Understanding Orofacial Defects via BMP-2 Knockout in *Xenopus laevis* embryos

1. **Abstract**

Birth defects are among one of science’s most disconcerting and elusive problems, accounting for over 20 percent of all infant deaths in the United States alone (1). Cleft lip and palate is one of the most common birth defects, and tends to affect more impoverished nations, where healthcare and treatment may not be as readily available or feasible as in the U.S. (2, 3). Unfortunately, as is the case with many other birth defects and genetic anomalies, the biological mechanisms that cause cleft lip and palate are poorly understood, and aside from post-natal treatment, there is no definitive cure. This research proposal aims at continuing the work of Wahl et al (2018), and improving our understanding of the genetic interactions responsible for cleft lip and palate formation.

1. **Introduction**

Orofacial clefting constitutes a significant portion of birth defects, with a wide range of abnormalities, causing oral, facial, and craniofacial malformations (3, 4). Orofacial clefts are separated into those that affect the lip and/or palate (CL/P), and those that affect only the palate (CPO), with CL/P being the most predominant cleft type (4). Although many of the genes, proteins, and ligands responsible for proper orofacial development are known (WNTs, BMPs, FGFs, SHH proteins, Retinoic Acid), our understanding of the complex interactions between these signaling pathways is crude (5). In an attempt to study orofacial development and clefting, scientists have performed experiments on model organisms such as frogs, mice, and zebrafish (5, 6, 7). A recent study by Wahl et al (2018) has emulated orofacial clefting in frog embryos via inhibition of Retinoic Acid signaling, and in a transcriptomic analysis, uncovered a network of genes associated with cleft palate (5). Of that network of genes associated with cleft palate, BMP-2 was one of the most altered genes, along with Wnt1 and RARg. To continue the findings of this novel research, a knock-out experiment on the gene BMP-2 will be performed in *Xenopus laevis* embryos using CRISPR/Cas9, and the resulting expression levels of Wnt1 and RARg will be measured using RT-PCR.

1. **Background of Cleft Lip and Palate, and its Physical and Emotional Implications**

Cleft lip and palate in humans is one of the most common occurrences of birth defects worldwide, involving improper fusion of the palate tissue during development, with a global incidence rate of about 1 per 600 births (3). The physical implications of a cleft lip and palate can involve impaired functioning of basic biological processes, such as: breathing, eating, hearing, and speech (4, 8). The emotional implications can involve issues with self-esteem due to concerns of physical appearance compared to others, which is emphasized in today’s society due to social media. Unfortunately, aside from about a dozen surgeries, and years of speech therapy, there is no definitive cure for cleft lip/palate, only treatment (8). Even surgical treatment can be troublesome, as improper timing of the procedure can cause inhibition of facial growth, and delayed speech development (8). All of these factors result in an unsettling reality for the millions of children and families affected by this common birth defect.

1. **Background of BMPs, the gene BMP-2, and the major steps in craniofacial development**

BMPs, or bone morphogenetic proteins, are a specific class of signaling molecules belonging to the transforming growth factor beta (TBF-beta) family (9). BMPs are primarily known for bone and cartilage development, however, they are also responsible for a vast range of developmental processes, including cell proliferation, differentiation, apoptosis, and morphogenesis (4, 9, 10, 11, 14, 15). There are many different types of BMPs, however, this research proposal will focus specifically on BMP-2, as it was found to be one of the most altered genes in Wahl et al’s transcriptome analysis of clefted frogs (5, 11). In addition, Sahoo et al (2011) conducted an experiment that sequenced and analyzed the DNA of three patients who had microdeletions within the BMP-2 coding region. All three patients exhibited similar clinical phenotypes of cleft palate and facial dysmorphism, suggesting that microdeletion of the BMP-2 gene in humans is associated with orofacial clefting (16). Furthermore, a review article identified the role of BMP signaling during the development of cranial neural crest cells, which are specialized cells that give rise to the craniofacial skeletal and cartilage tissues (10).

BMP signaling is a complex process that begins when a BMP dimer ligand binds extracellularly to Type 1 and Type 2 BMP Receptors (9).  The ligand binding process initiates autophosphorylation of the kinase domains bound intracellularly to the BMP Receptors. This leads to the recruitment and activation of transcription factors SMAD 1 and SMAD 5, forming a heterodimer, which is then trimerized with SMAD 4, and translocated to the nucleus (9).  The SMAD complex then binds directly to the DNA, and has the ability to regulate gene expression via chromatin remodeling proteins, and interactions with tissue-specific transcription factors (9).

This signaling cascade is important to note, as BMP signaling plays a key role in craniofacial development (12). The major steps in craniofacial development begins with the migration of neural crest cells into the pharyngeal arches via a morphogen gradient (12). Following that is the growth and convergence of facial prominences, which is caused by a BMP morphogen gradient (12). Then, Palate shelf fusion occurs, where TGF-beta signals epithelial cell apoptosis and mesenchyme cell migration (12).  Finally, BMP inhibitors initiate cell death for mouth creation, concluding the process of craniofacial development (12). The formation of the craniofacial region is initiated by a complex and intricate morphogenetic gradient, consisting of many different transcription factors, one of them being BMP-2 (12).

Disruption of any of these biological processes leads to orofacial clefting (4, 13). While we are cognizant of BMP-2’s role in development, we are still unsure of exactly how this signaling pathway integrates with other signaling pathways, transcription factors, and epigenetic regulators during palate development. To better understand the formation of cleft lip and palate, this experiment aims to discover the gene regulatory networks associated with BMP-2 during palate development, with the intent to discover key contributors responsible for orofacial clefting. With a deeper understanding of these key contributors, connections can be drawn between the complex gene regulatory network and the phenotype of orofacial clefting, perhaps leading the way to a cure for cleft lip and palate.

1. **Materials and Methods**

BMP-2 will be knocked out via microinjection of the CRISPR/Cas9 complex into 1-cell stage *Xenopus laevis* embryos. First, a sgRNA target sequence will be constructed, containing an –NGG PAM site, with a length of about 20 nucleotides complementary to a coding region of the BMP-2 gene. Then, the CRISPR/Cas9 complex containing the BMP-2 specific sgRNA and the Cas9 nuclease will be microinjected into the embryos. Afterwards, the sgRNA will bind to the target sequence on the BMP-2 gene, where the associated Cas9 nuclease will induce a double stranded break upstream of the PAM site region of the target sequence.  The two broken strands of DNA will repair via Non-Homologous End Joining, in which the two broken strands of DNA reconnect, except with random additions or deletions of nucleotides. This will induce a frameshift mutation, resulting in the desired non-functional BMP-2 gene. The embryos will then develop for three days, at which point they will undergo DNA extraction to verify if there was successful mutation or not. Then, a sample of the orofacial tissue from the frogs will be dissected, and after RNA isolation, will provide the RNA needed for RT-PCR. The resulting product of RT-PCR will undergo qPCR, where the delta-delta CT method will determine if BMP-2 knockout altered Wnt1 and RARg gene expression or not.

**Designing the sgRNA**

In order to construct the sgRNA, a web tool for CRISPR-based genome editing, CHOPCHOP, was utilized, where the target gene (BMP-2) and the organism of interest (*Xenopus laevis*) were selected for knock-out using CRISPR/Cas9. 

The tool scanned the entire genome of *Xenopus*,selected target sequences for BMP-2 knock-out, and ranked them based on: GC content, self-complementarity, and efficiency percentage. I opted to use the 3rd ranked target sequence, TGTCCGCAGGTCGACCATGGTGG, because it had a higher GC content, and zero self-complementarity when compared to the first two ranked target sequences. Previous studies have shown that CRISPR/Cas9 editing efficiency is markedly increased when the GC content is 65% or more (17). Additionally, others have observed that self-complementarity can disrupt efficient incorporation of the sgRNA into the targeted region (18). Although the third target sequence had a slightly lower efficiency percentage than the first two sequences, the difference was marginal. The combined factors of an optimal GC content, zero self-complementarity, and a relatively high efficiency percentage were the reasons why the third sgRNA target sequence will be used in this experiment.



**Procurement of *Xenopus laevis* embryos for control and experimental groups**

After the sgRNA is constructed, *Xenopus laevis* adults will be purchased from Nasco, which will then undergo breeding at VCU’s Dickinson lab for embryo production (5, 19). The embryos will be collected and placed in a petri dish containing 0.1X Modified Barth’s Saline (MBS), an embryo and tadpole culturing solution (5, 19). There will be two groups in this experiment: the control group, which is injected with a mismatched sgRNA designed not to target any functional genes, and the experimental group, which is injected with the sgRNA designed to target the BMP-2 gene.

**Microinjection of sgRNA & Cas9 protein into the control and experimental groups**

The respective sgRNA and the Cas9 protein will be co-injected into the two groups of 1-cell stage *Xenopus laevis* embryos using a nanoinjector and a light microscope, shown below (5).



 This will begin by placing the embryos in a custom acrylic injection tray (shown below), which contains tiny recesses that hold the embryos in place, facilitating injection (20).



Following successful microinjection of the CRISPR/Cas9 complex into the control and experimental groups, the embryos will be rinsed in 0.1X MBS to remove any dying embryos, as they can impair the growth of the healthy embryos (20). They will then be placed into a petri dish containing 0.1X MBS, where they will culture and incubate at 23 °C (20). The frog embryos will be incubated for 2 ½ - 3 days, at which point craniofacial development, as well as potential orofacial clefting due to BMP-2 knockout has begun (5).



**Assessing embryos for mutations in BMP-2 via PCR**

After the embryos are cultured for 2 ½ - 3 days, the control and experimental groups will be subdivided into two more groups, with both subdivisions containing control and experimental embryos. The first subdivision will test for mutations in the BMP-2 gene. This will be done by extracting the DNA, which will then undergo PCR, where the PCR products will be interpreted using agarose-gel electrophoresis. The DNA will be extracted similarly to how CRISPR was injected, using a nanolinter injector in conjunction with a light microscope and the acrylic tray full of embryos (20). After successful DNA extraction, the DNA will undergo PCR. The PCR products are run on agarose gel electrophoresis (shown below), where the DNA can be separated based by size. Visualization of the resulting PCR products on agarose gel, compared to PCR products of the healthy gene can help determine if there was a mutation or not. This will ensure there were no mutations in the control group, and ensure there was successful BMP-2 knockout in the experimental group. This will serve as a critical step in the experiment, as improper mutations may significantly alter the results of the research.



**Microdissection of orofacial tissue in control and experimental groups**

The other subdivision will undergo orofacial microdissection and RNA extraction, another important step in obtaining high-quality RNA for RT-PCR. The RNA will be extracted by placing the control and experimental embryos in a Petri dish containing 0.1X MBS, which is then chilled at a temperature of 15 °C (5). The area around the oral region of the frog embryos will be dissected using a dispensable scalpel, shown below (15 T Sklar Instruments, Cat. No 06-3120) (5).



**RNA isolation from dissected orofacial tissues using TRIzol reagent**

After microdissection, the facial tissues will undergo RNA isolation using TRIzol reagent. TRIzol is an effective reagent used to isolate RNA from proteins and DNA (21). TRIzol contains a solution of phenol and guanidinium isothiocyanate, which separates the RNA, DNA, and proteins based on solubility properties following reaction with the solution (21). The extracted facial tissues will be placed in 50mL centrifuge tubes, which will then be treated with TRIzol reagent, in a ratio of 1mL TRIzol reagent per 50 to 100mg of facial tissue (22). It is important to note that the tissue sample volume should not exceed 10% of the TRIzol reagent volume (22). Then, a phase separating agent, chloroform, is added, causing phase separation of the aqueous layer, containing RNA, the interphase layer, containing DNA, and the organic layer, containing the denatured proteins and lipids (23).



The ratio of chloroform added is 0.2mL of chloroform per 1mL of TRIzol reagent (22). The samples will then be vortexed for 15 seconds, and incubated at 23 °C for 2-3 minutes (22). Then, the samples will be centrifuged at 12,000 x g for 15 minutes, at a temperature range between 2-8 °C (22). The top layer of aqueous phase containing RNA will be carefully transferred into a new centrifuge tube (22). Following phase separation, the RNA in the aqueous phase is collected by isopropanol precipitation (23). The ratio of isopropanol used is 0.5mL of isopropanol per 1mL of TRIzol reagent (22). The isopropanol-RNA mixture will then be incubated for 10 minutes at a temperature range between 15-30 °C (22). Following incubation, the mixture will undergo centrifugation at 12,000 x g for 10 minutes, at a temperature range between 2-4 °C (22). The RNA will then precipitate, and form a pellet on the bottom of the centrifuge tube. The supernatant is removed, and the RNA pellet is washed once with 75% ethanol, with a ratio of 1mL of 75% ethanol per 1mL of TRIzol reagent used. The samples are vortexed and then centrifuged at 7,500 x g for 5 minutes at a temperature range between 2-8 °C (22). The washing procedure is repeated again, and then the excess ethanol is removed via air-drying the RNA pellet for 5-10 minutes (22). After the pellet is washed in ethanol and air-dried, the isolated RNA can be sequenced for RT-PCR.

**Using RT-PCR for cDNA synthesis**

RT-PCR, or Reverse Transcriptase PCR, will be the method used for analyzing expression levels of Wnt1 and RARg in response to BMP-2 knockout, as it is especially useful for gene expression measurement (24). Since gene expression is produced by RNA, being able to quantitatively measure the count of RNA’s can lead to extremely accurate observations of gene expression (25, 26). RT-PCR’s unique ability is that it can amplify templates of single stranded mRNA’s, in comparison to regular PCR only amplifying templates of double stranded DNA (24). RT-PCR is a two-step process, with steps similar to traditional PCR, but with a few distinct changes. The first step involves a reaction between the isolated RNA and the enzyme reverse transcriptase, which synthesizes single-stranded complementary DNA, or cDNA, in a process called reverse transcription (24, 27, 28). In a centrifuge tube, an RNA/primer mixture will be prepared: 5µg of isolated RNA, 3µl of random hexamers, 1µl of 10 mM of dNTP mix, and 10µl of DEPC-treated H2O (22). Then, the sample will be incubated at 65°C for 5 minutes, and then chilled on ice for 1 minute (22). Then, in another centrifuge tube, a reaction master mixture will be created: 2µl of 10x reverse-transcriptase buffer, 4µl of 25 mM of Magnesium Chloride, 2µl of 0.1 M DTT, and 1µl of RNAaseOUT (22). The two mixtures will be combined and then placed at room temperature for 2 minutes. Then, 1µl of the enzyme reverse transcriptase will be added to the tube, which will then incubate at 25°C for 10 minutes (22). The mixture will then be incubated at an increased temperature of 42°C for 50 minutes, then at 70°C for 15 minutes, and then chilled on ice. Finally, 1µl of RNase H is added, and the mixture is incubated one last time at 37°C for 20 minutes (22). The result is cDNA, which must be stored at -20°C until qPCR is performed. Once the cDNA is created, Wnt1- & RARg-specific primers and DNA polymerase are used to transform the cDNA into traditional double-stranded DNA (24, 28).

**Constructing high-quality primers for Wnt1 and RARg**

Constructing high-quality primers is critical to efficient reverse transcription and amplification (29). Utilizing the NCBI tool Primer Blast, primers for Wnt1 and RARg were constructed. First, the Pubmed gene database was accessed, where the genes of interest (Wnt1, RARg), as well as the organism (*Xenopus laevis*) were entered into the search function. The resulting gene of interest was selected, and the NCBI RefSeq gene ID was identified (NM\_001101736.1 for Wnt1, and NM\_001088194.1 for RARg). Then, the option “Pick Primers” was selected, which prompted the Primer Blast tool, where the RefSeq gene ID was entered, as well as primer parameters, such as PCR product size (Min of 70 and Max of 200). There are many parameters that correspond to high-quality primers, such as: 18-22 base pairs, a melting temperature range of 55-65 °C, and a GC content of 40-60% (29, 30). Additionally, there are many parameters that are to be avoided when constructing high-quality primers, such as: hairpins in the DNA, more than 4 di-nucleotide repeats, more than 4 of the same nucleotide in a row, and GC-rich 3’ ends (29, 30). Using this information, and the primers constructed by the NCBI tool Primer Blast, the following primers were chosen for Wnt1 and RARg, with reasoning below.

Primer pair 1 is the primer selected for Wnt1. Compared to the 9 other primer pairs, this one had: the ideal amount of base pairs (20 on both the + and the – strand), optimal Tm range (60.47 for the + strand, 60.18 for the – strand), GC content (50% for the + strand, 55% for the – strand), the lowest amount of self-complementarity, did not contain any nucleotide repeats that exceeded four base pairs, and did not contain any di-nucleotide repeats of four or more. 

Primer pair 7 is the primer selected for RARg. Compared to the 9 other primer pairs, this one had the ideal amount of base pairs (20 on the + strand, 19 on the – strand), ideal Tm (59.67 on the + strand, 60.04 on the – strand), ideal GC content (60% on the + strand, 55% on the – strand), had one of the lowest amounts of self-complementarity when compared to other primers, did not contain any nucleotide repeats that exceeded four base pairs, and did not contain any di-nucleotide repeats of four or more.


Once the primers have been constructed, the following 25-50µl mixture will be prepared : 12.5µl SYBR Green Mix (2x), 0.2µl of cDNA, 1µl of the gene-specific primer pair (5pmol/μl each primer), and 11.3µl of H2O (31). The mixture will then undergo qPCR.

**Using qPCR to measure gene expression levels of Wnt1 and RARg**

qPCR, or quantitative real-time PCR, is very similar to traditional PCR, except in addition, the DNA is tagged using a fluorescent-dye (SYBR Green Mix), and in between each thermal cycle, the fluorescence is measured (24, 28, 31, 32). As qPCR progresses, and with each duplication of the DNA, the fluorescent signal is equally duplicated (31). Since there are initially only a few template strands, the fluorescent signal is faint, and cannot be measured (31). However, usually after about 15 cycles, the threshold at which fluorescence can be detected is deemed the Threshold Cycle, or Ct (31). Once the Ct value is reached, the quantification of gene expression can begin (31).



**Analysis of qPCR using Relative Quantification and the delta-delta CT analysis test**

In relative quantification, the Ct values between the control and the experimental groups will be compared, resulting in a quantification of gene expression changes (33). This is done by utilizing a reference gene within the *Xenopus* genome, which acts as an internal control for normalizing the data (34). An experiment by Mughal et al (2018) identified 14 candidate reference genes in *Xenopus laevis*. Of those 14, the gene slc35b1, a nucleotide sugar transporter gene, was chosen, due to it having a completely different function than BMP-2, Wnt1, and RARg (34). It is important that the reference gene should exhibit constant expression, and should not change gene expression levels when presented with any experimental treatment (33). Following the selection of a reference gene, four Ct values are calculated: one for the control reference gene, one for the control target gene, one for the experimental reference gene, and one for the experimental target gene (33, 35). The difference in Ct values of the control group gives the delta CTC value, and likewise, the difference in Ct values of the experimental group gives the delta CTE value (33, 35). Then, the delta-delta Ct value will be calculated, with equation being 2^(delta CTE value)/2^(delta CTC value) (33, 35). This will then give us the relative expression change in the experimental group.



1. **Results**

Although no results are obtained due to the nature of this being a proposed research experiment, potential results will be discussed. Following successful BMP-2 knockout, RT-PCR, qPCR, and delta-delta Ct analysis, the relative gene expression for both Wnt1 and RARg could end up decreasing. This would likely be due to high Ct values, as a minimal amount of RNA produced by the decreased gene expression would require a large number of amplification cycles before the threshold cycle (Ct) is reached, translating to a high Ct value. Additionally, the relative gene expression for both Wnt1 and RARg could end up increasing. This would be shown by having very low Ct values, as a large amount of RNA’s produced by the increased gene expression requires very little amplification before the threshold cycle (Ct) is reached, translating to a low Ct value. Another possible result is that the relative gene expression for both Wnt1 and RARg could end up staying the same. This would be shown by having Ct values equal to that of the reference gene, or control. Since the level of RNA’s did not differ, the Ct value would remain at the typical threshold, translating to no change in gene expression. These potential discoveries will further our knowledge of the important roles that BMP-2, Wnt1, and RARg play during orofacial development, as well as shedding light on the interactions amongst these genes. This information is invaluable, as many of the genetic interactions are still yet to be uncovered. The uncovered interactions between these genes could pave the path towards better understanding orofacial clefting, craniofacial development, and cleft lip and palate.

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