis and candidate gene approaches have identified genes that are involved in age related cataracts as well. Like many complex diseases, age related cataract is a multifactorial disease which results from labyrinthine
interactions of several environmental and genetic factors. These interactions are not completely understood, and the genetic contribution to the disease varies (Skiljic et al. 2015). For example, in a twin study which was performed to establish the relative contribution of environmental versus genetic factors in age related cataract, the genetic contribution varied depending on the type of cataract. In this study, 226 monozygotic and 280 dizygotic female twin pairs between 49 to 79 years were utilized and the cataract was graded by the clinical and objective grading system. In cortical cataract, the genetic contribution (additive and dominant genetic effect) was 58% whereas the environmental factors accounted for 26% in digital grading. However, in nuclear cataracts, the genetic contribution was estimated to be 48% and the environmental contribution was 14% (Hammond et al. 2000, Hammond et al. 2001).

Additionally, inherited genetic abnormalities can increase the risk of age related cataracts (Benedek 1997). A recent study has shown that three single nucleotide polymorphisms in the EFNA5 gene, which encodes Ephrin-A5 protein, increase the risk of ARC in the Chinese population (Lin et al. 2014). Several meta-analyses have revealed polymorphisms in genes which results in age related cataract formation. For example, polymorphisms in XRCC1 gene, which encodes X-ray repair cross-complementing protein 1 (Arg399Gln) are associated with the development of age related cataract in Han Chinese (Luo et al. 2011, Liu et al. 2015). Furthermore, polymorphisms in GSTT1 which encodes glutathione S-transferase theta 1 (Liao et al. 2015), HSP70 (Heat shock protein 70) (Zhang et al. 2013), Ezrin (Lin et al. 2013), and TDRD7 (Tudor domain-containing protein 7) (Zheng et al. 2014) are associated with age related cataracts. Another study has identified two new loci for age related nuclear cataract, one on chromosome 3q25.31 in KCNAB1 gene, which encodes voltage-gated potassium
channel subunit beta-1 and the second locus in chromosome 21 in the proximity of CRYAA (Crystallin alpha A). As the severity of the cataract increases, KCNAB1 expression seem to upregulate and CRYAA seem to downregulate in the lens epithelium (Liao et al. 2014). Though many genes regulating ARC have been identified using meta-analysis and candidate gene approaches, the likely interaction of these genes with environmental factors makes it difficult to identify or study genes solely responsible for human ARC. Hence, using animal models for studying the age related conditions is advantageous as it minimizes the environmental factors such as UV light exposure that can potentially contribute to the cataract and hence attenuates the genetic variations due to these factors.

1.7 Using Animal Models for Studying Age Related Cataracts

It is indeed difficult to obtain sufficient human cataractous lenses to perform experiments. Cataractous lenses are obtained only from the cadavers and prevalence of cataract surgery in the first world leaves few cadavers with untreated cataract. Further, cataract surgery on human subjects disrupts lens fibers so these cells are unavailable for analysis. Mouse models have been historically used to study cataract (Wolf et al. 2000, Graw 2009, Puk et al. 2010, Fan et al. 2012), for example, knockout of the glutathione peroxidase-1 (GPX1) gene results in significantly more age related cataracts (Wolf et al. 2005), but in general, mouse cataract models have provided a better understanding of lens development than the aging process. There have always been efforts to identify and generate better mouse models to study age related cataracts (Pendergrass et al. 2005, Puk et al. 2010). These mouse models will help us identify and understand the genes that are more specific to the pathology of ARC.
1.8 Identifying Genes Associated with Age Related Cataracts

Our lab postulated that genes associated with age related cataracts are likely preferentially expressed in the lens and that they probably do not cause other ocular pathologies. Genes specific to cataracts can be identified in individuals with cataracts who are otherwise healthy and of normal physiological condition compared to their age. Cataracts resulting from complications in other systems will not be helpful in identifying genes specific to age related cataracts because the manifestation of cataract is a by-product of anomalies in those systems or part of the syndrome. For example, mutations in either COL4A3, COL4A4 or COL4A5 are known to cause Alport syndrome which affects the kidneys, and is also associated with lens capsule abnormalities and cataract formation (Longo et al. 2004, Pescucci et al. 2004, Crockett et al. 2010, Firtina et al. 2009). In this example, these genes may not be cataract specific genes. Also, as cataract often affects lens fibers, fiber cell specific or preferred genes would be good candidates for studying this phenomenon.

In order to identify fiber cell preferred genes, a previous lab member, Dylan Audette, performed RNA sequencing to identify differentially expressed genes in animals which fail to make lens fibers (Audette at al. 2016). In Prox1 mutant mice, the lens fibers fail to develop after lens vesicle closure, as seen from the absence of eosinophilic primary fibers when compared to their wildtype counterparts (Figure 1.7: A-F)
Figure 1.7  *Prox1* deletion from the early lens arrests its development at the LV. (A-F) Mouse eye sections at E12.5 (A, D), E13.5 (B, E) and E15.5 (C,F) stained with Hematoxylin and Eosin. In WT, primary lens fibers (LFs; pink) were evident by E12.5 (A), with secondary fibers produced at E13.5 (B) and E15.5 (C). In *Prox1* cKO lenses, the posterior-most cells never elongate into eosinophilic primary (D) or secondary fibers (E, F). (A-F) Blue, Hematoxylin; pink, Eosin. a, anterior; p, posterior; r, retina; e, lens epithelium; f, LFs. Scale bars: 200 µm in A-F. Image and the figure legend adapted from Audette et al. 2016

RNA-sequencing was performed on E13.5 lenses to determine the lens transcriptome after the removal of *Prox1* protein from the lens, which revealed 624
differentially expressed genes. This thesis aims to study a gene, CD24, which was 4.5 fold downregulated in Prox1 mutant mice (Audette et al. 2016).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>RPKM (reads per kilobase per million)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24</td>
<td>1584</td>
</tr>
<tr>
<td>Cx46 (Connexin 46)</td>
<td>278</td>
</tr>
<tr>
<td>CP49 (beaded filaments)</td>
<td>1641</td>
</tr>
<tr>
<td>Aqp0 (Aquaporin 0)</td>
<td>3954</td>
</tr>
<tr>
<td>Itgb1 (Integrin β1)</td>
<td>75</td>
</tr>
<tr>
<td>Cryba4 (βA4-Crystallin)</td>
<td>12375</td>
</tr>
</tbody>
</table>

**Table 1.1**  
Comparison of mRNA levels of CD24 with the mRNA levels of other important proteins in the lens of E15.5 wildtype lenses. The values are obtained from Dr. Dylan Audette.

This gene was not previously reported to be expressed in the lens but has been studied in several other systems (Fang et al. 2010). My thesis seeks to understand for the first time the role of CD24 in the lens.
1.9 CD24

Due to its heat resistance, Springer first named CD24 "heat stable antigen" in 1978 (Springer et al. 1978). In 1990, the mouse CD24 gene was cloned and found to encode a protein whose mature form comprises of only 27 amino acids (Kay et al. 1990). Soon after, the CD24 gene was identified in humans (Kay et al. 1991). In situ hybridization studies determined that the human CD24 gene is located on chromosome 6q21 (Hough et al. 1994). In both mouse and humans, many CD24 intronless pseudogenes have been identified and located on chromosomes 1, 15 and Y (Kristiansen et al. 2004). The mouse CD24 gene was mapped to chromosome 10 and initially referred as CD24a to uniquely differentiate it from the intronless retroposons located on chromosomes 8 and 14 (Wenger et al. 1993). The usage of the name CD24a is obsolete as the retroposons are not expressed (Fang et al. 2010).

The mouse CD24 cDNA has an open reading frame of 231 bp and a long 3’UTR (untranslated region) of 1.5 kb which plays an important role in its mRNA stability (Zhou et al. 1998). Comparison of the primary structure of mouse and human CD24 protein revealed several consensus N- and O- linked glycosylation sites (Figure 1.7) (Kristiansen et al. 2004). The human CD24 molecule has evolutionally acquired additional serine and threonine residues compared to the mouse protein (Aigner et al. 1997), conferring a typical mucin-like property on the molecule (Kay et al. 1991) due to the extensive glycosylation through GalNAc O-linkages provided by the additional serine and threonine residues (Kufe 2009). O-linked glycosylation sites are prevalent near the N-terminus of CD24, creating densely packed glycans at the tip of the molecule. Several glycosylation sites are present adjacent to the surface of the membrane (Kristiansen et al. 2004) and any aberration in the glycosylation of these membrane
glycans can result in cancer (Christiansen et al. 2014). Biochemical studies on murine models have determined that CD24 protein isolated from different tissues and/or cell types have molecular weights ranging from 20 to 70 kDa, due to the variation in the glycosylation of CD24, which is dependent on cell type (Alterman et al. 1990, Kay et al. 1991, Rougon et al. 1991, Wenger et al. 1991).

1.9.1 Expression of CD24

Figure 1.8  A model of CD24. The core protein is composed of 27 amino acids with many glycosylation sites which are variably used depending on the cell type and species analyzed. Image adapted form Kristiansen et al. 2004

Several studies have shown that CD24 is overexpressed in a plethora of tumor cells (Kristiansen et al. 2004). CD24 was found in several non-Hodgkin B cell lymphomas and leukemias (Abramson et al. 1981, Knowles et al. 1983, Oertel et al. 1988). Several tumors of the central nervous system including medulloblastomas, central neurocytomas, undifferentiated neuroblastomas and gliomas showed elevated
levels of CD24 (Poncet et al. 1996, Senner et al. 1999, Kristiansen at al. 2004). CD24 expression was found in several renal tumors including nephroblastomas and renal cell carcinomas (Droz et al. 1990a, b), hepatocellular carcinoma (Huang and Hsu 1995), and mesoblastic nephroma (Daniel et al. 2000). CD24 is expressed by several breast cancer cell lines (Yang et al. 1999), breast cancer (Kristiansen et al. 2003, Schabath et al. 2006, Athanassiadou et al. 2009) and ovarian cancer tissues (Kristiansen et al. 2002). CD24 is also expressed in a variety of other tumors such as small cell lung cancer (Jackson et al. 1992, Pass et al. 1998), prostate cancer (Kristiansen et al. 2004), pancreatic cancer (Sagiv et al. 2006), erythroleukemia, cholangiocarcinoma (Su et al. 2006, Agarwal et al. 2007), colorectal carcinoma (Nestl et al. 2001, Saito et al. 2002), nasopharyngeal carcinoma (Karran et al. 1995), bladder cancer (Gromova et al. 1999), urothelial carcinoma (Winkler et al. 2007, Choi et al. 2007) and Merkel cell carcinoma (Deichmann et al. 2003).

1.9.2 CD24 and its Functions in the Immune System

CD24 expression is higher in B-cell progenitor cells than mature resting B-cells, but is not expressed by terminally differentiated plasma cells (Hunte et al. 1998). CD24 knockout mice or chimeric mice are viable though and just exhibited a mild block in B-lymphocyte-poiesis (Kristiansen et al. 2004). The mean-life of erythrocytes is reduced in CD24 deficient mice, and these cells tend to aggregate and be susceptible to hypotonic lysis (Nielson et al. 1997). Also, CD24 deficiency resulted in the attenuation of late pre-B and immature B-cell populations in the bone marrow (Nielson et al. 1997, Lu et al. 2000). CD24 co-stimulates clonal expansion of CD4 T-helper cells, which plays a major role in the adaptive immune system (Liu et al. 1992, Hubbe and Altevogt...
Moreover, deficiency of CD24 accentuates clonal deletion (Carl et al. 2008). CD24 is also highly expressed at the surface of immature and activated T-cells, but its expression decreases in peripheral T-cells (Crispe and Bevan 1987, Hubbe and Altevogt 1994). It has been shown that while in a lymphopenic host, a functional CD24 gene is essential for homeostatic proliferation of T-cells (Li et al. 2004), while it acts as a negative regulator of T-cell homeostatic proliferation in dendritic cells (Li et al. 2006).

1.9.2.1 CD24 and Autoimmune Diseases

CD24-deficient mice were resistant to experimental autoimmune encephalomyelitis and thyroiditis suggesting a possible role for CD24 in autoimmune diseases (Bai et al. 2004, Chen et al. 2009). In vitro experiments have shown that CD24 expressed on microglia and astrocytes facilitates the activation and proliferation of pathogenic T-cells (Bai et al. 2004). Over expression of CD24 in the central nervous system under the regulation of a glial specific promoter increased the severity of experimental autoimmune encephalomyelitis (EAE) (Liu et al. 2007). Several polymorphisms in CD24 are linked to an increase in the risk of diseases such as multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus (SLE) (Fang et al. 2010). For example, the human CD24 gene displays an allelic polymorphism (Val to Ala exchange) in the C-terminus of the mature protein. In CD24V/V patients, there exists the risk and progression of multiple sclerosis and also the expression of CD24 on peripheral blood T-cells was higher than that of CD24A/A patients (Zhou et al. 2003) which was confirmed by a Spanish cohort (Otaegui et al. 2006) although not verified by other research groups (Goris et al. 2006). The CD24V/V genotype was seemed to be more
prevalent in populations with SLE (Sanchez et al. 2007), rheumatoid arthritis (Sanchez et al. 2008) and giant cell arthritis (Rueda et al. 2008). Additionally, CD24 interacts with SiglecG (Sialic acid-binding immunoglobulin-type lectins) (mouse), and Siglec10 (human), and selectively represses the host response to tissue injury. Danger associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) are host molecules which activate innate immune system in response to tissue injury. As CD24-SiglecG interaction does not affect host response to pathogen associated molecular patterns, it is proposed that the CD24-SiglecG pathway differentiates DAMPs from PAMPs (Liu et al. 2009).

1.9.3 CD24 and Tumor Biology

CD24 is an important marker for cancer prognosis and diagnosis (Kristiansen et al. 2004) as CD24 expression is higher in invasive carcinoma than in precancerous lesions. High CD24 expression correlates with several factors including, but not limited to, tumor size, lymph node positivity, poor prognosis, and histological grade (Kristiansen et al. 2003, Athanassiadou et al. 2009, Fang et al. 2010). Kristiansen et al (2003) showed that high CD24 serves as an independent prognostic marker in non-small cell lung cancer patients. Additionally, high CD24 expression was positively correlated with tumor lymph node metastasis, tumor grade and poor survival time in esophageal squamous cell carcinoma (Sano et al. 2009), cholangiocarcinoma (Su et al. 2006), urothelial carcinoma (Choi et al. 2006, Winkler et al. 2007), ovarian cancer (Kristiansen et al. 2002) and prostate carcinomas (Kristiansen et al. 2004). Antibody-blocking
experiments have shown that an anti-CD24 monoclonal antibody can suppress the growth of human pancreatic cell lines (Sagiv et al. 2006).

1.9.4 CD24 and its Ligands

The ligand specificity of CD24 seems to vary depending on both cellular context and its glycosylation pattern (Fang et al. 2010). P-selectin is the predominant ligand of CD24 in the immune system and cancer, where it fosters cell adhesion and cancer cell-rolling and colonization (Sammar et al. 1994, Aigner et al. 1997, Aigner et al. 1998, Friederiches et al. 2000). Under physiological conditions, CD24 facilitates the adhesion of monocytes and neutrophils to activated endothelial cells or platelets, both of which express P-selectin. However, lymphocytes showed no adhesion to activated endothelial cells or platelets, which suggests that the cell type specific glycosylation influences CD24-P-selectin interactions (Kristiansen et al. 2004).

In brain, CD171, also known as L1, seems to serve as a receptor for 2, 3-linked sialic acid on CD24. The tetrasaccharide carbohydrate that is usually attached to O-glycans, is commonly referred as “Lewis^X”. This tetrasaccharide is present in CD24 and interacts with receptors such as contactin and TAG-1 in brain tissue (Sammar et al. 1997, Kleene et al. 2001, Fang et al. 2010). These interactions serve to mediate CD24 induced inhibition of neural growth. CD24 protein also binds to danger associated molecular patterns (DAMPs) in hematopoietic cells, which forms a trimolecular complex with siglec G (mouse) and siglec 10 (human) to selectively repress damage-induced immune responses (Chen et al. 2009).
1.9.5 CD24 Signaling

CD24 also participates in a number of signaling pathways regulating tumor and other immune processes. In a recent study which investigated the effects of overexpression of CD24 in human gastric cancer cells, it was shown that the ectopic expression of CD24 increased the expression of EGFR. However, a knockdown of CD24 impaired the EGFR signaling cascade and cell migration velocity. CD24 by mediating RhoA activity, maintained EGFR stability in gastric cancer cells and its depletion resulted in lower ERK and Akt phosphorylation levels, suggesting a positive correlation between CD24 and EGFR levels (Deng et al. 2016). Also, knockdown of CD24 in breast cancer cells expressing HER2 (Human epidermal growth factor 2), downregulated Her2 expression and also suppressed the phosphorylation of Akt. Whereas, CD24 expression was increased upon the stable over expression of HER2 in HER2, estrogen receptor and progesterone receptor triple-negative breast cancer cells. Overall, this study indicates that CD24 expression promotes the expression of HER2 and further activation of PI3K-Akt signaling (Hosonaga et al. 2014).

In addition, CD24 participates in integrin signaling. Integrins are a heterodimeric family of proteins containing 18 α- and 8 β-subunits, whose combination can bind to a wide variety of ligands such as components of extracellular matrix and mediates cellular adhesion and migration processes (Hynes 2002). CD24 can regulate the binding of α4β1 (VLA-4) to VCAM-1 and FN40 (a fragment of fibronectin which has the α4β1-binding motif) in pre-B cells (Hahne et al. 1994). In a rat carcinoma system, CD24 expression promoted tumor cell proliferation and invasion by its interaction with P-selectin. In addition, CD24 stimulated cellular adhesion to fibronectin, collagens I and IV, and laminin by activating α3β1 and α4β1 integrin
(Baumann et al. 2005). The interactions of integrins with lipid rafts have been regarded as an important factor in regulating integrin activation. Transfection of CD24 into CD24-negative carcinoma cells induced the localization of β1 integrin into lipid rafts rich in cholesterol and sphingolipid domains, which does not happen otherwise (Runz et al. 2008). Also, CD24 recruits Src family protein tyrosine kinases (PTKs) via membrane rafts and mediates signal transduction by activating the mitogen-activated protein kinase pathway, which is involved in B-cell and T-cell development, apoptosis, cell binding and adhesion and stabilizing integrins (Fischer et al. 1990, Sammar et al. 1997, Krauss and Altevogt 1999, Salamone et al. 2001, Suzuki et al. 2001, Gekara and Weiss 2004, Schabath et al. 2006).

Although we know the role of CD24 in many other systems, its function is the lens is not studied or reported. This thesis has made several key findings about the expression and function of this gene in the lens which will be discussed in the results chapter.
Chapter 2
MATERIALS AND METHODS

2.1 Lens Enrichment Analysis by the Gene Prediction Tool iSyTE

The normal expression pattern of CD24 during lens development was predicted using the iSyTE database (Lachke et al. 2012). To identify genes with lens enriched expression, iSyTE utilizes in-silico subtraction in which lens microarray datasets obtained at various embryonic (E10.5, E11.5, E12.5, E16.5, E17.5, E19.5), new born (P0, P2, P4, P8, P12, P20, P28) and adult stages (P30, P42, P52, P56, P60) are compared to the embryonic whole body (WB) which contains combined embryonic datasets (E10.5, E11.5, E12.5), from which the entire eye was removed by microdissection (Lachke et al. 2012, iSyTE-2, Lachke lab Unpublished).

2.2 Animals

All animal experiments conducted in this study conform to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research, and also are approved by the University of Delaware Institutional Animal Care and Use Committee (Approval number: 1039). All mice were bred and maintained in a pathogen free environment under a 14/10-hour light/dark cycle at the animal facility of the University of Delaware.

2.2.1 CD24 Knockout Mice

C57BL/6<har> mice lacking the promotor region and the exon-1 of CD24, CD24<sup>tm1Pjln/tm1Pjln</sup>, henceforth referred as CD24 null mice, were obtained from Dr. Yang Liu, Children’s National Health System, Washington, DC. CD24 deficient mice
were homozygous for the targeted gene throughout their system (Nielson et al. 1997). All control mice used in this study are C57BL/6<sup>+</sup> mice. The embryonic staging was carried out by designating the day the vaginal plug was found in the dam as E0.5 (Embryonic day 0.5).

### 2.3 Isolation and Embedding of Samples for Immunofluorescence

Pregnant female mice were euthanized for specific embryonic time points in accordance with IACUC standards. The mouse is placed in a chamber and charged with dioxide at the rate of 2-3 L/min. Once the animal was unconscious, the flow rate was increased. Animals were exposed to CO<sub>2</sub> for an additional minute past the complete cessation of breathing. Double killing was performed by cervical dislocation following CO<sub>2</sub> administration. Then, the embryos were isolated, the head of the embryos were embedded in a Tissue-Tek<sup>®</sup> Cryomold<sup>®</sup> filled with Tissue-Tek<sup>®</sup> O.C.T. Compound (Optimum Cutting Temperature) (Sakura<sup>®</sup> Finetek, Torrance, California), and immediately placed at -80°C to freeze the samples. For analyzing adult lens samples, whole eyes were collected at different time points and were embedded in 15mm x 15mm x 5mm intermediate Tissue-Tek<sup>®</sup> Cryomold<sup>®</sup> filled with Tissue-Tek<sup>®</sup> O.C.T. Compound (Sakura<sup>®</sup> Finetek, Torrance, California) and immediately placed at -80°C to freeze the samples.

### 2.4 Cross-sectional (equatorial) Embedding of Samples for Super Resolution Microscopy

Two month old mice were euthanized in accordance with IACUC standards as described above. Whole eyes were collected at different time points and were embedded
equatorially in 15mm x 15mm x 5mm intermediate Tissue-Tek® Cryomolds® filled with Tissue-Tek® O.C.T. Compound (Sakura® Finetek, Torrance, California) and immediately placed at -80°C to freeze the samples.

2.5 Immunofluorescence

All immunofluorescence analysis was performed as previously described (Reed and Duncan 2001). Briefly, the embedded samples were subjected to cryosectioning using a Leica CM3050 S Cryostat. 16μm sections were collected on Fisher Colorfrost/Plus (ColorFrost Plus, Fisher Scientific Hampton, NH) slides at -15°C to -18°C for embryos and at -13°C to -15°C for adult lenses and stored at -80°C. For immunostaining, initially the samples were fixed in 1:1 acetone: methanol at -20°C for 20 minutes. Blocking buffer was prepared by dissolving 1% BSA (Bovine Serum Albumin, Sigma Aldrich) in 1X PBS solution, and the slides were blocked for one hour at room temperature. Primary purified rat anti-mouse CD24 antibody (Catalogue # 557436, BD Pharmingen, San Diego, CA) was diluted in the blocking buffer (1:100), applied onto the slides, and incubated in a humid chamber at room temperature for one hour. Then the slides were washed with 1X PBS for 10 minutes, thrice. For secondary antibody staining, slides were stained with 100μl of a cocktail containing a 1:2000 dilution of DRAQ5™ (Biostatus, Leicestershire, United Kingdom), and 1:200 dilution of AlexaFluor® 568 goat anti-rat IgG (H + L) (catalogue # A11077, Life technologies, Eugene, OR) secondary antibodies. For Wheat Germ Agglutinin (WGA) staining, 1:200 of AlexaFluor® 488 conjugated WGA, which binds N-acetyl-d-glucosamine and sialic acids, (Catalogue number, W11261, Molecular probes™, Life technologies, Eugene, OR) was added to the above secondary antibody cocktail to visualize cell membranes.
(Ohno et al., 1986). The slides were incubated for one hour at room temperature in a dark humid chamber followed by three, 10 minute washes with 1X PBS in a dark environment. Excess solution surrounding the tissue was wiped with a Kimberly-Clark® Kimwipe (Item # UX-25000-35, Vernon Hills, IL), and approximately 150μl of mounting media (10 ml of PBS containing 100 mg of p-phenylenediamine to 90 ml of glycerol, pH 8.0) was placed over the tissue, a coverslip added (Item # 12-544-C, Fisherbrand™, Fisher Scientific, Pittsburg, PA), and sealed with nail polish. The slides were stored at -20°C and analyzed within two weeks. For all the immunostaining analysis, lenses from three different animals each (3 biological replicates) were used. Human lenses were obtained from the San Diego Eye Bank and sections obtained from a 67 year old human lens were utilized for immunostaining analysis, using the same procedures described above for the mouse lens.

### 2.6 Confocal Microscopy

The slides were imaged using a Zeiss LSM 780 Confocal Microscope (Carl Zeiss Inc., Gottingen, Germany) equipped with a 405 nm diode laser, an Argon laser with 458/488/514 nm lines, DPSS 561 nm and HeNe 633 nm laser. All comparisons of staining intensity between the samples were done on sections stained simultaneously. The imaging was carried out under identical laser power and software settings to ensure the validity of intensity comparisons. In all the images, manipulations such as adjusting the brightness/contrast was done identically using Adobe Photoshop (Adobe, San Jose, CA) in both experimental and control images.
2.7 Super Resolution Structured Illumination Microscopy

Slides containing the equatorial sections of the lens (Cheng et al. 2016) were stained using the immunofluorescence protocol described above. Super resolution imaging was carried out using a Zeiss Elyra PS1 (Carl Zeiss Inc., Jena, Germany) equipped with a plan-Apochromat 63x/1.4 NA Oil objective lens. Image stacks were taken at 0.110μm z-interval with 25 images per plane and a total z distance of 5 – 15 μm (Scheiblin et al. 2014). Raw images were processed and reconstructed to obtain high-resolution structures and channels were aligned using ZEN software (Carl Zeiss Microimaging) based on structured illumination algorithms.

2.8 Morphological and Optical Analysis of Lens

Mice were euthanized at specific time points in accordance with IACUC standards as described above. Eyes were removed and lenses were dissected under a dissecting microscope. Lens transparency was assessed by placing lenses in Medium 199, (Mediatech Inc, Manassas, VA) at 37°C to prevent cold cataract formation. Photographs were taken under both bright-field and dark field conditions using a Cannon digital camera A420 mounted on a Zeiss Stemi SV 11 Apo Stereo microscope (Zeiss, Thornwood, NY). For morphological analysis, 2-, 4-, 6-, 9-, 12-month old lenses from three biological replicates (N=3) were utilized. Lens diameter was measured using Adobe Photoshop (Adobe, San Jose, CA). Statistical significance was assessed by unpaired t-test using GraphPad Prism 7.01 (GraphPad Software, Inc. La Jolla, CA), with data reported as mean ± S.E.M.
2.8.1 Refractive Analysis

For optical analysis, the lenses were dissected and placed on a 200-mesh electron microscopy grid under Medium 199 and photographed under bright field conditions using the same camera described above. For refractive analysis, 2-, 4-, 6-, 9-, 12-month old lenses from three biological replicates (N=3) were used.

2.9 Scanning Electron Microscopy

Eyes were enucleated and immersion fixed in 0.08M Sodium Cacodylate buffer pH 7.4 (Electron Microscopy, Hatfield, PA), 1.25% Glutaraldehyde (Electron Microscopy, Hatfield, PA) and 1% Paraformaldehyde (Electron Microscopy, Hatfield, PA) for five hours. Then, the lenses were excised and transferred to fresh fixative and incubated for another 48 hours. The lenses were washed with 1X PBS following fixation. The lens capsule and the superficial fiber cells were peeled to reveal the cortical fiber cells. Some lenses were peeled further to expose the nuclear fiber cells. The peeled lenses were subjected to an ethanol dehydration series (25%, 50%, 75% and 100% 10 min each) followed by overnight incubation in fresh 100% ethanol. The following day, the lenses were incubated with 100% fresh ethanol for 2.5 hours, twice. Then, the lenses were dried in 1:2 hexamethyldisilazane (HMDS)/ethanol for one hour, 2:1 HMDS/ethanol for one hour and finally in 100% HMDS for 30 minutes, twice. Then the samples were vacuum dried overnight and mounted on aluminum stubs. The samples were coated with Gold/Palladium using Leica EM ACE600 coating system and viewed under Hitachi S-4700 Field Emission Scanning Microscope (Tokyo, Japan).
2.10 Whole Mount Staining

Eyes were enucleated and immersed in 1X PBS. The whole lens was dissected and transferred to a petri-dish coated with 1:10 diluted SYLGARD® 184 silicone elastomer curing agent (Dow Corning Corporation, Midland, MI). The anterior side of the lens was placed face down and the whole lens capsule was peeled from the posterior side of the lens. After the removal of residual fiber cells, the capsule was washed with 1X PBS and subjected to the standard staining protocol.

2.11 Gel Electrophoresis

Three biological replicates of 3-month old wildtype (C57BL/6.har) and CD24 null lenses, each, were isolated and homogenized using ice-cold lysis buffer (50mM Tris-HCl, 150 mM NaCl, 1% NP-40 (Catalogue number # NP40S, Tergitol, Sigma-Aldrich, St. Louis, MO), 0.5% Na-deoxycholate, 0.1% SDS). 1X Halt protease and phosphatase inhibitor was added to the lysate (Thermo Scientific™, Rockford, IL). The cellular debris was separated using centrifugation at 14000g at 4°C for 30 minutes. Final protein concentrations were measured using the Pierce™ BCA protein assay kit (Catalogue number # 23225, Thermo Scientific™, Rockford, IL) as per the manufacturer’s protocol. 30 μg of protein was resolved using 4-20% Mini-PROTEAN® TGX™ Precast polyacrylamide gel at 100 V for 90 mins and the gel was stained with SimplyBlue Safestain (Invitrogen, Grand Island, NY) according to the manufacturer’s protocol.
3.1 Bioinformatics Analysis Using iSyTE Revealed a Novel Gene in Lens Biology – CD24

Previously in our lab, RNA sequencing was performed to analyze the fiber cell transcriptome in Prox1 mutants which fail to make lens fibers (Figure 1.7: A-F). This analysis revealed that Prox1 activates the expression of a large proportion of genes that are expressed preferentially in the lens (Audette et. al., 2016). Overall, 642 genes were differentially expressed in the Prox1 conditional knockouts at E13.5. Among the 356 downregulated genes, CD24, a heavily glycosylated cell adhesion molecule known for its function in immune cells (Kristiansen et al. 2004), was found to be 4.5 fold downregulated. CD24 had never been previously studied in lens, although it is expressed at very high levels at the mRNA level at E15.5 in wildtype lens (Manthey et al. 2014). The mRNA levels of CD24 (1584 RPKM (reads per kilobase per million)) was on par with that of beaded filaments (1641 RPKM), an important filament protein in the lens and 20 fold higher than β1 integrin (75 RPKM), an important protein involved in the development of lens (Table 1.1). This led us speculate that CD24 might be an important molecule in lens biology.

In order to determine the gene expression profile of CD24 in wildtype lenses compared to the average expression over the embryo, a bioinformatics tool, iSyTE (Lachke et al. 2012), was utilized which employs the use of in-silico subtraction in which the lens microarray datasets at various embryonic (E10.5, E11.5, E12.5, E16.5, E17.5, E19.5), new born (P0, P2, P4, P8, P12, P20, P28) and adult stages(P30, P42, P52, P56, P60) are compared to the embryonic whole body (WB) which contains combined
embryonic datasets (E10.5, E11.5, E12.5), from which the entire ocular tissue was removed by microdissection (Lachke et al. 2012, iSyTE-2, Lachke lab unpublished). This showed that CD24 was expressed preferentially in the lens from embryonic day 12.5 (onset of lens fiber cell differentiation) until adulthood. The lens enrichment scores were higher at E16.5 (8.14) and P30 (7.67) suggesting that CD24 might be more essential during these developmental timepoints than the others (Figure 3.1).

**Figure 3.1** Gene expression of CD24 using iSyTE. iSyTE data shows that CD24 is expressed in wildtype lenses from embryonic day 12.5 till adulthood. The numbers beneath each developmental time-point denotes the lens enrichment score (LE score) of CD24 gene which is a measure of its fold difference between the lens and the whole body (WB) reference (Lachke et al. 2012). This analysis was performed by Dr. Deepti Anand of Dr. Salil Lachke lab, University of Delaware, using the unpublished, updated version of iSyTE.
3.2 CD24 is Expressed in the Lens from Embryonic Day 12.5 (E12.5) to Adulthood

In order to determine the expression pattern of CD24 at various embryonic and adult timepoints, immunostaining was performed in lenses of wildtype animals. Co-staining was performed with wheat germ agglutinin (WGA) which is a lectin that binds to the carbohydrate residues of proteins found on the cell surface (Gallagher et al. 1985), which is an often used as a counter stain in immunofluorescent experiments in lens (Scheiblin et al. 2014). The expression of CD24 was observed at E10.5 (Figure 3.2: A) and WGA staining was brighter in the lens pit and the extracellular matrix surrounding the interior and exterior of developing lens vesicle cells (Figure 3.2: B). The CD24 expression is prevalent in the elongating lens fiber cells at E12.5 (Figure 3.2: D) and intense when compared to E10.5. WGA staining was also observed in the developing lens fibers at E12.5 (Figure 3.2: E). By E16.5, the expression of CD24 was confined to lens fiber cells and not identified in lens epithelium (Figure 3.2: G), while the WGA staining was observed in both epithelial and fiber cells (Figure 3.2: H).
Figure 3.2  Expression of CD24 in embryonic development in C57BL/6<sup>har</sup> mice. (A) CD24 expression alone in wildtype lens at E10.5 (B) WGA staining alone in wildtype lens at E10.5 (C) Merge of CD24+WGA in wildtype lens at E10.5 (D) CD24 expression alone in wildtype lens at E12.5 (E) WGA staining alone in
wildtype lens at E12.5 (F) Merge of CD24+WGA in wildtype lens at E12.5 (G) CD24 expression alone in wildtype lens at E16.5 (H) WGA staining alone in wildtype lens at E16.5 (I) Merge of CD24+WGA in wildtype lens at E16.5. Scale bar = 71μm. Red=CD24, Green=WGA, Blue=nucleus. lv- lens vesicle, a-anterior, p-posterior, e-epithelial cells, f-fiber cells, tz-transition zone, r-retina

It is evident that the expression of CD24 in a two-month old adult mouse lens is confined to fiber cells and cannot be detected in lens epithelial cells or lens capsule (Figure 3.3: A). The WGA expression can be visualized clearly in fiber cells and lens capsule, but whether or not it is expressed in epithelial cells cannot be clearly ascertained by this experiment (Figure 3.3: B). The co-localization of CD24 with WGA suggests that CD24 is preferentially found at the cell membrane (Figure 3.3: C).
Figure 3.3  Expression of CD24 in 2 month old C57BL/6 mice. (A) CD24 alone in 2-month old wildtype lens (B) WGA staining alone in 2-month old wildtype lens (C) Merge of CD24 + WGA staining in 2-month old wildtype lens. Scale bar = 38.5 μm. Red=CD24, Green=WGA, Blue=nucleus. c-capsule, e-epithelial cells, f-fiber cells, tz-transition zone.

3.3 CD24 is not Expressed in Lens Epithelial Cells and is Fiber Cell Specific in Mice

In order to determine whether CD24 is expressed in the epithelial cells, whole mount staining of the lens epithelium was performed in a two-month old C57BL/6 (wildtype) lens. In a whole mount epithelial preparation, imaged to avoid all fiber cell remnants (Figure 3.4: A-C), CD24 expression was undetectable in the epithelial cells of wildtype mice (Figure 3.4: A). WGA staining was detectable in the whole mount of epithelial cells (Figure 3.4: B) when compared to the cross section of the lens (Figure...
As we know that CD24 was expressed in the fiber cells, we used the whole mount containing the fiber cell tips (residual fiber cells) attached to the epithelial cells, as an internal control (Figure 3.4: D-F) where the CD24 expression was detectable in the residual fiber cells (Figure 3.4: D). Together, this confirms that CD24 is a lens fiber cell preferred protein and not expressed in epithelial cells in mice.

**Figure 3.4** Whole mount staining and immunofluorescent analysis of CD24 and WGA in the epithelial cells of a 2 month old C57BL/6<har> mice. (A-C) CD24 and WGA staining in whole mount of epithelial cells with complete removal of lens fiber cells (A) CD24 expression
alone in the epithelial cells (B) WGA staining in the epithelial cells (C) Merge of CD24+WGA+DRAG5 staining in the epithelial cells (D-F) CD24 and WGA staining in whole mount of epithelial cells with fiber cell tips (D) CD24 expression alone in epithelial cells with fiber cell tips (E) WGA staining in the epithelial cells with fiber cell tips (F) Merge of CD24+WGA+DRAG5 staining in the epithelial cells with fiber cell tips. Scale bar = 38.5μm. Red=CD24, Green=WGA, Blue=nucleus.

3.4 CD24 is Expressed in Both Epithelial and Fiber Cells in Humans

CD24 is known to be expressed in a wide variety of human tissues (Fang et al. 2010), although there has been no report about its expression in the human lens. In order to determine the expression of CD24 in human lens, immunofluorescent analysis was carried out on sections from a 67 years old human lens. In contrary to the mouse lens, where CD24 expression was confined only to fiber cells, CD24 was expressed in both epithelial cells and fiber cells (Figure 3.5: A). However, WGA staining was prevalent in both epithelial and fiber cells, same as the mouse lens (Figure 3.5: B).
Figure 3.5  Immunofluorescent staining of a 67 year old human lens for CD24 and WGA. (A) CD24 expression alone in a 67 year old human lens section (B) WGA staining alone of the same 67 year old human lens section (C) Merge of CD24+WGA+DRAQ5 staining. Scale bar = 38.5μm. Red=CD24, Green=WGA, Blue=nucleus.

3.5  CD24 is Also Expressed in Retina of Adult Mouse Eye

In order to determine whether CD24 is expressed in other ocular tissues, as a pilot study, immunofluorescent analysis was carried out in a 2-month old wildtype retinal section. It was found that the CD24 protein was intensely expressed at the outer plexiform layer, while the CD24 expression was detected in other retinal layers and sclera (Figure 3.6: A). WGA staining was prevalent in the inner and outer plexiform layer, and also in the sclera (Figure 3.6: B).
**Figure 3.6** Immunofluorescent staining of a 2-month old C57BL/6<sup>har</sup> retina for CD24 and WGA. (A) CD24 expression alone in a 2-month old mouse retinal section (B) WGA staining alone of the same 2-month old mouse retinal section (C) Merge of CD24+WGA+DRAQ5 staining. Scale bar = 38.5μm. **Red**=CD24, **Green**=WGA, **Blue**=nucleus. gc-ganglion cell layer, inl-inner nuclear layer, ipl-inner plexiform layer, j-junction of pigmented epithelium and photoreceptor layer, onl-outer nuclear layer, opl-outer plexiform layer, rpe-retinal pigmented epithelium, s-sclera.
3.6 CD24 Null Mice are Viable and Fertile

To understand the molecular functions of CD24, we obtained mice where the CD24 gene was constitutively inactivated by replacing the promoter and exon 1 by a neomycin cassette (Nielson et al. 1997) (Figure 3.6: A). Although CD24 null mice are known to have several immune system abnormalities (Fang et al. 2010, Bretz et al. 2014), in specific pathogen free conditions, they are viable and fertile with normally sized eyes (Figure 3.6: B).

**Figure 3.7** CD24 null mice (A) Diagram of the CD24 gene in both Wildtype and CD24 null mouse demonstrating that the deletion of part of the proximal promoter and exon 1 would be expected to result in a complete loss of protein (B) Representational picture of 2-month old Wildtype (C57BL/6<har>) and CD24 null mice.
3.7 CD24 Null Mice Lack CD24 Protein from the Lens

CD24 protein is inactivated in the entire system of the mouse when the exon 1 is replaced (Nielson et al. 1997). In order to confirm that the CD24 null mice lack CD24 protein in the lens, immunostaining was performed on two-month old wildtype and CD24 null mice. The M1/69 monoclonal antibody, which was raised in rat, specifically binds to CD24, and was purified by affinity chromatography, using the mouse antigen (Catalogue number # 557436, BD Pharmingen). As expected, CD24 expression is prominent in the wildtype lens throughout the lens fibers (Figure 3.7: A-B), while it is evident that the CD24 null lenses lack CD24 protein (Figure 3.7: C-D).
Figure 3.8  Immunofluorescent analysis of CD24 in 2 month old C57BL/6 and CD24 null mice. (A) CD24 expression alone in a 2-month old wildtype lens section (B) CD24+DRAQ5 staining in 2-month old wildtype lens section (C) CD24 expression alone in 2-month old CD24 null mouse lens section (D) CD24+DRAQ5 staining in 2-month CD24 null lens section. Scale bar = 35.5μm, Red = CD24, Blue =nucleus, ce = central epithelial cells, f- fiber cells.

3.8  CD24 Null Lenses Exhibit Normal Lens Morphology at 2 Months of Age but are Smaller in Size

In order to identify phenotypic anomalies in CD24 null lenses, gross morphological characterization was performed at various timepoints using simple light microscopy. The refractive properties were analyzed using a 200-mesh electron microscopy grid. At 2 months of age, CD24 null lenses appeared transparent under bright field imaging (Figure 3.8: A-B) and refracted a hexagonal grid similar to wildtype lenses (Figure 3.8: C-D). Upon measuring the lens diameter of both the wildtype and CD24 null lenses, the latter was found to trend towards being smaller in size, but this was not statistically significant (Figure 3.8: E).
Figure 3.9  Morphological and refractive analysis of 2 month old C57BL/6 and CD24 null mice. (A) A bright field image showing a 2-month old wildtype lens (B) A bright field image showing a 2-month old CD24 null lens (C) Refractive analysis using a 200-mesh electron microscopy grid of a 2-month old wildtype lens (D) Refractive analysis using a 200-mesh electron microscopy grid of a 2-month old CD24 null lens (E) Unpaired t-test between the lens diameter of wildtype and CD24 null lenses showing that CD24 null lenses trend towards being smaller but this was not statistically significant, $p = 0.2075$, $N=3$. Values are expressed as mean ± S.E.M. Scale bar = 0.5 mm.
3.9 CD24 Null Lenses Develop a Refractive Index Discontinuity Between 4-6 Months of Age

At 4 months, a refractive index discontinuity (as indicated by white arrows Figure 3.9: B) becomes evident by bright field imaging. The CD24 null lens seem to refract the hexagonal grid with minimal distortion, but not as clear as the wildtype lens (Figure 3.9: C-D). The lens diameter of the CD24 null lenses was significantly smaller than the wildtype lenses at 4 months (Figure 3.9: E). By 6 months, the refractive index discontinuity becomes more severe than at 4 months (Figure 3.10: B) and the CD24 null lens's ability to refract the hexagonal grid is compromised, resulting in distorted hexagons (Figure 3.10: D). The lens diameter CD24 null mice continue to be significantly smaller than its wildtype counterparts (Figure 3.10: E).
Figure 3.10  Morphological and refractive analysis of 4 month old C57BL/6 and CD24 null mice. (A) A bright field image showing a 4-month old wildtype lens (B) A bright field image showing a 4-month old CD24 null lens. Arrow heads indicate the refractive index discontinuity (C) Refractive analysis using a 200-mesh electron microscopy grid of a 4-month old wildtype lens (D) Refractive analysis using a 200-mesh electron microscopy grid of a 4-month old CD24 null lens (E) Unpaired t-test between the lens diameter of wildtype and CD24 null lenses showing that CD24 null lenses are significantly smaller, p=0.0002, N=3. Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistical significance. Scale bar = 0.5 mm.
Figure 3.11  Morphological and refractive analysis of 6 month old C57BL/6 and CD24 null mice. (A) A bright field image showing a 6-month old wildtype lens (B) A bright field image showing a 6-month old CD24 null lens. Arrow heads indicate the refractive index discontinuity (C) Refractive analysis using a 200-mesh electron microscopy grid of a 6-month old wildtype lens (D) Refractive analysis using a 200-mesh electron microscopy grid of a 6-month old CD24 null lens (E) Unpaired t-test between the lens diameter of wildtype and CD24 null lenses showing that CD24 null lenses are significantly smaller, p = 5.71E-6, N=3. Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistical significance. Scale bar = 0.5 mm.
3.10 CD24 Null Lenses Exhibit a Marked Refractive Index Discontinuity Around 8 Months Followed by Cataract at One Year of Age

To determine whether the lens phenotype gets worse with aging, morphological characterization and refractive analysis was performed at later ages. Around 8-9 months of age, CD24 null lenses exhibit a distinct refractive index discontinuity, henceforth referred as the "ring" (as it appears black/dark grey under the bright field imaging) (indicated by the white arrow heads Figure 3.11: B) which is apparent in the bright field imaging. At this time point, the ability to refract the hexagonal grid is further compromised compared to 6 months, especially around the region of the ring (Figure 3.11: D). The lens diameter of the CD24 null lenses was significantly smaller than the wildtype lenses at a p-value of 0.025 (Figure 3.11: E). By 11-12 months of age, the CD24 null lenses exhibit cataracts (Figure 3.12: B) and their ability to refract the hexagonal grid is almost lost. The lens loses its transparency and tends towards opacity (indicated by arrow heads), especially around the region of ring. (Figure 3.12: D). The lens diameter of the CD24 null lenses is significantly smaller than its wildtype counterparts with a p-value equal to 0.0027 (Figure 3.12: E).
Figure 3.12  Morphological and refractive analysis of 9 month old C57BL/6 $<$har$>$ and CD24 null mice. (A) A bright field image showing a 9-month old wildtype lens (B) A bright field image showing a 9-month old CD24 null lens. Arrow heads indicate the refractive index discontinuity referred as the "ring" (C) Refractive analysis using a 200-mesh electron microscopy grid of a 9-month old wildtype lens (D) Refractive analysis using a 200-mesh electron microscopy grid of a 9-month old CD24 null lens. Arrow heads indicate that the region around the ring has poor refractive properties and is opaque (E) Unpaired t-test between the lens diameter of wildtype and CD24 null lenses showing that CD24 null lenses are significantly smaller, $p = 0.025$, N=3. Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistical significance. Scale bar = 0.5mm.
Figure 3.13  Morphological and refractive analysis of 1-year old C57BL/6 and CD24 null mice. (A) A bright field image showing a 1-year old wildtype lens (B) A bright field image showing a 1-year old CD24 null lens. Arrow head indicate the position of cataract (C) Refractive analysis using a 200-mesh electron microscopy grid analysis of a 1-year old wildtype lens (D) Refractive analysis using a 200-mesh electron microscopy grid analysis of a 1-year old CD24 null lens. Arrow head indicate the cataract formation (E) Unpaired t-test between the lens diameter of wildtype and CD24 null lenses showing that CD24 null lenses are significantly smaller, p = 0.0027, N=3. Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistical significance. Scale bar = 0.5mm.
3.11 CD24 Null Lenses do not Increase in Size with Aging Unlike the Wildtype Lens

In order to compare the lens diameter of both wildtype and CD24 null lenses at various time points, the mean lens diameter was plotted against the time. It was found that over time, while both wildtype and CD24 null lenses continue to grow during adulthood, the CD24 null lenses tend to grow slower between 4 to 9 months of age, (Figure 3.13). Fitting the data points with a linear regression curve (blue line) showed that as the animal grows older, the lens diameter significantly increases in size in wildtype lenses (p=0.026). In contrast, while the lens diameter seems to increase in CD24 null lenses with age, unlike the wildtype lens, the increase was not significant (p=0.084).

![Graph showing comparison of wildtype and CD24 null mean lens diameter over time.](image)

**Figure 3.14** Comparison of C57BL/6 and CD24 null mean lens diameter at various timepoints. The values on the graph indicate the mean lens diameter at that specific time point. The blue line indicate a linear regression fit of the given data points.
Wildtype: $Y=0.03838X+2.428$, CD24 null: $Y=0.01973X+2.32$.
Asterisk (*) indicate statistical significance, N=3.

3.12 Fiber Cell Organization is Preserved in CD24 Null Lenses Until 4 Months of Age

As CD24 is preferentially expressed in the lens fibers, and CD24 null lenses exhibit refractive index discontinuities and loss of transparency, the organization of fiber cell cells at the ultra-structure level was characterized using scanning electron microscopy (SEM). At 2 months, both the wildtype and the CD24 null lenses exhibit similar fiber cell organization, nevertheless the lateral surfaces of the fiber cells seemed quite pitted in CD24 null lenses (Figure 3.14: A-B). Even at 4 months, the fiber cell organization seems to be generally preserved in CD24 null lenses similar to wildtype lenses (Figure 3.14: C-D).
Figure 3.15  Scanning electron microscopy analysis of 2 and 4-month old C57BL/6 and CD24 null mice. (A) An SEM image showing the ultra-structure of lens fibers of a 2-month old wildtype lens (B) An SEM image showing the ultra-structure of lens fibers of a 2-month old CD24 null lens (C) An SEM image showing the ultra-structure of lens fibers of a 4-month old wildtype lens (D) An SEM image showing the ultra-structure of lens fibers of a 4-month old CD24 null lens. Scale bar = 10μm
3.13 CD24 Null Lenses Exhibit Fiber Cell Disorganization from 6 Months Onward

In order to evaluate whether the fiber cell organization is maintained in later stages, scanning electron microscopy analysis was performed in 6-9 months old wildtype and CD24 null lenses. Since the "ring" structure becomes more evident after 6 months, it was expected that the fiber cell organization would be disrupted in CD24 null lenses. As expected at six months, in contrast to the finely organized fiber cells in wildtype lenses (Figure 3.15: A), the CD24 null lenses had abnormal fiber cell packaging ranging from the absence of membrane protrusions to the aggregation of fiber cells (Figure 3.15: B-C). Even at nine months, while the fiber cells of the wildtype lenses remained well ordered (Figure 3.15: D), the organization of CD24 null lenses was still abnormal, consistent with the previous time points (Figure 3.15: E-F).
Figure 3.16  Scanning electron microscopy analysis of 6 and 9-month old C57BL/6<sup>har</sup> and CD24 null mice. (A) An SEM image showing the ultra-structure of lens fibers of a 6-month old wildtype lens (B, C) SEM image showing the ultra-structure of lens fibers of a 6-month old CD24 null lens (D) An SEM image showing the ultra-structure of lens fibers of a 9-month old wildtype lens (E, F) SEM image showing the ultra-structure of lens fibers of a 9-month old CD24 null lens. Scale bar = 10μm.
3.14 CD24 and WGA are Both Enriched at the Short Sides of Lens Fibers

Since the fiber cell organization was disrupted in CD24 null lenses, I wanted to determine the localization pattern of CD24 in the fiber cells. In order to determine whether CD24 expression varies among the short sides, broader sides and vertices, equatorial sectioning was carried out to reveal the hexagonal geometry of lens fibers as previously described (Cheng et al. 2016). CD24 expression was prevalent in the shorter sides of the lens fibers (Figure 3.16: A, indicated by the arrow heads) compared to broader sides. Surprisingly, even the WGA staining was enriched in the shorter side of the lens fibers (Figure 3.16: B). Also, the super resolution imaging suggested that CD24 was also found vesicles (Figure 3.16: A).

![Figure 3.17 Cross sectional immunostaining and super resolution imaging of 2-month old C57BL/6 lens tissue with CD24 and WGA. (A) CD24 expression alone in a 2-month old wildtype lens section (B) CD24 expression alone in a 2-month...](image)
old wildtype lens section (C) Merge of CD24+WGA staining.
Scale bar = 38.5 μm. Red=CD24, Green=WGA.

3.15 WGA staining is Attenuated on the Short Sides of CD24 Null Lens Fiber Cells but not in Epithelial Cells.

In order to determine whether CD24 is a predominant glycoprotein in the lens, WGA staining was carried out in both epithelial and fiber cells of both wildtype and CD24 null lenses. There was a huge attenuation in the WGA staining in the lens fibers upon CD24 deletion (Figure 3.17: A-B), however, there was no difference in the WGA staining in the epithelial cells between the wildtype and CD24 null lenses although the epithelial cells looked bigger in CD24 null lenses. (Figure 3.18: A-B).
Figure 3.18  WGA staining of lens fibers of 2-month old C57BL/6 lens tissue with CD24 and WGA. (A) WGA staining in a 2-month old wildtype lens cross-section (B) WGA staining in a 2-month old CD24 null lens cross-section. Arrow heads point the WGA staining in the short sides of lens fibers. Scale bar = 38.5μm. Green=WGA.

Figure 3.19  WGA staining of lens epithelial cells of a 2-month old C57BL/6 and CD24 null lens with WGA. (A) WGA staining in a 2-month old wildtype lens epithelial cells (B) WGA staining in a 2-month old CD24 null lens epithelial cells. Scale bar = 38.5μm. Green=WGA.
3.16 There is no Detectable Difference in Crystallin Expression Between the Wildtype and CD24 Null Lenses

Morphological characterization and refractive analysis of CD24 null lenses suggested that the center of the lens is formed correctly and that the cortical fibers, though transparent, seem to have a markedly lower refractive index than the lens nucleus. Since smooth refractive index is a function of changing crystallin expression and post-translational modifications in the lens, we speculated that postnatally formed crystallins might not be appropriately synthesized or undergo normal maturational processes. To test this, 1D gel electrophoresis was performed to separate the protein extracted from 3-month old wildtype and mutant lenses. There was no obvious difference in the expression of crystallin between the wildtype (Figure 3.19: Lanes 2-4) and the CD24 null lenses (Figure 3.19: Lanes 7-9). The expression of crystallin was confined mostly between 19 to 26 KDa.

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Figure 3.20  Gel electrophoresis of protein extracted from 3-month old C57BL/6\textgreater{}har\textless{} and CD24 null lenses. Lanes (1, 9) Molecular weight of Ladder in kDa (2, 3, 4) protein samples obtained from three biological replicates of wildtype lenses (5) excess from lane 4 and 6 (7, 8, 9) protein samples obtained from three biological replicates of CD24 null lenses.
Chapter 4

DISCUSSION

Age related cataract is still the leading cause of blindness worldwide, representing more than 80% of the blind people over 50 years of age (Jefferis et al. 2010, Zetterberg 2015). Till date, the only proven treatment for cataract is surgical removal of cataractous lenses (Gupta et al. 2009) which is often followed by implantation of an intraocular lens. Sometimes cataract surgery involves aphakia (surgical removal of the lens) without the implantation of an intraocular lens, after which aphakic glasses can be used to focus in the absence of lens (Kim et al. 2012). Complications can occur following surgical cataract treatment including aphakic glaucoma (Comer et al. 2011, Ma, Lu et al. 2016), post-operative endophthalmitis (Ng et al. 2005) and posterior capsular opacification (Raj et al. 2007). Hence, identifying genes to which cataract prevention drugs can be targeted is still relevant. Existing anti-cataract agents were developed based on the understanding of the interaction of various environmental factors, such as UV radiation, with the lens (Liao et al. 2011). Recently, studies have identified pharmacological chaperones which restore transparency in R49C cryAA and R120G cryAB mouse models of hereditary cataracts (Makley et al. 2015) and amphipathic molecules such as lanosterol which prevents protein aggregation in animal lenses (Zhao et al. 2015). Identification and characterization of new genes associated with ARNC, can enhance the list of such drug targets (Mooney et al. 2010).

Recent studies have shown the involvement of various genes in congenital cataract which might have variants associated with age related cataracts. However, around 45 genetic loci of inherited forms of cataracts have been identified (Shiels and Hejtmancik 2013), very few genetic loci have been associated with age related cataracts.
Nevertheless, twin studies have demonstrated a clear genetic component to ARC’s etiology (Michael and Bron 2011, Shiels and Hejtmancik 2016, Truscott and Friedrich 2016) and meta-analysis studies has identified a few genes associated with age related cataracts (Liao et al. 2014, Liao et al. 2015). A meta-analysis usually involves a combination of a comprehensive systematic literature search using specific keywords with defined inclusion criteria. For example, a recent meta-analysis of Glutathione S-transferase polymorphisms involved the use of Medline, Embase, PubMed, Web of Science and China National Knowledge Infrastructure with the specific keywords and search criteria as described (Liao et al. 2015). Statistical analysis is performed to analyze the inconsistencies, if any, in these studies. This study concluded that GSTT1 null genotype resulted in increased cataract in Asians but not in Caucasians (Liao et al. 2015).

Although these studies have identified a few genes and genetic loci that can contribute to ARCs, the likely interaction of these genes with environmental factors makes it difficult to identify and study genetic factors associated with age related conditions. Several environmental factors can influence the formation of age related cataract, such as UV irradiation, smoking, alcohol consumption, and obesity which can be eliminated/minimized by the use of animal models such as mouse. The availability of less environmental factors to influence the genetic factors and also the ease of maintaining the animal colony, makes mouse models suitable for studying age related cataracts (Pendergrass et al. 2005, Wolf et al. 2005, Puk et al. 2010).

In order to identify genes associated with age related cataract, we postulated that the genes associated with age related cataracts are likely preferentially expressed in the
lens and that they do not cause any other ocular pathologies apart from cataract in adults. Similarly, genes that are expressed broadly in the body are more likely to yield pleiotropic effects (i.e. a defect in the gene can cause different clinical manifestations in different systems). Such genes are less likely to be involved in ARC development as ARC patients are often phenotypically normal otherwise. For example, mutations in either COL4A3, COL4A4 or COL4A5 genes are shown to cause Alport syndrome which majorly affects kidneys, and is also associated with lens capsule abnormalities and cataract formation (Longo et al. 2004, Pescucci et al. 2004, Crockett et al. 2010, Firtina et al. 2009). In most cases, but not all (Li et al. 1995), cataracts often affect lens fiber cells, hence we proposed that fiber cell specific preferred genes would be good candidates for studying ARCs.

Prior to this work, in an attempt to enhance the understanding of fiber cell differentiation and its regulation, an animal model which fails to develop lens fibers was utilized (Figure 1.7). RNA sequencing of these Prox1 mutant mice revealed many differentially expressed genes (Audette et al. 2016). Among the 624 differentially expressed genes, CD24 was found to be 4.4 fold downregulated in Prox1 mutant mice. In the wildtype control, the mRNA expression of CD24 was as abundant as that for the beaded filaments and 20 fold higher than β1-integrin which is vital for lens function (Simirskii et al. 2007, Scheiblin et al. 2014, Pathania et al. 2016). This led us speculate that CD24 might be an important molecule in the lens.

As a first step, we used a bioinformatics tool, iSyTE, to predict the gene expression pattern of CD24 during lens development. iSyTE (Lachke et al. 2012), utilizes an in-silico subtraction approach in which lens microarray datasets from various
embryonic, newborn and adult stages are compared to microarray datasets obtained for the embryonic whole body (WB), (which is derived from a pool of RNAs obtained from E10.5, E11.5 and E12.5 embryos) from which the entire eye was removed by microdissection (Lachke et al. 2012, iSyTE-2, Lachke lab unpublished). The fold difference in the expression of the gene as compared to the whole body, is given by the lens enrichment score. CD24 has positive lens enrichment scores from E12.5, which marks the onset of fiber cell differentiation (Figure 3.1).

Previously CD24 has been described as a small mucin-like glycoprophatidyl-linked cell adhesion protein which is expressed in both developing and regenerating tissues, granulocytes, B-cells, keratinocytes and renal tubules (Kristiansen et al. 2003). CD24 is found in a wide variety of tumors and plays roles in immune system regulation and autoimmune diseases (Fang et al. 2010). Notably, while this membrane protein is a ligand of P-selectin in some tissues (Sammar et al. 1994, Aigner et al. 1997), there is no P-selectin mRNA detected in the lens by RNA-seq (Manthey et al. 2014, Audette et al. 2016). CD24 also participates in integrin signaling in carcinoma cells (Runz et al. 2008), but the role of CD24 in the lens was not yet studied. Thus, this study was the first to investigate the role of CD24 in the lens.

4.1 CD24 is a Novel Lens Fiber Cell Marker

From the iSyTE data (Figure 3.1), it is clear that the lens enriched expression of CD24 begins in the lens at E12.5 when fiber cell differentiation begins, although CD24 is expressed earlier at E10.5. As this determination was based on microarray analysis of mRNA levels, I characterized CD24 expression at the protein level using
immunostaining. In accordance with the iSyTE data, the lens preferred expression of CD24 begins at E12.5 during the onset of fiber cell differentiation and is expressed in the differentiating lens fibers (Figure 3.2: D-F). The expression of CD24 in E12.5 is intense (LE score=3.84) when compared to E10.5 (LE score=-2.27) (Figure 3.2: A-C), but less intense than E16.5 (LE score=8.14) (Figure 3.2: G-I), in accordance to the lens enrichment scores of CD24 obtained from iSyTE. Also, the expression of CD24 was confined only to the lens fiber cells and not in epithelial cells (Figure 3.4: A). This is the first report of CD24 in the lens, proving that CD24 can be used as a novel fiber cell marker in mice. In addition, CD24 expression was identified in retina, further extending the scope of CD24 to other ocular tissues. Although, the expression of CD24 is detected throughout the retina, the staining was intense in the outer plexiform layer and the overall expression was similar to the expression profile as described before for CD24 in human retina (Lakowski et al. 2015). Performing immunostaining in different ocular tissues with sufficient replicates would provide better understanding about the expression of CD24.

4.2 CD24 is not Required for Lens Morphogenesis

Morphological characterization using bright field imaging and refractive analysis using a 200-mesh electron microscopy grid indicated that the CD24 null lenses formed normally as they were transparent and able to focus clearly before 4 months, demonstrating that CD24 is not required for lens development. But CD24 null lenses develop a refractive index discontinuity followed by cataract around 1 year, which suggests that CD24 might be associated with the regulation of age related processes in lens and that the loss of CD24 might dysregulate these processes.
4.3 CD24 Might Have Different Functions in Mouse and Human Lenses

Since the expression of CD24 was detected only in fiber cells until adulthood in mice (Figure 3.2, Figure 3.3), I hypothesized that CD24 might be a fiber cell preferred protein in the lens. To confirm this, whole mount epithelial staining was carried out on mouse lenses to check whether any detectable expression was seen in the epithelial cells (Figure 3.4), which revealed that CD24 protein is not expressed in mouse lens epithelial cells and it is indeed a fiber cell preferred protein. Contrary to the mouse lens, CD24 protein appeared to be expressed in both the epithelial and fiber cells of human lenses (Figure 3.5). More biological replicates of human lenses are required to confirm this data and whole mount staining of human epithelial cells would reveal the extent to which CD24 was expressed. One caveat of this experiment might be that mouse specific antibody was used for staining human lenses. An antibody raised against the human CD24 antigen would provide more concrete evidence on the expression of CD24 in human tissue.

Overall, this experiment shows that the CD24 protein is found in both mouse and human lenses, however, it may have a different role(s) in human lenses compared to mouse due to its apparent expanded expression pattern. Additional serine and threonine residues provide more GalNAc O-linkages, resulting in extensive glycosylation in the human protein compared to the mouse (Aigner et al. 1997, Kufe 2009), rendering the human protein more like a typical mucin (Kay et al. 1991). Overall though, these data suggest that CD24 might be a clinically significant protein which needs to be studied in detail in lens biology. Also, the human lens used to analyze CD24 localization came from a 67-year old human, while the mouse lens was around 2-months of age. Hence immunostaining in a younger human lens or an older mouse lens would
be useful to make a better age related comparison between the two species. Additionally, it might be useful to examine the expression of CD24 in the lens of different species to determine which expression pattern is more representative of mammalian lenses in general.

4.4 CD24 is Essential for Maintaining the Fiber Cell Organization of Aged Lenses

Morphological analysis of CD24 null lenses suggests that CD24 is likely not be required for the regulation of lens morphogenesis. Two month old CD24 null lenses appeared transparent under the bright- and dark-field microscopy and were able to refract the microscopic grid similar to wildtype lenses. However, around 4 months of age, the refractive index discontinuity referred as the “ring” begins to appear in the CD24 null lenses, which obstructs the transparency and the ability of the lens to refract light in later ages. Hematoxylin and eosin staining of both wildtype and CD24 null lenses would provide concrete evidence for the above speculation.

Since the lack of this fiber cell preferred protein results in a refractive discontinuity in the fiber cell region of adult lenses after 4 months of age as well as a loss of transparency after 9 months, the ultra-structure of lens fibers was examined using scanning electron microscopy. In accordance with the gross phenotypic characterization, the fiber cells of both the wildtype and CD24 null lenses were organized in discrete parallel layers which interdigitate with adjacent fibers via distinct membrane protrusions until 4 months of age (Figure 3.14). However, the organization of lens fibers is disrupted around 6 months and it worsens with age. While the wildtype lenses have distinct membrane protrusions, a loss of organized repeatable membrane
protrusions was prevalent in CD24 null lenses from 6-months of age onwards (Figure 3.15), which can be quite comparable to the disorganization of lens fibers seen in β1-integrin conditional knockout lenses (Scheiblin et al. 2014). This disorganization ranges from aggregation of lens fibers, overlapping of lens fibers, or mere disruption of membrane protrusions. This suggests that CD24 is required for maintaining the organization of lens fiber cells at later stages, by regulating lens homeostasis. In order to test this, the impedance and intracellular hydrostatic pressure of the wildtype and CD24 null lenses should be determined as described earlier (Scheiblin et al. 2014). The differences in these values might reveal differences in the gap junctional coupling which in turn affects the fluid homeostasis (Scheiblin et al. 2014).

4.5 Lack of CD24 Might Alter Fiber Cell Compaction and Hence the Critical Gradient of Refractive Index (GRIN) of the Lens

As we age, in addition to environmental insults, several intrinsic factors lead to lens opacity. The partial degradation of α and β crystallins and elevated acidity of γ crystallins were identified as a consequence of normal aging (Ueda et al. 2002), although, when this process is unregulated, it may lead to lens opacity (David et al. 1994). We know that crystallins are the major determinant of the lens refractive index which is a function of changing crystallin gene expression from embryogenesis until adulthood. Understanding the role of crystallin modification in the lens is tedious as the modifications are a part of the normal maturation process. Particularly, site specific proteolysis of β-crystallins in young animals lead to dense packing of crystallins during lens maturation (David et al. 1994, Lampi et al. 1998). Stringent regulation of this process is essential to maintain a smooth refractive index gradient in the lens (Ueda et
al. 2002). Gross phenotypic characterization of CD24 null lenses revealed that the ring structure is present near the cortical fiber cells resulting in a loss of refractive index continuity and transparency. This leads to the hypothesis that postnatally made crystallins are not appropriately synthesized and/or the crystallins in the newly formed lens fibers are not appropriately undergoing the maturational proteolysis which is necessary for a smooth refractive index gradient. To test this, 1D gel electrophoresis was carried which revealed that there are no gross differences in the crystallins between the wildtype and CD24 null lenses. Further analysis of lens crystallins using 2D gel electrophoresis or mass spectrometry will be necessary to determine whether defects in crystallin processing drive the refractive index discontinuity.

Alternatively, a recent article suggests that the critical gradient index (GRIN) is a function of loss of volume by inner fiber cells through the process of compaction (Bassnett and Costello 2016). Notably, CD24 null lenses show a poor GRIN and are significantly smaller in size compared to their wildtype counterparts (Figure 3.13). This suggests the absence of CD24 results in dysregulated fiber cell compaction, leading to an altered refractive index gradient.

In order to test this speculation, numerical quantification of the GRIN is also essential. Since the direct measurement of GRIN is not very practical, equivalent refractive index can be used in lieu of GRIN. Equivalent refractive index is a relative measurement of the contribution of GRIN to the refractive power of the lens which can be calculated by measuring the focal length of a lens, thickness, curvature and computing the corresponding refractive index for a normal lens (Bassnett and Costello 2016). Finding the correlation between the loss of inner cell mass of the lens and GRIN
(by measuring the equivalent refractive index) can provide evidence on the idea of altered fiber cell compaction.

### 4.6 CD24 Might be Involved in Various Signaling Cascades in the Lens

GPI anchored molecules including CD24 are known to participate in diverse signaling cascades, and have the ability to transduce activation signals to the nucleus (Robinson 1991, Brown 1993, Malek et al. 1994). Studies have shown the physical and functional interaction of diverse GPI-anchored proteins, including CD24, with integrins, which provide a transmembrane transducer necessary to link these proteins to the cytoplasm as they lack a cytoplasmic domain (Petty and Todd, 1996). Integrins are heterodimeric family of protein whose interaction with lipid rafts is important for their activation. Transfection of CD24 into CD24-negative carcinoma cells induced the localization of β1 integrin into lipid rafts rich in cholesterol and sphingolipid domains, which does not happen otherwise (Runz et al. 2008). Additionally, CD24 is known to interact with several integrin heterodimers and regulate their activity, by regulating its interaction with extracellular matrix proteins, in immune cells. For example, CD24 regulates the binding of α4β1 (VLA-4) to VCAM-1 and FN40 (a fragment of fibronectin which has the α4β1-binding motif) in pre-B cells (Hahne et al. 1994). Also, CD24 activates α3β1 and α4β1 integrin and mediate cellular adhesion to fibronectin, collagens I and IV and laminin (Baumann et al. 2005).

Integrin signaling plays multiple roles in the lens. In the lens, β1 integrin is expressed in both epithelial and fiber cells (Simirskii et al. 2013). Deletion of β1 from both the epithelial and fiber cells results in apoptosis of epithelial cells resulting in
resorption of the lens and microphthalmia (Simirskii et al. 2007). The fiber cells of the surface and middle layer of a normal lens, are adhered to the capsule through a β1 integrin containing adhesion complex (Lu et al. 2008). Also, β1 integrin is present in the interdigitating protrusions on the lateral surface of the lens fibers (Biswas et al. 2010), and might facilitate cell-cell contact of protein with the actin filaments of the lens fibers (Scheiblin et al. 2014). Gene knockout of β1 integrin from lens fibers compromised their spatial organization by the loss of contact with the capsule and dysregulated cell-cell communication between the lateral surfaces (Scheiblin et al. 2014). On the level of ultrastructure, loss of β1 integrin results in the disorganization of lens fiber cells resulting in the loss of distinct membrane protrusions and membrane furrows in aged mice (Scheiblin et al. 2014). Although the complete loss of CD24 does not affect the morphogenesis of lens till 2 months of age, CD24 null lenses exhibit a refractive index discontinuity as well as disorganization of lens fibers with attenuation of membrane protrusions, similar to β1 integrin knockout lenses. Since CD24-integrin interactions have been proposed in several systems, it is crucial to evaluate their interaction in the lens. It might be possible that CD24 and integrin interaction is essential for fiber cell organization during aging, and that the absence of one protein affects the other, hence resulting in the disruption of fiber cell organization.

Previous studies have shown that Notch signaling regulates the development and homeostasis of the lens (Saravanamuthu et al. 2012). A recent report showed that CD24 regulates notch 1 expression in MCF-7 cells. Notch 1 and activated Notch1 (NICD) decrease when CD24 was downregulated (Lim et al. 2014). It has also been shown that CD24 activates p38MAPK signaling and modulates Notch 1 expression by regulating mRNA stability (Leelawat et al. 2013, Lim et al. 2014). The notch signaling in the lens
transduces cell contact-mediated communication by interacting with its ligands Delta-like or jagged. In addition to the regulation of growth and differentiation in mammalian lens (Rowan et al. 2008), studies in self renewing tissues have shown the importance of Notch and Wnt signaling in adult tissue renewal and maintenance (Sato et al. 2012). This information suggests that CD24 might affect notch signaling, which alters the cellular maintenance and cell-cell contact, leading to a discontinuity in the lens and also to cataracts. But this may not be relevant in CD24 null lenses, since the disruption of Notch signaling would affect lens development, while the CD24 null lenses develop normally. This could be ascertained by measuring the expression levels notch and its ligands in the lens.

4.7 CD24 Might be a Predominant Sialoglycoprotein of Mouse Lens Fibers, but not in Epithelial Cells

Wheat Germ Agglutinin (WGA) is one of the best characterized lectins which belongs to a well conserved family of chitin binding lectins (Lienemann et al. 2009). The protein comprises of two identical polypeptide subunits, (MW=18KDa, each), which assemble as a dimer at neutral pH (Nagata and Burger 1974) and the subunit interface contains 8 carbohydrate binding sites (Wright 1992). The binding affinity of WGA to oligosaccharides varies depending on the number of GlcNAc residues (Lienemann et al. 2009). WGA binds to N-acetyl-glucosamine and N-acetyl-neuraminic acid residues (Ohno et al. 1986) and interacts strongly with N-linked oligosaccharides when compared to the O-linked oligosaccharides (Gallagher et al. 1985).
Since CD24 contains both N- and O-linked glycans (Bleckmann et al. 2008), lenses were co-stained with CD24 and WGA to determine their co-localization. Equatorial sectioning of lens tissue was performed to reveal the hexagonal geometry of the fiber cells with short side, broad side and vertices (Cheng et al. 2016). In lens fiber cells, CD24 expression was abundant in the short sides when compared to the broad sides, similar to the localization of the WGA binding (Figure 3.16 A-C). However, when CD24 was knocked out, the WGA staining was attenuated at the short sides of lens fiber cells when compared to the wildtype (Figure 3.17: A-B). This suggests that CD24 might be the predominant glycoprotein located on the short side of the lens fibers. Quantification of WGA staining in both wildtype and the mutant lenses is needed to further support this proposition.

Since the WGA staining was similar in both wildtype and CD24 null epithelial cells (Figure 3.18: A-B), and that the CD24 expression was not detected in those cells, it is likely that the glycoproteins of the epithelial cells are unique and may not be present in fiber cells. This finding may open up new avenues for research about the carbohydrate biology of lens.

Additionally, super-resolution imaging of CD24 expression suggested that it was also present in membrane vesicles (Figure 3.16). A recent study has shown that CD24 engagement resulted in the release of CD24-bearing plasma membrane-derived extracellular vesicles in B-cells (Ayre et al. 2015). At the same time, integrins have also been shown to participate in endocytic vesicular transport regulating cell adhesion and migration (Caswell et al. 2009). These information suggests that CD24-integrin signaling is critical in regulating several important processes in the lens and hence
further analysis of integrin signaling may provide further insight on the importance of their interaction.

4.8 Summary

This is the first report describing the role of CD24 in the lens. Both the bioinformatics analysis using iSyTE and immunofluorescence analysis revealed that the lens enriched expression of CD24 begins from embryonic day 12.5, although CD24 mRNA as well as protein is detected at E10.5. The expression of CD24 is also detected in adult mouse lenses, and I have shown that CD24 is expressed only in fiber cells and not in epithelial cells, hence CD24 is a fiber cell marker in mouse lens. Further, CD24 expression is prevalent in both human epithelial and fiber cells, suggesting evolutionary differences in its distribution. I also found that CD24 null mice develop a marked refractive index discontinuity around 6 months of age, followed by cataract, suggesting that CD24 null lenses can be used as an animal model of age related cataract. The fiber cells of juvenile and adult lenses until 4 months of age were well organized as identified by scanning electron microscopy, while the aged lenses cell show fiber cell disorganization. Notably, CD24 null lenses did not increase significantly in size in mid-adulthood, unlike wildtype, suggesting that they either failed to grow or had defects in gap-junctional regulation of ion exchange and/or water homeostasis. Additionally, WGA staining identified CD24 as a predominant glycoprotein in the lens fiber cells and not in epithelial cells. In the future, studies on CD24-integrin interaction may be crucial to determine the molecular function of CD24 in the lens. Conversely, RNA sequencing can be used to identify differentially expressed genes in CD24 null lenses, which can
provide more information about the genes and signaling cascades that CD24 regulates. Overall, CD24 is a novel protein for lens biology and may play an important role in the pathogenesis of age related cataracts.
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Appendix A

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Principal Investigator: Melinda K. Duncan

Common Name: mouse, chicken, rat

Genus Species: Mus musculus, Gallus gallus, Rattus rattus

Pain Category: (please mark one)

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1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.

2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).

3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.

4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.

5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.

6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.

7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.

8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.

9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.

10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.

11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*

12. I understand that by signing, I agree to these assurances.

*Melissa Duncan*  
Signature of Principal Investigator  
11/3/15  
Date
**NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL**

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
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<tbody>
<tr>
<td>1. Yan Wang</td>
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<tr>
<td>2. Dylan Audette</td>
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<td>3. Yichen Wang</td>
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<tr>
<td>4. Mahbubul Shihan</td>
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<td>5. Ramachandran Balasubramanian</td>
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<td>6. Patrick Ihejirika</td>
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<td>7. Troy Rubenstein</td>
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<td>8. Priyha Mahesh</td>
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The Animal Use Protocol form has been developed to facilitate review of requests for specific research, teaching, or biological testing projects. The review process has been designed to communicate methods and materials for using animals through administrative officials and attending veterinarians to the Institutional Animal Care and Use Committee (IACUC). This process will help assure that provisions are made for compliance with the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Please read this form carefully and fill out all sections. Failure to do so may delay the review of this application. Sections that do not apply to your research must be marked “NA” for “Not Applicable.”

This application form must be used for all NEW or THREE-YEAR RENEWAL protocols.

All answers are to be completed using Arial 12 size font.

All questions must be answered in their respective boxes and NOT as attachments at the end of this form.

Please complete any relevant addenda:
- Hybridoma/Monoclonal Antibodies (“B”)
- Polyclonal Antibodies (“C”)
- Survival Surgery (“D”)
- Non-Survival Surgery (“E”)
- Wildlife Research (“F”)

If help is needed with these forms, contact the IACUC Coordinator at extension 2616, the Facility Manager at extension 2400 or the Attending Veterinarian at extension 2980.
1. Principal Investigator Information:

<table>
<thead>
<tr>
<th>a. Name:</th>
<th>Melinda Duncan</th>
</tr>
</thead>
<tbody>
<tr>
<td>b. University/Company:</td>
<td>University of Delaware</td>
</tr>
<tr>
<td>c. Department:</td>
<td>Biological Sciences</td>
</tr>
<tr>
<td>d. Building/Room:</td>
<td>Wolf Hall, Room 327</td>
</tr>
<tr>
<td>e. Office Phone:</td>
<td>302 831 0533</td>
</tr>
<tr>
<td>f. Lab Phone(s):</td>
<td>302 831 0497</td>
</tr>
<tr>
<td>g. Home Phone:</td>
<td>410 620 5696</td>
</tr>
<tr>
<td>h. Mobile Phone:</td>
<td>302 593 5074</td>
</tr>
<tr>
<td>i. E-Mail Address:</td>
<td><a href="mailto:duncanm@udel.edu">duncanm@udel.edu</a></td>
</tr>
</tbody>
</table>

2. Protocol Status:

| a. □ New Protocol        OR □ Re-submission due to three (3) completed years. |
|                          | If re-submission, enter Protocol Number: 1039 |
| b. □ Research OR □ Teaching |
| c. □ Laboratory Animals OR □ Wildlife |
| If “Wildlife” please complete Addendum “F” |
| d. Proposed Start Date: December 1, 2015 |
| e. Proposed Completion Date: November 30, 2018 |
| f. Funding Source: National Eye Institute and Aniridia Foundation International |
| g. Award Number: EY015279; no number for Aniridia Foundation International |

3. Personnel involved in Protocol (Include Principal Investigator):

**Status:** Indicate Prof, Post-Doc, Grad Student, Lab Manager, Research Assistant, Technician, etc.
**Qualifications**: Include procedures this person is proficient in performing on proposed species and the time they have been doing the procedure. **Be specific** (e.g., sub-mandibular bleeding on mice-2yrs, performing castrations on mice and rats-1yr, tail-vein injections on mice-2yrs, etc.) **(If no experience, list who will train.)**

**Responsibilities**: Include all responsibilities this person will have with live animals on this protocol, including euthanizing animals.

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<thead>
<tr>
<th>Name</th>
<th>E-mail</th>
<th>Office Phone Number</th>
<th>Home/Cell Phone Number</th>
<th>Received Animal Facility Training</th>
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<tbody>
<tr>
<td>a. Melinda K. Duncan</td>
<td><a href="mailto:duncanm@udel.edu">duncanm@udel.edu</a></td>
<td>0533</td>
<td>302 593 5074</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Status**: Professor and Principal Investigator

Qualifications: Ph.D. in Biochemistry, 33 years experience working with animals including five years as a veterinary technician followed by 25 years experience in animal research using diverse animals including mice, chickens, rats, fish and rabbits as a graduate student, postdoctoral fellow and for the past 18 years as principle investigator of NIH funded research projects which use animals. Dr. Duncan is experienced with animal handling, breeding, genetics, diverse euthanasia methods, survival surgeries including partial heptectomy, unilateral nephrectomy, corneal wounding, cataract surgery in animal models, castration, vasectomy, embryo transfer into both ampulla and uterus, ovarectomy and thymectomy; rodent anesthesia, both IV and IP injections, and blood collection via infraorbital bleeding, tail vein collection, and trunk blood collection by guillotine.

Responsibilities: oversee project, train in animal procedures, kill and dissect animals

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<tr>
<td>b. Yan Wang</td>
<td><a href="mailto:Yanwang@udel.edu">Yanwang@udel.edu</a></td>
<td>0497</td>
<td>302-897-3712</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Status**: Lab manager and research associate

Qualifications: 11 years experience with rodent survival surgery including diverse ocular surgeries/injuries including those involving retroviral infusions into eye and tail vein injections, husbandry, 4 years prior experience working with rabbits, 5 years experience working with chicken embryos, MD degree, clinical ophthalmologist; experienced in euthanasia of rats, chicks (pre and post-hatch), mice, fish
Responsibilities: oversee animal colony, survival surgeries, timed matings, train students in animal procedures, sacrifice mice, rats, chicks

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<tr>
<td>c. Dylan Audette</td>
<td><a href="mailto:audette@udel.edu">audette@udel.edu</a></td>
<td>831-0497</td>
<td>1-401-556-5060</td>
<td>yes</td>
</tr>
</tbody>
</table>

Status: Ph.D. in Biological Sciences, ISEL preceptor (full time permanent UD employee), volunteer in laboratory to finish up papers from Ph.D.

Qualifications: Ph.D. in Biological Sciences from the Duncan laboratory, 7 years experience working with mice including embryology, colony maintenance, proliferation assays, dissection; experienced in euthanasia of mice and chicks (pre and post hatch) as well as IP injection

Responsibilities: maintain colony, timed matings, proliferation assays, sacrifice mice and chicks, train undergrads in animal procedures

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<tr>
<td>d. Yichen Wang</td>
<td><a href="mailto:ycwang@udel.edu">ycwang@udel.edu</a></td>
<td>0497</td>
<td>302-220-8126</td>
<td>yes</td>
</tr>
</tbody>
</table>

Status: Ph.D. Student in Biological Sciences

Qualifications: Masters of Medicine, trained opthalmologist, 4 years experience working with mice including embryology, colony maintenance, proliferation assays, dissections, experienced in euthanasia of mice as well as IP injection

Responsibilities: maintain colony, timed matings, proliferation assays, sacrifice mice, train undergrads in animal procedures,
e. Mahbubul Shihan  
shihan@udel.edu  
0497  
302-419-6422  
X  
Click here to enter text.

Status: Ph.D. Student in Biological Sciences

Qualifications: 1 year experience working with mice including embryology, colony maintenance, dissections, experienced in euthanasia of mice

Responsibilities: maintain colony, timed matings, proliferation assays, sacrifice mice, train undergrads in animal procedures

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<th>Office Phone Number</th>
<th>Home/Cell Phone Number</th>
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<tbody>
<tr>
<td>f. Ramachandran Balasubramanian</td>
<td><a href="mailto:ramb@udel.edu">ramb@udel.edu</a></td>
<td>0497</td>
<td>215-490-8666</td>
<td>yes</td>
</tr>
</tbody>
</table>

Status: MS student in Biological Sciences

Qualifications: 1 year experience working with mice including embryology, colony maintenance, dissections, experienced in euthanasia of mice

Responsibilities: maintain colony, timed matings, proliferation assays, sacrifice mice, train undergrads in animal procedures

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<tr>
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<tr>
<td>g. Patrick Ihejirika</td>
<td><a href="mailto:hpatrick@udel.edu">hpatrick@udel.edu</a></td>
<td>0497</td>
<td>484-522-1208</td>
<td>yes</td>
</tr>
</tbody>
</table>

Status: Masters student in Biological Sciences

Qualifications: Initial animal facility training, some basic training in animal handling; currently being trained by Yan Wang, Melinda Duncan and other lab members in animal procedures

Responsibilities: maintain colony, timed matings, proliferation assays, sacrifice mice, train undergrads in animal procedures once he gains competency