CADMIUM ALTERS EYE-RELATED GENE EXPRESSION

CADMIUM EXPOSURE ALTERS GENE EXPRESSION OF LENS, RETINA, AND EYE-RELATED GENES IN ZEBRAFISH AND HUMAN LENS EPITHELIAL CELLS

BY

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A THESIS

SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY & BIOMEDICAL SCIENCES

AND THE SCHOOL OF GRADUATE STUDIES

OF MCMASTER UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

Master of Science (2018) McMaster University

(Biochemistry & Biomedical Sciences) Hamilton, Ontario, Canada

TITLE: CADMIUM EXPOSURE ALTERS GENE EXPRESSION OF LENS, RETINA, AND EYE-RELATED GENES IN ZEBRAFISH AND HUMAN LENS EPITHELIAL CELLS

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NUMBER OF PAGES: xiii, 83

Lay Abstract

The eye is a sphere-like organ which is important for visualizing your surroundings. It is composed of many different structures such as the cornea, lens and retina. Many eye diseases have been characterized by abnormalities in eye structures; for example, a cataract occurs when the lens becomes cloudy and unable to focus light while macular degeneration is defined by progressive deterioration of the retinal macula region. While these diseases can occur through the natural aging process, certain environmental factors can increase risk. Exposure to cadmium, a toxic heavy metal which causes negative effects in animals, has shown to be associated with eye disease like cataracts and macular degeneration. In order to expand on this knowledge, we exposed both zebrafish and human lens cells to cadmium. By utilizing different experimental methods such as microarray analysis and RNA sequencing, eye-related genes which were affected by cadmium were revealed. Identifying the relationship between eye diseases, cadmium and gene expression will help identify the mechanism by which cadmium contributes to eye disease formation.

Abstract

Vision is a crucial aspect of life for humans and animals. Impaired vision can lead to reduced quality of life along with other complications. Cataracts are a leading cause of impaired vision and blindness worldwide. Cataracts develop as a process of aging, although several environmental and lifestyle factors increase the risk of this disease. The toxic metal cadmium (Cd) has been associated with cataract formation and other ocular diseases such as macular degeneration. Cadmium exposure experiments were conducted to investigate potential pathways or mechanisms by which Cd may contribute to cataract formation and ocular disease. Zebrafish larvae (72, 96, and 120 hours post fertilization), adult zebrafish (6-month male, 10-month male, and 10-month female) and the B3 human lens epithelial (HLE) cell line were acutely exposed to varying concentrations of Cd. Transcriptomic changes relative to control (0 µM Cd) were determined using microarray analysis for zebrafish larvae and RNA sequencing (RNA-Seq) for adult zebrafish and HLE cells. Gene Ontology (GO) enrichment analysis for the zebrafish larvae exposure (50 µM Cd for 4 or 8 hours) enriched the "retina development in camera-type eye" term, and genes involved in enrichment (*dnmt1*, *ccna2*, *fen1*, *mcm3* and *slbp*) were down-regulated. Gene set enrichment analysis (GSEA) for the 10-month male zebrafish exposure (50 µM Cd for 4 hours) enriched the "embryonic eye morphogenesis" gene set and significant

genes involved in enrichment (*tcf7l1a*, *pitx2*, *fzd8a*, *sfrp5*, *lmx1bb*, *mfap2*, *six3b*, *lum*, *phactr4b*, and *foxc1a*) were down-regulated. GSEA for the 10-month female zebrafish (50 µM Cd for 4 hours) enriched the "photoreceptor cell differentiation" gene set and significant genes involved in enrichment (*odc1*, *thrb*, and *ush2a*) were up-regulated. GO enrichment analysis for up-regulated genes in the HLE cell exposure (10 µM Cd for 4 hours) enriched the terms "eye development" (22 genes) and "lens development in camera-type eye" (*CITED2*, *SKIL*, *CRYAB*, *SLC7A11*, *TGFB2*, *EPHA2*, *BCAR3*, *WNT5B*, and *BMP4*). These results show cadmium is capable of altering transcription of eye-related genes in both zebrafish and human models, which may contribute to the formation of ocular disease. Many of these genes are involved in lens and retina development, yet they are also associated with diseases in these eye structures. Future studies could assess the consequences of altered transcription of these genes which could help elucidate the mechanisms of these changes and the overall effect of cadmium exposure on ocular disease. Ultimately, our study characterized the regulation of eye-related genes in response to Cd exposure and provided valuable knowledge laying the foundation for identification of the molecular mechanisms contributing to ocular diseases.

Acknowledgements

As my graduate career comes to an end, I look back fondly at the past two years. Through all the good times and the bad, there are so many people I would like to thank and acknowledge. Firstly, I would like to thank my amazing supervisor Dr. Andrew G. McArthur. Thank you for supporting and guiding me to the finish line. Thank you to my graduate committee, Dr. Judith A. West-Mays and Dr. Alison C. Holloway, and Dr. Joanna Y. Wilson for your support and lending me your knowledge and expertise.

Over these past two years the people I saw the most were definitely my coworkers. My Masters experience would not have been the same without seeing all of you on almost a daily basis. Thank you Brian for all your help through the years and for all the video game, political, and random conversations we had. Thank you Amos for all the programming help when I didnt know how to use a terminal and for all the laughs; 2 points for you. To Kara, thanks for being good lab mate and listening to my project struggles. To Kirill, thanks for being the one person I could always talk about One Piece with and for all the great squash games. To Arjun, if you think about it, we had some great conversations about basketball and hockey. Thank you to the entire McArthur lab for enriching my entire graduate experience.

I would like to thank all my friends from Milton and Hamilton for their support.

Thanks Danial and Asim for listening to my departmental seminar presentation and for all the fun times we had. To Tashfeen and Waleed, even though we do not see each other as often, whenever we do it always feels like old times. Thanks Cho, MJ and Sipos for all those homecoming trips and being the people I could relax and unwind with. Thank you Mathura and Abigail for your support during my masters. Finally, the biggest thank you to my parents for their support and for giving me the opportunity to pursue an education.

Declaration of Academic Achievement

All animal husbandry, cell culture, experimentation, dissections, and molecular biology were performed by members of Dr. Matthew J. Jenny's laboratory (Department of Biological Sciences, University of Alabama). Genomic techniques (RNA-Seq, microarrays) were outsourced as outlined below.

All bioinformatics and statistical analyses were performed by Krishna Srinivasan, excluding initial analysis of normalized microarray data which was previously conducted by Dr. Andrew G. McArthur (Department of Biochemistry & Biomedical Sciences, McMaster University).

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Chapter 1

Introduction

Xenobiotics are any foreign substances found in a biological system. Common forms of xenobiotics include pharmaceuticals and environmental contaminants such as heavy metals. Excessive use of natural heavy metals for human uses increases the concentration of these metals found in soil and water [1]. Although seemingly having a negative connotation, xenobiotics can have beneficial effects on an organism. Certain heavy metals, such as zinc, are beneficial in small concentrations and are vital for regular functioning, while others like cadmium have no functional use in the human body and causes deleterious outcomes if present past certain thresholds [2]. Metal regulation and homeostasis is an important aspect of life, as incorrect regulation can cause negative outcomes to many organs ranging from the heart to the eyes. The human eye is an incredibly complex organ that facilitates vision. Many factors are involved in maintaining its structure and function. A common metaphoric descriptor for the eye is a "camera-type eye" due to the lens, iris and extra-ocular muscles resembling the structure of a modern camera (Figure 1.1). A simplified summary of the process of converting light stimuli to perceivable images is as follows; the cornea

initially refracts incoming light, light is then further refracted by the lens onto the retina, and the electrical signal generated by the retina is sent via the optic nerve to the brain for interpretation. Trace metals, such as iron and zinc, in the eye must be carefully balanced for optimal function. Studies have reported associations between metals and ocular diseases; in a cross-sectional study in a Korean population, lower blood manganese and higher blood mercury levels were associated with glaucoma [3]. Unfortunately, the mechanisms by which metals influence ocular diseases is poorly understood.

1.1 The lens and retina

Two important structures in the vertebrate eye that facilitate vision are the lens and the retina. The lens is a transparent biconvex structure that focuses incoming light rays onto the retina [4]. Vertebrate lenses are composed of 4 distinct families of structural proteins, α, β, γ, and δ-crystallin, which account for 80 - 90% of the soluble proteins in the lens [5]. Invertebrate lenses (in cephalopods) are composed of different proteins such as S,O,L, and Ω-crystallin [6]. Crystallin proteins are responsible for maintaining the transparency and high refractive index of the lens; transparency is achieved by positioning crystallins in a similar arrangement as the particles in glass [7]. Unfortunately, the functions of the βγ-crystallins are not fully understood. βγ-crystallins are involved in maintaining lens transparency, but may also play a role in lens development and may function as stress proteins [8]. In contrast to βγ-crystallins, the α-crystallins are better described in the literature. The α-crystallin family is composed of two genes, α-A and α-B, which occur in a ratio of 3A:1B in the lens. The α-crystallins function to refract incoming light to maintain the transparency of

the lens; a high concentration of proteins is also required to maintain the necessary refractive index, which has shown to be as high as 450 mg/mL in the centre of the lens [9]. Interestingly, α-crystallins are similar to the small heat shock protein family and can also act as molecular chaperones that bind unfolded and denatured protein to prevent aggregation [10]. Maintaining protein balance is incredibly important for transparency of the lens, as protein aggregation can lead to the formation of cataracts.

The retina is a photosensitive membrane located in the posterior section of the eye where light signals are converted into electrical impulses [4]. Mammalian retinas possess 55 different cell types, each with differing function [11]. Two types of cells responsible for vision in humans are rods and cones. Rod cells are photoreceptors specialized for low light situations as they contain only one type of visual pigment, rhodopsin, whereas cones are specialized for colour vision as they contain opsins with maximal absorption of short and long wavelengths of light [11]. Maximal optical resolution occurs in the macula region of the retina at a point called the fovea [4]. Incoming light signals are relayed from the retina to the brain via the optic nerve for processing. Both the lens and the retina play an important role in vision and many ocular diseases are associated with abnormalities in these structures.

1.2 Zebrafish eye development

Initiation of zebrafish eye development is incredibly similar compared to other vertebrate species. The transcription factors Six3a and Pax6 are expressed in the anterior neural plate, designating a region for ocular tissue development [12, 13]. In this region, large masses of cells from the forebrain come together to produce an eye-mass, and the optic cup will have formed by 22 hours post fertilization (hpf) [14, 15]. Lens

development in zebrafish begins when the lens placode (thickened portion of the lens ectoderm) detaches from the surface ectoderm and forms a sphere-like mass of cells [14, 16]. The sphere-like mass of cells detaches from the surface at 24–26 hpf and the lens epithelium begins to form. The lens epithelial cells proliferate and eventually differentiate into lens fibre cells which compose the majority of the lens. After 72 hpf lens fibre cells begin losing organelles, which is required for optimal lens function [16]. Zebrafish corneal development is intertwined with lens development and is initiated with cells from the lens ectoderm. Corneal epithelial cells begin to differentiate and arrange themselves into distinct layers, while simultaneously cells from optic cup migrate to the cornea to produce the corneal endothelium [17]. The corneal epithelium detaches from the lens at approximately 30 hpf, and the three major layers of the cornea (epithelium, stroma and endothelium) are formed by 72 hpf [18]. Zebrafish retinogenesis begins when cells differentiate into retinal ganglion cells, which can be seen between 28–32 hpf [19, 20]. At 36 hpf the retina is composed of two epithelial layers, the pigmented epithelium and the retinal neuroepithelium [21]. After differentiation of the retinal ganglion cells both amacrine and interneruons begin to differentiate, and retinal lamination is apparent by 48 hpf [15]. Muller glia cells are the last to differentiate, and do not seem to be functional until 72–96 hpf [22]. Zebrafish retinae possess both rods and cones. Cone photoreceptors are arranged in a crystalline-like manner and cone opsins are arranged in an fashion while rod photoreceptors are arranged around the cones and rod opsins are arranged more sporadically [23]. By 72–96 hpf, the cells in the central retina are differentiated, and many different cell types can be identified [22].

1.3 Cadmium and cataracts

Cadmium is a heavy metal produced as a by-product of extracting and refining zinc from sulphide ore. Unlike zinc, cadmium has no beneficial role in mammalian biological functions and is toxic at relatively low concentrations; the LD50 of cadmium in mice was found to be 15 – 17 µM/kg [24, 25]. Approximately 90% of cadmium exposure occurs through food for non-smokers who are not regularly exposed to cadmium due to their occupation [26]. Intestinal absorption of cadmium can be as high as 5%. Interestingly, it seems that absorption of cadmium is higher when internal iron stores are lower. In humans and rats, iron deficiency resulted in higher cadmium uptake in the small intestine that was mediated through increased expression of divalent metal transporter-1 [27, 28]. In an attempt to uptake more iron, there is an increased expression of divalent metal transport-1 but also a subsequent increase in cadmium uptake. Cadmium exposure has been associated with abnormal vision and ocular disease. Zebrafish embryos exposed to cadmium at environmentally relevant concentrations (0.05 – 2 parts per billion) had similar eye morphology compared to controls yet displayed a decreased optomotor response, thereby showing developmental cadmium exposure is subtle but also detrimental to vision [29]. Cadmium exposure has also shown to induce apoptosis of lens epithelial cells through MAPK signalling [30]. Li and colleagues found that in 20 cataract patients, all had apoptotic lens epithelial cells ranging from 4.4 - 41.8% [31]. Perhaps the mechanism by which cadmium causes cataracts is by inducing apoptosis of the lens epithelial cell layer, thereby reducing the number of differentiated lens fibre cells. Another theory is that cadmium binds to hemoglobin which reduces its ability to deliver oxygen efficiently [32]. Cigarette smoking has also been linked to increased likelihood of developing cataracts [33, 34].

Significant concentrations of copper, lead and cadmium were found to be accumulated in the lenses of smokers [35].

Cataractogenesis, the formation of a cataract, occurs when the transparency of the lens is compromised. Opacification of the lens greatly impairs vision by preventing light rays from being focused onto the retina. Cataracts can be classified by the location where opacification occurs; nuclear cataracts are defined by opacification in the centre of the lens (most common), cortical cataracts involve opacification of the lens cortex, and posterior subcapsular cataracts occur in the central posterior cortex. Cataracts may also be classified by other criteria including age, diabetic status and cold-induced. Cataracts are an incredibly prevalent ocular disease, as recent data collected in 2010 estimated that cataracts caused 33.4% of all cases of blindness worldwide [36]. Similar to many other diseases, the physiological and chemical mechanisms of cataract formation is not well understood and seems to be affected by many factors. Personal factors such as increasing age, sex, ethnicity and genetics as well as environmental factors such as cigarette smoking, ultraviolet exposure, and diabetes status have shown to be risk factors for cataract formation [33]. In mice, knockout of the α-A crystallin gene (*CRYAA*) induced the cataract phenotype, indicating that α-crystallin is necessary for lens transparency [37]. The α-B crystallin gene (*CRYAB*) has also been implicated in cataract formation. Berry and colleagues identified that an English family with a history of congenital cataracts had a deletion in exon 3 in the *CRYAB* gene resulting in a frameshift mutation [38], illustrating a genetic component for cataracts. Oxidative stress has also been shown to play a role in cataractogenesis. Reactive oxidative species breaks down lipids in cataractous lenses, possibly due to the decrease in activity of anti-oxidants [39]. Essential metal regulation is another

factor to be considered, as mature cataracts were found to have higher concentrations of zinc and iron compared to immature cataracts [40]. Based on the literature, a multitude of factors ranging from altered gene expression to regulation of metals influence cataract formation. The metallothionein gene family is of interest due to their vital role in metal regulation and homeostasis.

1.4 Metallothioneins

Metallothioneins (MT) are a class of small proteins characterized by their high affinity for heavy metals such as cadmium. MT is involved in biological functions such as metal regulation, heavy metal detoxification, and oxidative stress response. In mammals, the four metallothionein isoforms are MT-1, MT-2, MT-3, and MT-4 [41]. The most common MT, MT-1 and MT-2, are expressed in almost all tissue types and can be induced by stress and compounds such as metals, cytokines and reactive oxygen species [42]. MT-3 and MT-4 exhibit tissue-specific expression in the central nervous system and stratified squamous epithelia respectively [43, 44] and, in contrast to MT-1 and MT-2, cannot be induced by metals ions. Mammalian MT proteins contain 20 cysteine amino acid residues in many cys-x-cys arrangements, where x is any amino acid except cysteine. These cysteine residues facilitate the chelation of metal ions using both sulfhydryl and primary amine functional groups [45]. MT was found to be localized in the epithelium of the lens and cornea, as well as the pigmented layer of the retina in rats [46]. MT has shown to have a protective effect against metal toxicity and UV-A radiation in cultured lens epithelial cells [47]. It is possible that MT expression in the lens epithelium allows for initial protection from heavy metals in this layer in order to allow primary fibre cells to develop. Lens fibre cells arise from

the lens epithelial cells, therefore perhaps the protective mechanism is preserving this progenitor region. Interestingly, MT-2A was found to be up-regulated in age-related cataractous lenses compared to non-cataractous lenses [48]. Perhaps years of acute exposure to metals in the lens has a threshold, at which point the protective effect of MT is diminished. Due to the complex nature and multiple factors influencing this ocular disease, it is still unclear exactly how MT could be involved in cataractogenesis, and further research would have to be conducted to elucidate mechanisms.

1.5 MTF-1

Expression of MT proteins are increased in response to metals. Specifically, researchers identified a zinc-responsive transcription factor that binds to the metal response element (MRE) region of the MT gene promoter which was responsible for up-regulation of MT [49]. The novel protein was named MRE binding transcription factor-1 (MTF-1) but is also known as metal-responsive transcription factor-1 or metal-regulatory transcription factor-1. Human MTF-1 is a 753 amino acid protein composed of six Cys_2-His_2 zinc finger motifs and three transactivation domains (acidic, proline-rich, and serine/threonine-rich) and is evolutionarily conserved. MTF-1 is localized in the cytoplasm under normal conditions but is rapidly transported to the nucleus in response to heavy metals, oxidative stress or heat shock [50]. Zinc fingers 1-3 were shown to contain a nuclear import signal for MTF-1, whereas the nuclear export signal is contained in the acidic transactivation domain [51]. MTF-1 binds to target genes at the MRE, which share a core consensus sequence of 5′-TGCRCNC-3′ [52, 53]. MTF-1 is responsible for basal and metal-induced expression of the MT gene family and zinc homeostasis [52]. MTF-1 is necessary for embryonic hepatogenesis

in mice, and MTF-1 knockout mice die from liver degradation [54]. Nuclear export of MTF-1 is mediated by chromosomal maintenance-1 (crm1), an exporter protein responsible for transporting other proteins with a leucine export signal. Inhibition of crm1 results in MTF-1 aggregating in the nucleus [51].

The role of MTF-1 in the eye is not particularly prevalent in the literature; however, a few studies conducted in zebrafish illustrate potentially novel functions of MTF-1. Chen and colleagues have shown that in zebrafish, MTF-1 is expressed throughout early larval development with strong expression ~ 22 hours post fertilization in the cerebellum, neural tube, retina and lens regions [55]. Inhibition of MTF-1 signalling in zebrafish embryos using a constitutively dominant-negative MTF-1 resulted in the down-regulation of many crystallin genes (α, βγ-crystallins) as well as other genes related to structural components of the eye [56]. Yet, expression levels of MTF-1 between normal and age-related cataractous lens showed no significant differences [57]. It is possible that basal levels of MTF-1 can regulate genes involved in cataractogenesis, which could explain why its transcript levels are unchanged. The previous studies identified a slight correlation between MTF-1 expression and the eye; however additional research is needed to verify if MTF-1 is involved in eye-related gene expression.

1.6 Zinc and MTF-1

MT and MTF-1 have also been implicated in zinc homeostasis. If the intracellular concentration of zinc increases past a threshold value, MTF-1 is activated and the expression of MT increases resulting in more MT proteins sequestering zinc [58]. Biochemical events or processes that require free zinc, such as oxidative signalling,

result in the release of zinc from MT. Therefore, MTF-1 must have some zinc sensing capabilities. The dissociation constant of MTF-1 for zinc is approximately 2 µM, which indicates relatively high affinity [59]. Even though MTF-1 as a whole has a high affinity for zinc, the individual zinc finger motifs have varying affinities for zinc; from strongest to weakest the order is F4 >F2 = F5 >F6 = F3 = F1 [60]. A proposed mechanism of zinc detection (Figure 1.2) involves increased intracellular zinc binding to the zinc fingers of MTF-1, MTF-1 binding to DNA to induce transcription of MT genes, and MT sequestering zinc thereby inactivating MTF-1 [59, 61]. Bittel and colleagues conducted an experiment using HeLa and Hepa cell lines to assess the effect of metals on DNA binding activity of MTF-1. Of the many transition metals tested (zinc, cadmium, nickel, silver, cobalt and copper) only zinc was found to increase MTF-1 DNA binding activity significantly [62], which suggests that MTF-1 is responsive to zinc alone and the right balance of zinc is crucial for cell functioning. However, studies conducted on fish have reported conflicting results, with zebrafish and pufferfish MTF-1 binding to the MRE with both zinc and cadmium exposure [63, 64]. Perhaps the mechanism by which cadmium leads to increase MTF-1 DNA binding is by displacing MT-bound zinc, causing a rise in free zinc levels which results in MTF-1 activation.

1.7 Genomic examination of gene expression using RNA-Seq

RNA sequencing (RNA-Seq) utilizes a high-throughput assay to identify or quantify RNA transcripts from an experiment [65]. The presence of a transcript or differential

gene expression can be tested using the data generated by RNA-Seq. The mRNA extracted from experimental samples is converted into complementary DNA (cDNA). A library is created by fragmenting the cDNA, and attaching sequencing adaptors to the resulting fragments. The fragments then undergo high throughput DNA sequencing, producing either single end or paired end reads. Reads can then be aligned to a reference genome to quantify transcript abundance. RNA-Seq is increasingly being used in the field of toxicology to assess transcriptional changes in response to a wide variety of compounds ranging from pharmaceuticals, narcotics, and nanoparticles [66, 67]. The advantages of using RNA-Seq over DNA microarrays are identification of transcripts that do not map to existing genomic sequences, identifying intron-exon boundaries and splice junctions, and low levels of background signal [68]. RNA-Seq is an invaluable methodology and is vital for discovering and understanding the transcriptional changes in toxicological experiments.

1.8 Research question and hypothesis

Cadmium exposure and accumulation has been associated with cataracts [32, 35], yet the mechanisms for development remain largely unknown. In this study, we exposed zebrafish larvae, adult zebrafish, and B3 human lens epithelial (HLE) cells to cadmium and assessed transcriptional changes. For cadmium-exposed zebrafish larvae, we hypothesize changes in developmental gene transcription as larvae are still growing and developing. For cadmium-exposed adult zebrafish, we hypothesize changes in eye maintenance gene transcription as initial eye development has been completed. For cadmium-exposed HLE cells, we hypothesize changes in transcription of lens development or maintenance genes and potentially the crystallin gene family

as the respective proteins are vital for lens structure and function. Overall, we predict that cadmium exposure results in altered transcription of eye-related genes which may ultimately lead to cataract formation or contribute to development of other ocular diseases.

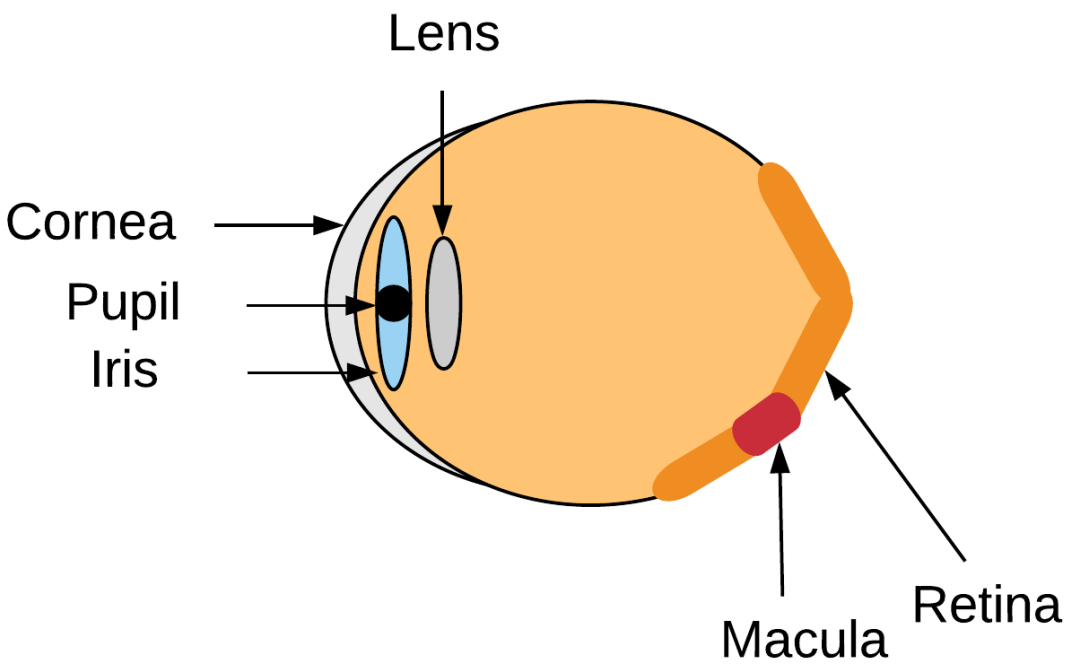


Figure 1.1: **Structural components of the eye.** Incoming light rays are initially diffracted by the cornea which controls the amount of light entering the eye. Light then passes through a perforated hole in the iris called the pupil. The lens functions to diffract light onto the retina, at which point an electrical signal is produced and delivered to the brain for interpretation.

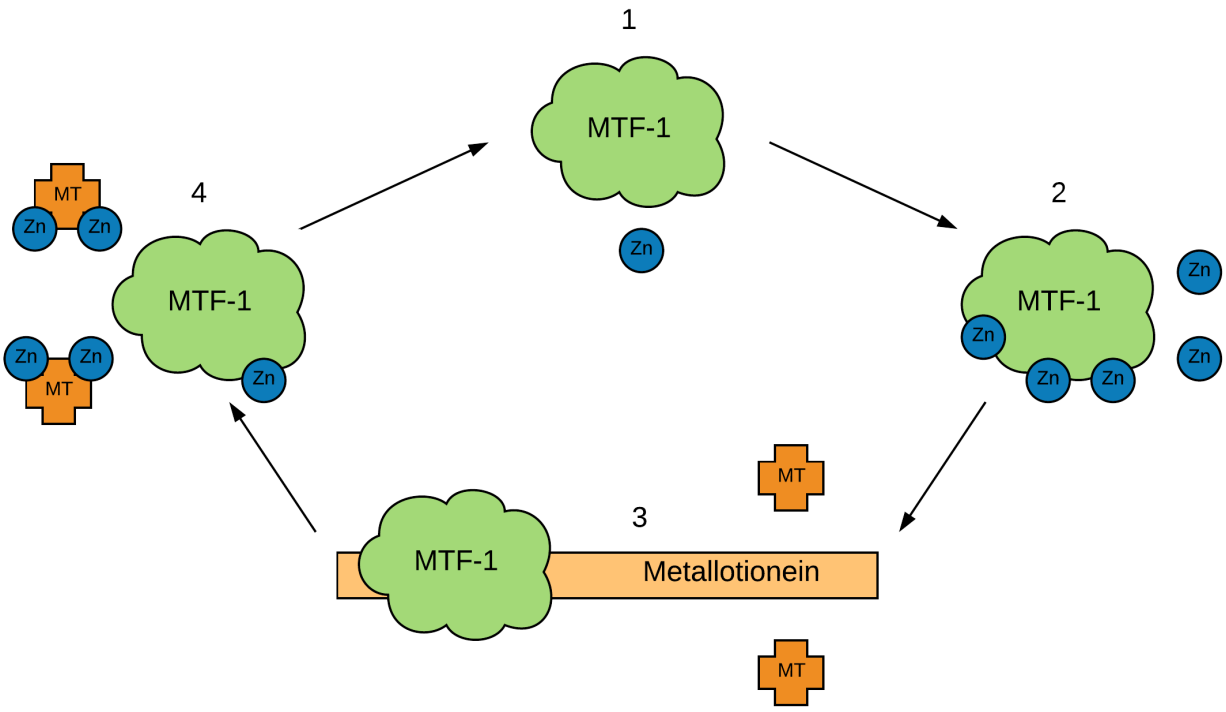


Figure 1.2: **Zinc sensing capability of MTF-1.** A) In normal concentrations of zinc (Zn), MTF-1 is relatively inactive. B) When the Zn concentration is increased, Zn ions bind to the zinc finger motifs of MTF-1 [60]. The binding of Zn increases the DNA binding activity of MTF-1 [62]. C) MTF-1 binds to the metallothionein (MT) promoter and up-regulates the expression of the MT proteins [58]. D) The MT proteins sequester Zn ions, thereby decreasing the concentration and inactivating MTF-1 [59, 61].

Chapter 2

Materials and Methods

2.1 Microarray experiment of cadmium-exposed zebrafish larvae

Zebrafish larvae at 3 different developmental time points (72, 96, and 120 hpf) were reared. For each time point, 4 replicates of 50 pooled larvae were transferred to a petri dish and exposed to 50 µM of Cd (Sigma-Aldrich, St. Louis MO, USA) for either 4 or 8 hours (4h, 8h); each developmental time point and exposure duration combination had corresponding, replicated Cd-free controls. The 96 hpf 4h Cd group only had 3 replicates. Figure 2.1A summarizes the experimental design for the microarray experiment. After Cd exposure the larvae were pooled, flash frozen in liquid nitrogen, and stored at -80℃. Total RNA was isolated using TRIzol™ reagent (Invitrogen Corp, Carlsbad CA, USA) using the standard extraction protocol. The microarray hybridizations were conducted by Cogenics Inc. (Morrisville NC, USA).

Approximately 1-2 µg of total RNA was shipped and quality was assessed using spectrophotometry and an Agilent Bioanalyzer (Agilent Technologies, Santa Clara CA, USA). The universal reference RNA for all hybridizations originated from the total RNA of the zebrafish ZF4 fibroblast cell line. Using 500 ng of total RNA, the Low RNA Input Linear Amplification Kit (Agilent Technologies, Santa Clara CA, USA) was used to produce fluorescently labelled cRNA with either Cy3 (universal reference) or Cy5 (experimental replicate) tagged nucleotides. Approximately 750 ng of both Cy3 and Cy5 labelled cRNA was hybridized to the Agilent zebrafish genome oligonucleotide microarrays. Following hybridization using the standard Agilent protocol, the array was washed, scanned, and data was extracted using the Feature Extraction software version 9.5 (Agilent Technologies, Santa Clara CA, USA).

2.2 RNA-Seq experiment of cadmium-exposed adult zebrafish

Adult male and female zebrafish of 6- and 10-months of age were used. For each age, six individual fish (3 males and 3 females) were placed in a 4 L beaker containing 3 L of fish water (360 mg Instant Ocean Sea Salt and 11 mg of $NaHCO_3$ per liter of deionized water; pH = 7.4, conductivity = 750 µ-siemens, temperature = 28.5°C) and were exposed to 50 µM of Cd for 4-hours. For each age, a total of 6 beakers (3 Cd-free control and 3 Cd treated) were used. Figure 2.1B illustrates the experimental setup. At the conclusion of the exposure, the fish were anesthetized using 150 mM of MS-222 in buffered fish water and euthanized through cervical transection. Entire zebrafish eyes were removed, with eyes from males and females from a single beaker being

pooled separately to form single male and female biological replicates, respectively. Dissected eyes were flash frozen in liquid nitrogen and subsequently stored at -80℃. Total RNA was isolated using TRIzol™ reagent (Invitrogen Corp) using the standard extraction protocol. All RNA samples, excluding the 6-month females, were sent to Hudson Alpha Institute for Biotechnology (Huntsville AL, USA) for RNA sequencing. The RNA concentration and quality was assessed using a Qubit 2.0 fluorometer (Invitrogen Corps, Carlsbad CA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA, USA). Messenger RNA (mRNA) libraries were constructed using a directional protocol with poly(A) selection using the following New England Biolabs (Ipswich MA, USA) reagents: NEBNext Poly(A) mRNA Magnetic Isolation Modul, NEBNext First Strand Synthesis Module. NEBNext Second Strand Synthesis Module (with dUTP), NEBNext End Repair Module, NEBNext dA Tailing Module, and NEBNext Quick Ligation Module. Platinum PFX DNA polymerase (Invitrogen Corp, Carlsbad CA, USA) was used for complementary DNA (cDNA) synthesis. Library quality was examined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA, USA) and library quantity was examined using Kapa Biosystems Library Quantitation kit (Kapa Biosystems, Wilmington MA, USA). Paired-end 50 bp sequencing was performed using the Illumina HiSeq 2500 (Illumina Inc., San Diego CA, USA) with v4 chemistry. Sequencing quality was assessed using Illumina Real Time Analysis software.

2.3 RNA-Seq experiment of cadmium-exposed human lens epithelial cells

HLE B3 cells were cultured in Eagles minimum essential medium (with 20% fetal bovine serum and 100 µg/mL of penicillin/streptomycin) at 37℃ with CO_2 levels less than 5%. Using three 12 well plates, 250,000 HLE cells were distributed per well. After an incubation period of 24-hours the media solution was replaced with serum free media. For each plate, 6 wells were exposed to 10 µM of Cd for 4-hours while the remaining wells served as controls as shown in Figure 2.1C. After the 4-hour exposure, treatment and control wells were pooled separately per plate, each representing a single biological replicate. Total RNA was isolated using TRIzol™ reagent (Invitrogen Corp, Carlsbad CA, USA) using the standard extraction protocol and sent to Hudson Alpha Institute for Biotechnology (Huntsville AL, USA) for RNA sequencing. mRNA library construction and sequencing was conducted described above.

2.4 Animal care and husbandry

All animal experiments used the AB wild-type strain of zebrafish. Breeding groups of adult zebrafish (~ 200 fish) were maintained in a Mass Embryo Production System (Aquatic Habitats, Apopka FL, USA) at a male to female ratio of 1:2. Zebrafish were fed twice per day using Zeilgers Zebrafish Diet (Pentair, London, UK). Zebrafish were given enough food to feed for approximately 5-10 minutes, and excess food was removed. Approximately 3000 zebrafish embryos were generated by breeding fish in the system for 1-hour. Embryos were immediately washed and transferred into large

petri dishes at a density of 100 embryos per 100 mL of 0.3X Danieaus solutions at a temperature of 28.5℃. At 4 hpf embryos were screened for developmental defects, and healthy embryos were maintained in solution using a 14-hour light/ 10-hour dark cycle with Danieaus solution changed every 24-hours. The procedures used in this experiment were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Alabama, Tuscaloosa, Alabama, USA.

2.5 Microarray statistical analysis

Raw microarray data was collected using Agilents Feature Extraction Software (Version 9.5) with background subtraction using both local and global normalization methods, and dye normalization using linear/lowess algorithms. The log ratio of Cy5 to Cy3, log(Cy5/Cy3), was calculated for individual probes. Statistical analysis of normalized data was performed using MEV software package [69]. Prior to analysis, Cy5/Cy3 ratio variables (experimental RNA/universal RNA) were log transformed (base 2) and median centered. The design of the experiment required the use of a two-way ANOVA for age (72, 96 and 120 hpf) versus treatment (4h control, 8h control, 4h Cd-treated, 8h Cd-treated). A calculated distribution was used ($p < 0.01$, 1000 permutations) with no correction for multiple comparisons. Treatment and age/treatment interaction probe sets were combined into a single set of significant probes. Duplicate probes were averaged before further analysis, resulting in a list of unique probes IDs. Rank Product analysis [70] was conducted between each treatment and the corresponding control (two class unpaired, 1000 permutations, α

< 0.01, FDR = 10%). Probes were filtered based on identification as significant in at least one Rank Product test. BioMart was used to confirm if probes mapped to an Ensembl gene identifier (Ensembl release 89), resulting in a final list used for clustering. Cluster analysis, based on expression patterns across samples, was performed for the final set of significant probes using Cluster 3.0 [71]. A $\log_2$ transformation was applied, as the data existed as fold change ratios between treatment and control samples. Hierarchical clustering using centered correlation and the average linkage clustering method was performed. Java Treeview [72] was used for visualization of the heatmap and to obtain clusters of co-expressing probes.

2.6 RNA-Seq data analysis pipeline

Sequence processing of both zebrafish and HLE reads were conducted using the McArthur Lab instance of Galaxy [73]. Raw sequencing reads (~ 30 million paired-end per sample; 50 base pairs each) were downloaded from Hudson Alpha and subsequently uploaded to the Galaxy server in FASTQ format. Quality parameters such as per base sequence quality, per base sequence content, sequence duplication levels and many others were assessed using FASTQC [74]. All files passed recommended quality control parameters, therefore the FASTQ files passed through the pipeline unaltered. Reads were aligned to the Ensembl zebrafish (GRCz10) genome or the Ensembl human (GRCh38) genome using HISAT2 [75]; HISAT2 parameters were set to default except for disable spliced alignment = false, strand information = first strand (R/RF), and maximum fragment length = 600. The number of reads mapping to each gene was identified using htseq-count [76], with stranded = reverse, force sorting of SAM/BAM by name = yes and all other parameters default. For

both zebrafish and humans, GTF gene annotation reference files were obtained from Ensembl.

2.7 Zebrafish RNA-Seq statistical analysis

Statistical analysis of zebrafish data was conducted using two different methods. The first method involved using gene count tables obtained from htseq-count as input for DESeq2 normalization and statistical testing [77], using a single factor (treatment) with two factor levels (Cd and control) with all other parameters set to default. Normalization of read counts in DESeq2 is based on size factors calculated from the input dataset, and differential gene expression is determined by fitting data to a negative binomial generalized linear model in which likelihood ratio tests analyze deviance instead of variance. DESeq2 was used separately for the 6-month males, 10-month males, and 10-month females resulting in 3 lists of genes with associated p-values for gene set enrichment analysis (GSEA), as outlined below.

The second method of statistical analysis involved combining the htseq-count gene count tables into a single document and normalizing counts using the DESeq2 R package. Normalized counts were imported into Microsoft Excel and all 0 counts were changed to 1 to facilitate downstream log transformations. Two separate tables were made from the normalized counts, one with data from 6-month males and 10-month males and another with 10-month males and 10-month females. Individual lists were imported into MEV and the data was $\log_2$ transformed and median centered. Two separate two-way ANOVAs were performed; 6-month males vs. 10-month males and 10-month males vs. 10-month females. For the first ANOVA, the factors were treatment (Cd-treated, Cd-free control) and age (6-month, 10-month). For the second

ANOVA, the factors were treatment (Cd-treated, Cd-free control) and sex (male, female). Both ANOVAs were performed using a calculated distribution ($p < 0.01$, 1000 permutations) with no correction for multiple comparisons. For each ANOVA, treatment significant and interaction significant terms were isolated and exported into a new MEV session. Rank product analysis was conducted between treatment and the corresponding control for 6-month males and 10-month males for the first experiment and 10-month males and 10-month females for the second experiment (two class unpaired, 5000 permutations, $\alpha < 0.01$, FDR = 10%). All unique significant gene identified by the Rank Product were clustered as described above, and groups of genes were input into DAVID for Gene Ontology (GO) enrichment, as outlined below.

2.8 HLE RNA-Seq statistical analysis

The htseq-count outputs were also used as input for DESeq2 in Galaxy for differential gene expression analysis. Significant genes, with an adjusted p-value (Benjamini-Hochberg) of < 0.05, were identified from the output, and the normalized read counts for these genes were isolated. All 0 values were replaced with a value of 1 to facilitate down-stream log transformation. Only genes in which all samples had > 10 reads were used. Normalized counts for each gene were averaged over all 3 replicates of either control or Cd treated and the ratio of Cd/control was calculated. Up-regulated and down-regulated genes were defined as having a Cd/control ratio of > 2 and < 0.5 respectively. Up-regulated and down-regulated genes were separately input into DAVID for GO enrichment, described below. DESeq2 output was also converted into a ranked list and used with GSEA as outlined below.

2.9 DAVID Gene Ontology enrichment

The Database for Annotation, Visualization and Integrated Discovery (DAVID) [78] was used for GO enrichment analysis for both microarray and RNA-Seq experiments. Agilent probe IDs were entered into a list in DAVID using the default whole genome zebrafish background for the microarray, while Ensembl gene identifiers and the default zebrafish or human genome background was used for RNA-Seq. Parameters used in DAVID included a minimum gene count of 2, calculation of both Benjamini p-value and fold enrichment, and an EASE value of 0.05. Enriched GO terms were subsequently exported into an Excel spreadsheet.

2.10 Gene set enrichment analysis

A python script to convert the DESeq2 output into a ranked list of all genes was used for each examined sub-experiment. Ranking scores were generated using rank score, as previously described [79, 80].

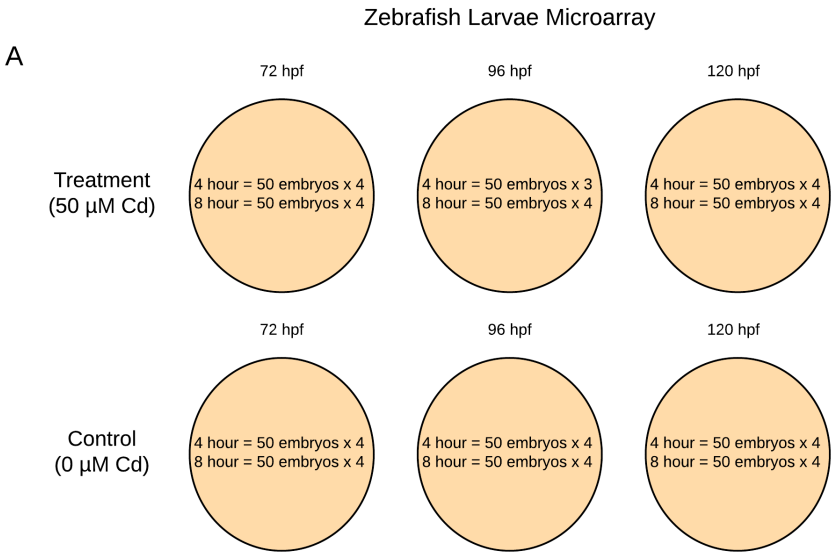
$$Rank\ Score = -\log_{10}(p)(\text{sign}(\log(\text{FC})))$$

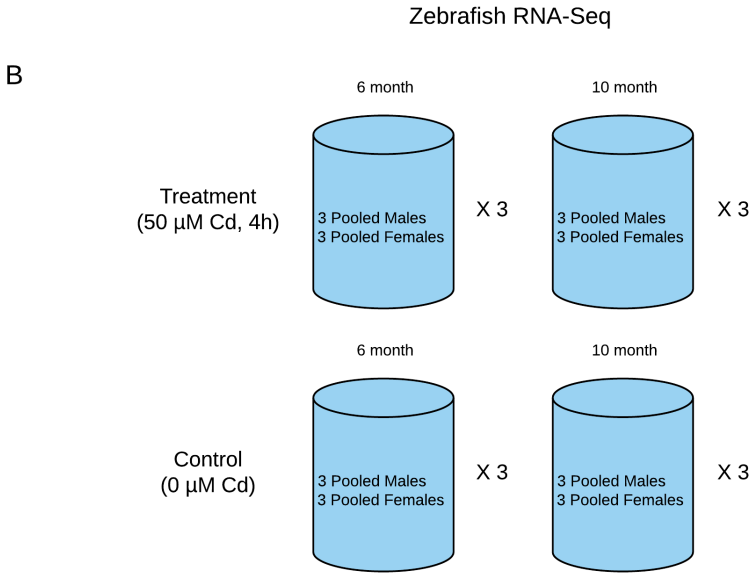
where,

$$\text{FC} = \text{Fold Change}$$

$$p = \text{p-value}$$

The ranked lists were used as input for GSEA [81]. GSEA was run using 1000 permutations with a zebrafish gene set database obtained from the Molecular Signatures Database using their GO2MSIG tool [82], or a human gene set database obtained from the Bader Lab at the University of Toronto (`http://download.baderlab.org/EM_Genesets/`). The results of GSEA were visualized using Cytoscape [83] using a false discovery rate (FDR) cut-off of 25%.


Zebrafish Larvae Microarray
A
72 hpf
96 hpf
120 hpf
Treatment
(50 µM Cd)
4 hour = 50 embryos x 4
8 hour = 50 embryos x 4
4 hour = 50 embryos x 3
8 hour = 50 embryos x 4
4 hour = 50 embryos x 4
8 hour = 50 embryos x 4
72 hpf
96 hpf
120 hpf
Control
(0 µM Cd)
4 hour = 50 embryos x 4
8 hour = 50 embryos x 4
4 hour = 50 embryos x 4
8 hour = 50 embryos x 4
4 hour = 50 embryos x 4
8 hour = 50 embryos x 4


Zebrafish RNA-Seq
B
6 month
10 month
Treatment
(50 µM Cd, 4h)
3 Pooled Males
3 Pooled Females
X 3
3 Pooled Males
3 Pooled Females
X 3
6 month
10 month
Control
(0 µM Cd)
3 Pooled Males
3 Pooled Females
X 3
3 Pooled Males
3 Pooled Females
X 3

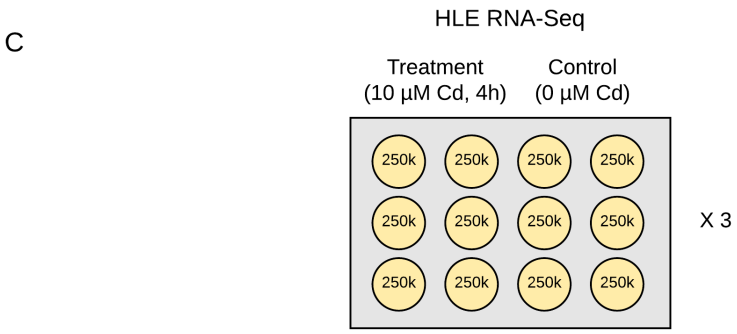

HLE RNA-Seq
C
Treatment
(10 µM Cd, 4h)
Control
(0 µM Cd)
250k
X 3

Figure 2.1: **Overview of the 3 separate experiments conducted.**

A) For the zebrafish larvae microarray, zebrafish larvae aged 72, 96 or 120 hours post fertilization (hpf) were exposed to 50 µM of Cd for either 4 or 8 hours, with each combination having a control group. A single replicate consisted of 50 pooled embryos, and all treatments (excluding 96 hpf 4h Cd treatment) consisted of 4 replicates. B) For the zebrafish RNA-Seq, adult zebrafish aged 6-months and 10-months were contained in glass beakers and exposed to 50 µM of Cd for 4-hours. Three males and 3 females of the same age were housed together in a single beaker for treatment and control conditions. The three males in a beaker were treated as a single replicate as were the three females. Each group contained 3 replicates, however the 6-month females were not further processed. C) For the HLE RNA-seq, 250,000 HLE cells were distributed into each well of a 12-well plate. The first 6 wells were exposed to 10 µM of Cd for 4-hours, serving as a single replicate, while the next 6 were used for control. Each group contained 3 replicates.

Chapter 3

Results

3.1 Microarray experiment of cadmium-exposed zebrafish larvae

3.1.1 Filtering and cluster analysis

After normalization and transformation, two-way ANOVA ($\alpha < 0.01$) identified 15475 probes as significant during development (hpf), 9004 probes as Cd treatment significant, and 2262 probes significant for interaction of development and Cd exposure. Combined, 9575 probes were significant for Cd treatment or Cd x hpf interaction and were selected for further analysis. Values for probes present in duplicate on the microarray were averaged, resulting in values for 5675 unique probes. Rank product analysis ($\alpha < 0.01$, FDR = 10%) was conducted to narrow down the list of significant probes since two-way ANOVA does not include false discovery correction: 1246 probes were significant in at least one rank product test. A final filtering step involved matching probe names to Ensembl gene identifiers, resulting in a list of 733 probes.

Cluster analysis for probes with similar expression patterns was visualized using a heatmap (Figure 3.1). Five clusters of probes with varying intensities were identified. Cluster 1 contained 152 probes, the majority of which were down-regulated between Cd and control, regardless of developmental time point or length of exposure. The 96 hpf 8h and both 120 hpf treatments show stronger down-regulation compared to the other treatments. The 120 hpf 8h treatment shows slightly stronger down-regulation compared to the 4h treatment and the same developmental time point. Cluster 2 contained 52 probes with the majority of the 96 hpf 4h Cd exposure being up-regulated, and a small up-regulated section in the 120 hpf 4h treatment. Cluster 3 contained 233 probes with the majority of probes in all treatments being up-regulated. The majority of probes in both 120 hpf treatments (4 or 8 hr Cd exposure) had the strongest up-regulation, while both 72 hpf treatments had weak up-regulation. Cluster 4 contained 177 probes, with strong down-regulation in the 96 hpf 4h treatment and a small subset of probes in the 120 hpf 4h condition was strongly up-regulated. Both 72 hpf treatments showed no discernible pattern for the majority of these probes. Cluster 5 contained 60 probes with differing expression patterns between conditions. The 72 hpf 4h treatment had the majority of probes up-regulated while the 72 hpf 8h treatment has many probes down-regulated.

3.1.2 Zebrafish larvae Gene Ontology enrichment

GO enrichment analysis was conducted for each cluster of probes in Figure 3.1 using DAVID, retaining only terms with a false discovery rate of less than 5% (Benjamini value of < 0.05) (Table 3.1). GO enrichment analysis of cluster 1 resulted

in 112 enriched GO terms, 2 of which were eye-related. The GO terms "retina development in camera-type eye" (GO:0060041) and "camera-type eye development" (GO:0043010) had a fold enrichment (above background) of 5.5 and 4.2, respectively. Genes involved in the enrichment of these eye-related GO terms are found in Table 3.2. DNA (cytosine-5)-methyltransferase-1 (*dnmt1*), cyclin A2 (*ccna2*), flap endonuclease-1 (*fen1*), minichromosme maintenance complex-3 (*mcm3*) and stem-loop binding protein (*slbp*) were all found to be down-regulated in the 120 hpf 8h treatment. Only *ccna2*, *fen1*, *mcm3* and *slbp* were down-regulated in the 96 hpf 8h treatment. *dnmt1*, *fen1*, and *mcm3* were down-regulated in the 120 hpf 4h treatment and *fen1*, *mcm3* and *slbp* were down-regulated in the 96 hpf 4h treatment. None of the genes were found to be down-regulated in either of the 72 hpf treatments. GO enrichment analysis of cluster 4 resulted in 22 enriched GO terms. No eye-related terms were present, but many terms involving embryonic development or developmental processes were significantly enriched. GO enrichment analysis for clusters 2, 3, and 5 resulted in no GO terms being enriched.

3.2 RNA-Seq experiment of cadmium-exposed adult zebrafish

3.2.1 RNA-Seq read statistics

All raw DNA sequencing results passed the recommended FASTQC quality control parameters, allowing analysis of all data without filtering or trimming, resulting in ~ 30 million read pairs per replicate (Table 3.3), with one notable exception: 183,535,190 read pairs for 10-month males control replicate #2. For all replicates, the HISAT2

read pair mapping rate was ~ 90% or greater with annotation mapping rate ranging from 65 – 69%.

3.2.2 Zebrafish gene set enrichment analysis

The 6-month male zebrafish data underwent GSEA, resulting in 15 up-regulated and 0 down-regulated genes sets. Many of the up-regulated gene sets involved metal, including terms such as "metal ion transport" and "response to metal ion", however no eye-related gene sets were enriched in the 6-month males.

Analysis of the 10-month male zebrafish data using GSEA resulted in 15 up-regulated and 3 down-regulated gene sets. Metal-related gene sets were up-regulated, and 11 of the up-regulated gene sets were identical to the 6-month male zebrafish treatment. In the down-regulated gene sets 2 eye-related terms ("embryonic camera-type eye formation" and "embryonic eye morphogenesis") were present. Enrichment plots for the eye-related gene sets are shown in Figure 3.2. The core genes involved in enrichment of the male eye-related gene sets are listed in Table 3.4. The core genes for "embryonic camera-type eye formation" were paired-like homeodomain transcription factor-2 (*pitx2*), frizzled class receptor-8a (*fzd8a*), secreted frizzled-related protein-5 (*sfrp5*), LIM homeobox transcription factor-1, beta b (*lmx1bb*), phosphatase and actin regulator-4b (*phactr4b*) and forkhead box c1a (*foxc1a*), while the core genes for "embryonic eye morphogenesis" additionally included transcription factor 7-like-1a (*tcf7l1a*), microfibril associated protein-2 (*mfap2*), six homeobox-3b (*six3b*), lumican (*lum*), zinc finger protein-703 (*znf703*), chaperonin containing TCP1 subunit-3 (*cct3*), and LIM homeobox transcription factor-1, beta a (*lmx1ba*). The Cd/Ctrl fold change for the genes range from 0.63 to 0.89. All genes (excluding *znf703*, *cct3* and *lmx1ba*)

had a Benjamini value < 0.05.

GSEA of the 10-month female zebrafish data resulted in 43 up-regulated and 0 down-regulated gene sets. Metal-related terms were once again present in the up-regulated gene sets, with the same 8 up-regulated gene sets identical to the 6-month male and 10-month male zebrafish treatments. Unlike the results for males, gene sets for "eye photoreceptor cell development", "photoreceptor cell development", "eye photoreceptor cell differentiation", and "photoreceptor cell differentiation" were up-regulated. Enrichment plots for the photoreceptor gene sets are outlined in Figure 3.2. Core gene involved in enrichment of the photoreceptor gene sets are presented in Table 3.5. Only ornithine decarboxylase-1 (*odc1*), thyroid hormone receptor beta (*thrb*), usherin (*ush2a*), adhesion g protein-coupled receptor V1 (*adgrv1*), synaptojanin-1 (*synj1*), and cone rod homebox (*crx*) were core genes for "eye photoreceptor cell development", while all genes excluding CDP-diacylglycerol–inositol 3-phosphatidyltransferase (*cdipt*) and retinal homebox-1 (*rx1*) were associated with "photoreceptor cell development". All genes excluding phosphatidylinositol transfer protein beta (*pitpnb*) were associated with "eye photoreceptor cell differentiation", while all 10 genes were core genes for the "photoreceptor cell differentiation" gene set. The Cd/Ctrl fold change for the genes range from 1.13 to 2.43 yet only three of these genes (*odc1*, *thrb* and *ush2a*) had a Benjamini value < 0.05.

3.2.3 Zebrafish statistical analysis using two-way ANOVA and Rank Product

Two-way ANOVA between the 6-month males and 10-month males resulted in 3356 treatment significant, 4032 age significant, and 698 treatment x age interaction significant genes. The combination of treatment and treatment x age interaction resulted in 6642 significant genes. Two-way ANOVA between the 10-month males and 10-month females resulted in 3236 treatment significant, 5380 sex significant, and 742 treatment x sex interaction significant genes. The combination of treatment and treatment x sex interaction resulted in 7732 significant genes. Rank Product analysis resulted in a total of 610 unique genes which were found to be significant in at least 1 Rank Product test. Cluster analysis of gene expression was visualized using a heat-map, shown in Figure 3.3. Four clusters of genes were identified and labelled. Cluster 1 contained 111 genes, with the majority of genes being strongly up-regulated in the 10-month male Cd treatment relative to control. Cluster 2 contained 128 genes with many genes being down-regulated in the 6-month males while genes in the 10-month males were up-regulated. Cluster 3 contained 269 genes with the majority of genes in the 10-month females showing strong down-regulation and slightly weaker down-regulation in the 10-month males. Cluster 4 contained 102 genes with down-regulation in the 10-month males. GO enrichment analysis results for the clusters are shown in Table 3.6. Genes in cluster 1 enriched 23 GO terms, while genes in all other clusters did not enrich any GO terms. Enriched GO terms for cluster 1 included terms relating to iron ions and metal ion homeostasis, however no eye-related GO terms were enriched.

3.3 RNA-Seq experiment of cadmium-exposed human lens epithelial cells

3.3.1 RNA-Seq read statistics

All raw DNA sequencing results passed the recommended FASTQC quality control parameters, allowing analysis of all data without filtering or trimming, resulting in ~ 30 million read pairs per replicate (Table 3.3), with two notable exceptions: 19,209,331 read pairs for control replicate #3 and 41,186,629 read pairs for cadmium replicate #3. For all replicates, the HISAT2 read pair mapping rate was ~ 95% or greater with annotation mapping rate ranging from 79 – 81%.

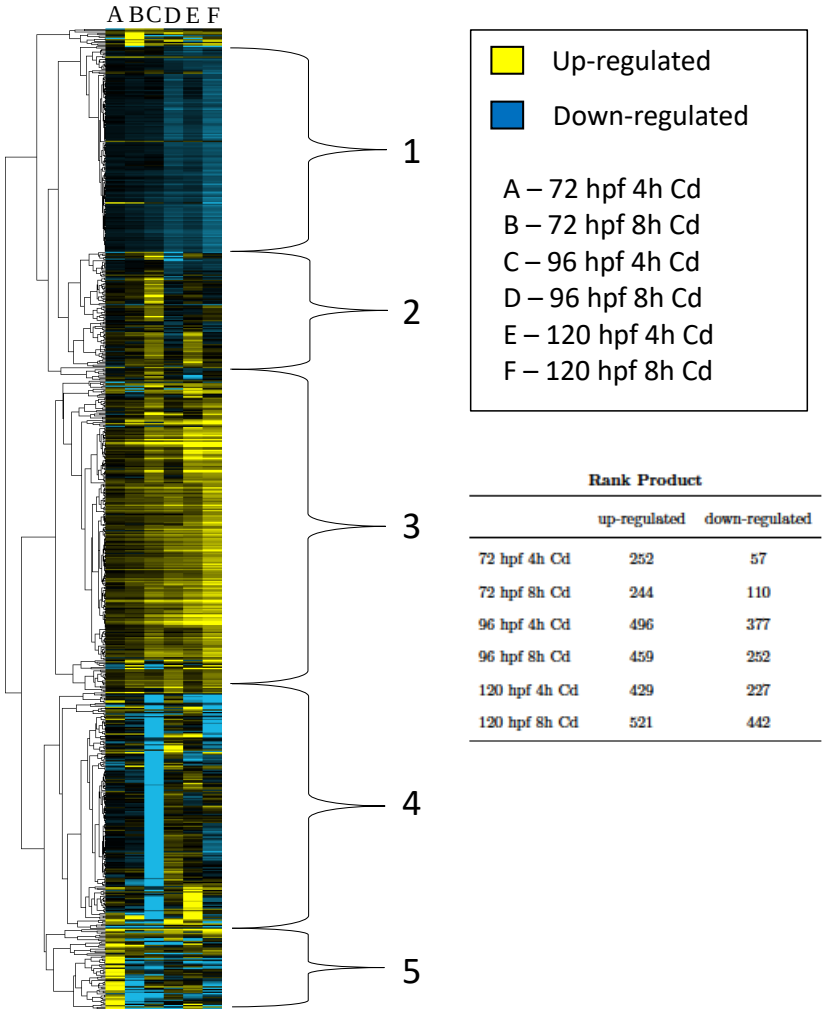
3.3.2 HLE Gene Ontology enrichment

DESeq2 analysis of the HLE cell data identified 8197 genes as significant in Cd treated cells compared to control. After filtering for genes with less than 10 reads in one or more replicates, 7583 genes remained for further analysis. Only 9 genes which were filtered out had less than 10 reads for all the control samples and more than 100 reads for the Cd-treated samples. These genes include different metallothioneins (*MT1XP1*, *MT1P1*, *MT1M*), heat shock proteins (*HSPA7*) and others (*XIRP1*, *OROL1*, *C11orf96*, *PAQR9*, *ARC*). There were 767 up-regulated genes with 2-fold or greater increase and 200 genes with 2-fold or greater decrease in normalized Cd/Ctrl read counts. GO enrichment analysis identified a total of 1318 enriched GO terms for the up-regulated genes (Table 3.7). Three eye-related terms were enriched: "camera-type eye development" (GO:0043010) , "eye development" (GO:0001654), and "lens development in camera-type eye" (GO:0002088). The genes involved in enrichment

of these eye-related terms are outlined in Table 3.8. All 22 genes were involved in enrichment of the "eye development" term. All genes, excluding PR domain zinc finger protein-1 (*PRDM1*), were involved in enrichment of the "camera-type eye development" term and only Cbp/p300-interacting transactivator-2 (*CITED2*), SKI like proto-oncogene(*SKIL*), crystallin alpha B (*CRYAB*), solute carrier family-7 member-11 (*SLC7A11*), EPH receptor A2 (*EPHA2*), breast cancer anti-estrogen resistance-3 (*BCAR3*), Wnt family member-5B (*WNT5B*), and bone morphogenetic protein-4 (*BMP4*) enriched the "lens development in camera-type eye" term. The transforming growth factor beta-2 (*TGFB2*) is also involved in lens development, however this gene was missed during enrichment analysis. GO enrichment analysis of the down-regulated genes resulted in 4 terms: "nucleic acid metabolic process" (GO:0090304), "RNA metabolic process" (GO:0016070), "nucleobase-containing compound metabolic process" (GO:0006139) and "heterocyclic metabolic process" (GO:0046483).

3.3.3 HLE gene set enrichment analysis

GSEA analysis of the HLE RNA-Seq data resulted in 66 up-regulated gene sets and 70 down-regulated genes sets. Neither the up-regulated or down-regulated gene sets contained any eye-related terms.

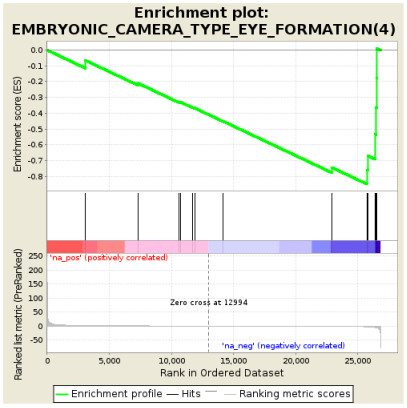


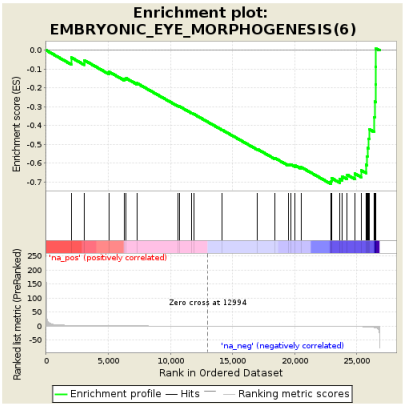
Rank Product

	up-regulated	down-regulated
72 hpf 4h Cd	252	57
72 hpf 8h Cd	244	110
96 hpf 4h Cd	496	377
96 hpf 8h Cd	459	252
120 hpf 4h Cd	429	227
120 hpf 8h Cd	521	442

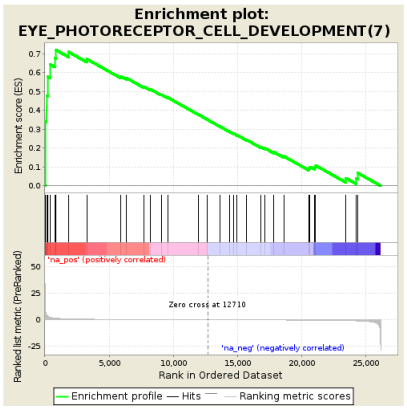
Figure 3.1: **Clustered heat-map of zebrafish larvae microarray probes.** Data shown is the ratio of Cy3/Cy5 between cadmium exposed zebrafish larvae and control larvae. Prior to clustering, data was $\log_2$ transformed. The number of significant genes found by Rank Product for each treatment is shown in a table. Each of the 733 probes shown were significant in at least 1 rank product test and map to an Ensembl gene identifier. Probes were clustered based on similar expression between the different experimental conditions using hierarchical clustering with a centered average linkage method. Yellow indicates up-regulation, blue indicates down-regulation and black indicates no change in probe expression between cadmium treated larvae and their respective controls. Five clusters of probes have been identified due to unique expression patterns. Probes in the clusters were used in DAVID for Gene Ontology enrichment analysis (Table 3.1).

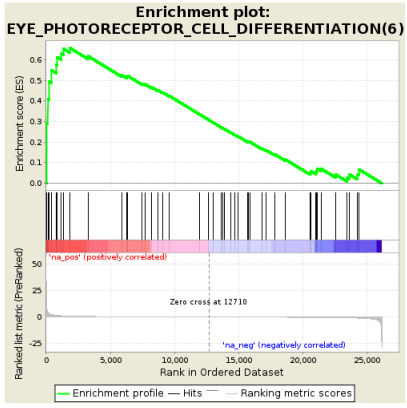
10-Month Males

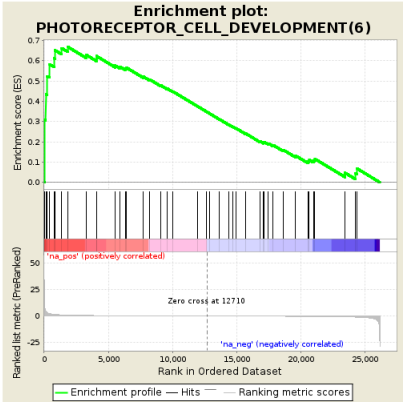




10-Month Females







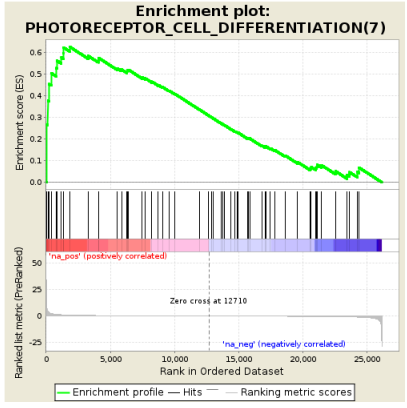
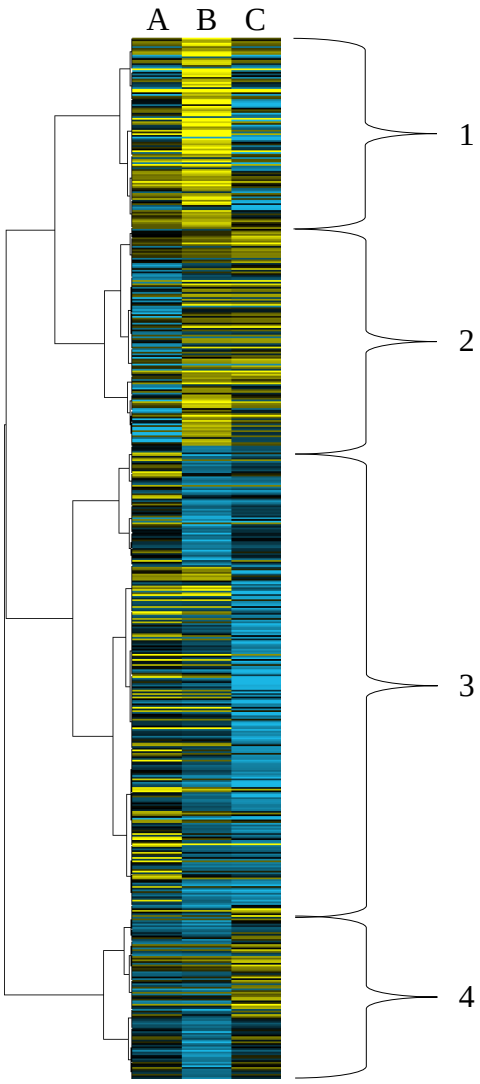


Figure 3.2: **Significant eye-related gene sets in 10-month male and female zebrafish identified using Gene Set Enrichment Analysis (GSEA).**

Eye-related enrichment plots for 10-month male and female zebrafish were generated using GSEA, which requires a ranked list of every gene in an experiment. Using the ranked list along with a curated list of gene sets, GSEA (using the Kolmogorov-Smirnov test) determines which gene sets are enriched based on the position of genes (of a gene set) in the ranked list (indicated by black bars). Enrichment plots for gene sets illustrate enrichment scores for gene sets and shows the number and rank of genes involved in enrichment. For 10-month males, the enrichment plots show down-regulation of both gene sets, as the maximum enrichment score occurs at the lower end of the gene list. For the 10-month females, the enrichment plots show up-regulated gene sets as enrichment is mainly occurring near the top of the gene list.



Up-regulated

Down-regulated

A – 6 Month Male
B – 10 Month Male
C – 10 Month Female

Rank Product

6-month vs. 10-month Male		
	up-regulated	down-regulated
6-month Male	72	102
10-month Male	116	158
10-month Male vs. 10-month Female		
	up-regulated	down-regulated
10-month Male	127	153
10-month Female	121	157

Figure 3.3: **Heat-map of significant zebrafish genes identified using two-way ANOVA and Rank Product.** Cluster analysis of probes is shown as the ratio of normalized read counts for cadmium exposed zebrafish to corresponding controls. The number of significant genes found by Rank Product for each treatment is shown in the table. A total of 610 unique genes were found to be significant in at least 1 rank product test. Genes were clustered based on similar expression between the three different experimental conditions using hierarchical clustering with a centered average linkage method. Yellow indicates up-regulation, blue indicates down-regulation and black indicates no change in gene expression relative to control. Four groups of probes have been identified due to unique expression patterns. These genes clusters were used in DAVID for Gene Ontology enrichment (Table 3.6).

Table 3.1: **Enriched Gene Ontology (GO) terms for the cadmium treated zebrafish larvae microarray experiment.** Clusters of probe IDs obtained from the heatmap (Figure 3.1) were input into DAVID for GO enrichment analysis, using an EASE value of 0.05. The enriched GO term, GO accession, the respective cluster of the input probes, the number of genes involved in enriching the term, the fold enrichment, and the Benjamini value are listed. In addition to all eye-related GO terms, the top 10 terms (based on Benjamini value) for the clusters are listed.

Enriched GO Term	GO Accession	Cluster	Number of Genes	Fold Enrichment	Benjamini Value
DNA-dependent DNA replication	GO:0006261	1	20	45.6	2.40E-24
DNA replication	GO:0006260	1	30	38	7.40E-36
DNA metabolic process	GO:0006259	1	46	16.6	5.50E-41
DNA repair	GO:0006281	1	24	13.7	1.10E-17
cellular response to DNA damage stimulus	GO:0006974	1	27	12	1.10E-18
cell cycle process	GO:0022402	1	29	11.5	1.10E-19
cell cycle	GO:0007049	1	34	8.1	4.00E-19
cellular response to stress	GO:0033554	1	29	7.9	1.00E-15
retina development in camera-type eye	GO:0060041	1	7	5.5	2.00E-02
camera-type eye development	GO:0043010	1	8	4.2	3.00E-02
nucleobase-containing compound metabolic process	GO:0006139	1	61	2.9	1.80E-15
organic cyclic compound metabolic process	GO:1901360	1	63	2.8	9.10E-16
embryonic morphogenesis	GO:0048598	4	19	3.3	1.80E-03
embryo development	GO:0009790	4	29	3.2	6.70E-05
cell differentiation	GO:0030154	4	35	2.2	1.10E-03
cellular developmental process	GO:0048869	4	37	2.1	1.90E-03
multicellular organismal process	GO:0032501	4	65	1.8	1.10E-04
developmental process	GO:0032502	4	60	1.8	3.80E-04
anatomical structure development	GO:0048856	4	59	1.8	4.70E-04
single-multicellular organism process	GO:0032501	4	57	1.8	6.90E-04
single-organism developmental process	GO:0032502	4	58	1.8	7.10E-04
multicellular organism development	GO:0007275	4	54	1.8	8.30E-04

Table 3.2: **Genes involved in enrichment of "retina development in camera-type eye" and "camera-type eye development" GO terms.** Eye-related genes are listed along with cadmium (Cd) to control (Ctrl) expression ratios for the six different treatments. Bolded Cd/Ctrl ratios indicates that the gene was found significantly down-regulated in a rank product test for that treatment. Standard deviation is not listed due to complications caused by averaging duplicate probes. Due to DAVID misidentifying older probes, IDs were first converted into gene names then used in DAVID to identify the eye-related genes.

Gene Name	Cd/Ctrl 72 hpf 4hr	Cd/Ctrl 72 hpf 8hr	Cd/Ctrl 96 hpf 4hr	Cd/Ctrl 96 hpf 8hr	Cd/Ctrl 120 hpf 4hr	Cd/Ctrl 120 hpf 8hr
dnmt1	0.73	0.71	0.69	0.58	**0.59**	**0.47**
ccna2	0.87	0.91	1.03	**0.58**	0.72	**0.51**
fen1	0.83	0.70	**0.60**	**0.44**	**0.52**	**0.32**
mcm3	0.79	0.69	**0.58**	**0.46**	**0.47**	**0.27**
slbp	0.87	0.85	**0.67**	**0.57**	0.64	**0.36**

Table 3.3: **RNA-Seq data analysis pipeline summary statistics.** Data for 6-month male zebrafish (6m M), 10-month male zebrafish (10m M), 10-month female zebrafish (10m F), and human lens epithelial (HLE) cells are shown for both control (Ctrl) and cadmium treated (Cd) conditions, with indication of individual replicates. The main statistics are the initial number of raw paired reads, percentage of reads aligning to the genome using HISAT2, number of read pairs mapped to a single location (e.g. single copy genes), the number of read pairs mapped to annotated genes using htseq-count, and the overall percentage of raw reads that mapped to annotated genes.

Condition	Paired Reads	HISAT2 Alignment	Mapped to Single Location	htseq-count Read Pairs	Overall Annotation Mapping Rate
6m M Ctrl1	24,402,369	91.52%	20,296,613	16,994,925	69.64%
6m M Ctrl2	34,188,281	91.15%	28,199,490	23,499,975	68.74%
6m M Ctrl3	35,128,332	91.36%	28,943,094	24,436,717	69.56%
6m M Cd1	31,942,104	91.11%	26,392,806	22,078,002	69.12%
6m M Cd2	44,390,708	90.98%	36,617,532	30,554,864	68.83%
6m M Cd3	36,643,425	90.58%	30,014,714	24,934,425	68.05%
10m M Ctrl1	35,079,858	90.39%	28,614,580	23,376,678	66.64%
10m M Ctrl2	183,535,190	89.93%	148,548,650	122,124,315	66.54%
10m M Ctrl3	43,497,258	90.07%	35,328,398	28,974,147	66.61%
10m M Cd1	62,339,982	90.35%	50,767,559	41,337,209	66.31%
10m M Cd2	31,489,275	90.10%	25,516,262	20,843,059	66.19%
10m M Cd3	31,412,826	90.62%	25,668,130	20,930,252	66.63%
10m F Ctrl1	32,159,602	89.25%	26,069,861	21,523,555	66.93%
10m F Ctrl2	31,118,640	90.25%	25,381,169	20,824,409	66.92%
10m F Ctrl3	30,066,502	89.93%	24,344,560	19,727,409	65.61%
10m F Cd1	32,108,570	89.97%	26,030,359	21,113,699	65.76%
10m F Cd2	28,723,568	90.43%	23,465,166	19,176,799	66.76%
10m F Cd3	40,638,341	89.98%	32,926,650	26,818,086	65.99%
HLE Ctrl1	31,194,384	95.93%	28,050,206	25,185,221	80.74%
HLE Ctrl2	30,598,947	96.47%	27,644,224	24,838,433	81.17%
HLE Ctrl3	19,209,331	95.41%	17,182,007	15,485,249	80.61%
HLE Cd1	32,071,970	96.19%	28,552,087	25,431,689	79.30%
HLE Cd2	30,768,684	96.78%	27,586,589	24,575,217	79.87%
HLE Cd3	41,186,629	96.55%	36,817,376	32,729,764	79.47%

Table 3.4: **Core genes involved in enrichment of "embryonic camera-type eye formation" and "embryonic eye morphogenesis" gene sets in 10-month male zebrafish.** Fold change of normalized read counts between cadmium treated (Cd) and control (Ctrl) treatments and the Benjamini value provided by DESeq2 is listed for each gene. Standard deviation for fold change was calculated using a variance formula outlined in van Hees 2002 [84]. Bolded genes were involved in core enrichment for both gene sets, while all other genes were only involved in core enrichment for "embryonic eye morphogenesis".

Gene Name	Fold Change (Cd/Ctrl)	DESeq2 Benjamini Value
tcf7l1a	0.67 ± 0.40	8.52E-06
pitx2	0.69 ± 0.40	1.52E-05
fzd8a	0.70 ± 0.40	1.89E-05
sfrp5	0.63 ± 0.37	5.28E-05
lmx1bb	0.63 ± 0.37	1.19E-04
mfap2	0.63 ± 0.37	3.13E-03
six3b	0.85 ± 0.49	4.13E-03
lum	0.82 ± 0.46	6.09E-03
phactr4b	0.84 ± 0.50	7.45E-03
foxc1a	0.77 ± 0.44	9.05E-03
znf703	0.86 ± 0.50	1.49E-01
cct3	0.89 ± 0.52	2.10E-01
lmx1ba	0.78 ± 0.42	2.14E-01

Table 3.5: **Core genes involved in up-regulated enrichment of photoreceptor gene sets in 10-month female zebrafish.** For each gene, the fold change of normalized read counts between cadmium treated (Cd) and control (Ctrl) treatments is listed along with the Benjamini value provided by DESeq2. Standard deviation for fold change was calculated using a variance formula outlined in van Hees 2002 [84]. The final four columns indicate if a gene was a core gene involved in enrichment of the specified gene set, with an x signifying presence of the gene.

			Enriched Gene Sets			
Gene Name	Fold Change (Cd/Ctrl)	DESeq2 Benjamini Value	Eye Photoreceptor Cell Development	Photoreceptor Cell Development	Eye Photoreceptor Cell Differentiation	Photoreceptor Cell Differentiation
odc1	2.43 ± 0.99	1.33E-09	x	x	x	x
thrb	1.41 ± 0.62	1.25E-03	x	x	x	x
ush2a	1.34 ± 0.68	1.47E-02	x	x	x	x
adgrv1	1.24 ± 0.60	8.24E-02	x	x	x	x
syjn1	1.15 ± 0.61	2.78E-01	x	x	x	x
crx	1.15 ± 0.57	3.11E-01	x	x	x	x
cdipt	1.13 ± 0.60	4.68E-01			x	x
pitpnb	1.13 ± 0.59	5.16E-01		x		x
rx1	1.15 ± 0.53	5.19E-01			x	x
bbs5	1.15 ± 0.60	6.52E-01		x	x	x

Table 3.6: **Enriched Gene Ontology (GO) terms for the cadmium treated adult zebrafish using two-way ANOVA and Rank Product.** Clusters of probe IDs obtained from the heatmap (Figure 3.3) were input into DAVID for GO enrichment analysis, using an EASE value of 0.05. The enriched GO term, GO accession, the respective cluster of the input probes, the number of genes involved in enriching the term, the fold enrichment, and the Benjamini value are listed.

Enriched GO Term	GO Accession	Cluster	Number of Genes	Fold Enrichment	Benjamini Value
sequestering of iron ion	GO:0097577	1	4	90.4	7.70E 03
intracellular sequestering of iron ion	GO:0006880	1	4	90.4	7.70E-03
negative regulation of response to wounding	GO:1903035	1	3	62.1	4.40E-02
cellular iron ion homeostasis	GO:0006879	1	4	43.2	2.00E-02
iron ion transport	GO:0006826	1	4	43.2	2.00E-02
cellular transition metal ion homeostasis	GO:0046916	1	4	31.1	2.70E-02
maintenance of location in cell	GO:0051651	1	4	28.4	2.80E-02
sequestering of metal ion	GO:0051238	1	4	26.2	3.30E-02
iron ion homeostasis	GO:0055072	1	4	24.9	3.20E-02
cholesterol metabolic process	GO:0008203	1	4	24.9	3.20E-02
secondary alcohol metabolic process	GO:1902652	1	4	24.2	3.20E-02
transition metal ion transport	GO:0000041	1	5	20.4	2.60E-02
steroid biosynthetic process	GO:0006694	1	4	20.3	4.50E-02
sterol metabolic process	GO:0016125	1	4	19.5	4.50E-02
transition metal ion homeostasis	GO:0055076	1	4	18.8	4.80E-02
leukocyte migration	GO:0050900	1	5	15.7	3.10E-02
lipid transport	GO:0006869	1	6	10.4	3.50E-02
lipid localization	GO:0010876	1	6	9.7	3.10E-02
response to wounding	GO:0009611	1	8	9.2	9.20E-03
response to oxygen-containing compound	GO:1901700	1	7	6.9	3.10E-02
response to external stimulus	GO:0009605	1	12	3.8	3.70E-02
cellular response to chemical stimulus	GO:0070887	1	11	3.6	3.80E-02
response to stress	GO:0006950	1	14	2.8	4.60E-02

Table 3.7: **Enriched Gene Ontology (GO) terms for up-regulated genes in Cd treated human lens epithelial (HLE) cells.** Up-regulated genes with a > 2-fold change between normalized Cd/Control read counts were input into DAVID for GO enrichment analysis, using an EASE value of 0.05. The enriched GO term, GO accession, the respective cluster of the input probes, the number of genes involved in enriching the term, the fold enrichment, and the Benjamini value are listed. In addition to all eye-related GO terms, the top 10 terms (based on Benjamini value) for the clusters are listed.

Enriched GO Term	GO Accession	Number of Genes	Fold Enrichment	Benjamini Value
lens development in camera-type eye	GO:0002088	8	3.7	3.40E-02
regulation of apoptotic process	GO:0042981	139	3.1	3.60E-31
regulation of programmed cell death	GO:0043067	140	3.1	2.80E-31
regulation of cell death	GO:0010941	144	2.9	1.00E-30
response to organic substance	GO:0010033	209	2.3	3.10E-31
camera-type eye development	GO:0043010	21	2.2	9.10E-03
regulation of response to stimulus	GO:0048583	247	2.1	1.70E-32
eye development	GO:0001654	22	2.0	2.10E-02
negative regulation of biological process	GO:0048519	303	2.0	1.30E-40
negative regulation of cellular process	GO:0048523	285	2.0	4.90E-38
regulation of cellular metabolic process	GO:0031323	329	1.7	7.50E-32
regulation of metabolic process	GO:0019222	340	1.7	8.40E-32
regulation of primary metabolic process	GO:0080090	325	1.7	4.30E-31

Table 3.8: **Genes involved in enrichment of eye-related Gene Ontology (GO) terms.** For each gene, the fold change of normalized read counts between cadmium treated (Cd) and control (Ctrl) treatments is listed along with the Benjamini value provided by DESeq2. Standard deviation for fold change is was calculated using a variance formula outlined in van Hees 2002 [84]. The final four columns indicate if a gene was a core gene involved in enrichment of the specified gene set, with an x signifying presence of the gene. *TGFB2* was not present in the lens development GO term, but was manually curated as it has a role in the process.

			Enriched GO Terms		
Gene Name	Fold Change (Cd/Ctrl)	DESeq2 Benjamini Value	Camera-type Eye Development	Eye Development	Lens Development in Camera-type Eye
CITED2	3.70 ± 2.15	0.00E+00	x	x	x
JUN	6.71 ± 4.05	0.00E+00	x	x	
SKIL	3.04 ± 1.78	0.00E+00	x	x	x
JMJD6	3.47 ± 2.03	0.00E+00	x	x	
CRYAB	4.92 ± 2.86	0.00E+00	x	x	x
INHBA	10.29 ± 6.18	0.00E+00	x	x	
SLC7A11	5.50 ± 3.20	0.00E+00	x	x	x
TGFB2	3.44 ± 2.00	1.56E-270	x	x	x
EPHA2	2.65 ± 1.57	1.03E-222	x	x	x
VEGFA	3.19 ± 1.90	1.51E-207	x	x	
RARA	2.69 ± 1.59	1.71E-110	x	x	
SOX9	2.21 ± 1.27	5.30E-104	x	x	
BCAR3	2.31 ± 1.32	2.97E-73	x	x	x
FOXC2	3.50 ± 1.96	1.86E-61	x	x	
ZEB1	2.10 ± 1.24	1.81E-58	x	x	
KLF4	7.36 ± 4.34	1.29E-54	x	x	
WNT5B	2.29 ± 1.33	3.02E-33	x	x	x
RDH10	2.07 ± 1.24	1.15E-32	x	x	
BMP4	2.05 ± 1.16	1.80E-16	x	x	x
TBX2	2.25 ± 1.47	4.80E-14	x	x	
PRDM1	3.03 ± 1.89	8.83E-12		x	
FOXC1	2.13 ± 1.21	1.55E-09	x	x	

Chapter 4

Discussion

Research on cadmium toxicity in mammals and aquatics species is important due to the increasing environmental concentrations and the negative effects associated with cadmium. Cadmium can cause damage to multiple organs such as the liver, kidney and the eyes. Cadmium has been associated with ocular diseases such as cataracts [32] and has shown to accumulate in the retina, possibly contributing to age-related macular degeneration [85]. Mechanisms by which cadmium contributes to these diseases remains largely unknown. Perhaps altered transcription of genes involved in the ocular system are responsible for the onset of disease. In this study, we have used multiple models (zebrafish larvae, adult zebrafish, and HLE cells) to assess the impact of cadmium on gene transcription, and aim to increase the knowledge between cadmium, gene transcription and ocular disease.

4.1 Cadmium exposure down-regulates retina-related genes in zebrafish larvae

The microarray experiment identified five down-regulated eye-related genes which were involved in enriching the "retina development in camera-type eye" GO term. The first gene of interest was *dnmt1*, which was found to be down-regulated in both 120 hpf treatments. *dnmt1* encodes an enzyme responsible for cytosine methylation and may play a role in gene silencing [86, 87]. In zebrafish, patterns of DNA methylation are thought to be set during early embryonic development. During the mid-blastula stage of development, the zebrafish embryo methylome was found to be identical to the sperm, suggesting paternal inheritance of the methylome [88]. Therefore, any alterations in paternal *dnmt1* expression and methylome will be passed on to the offspring. Interestingly, *dnmt1* has been shown to play a role in retina development. Knockdown of *dnmt1* in zebrafish embryos caused defects in retinal lamination, retinal pigmented epithelium differentiation, and photoreceptor differentiation and morphology [89, 90]. In hypomethylated *dnmt1*-mutant mouse retinas, differentiation of progenitor cells into photoreceptors was inhibited suggesting that methylation is an important factor for this process [91]. Studies have shown that cadmium has an inhibitory effect on DNA methyltranserases; cadmium exposure was found to inhibit hepatic DNA methyltransferases activity in rats [92], which agrees with the finding that Argentinian women exposed to environmental cadmium had hypomethylated DNA [93]. Perhaps the mechanism by which cadmium contributes to ocular disease is by inhibiting DNA methyltransferases such as *dnmt1*, resulting in hypomethylated DNA which in turn alters gene expression.

The other four differentially expressed retinal-related genes were *ccna2*, *fen1*, *mcm3*, and *slbp*. *ccna2* encodes a protein which is expressed during cell cycle progression in dividing cells, *fen1* encodes an endonuclease involved in DNA repair which excises 5 flap overhangs [94], the *mcm3* gene product is involved in genome replication, and *slbp* is involved in synthesis and degradation of histone proteins during DNA replication. These genes were down-regulated in different conditions within the microarray experiment, with *ccna2* down-regulated at the 96 hpf 8h and 120 hpf 8h treatments, both *fen1* and *mcm3* down-regulated at all 96 hpf and 120 hpf treatments, and *slbp* down-regulated at both 96 hpf 4h and 8h exposures and the 120 hpf 8h treatment. Research pertaining to the involvement of *ccna2*, *fen1*, *mcm3*, and *slbp* in the retina is scarce. One study reported that independent mutations in *ccna2*, *fen1*, and *mcm3* in zebrafish embryos resulted in smaller eyes and central retinal degeneration, possibly due to repression of retinal progenitor proliferation [95]. Interestingly, cyclin dependent kinase (Cdk) Cdk1-cyclin A forms a complex with *fen1 in vitro* and is capable of phosphorylating *fen1*, resulting in a reduction of endonuclease activity [96]. Another study found *mcm3* to be strongly expressed in the *Xenopus* retinal stem cell compartment [97], suggesting *mcm3* is a marker for retinal cell proliferation. It was also discovered that *slbp* is required for retinal stem cell maintenance and retinal progenitor proliferation in zebrafish [98]. All four of these genes are primary involved in DNA replication, yet they clearly possess an additional role in the development of retinal stem cells. Altered transcription of these genes could have negative consequences for retinal development and our results are the first illustrating such a transcriptional response to cadmium. These results suggest cadmium could induce retinal disease, yet further research is required for confirmation.

Cluster analysis, shown in Figure 3.1, showed little to no change for the majority of probes for both 72 hpf treatments. At first glance, it was peculiar that the 72 hpf group did not to respond to cadmium as much as the other time points; compared to adults, younger animals are more susceptible to many (but not all) toxicants [99, 100]. Developing embryos are constantly undergoing changes in gene expression as well as molecular and physiological composition, which makes identifying susceptible cells difficult [101]. Examples in the literature show a protective effect of embryos compared to hatched larvae. Zebrafish embryos, approximately 1-3 days post fertilization, were found to be more resistant to cadmium than larvae at 7-8 days [102]. An explanation for this toxicant resistance involves differences in ion uptake between the developmental stages. Following hatching, larvae rapidly uptake calcium which is required for further growth and development [103]. However, due to calcium and cadmium having similar size and binding targets, cadmium-exposed larvae rapidly uptake cadmium, leading to increased cadmium and decreased calcium concentrations [104, 103]. The protective effect of embryos is attributed to the hardening of the chorion (outer layer of embryo) which decreases permeability; embryos exposed to wastewater effluent before ~1 hpf were more susceptible, showing chorion hardening occurs around this time [105]. Based on the literature, it is plausible that the 72 hpf zebrafish used in our experiment had a lower ion uptake rate compared to the 96 and 120 hpf treatments, resulting in a decreased response to cadmium.

4.2 Cadmium exposure down-regulates eye development-related genes in adult male zebrafish

RNA sequencing, GO enrichment analysis and GSEA of the cadmium-exposed 6-month male zebrafish treatments resulted in expected enrichment of terms involving metal regulation, yet no eye-related terms were enriched. As the results of the 6-month males did not show eye-related enrichment, it was unexpected that GSEA of the 10-month males resulted in down-regulated enrichment of the gene sets "embryonic eye formation" and "embryonic eye morphogenesis". The majority of the core genes involved in enrichment of these gene sets (Table 3.4) encode transcription factors. Ten of the 13 genes listed had an FDR (Benjamini value) of less than 0.05 and thereby warrant further discussion. Many of these genes have been associated with eye disorders in the literature. Mutations in the developmental transcription factor genes *pitx2* and *foxc1* have been associated with Axenfeld-Rieger syndrome (ARS), which is characterized by abnormalities in the iris and the anterior segment of the eye [106]. In cells affected with the disorder, the *pitx2* allele causing the disease was found to be transcriptionally inactive [107], while another study identified a missense allele (V45L) which reduced DNA binding while increasing transactivation [108]. Tumer and Bach-Holm outline many other mutations in *pitx2* which were associated with ARS [109]. Homozygous null *pitx2* mice developed optic nerve coloboma, and also failed to develop functional ocular muscles, while mice heterozygotes for the null allele displayed an ARS phenotype [110]. These studies indicate that precise expression of *pitx2* is required, as increased or decreased activity during development can have negative outcomes [106]. Similarly, *foxc1* is also associated with ARS. Homozygous

null *foxc1* mice were lethal, however they have been shown to develop multiple ocular abnormalities such as open eyelids, unformed anterior chamber, and attachment of the lens to the cornea [111, 112]. Missense mutations in *foxc1* in humans have shown to alter protein stability, DNA binding, and transactivation of target genes [113, 114]. Our results have shown cadmium exposure in 10-month male zebrafish down-regulates the primary genes associated with ARS. Cadmium-induced down-regulation of *pitx2* and *foxc1* results in a lower amount of functional protein, which could be a similar outcome in genetically inherited ARS. It would be interesting to see if cadmium exposure can induce an ARS disease phenotype during development.

Other genes listed in Table 3.4 have a role in eye development as described in the literature. For example, *tfc71la* is important for the formation of the zebrafish eye as double negative mutants failed to develop the organ [115]. The role of *sfrp5* in eye development is slightly unclear. *sfrp5* (along with *sfrp1a*) is expressed in the developing retina and was shown to facilitate Wnt and BMP signalling, both important for retinal patterning [116], however previous studies have shown that the frizzled and frizzled-related proteins inhibit Wnt and BMP signalling [117, 118]. *six3b*, a transcription factor involved in the specification of the forebrain is also vital for eye development. Overexpression of *six3* in mice resulted in the formation of optic vesicle-like structures [119], while inactivation of *six3* in medaka prevented eye formation [120]. *six3* functions as a transcriptional repressor of *bmp4*, maintaining low levels which allow for specification and cell proliferation in the anterior neuroectoderm [121]. Interestingly, cadmium exposure was found to affect retina development in developing zebrafish, as embryos exposed to cadmium had smaller eyes, a lower

number of retinal progenitor cells and retinal ganglion cells, and an absence of photoreceptors [122]. Cadmium exposure may cause a chain reaction of events that could result in eye defects; cadmium exposure reduces expression of *six3*, which results in an increased expression of *bmp4* thereby preventing cell specification and proliferation. The keratin proteoglycan *lum* has been shown to be important for maintaining corneal transparency, as the cornea is composed of a unique layer of collagen which normally remain optically transparent but swells when exposed to fluid [123]. Mice with a homozygous null mutation in *lum* developed corneal opacification and had abnormal collagen fibril matrices [124]. These results, along with other studies, have shown the importance of *lum* for collagen fibril assembly in the cornea [125, 126]. An avenue for further research would be to examine how cadmium impacts collagen in the cornea, as cadmium-exposed rats showed defects in bone collagen crosslinking and maturation [127]. Overall, our results showed cadmium exposure of 10-month male zebrafish resulted in the down-regulation of genes associated with eye disease, development and maintenance.

4.3 Cadmium exposure up-regulates photoreceptor-related genes in adult female zebrafish

The results of the 10-month female GSEA were also unexpected as they were remarkably different when compared to the 10-month males; up-regulated enrichment of gene sets such as "photoreceptor cell development" and "photoreceptor cell differentiation" was observed. There were 10 relevant genes (listed in Table 3.5) from the enriched genes sets, however only 3 had an FDR < 0.05. The gene *odc1* had the highest fold

change between treatments and has been shown to have an important role in the synthesis of polyamines [128]. Polyamines and *odc1* are vital for photoreceptor outer segment development, as zebrafish *odc1* mutants had photoreceptors with an improperly formed outer segment [95]. Similarly, depletion of polyamines in the rabbit retina (using an inhibitor of ornithine decarboxylase) resulted in disruption of the formation of the outer nuclear layer of cone photoreceptors [129]. The thyroid hormone receptor *thrb* also has an unexpected role in the development of photoreceptors. In human and rat retinal cultures, exposure to *thrb* ligand facilitated the differentiation of retinal progenitor cells into rods or cones depending on the concentration [130, 131]. More recent research has shown that *thrb* is important for expression of opsins in cone photoreceptors; knockout of *thrb* in mice resulted in a loss of M-opsin, with cones only containing S-opsin in an irregular distribution [132]. The gene *ush2a*, also known as usherin, encodes a matrix protein that has been implicated in Usher syndrome which is characterized by vision and hearing loss. Usher syndrome is a primarily genetic condition in which mutations in *ush2a* result in retinitis pigmentosa [133]. Studies have shown that *ush2a* is important for maintenance of photoreceptors; in mice, disruption of *ush2a* resulted in photoreceptor degradation and hearing impairment [134]. The different components required for photoreceptor differentiation and maintenance vary wildly and include enzymes, receptors and matrix proteins. Our results show that cadmium alters transcription of these genes and could potentially play a role in retinal diseases.

The differing results of GSEA between the 10-month male and 10-month female zebrafish was unexpected. The 10-month males showed down-regulation of eye

development gene sets while the 10-month females only had up-regulation of retinal/photoreceptor specific gene sets. For zebrafish, eye structures in the anterior segment are present by 72 hpf, when vision starts to develop [135] and will continue to develop past sexual maturity at ~ 3 months [16]. Interestingly, differences between male and female juveniles are undetectable because all juvenile possess undifferentiated ovary-like gonads. In presumptive males, apoptosis of the oocytes and development of spermatocytes is completed by 30 days post fertilization while in females the oocytes mature into ovaries [136]. Due to this unique form of sexual differentiation, males and females undergo the same process of initial eye development during early life. Perhaps differences in eye maintenance and visual behaviour, rather than development, can help explain the contrasting results in 10-month zebrafish. For example, male and female zebrafish have shown differences in visual behaviour, as male zebrafish use their left eye more during aggressive acts than compared to females [137]. Currently, the cause of the difference in eye-related gene expression between the 10-month male and female zebrafish is unclear, and further research could expand upon this observation.

4.4 Cadmium exposure up-regulates lens development genes in HLE cells

RNA sequencing and GO enrichment analysis of up-regulated genes in cadmium exposed human lens epithelial cells identified 22 genes which were involved in enriching the "eye development" GO term, 9 of which also enriched the "lens development in camera-type eye" and will be the focus of this discussion. Prior to the start of the

experiment, we hypothesized that the structural crystallin proteins would be significantly altered due to cadmium exposure. Yet, only the crystallin gene *CRYAB* was present in the gene list. The expression of *CRYAB* in the lens is carefully controlled by *PAX6* and retinoic acid nuclear receptors [138], and mutations in *CRYAB* have been shown to induce cataracts [38]. *CRYAA* is another member of the crystallin family, specific to the lens, that is vital for maintaining lens transparency. Unfortunately, the HLE B3 cell line does not expresses *CRYAA* past 11 propagations therefore we are unable to determine the effect cadmium has on this gene [139].

In addition to the crystallins, various other transcription factors, receptors, and signalling proteins are involved in lens development and maintenance. *CITED2* is specifically required for lens morphogenesis, as expression of *CITED2* was found in the surface ectoderm, lens pit and lens epithelial cells, yet was absent in differentiated lens fiber cells [140]. *SKIL* null mice had several ocular disorders including microphthalmia, lens dysgenesis, and various other anterior segment problems [141]. The *EPHA2* receptor has a potential role in lens maintenance, as deletion of the gene lead to development of cortical age-related cataract in mice [142]. Expression of the growth factor *TGFB2* has been associated with cataracts in rats; *TGFB2* and *TGFB3* were found to be 10 times more potent than *TGFB1* in inducing cataracts in rat lenses [143]. *BMP4* is vital for initiation of lens induction in mice [144], while *WNT5B* is expressed in the lens epithelium and is involved in fiber cell differentiation via stimulation of FGF growth factors [145]. Finally, a mutation in mouse *BCAR3* has shown to be associated with cataracts and abnormal lens physiology [146]. Perhaps the relationship between cadmium and cataracts involves cadmium-induced up-regulation

of these genes causing disruption of lens development and maintenance thereby contributing to cataract formation.

Cadmium exposure up-regulates multiple lens-related genes in HLE cells by at least 2-fold, yet the mechanism and interactions of genes remains poorly understood. An example of uncertainty is *FOXC1*, which was found to be up-regulated in cadmium exposed HLE cells, but down-regulated in cadmium exposed 10-month male zebrafish. Perhaps the differences between human and zebrafish models can explain the contrasting results, as only one cell type (epithelial cells) were used for humans. Another important point to note is that all the genes previously mentioned eye-related genes were identified using lists of genes with either GO enrichment analysis or GSEA. Because these methods look for enrichment of terms above a background level, it is possible that other eye-related genes which are differentially expressed in the data are omitted. In Figure 3.1 clusters 2,3, and 5 did not enrich any GO terms, however individuals genes in those clusters could be associated with an eye-related GO terms. For example, one study showed that cadmium up-regulated both retinoblastoma binding protein 6 and crystallin lamda 1 in zebrafish embryos [147]; looking at the individuals probes in Figure 3.1 our results show different retinal and lens-related gene expression (SRY-box containing gene 2 being down-regulated and crystallin gamma S4 being up-regulated) in at least one condition. We may also be overlooking other interesting biological processes that could be related to eye disease, such as apoptosis. Therefore, manual curation of all the genes in our experiments is required in order to truly identify the eye-related genes transcriptionally altered by cadmium. Nevertheless, more research concerning cadmium, cataracts, the lens and retina must be conducted to elucidate mechanisms and interactions which contribute to disease onset.

Chapter 5

Conclusions and Future Directions

5.1 Conclusions

Cadmium is a toxic heavy metal that negatively affects many organs systems. In particular, many structures in the ocular system accumulate cadmium, which seems to be associated with ocular diseases. We hypothesized that cadmium alters transcription of eye-related genes, potentially contributing to ocular disease formation. In developing zebrafish larvae, cadmium exposure resulted in down-regulation of genes involved in retina development. In adult 10-month male zebrafish, genes involved in eye morphogenesis were down-regulated while in adult 10-month female zebrafish genes involved in retina/photoreceptor development were up-regulated. In human lens epithelial cells, genes involved in eye development and specifically lens development were up-regulated. These experiments have shown that cadmium exposure does have an impact on eye-related gene transcription even though slightly different outcomes were observed depending on the model used. Differences between the zebrafish and HLE cells could be attributed to the differences in using *in vivo* vs.

in vitro testing methods for cadmium exposure. Male and female zebrafish also respond differently to cadmium exposure, identifying potential sex differences in eye maintenance. Regardless of the differences, each model had altered transcription of multiple genes which have been associated with an ocular disease. Further research on the interaction between cadmium and these genes could be beneficial for understanding cadmium-related ocular diseases. Overall, this study provides insight on the transcriptional response of eye-related genes to cadmium yet the underlying mechanisms and contributions of these genes to ocular disease remain poorly understood and require further research.

5.2 Future directions

Our results show that cadmium exposure influences transcription of many eye-related genes. Many of these genes are implicated in ocular diseases such as cataract and retinal degeneration, however most studies in the literature use knockouts or mutations to inactivate the gene. Instead of using knockouts, it would be interesting to observe the impact of prolonged overexpression or underexpression of these genes in relation to ocular diseases. Also, more knowledge is required on the mechanism by which cadmium contributes to ocular diseases. Cadmium is known to accumulate in many eye structures naturally during aging and has been associated with diseases. Future experiments could focus on identifying a cadmium-induced regulator which may contribute to the observed differential gene expression. For example, cadmium-induced MTF-1 could potentially bind to these gene targets, thereby altering expression. Another aspect to explore is the relationship between cadmium, eye-related genes, sex and organism, as we observed differences in cadmium-induce gene expression in HLE

cells, male zebrafish, and female zebrafish. It would be interesting to identify if any of these factors can influence cadmium-related ocular disease. For humans, using primary HLE cells or other cells types rather than the B3 cell line could provide mechanistic insight; primary cells can differentiate into lens fibre-like structures under the right conditions [148], thereby providing knowledge on cadmiums effect on differentiating lens fibres. Futures studies could also address the potential sex differences by replicating the cadmium exposure experiment in another model organism (eg. mice) and sequencing whole eyes. By utilizing other model organisms, we can determine if the observed sex specific differences are unique to zebrafish or occur in other species. Ultimately, the relationship and mechanisms between cadmium and ocular disease is still poorly understood, and further research is required to elucidate the impact of cadmium on the eye.

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