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The Effects of Inhibiting Wnt Secretion and Activity on Cranial And Neural Development

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ABSTRACT

The Effects of Inhibiting Wnt Secretion and Activity on Cranial and Neural Development

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Wnt signaling has been shown to have several roles in the development of sensory neurons, particularly in the ophthalmic portion of the trigeminal nerve. Many of these studies have relied on the conclusion that Wnt is necessary but not sufficient for the induction and maintenance of the neural precursor cells that develop in the ophthalmic placode. Wnt had been inhibited in the ophthalmic placode using a dominant negative t-cell factor (TCF) and resulted in the loss of Pax3 expression (indicative of undifferentiated placode cells) in all targeted cells, suggesting a loss of specification/commitment of these cells to the sensory neuron fate. This study aimed to build on that conclusion by identifying the source of Wnt signaling that allowed for the maintenance of these placode cells. To investigate this, chick embryo *ex ovo* cultures were used and treated with small molecule chemical Wnt inhibitors to globally knock out Wnt signaling. The embryos were then sectioned and stained for cell markers of undifferentiated placode and differentiated neural cells (Pax3 and Islet1, respectively). Also used was a conditional knockout of *Porcn*, a gene critical to post-transcriptional modification of the Wnt ligand, using Wnt1-cre as a driver; this allowed for the knockout of Wnt secretion from the dorsal neural tube as well as neural crest cells. The data showed a decrease in placode cell differentiation but did not indicate a necessity for Wnt in maintenance of the ophthalmic placode cells—there was no loss of Pax3 expressing cells in the ectoderm. This suggested that maintenance of the ophthalmic placode could be through alternate pathways. Data is also presented describing how loss of *Porcn* in Wnt1 expressing cells impacts craniofacial development, where the mouse mutant used in this study displayed the absence and underdevelopment of cranial neural crest structures.

Keywords: wnt, ophthalmic, placode, craniofacial defects, Pax3, trigeminal

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INTRODUCTION

Cranial development and neural development in embryogenesis are both intricate processes guided by a variety of pathways and signaling gradients. In addition to various divisions, invaginations, folding, and other cell movements, there is very specific induction and differentiation of cell populations to correctly develop each structure. Because of the many factors involved in proper development, it's hard to know what is responsible when abnormalities occur. While there are many pathways that are active in and manage the specific development of both general cranial and cranial neural structures, my goal was to examine the source and roles of Wnt family members specific to cranial neuron development.

The major research focus of the Stark Lab at BYU has been to study sensory neuron formation in vertebrates. Several studies from the group examined the role of various pathways, such as FGF, Notch, and Wnt, to see how they contributed to the development of sensory neurons (Lassiter *et al.,* 2007, Voelkel *et al.,* 2013, Lassiter *et al.,* 2009). One particular study that was surprising was that of the Wnt pathway and its role in the development of the ophthalmic trigeminal placode; Wnt was seen to be necessary for not only induction but also maintenance of the placode structure. While Wnts from the midbrain region have been implicated in trigeminal placode cell development, they have additionally been seen to be critical in the formation of the neural crest (NC) from this region (Lassiter *et al.,* 2007, Adams 2012, Canning *et al.,* 2008, Brault *et al.,* 2001; Chang and Hemmati-Brivanlou, 1998; Dorsky *et al.,* 1998; Garcia-Castro *et al.,* 2002; Ikeya *et al.,* 1997; LaBonne and Bronner-Fraser, 1998; Lewis *et al.,* 2004; Saint-Jeannet *et al.,* 1997)). As you will see in this thesis, I happened upon a potentially important NC phenotype while investigating Wnt function in sensory neuron formation.

Wnt family members are generally segregated into two categories: the canonical/β-catenin-dependent and the non-canonical/β-catenin-independent pathway. Wnt is a paracrine pathway and thus a ligand is prepared and secreted from one cell and, in the canonical pathway, binds to co-receptors frizzled and low-density lipoprotein receptor-related protein (LRP) on another cell. This results in the phosphorylation and translocation of disheveled to the membrane and Axin binding to LRP5/6, thus dissociating the destruction complex (which normally targets β-catenin for degradation in the absence of Wnt). β-catenin then enters the nucleus and associates with t-cell factor (TCF)/lymphoid-enhancing factor (LEF) receptors to activate transcription (Fig 1).

In past experiments, various methods have been used to interrupt the Wnt pathway and determine how it's involved in neural development (Lassiter *et al.,* 2007, Adams 2012). A prime area to study and isolate development of sensory neurons in particular has been the trigeminal ganglion (cranial nerve V), which mediates touch, pain, and temperature of the face. It forms from neural crest cells and two overlapping placodes: the ophthalmic (opV) and maxillo-mandibular (mmV) placodes (D'Amico-Martel and Noden, 1983; Hamburger, 1961). The ophthalmic lobe of the trigeminal nerve specifically innervates the skin of the head as well as the eye muscles and nose (Baker and Bronner 2001). The earliest expressed marker of these specified cells (Pax3) was pinpointed and used to define important stages of development in the opV placode—induction, specification, commitment, and differentiation (Stark *et al.,* 1997). Pax3 expression was then used as evidence of induction and maintenance of the opV placode in response to inhibiting various signals. Lassiter *et al.* looked at the effects on Pax3 expression when all neural tube (NT) signals were blocked and found that NT signaling was necessary in opV placode induction (2007).

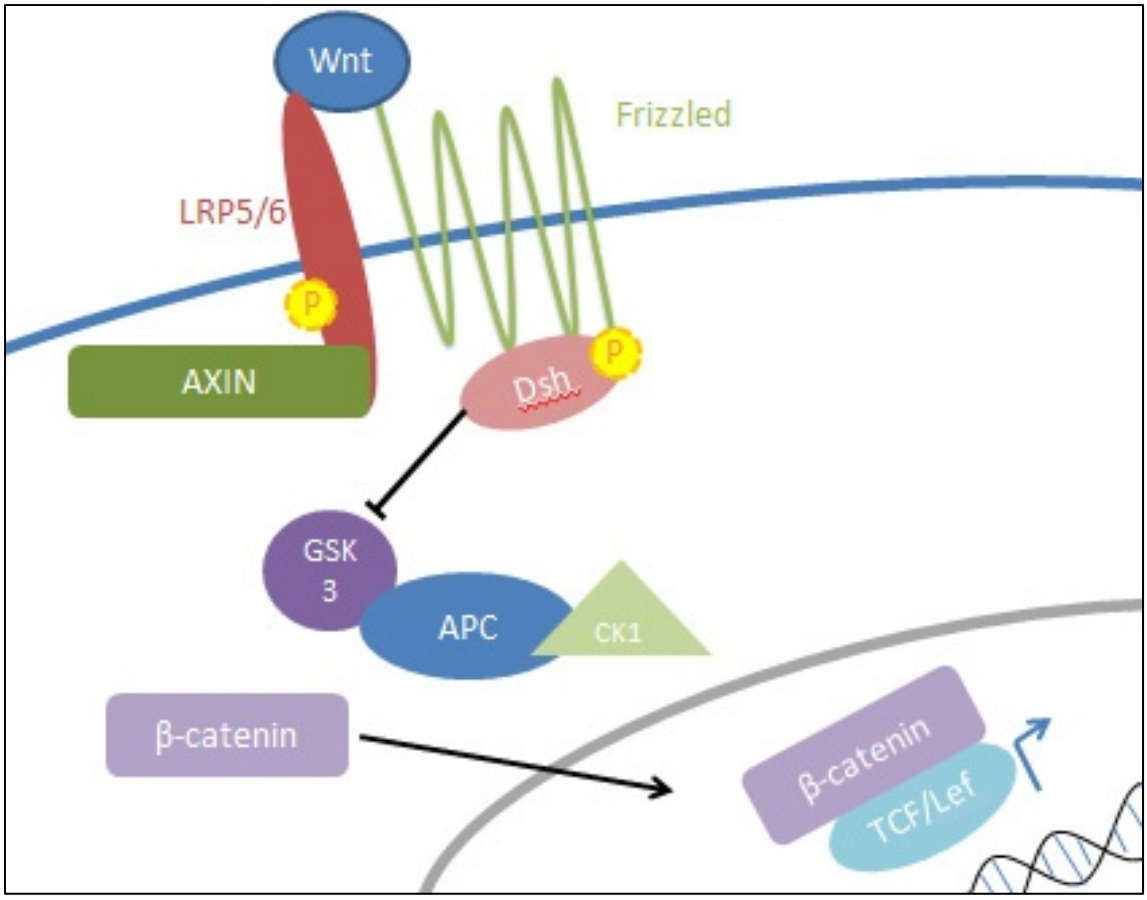


Figure 1: Canonical Wnt Pathway.

When the Wnt ligand is present, it binds to co-receptors frizzled, a 7-pass transmembrane G-protein coupled receptor, and low-density lipoprotein receptor-related protein (LRP) 5/6. Disheveled (Dsh) is then phosphoryled and translocated to the membrane and Axin binds to LRP5/6; this dissociates the destruction complex (normally composed of GSK3, APC, CK1 and Axin) and allows for β-catenin to persist. β-catenin enters the nucleus and associates with t-cell factor (TCF)/lymphoid-enhancing factor (LEF) receptors to activate transcription

Several Wnt family members had been shown to be expressed throughout the neural tube, some as early as 3 somite stage (ss), with corresponding Frizzled receptor expression in the cranial ectoderm and thus were used as candidates for induction of the opV placode (Lassiter *et al.,* 2007, Stark *et al.,* 2000). By misexpressing a dominant negative (DN) TCF4 in placodal ectoderm at various stages, Lassiter *et al.* found that placode cell formation was disrupted, suggesting a requirement for Wnt in placode cell induction and maintenance (indicated by loss of Pax3 expression) (2007). They also tested for sufficiency of the Wnt pathway by blocking all NT/ectoderm interaction while at the same time adding in dominant active B-catenin and observed no rescue, thus concluding that Wnt was necessary but not sufficient in opV placode development. Recent work produced by Jason Adams investigated the role of specific Wnts—Wnt1 and Wnt3a—in opV placode development by producing knockouts of each, individually, in mice. Neither mutant strains showed any decrease in Pax3 expression. However, a double-knockout of Wnt1 and Wnt3a showed diminished expression of Pax3 as well as diminished development of the trigeminal nerve (Adams 2012, Ikeya *et al.,* 1997). This was an important discovery, since it left open the possibility that other Wnts were involved in the maintenance of the opV placode.

Thus I looked at the manipulation of the gene Porcupine (*Porcn*) which codes for a membrane-bound O-acyl transferase required for the post-transcriptional modification of all Wnt proteins and thus ligand secretion. Mice from a Sox1-cre driver line were bred with *Porcn* mutant mice to create a conditional knockout of *Porcn* in the NT (based on the expression of Sox1). These mice showed no significant change in Pax3 expression. Upon investigation, it was shown that, while *Porcn* was eliminated from the majority of NT cells, it was not knocked out in all cells in the NT, particularly in a few cells in the dorsal neural folds where Wnt1 is expressed

(Adams, 2012). The results left a few questions: what is the source of Wnt expression required in maintenance of the opV placode? Is Wnt diffusing from the NT or is it expressed endogenously (i.e. within the ectoderm) to maintain the opV placode?

Our aim was to broaden our inhibition of Wnt and observe the effects in vitro to define if it is an endogenous signal or not. I did so by using two different chemical inhibitors (IWP-2 and XAV939) in embryo tissue and explant culture: Inhibitor of Wnt Processing (IWP)-2 inactivates *Porcn*, inhibits palmitoylation of Wnt, and prevents Wnt-dependent phosphorylation of Lrp6 and disheveled in addition to causing accumulation of B-catenin (Chen et al., 2009); XAV939 inhibits Tankyrase, a Poly-ADP-ribosyltransferase, resulting in a prolonged half-life for Axin2 and increased degradation of B-catenin (Karlberg et al., 2010, Huang et al., 2009). While no gross morphological effects were observed, the treatments led to a decrease in differentiated placode cells. This might suggest that Wnt expression is not required in maintenance of the opV placode as previously thought, but rather plays a role in allowing the cells to differentiate. Alternatively it could provide the foundation of a new model of sensory neuron differentiation wherein Pax3 is regulated by an alternate TCF-mediated pathway.

To clarify these potential models, I sought to more severely remove Wnt secretion in the NT by using a new cre driver line, Wnt1-cre, and crossing it with a *Porcn* mutant. This knocked out Wnt secretion from the NT as well as neural crest cells (NCC). While placode cell differentiation did not seem to be disrupted, severe craniofacial abnormalities were observed. These results led us to propose that Wnt, secreted from NCC, is required in craniofacial development, specifically those derivative structures from the first, second, and third pharyngeal arches.

MATERIALS AND METHODS

Obtaining of Chicken Embryos and Incubation

Fertilized chicken eggs were obtained from Dunlap Hatchery in Caldwell, ID and were incubated without rotation at 38℃ from 30-40 h to the 8-10 and 10-12 somite stage. When the embryos had developed to the desired stage, an adapted version of the chick whole-embryo culture developed by Susan Chapman *et al.* was used (Chapman 2001).

Explant Preparation

The eggs were cracked into a petri dish with the embryo top of the yolk. About 30 ml of thin albumin was removed from the petri dish with a 10 ml syringe. The thick albumin that surrounds the embryo and blastoderm was carefully moved away with a Kimwipe and a ring of Whatman no 2 filter paper with an outer diameter of 1.0 inch and an inner hole cut to be about 0.5 inch in diameter. The ring was placed around the embryo so that the embryo was centered in the ring. Scissors were used to cut out the vitelline membrane around ring's circumference. After the vitelline membrane has been detached from the yolk, it was removed and a second ring was placed on the ventral side of the embryo creating a sandwich of two rings with the embryo and vitelline membranes in the center.

Agar-Albumin Culture Dishes with Chemical Inhibitor

Agar culture dishes were prepared per Darnell and Schoenwolf's protocol (2000). 0.72 g of Bacto-Agar was mixed with boiling saline solution (7.19 g NaCl into 1 L distilled autoclaved water) and put in a water bath at 45℃ for 20 min. Meanwhile, 120 mL of thin albumin was collected from fertilized chicken eggs and Wnt antagonist XAV939, dissolved in

dimethylsulfoxide (DMSO) was added in order to achieve a concentration of 100 µM while an equivalent amount of DMSO was added to the control culture solution. After 20 min, the Bacto-Agar/saline solution was removed from the water bath and mixed with 120 µL Penicillin/Streptomycin by swirling for 30-60 sec in a flow hood. This solution was then aliquoted into separate tubes and mixed thoroughly with the previously measured DMSO or XAV939 in the respective individual tubes. Thin albumin (2:1 ratio with the agar/saline solution) was added and mixed well but gently enough to prevent the formation of bubbles. 2 mL of the solutions was placed in separate wells in 6-well dishes. Each well was then covered and allowed to set up for 3 h prior to use. The rings containing the embryos were laid gently in the separate agar wells containing DMSO or Wnt-antagonist. These embryos were allowed to incubate at 37℃ for a total of 24 h.

Collection

Following the incubation period embryos were cut out of the ring and vitelline membrane in a solution of 1X PBS. These same embryos were fixed in 1ml of 4% formaldehyde for one hour at room temperature or at 2-8℃ over night. Following fixation, embryos were washed three times with PBS, placed in 5% sucrose/PBS for 4h at room temperature, and then stored in 15% sucrose/PBS at 2-8℃ until sectioning.

Cryosectioning

Embryos were prepared for cryosectioning by embedding them in gelatin consisting of 7.5 g gelatin (Sigma) and 15 g sucrose filled to a volume of 100 ml with 1xPBS. Each embryo, in 15% sucrose/PBS, was heated alongside the 15% gelatin to 37.5℃ for 20-30 min to equilibrate temperatures. The embryo was then removed from the 15% sucrose/PBS with tweezers or a

capillary pipette and placed into the warmed gelatin to sit for 2-3 h. Warmed gelatin was then placed in a mold and the embryos were removed again with tweezers or a capillary pipette and placed into a gelatin-filled mold. After positioning the embryo, the gelatin block was allowed to sit for at least 30 min at room temperature and then stored in the refrigerator or taken and prepared for sectioning. Each gelatin block was flash frozen in liquid nitrogen and sectioned at 10 μM with a cryostat, with the sections mounted in sequence on Superfrost Plus glass slides.

Immunohistochemistry

Embryo sections were rehydrated by placing them in PBS at room temperature for 30 min. Gelatin surrounding the embryo sections was removed by placing slides in 37℃ PBS for 5-10 min and then switching the slides to a new slide bath with warmed PBS for another 5-10 min, or until gelatin was dissolved and no longer visible. The cryosections were then stained for the expression of molecular markers Pax3, Islet1, and DAPI. Pax3 was used to mark cells that had been specified to the opV cell fate (Stark et al., 1997). In addition, Islet1 was used to stain cells that have undergone cell fate and committed to specify sensory neurons in the trigeminal ganglion (Sun et al., 2008). Pax3 primary antibody (mouse IgG2a 488 secondary) is diluted to a 1:500 concentration with PBS/BSA/Tween and Islet1 (mouse IgG2b 546 secondary) to a 1:500 concentration with the same mixture. Each antibody solution was allowed to sit at room temperature for 10 min before application. 300μl was applied to each slide and stored at 25℃ for 4 h or overnight at 4℃. Each slide was then washed with PBS and covered with 300μl of secondary antibody diluted (1:1000) in PBS/BSA/Tween and stored for one hour at 25℃. Following the application of the secondary antibody, the slides were again washed with PBS and cover-slipped. Fluorescent images of the staining were taken with an Olympus BX61 microscope.

Collagen Matrix Cultures

After incubating the eggs to somite stage 8-10, the eggs were cracked and placed into a petri dish. The embryo was cut out and placed into PBS with 1% penicillin-streptomycin. The head of the embryo was removed and placed on ice in DMEM with 1% penicillin-streptomycin. Explants were stored on ice in medium until ready to be placed in collagen gel culture. Collagen matrix gels were prepared as previously described (Artinger and Bronner-Fraser, 1993) except commercially produced collagen was used (Collaborative Research). The bottom layer (20µLcollagen) was allowed to set for 5-7 min at room temperature before the explants were added. The top layer (4µLcollagen) was allowed to set for 10 min at room temperature before addition of medium plus N-2 supplements (Gibco-BRL). Wnt inhibitors XAV939 and IWP-2 were added to N-2 supplement media in concentrations from 5µM to 100µM. A corresponding concentration of 0µM of DMSO was added to N-2 supplement media for the control. Cultures were incubated at 38℃, 5% CO2 for 24 h.

Preparation and Collection of Mouse Embryos

The Wnt1-cre driver line was obtained from the Jackson Labs (stock #022137) and was cared for and breeded here with help from the Barrow Lab at Brigham Young University. A conditional knock-out had been made using a female mouse heterozygous for a X-linked *Porcn* knock-out obtained from the University of Utah (Barrott *et al.,* 2011). When bred with the Cre driver lines, it created a conditional knock-out of *Porcn* where Wnt1 is expressed (namely, in the dorsal folds of the neural tube). The embryos were allowed to grow until embryonic day 8.5 (E8.5) and embryonic day 9.5 (E9.5). Then, the mother was sacrificed and the embryos were removed from the uterus and fixed in paraformaldehyde. Sections of yolk sac were cut from each embryo and placed in a tube with 2µLproteinase K and 100µLear lysis buffer, incubated

overnight and then boiled. These were used to genotype the mouse embryos. Whole mount imaging was done using bright field microscopy.

Genotyping

PCR was done on the yolk sac DNA (preparation described above) to determine the sex of the embryos. Previously designed primers were used to amplify the SRY region of the prepared DNA. Presence of the SRY and Cre genes indicated mutants in the litters.

Whole Mount In Situ Hybridization

A dioxygenin (DIG)-labeled RNA antisense probe was synthesize from a plasmid of *Axin2* (obtained from Clare Baker, Cambridge University, England). Whole mount *in situ* hybridization was performed in chick embryos described Henrique et al. (1995). Briefly, formaldehyde fixed embryos of appropriate developmental stages were buffered and exposed to a DIG-labeled antisense RNA probe, which recognized the *Axin2* mRNA transcripts. After removal of the nonspecifically adhering probe, the embryos were incubated with an alkaline phosphatase (AP) -labeled anti-DIG antibody, followed by a chromogenic substrate for AP. The embryos were removed and washed in with 1%Tween/PBS and imaged with bright-field microscopy.

Skeletal Preps

Fetuses were obtained at 18.5 days post conception (dpc) by caesarean section, eviscerated, and fixed in 95% alcohol for 5 days. The fat pads are dissolved by incubating the fetuses in acetone for 48 h. The fixed fetuses were then incubated in alizarin red/alcian blue stain solution at 37℃ for 7-9 days and then washed 3 times in water. The fetuses were then incubated

with Sodium tetra-borate with Trypsin (30% Sodium Borate, 1% trypsin) at 37℃ for 5-6 h. They were cleared in grades KOH:glycerol solution--80% KOH:20%glycerol, 50% KOH: 50% Glycerol, 20% KOH: 80% Glycerol, and lastly 100% glycerol for 48 h each.

Statistical Analysis

Sections were randomly selected using Research Randomizer Form 4.0V (http://www.randomizer.org/form.htm) and cell counts were done using Olympus Cellsens software. The populations of cells counted included Pax3 antibody expressing cells, Islet1 antibody expressing cells, and Pax3/Islet1 antibody coexpressing cells in the ectoderm and mesenchyme. SAS software, version 9.4 (SAS Institue Inc., Cary, NC), was used to do a One-way ANOVA and Tukey-Kramer test. One-way ANOVA was used to determine if the means of all of the groups were equal or not. Tukey-Kramer test was used to show if and which means differed significantly in the ANOVA. P-values of ≤ 0.05 were used to indicate statistical significance.

RESULTS

Global Inhibition of Wnt Activity Points to Wnt Involvement in Differentiation/Delamination Only

In chicken embryos, placode induction occurs as early as the 4 ss (Stark *et al.,* 1997, McCabe *et al.,* 2009). Past research has shown that opV placode cells are generally committed over a protracted period beginning at about the 7-8ss and peaking h later at the 10-15ss (Stark *et al.,* 1997). Lassiter *et al.,* found that Pax3 expression was strongly reduced in the placode when electroporated with DN-TCF4, so I attempted to duplicate the result by chemically and globally inhibiting Wnt and looking at Pax3 expression in the ectoderm and mesenchyme in the midbrain region of the head.

I explanted chick embryos at the 8-10ss onto an agar/albumin (EC) culture medium treated with DMSO or XAV939. The embryos were then incubated for 24 h and observed for viability and morphological defects. All embryos were still alive but no obvious morphological differences were observed between the Wnt-antagonist treated embryos and the control embryos. Embryos were then cut out of the vitelline membrane and fixed in 4% formaldehyde. After embedding the embryos in 15% sucrose gelatin, I cryosectioned them coronally through the region of interest and stained them using antibodies to Pax3 (marker for placode cells) and Islet1 (marker for differentiated neural cells). Total cell counts from five random sections through the placode region of embryos from each treatment type were compared and analyzed for statistical significance. Those embryos that showed unhealthy morphology were not included in cell counts/analysis. The experimental samples showed a decreasing trend in the number of cells expressing both Islet1 and Pax3 in the mesenchyme (Fig 2C, 2F, Fig 3B). While the difference was not statistically significant (Table 1), it did seem to support previous findings that Wnt was involved in the delamination and differentiation of the placode cells, as seen by a reduction in the

number of delaminated Pax3/Islet1 expressing cells (Lassiter et al., 2007). These results did not replicate the previous findings that Wnt was required to maintain Pax3.

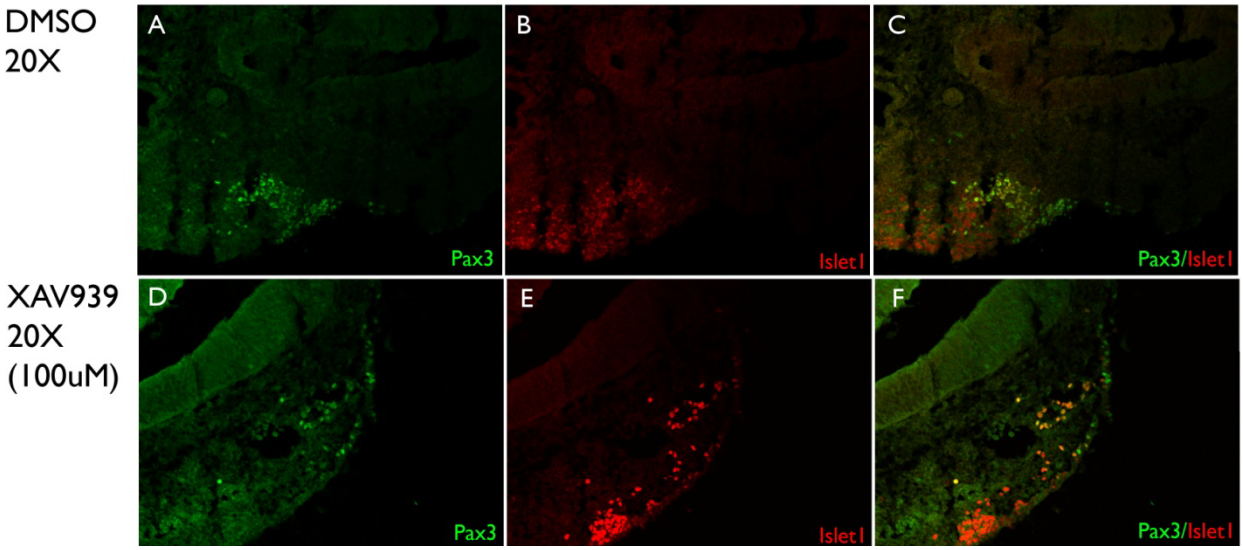
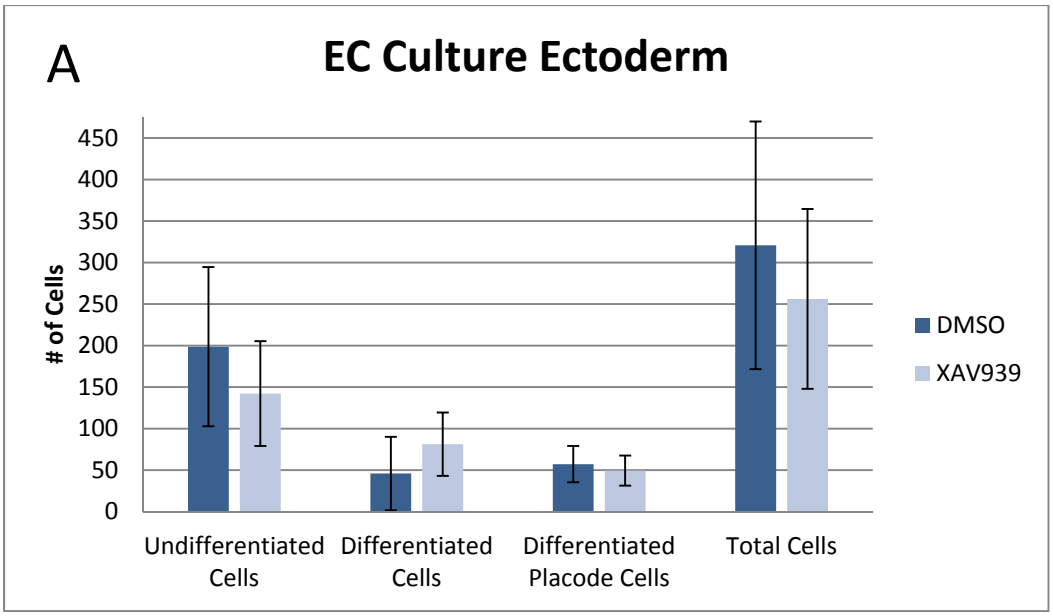


Figure 2: Inhibition of Wnt Signaling Via XAV939 in the Trigeminal Placode.

The sections are of the opV placode from embryos collected at 8-10ss and cultured on Agar-albumin dishes for 24 h. The sections were then stained for Pax3 and Islet1. (A-C) 20X magnification of placode ectoderm and underlying mesenchyme of control embryos. (G-I) 20X magnification of placode ectoderm and underlying mesenchyme of XAV939-treated embryos. Many Pax3+/Islet1+ cells can be observed in both the treated and control embryos.



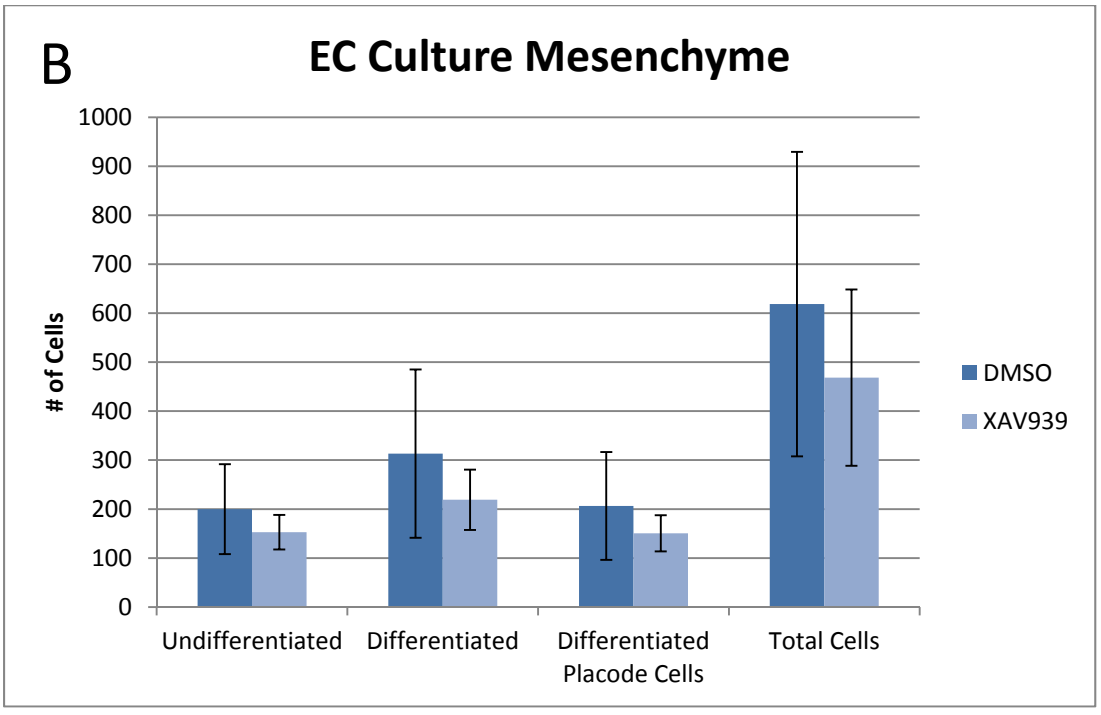


Figure 3: Average Cell Counts Of Pax3+ (Undifferentiated Placode Cells), Islet1+ (Differentiated Cells), Pax3+/Islet1+ (Differentiated Placode Cells) and Total Cells Counted.

(A) Cell counts in the placodal ectoderm. (B) Cells counts in the mesenchyme. While there is a trend toward a reduced number of Pax3+/Islet1+ cells in the mesenchyme between the control and experimental embryos, this reduction is not statistically significant (p-value = 0.0695). Error bars represent the standard error of the mean. DMSO (n=5), XAV939 (n=5).

Table 1: Analysis Of Variance of Pax3+/Islet1+ Cells in the Mesenchyme.

Source	DF	SS	MS	F-value	p-value
Model	1	1556.82	1556.82	3.45	**0.0695**
Error	48	21669.68	451.45		
Corrected Total	49	23226.50			

Collagen Cell Culture Confirms Trend Suggesting Wnt's Role in Differentiation and Delamination

To control for the possibility that our technical approach did not effectively deliver the Wnt inhibitor to the dorsal embryo, I repeated the experiment using the collagen matrix tissue culture (CMC) method and an additional Wnt inhibitor, Inhibitor of Wnt Processing (IWP-2). I chose to use the two inhibitors in this experiment to establish whether the drug delivery was successful, hoping to see specific phenotypes. I collected embryos from 8-10ss and removed them from the vitelline membrane. I removed the heads, making an incision right above the first somite, and placed them in tissue culture media. A mound of collagen was placed in the middle of each well in a 12-well culture plate and allowed to set up. The embryo heads were then carefully placed on the center of the collagen mound and covered with more collagen (treated with 10x DMEM and pH balanced with sodium bicarbonate). The embryos were then surrounded with 2 ml of N-2 supplemented DMEM culture media treated with either DMSO, 20 μM XAV939, or 5 μM IWP-2. These were then incubated for 24 hours. These embryos were processed as described previously with the EC culture embryos and analyzed for the number of cells expressing Pax3 and Islet1 in the ectoderm and mesenchyme (Fig 4).

There were no distinct morphological differences between any of the embryo heads. However, immunohistochemistry and statistical analysis showed that there was a significant difference ($p \leq 0.05$) in dual Pax3 and Islet1 expression in the mesenchyme in both experimental treatments (XAV939 and IWP-2) versus the control embryos, following the trend established with the EC cultured embryos (Fig 4C, 4F, 4I, Fig 5, Table 2). These results only hint at the previous conclusion that Wnt is involved in differentiation and delamination; Lassiter *et al.* observed a complete loss of Pax3 expression (ophthalmic placode cells) in both the ectoderm and mesenchyme, whereas I observed only a *reduction* in the number of differentiated placode cells

that had delaminated into the mesenchyme (2007). This leaves open the possibility that some other signal is involved in differentiation and delamination or that Wnt was not being sufficiently inhibited. Since there was no difference in the results between IWP-2 and XAV939, I opted to move forward using XAV939.

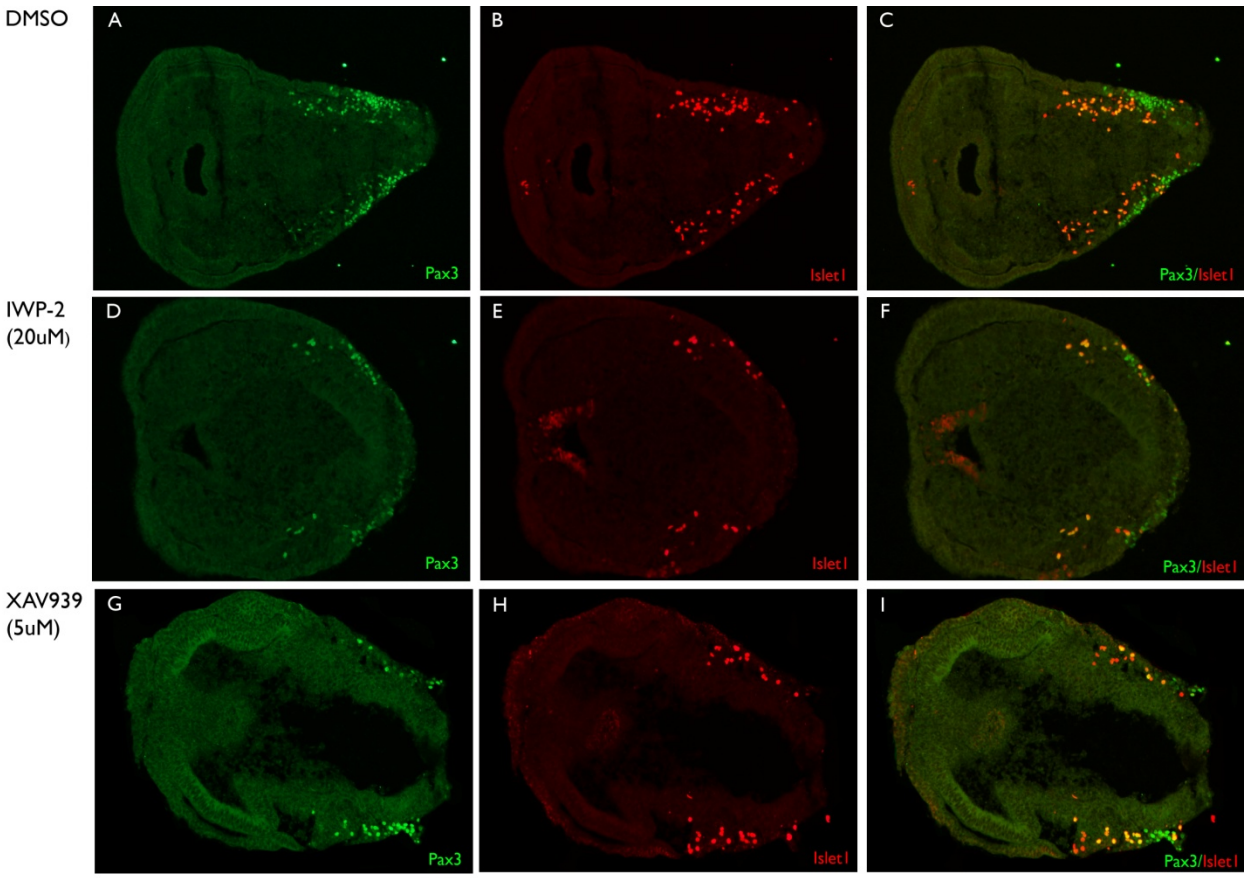


Figure 4: Inhibition of Wnt Signaling Via XAV939 and IWP-2 in the Trigeminal Placode.

The two different Wnt inhibitors used showed that inhibiting Wnt secretion and activity throughout the embryo head prevented the differentiation of the placode cells. The sections are of the opV placode from embryo heads collected at 8-10ss and cultured in collagen matrix and tissue culture media for 24 h. The sections were then stained for Pax3 and Islet1. (A-C) Control embryos treated with DMSO (20µM). (D-F) Experimental embryos treated with 5 µM XAV939. (G-I) Embryos treated with 20µM IWP-2. There appears to be a reduced number of Pax3+/Islet1+ cells in the mesenchyme between the control and both sets of experimental embryos (C, F, I).

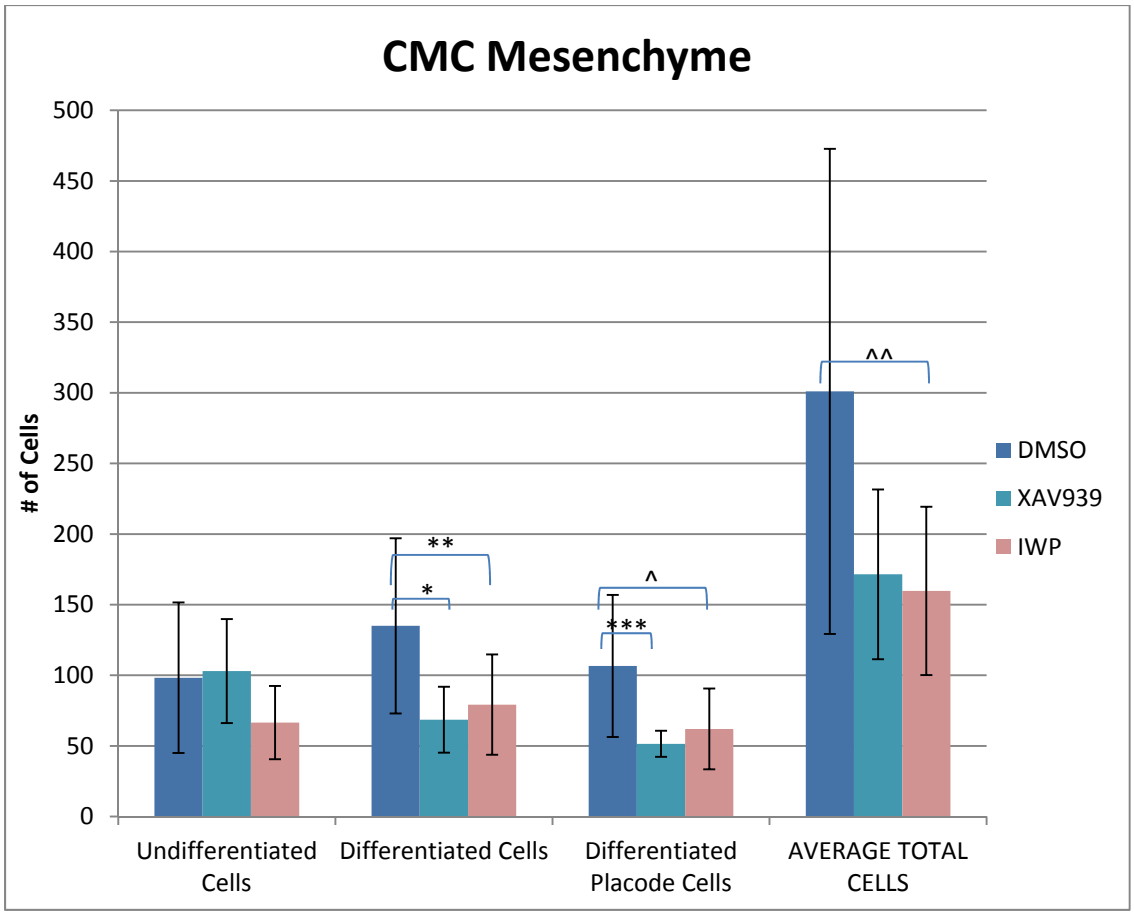


Figure 5: Average Cell Counts Of Pax3+ (Undifferentiated Placode Cells), Islet1+ (Differentiated Cells), Pax3+/Islet1+ (Differentiated Placode Cells), and Total Cells Counted.

Average cell counts in the mesenchyme. There was a significant reduction of differentiated placode cells found in the mesenchyme. *, **, ^, ^^ indicates $p \leq 0.05$ for the specified groups. Error bars represent the standard error of the mean. DMSO (n=5), IWP-2 (n=4), XAV939 (n=2).

Table 2: Analysis of variance of Pax3+/Islet1+ cells in the mesenchyme (groups include DMSO, IWP, and XAV939 treatment)

Source	DF	SS	MS	F-value	p-value
Model	2	1291.37	645.68	4.79	**0.0123**
Error	52	7006.34	134.74		
Corrected Total	54	8297.71			

We checked the effectiveness of the inhibitor XAV939 by performing whole mount *in situ* hybridization. The embryos used were heads that had been collected at 8-10ss and cultured in XAV939 through the CMC and compared with embryos that were incubated for 2 days (to mimic the same stage at which the cultured heads were collected). I used *Axin2* as an antisense probe to indicate the activity of Wnt. XAV939 specifically targets Axin2 protein, rather than mRNA, so while the Axin2 protein levels would normally be increased if it was effective, the mRNA levels should decrease as Wnt has been seen to be a transcriptional inducer of Axin2 (Chen *et al.,* 2009, Jho *et al.,* 2002).

The *in situs* showed that there was little to no Axin2 mRNA in the treated embryos qualitatively compared to the control embryos (Fig 6A-D) suggesting little Wnt canonical activity. Even in the control embryos, however, it seemed that there was scant Wnt activity in the placode region, as indicated by Axin2 levels. Sections of the embryo in the opV placode region showed good expression however of Axin2 in the dorsal ectoderm (Fig 6H) and in the otic placode region (6E, 6F) in the controls. The embryos treated with XAV939 showed no staining at all (Fig 6G, 6H). This indicates the inhibitor effectively blocked Wnt signaling in our experimental approach. Synthesizing these data with what was found by Lassiter *et al.* yields the possibility that Pax3 expression and placode identity is regulated independent of Wnt/β-catenin through TCF at both the induction and maintenance stages.

Overall, these experiments showed that inhibition of Wnt activity throughout the entire head of the embryo still only resulted in a decrease of differentiated placode cells in the mesenchyme and no difference in the undifferentiated (Pax3-expressing) cells in the ectoderm, a different result from what had been previously reported (Lassiter *et al*., 2007).

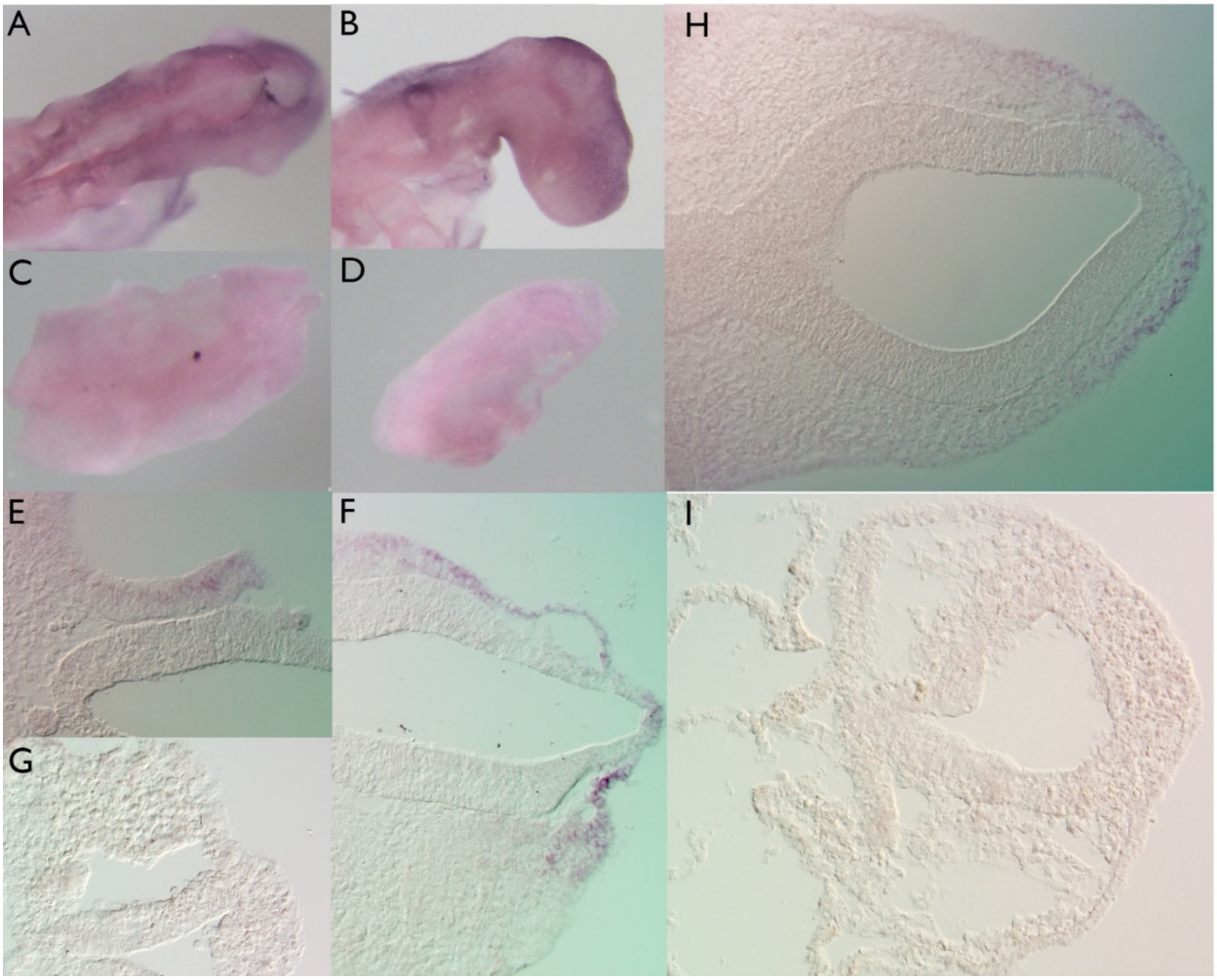


Figure 6: Staining of Axin2 Expression Showing Spatiotemporal Activity of Wnt Signaling on Experimental and Treated Chicken Embryos

In situ hybridization staining for the scaffold protein Axin2, which is often used as a readout for canonical Wnt signaling, appears to be scant in the experimental embryos versus the control embryos. Axin2+ ectoderm can be seen in the dorsal head ectoderm of the controls, especially in specific regions near the otic vesicles. 48-hr embryos were collected as controls (A,B, E, F, H) to compare with 8-10ss embryos treated with XAV939 and culture as per the CMC protocol for 24 h (C, D, G I). Axin2 staining in sections of wild type otic region (E) and the anterior edge of the otic region (F). Axin2 staining in a section of the otic region in the treated embryos (G). Axin2 staining in a section of the dorsal ectoderm in the midbrain in a wildtype embryo (H). Axin2 staining in a section of the dorsal ectoderm in the midbrain in a treated embryo (I).

Wnt Secretion from NCCs an Important Factor in Developing Structures from NCC Streams 1, 2, and 3

Our other goal conditionally knockout Wnt secretion from the dorsal NT to see if it was the source of Wnt signals affecting the opV placode. This was a follow-up to work done by Jason Adams but was more specifically and severely knocking out Wnt secretion in the NT and NCCs. I crossed a Wnt1-cre male with a *Porcn* mutant female; Wnt1-cre:*Porcn* mutants were identified by the presence of the *Sry* and *Cre genes* in their genotypes. Mice were collected at E8.5 and E10.5 and observed for morphological differences but none were observed and there were relatively few mutants produced in each litter. These embryos were also processed through cryosectioning and immunohistochemistry but little to no differences were seen in Pax3 and Islet1 expression (data not included). At this time, I was using a Wnt1-cre driver line from Dr. Fuhrmann (University of Utah) and it wasn't known to be the most effective Wnt1-cre available, which could have contributed to the lack of defects seen early on. I wanted to check to see if any abnormal morphologies could be observed at birth or whether it was a lethal mutation, so I let the embryos go to birth. One mutant was obtained that showed micrognathia and abnormal presentation of the ears that seemed consistent with phenotypes of craniofacial disorders.

We obtained a new Wnt1-cre male and female from Jackson Labs (Stock # 022137), due to new facility requirements. After breeding the new Wnt1-cre line with our *Porcn* knockout mice, I continued to find mutants who presented the previously observed phenotype. Mice were collected as newborns. Skeletal preps were then performed on these fetuses to compare bone and cartilage formation between the mutant and wildtype mice. The abnormalities in the mutant mice were very similar to those seen in Wnt1-cre conditional knockouts of β-catenin from Brault *et al*. (2001). The trunk skeleton, it seemed, had no abnormal structures or malformations compared to the wild type; this included limbs and vertebral structures up until the atlas bone. In the head

region, however, most of the bone structures that were derived from cranial NCCs were absent in the mutant mice. In fact, there was almost no ossification in the head except for the exooccipital bones (compare Fig 7A and 7B). Cartilage from the mandible was present but other structures from pharyngeal arch 1—the maxilla, incus, malleus, and tympanic ring—were missing (Fig 7A-D, 7E, 7F). The stapes and the lesser horn of the hyoid bone from pharyngeal arch 2 (Fig 7E-7H) and pharyngeal arch 3 structures, the lower rim and greater horn of the hyoid bone, were missing as well (Fig 7G-7J). All that was evident of the hyoid bone in the mutant was cartilage from the body of the hyoid (Fig 7H). These results suggested a strong necessity for Wnt secretion either from the NCCs or the dorsal NT.

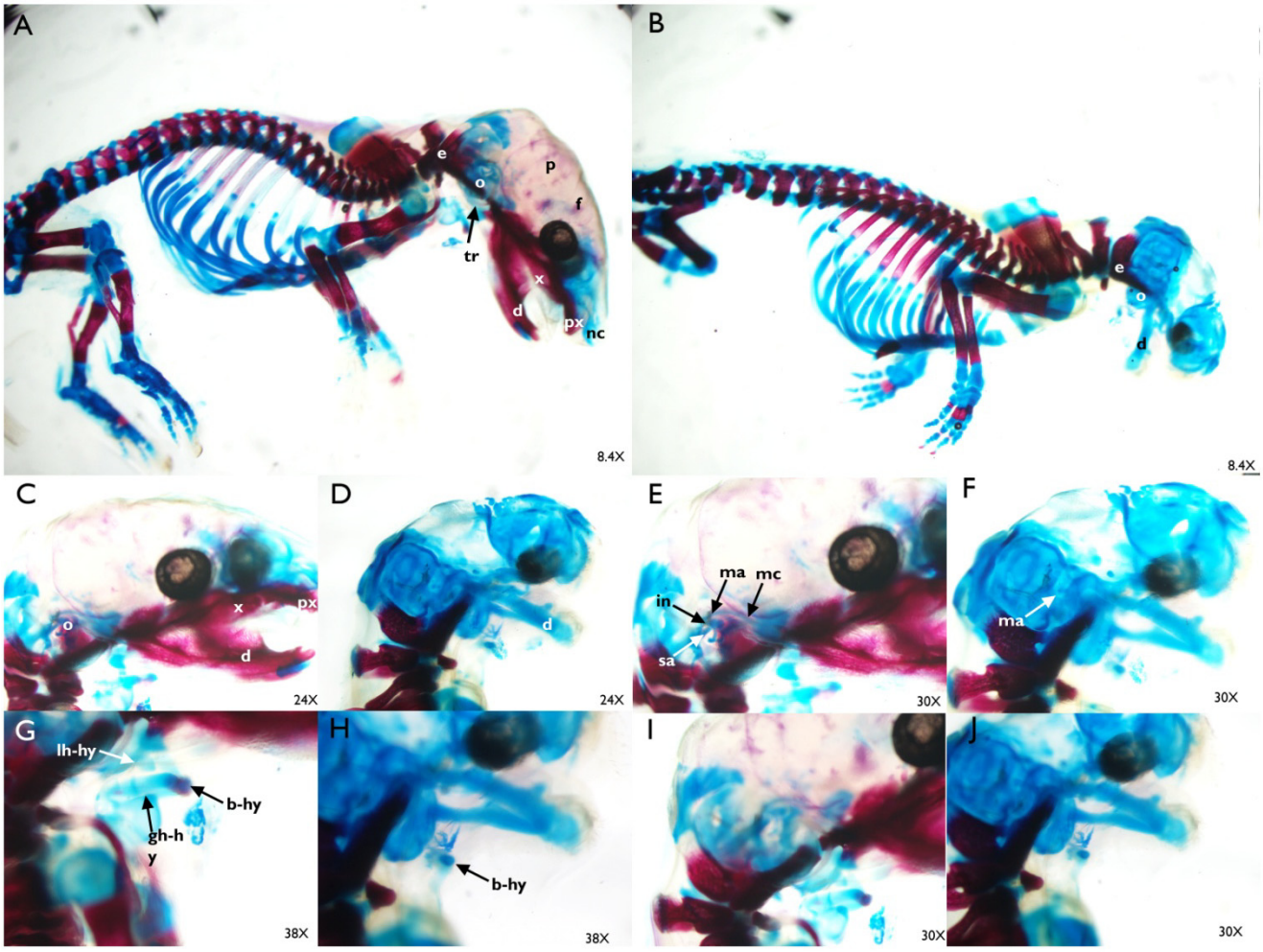


Figure 7: Alizarin Red/Alcian Blue Staining of the Collagen and Bone Structures in Wnt1-cre: *Porcn* Mice

Skeletal preparations of 18.5 dpc wild-type (A, C, E, G, I) and *Porcn* mutant (B, D, F, H, J) fetuses stained with Alizarin Red and Alcian Blue. (A,B) Lateral views of the full skeleton. (C-J) Zoomed-in views of the head and neck region to show defects. In the mutant, most of the skeletal structures derived from cranial NCCs are missing except the exoccipital (e) bones and cartilaginous templates for the mandible (d), the maxilla (x), the frontal bone (f), the parietal bone (p) the body of the hyoid (b-hy), the malleus (ma), and the otic capsule (o). The tympanic ring (tr), lesser (lh-hy) and greater horns of hyoid bone (gh-hy), stapes (sa), and incus (i) all seem to be missing from the mutant. b-hy, body of the hyoid bone; d, mandible; e, exoccipital bones; f, frontal bone; gh-hy, greater horn of the hyoid bone; i, incus; lh-hy, lesser horn of hyoid bone; m, malleus; mc, Meckel's cartilage; nc, nasal capsule;o, optic capsule; p, parietal bone; px, incisive (premaxillary) bone; sa, stapes; tr, tympanic ring; x, maxilla.

DISCUSSION

Wnt is an important factor in sensory neurogenesis as has been seen in past research (Lassiter *et al.,* 2007, Canning *et al.,* 2008, Litsiou *et al.,* 2005, Shigetani *et al.,* 2008). It is thought to be a potential inducing signal from the NT, although this source of Wnt is no longer required after the initial induction of the opV placode. Wnt has been suggested to be required for maintenance of Pax3 expression and thus opV placode cells (Lassiter *et al*., 2007). There have also been several studies that have linked canonical Wnt signaling to Pax3 expression through the caudal-related homeobox (Cdx) family and msh homeobox 1 (Msx1) acting on various enhancers for the Pax3 gene (Miller *et al.,*. 2007, Bang *et al.,* 1999, Moore *et al.,* 2013, Zhao *et al.,* 2014, Sanchez-Ferras *et al.,* 2014). In this study, however, global inhibition of Wnt signaling by known Wnt inhibitors surprisingly did not eliminate Pax3 expression or neuronal differentiation in the opV placode. While prior studies point to Wnt being an upstream regulator of Pax3 expression, the results presented here have made the role of Wnt unclear.

In considering how the seemingly contradictory data sets might be reconciled, the regulation of TCF/Lef was more closely evaluated. It is perhaps possible that the previous studies, which have focused heavily on the activity of transcription activator TCF/Lef, have not examined the possibility of Wnt/β-catenin-independent regulation of TCF. There have been a few studies that have seen activation of TCF/Lef in other developmental processes and systems through alternate routes other than Wnt signaling (Fuhrmann *et al.,* 2009, Sprowl and Waterman 2013, Grumolato *et al.,* 2013). They have demonstrated a few connections between the TCF family and the activating transcription factor (ATF) family. Interestingly, ATF5 has been found to be expressed highly in the ventricular zone of the developing central nervous system and has known roles in the progression of neural progenitor cells to become neurons (Angelastro *et al.,*

2003, Mason *et al.,* 2005). It has also been seen to modulate signals required for the development of other neural structures such as oligodendrocytes and astrocytes (Mason 2005, Angelastro *et al.,* 2005). More specifically, there has been evidence provided showing specific binding interactions of ATF1 and TCF1 in hematopoietic tumor cells as well as a proven interaction between ATF5 and TCF4 (Grumolato *et al.,* 2013, Xiongjun *et al.,* 2013). Thus, future studies could investigate the maintenance of Pax3 via TCF regulation without contributions from Wnt signaling, or Wnt-independent regulation of TCF target genes.

The other interesting experimental result was the presentation of severe abnormalities in NC structures from the *Porcn* knockout mice. The novel part of the work was that the Wnt1-cre:*Porcn* mouse would be suppressing *secretion* of all Wnt ligands rather than *activity* of just the canonical Wnt pathway (as was done with the β-catenin conditional knockout). It provides a novel approach (inhibiting Wnt secretion via a *Porcn* knockout) that validates the predominant hypothesis that Wnt from the dorsal NT is required for induction and expansion of NCCs (Brault et al., 2001; Chang and Hemmati-Brivanlou, 1998; Dorsky et al., 1998; Garcia-Castro et al., 2002; Ikeya et al., 1997; LaBonne and Bronner-Fraser, 1998; Lewis et al., 2004; Saint-Jeannet et al., 1997). It also raises the possibility that Wnt secretion from the NCCs themselves (targeted by using Wnt1-cre as a driver) plays a role in the formation of these structures, especially since there were a few differences in abnormal structures between our Wnt1-cre:*Porcn* mutants and the Wnt1-cre:β-catenin knockouts (Brault *et al.,* 2001).

Follow-up work on the Wnt1-cre:*Porcn* mice could also provide good evidence on the neural development in those embryos as well as further elucidating the role of Wnt in craniofacial development. Work has been done in which Wnt1-cre was used as a driver line to inactivate β-catenin; it resulted in a more extreme phenotype of craniofacial defects as well as

abnormal development of the trigeminal ganglion—all connecting parts with the hindbrain were lost in the mutants and the mutant ganglion appeared to be much smaller than the wildtype (Brault *et al.,* 2001). The interesting point is that, in spite of the loss of canonical Wnt activity as early as E9 in the β-catenin knockout mice, the ganglion was still able to develop, which could be due to the continued activity of other Wnts outside of the dorsal neural tube. An *in vivo* study of the *Porcn* knockout and the trigeminal nerve could provide additional comparative information regarding the differential effects of Wnt activity versus Wnt secretion. It could also shed more light on the result in our chicken tissue cultures that there was no significant difference between the two different Wnt inhibitor treatments; secretion and activity of Wnt seemed to affect the embryos similarly in the placode.

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CURRICULUM VITAE

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EDUCATION

May 2019	D.O. Medicine: Campbell University School of Osteopathic Medicine
Aug 2014	M.S Physiology and Developmental Biology: Brigham Young University
Aug 2009	B.S. Physiology and Developmental Biology: Brigham Young University Minor: Editing

WORK EXPERIENCE

Sep 2012- Jun 2015 Research Assistant: *BYU* Provo, UT

- Mastered lab techniques such as PCR, In Situ Hybridization, and embryo collection
- Worked cooperatively with teams to gather data for both
- Mentored undergraduate students in lab work and setting up experiments

Sep 2011-Apr 2015 Teaching Assistant: *BYU* Provo, UT

- Helped with the following courses: PDBIO 562 Reproductive Physiology, PDBIO484 Embryology, PDBIO 482 Developmental Biology, ELANG 410R Technical Editing/Editing for Scholarly Publishing
- Assisted all the professors in grading, taught when the professor was absent, and held regular review sessions for the students
- Class numbers were anywhere between 23 and 44 students

Sep 2006 – May 2012 Master Control Operator: *BYU Broadcasting* Provo, UT

- Prevented and solved on-air problems regularly and at all hours of the day
- Helped train new operators in managing broadcasting schedules and fixing discrepancies on-air
- Edited the training manual

May 2008-Aug 2008 Lab Technician Assistant: *USDA-ARS Pasture Lab* Provo, UT
May 2007-Aug 2007

- Collected and analyzed data for the primary researchers
- General care of research plots

OTHER EXPERIENCE

Aug 2013-Jun 2014 Cancer Resource Center Volunteer Chair: *Utah Valley Regional Medical Center (UVRMC)* Provo, UT

- Responsible for scheduling and training about 10 volunteers
- Organizing events to unite the volunteers and help improve volunteer work
- Meeting with the American Cancer Society Staff regularly to manage volunteers

Jun 2013-Jul 2014 Wound Care Volunteer: *UVRMC* Provo, UT

- Ushered patients into the exam rooms to take vital signs and prepare patient for the nurse
- Clean, disinfect, and set up rooms after each patient
- Aided the secretaries with their work when needed

Jan 2012-Dec 2013 Cancer Resource Center Volunteer: *UVRMC* Provo, UT

- Data Entry for program and information requests
- Answering questions for the patients and their families and helping them find information
- Helping the patients feel welcome and happy.

Sep 2008 – Jun 2012 Assistant Lab Researcher: *BYU* Provo, UT

- Team leader for PCR and genotyping mice
- Aided in embedding, sectioning, and staining mouse embryos
- Trained team and other individuals in PCR and gel electrophoresis procedures

AWARDS

Aug 2012-May 2015 Research Assistantships (7): *Department of Physiology and Developmental Biology (BYU)* Provo, UT

Aug 2012-Jan 2015 Teaching Assistantship (5): *Department of Physiology and Developmental Biology (BYU)* Provo, UT

PUBLICATIONS

2015-2016 In progress: Comparative morphological and molecular evidence supports an essential role for embryonic timing in determining anterior-posterior limb position. Stark, M. Prestwich, T. Hulet, J. Stephens, T.