LIBERTY UNIVERSITY

CRISPR Cas9 mediated GFP Tagging of CD79a in *Danio rerio*

Asia Eskaros, Daniel Leonard, Gavin Litchford, Hunter Hall, Leeah Mills, Oluwakonyinsola Adebayo, Dr. Lindsey Stevenson

Abstract and Background

Background: The location of B cell development in mammalian models is adequately described in present literature. However, it has yet to be determined where early B cell development first takes place in *Danio rerio*, commonly known as zebrafish. *Danio rerio* are a common model for studying the immune system as they possess both and adaptive and innate immune systems. However, the immune development pathways of these organisms are not fully understood. Previous research indicates that early B cells may be detectable 7 days post-fertilization: a claim that has not been adequately investigated. **Methods:** Therefore, this team seeks to investigate this prospect by employing CRISPR-Cas9 technology to fluorescently tag B cells and track the location of B cells throughout *Danio rerio* development using fluorescent microscopy. **Conclusions:** This project is still in its fledgling steps but will be continued throughout the coming semesters.

Introduction and/or Research Question

B cells are antigen-specific leukocytes that are derived from the lymphoid lineage of hematopoietic stem cells. These cells synthesize and excrete immunoglobulins – aka antibodies - which facilitate humoral immunity, thus combating invading pathogens. There are many forms of B cells depending on the stage of maturity, beginning with the initial pre-pro B cell, early pro-B cell, late pro-B cell, large pre-B cell, small pre-B cell, immature B cell, and finally reaching the naïve B cell stage (Fig. 2). The early pro-B cell is the earliest form of the B cell that can be identified and tracked to locate maturing B cells during the development and adulthood of organisms; this specific form is ideal since it is the first of the B cell stages to exhibit the surface co-receptors Igα and Igβ, as well as the first to undergo D to J cassette recombination on the heavy chain of its B cell receptor. The Igα and Igβ receptors are of particular importance since they are expressed on the cell surface extremely early in the stages of B cell development, in addition to being exclusively unique to B cells. Forms of B cells prior to this stage lack obvious differentiation from antecedent hematopoietic stem cells.

During mammalian development, developing B cells can initially be found in the yolk sac of a fertilized embryo. As gestation proceeds, developing B cells eventually are relegated to the bone marrow of the fetus, where they continue to reside for the remainder of the lifetime of the mammalian organism. In adulthood, naïve B-cells that have undergone proper B-cell development in the bone marrow will subsequently exit and relocate to secondary lymphoid tissue – such as the spleen, a lymph node, or more generalized lymphatic tissue associated with a specific organ or region – where they can then participate in humoral immunity. Today, this process of tracking B-cell development concerning their physical location within developing mammals is well described in contemporary literature, especially in humans. However, literature is scarce when it comes to fish, particularly in zebrafish (*Danio rerio*). Literature describes the presence of B cells in the pancreas of zebrafish 10 days post fertilization (dpf), while simultaneously suggesting that B cells may be detectable as early as 7 dpf (1).

Zebrafish are model organisms that are crucial to our understanding of eukaryotic development, and due to this gap in knowledge, we aim to identify and track B-cell development in zebrafish by utilizing CRISPR-Cas9 technology (Fig. 1) to mark the Igα coreceptor – known as CD79a in zebrafish - with green fluorescent protein (GFP). This technique will allow for earlier visualization compared to previous methodologies since CD79a is one of the first surface proteins expressed on developing B cells. Another important feature of CD79a is that it remains embedded in the B cell membrane during the entire life cycle of the cell, meaning that the GFP-tagged cell can be tracked beyond its maturation stages, which allows for prolonged and extensive observation. This project will therefore elucidate the previously obscure location of developing B cells in developing zebrafish embryos, which will contribute to immunological comprehension of this specific model.

Methods

Transfection by CRISPR/Cas 9
Gametes will be collected from both male and female fish. The oocytes will be sorted to ensure viability and stored in a sorting medium. The viable oocytes will be injected with a mix of Cas 9, sgRNA, True Tag Donor DNA and phenol red. The microinjection will be done by pulled needles attached to a Hamilton syringe. The oocytes will then be cultured in a pH adjusted storage medium for 30 minutes. The cultured oocytes will be fertilized with the collected sperm. The fertilized eggs will be incubated at 28 °C and maintained. The successful transfection will be verified by PCR. The fish will be raised to sexual maturity.

Back crossing
The GFP gene when transfected is a dominant gene. The transfected fish, Gg, will be raised to sexually maturity. The heterozygous fish will be crossed to produce F1: heterozygous (Gg), homozygous dominant (GG) and homozygous recessive (gg). The F1 generation will be raised, screened and separated according to genotype. The homozygous dominant (GG) fishes will be crossed to have produce more homozygous dominant offspring.

Fish raising
The fish will be kept in a circulating system with temperatures maintained between 26 to 28.5 °C and pH maintained between 6.8 to 7.5. The lightening conditions will be 10 light: 14 dark. The fish will be dry fed 4-5% of its biomass twice daily during the week and once daily on the weekends.

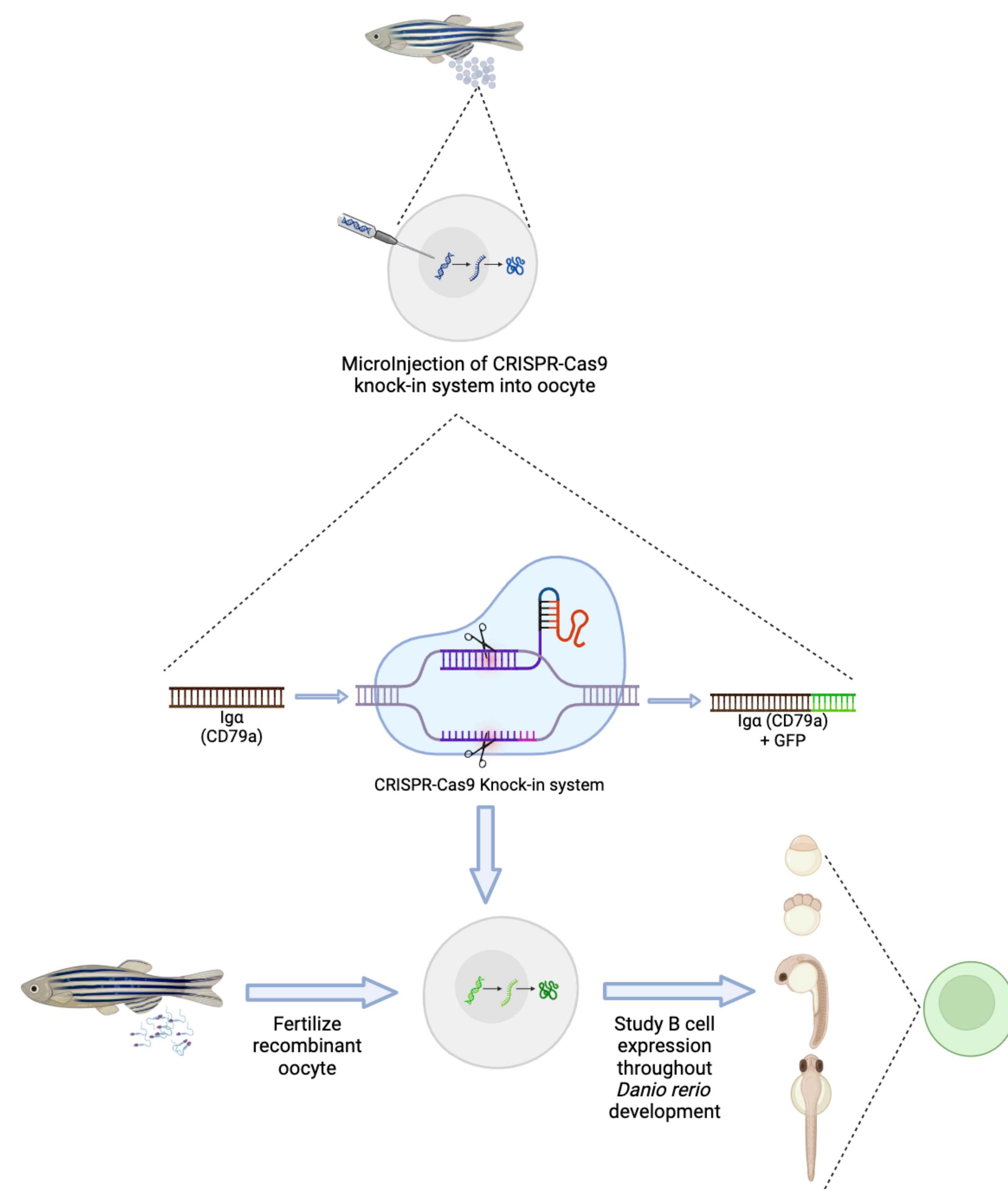

Figure 1. Schematic for CRISPR-Cas9 mediated tagging of Igα (CD79a) in *Danio rerio* B cells. The CRISPR-Cas9 system will be used to tag the Igα gene with GFP, which will cause the B cells in the developing zebrafish to fluoresce green. This system will be injected into *Danio rerio* oocytes. Once successfully injected, the recombinant oocyte will be fertilized with manually obtained *Danio rerio*. These fertilized embryos will be raised and the location of the B cells throughout the development of *Danio rerio* will be determined using fluorescent microscopy.

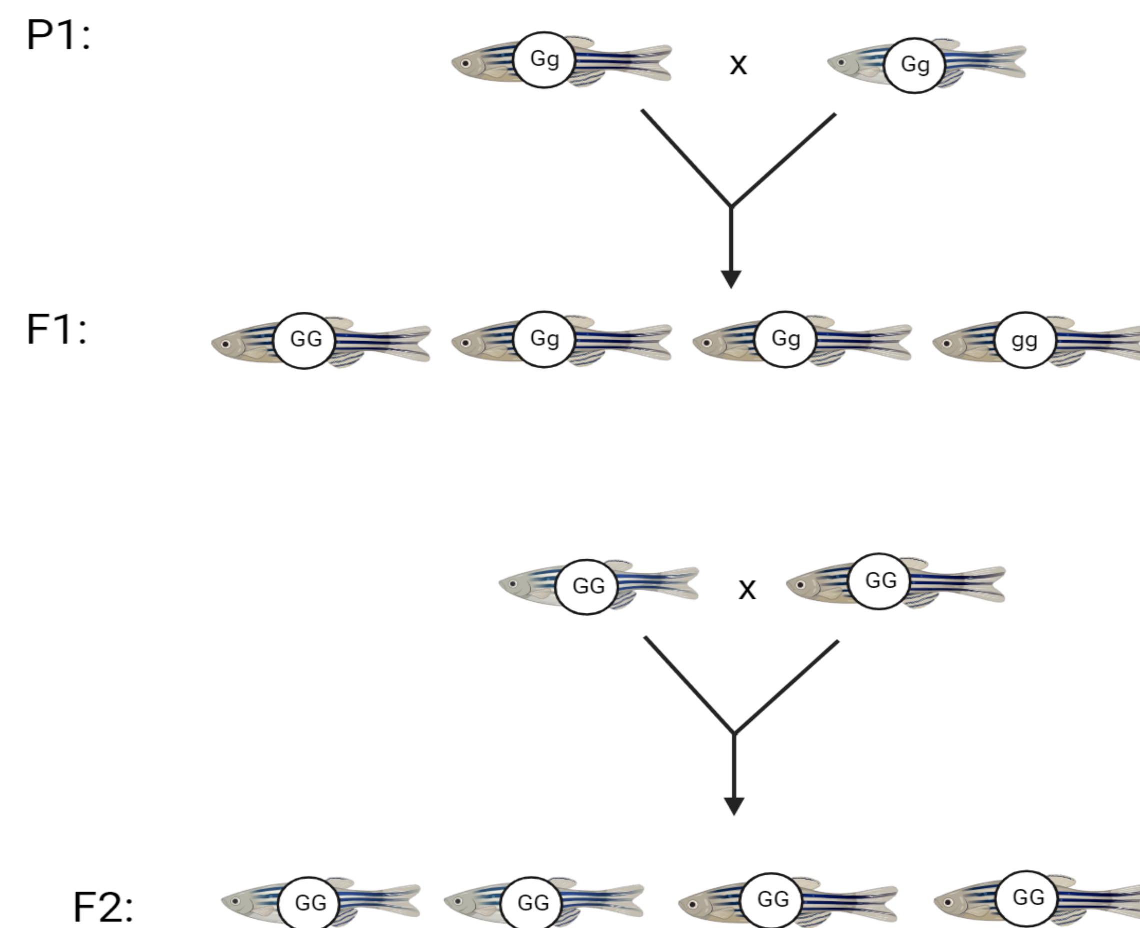

Figure 2. Schematic of the backcrossing of the successfully transfected zebrafish, P1. The GFP gene is represented as G, a dominant gene. The F1 generation is produced from the cross of two successfully transfected sexually matured fishes. Two fishes from the homozygous dominant percentage were crossed to produce more homozygous dominant fishes, F2.

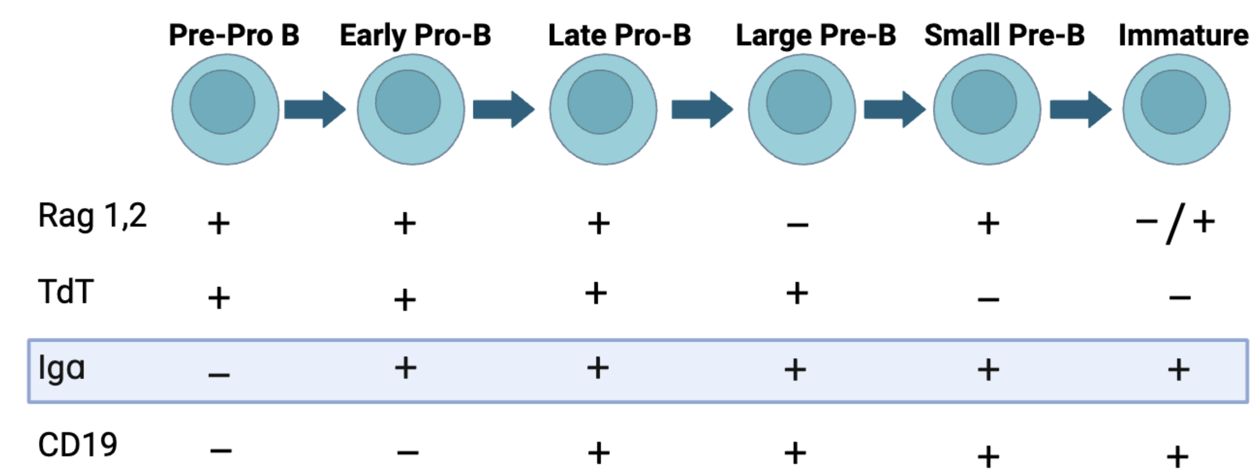

Figure 3. Stages of B cell development.
The maturation of a B cell encompasses multiple stages defined by various aspects of development within the cell. Expression of the Igα coreceptor begins early in B cell development and is expressed constitutively throughout the life of the cell, making it an ideal candidate for tracking the location of developing and adult B cells in zebrafish.

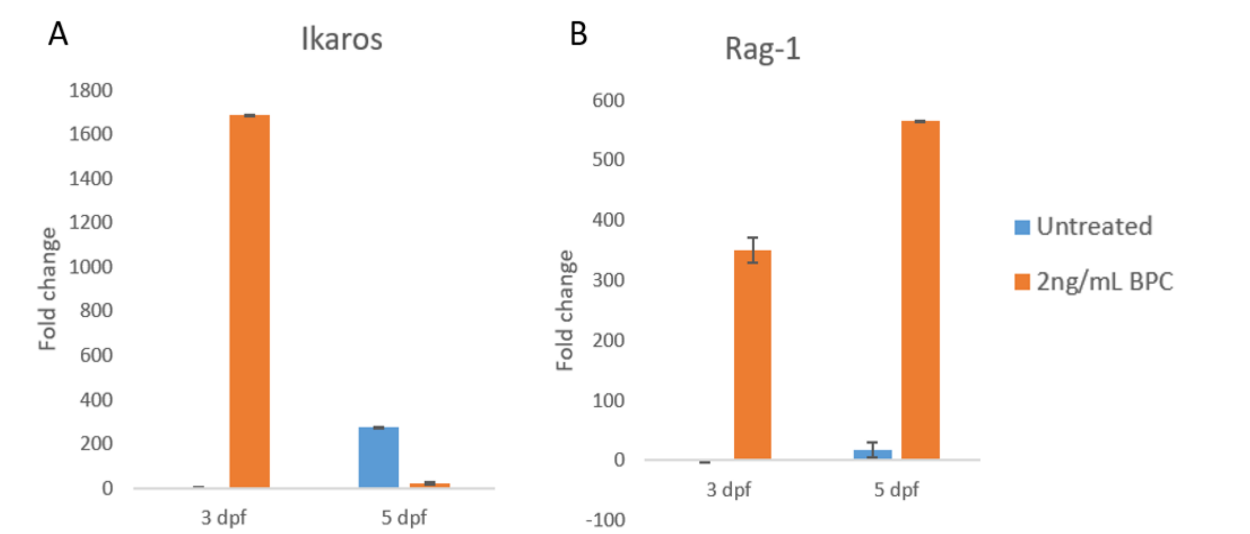

Figure 4. Ikaros and RAG-1 3 and 5 dpf.
Ikaros (A) and Rag-1 (B) expression in zebrafish embryos either exposed to 2 ng/ml BPC or no BPC, at 3 and 5 dpf. Zebrafish were exposed to BPC immediately after fertilization (0 dpf), with water changes every 24 hours.

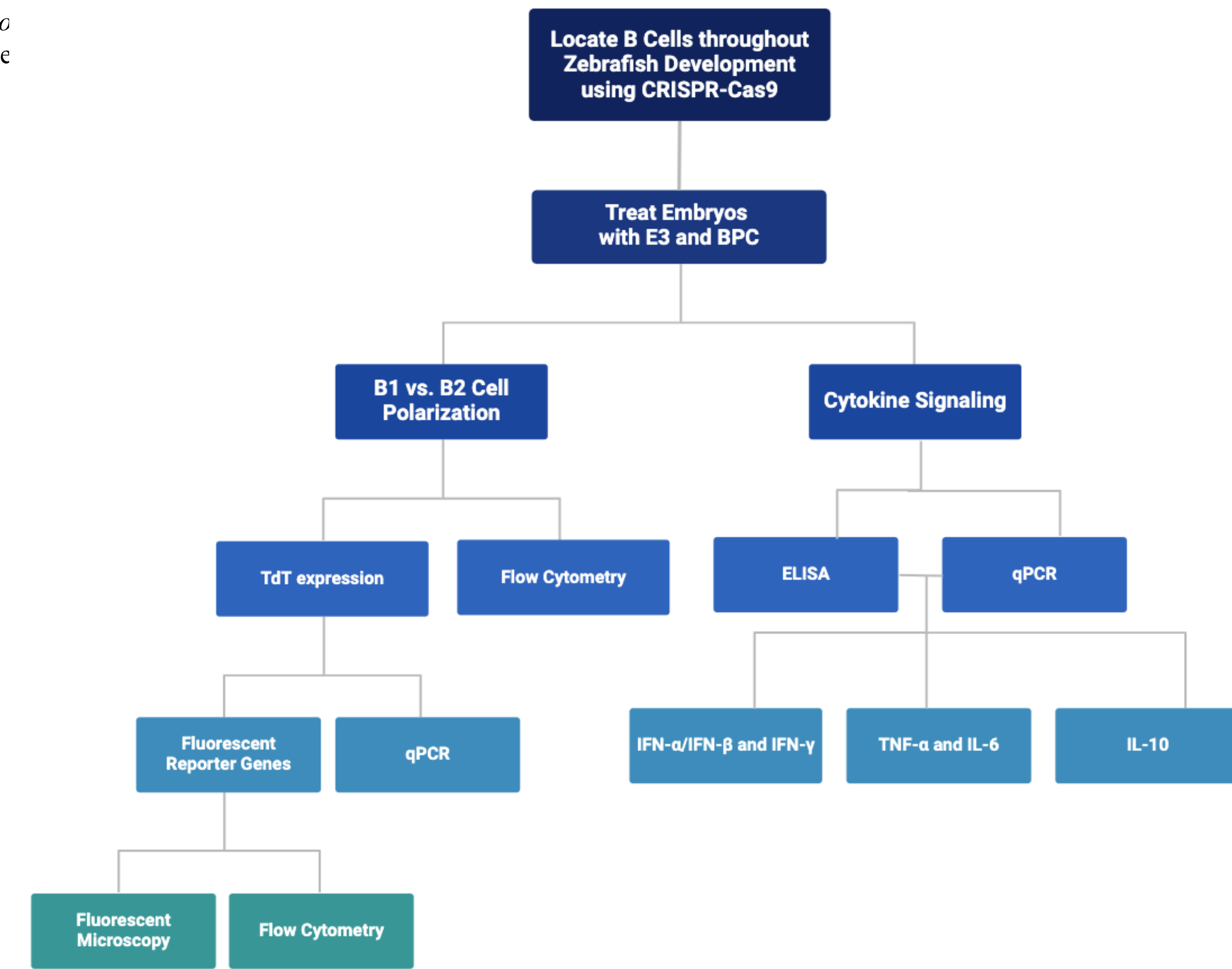

Figure 5. Future Directions for Studying the Effect of BPC on Immune Development.
To isolate the effect BPC on B-cells in immune development, the location of B cells throughout development will be determined using CRISPR-Cas9. Then, two different methods will be used to study the effect of BPC on B1 vs. B2 cell polarization. B1 vs. B2 cells will be gated for in Flow Cytometry using CD19/CD5 double positive vs. CD19 positive/CD5 negative cells respectively. Unlike B2 B cells, B1 B cells do not utilize TdT during their V(d)J Recombination. Thus, rtPCR will be used to quantify overall TdT expression in fish exposed to BPC. We expect to see increased RAG-1 expression without accompanying TdT expression, in comparison to their untreated counterparts. Any differences will be confirmed using fluorescent reporter genes. To study the effect of BPC on cytokine signaling, rtPCR of IFN-β ,IFN-α, IFN-γ, IL-6, TNFα, and IL-10 will be conducted following the exposure of BPC. In order to assess the affect of BPC on the immune system's ability to respond to infection, ex-vivo cells, taken from fish either exposed to BPC or not, will be either stimulated by a PAMP, such as LPS, or by viral infection. ELISA and qPCR will be used to assess cytokine expression.

Methods (cont.)

DNA extraction and Testing
The DNA of the zebrafish will be tested after transfection and for sorting at 3 to 5 days post fertilization (dpf). The fish will be anesthetized with Tricaine. The A P1000 tip will be cut to approximately 2mm and used to pick up the anesthetized larva. A microscalpel will be used to gently separate the distal portion of the caudal fin in a gap in pigmentation. The separated portion of the caudal fin will be transferred onto a piece of paper and then inserted into a PCR well containing sodium hydroxide. The larva will be transferred into a well labelled 96-well tissue plate containing E3 media. The wells will be labelled per their respective extracted caudal fin sample. The PCR plate containing the caudal fin sample will be sealed and centrifuged for 1 minute. The PCR plate will then be placed in a thermocycler at 95°C for 5 minutes and cooled to 4 °C for 10 minutes. Tris-HCL, pH 8 will be added to each sample and vortexed. The plate will be centrifuged at 1,500 for 5 minutes at room temperature. The DNA supernatant will be used for PCR. The PCR will be done according to the Invitrogen True Tag Donor DNA kit.

Results

In the past, this team found that when *Danio rerio* embryos are exposed to BPC, which has been shown to be an estrogenic compound, they express Ikaros and Rag-1 earlier than control embryos (Figure 4). Ikaros and Rag-1 are inherent to B cell development. Thus, the team hoped to study how BPC exposure affected B cells throughout *Danio rerio* development. However, there was no literature about where B cells are normally found throughout development of *Danio rerio.* Thus, much literature review was conducted, and the team was able to write up a protocol for tagging B cells (CD79a) with GFP. After successfully completing the lab protocol, the team was able to write up a protocol for IACUC approval. Once approved by the IACUC committee, this team will begin working towards obtaining quantitative data regarding the location of B cells throughout *Danio rerio* development.

Future Work

This research team intends to track B cells throughout the development of zebrafish by performing CRISPR-Cas9 mediated GFP tagging of the Ig alpha coreceptor (CD79a in *Danio rerio*), which is expressed early in B cell development and constitutively throughout the cell's life. This will allow us to determine in which specific tissues B cells are located, over the lifespan of zebrafish, which has yet to be fully elucidated. Knowledge of the specific tissue where B cells are located will aid in future projects.

After transfection, we plan on exposing adult zebrafish to various concentrations of BPC to investigate how exposure to BPC affects B cell development. We have previously observed that BPC exposure corresponds with a premature upregulation of Ikaros, which is vital for B and T cell development. We will first investigate the effect BPC exposure has on the numbers of B cells present in zebrafish during various stages of development. This will be done by using flow cytometry to quantify the number of B cells present in a single cell suspension generated from the tissues we find to contain large reservoirs of B cells. After determining the numbers of B cells present, we will investigate the effect of BPC exposure on B cell polarization. BPA and its derivative BPC have been previously shown to be estrogenic. It is well known that increased estrogen levels, found primarily in females, is correlated with increased numbers of B1 B cells found in the body, in contrast with the classical and more prevalent B2 B cell. To determine B1 vs. B2 cell polarization, we will use flow cytometry to quantify the expression of double positive CD19/CD5 vs. CD19 positive/CD5 negative cells, which correspond to B1 and B2 cells, respectively.

Additionally, the presence of high levels of B1 B cells has been correlated with increased risk for developing autoimmune disorders, as B1 B cells sustain themselves by binding weakly to self antigens. We plan to begin by investigating the effect of BPC on general immune signaling. We will target some basic pro- and anti-inflammatory cytokines. Targeted cytokines include the Type I and Type II Interferons, IFN-α/IFN-β and IFN-γ, respectively; the pro-inflammatory cytokines IL-6 and TNFα; and the anti-inflammatory cytokine IL-10. ELISA and qPCR will be used to assess cytokine expression.

References and/or Acknowledgments

Danilova, N., & Steiner, L. A. (2002). B cells develop in the zebrafish pancreas. Proceedings of the National Academy of Sciences, 99(21), 13711–13716.