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NOVEL ROLES OF ADHESION G PROTEIN-COUPLED RECEPTORS IN
CARDIOVASCULAR DEVELOPMENT

by

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A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Health and Human Physiology

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All requirements for graduation with Honors in the
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Novel Roles of Adhesion G Protein-Coupled Receptors in Cardiovascular Development

Abstract

Congenital heart disease is the most common congenital birth defect, affecting 1.35 million newborns every year. Though therapeutic techniques have been developed to assist those afflicted, new issues arise as those that have been treated may have a higher likelihood to pass on their cardiovascular defect to their children. Adhesion G protein-coupled receptors are an increasingly studied member of the G protein-coupled receptor superfamily. aGPCRs have a wide array of molecular mechanisms that it affects; however, their role in the development of organ systems, specifically the cardiovascular system, is the focus of our research. When screening for aGPCRs within the cardiovascular system, the *adgrl* gene family was one family found to be expressed. Previous research has shown that various *adgrl* isoforms, such as *adgrl2*, play roles in cardiovascular development. Digoxigenin-UTP RNA probes developed through TA-vector cloning and transformation into *E. coli* were used to reveal mRNA expression in *in-situ* hybridization (ISH). ISH reveals that the *adgrl* isoforms 1 and 3 are expressed in the cardiovascular system, similar to *adgrl2*. Using CRISPR/Cas9 technology, *adgrl1* and *adgrl3* mutant lines were generated and, once stable, will be screened and assessed for deviant phenotypes to elucidate their role in the cardiovascular system. Additional efforts have demonstrated another gene family, *bai*, is expressed in dorsal forerunner cells, which influence the flow of fluid within Kupffer's Vesicle, establishing asymmetry within the brain, gut, and heart. Mutant *bai2*^{-/-} and *bai2l*^{-/-} zebrafish initially demonstrated similar deviations in left-right asymmetry randomization but were eventually lost in successive generations. When stable homozygous mutants for both mutations were developed, no deviant phenotypes were observed, potentially due to their redundant roles. To assess this possibility, *bai2*^{-/-} and *bai2l*^{-/-} mutants were crossed and double homozygous mutants' phenotypes will be assessed when mature.

Introduction

Congenital heart disease is the most common congenital defect, affecting 1-2% of newborns, often leading to infant mortality (Fahed, Gelb, Seidman, & Seidman, 2013). Congenital heart disease accounts for more than a third of all congenital birth defects with 1.35 million children a year being born with a congenital heart defect (Fahed et al., 2013). Modern

medical interventions have been able to treat newborns with congenital heart disease; however, they often encounter further cardiovascular maladies later in life, such as cardiac arrhythmias (Ransom & Srivastava, 2007). Furthermore, there is also concern that those affected with congenital heart defects have a higher likelihood to pass the defect onto their children, thus replicating the deadly cycle (Ransom & Srivastava, 2007). Congenital heart disease arises within early heart development as the result of both environmental factors such as teratogens as well as genetic abnormalities (Fahed et al., 2013).

Previous studies have implicated that adhesion G protein-coupled receptors (aGPCRs) play a key role in heart development (Doyle et al., 2006). Consisting of 33 family members in humans, aGPCRs are characterized by autocatalytic processing at a GPS Autoproteolysis INducing (GAIN) domain, multiple domains in long N termini, and strong evolutionary conservation (Hamann et al., 2015). aGPCRs have a wide array of roles within the organism, including but not limited to establishing cell polarity, cell adhesion and migration, cell size, tumorigenesis, and development of organ systems (Hamann et al., 2015). Indeed, previous efforts have demonstrated that knocking out certain aGPCRs leads to serious and sometimes fatal complications within specific organ systems (Hamann et al., 2015). For example, one aGPCR, *adgrg6*, leads to highly lethal mutations such as myocardial wall thinning and mitochondrial dysfunction in embryos when it is knocked out. (Patra et al., 2013). Since aGPCRs are tightly linked to the proper development of the cardiovascular system, it is critical to elucidate their function in order to develop therapeutic techniques to provide more competent, individualized care for those with congenital maladies.

aGPCRs were screened for in cardiac cells, and it was found that two gene families have high expression levels: *adgrl* and brain-specific angiogenesis inhibitor (*bai*). Both families have multiple isoforms that contribute to different aspects to cardiovascular morphology (Hamann et al., 2015). The *adgrl* gene family is characterized by long N termini containing a rhamnose-binding lectin (RBL) domain, hormone receptor motif (HRM), an olfactomedin domain, seven putative transmembrane domains, and a GAIN domain. In addition, they contain the latrophilin receptor, a unique characteristic to the family (Hamann et al., 2015). The *adgrl* gene family has been shown to be expressed in the nervous system and cardiovascular system (Hamann et al., 2015). Presently, the roles of only a few members of *adgrl* have been established in the cardiovascular system. For example, a study demonstrated that knocking out *adgrl2* leads to

lethal mutations that inhibit atrioventricular cushion EMT through diminishment of mesenchymal cells in mice (Doyle et al., 2006). The atrioventricular canal endothelial cells form heart valves after an epithelial-mesenchymal transition (EMT) and, when *adgrl2* is knocked out, leads to a reduction in migration ability in endothelial cells (Doyle et al., 2006). Another isoform, *adgrl4*, is expressed in endothelial cells and regulate angiogenesis in Zebrafish (Hamann et al., 2015). In the nervous system, the roles of *adgrl1* and *adgrl3* have already been established. *Adgrl1* and *adgrl3* deletions are risk factors for nervous system ailments such as mental retardation and hyperactivity; however, their function in the cardiovascular system is still unknown (Bonaglia et al., 2010).

The *bai* gene family is characterized by leucine-rich repeat N termini, an HRM, seven transmembrane domains, and long C termini (Hamann et al., 2015). Original research illustrates that the *bai* gene family is predominant in the neurons of the nervous system; however, novel data demonstrate that the deletion of *bai* isoforms also results in cardiovascular defects (Duman et al., 2013). *Bai2* is an isoform found to be expressed in dorsal forerunner cells (DFCs), cells that form Kupffer's Vesicle (Bakkers, Verhoeven, & Abdelilah-Seyfried, 2009). Kupffer's vesicle is a ciliated organ that acts as the primary left-right organizer for the brain, gut, and heart by influencing the direction of the flow of fluid within the organ. (J. J. Essner, J. D. Amack, M. K. Nyholm, E. B. Harris, & H. J. Yost, 2005). The directionality of fluid-flow within Kupffer's Vesicle induces the expression of nodal-related genes that are responsible for the development of asymmetrical organs, a requirement for proper organism development (Jeffrey J. Essner, Jeffrey D. Amack, Molly K. Nyholm, Erin B. Harris, & H. Joseph Yost, 2005). Asymmetrical formation of organs during embryogenesis is a highly conserved process throughout evolution and, when disrupted, can lead to birth defects (Blum, Feistel, Thumberger, & Schweickert, 2014). Two mutant lines were generated by a postdoctoral fellow: *bai2^{-/-}*, a 16 base pair insertion causing a frameshift at the GPS domain, and *bai2l^{-/-}*, a four base pair insertion located at the N terminus that causes a frame-shift and subsequent truncated protein. After injection, F₀ mutants were crossed to generate mutant lines. Initially, both mutations demonstrated similar defects in left-right asymmetry; however, the defect is gradually lost in later progenies. Both mutations have been reinjected into embryos and were allowed to grow to maturity and examined for phenotypic variations.

My research goal is to elucidate the roles of *adgrl* and *bai* gene families in heart development and LR asymmetry. Specifically, I will assess the expression patterns of isoforms *adgrl1* and *adgrl3* and their role in heart development through mutant generation via CRISPR/Cas9 technology. I expect *adgrl1* and *adgrl3* are also present in the heart and have a key function in heart development, when deleted, caused lethal mutations similar to results seen for *adgrl2* in mice. Moreover, I postulate that *bai2* mutants will exhibit left-right asymmetry defects when *bai2*, as well as purported compensatory genes, are knocked out. Once stable mutant lines are generated and maintained, I expect to see variations in heart formation and LR asymmetry when comparing mutants and wild type phenotypes. Finally, once *bai2* and other compensatory genes are reinserted into the mutant line, I propose that LR asymmetry will dissipate in most offspring, confirming the role of *bai2* in cardiac laterality.

Materials and Methods

Zebrafish Strains and Maintenance

AB*/Tuebingen, transgenic *Tg myl7:EGFP*) (Huang, Tu, Hsiao, Hsieh, & Tsai, 2003), and *Tg(sox17:EGFP)* (Mizoguchi, Verkade, Heath, Kuroiwa, & Kikuchi, 2008) zebrafish strains were maintained as described (Xu, Echemendia, Chen, & Lin, 2011). Embryos utilized for experimental purposes were obtained from NIH Zebrafish strains. All embryos were obtained through mating and were grown at 28.5°C or 32°C and were staged according to morphology and hours post fertilization.

Database Research

Potential zebrafish homologs of *adgrl* were identified using the following databases: NCBI, Ensembl, and The Zebrafish Model Organism Database. To determine homology between the human and zebrafish *adgrl* transcripts, Clustal Omega online software aligned available database amino acid information and quantified the percent similarity of each species.

Cloning Zebrafish Partial *adgrl* Genes for *in-situ* hybridization

To examine the expression of the *adgrl* isoforms, cloned partial genes were used to generate antisense RNA probes for *in-situ* hybridization. Primers were designed to span ~1 kb of Human Genetics (table 1).

	Forward	Reverse
<i>Adgrl1</i>	5'-CACTGCTGACACAGGTC-3'	5'-TTAGTGATCTCACCTTCTGGG-3'
<i>Adgrl3</i>	5'-GCTCGGGATACAAGTGCC-3'	5'-GCCAGCCGAAAGCTCATC-3'

Table 1: Forward and reverse primers for *adgrl1* and *adgrl3*.

Amplification of the target region was completed through polymerase chain reaction of cDNA obtained from wild-type 30 hour and 5 day zebrafish RNA using RT-PCR. A 20 μ L reaction containing 1 μ L cDNA and 0.5 μ L of forward and reverse primer (10mM), 1.6 μ L dNTP (2.5mM), 1 unit of taq polymerase, and 1 unit of taq buffer was placed in a thermocycler and utilized the appropriate annealing temperatures. The PCR product was fractionated by a 1% agarose gel electrophoresis to assess for the correct size. Finally, the PCR product was purified using an IBI Scientific PCR Clean-up and Gel Extract Kit.

TA Vector Insertion

The purified PCR product was ligated into a pGEM-T Easy Vector. A 5 μ L reaction was created with 0.5 μ L T4 DNA ligase (3 Weiss units/ μ L), 2.5 μ L 2x Rapid Ligation Buffer, 1 μ L PCR product, 1 μ L pGEM-T Easy Vector (50ng), and 0.5 μ L homemade ddH₂O. DH5 α competent cells stored at -80°C were thawed on ice. 50 μ L of competent cells were added to the ligation reaction and placed on back on ice for 20 minutes. The cells were then heat shocked for 45-50 seconds and immediately put back on ice for 2 minutes. Cells were plated onto lysogeny broth (LB) plates containing the appropriate antibiotic and incubated over night at 37°C overnight. Cells showing proper insertion of the PCR product appeared white on the plate. White colonies were collected and grown in a 15 mL tube with a 1:1 ratio of appropriate antibiotic and LB medium at 37°C overnight.

Restriction Enzyme Digestion

To test if plasmid contains the correct plasmid and to determine the direction of the sequence, the plasmid was digested. 2 μL of restriction enzyme (sp6 or T7 depending on the directionality of the sequence) as well as 10 μL of the appropriate buffer were added to 10000ng of the plasmid. Then, ddH₂O was added to create a 100 μL reaction and was placed in a 37°C water bath for 2 hours. Finally, the plasmid ran on a 1% agarose gel for confirmation. Linearized plasmids were sequenced to determine the directionality of the insert.

RNA Probe Synthesis, Purification, and Verification

Digoxigenin-UTP-labeled RNA probes were synthesized according to Roche DIG RNA Labeling Kit and protocol. Briefly, 1 μg of linearized DNA (quantified by UV light absorption), 1 μL transcription buffer, 2 μL NTP-DIG-RNA, 1 μL RNase inhibitor, 2 μL enzyme mix, and nuclease-free water to 20 μL were added to a 1.5 mL microcentrifuge tube and mixed by pipetting. The mixture incubated at 37°C for two hours. After two hours, 1 μL TURBO DNase was added to the sample and incubated for 15 minutes at 37°C to remove extraneous template DNA. To purify the RNA, 2 μL 0.2 M EDTA, 2.5 μL 4M LiCl, and 75 μL cold 100% ethanol were added to the reaction and spun down at 12,000 rpm for 30 minutes at 4°C. The supernatant was extracted and the RNA pellet was re-suspended and washed with 70% ethanol and spun down again for 3 minutes to pellet the RNA. Finally, the sample was eluted with 50 μL diethylpyrocarbonate (DEPC) water at pH 8 instead of elution buffer or ddH₂O⁴. DEPC water is used as an eluent due to its RNase-free property and reduces the risk of RNA being degraded by RNases. RNA concentrations were determined by UV light absorption and were confirmed by fractioning on a 1% agarose gel.

Whole Mount *In-situ* Hybridization

To determine the amount and location of gene location, *in-situ* hybridization was performed on different developmental stages, including but not limited to eight-cell, 16-cell, 32-cell, shield, 22-somite, 1-day post-fertilization, 2-days post-fertilization, and 3-days post-fertilization. Sense and antisense RNA probes for *adgr11A* and *adgr13* were synthesized according to protocol. *In-situ* hybridization was performed as described (Lin et al., 2005).

Cloning-Free gRNA Synthesis

Cloning-free gRNA synthesis was used to generate gRNA for CRISPR/Cas9 targeting. Using previous genomic information obtained from database mining, gRNA sequences were chosen based on the probability of off-site targeting. Potential off-site targets were detected using ZiFiT targeting software and the gRNA with the lowest probability of off-site targeting was chosen. A clamp, T7 promotor sequence, NGG sequence, guide sequence, and overlap sequence were assembled into a specific oligonucleotide and was annealed to a generic oligonucleotide with a complementary overlap sequence (table 2). A 20 μ L annealing reaction consisted of 1 μ L 20x taq buffer, 0.8 μ L 2.5 mM dNTPs, 2 μ L 10 μ M specific oligonucleotide, 2 μ L 10 μ M generic oligonucleotide, 0.3 μ L 100% DMSO, 1 μ L homemade taq polymerase, and 2.9 μ L H₂O. The mixture was denatured at 95°C for 2 minutes, annealed at 50°C for 10 minutes, extended at 70°C for 10 minutes, denatured again at 95°C for 5 minutes, and was removed to room temperature and allowed to cool. Confirmation of proper annealing was obtained on a 3.5% agarose gel.

	Oligonucleotide A
<i>Adgr11</i>	5'-GCGTAATACGACTCACTATAGGCAGCAAGTTTGCTGCAGGGTTTTAGAGCTAGAAATAGC-3'
<i>Adgr13</i>	5'-GCGTAATACGACTCACTATAGGTGGACGAAAACGGCCTCTGTTTTAGAGCTAGAAATAGC-3'

Table 2: Specific oligonucleotide ordered for both *adgr11* and *adgr13*. Each oligonucleotide contains a clamp, T7 promotor sequence, PAM site (NGG sequence), guide sequence, and overlap sequence.

gRNA Injection and Mutation Analysis

1nL of a mixture containing 80ng/ μ L of *nls-zCas9-nls* mRNA and 40 ng/ μ L gRNA created in the aforementioned protocol was injected into the blastomere of the wild-type embryos at 1-cell stage. To determine if gRNAs created mutations, genomic DNAs from pools of 5 injected embryos were isolated and amplified using primers flanking the region where the potential mutations could occur (table 3). Potential mutations were analyzed by polyacrylamide gel electrophoresis (PAGE) as polymorphisms run at different rates. (table 3).

	Forward	Reverse
<i>Adgrl1</i> gRNA	5'-CCGTCCTTCTATGCGTT-3'	5'-GTCTTCCCATGAAGCATACTC-3'
<i>Adgrl3</i> gRNA	5'-AAACTCCCGCACCGAGTG-3'	5'-GTCCCACGTTCCCTCGAC-3'

Table 3: Flanking primers for the gRNA site within the genomic DNA. Flanking primers are used to verify the efficacy of CRISPR/Cas9 technology and amplify the target region for mutation analysis.

Genotyping

Groups of embryos or tails of adult fish were used to isolate genomic DNAs. The embryos were combined into groups of five and lysed in 0.5 µL proteinase K with 10 µL of lysis buffer and were incubated for eight hours at 55°C and thirty minutes at 98°C to denature the proteinase K. Samples underwent similar PCR conditions as before and were analyzed via PAGE gel. Samples were electrophoresed 2% agarose gel, cleaned using an IBI Scientific PCR Clean-up and Gel Extract Kit⁴, and were sent for sanger sequencing at the Iowa Institute of Human Genomics if they demonstrated a novel mutation.

RNA Isolation and RT-PCR Gene Expression Analysis

To determine the relative expression of the *adgrl* gene family across model organisms, adult mouse heart sample was reverse transcribed to generate cDNA. Using 1 mL Ambion TRIzol Reagent, a solution highly effective in RNase activity inhibition and dissolving cell components, the sample was homogenized (Ambion). To separate aqueous and organic phases, 0.2 mL chloroform per 1 mL TRIzol Reagent was added, shaken vigorously, and allowed to incubate at room temperature for 3 minutes. The sample was centrifuged at 12,000 x g for 15 minutes at 4°C, permitting the solution to separate into a lower organic phase, interphase, and an upper aqueous phase, where the RNA was located. The aqueous phase was removed and transferred into a new tube. The RNA was isolated from the aqueous phase using 0.5 mL 100% isopropanol per 1 mL TRIzol Reagent and incubated at room temperature for 10 minutes. It was centrifuged for 10 minutes at 12,000 x g at 4°C. Once an RNA pellet formed, the pellet was washed with 1 mL 75% ethanol per 1 mL TRIzol Reagent, briefly vortexed to re-suspend the pellet, and centrifuged at 7,500 x g for 5 minutes at 4°C. the wash was discarded, and 30 µL of RNase-free water was added to re-suspend the RNA pellet. The sample was incubated in a 55°C water bath for 15 minutes and stored at -80°C. (). To synthesize cDNA from the isolated RNA,

an Invitrogen Cloned AMV First-Strand cDNA Synthesis Kit was used. A mixture of 1 μ L random hexamer, 2 μ L 10mM dNTP, and 9 μ L RNA were added to a 0.5 mL tube. The mixture was placed in a 65°C water bath for 5 minutes to denature the RNA and was immediately placed on ice. 4 μ L 5cDNA buffer, 1 μ L .1M DTT, 1 μ L RNase Out, 1 μ L DEPC-treated water, and 1 μ L AMV were added to the previous mixture and placed in a thermocycler at 25°C for 10 minutes, 55°C for 60 minutes, and 85°C for 5 minutes. The newly prepared cDNA was stored in -20°C.

Results

Identification of Genes

Database research confirmed the existence of four isoforms of *adgrl* within the human genome. It was found that zebrafish contain homologs to the human and mouse *adgrl* gene sequences, *adgrl1*, *adgrl2a*, *adgrl2b*, *adgrl3*, and *adgrl4*.

Located on chromosome 3 in zebrafish and chromosome 19 in humans, *adgrl1* (Ensembl ID ENSDART00000124480.2) contains 1522 amino acids with an open reading frame (ORF) and is 71% and 85% similar to the human and mouse homolog, respectively (Fig. 2A). In addition, *adgrl3* is located on chromosome 7 in zebrafish and chromosome 4 in humans. It also contains an ORF encoding 1347 amino acids and shares 69% and 70% similarity with the human and mouse homolog, respectively (Ensembl ID ENSDART00000171848.1) (Fig. 2B).

Utilizing the available database sequences, primers were developed and used to amplify previously generated cDNA from 30-hour post-fertilization and 5-day wildtype zebrafish, our results showed bands at the expected size, indicating that *adgrl3* is expressed in the zebrafish genome at these stages (Fig. 1). Amplicon size for *adgrl1* is 945 base pairs while *adgrl3* is 771 base pairs.

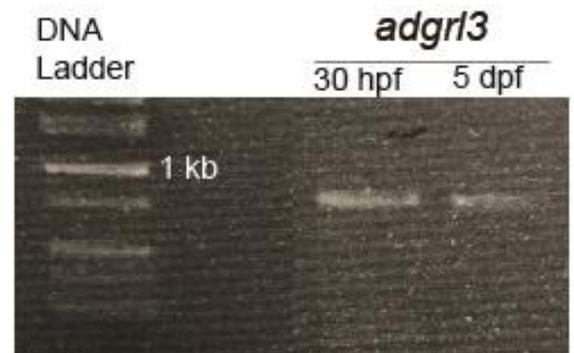


Fig. 1. 1% Agarose gel confirming the presence of *adgrl3* in zebrafish at 30 hpf and 5 dpf. Amplicon size is ~771 base pairs.

Linearized Plasmid Confirmation

Purified PCR product was transformed into DH5 α competent cells and were purified successfully. Confirmation by agarose gel electrophoresis (Fig. 3) shows that the plasmid was approximately 3,000 base pairs. Sequencing of *adgrl1a* showed the insert had antisense directionality and was linearized with Sall with T7 transcription buffer. Conversely, *adgrl3* sequencing showed in-sense directionality and was cut using ApaI with SP6 transcription buffer.

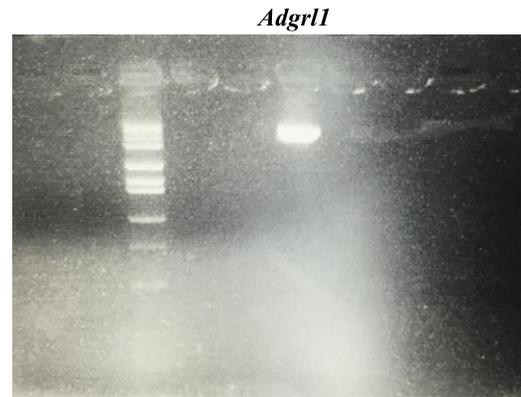


Fig. 3. 1% Agarose gel confirming the proper linearization of *adgrl1* into the vector. Product size shown ~3000 base pairs.

RNA Probe Verification

RNA was successfully synthesized and purified using the methods mentioned above. *Adgrl1* shows successful RNA probe creation; however, *adgrl3* shows the presence of multiple bands when fractionated by a 0.8% agarose gel. *Adgrl3* was remade with new reagents to ensure proper techniques and protocols were followed, but agarose gel fractioning showed the same results. Overall, both *adgrl1* and *adgrl3* had the proper RNA probe size (~300bp) (Fig. 4).

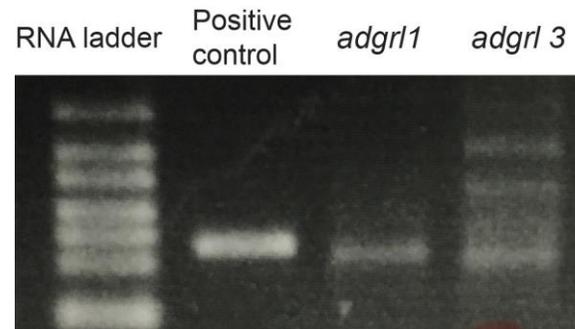


Fig. 4. 0.8% Agarose gel containing (from left to right): an RNA ladder, a positive control, *adgrl1*, and *adgrl3*. RNA probe size ~300bp for both isoforms.

Expression of *adgrl1* and *adgrl3*

Whole-Mount *in-situ* hybridization revealed information about the expression location and timing of *adgrl1* and *adgrl3*. *Adgrl1* showed expression levels within the eight-cell and 16-cell stages, demonstrating a maternal deposition of the gene (Fig. 5A,5B). As the embryo undergoes gastrulation, *adgrl1* is found to be expressed throughout the entirety of the animal pole, hinting at a possible role in early development (Fig. 5C). As gastrulation and somitogenesis occurs, *adgrl1* localizes to the hindbrain, midbrain, and forebrain, as seen in Fig. 5D. as well as near the location of the heart. Strikingly, *adgrl1* also hybridized in the location of the heart cone, a primitive stage of the heart (Fig. 5E). One can clearly see the expression levels within the brain, heart, and blood vessels at 26-hours post fertilization (Fig. 5F), as illustrated by the dark staining around the head and throughout the vasculature on the back. At day 2, staining is confined to the brain and heart and slight staining in the vessels (Fig. 5G,5H). Finally, at day 3, staining seems to be isolated in the heart, midbrain, and hindbrain (Fig. 5I).

Similar to *adgrl1*, *adgrl3* was found to be expressed in early blastula, eight-cell and 16-cell stages, once again implying maternal contribution (Fig. 6A,6B). It is expressed throughout during gastrula (Fig. 6C) and localizes in the heart and brain throughout somitogenesis. Specifically, *adgrl3* is highly expressed within the heart cone and brain at 22-somite, as seen in the dorsal view (Fig. 6D,6E). As the embryo matures, *adgrl3* becomes more confined to the brain and cardiovascular system, as seen in Fig. 6F-6H, 6J-6K, until its high expression is clearly visible at day 3 (Fig. 6I).

***Adgrl* Expression in Mouse Heart**

As previously mentioned, *adgrl2* (latrophilin-2) was found to be expressed within mouse endothelial cells in the atrioventricular canal. To determine whether other *adgrl* isoforms are present in the mouse heart, primers were developed for *adgrl1*, *adgrl2*, *adgrl3*, and *adgrl4*. It was shown that *adgrl2* and *adgrl4* were highly expressed in mouse cardiac tissue when compared to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

***Bai2* Phenotype Expression**

Previously injected *bai2* mutants from post-doctoral candidates have generated F₁ mutants that have been assessed for a deviant phenotype. The F₁ generation were maintained and

generated for the two mutations, and *bai2^{-/-}* and *bai2l^{-/-}*. *Bai2l^{-/-}* F₁ mutants were crossed and 24 embryos were collected. The embryos were assessed for the mutant phenotype at 22-somite. No mutants exhibited heart laterality defects. When *bai2^{-/-}* homozygous mutants were crossed, all 83 embryos demonstrated normal left-side cardiac laterality.

Adgrl Mutation Analysis

F₀ injected mutants were screened to determine the efficacy of the injection procedure. Fig. 7 shows the F₀ injection results of *adgrl1* and *adgrl3* when compared to controls. Mutant polymorphism bands can be clearly seen in all four samples in *adgrl1*, illustrating a high efficacy rate. Conversely, *adgrl3* injected mutants demonstrated a low efficacy as only one of the four samples demonstrated mutations. However, the all injected F₀ progeny was allowed to continue to grow to see if there would be any mutations shown in F₁ progeny.

Currently, *adgrl* injected Zebrafish have demonstrated transmissible mutations as seen in their a F₁ progeny when crossed with the wildtype genotype. Fig. 8 illustrates one type of mutation that from *adgrl3* F₁ progeny on a 10% PAGE gel that was been submitted for sanger sequencing. Sequencing results determined that the mutation in Fig. 8 was a frame-shift that was observed on

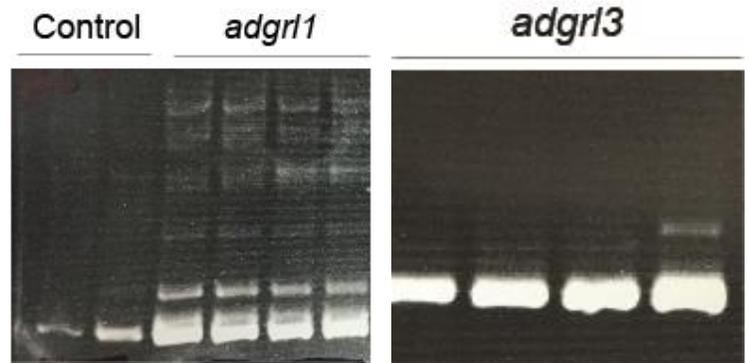
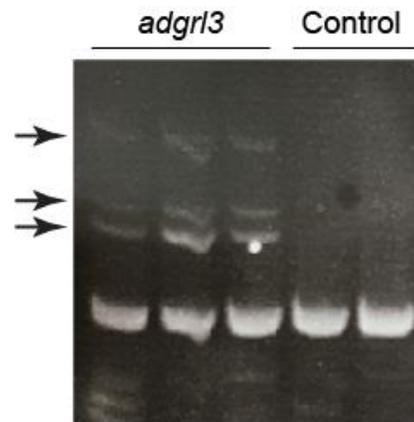


Fig. 7. F₀ mutant injection confirmation of *adgrl1* and *adgrl3*. Efficacy of injection can be seen when compared to control samples. *Adgrl1* demonstrates multiple polymorphisms as visualized by the extemporaneous bands above the control DNA in all samples. Conversely, *adgrl3* shows only one extemporaneous band above the control DNA, illustrating a low efficacy.



Wild-type	GGTGGACGAAAACGGCCT	CTGGGTGATTTACGCGACGGA
	G G R K R P	L G D L R D G
Mutant	GGTGGACGAAAACGGCCT	TCTGTCTGGGTGATTTACGCGACGGAG
	G G R K R P	S V W V I Y A T E

Fig. 8. Mutation analysis of F₁ *adgrl3* mutants via 10% PAGE gel. Three extra bands appear in samples 1,2, and 3 when compared to controls. Below, sequencing results demonstrated a 5 base pair insertion in the mutant had occurred, causing a frame-shift mutation as denoted in red.

the PAGE gel. The frame-shift was a 5 base pair insertion, creating new serine and valine amino acids (Fig. 8).

Discussion

***Adgrl* Gene Family**

As previously discussed, it has been demonstrated that all three isoforms, *adgrl1*, *adgrl2*, and *adgrl3* exist within the Zebrafish genome and have been successfully amplified, targeted, and located within the organism. Using whole mount *in-situ* hybridization, I confirmed the presence of *adgrl1* and *adgrl3* genes in the cardiovascular system. It appears that *adgrl1* is expressed ubiquitously throughout the embryo in blastula and gastrula stages, then is confined to the nervous and cardiovascular system. I have injected wildtype embryos with gRNA to create mutations in *adgrl1*. When the F₀ line grows to maturity, they will be screened to assess the type of mutation that occurred. The F₀ generation will then be in-crossed to generate F₁ mutant progeny that will be assessed for phenotypic variations.

Adgrl3 expression appears to be similar to that of *adgrl1* in many aspects including temporal and spatial characteristics; however, it appears that *adgrl3* is more strongly expressed in the cardiovascular system than in the nervous system. In contrast to *adgrl1*, *adgrl3* exhibits higher expression levels in the cardiovascular system, illustrated by the darker staining seen in ISH. This is clearly evident when one compares the anterior images of the day 2 embryos, Fig. 5G and Fig. 6K, for *adgrl1* and *adgrl3*, respectively. It is clear that, though there is expression of *adgrl1* in the heart, the level of expression differs vastly between the two isoforms. The level of expression within the heart for *adgrl3* is strong enough that it is possible to clearly see the atria and ventricle; leading to the belief that there is a substantially higher level of expression of *adgrl3* than *adgrl1* in the heart and implying that *adgrl3* may play a more dominant role in heart development than *adgrl1*. Like *adgrl1*, a mutant generation of zebrafish are being screened for sequence mutations and will be used to establish a stable line of mutants for phenotypic observation. One *adgrl3* mutation has already been observed in the F₀ injected mutants. Five base pairs have been inserted into this sample and may generate a stop codon; however, since the entirety of the genomic sequence is not known, it may be difficult to determine whether a

premature stop codon has been generated. As such, this mutant will be used to grow F₁ progeny, generate stable mutant lines, and monitored for any mutant phenotypes.

Bai Gene Family

As aforementioned, all F₁ *bai2* mutant embryos did not show any deviation in the phenotype, suggesting that either the mutation did not result in a frame-shift mutation and subsequent truncated protein, allowing the protein to continue functioning normally or other genes from the *bai* gene family were compensating for the loss of function from the mutation. To ensure there was a frame-shift mutation, the mutant DNA was sequenced. The sequence demonstrated that the mutation had worked as intended, thus leading to another explanation: the two mutations, since they display similar phenotypic defects in embryos, may be playing redundant roles and, when one site is mutated, the other allows the organism to grow normally. To determine whether they are redundant, *bai2*^{-/-} and *bai2l*^{-/-} mutants were crossed with each other. Double homozygote mutants will, when mature, be genotyped, sequenced, and observed for any possible phenotypic variation.

The goals of this research project are as follows: assessing the expression patterns and locations of *adgrl1*, *adgrl3*, and *bai2* isoforms and generating mutant lines to determine their respective roles in heart development. The results have demonstrated that *adgrl1* and *adgrl3* isoforms are present within the cardiovascular system and support the decision to generate mutant lines for further study. Moreover, they have demonstrated a lack of phenotype in *bai2*^{-/-} and *bai2l*^{-/-} mutants, possibly due to the two genes playing redundant roles. I will be continuing to develop double homozygous mutants to rule out the possibility of redundancy. As the project develops, I expect to have stable *adgrl1*, *adgrl3*, and *bai2*^{-/-}/*bai2l*^{-/-} mutant lines from which to assess their functions in cardiovascular development.



Fig. 5. expression of *adgrl1* during zebrafish embryogenesis. **A-I:** Whole mount *in-situ* hybridization revealed expression patterns at various stages: eight-cell (A), 16-cell (B), shield (C), 22-somite (D-E), 26-hour post-fertilization (F), day 2, and day 3 (I).

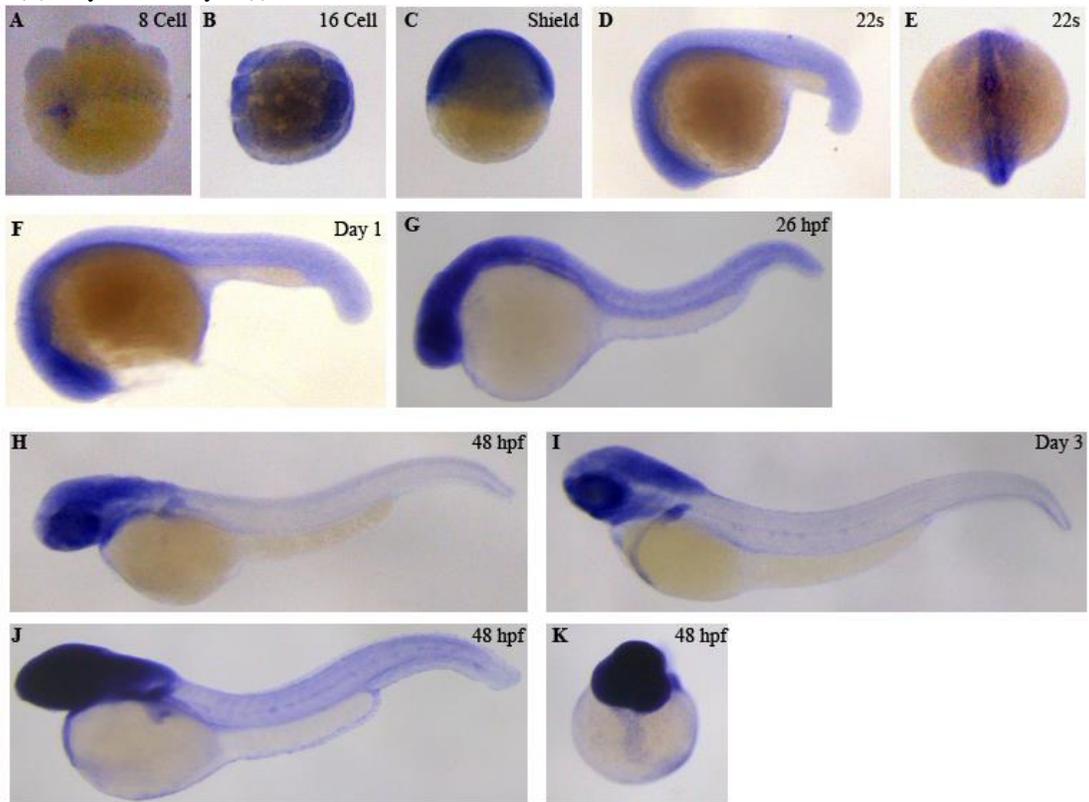


Fig. 6 expression of *adgrl3* during zebrafish embryogenesis. **A-K:** Whole mount *in-situ* hybridization revealed expression patterns at various stages: eight-cell (A), 16-cell (B), shield (C), 22-somite (D-E), day 1 (F), 26-hour post-fertilization (G), day 2 (H), day 3 (I), 48-hour post-fertilization (J-K).

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