CHARACTERIZATION OF MIGRATING NEURAL CREST CELLS FROM HINDBRAIN EXPLANTS IN ZEBRAFISH

by

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Our lab was interested in creating a reproducible mechanism for obtaining neural crest cells in culture from cultured hindbrain explants of zebrafish. We have characterized the population of cells migrating from the explants as neural crest using a genetic variant of zebrafish with a GFP tag SOX-10, as well as immunostaining using HNK-1, a known neural marker for neural crest cells. We have also devised a modified protocol of in-situ hybridization that can be used for cells in culture for determining specific genes cells express in culture.

Based on previous studies by Bingham et al., hindbrain explants have been extensively studied for preservation of their native (endogenous) cellular environments as well as the behavior of individual cell types. Biopidy ceramide labeling of live embryos has been used to extensively monitor and record cell shapes and organization in the developing zebrafish neural tube (Cooper et al). Studies by Bingham et al. have shown that morphogenesis of the neural tube in the zebrafish hindbrain is unaffected by the explantation of the hindbrain into a culture medium. The expression of krox20 and hoxb1a, which are expressed in the developing embryo in the hindbrain in rhombomeres

3 and 5 and rhombomere 4 respectively have been found to be unaffected when the hindbrain is explanted into a culture media. It has also been shown that valine expressing neural crest cells migrate normally out of r6. These results demonstrate that morphogenesis and patterning of the explanted hindbrain occur normally following the explanation of the hindbrain into a culture media (Bingham et al). Our lab has carried out dissections to remove the hindbrain from the developing embryo 15-18 hours post fertilization. We have found that it is possible to dissect the hindbrain out of a developing embryo and have cells migrate from these explants. We have also shown that these cells express Sox10 and are stained when using an antibody for HNK-1, which is known to be a neural crest cell marker. Our lab has also developed a working protocol of in-situ hybridization on cells in culture for Caco 2 and HT-29 using RNA probes for human beta actin and Hox A10.

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LIST OF SYMBOLS AND ABREVIATIONS

μg- micrograms

μL- microliter

mL- milliliter

NC- neural crest

NCC- neural crest cell

CNCC- cranial neural crest cells

GFP- Green Flourescent Protein

Sox- Sry-related HMG box

Hox- Homeobox

R- rhombomere

Background

Neural crest cells are cells that delaminate and migrate extensively from a transient embryonic cell population of neural-ectoderm. These cells migrate and populate the face, pharyngeal aches, the gut, the heart, among many other regions, participating in production of bones, cartilage, nerves, blood vessels, and connective tissue (Dupin 2007). Their extensive migration and ability to differentiate into a large variety of cell types have made the neural crest (NC) a main topic in developmental embryological studies, and they have even been referred to as the fourth germ layer. zebrafish (Danio rerio) have long been extensively used as the ideal species for observing development due to their transparency and relatively short development phase. It is for this reason many developmental biologists have used zebrafish while studying NCCs.

During development in vertebrates, the central nervous system develops from a specialized region of the ectoderm called the neuroectoderm or the neural plate, which is formed in the process of gastrulation. In most teleosts, ectodermal epithelial cells thicken and columnize to form a classic neural plate, and eventually into a neural keel (Kimmel 1995). Following the neural keel formation the neural tube begins to form and separate from the surrounding ectodermal epithelium in a process called neurulation. Figure 1 depicts the process of neurulation leading to the neural tube formation. Formation of the neural tube leads to a population of cells found at the dorsal margin of the neural fold that undergo an epithelio-mesenchymal transition. These cells are called the neural crest (NC) and are responsible for the production of neural crest cells (NCCs). These NCCs receive signals from the neural plate border as well as the non-neural ectoderm and

mesoderm that cause them to lose adhesion molecules and to migrate extensively. Rostral caudal positioning along the neural tube determines which signals these cells will receive and how extensively they will migrate (Table 1 lists many of the fates of NCCs) (Duband 2009).

Discovery of the Neural Crest

In 1868 while studying embryo development in the chicken, Wilhelm His identified a band of cells between the developing neural tube and the future dorsal epidermal ectoderm that was responsible for the production of spinal and cranial ganglia (Hall 2008). His identified this band as *Zwischenstrang,* the intermediate chord. Wilhelm was the first to provide an explanation for the process of embryonic development, physiology of embryonic development and characterization of germinal regions for predetermined organ-formation. In the 1890's a woman by the name of Julia Platt claimed that the cartilages of the craniofacial and pharyngeal arch skeletons and the dentine-forming cells (odontoblasts) of the teeth of the mudpuppy, *Necturus maculosus*, arose from the ectoderm adjacent to the neural tube (Platt 1893, 1894, 1897). It wasn't until the 1920's and 1930's that Platt's conclusions about the neural crest being the source of mesenchyme, connective tissue and cartilage were demonstrated in studies done by Landacre (1921), Stone (1926, 1929) and Raven (1931, 1936). Presently, the neural crest is referred to by many developmental biologists as the fourth germ layer confined to vertebrates (Hall 2006).

Neural Crest Regions

In vertebrates the NC is divided into 4 regions (Figure 2), each region responsible for producing cells that will populate and differentiate into specified cell types. The trunk neural crest, which is responsible for producing cells that that will take one of two pathways during migration. Cells that delaminate from this region can migrate dorsolaterally into the ectoderm to the ventral midline and will differentiate into the pigment-synthesizing melanocytes. Additionally cells that delaminate from this region can migrate ventrolaterally through the anterior of the sclerotome and will remain in the sclerotome (pre-vertebral cartilage of the spine) to become the dorsal root ganglia containing the sensory neurons, the sympathetic ganglia, the adrenal medulla and nerve clusters surrounding the aorta.

The vagal and sacral neural crest is another region of the neural crest, which is responsible for generating the parasympathetic ganglia of the gut. Failure in development of this region of the neural crest will result in the inability of the organism to undergo peristaltic movements.

The cardiac neural crest, which is located between the cranial and trunk neural crest is primarily responsible for producing the entire musculoconnective tissue wall of the larger arteries that arise from the heart. It is also responsible for producing cells that will populate the septum, which is necessary in separation of the pulmonary circulation from the aorta. This region of neural crest is also responsible for producing cells that can develop into melanocytes, neurons, cartilage, and connective tissues.

The fourth region of the neural crest is referred to as the cranial neural crest, or cephalic neural crest, which is located in the anterior portion of the head. Cells that

migrate out from the CNC will migrate dorsolaterally to produce the craniofacial mesenchyme that differentiates into the cartilage, bone, cranial neurons, glia, and connective tissues of the face. These cells enter the pharyngeal arches and pouches to give rise to thymic cells, odontoblasts of the tooth primordia, and the bones of gill support structures and the jaw. The CNC is the only portion of the neural crest that is responsible for the production of bone and cartilage (Figure 3).

Fate Mapping Studies on Cephalic Neural Crest

Many studies have been conducted to determine and test the cell fates within the neural crest in many organisms. Transplant studies have been conducted to determine the fate of cells that migrate from different regions of the neural crest. Studies done by Le Douarin (1969), used developing embryos from quail and duck. Regions of the neural tube were excised out of one embryo and placed in the same region of another embryo to form a quail-chick chimera (Figure 4). These chimeras were then allowed to develop, and differences in development could be visualized after hatching. Based on the region of the neural tube being replaced, and the phenotypic outcome of the chick's, regions of the neural crest could be characterized as to what portion of the developing embryo they contributed to.

In one of Le Douarin's studies, the cephalic neural crest from duck and quail were used to form chimera. Results showed that quail embryos transplanted with duck cephalic neural crest cells formed beaks that more closely resembled duck beaks. Reciprocally, duck embryos transplanted with quail cephalic neural crest cells formed beaks that more closely resembled quail beaks (Figure 5). These studies were among the

first to show that the cephalic neural crest was responsible for making up the bones and cartilage of the facial skeleton, and to effectively map the regions of the neural crest.

Later studies by Daghiani(1987) used electron microscopy and transplantation studies in *Xenopus* to show that regions of the neural crest as well as neural crest cell derivatives and migration patterning were conserved between different species of birds, but also across different vertebrate species.

Hindbrain Patterning

During the early stages of vertebrate development, three streams of CNCs migrate from a structure in the developing head referred to as the hindbrain (Creuzet 2006). The hindbrain is transiently divided into seven distinct neuroepithelial segments called rhombomeres. Each rhombomere takes on distinct molecular and cellular properties, which causes cells in these regions to express a different set of gene expression. Differentiation and cell fate studies have shown CNCs migrate from the ectoderm overlaying the dorsal neural tube in three distinct streams. Cells that migrate out of rhombomere 2 migrate into the first pharyngeal arch (mandibular arch) forming the bones of the jaw, Meckel's cartilage, and the palatoquadrate. Cells migrating out of rhombomere 4 populate the second pharyngeal arch (hyoid arch) responsible for formation of the basihyal, ceratohyal, and hyosymplectic cartilage responsible for making up the hyoid bone. Cells migrating out of rhombomere 6 migrate into the third and fourth pharyngeal arches to form the support structures for the gill (Figure 3)(Creuzet 2006).

NCC Plasticity

The plasticity of migrating NCCs has been studied extensively for the possible characterization of NCCs as stem cells. Studies done by Schilling (2001) removed single and groups of cranial neural crest cells from developing zebrafish embryos. Contrary to previous hypothesis that neural crest cells had predetermined expression patterns, Schilling found that transplanted cells were capable of changing their gene expression depending on the cell expression patterns of the surrounding cells. These studies seem to be consistent with similar transplant studies performed in chick, and mouse (Schilling 2001).

Researchers have focused their attention on neural crest cells because of the extensive range of cell types they are capable of differentiating into as well as their essential role during vertebrate embryonic development. Although the role of neural crest cells *in vivo* has been extensively studied, the factors influencing their differentiation into a variety of cell types still needs to be completely characterized. These studies will be facilitated if we were able to maintain and propagate NCC in culture. Such a cell culture system will be a useful tool to study their differentiation and characterize the factors involved *in vitro*. Such system will also permit to analyze the effect of pharmaceutical drugs on NCC, differentiation and possibly migration.

Previous Explant Studies

Although zebrafish embryos are transparent and superficial developmental processes can easily be visualized, it is difficult to observe many of the later deep tissue developmental processes in the embryo. Late developmental processes also became

problematic to view because of movement within the embryo. Langenberg et al devised a technique to visualize and image live brain development and organogenesis using whole explanted zebrafish embryos. Langenberg began by removing the chorion and the yolk sack and dissecting the entire head region. He then embedded the whole head explant into a bed of agar and positioned it according to what he wanted to observe (Figure 6). He found that removal of the chorion, the detachment of the embryo from the yolk sack and removal of the head had no developmental effects on organogenesis. The success of this technique led Bingham *et al*. to devise a new technique to view the extensive migration of the neural crest cells from the neural crest. Bingham et al. (2003) found it was not only possible to remove a whole intact head regions of the embryo, but it was also possible to simply excise a specific portion of the head, mount in agar and grow in a culture dish. Studies by Bingham showed that removal of specific segments of the developing embryo did not affect the cellular migration patterns of neural crest cells within the explant (Figure 7). The success of this technique opened the doors to many developmental biologists interested in the study of the migration of neural crest cells, or the effects of pharmaceuticals on the migration and differentiation of neural crest cells.

Visualizing streams of NCCs

The importance of NCCs has lead to the development of many techniques used to visualize these cells both *in vitro* and *in vivo*. Early research lead to the development of HNK-1, an antibody that detects a carbohydrate compound found in cell adhesion molecules. Expression studies done by Morikawa *et al. (2001)* has shown HNK-1 to be expressed on the surface of migrating NCCs. This immunohistochemical method of

detecting NCCs is a useful and valuable technique for distinguishing NCCs from other cell types both in culture and *in vivo*, but falls short when trying to distinguish between the three different streams of neural crest cells that migrate out of the hindbrain.

Sry-related HMG box (*Sox*) genes code for an important group of transcription factors. These Sox genes can be grouped into many different subfamilies based on their sequence and function. In vertebrates, *Sox*E genes, which include *Sox*8, *Sox*9, and *Sox*10, have been best studied due to their activity in developmental processes, and can be linked to many congenital diseases. Although each of these *Sox*E genes have been found to be expressed during development, *Sox*10 function has been examined in all major vertebrate model systems. *Sox*10 is highly conserved across the vertebrates studied, including zebrafish, and highly dynamic. Across all vertebrates studied, *Sox*10 is expressed in all pre-migratory NCCs including both medial and dorsolateral migration pathways. Although *Sox*10 gene expression is rapidly downgraded post-migration (with the exception of glial cell derivatives), it has been used widely as a genetic marker for pre-migratory NCC. Thus, visualization of the *Sox*10 gene can be used at early stages of development to more clearly distinguish NCCs from other cell types in vivo, however like HNK-1, *Sox*10 expression does not allow us to distinguish between the three streams of CNCs.

In the developing embryo's hindbrain, each rhombomere express a different combination of genes (Figure 8). For example cells in rhombomere (r) 2 express *hoxa2*, cells in r4 express *hoxa2*, *hoxb1*, *hoxb2*, while cells in r6 express *hoxa2* and *hoxb3*. In most instances NCC express a similar set of genes than the compartment they originate from (Figure 9). In the hindbrain of a developing zebrafish embryo, *hoxa2* expression is

seen in the NCCs emigrating out of r4 and r6. In the posterior hindbrain *hoxb3* is expressed again in both the posterior rhombomeres (r5, 6 and 7) as well as the NCCs migrating from r6, but is not expressed in the NCCs emigrating out of r4 or r2. The difference in expression between these two genes can be used to distinguish cells based on which of the three streams of NCCs they belong. Those cells, which have emigrated from r2, will express neither the *hoxa2* gene nor the *hoxb3* gene. Cells, which have emigrated from r4, will express only the *hoxa2* gene, and those cells that have emigrated from r6 will show expression for both *hoxa2* and *hoxb3*. These differences in gene expression can be used as a tool in characterizing and distinguishing between the different streams of CNCs.

Importance of Neural Crest Cells in Culture

Although many techniques have been used to suggest possible modes of differentiation of neural crest cells during development, there have been no studies to show which populations of neural crest cells are able to be cultivated from hindbrain explants. Characterization of these migrating cells is necessary for further studies to continue on the testing of developmental processes dealing with neural crest cells in zebrafish. Once cultures of neural crest cells can be maintained further differentiation studies can be done to characterize developmental pathways. These developmental pathways can open the door to study many developmental defects, or pharmaceutical interactions and their effects on developmental pathways.

Objective 1

My first objective is to develop a NCC culture system from explanted zebrafish embryo hindbrains.

Objective 2

In order to characterize the different type of NCC migrating out of the explants, I will develop an in situ hybridization method for cells in culture to analyze the genes expressed in the cells migrating out of the embryonic hindbrain explants. The method will be first developed using cells we are routinely maintaining in culture in the laboratory, such as Caco-2 or HT29 (2 human colon cancer cell lines).

Materials and Methods:

Zebrafish:

Maintenance and Care:

Wild type zebrafish were purchased from Carolina Biological, and sox-10-GFP transgenic zebrafish were obtained as embryo's from Dr. Shilling's laboratory at University of California at Irvine. Transgenic embryos were raised to adulthood according to protocols found in *The Zebrafish Book* (Westerfield, 2000). Following adulthood, the transgenic line was treated to the same care protocol as the wild type (D237). The wild type zebrafish strain was separated into three ten gallon tanks, each tank containing ten males and ten females. Tank water was prepared using Instant Ocean® salt stock (.2g/L) according to *The Zebrafish Book* (Westerfield, 2000). Tanks were kept at 28.5°C with a pH of 7.6. Ammonia levels were monitored using API® ammonia test kits to ensure all tanks maintained a concentration of ammonia of ~0parts per million. Once a week ¼ of the water in the tank was replaced with fresh stock water and biological filters were gently rinsed of debris. All fish were maintained on a constant photoperiod of fourteen hours of light and ten hours of darkness. Feedings occurred three times a day during their light cycle using a mixture of high protein ground food (Kyowa B, Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan) and live young artemia (GSL Brine Shrimp, Ogden Utah).

Spawning:

The day before spawning the fish were fed three times using only live young artemia. After the last feeding a large mouse cage (6''x10''x3'') with a false bottom (Figure 10) was completely filled with one half inch diameter marbles. After the third feeding of the day a quarter of the tank water was replaced with new stock water. The mouse cage was placed in the tank and allowed to sit overnight through the dark cycle on as seen in Figure 10. One hour after lights were turned on, the mouse cage was removed from the tank the false bottom with the marbles was removed and rinsed with distilled water. The runoff was collected in the bottom of the mouse cage. The contents of the mouse cage, containing the fertilized eggs, was run through a fine mesh net and was transferred to a 155x15 mm petri-dish containing a solution of 10% Hank's embryo media. The Hank's solution was prepared according to *The Zebrafish Book* (Westerfield, 2000). Eggs were isolated from waste and staged using a dissecting microscope. The isolated eggs were placed in fresh 10% Hank's containing methylene blue and incubated at 28°C until the embryos reached the 14 somite stage.

Embryo Dissection:

Preparation:

The day before dissections were performed, DMEM growth media containing 15% Fetal Bovine Serum and supplemented with 100ug/ml Penicillin/Streptomycin (pen/strep) cocktail and 100µg/ml of Fungizone was prepared and stored at 4°C. A twenty µg/mL solution of human fibronectin in PBS was also prepared and stored at 4°C.

The day of the dissection a sterile 24 well tissue culture plate was fitted with glass cover slips in each well. Three hundred uL of the human fibronectin solution (20µg/ml) was placed in each well covering the cover slip and placed in a 37°C incubator for 1 hour. Immediately before the dissection, the tissue culture plates were removed from the incubator, the fibronectin solution removed and replaced with 500 uL of prepared media.

Fifteen hours post fertilization (hpf), the developing embryos were removed from the incubator, washed with fresh 10% Hank's solution without the addition of methylene blue and were placed on ice until the dissection was to be carried out. The dissection was performed with a micro-scalpel and a tungsten needle that were sterilized by immersion in 100% ethanol until the dissection was to be carried out.

Dissections:

Dissections were carried out in a 35 mm petri dish. One mL of fresh media was was deposited in three separate spots in the petri dish to form three "bubbles". One mL of 10% Hank's solution containing approximately 10 embryos was placed to form a bubble in a separate area on the petri dish. Figure 11 shows a depiction of the set up used. Using a tungsten needle and a micro-scalpel one embryo was dechorionated in the Hank's 10% bubble on the petri dish. The embryo was dechorionated it was transferred using a transfer pipette to the first bubble of fresh media. Dissection was performed in this bubble using the otic vesicle as a landmark to determine the position of the hindbrain. A tungsten needle was used to make cuts to remove the head and the trunk regions surrounding the otic vesicle as seen in Figure 12. Once this segment was removed the

tungsten needle was used to scrape away some of the ventral ectoderm. Once the explant was completely removed from the embryo, it was transferred to the second bubble of fresh media using a transfer pipette. This step was repeated, transferring the explant to the third bubble of clean media before the explant was finally placed on top of the fibronectin-coated coverslip in the 48 well culture dish using a transfer pipette.

Dissections were carried out in this manner until the ten embryos had been dissected. Each well in the tissue culture plate contained no more than 4 explants. Once the ten embryos had been dissected the petri dish was washed using distilled water and dried using a wipe. Ten additional embryos were transferred to the cleaned petri dish and the dish was set up using the same procedure previously indicated. Dissections continued until embryos matured past sixteen hours post fertilization.

Controls:

Initial experiments were performed using whole embryos to ensure natural embryo development after dechorionation. Embryos were allowed to develop to 15hpf. After 15hpf embryos were dechorionated and placed in a cleaned petri dish. Once embryos were transferred to the petri dish they were placed in a 28°C incubator and allowed to develop for 24 hours.

Cell Culture:

Explant:

Once the dissection was completed the culture plate was immediately transferred to a 28°C incubator in 5%CO2 for 24 hours. After 24 hours each coverslip was transferred to a larger petri dish containing prepared media. Cell migration was observed using bright field and fluorescence microscopy. Explants were monitored for up to seven days to gage cell migration and possibly cell proliferation. The media was changed once every other day.

Caco2 Media:

Cells were stored in DMEM supplemented with 20% fetal bovine serum with a 1% penicillin/streptomycin (pen/strep) cocktail. After preparation, media was filtered sterilized and stored at 4°C.

Caco2 Maintenance:

Cells were obtained from Dr. Scemama (??) and maintained in a 37°C incubator in 5%CO2. Cells were grown in 75mm flasks until cells became confluent. Cells were routinely passaged every week.

Immuno-Staining:

Cells were fixed in a 4% PFA solution in 1X PBS for 10 minutes. A vector ABC staining kit for HNK-1 was used to characterize NCC according to manufacturer.

Briefly, cells were incubated for thirty minutes in a 0.3% solution of peroxide in distilled water. Cells were incubated in buffer provided by the kit for 5 min. Cells were then incubated in blocking solution provided by the kit for twenty minutes. Cells were then incubated for 30 minutes with primary HNK-1 mouse antibody diluted 1:2000 in buffer provided by kit. Cells were then washed in buffer for 5 minutes. Cells were then incubated for thirty minutes in biotinylated secondary anti-mouse antibody solution. Cells were then washed in buffer for five minutes. Cells were then incubated with the ABC reagent for thirty minutes. Cells were again washed in buffer for five minutes. DAB solution was used to develop the antibody staining until cells were brown.

In-Situ Hybridization:

Solutions:

PBS: .1X phosphate buffer + .9% NaCl

Triton X-100 (.3%): 300 μL Triton X-100 (Sigma) in 100mL PBS

50 mM EDTA: 1.46g EDTA to 100mL of .1M Tris buffer pH 7.5

Diluent for proteinase K: 50mM Tris with 2mM calcium chloride

Proteinase K Stock: 500μg proteinase K powder in 1mL diluent

Proteinase K working: 100μL stock in 5mL diluent (10μg/ml solution)

4% paraformaldehyde(PFA): 4.0g PFA in 100mL PBS

RNA Probes:

Fluorescein-labeled antisense probe was generated for human β-actin by enzyme digestion of the pCR II vector with EcoRV and synthesis with RNA polymerase SP6 (Blader et al., 1997) and for human HOX A10 by enzyme digestion using Xho1 and synthesis with RNA polymerase T7 (Blader et al., 1997).

Day 1:

Caco2 cell line was used to perform *in situ* hybridization. Cells were grown on coverslips in a 48 well tissue culture dish to confluency. Cells were first fixed to coverslips using 2.5% glutaraldehyde in .1X PBS for thirty minutes. They were then washed in a 4.5% sucrose solution in PBS for fifteen minutes four times. The cells were then placed in .1X PBS for five minutes and then placed in a .3% TritonX-100 solution for fifteen minutes at room temperature. A PBS wash was then performed for three minutes twice while shaking. A 15 μg/ml solution of proteinase K was added to the cells for fifteen minutes at room temperature. Cells were then fixed again in 4%PFA for five minutes. After fixation cells were rinsed in .1X PBS for three minutes twice while shaking. A .25% solution of acetic anhydride in .1X PBS was added to each of the wells for ten minutes while shaking. Cells were then washed in 50% formamide in 2x standard sodium citrate (SSC) for ten minutes and then placed in 37°C. Cells were then placed in a hybridization buffer composed of 50% formamide, 10% 20x SSC, 25% 1M tris pH 7.5, 15% deionized water, containing 200ng/ml of appropriate probe and incubated overnight at 37°C while shaking.

Day 2:

Cells were washed in 4XSSC for fifteen minutes three times shaking the last five minutes of the wash. A blocking solution containing 1% FBS and .01% Sheep Serum in PBT was used for fifteen minutes at room temperature. Cells were then washed in .1X PBS solution composed of phosphate buffered solution supplemented with .08% NaCl, for three minutes twice. Cells were then incubated at 37°C while shaking with a 1/2500 dilution of fluorescein Fab fragment by sigma diluted in the blocking serum for one hour. Cells were then washed in .1X PBS for 10 minutes four times. After the wash cells were equilibrated in AP buffer composed of 2M tris pH 9.5, 1M $MgSO_4$, 5M NaCl, 20% Tween 20 in water, for two minutes.

Visualization:

Developing was performed using NBT/BCIP tablets in AP buffer under foil rocking gently for two hours. Cell staining was continuously monitored. The reaction was stopped by washing the cells two times in PBT for ten minutes two times at room temperature. Coverslips with the cells were then transferred to a microscope slide and mounted using 70% glycerol.

Results

Control Sox10-GFP Development

Embryos expressing sox-10-GFP were allowed to develop and images were taken every 12 hours for 72 hours. Figure 13 shows a 24hpf embryo under brightfield (a), using a GFP filter (b), and then a merged image to show where GFP expression is located in the developing embryo. Figure 14 shows an embryo at 72hpf under brightfield (a), using a GFP filter (b) and a merged image to show where GFP expression is located in the embryo 72hpf. These images show clear expression of GFP in the basihyal, ceratobranchial, ceratohyal, hyosymplectic, Meckel's cartilage, ,palatoquadrate as well as the gill cartilages and the pectoral fins. Expression can also be seen in regions of the lower tail region surrounding the neural tube.

Development of the Dissection Method

Control of dechorionation:

Initial experiments were carried out using Leibovitz L-15 media supplemented with 10% FBS. Fifteen hpf embryos that had been dechorionated and placed in this media for 24 hours fully developed and continued to develop for 72 hours (Figure 15).

Media Development:

The first series of dissections were carried out in Dr. Scemama's lab using a dissecting microscope. The cranial region of the embryo was excised, using the otic vesicle as a landmark, cutting directly on either side of it. These explants were placed in

individual wells of a 24 well tissue culture dish containing Leibovitz L-15 media containing 10%FBS. After 24 hours every dissection had become contaminated, and no visible migration from the explant could be observed. The explants did not attach to the bottom of the plates.

To limit contamination, we used a dissecting microscope in a laminar flow hood. Again 24 hours after dissections every well was contaminated. The source of contamination was thought to be from the embryo itself so modification of pre-incubation period was made.

After collection, the embryos were treated with a .01% solution of Chlorox for 5minutes. The Chlorox was replaced with 10% Hank's solution three times to ensure all Chlorox was removed. The embryo's were then placed in 10% Hank's solution with an addition of Methylene Blue and stored at 28°C. After 15 hours of incubation the chorion of the embryos appeared shriveled and embryos appeared affected by the Chlorox wash. Dissections were done under a laminar flow hood and in open air. After dissections, no visible migration could be seen and explants had not attached to the bottom of the wells. After 24 hours all wells were contaminated.

In addition to the Chlorox treatment, a less stringent method was also tested. It was considered that maybe the contamination was coming from the media that had been sitting in the incubator. Prior to dissections, but after the 15 hour incubation period, all of the 10% Hank's media was removed and replaced with fresh media. Results showed that although it delayed contamination further than the other tested methods, contamination still resulted and no visible adherence or migration could be seen. The results for these experiments are summarized in table 2.

In order to stop contamination, antibiotics were going to be needed in small concentrations. Experiments were done testing the minimum concentration of a penicillin and streptomycin cocktail necessary to prevent bacterial contamination to prevent any adverse interactions on developmental processes. Results of these experiments showed that a concentration of at least 5% (250µg/ml) in solution was necessary to prevent bacterial contamination. Such a high concentration compared to other cell culture protocols, led to experiments on different antibiotics. Gentamicin was used in a solution of .01% weight by volume and found to prevent bacterial contamination.

Dissections were carried out using media with the supplemented 50µg/ml Gentamicin. After 24 hours there was no contamination, however there were also no cells adhering to the bottom of the petri dish. After 48 hours every well had contamination. Observation of the contaminants under the microscope led us to believe they were yeast.

Media was supplemented with Fungizone to a final concentration of 50µg/ml. Dissections were carried out using the media supplemented with both Gentamicin and Fungizone. After 24 hours no contamination was seen, however none of the explants had adhered to the plate. All explants developed contracting cells in the area that would be consistent with heart development. 48 hours after dissections there was still no contamination, however explants had balled up and turned black, indicating cell death. No cell migration could be visualized. Summary of these results can be seen in table 3.

Explant Adherence:

Coverslips were used in each of the wells on the tissue culture plate. The coverslips were incubated with varying concentrations of fibronectin ranging from 0 to 100µg/ml in PBS (table 4). After 24 hours there was no contamination and explants in the wells containing a concentration of 50µg/ml or greater had stuck to the coverslips. All explants contained contracting cells, again in the area that would correspond to the development of the heart. After 24 hours cell adhesion could be visualized, however migration was sparse. In the wells with coverslips that had been treated with a concentration greater than 50µg/ml of fibronectin, there was a lot of debris from the breakdown of the fibronectin matrix. The rhythmic beating cells lasted in culture for up to 72 hours before cells started lifting and dying. Results of these experiments are summarized in table 4.

Dissection Protocol:

Contamination was believed to be caused by the embryo's themselves so dissection protocols were modified to try to prevent this. As seen in figure 11 a series of washes of media were set up on the petri dish at the time of dissection to dilute and wash off any contamination found within the embryo. Embryo's were dechorionated in the fresh Hank's media and then transferred to the first wash of media. In this first wash of media on the petri dish, dissections were carried out. Once the explant was removed from the embryo, it was transferred using a micro-pipette to the next wash on the dish. It was allowed to sit for a thirty seconds and then transferred again using a different micro-

pipette to the third wash. Following this wash another micro-pipette was used to transfer the explant to a well of the tissue culture plate.

After 24 hours, explants could be seen adhering to the coverslips and cell migration again could be seen in sparse amounts. After 72 hours there was no contamination, however the explants began to detach from the coverslips. After 5 days all the explants had lifted off of the coverslips, however there was still no contamination that could be visualized.

Dissection of the Embyro:

Initial dissections carried out left explants with intact heads. Inconsistent and sparse cell migration was believed to be caused by cells being trapped in the head region of the explant (undergoing normal migration). The next set of dissections removed the head region above the otic vesicle (Figure 12). Although these dissections gave more migration on occasion, results were variable.

In the normal NCC migration pattern of the developing embryo, NCCs migrate dorsolaterally as well as dorsoventrally. Therefore again it was believed that the explant being removed contained NCCs that were being trapped inside the ectoderm according to normal migration patterns. A new set of dissections were carried out according to previous landmarks, however after the explant was excised from the embryo, a tungsten needle was used to scrape away some of the ventral ectoderm to allow cell migration out of the explant. These dissections showed much more consistent migration out of the explant, and cell numbers increased. Results are summarized in table 5.

Objective 2:

Development of *In Situ* Hybridization Method

Hybridization Buffer:

Initial hybridization buffer was prepared as follows:

100mL Formamide

100mL 4X SSC

7.88g Tris salt

Solution was stirred and heated to 37°C

.5g BSA

.5g Ficol-400

.5g PVP-360

1g Sodium Pyrophosphate

1g SDS

Components were mixed together while heated for 60min until everything was in solution.

This recipe was obtained from Dr. Gwen V. Childs at the University of Arkansas protocol for *in-situ* hybridization on thin tissue sections (2006).

Protocol:

Initial protocol was tested on HT-29 Human colon cancer cell line. Cells were fixed in 4%PFA prior to *in-situ* protocol. After the initial testing using cells fixed in 4%PFA, cells had become detached from the coverslips so we tested a different fixative

(2.5% glutaraldehyde) to see if the fixative would make a difference in cells staying attached to the coverslip. Figure 22 shows coverslips where cells were initially fixed with 4%PFA and coverslips which were initially fixed with 2.5% glutaraldehyde. Protocol was modified to fix cells initially in 2.5% glutaraldehyde.

Experiments were performed to test the optimal concentration of proteinase K needed for the protocol. Each experiment was done using the probe for human actin. No staining of the cells was observed for proteinase K concentrations of 0, 2.5 or 5 µg/ml. While we observed staining in every cells for concentration ranging from 10 to 20 µg/ml. Because we did not noticed significant differences between the 10 to 20 µg/ml concentrations, we decided to use the lowest dose of10 µg/ml in the rest of the experiments.

Sensitivity:

To develop the protocol in our lab, we used a probe targeting the mRNA encoding β actin, a gene we knew will be strongly expressed in every cells. To test the sensitivity of the assay, we performed the same experiment with a probe targeting *Hoxa10* gene. Figure 26 shows the results of these tests. Arrows represent staining inside the nucleus of cells expressing *Hoxa10*, where arrow heads show the cells that have not been stained or are not expressing *Hoxa10* in the nucleus. These images show that not every cell expresses the gene for *Hoxa10*, and those that do express the gene, the staining is only found in the nucleus. The absence of staining in the cytosol may be due to the low concentration of mRNA present.

Additional Cell Lines:

In addition to testing HT29 cells, it was necessary to see if this technique was able to be applied to other cells in culture. We used an additional colon cancer cell line Caco2 to show this technique could be used across cell lines. Actin staining on Caco2 cell lines also shows positive results for the anti-sense probe for beta actin. Negative controls shows no staining indicating the stained portions were not due to background, but due to the binding of our probe to the actin mRNA suggesting this technique can be used on other cell lines.

Discussion

The purpose of this research was to develop a reproducible method for cultivation of cranial neural crest cells as well as a method for distinguishing cell populations in cell culture. The research reported in this thesis has demonstrated that our protocol developed for cranial neural crest cell cultivation is viable and reproducible. We have shown using a GFP-SOX10 transgenic line of zebrafish that cells migrating out of the cranial explants show GFP expression, which is consistent with previous documented results showing SOX10 expression in cranial neural crest cells (Baroffio 1991). We have also shown cells, as well as explants, expressing the neural cell marker HNK-1 consistent with previous documented results showing neural crest cells expression of HNK-1(B. Sadaghiani 2009). Using β-actin as a positive control, we further develop a method to distinguish cell populations in cell culture using *in*-situ hybridization based on differential gene expression. We tested the sensitivity of our assay by analyzing the expression of a gene expressed at lower levels than β-actin.

Development and analysis of explant protocol

Previously, Langenberg *et al*. showed that it was possible to dechorionate zebrafish embryo, remove their yolk sack and have the embryo development continue through organogenesis and cellular migration. Bingham *et al*. showed that it was also possible to take that whole embryo and excise specific portions of it out and allow it to develop with normal cellular migration. Although each study showed that explantation of the developing embryo was possible, both studies had embedded their explants in agar

and solely visualized developmental processes that took place inside of the developing embryo.

Dissections began with excision of the head region as seen in the first column of table 5. These dissections led to full development for up to 48 hours in the explant, but no cells had migrated from the explant and no explant had attached to the coverslips on the bottom of the wells. There was also a lot of contamination problems following 24 hours of incubation. Future dissections were carried out with a more specific landmark, the hindbrain (Figure 12). Again with the exception of a few cells migrating out of the explants but not sticking to the coverslips, for the most part dissections were unsuccessful.

Contamination problems were approached on two fronts, the first being the actual dissection technique and sterility, and the second being additives to the media. At first the contamination seemed to be bacterial, so dissections were moved to under a fume hood where gloves and a mask were worn. Everything, including the dissecting microscope were sprayed with seventy percent ethanol and allowed to air dry. The embryos were treated with a Chlorox bath prior to bringing them under the fume hood for dissections to try and ward off any bacteria getting in through the embryo media. The Chlorox made the embryos brittle and affected the development and was stopped. Instead new 10% Hank's media was used to replace the media the embryos had been developing in for 15 hours. Although this kept contamination down for about 24 hours, contamination occurred in every well post 24 hours. Table 2 shows the results of experiments that were done both under a laminar flow hood, and in open air. The results showed that although exchanging the 10% Hank's media did not ward off all bacterial

contamination, it prolonged the cultures longer than any other treatment. Table 2 also shows that the best results were obtained under a laminar flow hood.

Our next stage of dealing with contamination was on additives to the media. Although many antibiotics affect developmental processes the addition of small concentrations of antibiotics were necessary to prevent contamination. We added a cocktail of penicillin and streptomycin at first at very low concentrations (1% in solution). These low concentrations had little effect on keeping bacterial contamination down. As much as a 5% concentration of the cocktail had to be used in order to keep contamination at bay. As it is known that pen/strep cocktails in high concentrations can have an effect on cellular morphology and possibly differentiation of cells, this supplement was much too high. Gentamicin is a more broad-spectrum antibiotic than penicillin and streptomycin. Although it has been shown to be lethal to cells in culture at high concentrations, at low concentrations it is more effective than the pen/strep cocktail. We used a very small concentration of Gentamicin (>.01%), which stopped bacterial contamination completely for seven days, however 24 hours later we found yeast contamination. Another additive was necessary for the prevention of fungal contamination and so the media was supplemented with fungizone. Table 3 shows the results of these experiments. All experiments reported on Table 3 were conducted under a laminar flow hood, and all dissections were carried out using the replacement of Hank's media procedure as previously discussed. Contamination was prevented for up to seven days although the adhesion of the explant and cellular migration was non-existent.

Fibronectin has been used in many cell-culturing techniques to help cells adhere to smooth surfaces more easily. Different concentrations of fibronectin were tested for

the optimal concentration of fibronectin needed for the protocol (Table 4). Optimization was determined by cellular adherence vs contamination found in the media as fibronectin begins to break down after 24 hours. Wells that had coverslips treated with fibronectin in concentrations of over 50ug/ml showed explant adhesion and sparse cellular migration. After 24 hours wells were monitored for debris from the breakdown of fibronectin. It was found that above 50ug/ml debris from the breakdown of the fibronectin matrix was excessive and a threat to the cells cultured on the plate once the excess began to lift into the media. 50ug/ml of fibronectin was used in all further dissections. Table 4 summarizes all of these results. All experiments from table 4 were conducted using the optimal media found in table 3 as well as the optimal techniques found in table 2 and discussed in previous sections.

With the addition of the fibronectin coating on the coverslips, we were now capable of getting the explants to adhere. The adhesion of the explant however did not lead to many gains for the cultivation of cranial neural crest cells. After many hours of dissections and fine-tuning of the boarders and boundaries of our landmarks there was still little migration to show. When studying the migration patterns of neural crest cells out of the hindbrain, it occurred to us that maybe the cells were migrating, however because the explant was whole, the cells were migrating inside the explant and were unable to breach the ectoderm and therefore not able to migrate out on to the coverslips. After visualizing the GFP-Sox10 embryos at 13 and 14 hpf, it was clear that all of the neural crest cells began their journey at the ventral portion of the hindbrain. 16 and 17 hpf the neural crest cells could be seen migrating dorsally. A modification of the dissections were made to scrape away some of the dorsal ectoderm to allow for cellular

migration. With the addition of this piece our protocol allowed for cultivating and migrating cells from our explant. Table 5 summarizes the results found during these experiments. All experiments from table 5 were kept under the optimal conditions found in the previous sections.

Once our system was in place for collecting migrating cells, it was necessary to distinguish what type of cells we were in fact cultivating. The process of cell identification occurred in two ways, one using our GFP line of transgenic fish, and the other using a known neural crest cell marker for immunostaining.

Sox10 is a gene that has been linked to neural crest cells. It has been demonstrated to be an integral part for neural crest cell production and for them to remain in their undifferentiated states (Hong 2005). During development sox10 has been characterized in both early and late migrating neural crest cells, and has also been shown in glial cells of the adult zebrafish (Hong 2005). Therefore cells that express sox10 can be said to be either neural crest cells, or a derivative of neural crest cells. Schilling's lab created a transgenic line of zebrafish that coupled the GFP gene to a Sox10 promoter. Therefore those cells which express Sox10 would also express GFP and could be visualized under a GFP filtered microscope. Cells that fluoresce under the GFP filter can also be said to be neural crest cells, or neural crest cell derivatives.

We have shown that a large number of our cells that migrate from the explants express GFP and therefore express or have expressed at one time sox10. Figure 18 shows an explant that was allowed to develop for 24 hours as well as cells that have migrated from this explant. Figure 18A is a brightfield image of this explant at 100X which shows adhesion of the explant as well as migration. Figure 18B shows the same brightfield

image under a GFP filter showing GFP expression. Figure 19 shows merged brightfield and GFP filtered images, further showing these cells are expressing GFP. These figures indicates that cells that have migrated from this explant are expressing, or have expressed GFP indicating they have or are expressing Sox 10.

In development, the ectoderm is split into segments, the ectoderm, the neural ectoderm, and the ectoderm. During neurulation, the neural ectoderm invaginates bringing the non-neural ectoderm regions together. This process of neurulation creates the neural tube, and a region found at the top of the invagination of neural ectoderm, which will become the neural crest and neural crest cells (Figure 16). HNK-1 is a monoclonal antibody that recognizes an antigen that is specific for tissues of neuro-ectodermal origin, which is why HNK-1 has been used as a marker for the neural crest and neural crest cells. Figure 20 and figure 21 show examples of cell explants and migrating cells that have been stained with HNK-1. We have shown that cells that migrate from our explant as well as portions of our explant stain positive for HNK-1.

Together with the results from the sox10 visualization, we can strongly suggest that the cells we have migrating onto coverslips in culture are of neural crest origin. Further studies need to be done in order to fully and correctly characterize these cell populations to determine what cells have migrated out of the explants, and what state they are in once adhered to the coverslip (differentiated or undifferentiated).

After seven days in culture the cells would all begin to detach and die off without dividing or replicating. Our lab tested the addition of L-glutamine, a known additive to media to help stimulate division. We tested concentrations as high as 10% by volume and in each case we were unable to stimulate cell division. Our lab also investigated the

possibility of addition of neurogenin1, known to stimulate division in neural crest cells in vivo. Again with concentrations as high as 200ng/ml we were unable to stimulate division. Further studies need to be done on prolonging the cells to remain in culture and replicate. There are many studies done on proliferation of neural crest cells *in vivo*, but we did not succeed to get these cells to proliferate *in vitro*. Additional studies need to be done on these cells in culture in order to develop a more stable environment for cell proliferation, or the creation of an immortalized cell line.

Development of in-situ hybridization on cells in culture

Our labs initial intention was to develop a system of *in situ* hybridization in cell culture that could be used on our explants to characterize cell populations migrating from our explants. Methods for *in situ* hybridization on whole mount embryos were taken from Dr. Capehart, and Dr. Scemama, and methods for *in situ* hybridization on thin section slices were taken from Dr. G Childs (2006) and combined to create a hybridization buffer as well as a starting point for a protocol.

A probe was developed for beta actin using amplified cDNA from HT-29 cell lines. The probe was tested using a dot blot assay and found to be at a concentration of 250ng/ul. The same procedure was used to create a probe for human HoxA10.

Investigation into the components of the protocol led to a hypothesis that the initial PFA fixation was not sufficiently keeping the cells intact during the hybridization step and the subsequent washes. After investigation of other alternatives, our lab hypothesized that if cells were initially fixed with a glutaraldehyde fixative, and then secondarily fixed with PFA the cells would remain attached to the coverslips. Testing of

this hypothesis led to positive results for cells that had been first fixed with glutaraldehyde, however staining remained spotty and insufficient when using the beta actin probes.

Further investigation into the components of the protocol led to the belief that the concentration of the proteinase K was not sufficient to allow every cell to be permeabilized thus allowing the probe to infiltrate the cell and hybridize with the complimentary RNA. Testing began on different concentrations of proteinase K starting from the removal completely of proteinase K to the addition of a 20ug/mL solution. Over perforation of the cells could result in non-specific staining as well as cells being lifted off of the coverslips, so optimization testing was done to show the most optimized solution for proteinase K. As indicated in our results, 10ug/mL led to full staining of every cell, while keeping the cells attached to the coverslip. Below 10ug/ml, staining was insufficient and spotty and above 10ug/ml cells began to detach. Figure 24 shows positive results for *human beta actin* staining as well as the negative control. This image also shows the ability of the technique to maintain cells on the coverslips during the frequent washes and hybridization.

Once the protocol had been sufficiently worked out to produce positive results for beta actin in the majority of the cells, sensitivity testing was done to see how specific this method for *in situ* hybridization could be. A probe for Hox A10 was used to show that genes that were not as widely expressed across the cell line could also be used to characterize individual cells. Our results indicate that Hox A10 is capable of being used as a probe to gain positive results on HT-29 cells. As seen in figure 25 the full arrows indicate staining inside the nucleus of cells, where the arrowheads indicate cells which

had no staining inside the nucleus. Although our results indicate some cells with staining inside the nucleus and some cells that do not show staining, our results do not show if the fact that some cells do not show staining indicates that the cells are not expressing Hox A10 because of a possibility of incomplete staining. Further testing needs to be done on the sensitivity and accuracy of this protocol before it can be sufficiently used to show accurate results.

Our lab wanted to show that this positive testing we were getting from human beta actin on HT-29 cells could be transferred across cell lines. We hypothesized that if we had in fact created a probe for beta actin, and our *in situ* hybridization protocol had been worked out, then we should be able to get positive results for beta actin on another human cell line. We tested the Caco2 cell line with our human beta actin probe and again received positive results. These positive results indicate that this method for cell characterization might be one day perfected and used as an additional method for cell characterization on cells in culture.

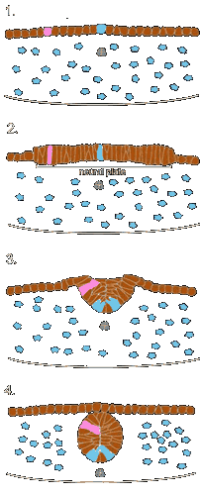
Conclusion

The studies presented in this thesis are an important addition to the scientific community and could possibly lead to many exciting endeavors in developmental understanding. It is in understanding processes and pathways that occur during development that we can begin to troubleshoot problems that arise in developmental processes. Characterization of these pathways in zebrafish is the beginning in understanding similar pathways in higher organisms. Treatments for many developmental defects involving cranial neural crest cells such as cleft pallet, Pierre Robin syndrome, Warding Shah syndrome and many others can be found simply by understanding what goes wrong during these developmental processes in neural crest cell migration and differentiation.

Characterization of the differentiation of cranial neural crest cells can also lead to advances in the treatment for Alzheimer. Understanding of the differentiation of neural crest cells into neurons and understanding neurogenesis that goes on within these cells can lead to possible advances and possible treatments for the regeneration of neurons within adults.

Although the suggestions made in this thesis for future application of this research is in the distant future, this research is the groundwork that will be necessary for development of future progression in developmental studies.

Figure 1



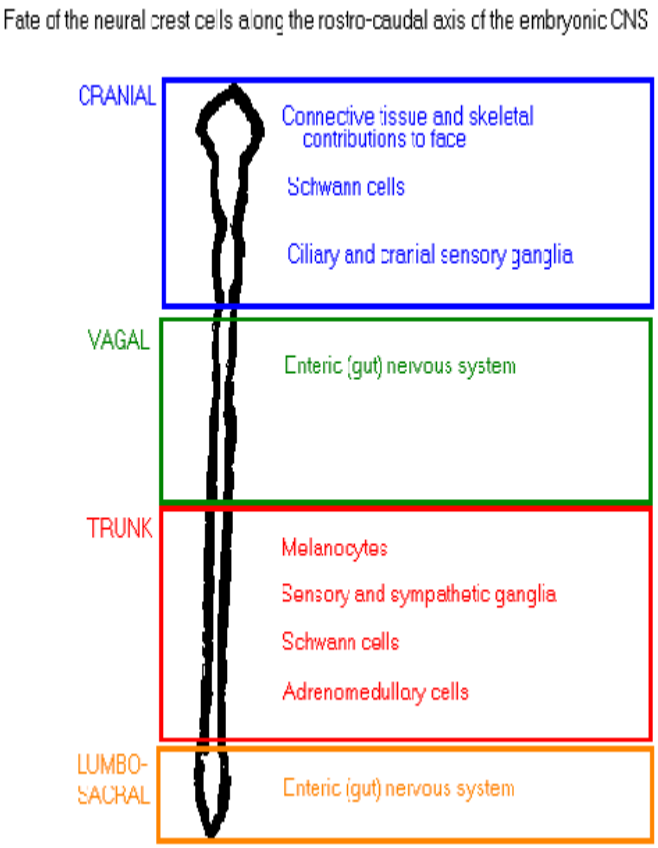
Neurulation and Neural Development: *The process of neurulation: 1) and 2) ectodermal epithelium condensation and columnization; 3) and 4) neural keel formation and separation from the surrounding ectodermal epithelium (edited from Gilbert 2000)*

Table 1

Arise From NC	Differentiates into
• Neurones	Peripheral nervous system (PNS): sensory, sympathetic and enteric ganglia
• Glial cells	PNS satellite glial cells and Schwann cells of peripheral nerves
• Pigment cells	Skin melanocytes
• Endocrine cells	C cells of the thyroid, Catecholaminergic cells of the adrenal gland
• Mesenchymal cells in head and neck	Cartilages and bones Odondoblasts Dermis Connective tissues in muscles and glands Meninges of the forebrain Vascular smooth muscle cells Adipocytes

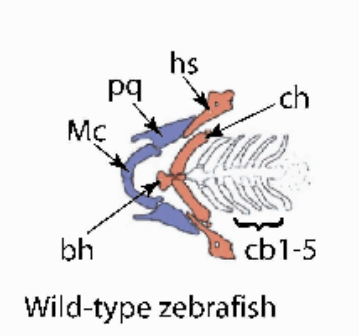
Table 1: *List of some of the cell types that can arise from Neural Crest Cells (neurones, glial cells, pigment cells, endocrine cells, and mesenchymal cells)(Column 1) and some of the cell types those derivatives can differentiate into (column 2)*

Figure **2**



Regions of the Neural Tube and Cell Derivatives from these Regions: *Different regions of the neural crest and what cell types they are responsible for differentiating into based on location within the embryo (edited from Gilbert 2000)*

Figure 3



Craniofacial Bones and Cartilages Derived from the Cranial Neural Crest*: Bones colored in blue represent PA1-derived bones. Bones colored in red represent PA2-derived bones. Bh, basihyal; cb, ceratobranchial; ch, ceratohyal; hs, hyosymplectic; Mc, Meckel's cartilage; pq, palatoquadrate;(Schilling 2001)*

Figure 4

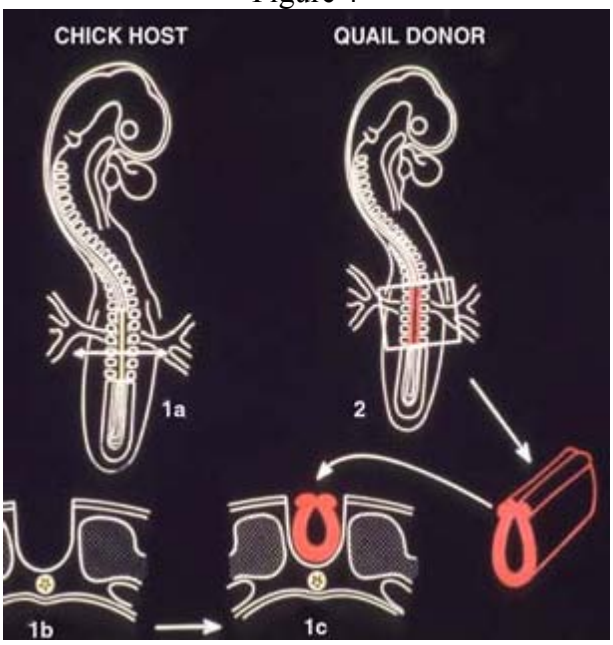
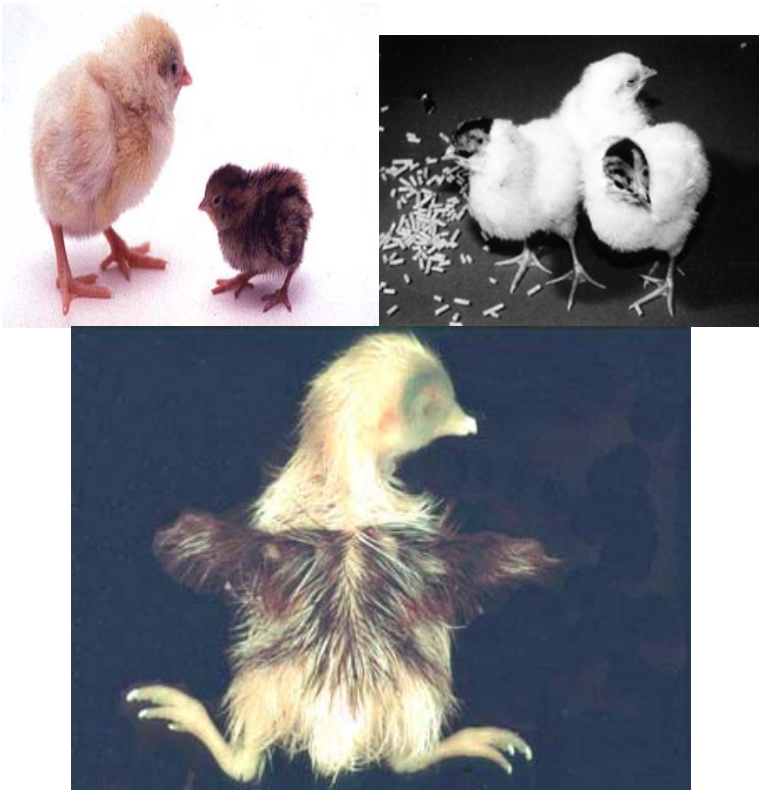


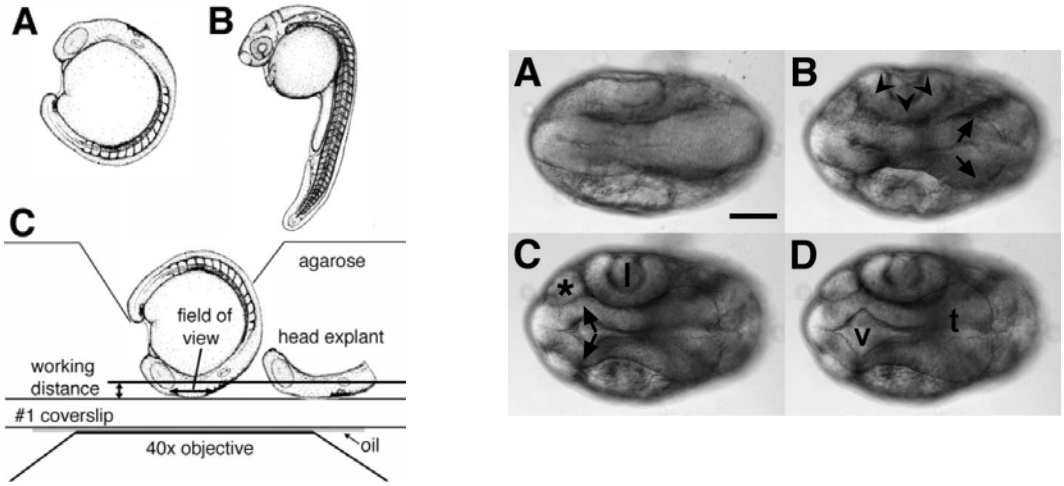
Figure 4: *Transplantation method done on quail and chick embryos. Part of the developing embryo's neural tube was dissected out and fused with a host embryo. The resulting organism is a chimera of both embryos. These studies dissected different portions of the neural tube to distinguish each portions role in the developmental process (Le Douarin 1969).*

Figure 5



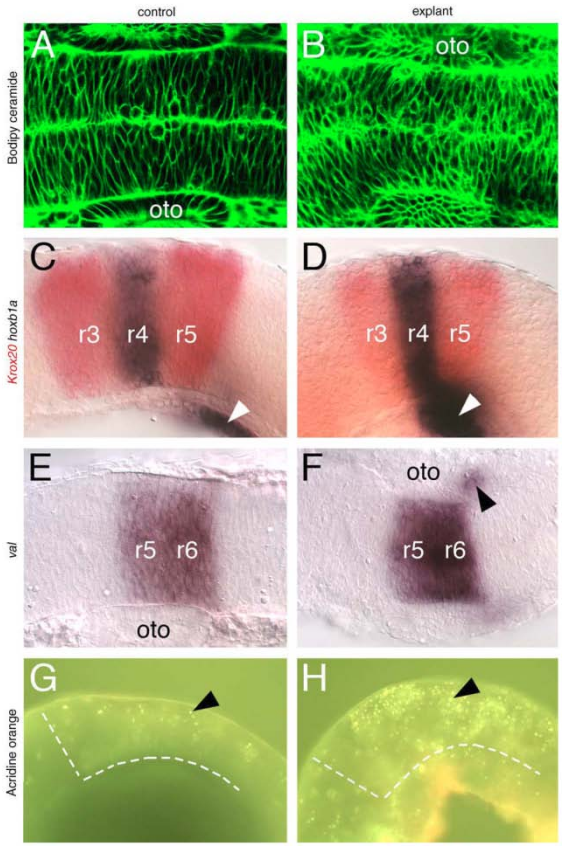
Chick Quail Chimera Formed from Neural Crest Transplant Studies: *Top left shows the organisms used in Le Douarin's study a normal chick compared to a normal quail hatchling. The top right shows the chimera formed after a segment of the cranial neural tube had been transplanted from a quail into a chick embryo. The facial features resemble that of a quail while the body resembles that of a chick. The bottom photo is another chimera where the trunk region of the neural tube from a quail was transplanted into the embryo of a chick. The resulting hatchling resembles the midsection of a quail and the rest of a chick. These studies show what regions are responsible for what parts developmentally (Le Douarin 1969).*

Figure 6



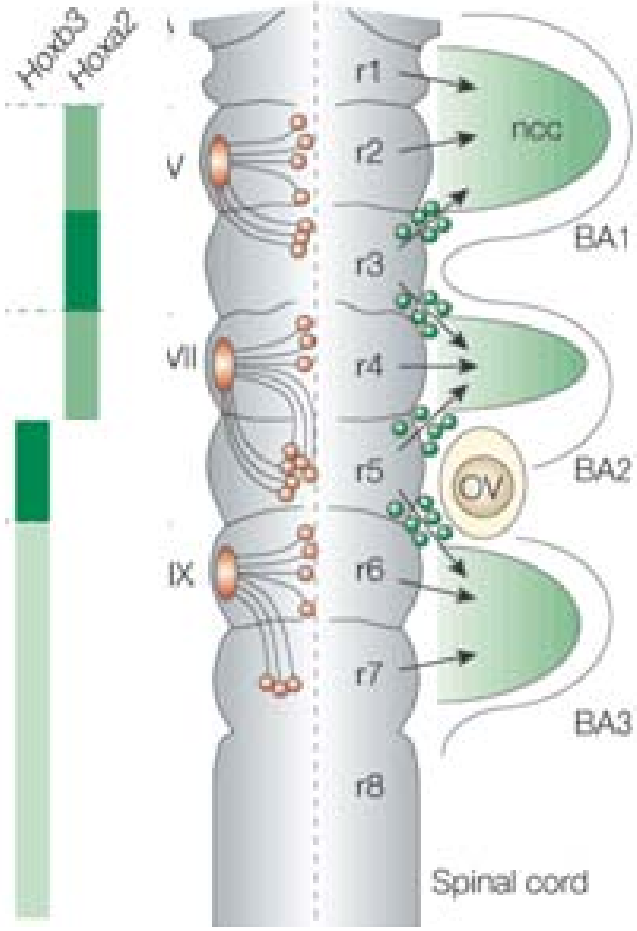
Langenberg et al Explant Experimental Procedures and Results: *The image to the left indicates the procedure followed by Langenberg et al to create whole head explants mounted in agarose to visualize organogenesis of the brain. The image to the right shows the normal developmental processes of organogenesis of the brain in the head explants of zebrafish embryos (Langenberg 2003).*

Figure 7



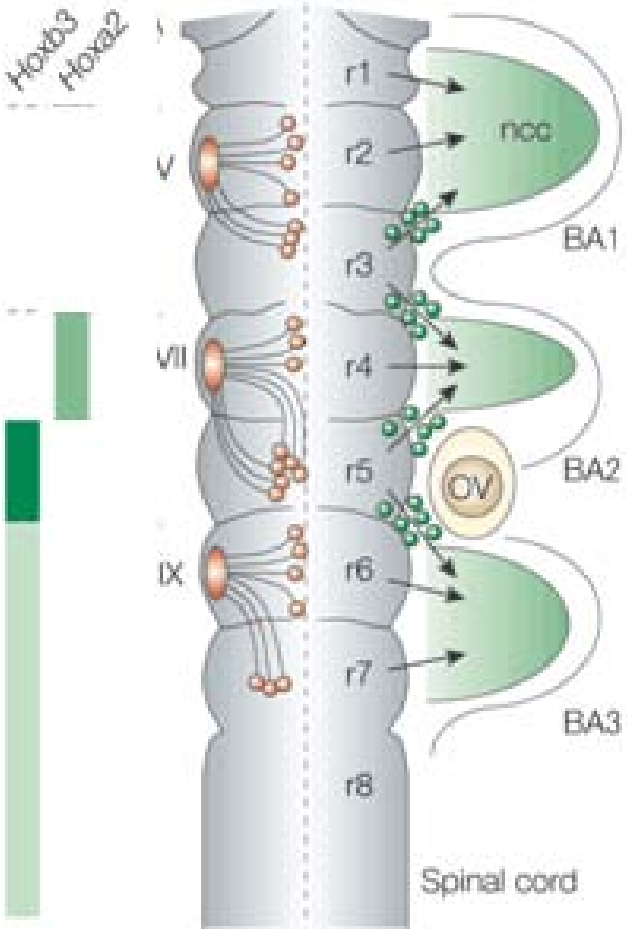
Bingham et al Experiments on Explantation and Gene Patterning on Explants: *This figure is a study done by Bingham et al testing the morphology, migration, and development of wild type zebrafish embryos vs explanted zebrafish embryos. The column to the left shows a normal developing embryo where the column to the right shows the explant. In all cases there is no clear difference in gene patterning, morphology or migration between the wild type and the explants (Bingham et al 2003).*

Figure 8



Gene Expression in Hindbrain Rhombomeres: *Hox expression in the rhombomeres of the developing mouse embryo (Edited from Santagati 2003).*

Figure 9



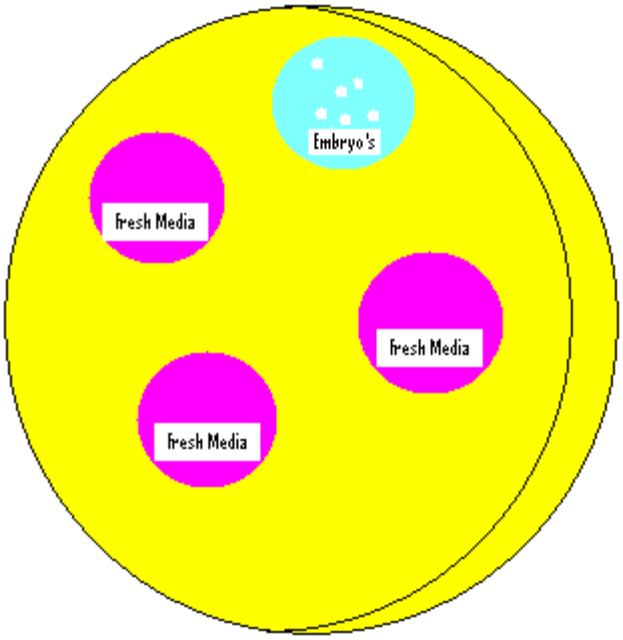
Gene Expression of Cells Migrating out of Hindbrain: *Hox expression of the cranial neural crest cells that migrate from the rhombomeres of the mouse hindbrain (Edited from Santagati 2003).*

Figure 10

Egg Collection System in Tank

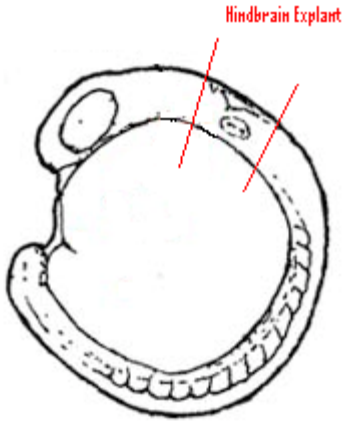
Egg Collection System: *Egg collection system that was used in the tanks to collect embryos for dissection experiments. The top left shows the bottom of the mouse cage which is solid followed by the image to the right of that showing the grated insert with pores that allow eggs to fall through.*

Figure 11



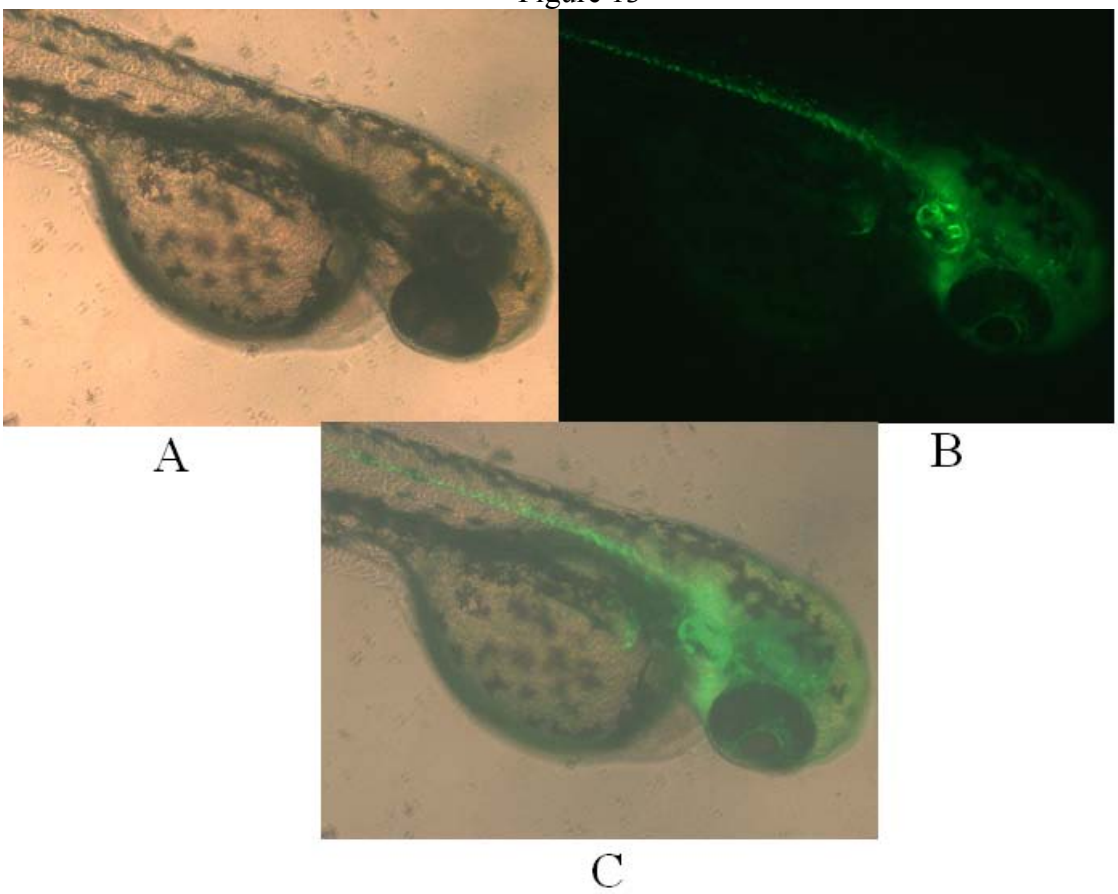
Petri Dish Setup for Dissections: *Setup of the petri dish used during zebrafish hindbrain dissections. The blue indicates embryos in 10% Hank's solution. The purple indicates fresh media. The embryos were dechorionated in the blue and brought to one of the purples where the dissections took place. Once dissected out, the explant was moved to a second, and a third wash station before being placed in a well in a tissue culture dish. The embryos were then placed in dissecting wells and allowed to develop for 24 hours.*

Figure 12



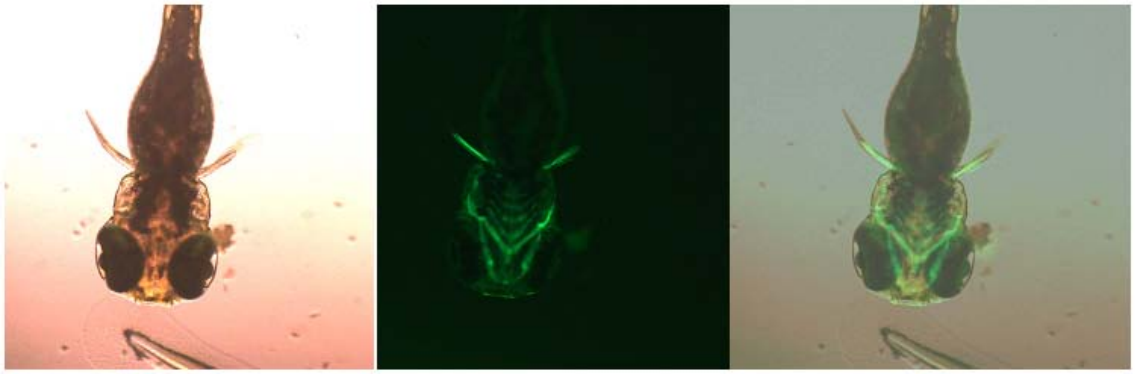
Schematic of Explant Dissections*: Portion of the developing embryo that was used as the hindbrain explant during dissections. The red indicates a cut that had been done to the embryo. In between the red hash marks is the otic vesicle which was used as a dissecting landmark.*

Figure 13



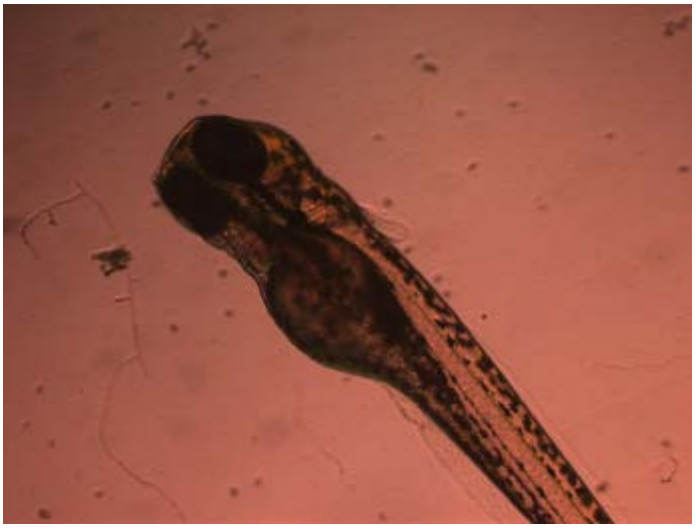
GFP Expression in 24hpf Zebrafish Embryo: *A) 24hpf embryo under brightfield, B) same embryo under a GFP filter, and C) a merged image showing GFP expression in the developing embryo 24hpf*

Figure 14



GFP Expression in 72hpf Zebrafish Embryo: *A) 72hpf embryo under brightfield, B) same embryo under a GFP filter and C) merged image of the two. C shows GFP expression in the developing embryo 72hpf.*

Figure 15



Developing Embryo 24hpf in Dissecting Media: *Embryo that was dechorionated after 15hpf and allowed to develop 24hpf in dissecting media.*

Table 2

	No Hood	**No Hood + .01% Chlorox**	**No Hood + Exchange 10% Hank's**	**Hood**	**Hood + .01% Chlorox**	**Hood + Exchange 10% Hank's**
Contamination (After 24h)	X	X	X	X	X	Prolonged
Adhesion	No	No	No	No	No	No
Migration	No	No	No	No	No	No

Table 2: *Table shows the results of a group of experiments testing for affect on contamination, adhesion, and migration. No Hood refers to experiments that were not done under a laminar flow hood, .01% Chlorox refers to a rinsing of the embryos before incubation with a Chlorox solution (see materials and methods), and Exchange of 10% Hank's refers to an exchange of the old media the embryos were developing in prior to dissection with new media before dissections were carried out.*

Table 3

	1% Pen/strep	+5% Pen/strep	.01% Gentomyocine	.01% Gentomyocine +.01% Fungizone
Contamination (Bacterial)	**Yes**	**No**	**No**	**No**
Contamination (Fungal)	**Yes**	**Yes**	**Yes**	**No**
Adhesion	**No**	**No**	**No**	**No**
Migration	**No**	**No**	**No**	**No**

Table 3: *Table summarizes the results of experiments done testing different concentrations of antibiotic cocktails on contamination, adhesion, and migration.*

Table 4

	0µg	25µg	50µg	75µg	100µg
Contamination of media	No	No	No	Yes	Yes
Adhesion	No	No	Yes	Yes	Yes
Migration	No	No	Some	Some	Some

Table 4: *Summarizes experiments done on the concentration of fibronectin in µg/ml of PBS 'vs' contamination of the media by the fibronectin excess, adhesion of the explant, and migration from the explant.*

Table 5

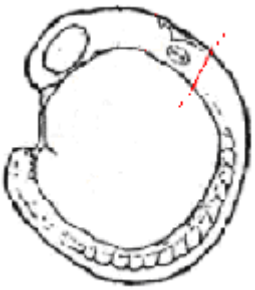
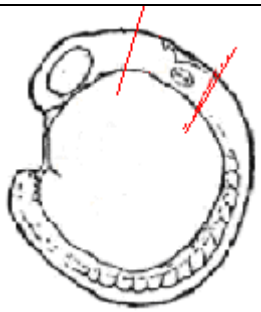
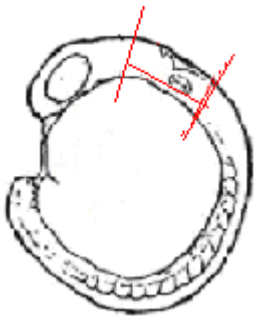
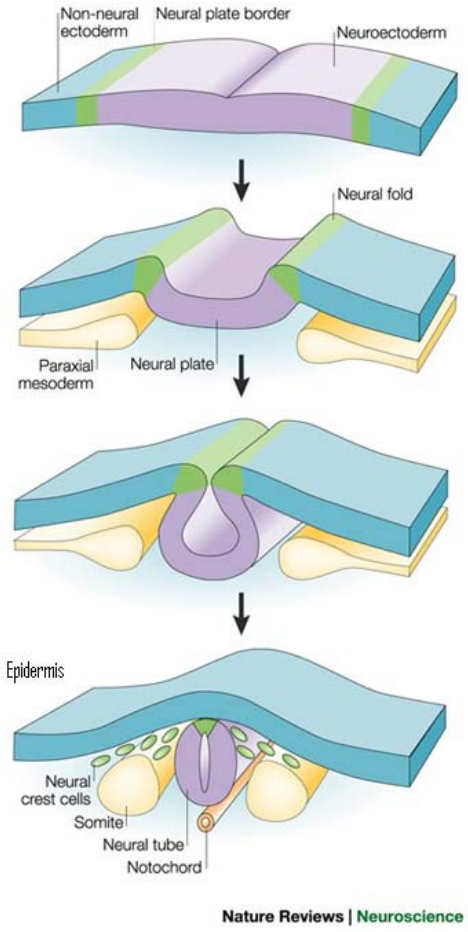
			
Contamination	**No**	**No**	**No**
Adhesion	**Yes**	**Yes**	**Yes**
Migration	**No**	**Little**	**Yes**

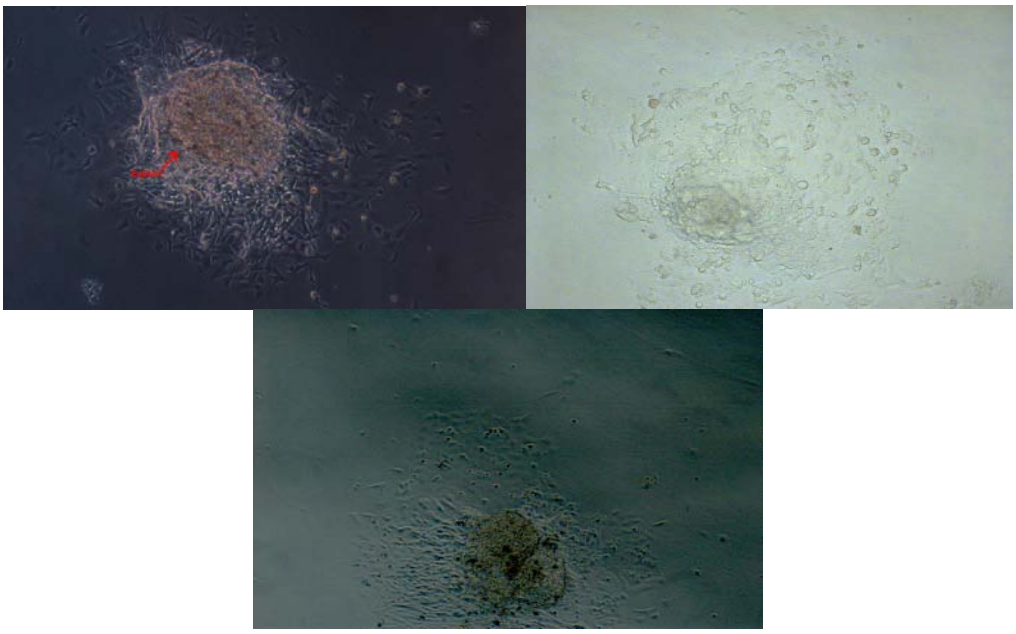
Table 5: *Summarizes the results of contamination, adhesion, and migration on the explant location. The first dissection landmark was just behind the otic vesicle, the second was in front of and behind the otic vesicle, and the third was using the same as the second, but scraping away some of the ectoderm on the ventral side.*

Figure 16



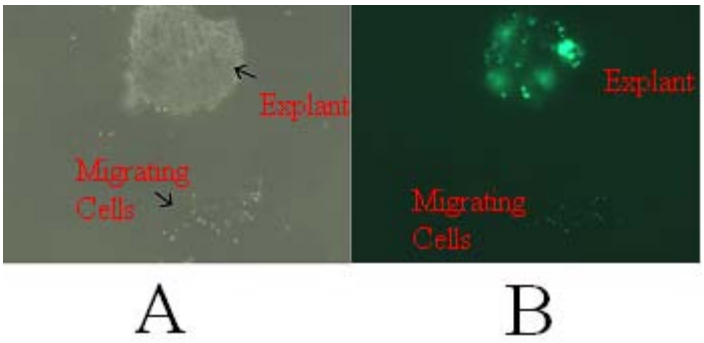
Process of Neurulation and Production of the Neural Crest and Neural Crest Cells: *This image shows the differentiation and formation of the neural crest, neural crest cells and the neural tube in a process called neurulation which occurs by the invagination of the neural plate. This invagination results in the coming together of two portions of neural ectoderm which results in the formation of the neural crest (image edited from Gilbert 2000).*

Figure 17



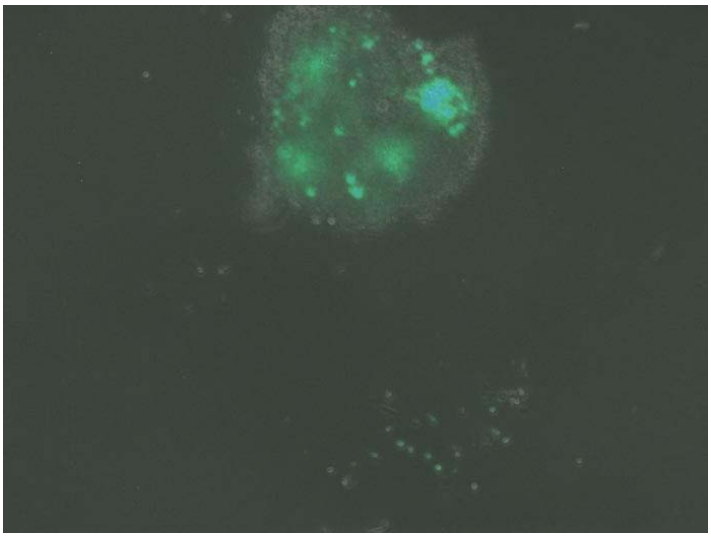
Explant with Migrating Cells: *Three different examples of zebrafish hindbrain explants that had been dissected 15hpf and allowed to develop in media for 24 hours. These images show migrating cells emigrating from adhered explants.*

Figure 18



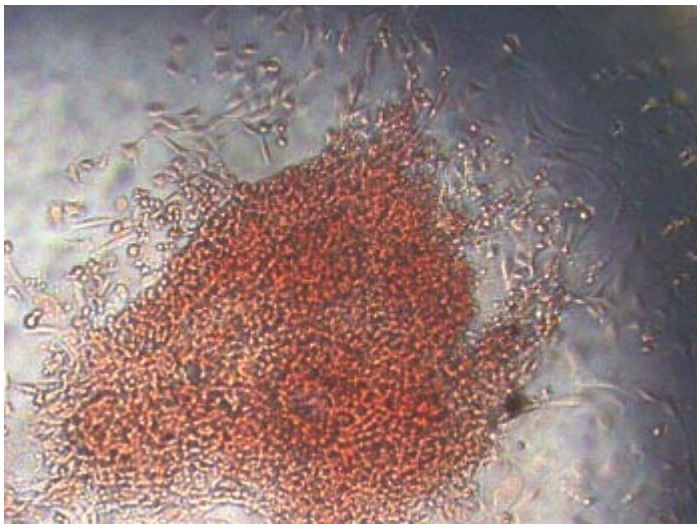
GFP Expression in Explant and Migrating Cells: *Explant from a 15hpf sox 10-GFP transgenic zebrafish embryo after placed in media and allowed to develop for 24 hours after dissection. Figure A) shows a brightfield image of explant 24 hours after dissection. Figure B) Shows the same image under a GFP filter. These images show migrating cells which are expressing GFP.*

Figure 19



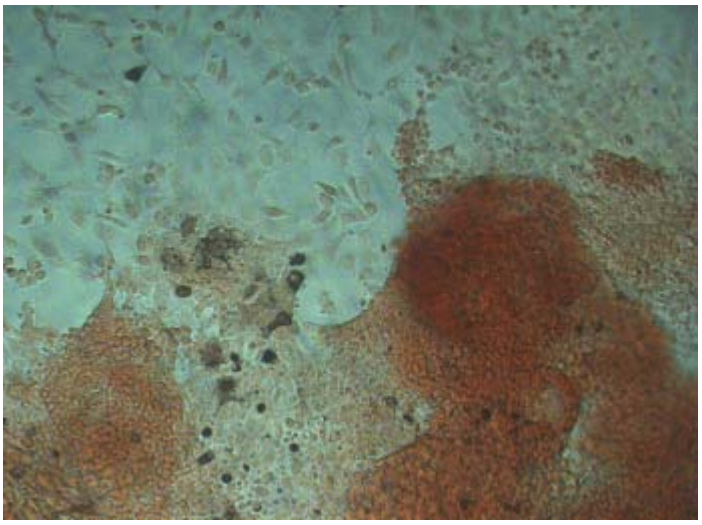
GFP Overlay of Brightfield and GFP Filtered Images: *Hindbrain explant from a sox 10-GFP transgenic zebrafish embryo after allowed to develop 24 hours in media. This shows a merged image of the brightfield and the GFP filtered images. The overlap shows a large majority of cells that have or are expressing GFP.*

Figure 20



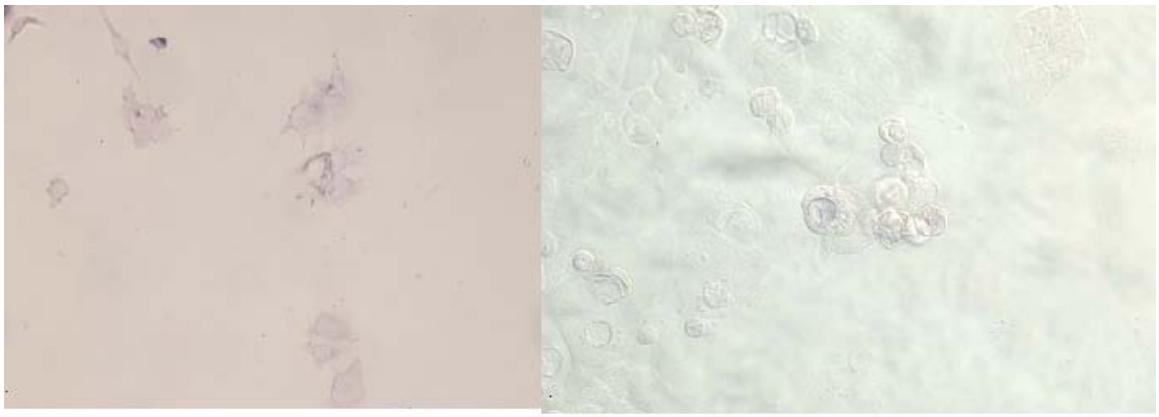
HNK-1 Stained Explant and Migrating Cells: *Hindbrain explant from a sox 10-GFP transgenic zebrafish embryo 15hpf and allowed to develop in media 24 hours after dissection. Image shows an explant with migrating cells that have all been stained with HNK-1.*

Figure 21



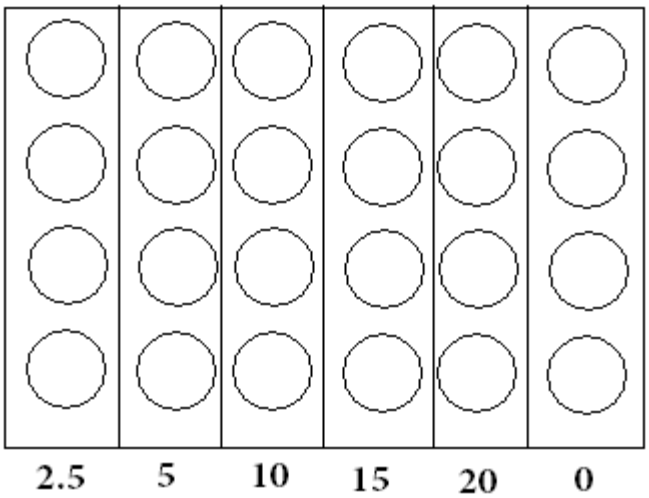
HNK-1 Stained Explant and Migrating Cells: *Hindbrain explant from a sox 10-GFP transgenic zebrafish embryo 15hpf and allowed to develop in media 24 hours after dissection. This image shows an example of an explant with migrating cells stained with HNK-1*

Figure 22



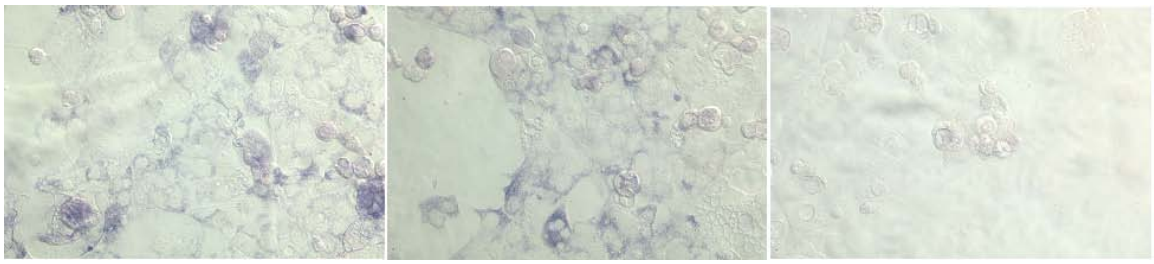
Remaining Cells After Pre-fixed in 4%PFA and 2.5% Glutaraldehyde and Exposed to In Situ Protocol: *The image on the left shows a coverslip with HT-29 cells that have been fixed with 4%PFA initially. The image to the right show a coverslip with HT-29 cells that have been fixed with 2.5% glutaraldehyde. After initial fixation protocol for in situ hybridization was performed.*

Figure 23



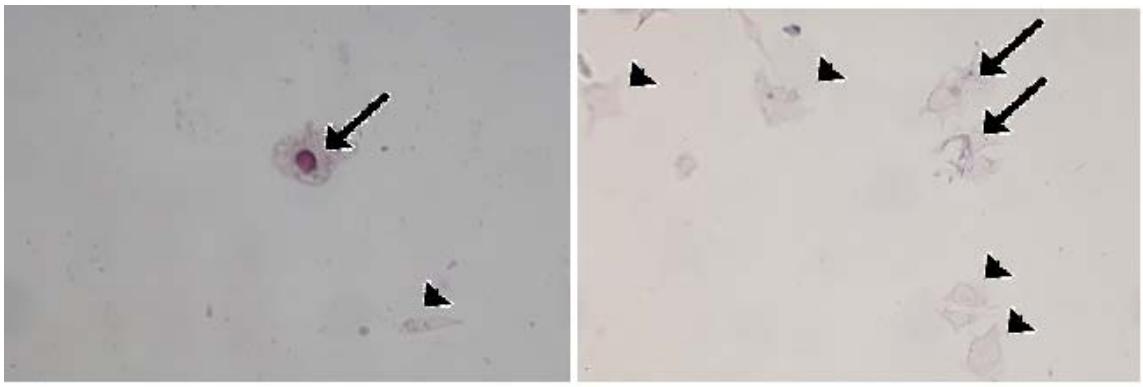
Experimental Setup Testing Concentrations of Proteinase K: *This shows a set up for an experiment testing the concentration of proteinase K (along the bottom) in µg/ml in the protocol for in situ hybridization.*

Figure 24



Positive Staining of Human Beta Actin on HT-29 Cells: *The first and the second image show positive staining for human beta actin on the HT-29 human colon cancer cell line. The third image shows a negative control for human beta actin.*

Figure 25



Positive Nucleus staining of hox a10 on HT-29 Cells: *HT-29 human colon cancer cells that have been exposed to an in situ hybridization protocol with probes for Hox A10. The arrows show positive staining in the cell nucleus while the arrow heads show cells without staining.*

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