Iridophore Expression in Mutant oca2 Zebrafish (Danio Rerio)

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Abstract:

The gene oculocutaneous albinism 2 (oca2) is crucial for the proper development of color pattern in fish, and eye, hair and skin color in humans. When this gene is mutated (not functioning) in humans, albinism occurs, which makes those affected by this disease more susceptible to skin cancers caused by ultraviolet beta induced damage to DNA. Within zebrafish (Danio rerio) there are three cells which produce pigment: brown/black melanophores, iridescent iridophores, and yellow xanthophores. When oca2 is mutated the zebrafish exhibit a decrease in the number of melanophores but an increased number of iridophores. This result suggests that the oca2 gene is involved in regulating iridophore production. The goal of this experiment was to determine the mechanism by which oca2 influences the production of iridophores. It was determined that oca2 is not involved in regulating the number of iridophores at initiation (cell fate choice). This result may suggest that this gene is rather involved in regulating a second means for controlling cell number by cell division. To address this question, cell division assays will be completed over the summer to determine how iridophore cell proliferation is impacted when oca2 is mutated. Further understanding the mechanisms by which this gene regulates pigmentation within zebrafish will grant a better understanding of the importance and function of this gene within humans.
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Introduction:

Significance of OCA2 in Humans

Oculocutaneous Albinism (OCA) is a developmental disease which results in a lack of pigment in the skin, eyes, and hair in humans. World-wide about 1 in every 17,000 people will have OCA (Gronskov et al. 2007). The most prevalent form of this disease is caused by a mutation in the gene OCA2. Within the United States every 1 in 36,000 people are affected by this disease (Ray et al. 2015). Due to the severe hypopigmentation or total lack of pigmentation this mutation causes, those affected are highly susceptible to skin cancers caused by ultraviolet (UV) radiation (Ray et al. 2015). This is because the black pigment or melanin produced by melanocytes, the pigment cell in humans, is essential for protection against ultraviolet beta induced damage to DNA (Brenner & Hearing 2008). Impacts to eye function are experienced as well, such as a lack of depth perception and decrease in visual acuity due to the abnormalities in the connections of optic nerve fibres during development (Biswa & Lloyd 1995).

Oculocutaneous albinism 2 is a recessive trait, meaning that both parents must carry the defective version of the OCA2 gene for the albinism phenotype to manifest in their offspring. This means that people who are heterozygous for the OCA2 gene, carrying one mutant copy and one functioning copy of OCA2, will not express the developmental defects (Lund & Gaigher 2002). This is because only one functioning copy of the OCA2 gene is required for proper melanin synthesis to occur. However, the disease can still be passed on to their children. Approximately one fourth of the children from heterozygotes are expected to inherit the mutated allele from both parents and therefore have the disease (Lund & Gaigher 2002).
Molecular Function of \textit{OCA2}

Melanocytes synthesize melanin in specialized organelles referred to as melanosomes. These melanosomes undergo four stages of development, starting as nonpigmented vacuoles and then maturing into active melanin synthesizing organelles (Wasmeier et al. 2008). The \textit{OCA2} gene produces a protein referred to as the P protein, or as OCA2, which is crucial for proper melanosome function (Brilliant 2001). This protein is characterized as a 12 domain transmembrane protein with amino acid sequences homologous to ion permeases (Sitaram et al. 2009). An ion permease is a protein which acts as a membrane transport protein in the membrane of the cell itself or in the membranes of organelles (Madej 2014). They regulate the transport of ions, such as chloride, in and out of a cell or organelle (Gadsby 2009). This is important because many reactions that take place within a cell are dependent upon ions, and the cell can respond differently to different levels of a given ion (Gadsby 2009).

Currently it is unknown the exact mechanism by which this protein regulates melanin synthesis. However, the P protein has been implicated to be essential for the trafficking of tyrosinase to melanosomes as depicted in Figure 1 (Costin et al. 2003, Kondo et al. 2014). Tyrosinase is an enzyme which must be present in melanosomes for melanin synthesis to occur (Ni-Komatsu & Orlow 2006). Within cells with defective \textit{OCA2}, tyrosinase is still functionally active but is not delivered to early melanosomes and is instead excreted from cells (Costin et al. 2003). This displays that the P protein is involved in the trafficking of tyrosinase from the Golgi apparatus to early melanosomes (Kondo et al. 2014). The P protein localizes with the protein complex BLOC-1 which may indicate that the P protein traffics tyrosinase by associating with BLOC-1 within melanocytes to direct proteins to melanosomes (Kondo et al. 2014). Protein complexes such as BLOC-1 ensure that proteins needed by melanosomes are separated from
ones destined for endosomes (Wasmeier et al. 2008). In non-melanocyte cells the P protein is destined for lysosomes (Sitaram 2009).

The P protein is also proposed to serve another vital function as depicted in Figure 2: regulating the pH of melanosomes (Brilliant 2001). The regulation of pH allows for melanin synthesis to be regulated. This is because tyrosinase is inactive at a pH < 5.5, therefore at pH levels under 5.5 melanin synthesis will not occur (Ancans et al. 2001). The P protein regulates pH by associating with an anion transporter, aiding in the neutralization of pH within melanosomes to promote melanin synthesis (Bellono et al. 2014). It is believed to work in junction with a proton transporter, such as H+/ATPase (Brilliant 2001, Bellono et al. 2014). The ratio of anions to protons would determine the pH, and therefore regulate when tyrosinase can function. In junction with this, the majority of P protein produced localizes to melanosomes, which also supports the idea that it serves as a transmembrane protein in the melanosomes themselves (Sitaram et al. 2009).
Figure 1. Simplified model for tyrosinase trafficking. A) The OCA2 gene is mutated, resulting in no P protein being produced. This results in the tyrosinase being exported to the outside of the melanocyte into the extracellular matrix. B) The OCA2 gene is successfully translated to P protein. The P protein aids in the transport of the tyrosinase to the melanosome allowing for melanin synthesis to occur, which causes the melanosome to be darker in appearance.
Figure 2. Simplified model for melanosome pH regulation. A) The P protein (depicted by the yellow circle) associates with an anion transporter enabling anions to cross the melanosome membrane. H+/ATPase is proposed to function as the proton transport protein. The flow of anions and protons allows for the pH of the melanosome to permit tyrosinase function and thus melanin synthesis can occur. B) Mutant OCA2 results in no P protein associating with the anion transporter, disabling its function. Protons accumulate within the melanosome with no anions to neutralize them, resulting in the inactivation of tyrosinase which halts melanin synthesis.
oca2 in Zebrafish

In zebrafish the loss of dark pigmentation is experienced when their version of the OCA2 gene, oca2, is mutated (Figure 3). This mutation is characterized by the presence of a deletion at amino acid sequence 263 along with a single base missense mutation at amino acid 273 leading to the wrong amino acid being produced (Beirl et al. 2014). This means that one of the parts of the DNA in the gene was encoded wrong, leading to the wrong peptide sequence being synthesized resulting in a non-functional p protein. This mutation therefore results in the zebrafish producing a significantly reduced amount of melanophores (zebrafish melanocytes) than in their wild-type (non-mutant) counterparts (Beirl et al. 2014). Similarly to mammalian melanocytes, zebrafish melanophores experience impacts to melanin synthesis and melanosome maturation when this protein is defective. In particular both in mammals and zebrafish the dark pigment cells are observed to secrete tyrosinase when oca2 is mutated indicating that the p protein is required for proper trafficking of tyrosinase to melanosomes (Beirl et al. 2014, Kondo et al. 2014). Furthermore, oca2 also appears to function by regulating the pH of melanosomes because reducing the flow of protons (and therefore decreasing the acidity) into melanosomes in mutant oca2 cells resulted in restoration of melanin synthesis (Beirl et al. 2014). Again, this is similar to what has been found to occur in melanocytes when OCA2 is mutated (Ancans et al. 2001, Chen et al. 2004). This further supports how the synthesis of dark pigment cells is known to be highly conserved between zebrafish and humans (Beirl et al. 2014). Due to how the function of the oca2 gene is largely conserved, zebrafish are a good model to examine the functions of oca2, and possibly for finding means to treat this disease in humans.

Understanding more about this mutation will also shed further light onto how color patterns are formed within zebrafish. Color patterns are generated through chromatophores, cells
which contain pigment. Currently it is known that there are three chromatophores in zebrafish: melanophores, xanthophores and iridophores. Melanophores function as the dark pigment cells, forming the black stripes on zebrafish, (Irion et al. 2013). The xanthophores and iridophores fill in the space between the black stripes. The iridophores are characterized as shiny cells which are highly reflective due to the presence of guanine within them. Xanthophores are dark orange in color and overlay the iridophores, (Irion et al. 2013). Color patterns are present in all animals and generally have principal functions beyond simply protecting the organism from radiation. For example, they can be used for communication within a species and as a means of camouflage to escape predators, (Irion et al. 2013). When oca2 is mutated in zebrafish, not only is there a decrease in melanophores, but an increase in iridophores (Beril et al. 2014). The disruption of proper expression of two chromatophores when this gene is mutated points to its importance in proper color pattern formation.
Figure 3. Comparison of oca2 mutant and wild-type zebrafish. (Borrowed from Beirl et al. 2014.) G) Wild-type zebrafish. H) oca2 mutant zebrafish I) Close up of the melanophore stripe in the wild-type fish J) Close up of the melanophore stripe in the oca2 mutant fish

Iridophore Development

Neural crest cells are multipotent embryonic cells which act as precursors for a variety of different cell types including chromatophores in zebrafish. The cells go through steps that cause the number of possible cell types a given cell can become to be more and more restricted (Petratou et al. 2018). For example, for neural crest cells to become any chromatophore, the gene sox10 must be expressed in the cell (Singh & Nüsslein-Volhard 2015). However, within cells fated to be melanophores sox10 will be downregulated, that is, decreased in expression, when the gene microthalmia-associated transcription factor (mitfa) begins to be expressed. In contrast, iridophore development is dependent on a sustained expression of sox10 (Petratou et al. 2018). A number of the same genes are involved in the development of iridophores and melanophores, which suggests genes affecting the development of one may impact the other as well (Petratou et al. 2018). Genes such as mitfa, vacuolar protein sorting 11 (vps), and kit need to be expressed
within a cell for the proper development of melanophores. Likewise, when any of these genes is malfunctioning in iridophores changes to their development and or proliferation occur as well (Cooper et al. 2018).

In one study it was found that during the differentiation of chromatophores into their respective cell types there exists a bi-potent precursor for dark pigmented melanophores and iridescent iridophores. These bi-potent precursors are characterized by the presence of the \textit{foxd3} and \textit{mitfa} genes (Curran et al. 2010). These bi-potent precursor cells are fated to be melanophores when the \textit{mitfa} gene is expressed, and iridophores when \textit{foxd3} is expressed, which produces transcription factors that repress the \textit{mitfa} gene (Curran et al. 2010). This study also demonstrated that the gene \textit{pnp4a} could be used to as a marker for iridophore expression (Curran et al 2010). The pattern of expression of \textit{pnp4a} appears in the same regions that iridophores are present. This gene produces an enzyme involved in purine synthesis, which allows for guanosine to be turned into guanine. This is essential for iridophore formation because these shiny cells are composed of accumulated guanine crystals (Curran et al 2010). Another study examined a mutation in the \textit{nacre} gene, which caused a complete loss of skin melanophores along with a significant increase in iridophores, similar to the phenotype of mutant \textit{oca2} zebrafish, (Lister et al. 1999). Within this study it was suggested that melanoblasts may switch fate to iridoblasts when \textit{nacre} is not properly expressed (Lister et al. 1999).

The structure of iridophores may reflect that of melanophores as well. Iridophores are known to contain reflective platelets, or iridosomes, which contain guanine (Oshima & Kasai 2002). These platelets are found alone or in stacks and they provide the iridescent colouration of iridophores (Oshima & Kasai 2002). Currently, it is unknown the maturation process of this organelle or how exactly it functions. It has been speculated that this organelle functions in a
manner analogous to melanosomes found in melanophores. For instance, the gene \textit{gpnmb} which has been suggested to produce proteins found in the melanosomes, is highly expressed in iridophore cells (Higdon et al. 2013). In further support of this idea, reflecting platelets were found to form from stage two melanosomes within white cells, a pigment cell type expressed in the frog species \textit{Xenopus laevis} (Fukuzawa 2015). This may indicate that reflective platelets in iridophores could form from a pathway similar to melanosomes in melanophores. This follows the idea that all three chromatophores in zebrafish come from a common precursor cell which contains an organelle which can give rise to melanosomes, reflective platelets and pterinosomes (found in xanthophores) (Bagnara et al. 1979). Due to the likely similar mechanisms of function and origin of reflecting platelets to melanosomes the examination of iridophore development could expand what is known of melanocyte function as well.

**Color Pattern Formation in Zebrafish**

The current model used for describing color pattern formation is referred to as the Turning Mechanism. Put simply, this model describes how two diffusible substances interact while diffusing at different rates, (Watanabe & Kondo 2015). This model asserts that color patterns are induced by the interactions between each chromatophore (Watanabe & Kondo 2015). This would suggest that the misexpression of one chromatophore would lead to abnormal behaviors in others. This has currently been displayed with the three chromatophore types present in zebrafish (Singh & Nüsslein-Volhard 2015).

The presence of iridophores themselves play a role in the development of proper pattern formation. It has been demonstrated that iridophores promote melanophore aggregation at a distance and repel them when close by, (Frohnhöfer et al. 2013). Iridophores also can attract xanthophores to create the proper pattern, (Singh & Nüsslein-Volhard 2015). When one
chromatophore is absent it can lead to the others behaving abnormally, (Singh et al. 2014, Watanabe & Kondo 2015). For example, mutations in the *rose* and *shad* genes lead to a lack of iridophores, which causes a subsequent decrease in melanophores, (Frohnhofer et al 2013). It has also been demonstrated that when melanophores are absent, iridophores will proliferate into regions outside of their regular boundaries, (Frohnhofer et al. 2013). Since chromatophores are dependent upon one another for proper development, it is possible that in *oca2* both iridophores and melanophores are being impacted due to changes in their interactions with one another.

**Research Question:**

As stated earlier, when *oca2* is mutated in zebrafish there is a decrease in melanophores and an increase in iridophores. In this project the aim was to determine the mechanism by which *oca2* is influencing iridophore expression. It was hypothesized that *oca2* is involved in regulating which cells become iridophores and melanophores (Figure 4). To determine if this was true the expression of two genes were examined: *pnp4a* and *mitfa*. When *pnp4a* is expressed in a cell that means the cell is fated to be an iridophore (Curran et al 2010). Cell specification, or cell fate, describes when a cell is locked into a trajectory towards a specific cell type. Due to this it was hypothesized that in *oca2* mutant zebrafish there would be an upregulation of the *pnp4a* gene leading to more cells expressing this gene. Therefore, more cells would differentiate into iridophores. It is also expected that the *mitfa* gene, which is important in melanin synthesis and inhibiting iridophore expression will be expressed significantly less in the *oca2* mutant zebrafish than in the wild type zebrafish (Curran et al. 2010, Cooper 2017). Since *mitfa* is essential for melanophore specification, it was expected to be downregulated in *oca2* mutants (Beirl et al. 2014).
Figure 4. Hypothesized regulation of *pnp4a* and *mitfa* by *oca2*.

**Methodology:**

**Fish Husbandry:**

Adult zebrafish were maintained on a 14 hour light/10 hour dark cycle at approximately 29.0°C and a pH of 7. They were fed twice per day, seven days a week with dry food and brine shrimp. Wildtype and *oca2* mutant fish were obtained from heterozygous fish carrying mutant allele *oca2*<sup>W77</sup>. These fish were set-up in pairs to naturally spawn and the fertilized eggs were collected the next day. The embryos collected were sorted and placed in petri dishes with embryo media and were grown to target stages in an incubator at 28.5°C. Fish were staged according to Kimmel et al. (1995). Approximately 40-60 larval fish were used per experiment. This laboratory is monitored by the Washington State University Institutional Animal Care and Use Committee (IACUC) and IACUC policy will be followed in terms of caring for the fish. Only approved humane euthanasia practices are used.
**Whole Mount In-situ Hybridization:**

In-situ hybridization is the process by which complementary DNA or RNA strands are introduced to an organism enabling researchers to view the pattern and level of expression of a given gene within the organism. In this experiment, RNA probes were used that are complementary to the mRNA produced by the *pnp4a* and *mitfa* genes. To prepare fish for the experiment they were fixed for 6 hours in 4% PFA in PBS at room temperature. Fixation is done to preserve tissues from decay and to prevent any further biological reactions to take place in the tissues (Thavarajah et al. 2012). The protocol described by Thisse & Thisse (2014) was followed, with minor adjustments to the procedure as was necessary. In-situ were done for varying ages of embryos namely: 24 hpf (hours post fertilization), 27 hpf, 48 hpf, and 72 hpf.

**Brightfield Microscopy:**

Fish that had successfully undergone an in-situ were viewed under a SMZ1500 microscope for analysis. The positive signal for a given gene (*mitfa* or *pnp4a*) was compared between *oca2* mutant and wildtype zebrafish. Pictures were taken of representative fish per each experiment using NIS-elements software. At 24 hpf fish could not be separated into *oca2* mutant and wildtype because the difference in phenotype is not apparent at this stage. Therefore, if any differences in gene expression were found between fish the number of those differing were counted. If more than one fourth of the fish had the same expression pattern, the pattern was deemed to not stem from the *oca2* mutation. This is because the mutation is recessive resulting in an expected ratio of 1:3 mutants to wildtype fish per spawning.
Results:

*oca2* mutant zebrafish express similar amounts of *pnp4a* compared to wild-type zebrafish

Successful in-situ were performed using the *pnp4a* probe for 24 hpf, 27 hpf and 72 hpf. From these varying ages there was no difference in expression found between *oca2* mutants and wild-type zebrafish. Figure 5 can be referred to for an example of zebrafish anatomy terminology. Fish at 24 hpf expressed *pnp4a* primarily bordering the eyes, the yolk sac and in the midbrain and regions (Figure 6). Less concentrated *pnp4a* expression was found down the spine on the dorsal side of the fish. Dotted expression was also found at the very tip of the tails of the fish. There were fish that appeared to have not as concentrated expression in the head region than others, however since this accounted for approximately one third to half of the fish in each sample this could not be attributed to the *oca2* mutation.

For *oca2* mutant fish staged at 27 hpf *pnp4a* expression was concentrated bordering the yolk sac and yolk extension. There was also expression found posteriorly located on the dorsal side of the fish. *pnp4a* expression was also found dotted across the spine of the fish. For wild-type fish staged at 27 hpf *pnp4a* expression was also found in around the yolk sac and yolk extension. Similarly, to the *oca2* mutants the expression was also found more posteriorly located on the dorsal side of the fish.

For *oca2* mutant fish staged at 72 hpf *pnp4a* expression was found running down the spinal cord of the fish. There was also *pnp4a* expression running down the ventral side of the fish, bordering the yolk sac and yolk extension and extending down to the tail tip of the fish. *pnp4a* expression was also concentrated encircling the eye lens of the fish as well as on the tips of the developing external gills. For wild-type fish staged at 72 hpf *pnp4a* expression was also
found running down the spinal cord of the fish, as well as down the ventral side of the fish.  

`pnp4a` expression was also located on the tips of the developing external gills and bordering the eye lens of the fish.

**oca2 mutant zebrafish express similar amounts of mitfa compared to wild-type zebrafish**

Successful in-situ were performed using the `mitfa` probe for 24 hpf for both `oca2` mutants and wild-type, and 27hpf for wild-type only as seen in Figure 7. At 24 hpf there was no difference in expression found between `oca2` mutants and wild-type zebrafish. The expression of `mitfa` at 24 hpf was similar to that of `pnp4a` expression, where the signal was concentrated in the head region. Specifically, in the hindbrain and midbrain regions. For about half of the sample there appeared to be less expression of `mitfa` in these regions, but they could not be attributed to the `oca2` mutation. `mitfa` expression was also found more posteriorly located on the dorsal side of the fish. The expression was a dotted pattern with almost equal spacing between each patch of gene expression. Gene expression was also concentrated bordering the yolk extension. For wild-type at 27 hpf `mitfa` expression ran down the spinal cord of the fish and bordered the yolk extension.
Figure 5. Zebrafish anatomy. Picture of fish taken at 24hpf, key regions of fish labelled.
Figure 6. Expression of *pnp4a* for 24, 27 and 72 hpf. Blue arrows indicate examples of areas where *pnp4a* is being expressed A) 72 hpf. Wild-type. Full body and close-up of posterior region around yolk extension. B) 72 hpf. *oca2* mutant. Full body and close-up of posterior region around yolk extension. C) 27 hpf. Wild-type D) 27 hpf. *oca2* mutant E) 24 hpf. Full body and close up of head region.
Figure 7. *mitfa* expression in 24 hpf and 27 hpf. Black arrows indicate examples of regions where *mitfa* is being expressed. A) 24 hpf B) 27 hpf. Wild-type.
Discussion and Future Directions:

The results of this study indicate that \textit{oca2} does not regulate the specification/cell fate of iridophores or melanophores. This finding aligns with what has been found previously concerning the role of \textit{oca2} with melanophores. The specification of melanoblasts, the precursor cells to melanophores, is not impacted by mutations to the \textit{oca2} gene (Beirl et al. 2014). The differentiation of the melanoblasts into melanophores however is impacted by the loss of \textit{oca2} function (Beirl et al. 2014). Differentiation of a cell occurs when the cell takes on new characteristics to be a particular cell type. Therefore, this may suggest that like in the case of melanoblasts, iridoblasts, the precursors for iridophores, are not regulated by \textit{oca2}.

Since \textit{oca2} does not regulate the cell fate of iridophores, the next step in my research will be to determine if \textit{oca2} regulates the proliferation of iridophores. Proliferation refers to the growth and division of cells. Since \textit{oca2} mutants have more iridophores I am predicting that there will be an increase in iridophore proliferation within the mutants compared to wild-type. If this is true, there are various pathways that could be explored to see how exactly \textit{oca2} is regulating proliferation. For example, genes required for iridophore growth and division could be examined as well for differential expression using whole mount in-situ hybridization to try and elucidate how exactly \textit{oca2} is regulating proliferation.

Currently what is known about the proliferation of iridophores is limited, but there have been a few genes found to be essential for proliferation in iridophores. For example, it is known that iridophores are dependent on the expression of \textit{leukocyte tyrosinase receptor (ltk)} gene for specification and proliferation (Fadeev et al. 2016). To understand if \textit{oca2} is possibly interacting with such genes will further reveal the exact mechanisms by which the p protein works.
Unravelling the function of the gene *oca2* in respects to iridophores will shed light onto the importance of *oca2* in other cells beyond that of melanophores. If *oca2* does indeed play a significant role in iridophore development and survival, it may reveal how the version of this gene within humans, *OCA2*, when mutated, does not merely decrease pigment production but could also be affecting other cellular pathways that have yet to be explored (i.e. cell proliferation pathways). If so, continuing to use zebrafish as a model to further define functions of *oca2* within a living system will be beneficial to understand the function of this gene within humans, and may aid in finding compounds that provide treatment for the oculocutaneous albinism II disease.
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