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# Next Generation Sequencing Reveals Gene Expression Patterns in the Zebrafish Inner Ear Following Growth Hormone Injection

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NEXT GENERATION SEQUENCING REVEALS GENE EXPRESSION  
PATTERNS IN THE ZEBRAFISH INNER EAR FOLLOWING GROWTH  
HORMONE INJECTION

A Thesis  
Presented to  
The Faculty of the Department of Biology  
Western Kentucky University  
Bowling Green, Kentucky

In Partial Fulfillment  
Of the Requirements for the Degree  
Master of Science

By  
Gopinath Rajadinakaran

August 2012

NEXT GENERATION SEQUENCING REVEALS GENE EXPRESSION PATTERNS IN  
THE ZEBRAFISH INNER EAR FOLLOWING GROWTH HORMONE INJECTION

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The mother who hears her son called a “wise man” will rejoice more  
than when she did at his birth.

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NEXT GENERATION SEQUENCING REVEALS GENE EXPRESSION  
PATTERNS IN THE ZEBRAFISH INNER EAR FOLLOWING GROWTH  
HORMONE INJECTION

Gopinath Rajadinakaran

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71 Pages

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Loss of hair cells due to acoustic trauma results in the loss of hearing. In humans, unlike other vertebrates, the mechanism of hair cell regeneration is not possible. The molecular mechanisms that underlie this regeneration in non-mammalian vertebrates remain elusive. To understand the gene regulation during hair cell regeneration, our previous microarray study on zebrafish inner ears found that growth hormone (GH) was significantly upregulated after noise exposure. In this current study, we utilized Next Generation Sequencing (NGS) to examine the genes and pathways that are significantly regulated in the zebrafish inner ear following sound exposure and GH injection. Four groups of 20 zebrafish each were exposed to a 150 Hz tone at 179 dB re 1 $\mu$ Pa RMS for 40 h. Zebrafish were injected with either salmon GH, phosphate buffer or zebrafish GH antagonist following acoustic exposure, and one baseline group received no acoustic stimulus or injection. RNA was extracted from ear tissues at 1 and 2 days post-trauma, and cDNA was synthesized for NGS. The reads from Illumina Pipeline version SCS 2.8.0 were aligned using TopHat and annotated using Cufflinks. The statistically significant differentially expressed transcripts were identified using Cuffdiff for six different pairwise comparisons and were analyzed using Ingenuity Pathway Analysis. I found significant regulation of growth factors

such as GH, prolactin and fibroblast growth factor receptor 2, different families of solute carrier molecules, cell adhesion molecules such as CDH17 and CDH23, and other transcription factors such as Fos, FosB, Jun that regulate apoptosis. Analysis of the cell proliferation network in the GH-injected condition compared to buffer-injected day 1 showed significant up-regulation of GH while down-regulation of apoptotic transcription factors was found. In contrast, the antagonist-injected condition compared to the GH-injected condition showed an opposite pattern in which up-regulation of apoptotic transcription factors were found while GH was down-regulated. A number of other transcripts (e.g., POMC, SLC6A12, TMEM27, HNF4A, CDH17 and FGFR2) that showed up-regulation in GH-injected condition showed down-regulation in antagonist-injected condition. These results strongly suggest that injection of exogenous GH potentially has a protective role in the zebrafish inner ear following acoustic trauma.

## Introduction

### *Anatomy of the teleost auditory system:*

Hair cells are mechanosensory cells found in both vertebrates (Avallone *et al.*, 2008; Harris *et al.*, 2003; Jones & Corwin, 1996; Song *et al.*, 1995) and invertebrates (Budelmann, 1994). They are present in the auditory and vestibular systems of all vertebrates, and the lateral line system of aquatic vertebrates such as fish and amphibians (Hudspeth & Corey, 1977; 1979; Bracho & Budelli, 1978). All together this system is called the acoustic-lateralis system which helps to detect sound, body acceleration and water movement (Hudspeth & Corey, 1977; Popper, 2005). The hair cells present in this system receive sounds and vibrations from the surroundings and convert them into a neural stimulus which is sent to the brain. The cell body of the hair cell gives rise to ciliary projections called stereocilia and a true kinocilium. While this is true in most vertebrates, hair cells of the mammalian cochlea lack kinocilia (Hudspeth & Corey, 1977). In non-vertebrates, the hair cells lack stereocilia but the kinocilia are present in vast numbers ranging between 1 and 700 (Hudspeth & Corey, 1977; Budelmann, 1994). In vertebrates, the kinocilium is the longest ciliary projection in a hair cell and the movement of stereocilia towards the kinocilium excites hair cells while movement to the opposite side causes inhibition.

Unlike mammals, fishes have no external or middle ears but they have a pair of inner ears located adjacent to the brain inside the cranial cavity (Hastings & Popper, 2005; Popper & Hastings, 2009). The auditory system of fish is organized into pairs of three sensory otolithic endorgans called the saccule,

lagena and utricle. The sensory epithelia of these organs are called maculae which contain a high density of mechanoreceptor hair cells. Each otolithic endorgan encases a bony structure called an otolith made of calcium carbonate and a protein matrix (Takagi & Takahashi, 1999). Each otolith overlies the sensory macula of one of the auditory endorgans. These otolithic compartments are filled with endolymphatic fluid and are interconnected by semicircular canals between all the three organs. They have two main functional roles in fishes: first, to measure the position of the head relative to gravity and the acceleration of the body in all possible directions, and second, to detect sounds. Like the otolithic organs, hair cells in vestibular canals are also involved in detecting angular acceleration (Bang *et al.*, 2001; Hastings & Popper, 2005).

*Hair cell damage:*

Hair cells are susceptible to damage and subsequent loss from a variety of insults resulting in deficits in hearing and balance. The functions inherent to the inner ear such as the head movement, orientation in space, and detection of sounds (Bang *et al.*, 2001; Matsui & Ryals, 2005) and to the lateral line system such as low frequency vibration and current detection (Hama 1965) are severely impaired due to hair cell loss and hence causes hearing and balance disorders (Matsui & Ryals, 2005; Suli *et al.*, 2012).

Hearing loss can be caused by a number of factors, including damage to hair cells and auditory nerves (i.e., sensorineural hearing loss; Tarlow *et al.*, 1991; Ryan, 2000), genetic defects (Matsui & Ryals, 2005), and age-related

hearing loss (Huh *et al.*, 2012). Hair cell damage can result from loud acoustic exposure (Poche *et al.*, 1969; Matsui & Ryals, 2005; Smith *et al.*, 2004a; 2004b; 2006) or exposure to ototoxic chemicals (Wright, 1973; Matsui & Ryals, 2005). Although other possibilities exist that may cause damage to hair cells, ototoxic exposure and acoustic trauma are the two most widely used methods to understand the molecular mechanisms of hair cell damage. The ototoxic chemicals that have been examined include a wide variety of compounds such as heavy metals: copper (Hernandez *et al.*, 2006, 2007; Olivari *et al.*, 2008); aminoglycoside antibiotics: gentamycin, streptomycin, kanamycin, tobramycin, and neomycin (Hu *et al.*, 1991; Hutchin & Cortopassi, 1994; Lombarte *et al.*, 1993; Ma *et al.*, 2008; Murakami *et al.*, 2003; Owens *et al.*, 2007; 2008; 2009); platinum based drugs: cisplatin (Ton and Parng, 2005; Muldoon *et al.*, 2000) and carboplatin (Neuwelt *et al.*, 1996).

The effects of hair cell damage using these two methods have been studied in many model organisms. The mammalian models include guinea pig (Tarlow *et al.*, 1991), rat (Liu *et al.*, 2011), mouse (de Jong *et al.*, 2012), and non-mammalian models include chick (Stone & Cotanche, 1992), zebrafish (Harris *et al.*, 2003; Schuck & Smith, 2009; Sun *et al.*, 2011), goldfish (Smith *et al.*, 2004a; 2004b; 2006), salamander (Balak & Corwin, 1990; Jones & Corwin, 1996), lizard (Avallone *et al.*, 2008), and bullfrog (Baird *et al.*, 2000). The use of cell culture is also in practice to study the damage of hair cells (Warchol & Corwin, 1996; Alharazneh *et al.*, 2011).

Following hair cell loss, regeneration occurs in non-mammalian vertebrates but not in the mammalian auditory system (Taylor & Forge, 2005; Roberson & Rubel, 1995; Yamashita & Oesterle, 1995; Avallone *et al.*, 2008; Smith *et al.*, 2006; Stone & Cotanche, 1992; Montcouquiol & Corwin, 2001). Since non-mammalian vertebrates such as birds (Matsui & Ryals, 2005), amphibians (Balak *et al.*, 1990), and fish (Sun *et al.*, 2011) have the capability of regenerating lost hair cells, they have been used as model systems to help scientists understand the hair cell regeneration process (Ryan, 2000). Understanding the cellular mechanisms of hair cell regeneration in these organisms may lead to the development of potential therapeutics or prophylactics for deafness in humans.

*Zebrafish as a model organism:*

Although a number of model organisms are available for studying hair cell damage, the zebrafish is widely used as a model organism in the field of otolaryngology as much is known about their hair cell degeneration and regeneration in this species (Guthrie 2008; Harris *et al.*, 2003; Mangiardi & Cotanche, 2005; Owens *et al.*, 2007; Olivari *et al.*, 2008; Yan *et al.*, 1991; Smith *et al.*, 2004a; 2004b; 2006; 2011). Studies with zebrafish hair cells show that they react similarly to those of mammals in response to ototoxins such as copper or aminoglycoside antibiotics (Guthrie 2008; Harris *et al.*, 2003; Olivari *et al.*, 2008). The molecular mechanisms controlling the development of the zebrafish inner ear is similar to that of humans although zebrafish lack the mammalian

equivalent of a cochlea (Brignull *et al.*, 2009). The forward genetics and antisense technology is well established for examining the development of the zebrafish inner ear which also makes it an excellent model (Nicolson, 2005). Zebrafish have a high fecundity rate and the young, translucent larvae facilitate live imaging of regenerating hair cells on neuromasts along the lateral line. All these features make the zebrafish an appropriate model for studying regeneration of hair cells. The main purpose of my research was to explore the molecular mechanisms of hair cell regeneration in zebrafish by examining patterns of gene expression following acoustic trauma.

*Characteristics of hair cells undergoing damage in fishes:*

When hair cells are damaged either with intense noise or with ototoxins, they undergo morphological changes. These changes include the formation of apoptotic bodies, activation of caspases and formation of reactive oxygen species in zebrafish, rodents, frogs and chickens exposed to ototoxins (Mangiardi & Cotanche, 2005). In the zebrafish and goldfish saccule, the changes in the cell structure following acoustic trauma included formation of thin or fused stereocilia, cuticular plates which lost all stereociliary bundles, lesions and scars (Schuck & Smith, 2009; Smith *et al.*, 2006). These morphological changes were also associated with a drastic reduction in the average hair cell density (Schuck & Smith, 2009; Smith *et al.*, 2006). The number of apoptotic cells in both saccule and lagena increased significantly after noise exposure and decreased over time in zebrafish and goldfish, but in an experiment with goldfish,



exposure to sound did not cause any significant damage to the utricles at any point of time during observation (Smith *et al.*, 2006) showing a differential sensitivity of hearing organs in fish. In addition to the morphological damage, the loss of hair cells in goldfish contributed to an increase in Temporary Threshold Shift (TTS) immediately after sound treatment (Smith *et al.*, 2004a; 2004b; 2006). Hence, loss of inner ear hair cells is indicative of hearing loss in fish.

#### *Hair cell regeneration in fishes:*

Regeneration of hair cells is facilitated by the presence of stem cell-like supporting cells around the hair cells. The supporting cells are present in the lateral line system of fish (Harris *et al.*, 2003). There is yet another population of cells called mantle cells found along the rim of neuromasts in the lateral line system (Hernandez *et al.*, 2007; Brignull *et al.*, 2009). The regeneration of hair cells can arise via two processes: mitotic proliferation or direct trans-differentiation (Brignull *et al.*, 2009; Hernandez *et al.*, 2006; Harris *et al.*, 2003). In mitotic proliferation, the supporting cells undergo cell division and this could be either symmetric or asymmetric. When the supporting cells divide equally into either two hair cells or two supporting cells, it is termed a symmetric division. In asymmetric division, one hair cell and one supporting cell result from a single division of a supporting cell. The asymmetric division replenishes the population of both progenies while a symmetric division is biased. In contrast, the trans-differentiation mechanism does not undergo any of these divisions; rather a

supporting cell is directly converted (changes its phenotype) into a hair cell (Brignull *et al.*, 2009).

These two different mechanisms operate through different signaling pathways and at different points in time. There is evidence that supporting cells which normally reside near the basal lamina of the cochlea move toward the lumen to undergo mitosis (Brignull *et al.*, 2009). Previous experiments with both goldfish and zebrafish showed hearing recovery which corresponded to an increase in the hair cell count following exposure to sound (Schuck & Smith, 2009; Smith *et al.*, 2006). Phalloidin-staining in zebrafish and goldfish inner ear following sound exposure confirmed that newly-regenerated hair cells show immature morphology with the formation of short hair bundles, and an increase in the total number of hair cells (Schuck & Smith, 2009; Smith *et al.*, 2006). BromodeoxyUridine (BrdU)-labeling showed an increase in the proliferation of hair cells following acoustic damage (Schuck & Smith, 2009). TUNEL-labeling for apoptotic cells showed an increase in cell death in both the saccule and lagena immediately following damage but a decrease in labeling over time post-trauma (Smith *et al.*, 2006). The hair cell recovery that followed sound exposure was accompanied by a decrease in temporary threshold shift, supporting the view that the regenerated hair cells are functional and improve hearing (Smith *et al.*, 2006). These studies show that hair cells undergo death immediately following acoustic-trauma and an increase in the number of hair cells was observed through mitotic proliferation.

A transcriptomics study by Schuck *et al.* (2011) on the zebrafish inner ear found that acoustic damage at two days post-trauma stimulates the expression of growth hormone (GH) transcripts coupled with a significant regulation of cell death and cell growth and proliferation functions. Another study, by Sun *et al.* (2011) found that peritoneal injection of external growth hormone in the zebrafish decreases apoptosis and increases hair cell regeneration at one day following sound-induced damage. These studies suggest that the expression of growth hormone might be important to the recovery of damaged hair cells or it may have an otoprotective role in zebrafish inner ears. The goal of the current study was to identify the genes, functional networks, canonical pathways and other transcription molecules that are specifically regulated by growth hormone following acoustic trauma using Next Generation Sequencing.

#### *Next Generation Sequencing:*

Transcriptomics is the study of whole mRNA of a cell to understand gene expression levels under different conditions. It is a powerful tool to identify specific networks of genes that are regulated under different natural or experimental conditions. To study transcriptomics, there are two widely used tools: microarray and Next Generation Sequencing (NGS). Each technology has its own advantages and disadvantages. Sample preparation is the first step in both methods. Purity of sample is a major factor that determines the quality of all downstream processing of mRNA. While microarrays have been widely used, they often have issues of consistency, reliability, sensitivity and specificity of the

data (MAQC consortium 2006). Microarray experiments can now be complemented by NGS technology.

NGS technology is a high-throughput technology which includes multi-step procedures. The steps include template preparation, sequencing, imaging, genome alignment and assembly. The advantages of using NGS over microarray analysis are that it is more cost-efficient (Wall *et al.*, 2009; Stiglic *et al.*, 2010; Meng *et al.*, 2012) and provides a drastic reduction in time to sequence large amounts of genomic sample (Stiglic *et al.*, 2010; Meng *et al.*, 2012). The novel sequences can easily be identified and this offers the advantage of knowing an unknown sequence which has not been previously reported and annotated (Wall *et al.*, 2009; Meng *et al.*, 2012). Transcripts of low abundance can still be efficiently sequenced using NGS (Liu *et al.*, 2011b; Meng *et al.*, 2012). This technology has several advantages of reproducibility, sensitivity and selectivity (Stiglic *et al.*, 2010). While it offers many advantages, it generates gigabytes of data. The bioinformatics tools that are currently available are able to handle this huge amount of sequence information (Meng *et al.*, 2012).

NGS technology can be used in the field of drug development as the miRNAs have the potential to regulate many genes (Liu *et al.*, 2011b). It is even now possible to assemble a whole genome *de novo* only with the short reads generated by these NGS platforms (Li *et al.*, 2010). Currently, this NGS technology can be obtained in three different platforms: Illumina, Roche 454 and SOLiD. The Illumina platform uses sequencing-by-synthesis, while Roche 454 uses pyrosequencing and SOLiD uses sequencing-by-ligation methods. Both

Illumina and SOLiD can be used to sequence short reads while Roche 454 can be used for long sequences and is best suitable for *de novo* sequencing. Among these three, Illumina is the most widely used platform for most sequencing projects as it has consistent data quality and proper read length (Liu *et al.*, 2011b).

Analysis of the hair cell transcriptome with no acoustic trauma gave clues about what genes are essential for the functioning of hair cells. The genes that are expressed in zebrafish hair cells can be categorized into channel proteins (involved in frequency tuning and to maintain resting potential), transporters, transcription factors, signal transduction molecules, cytoskeletal genes and transcripts for proper development of kinocilia (McDermott *et al.*, 2007). Although the transcripts necessary for the proper functioning of hair cells are known, it is still not clear as to what genes are differentially regulated when the hair cells undergo acoustic stress. If the zebrafish is going to be a model for understanding the process of hair cell regeneration, then the inner ear transcriptome during the process of hair cell regeneration following acoustic stress is needed. This was recently completed by Schuck *et al.* (2011) via microarray analysis. They found numerous genes that were significantly both up- and down-regulated in the zebrafish ear, but interestingly, growth hormone (GH) was endogenously up-regulated to more than 60-fold two days post-sound exposure. In a follow-up study, injection of growth hormone following sound exposure also significantly reduced apoptosis in the zebrafish inner ear and greatly increased hair cell regeneration (Sun *et al.*, 2011). Thus growth hormone may be a potent mitogen

that holds promise for the induction of hair cell regeneration or the prevention of hair cell loss. In this study, I analyzed differential gene expression profiles of the zebrafish inner ear under different treatment conditions (buffer, GH and GH-antagonist injections) following acoustic trauma at one and two days post-sound exposure.

*Properties of growth hormone:*

Growth hormone (GH) is a 22 KDa, single chain mitogen with two disulfide bonds. GH is responsible for growth, sea water adaptation, reproduction, immune function, and osmoregulation in fishes (Calduch-Giner *et al.*, 2001; Perez-Sanchez, 2000; Perez-Sanchez *et al.*, 2002). For example, in salmonids, GH can act as a phagocyte activating factor (Calduch-Giner *et al.*, 1997). In both juvenile and adult seabream, liver cells have 30-50 fold higher GH binding sites than found on cells in muscle, adipose tissue or brain, suggesting that it is an important organ for GH action (Perez-Sanchez, 2000). Binding sites for GH are also higher in fish such as rainbow trout and seabream, and it promotes growth in early stages of development in these juveniles (Perez-Sanchez, 2000). Growth hormone binding sites occur in many other tissues like muscle, gill, testis, adipose, hematopoietic cells and the central nervous system of many vertebrates including fishes (Calduch-Giner *et al.*, 2001). The action of endocrine hormones on mammalian immune system is found to be immunosuppressive. The immune system exerts a reciprocal response towards molecules such as growth hormone, prolactin, insulin, insulin growth factor-1, and thyroid hormones (Perez-

Sanchez, 2000). There is ample evidence which supports the notion that GH can act as erythropoietic, myelopoietic and lymphopoietic growth factors in *in vitro* cultures (Perez-Sanchez, 2000; Calduch-Giner *et al.*, 1997).

Factors such as the age of fish, daylight period or temperature of water have significant effects on the expression levels of growth hormone (Perez-Sanchez, 2000). For instance, growth hormone expression decreases in pituitary tissue with an increase in age of seabream (Marti-Palaca *et al.*, 1996). Increasing the daylight period helps stimulate pituitary growth hormone synthesis in seabream, goldfish and smoltifying salmon. The growth rate and the plasma GH levels drop when the temperature of water is decreased and vice versa (Ricordel *et al.*, 1995). In many fish species, handling and confinement stress rapidly reduces the plasma GH concentration (Perez-Sanchez, 2000).

Although growth hormone has pleiotropic effects as mentioned above, its role in hair cell regeneration in fish or any other organism has not been studied until recently. The aim of the current project is to examine the expression and regulation of growth hormone and other genes responsible for hair cell regeneration following acoustic damage. I found the significant regulation of cell proliferation and cell death pathways following injection with GH. GH and blocking GH with an antagonist, produces differential regulation of molecules such as transcription factors that are involved in apoptotic processes, cell adhesion molecules that control hair cell regeneration and solute carrier molecules.

## Materials and Methods

### *Experimental animals:*

Adult breeder zebrafish (*Danio rerio*) were obtained from Segrest Farms (Gibsonton, FL) and maintained in 170-L flow-through aquaria under conditions of constant temperature (25°C) and a 12-h light/12-h dark schedule. Fish total lengths ranged from 36 to 44 mm. All work was done under the supervision of the Institutional Animal Care and Use Committee of Western Kentucky University.

### *Sound exposure:*

Adult zebrafish were randomly assigned to treatment and control groups without bias for weight or length or sex. A total of eighty zebrafish separated into four groups, each with 20 zebrafish were exposed to a 150 Hz tone at 179 dB re 1  $\mu$ Pa RMS. The fifth group of 20 zebrafish which were not exposed to sound or any treatment was used as the control (C) group. The sound was generated by a B&K Precision function generator (4017A) connected to a 5.3 amp/200 watt Audio source monoblock amplifier and a University Sound UW-30 underwater speaker placed in a 19-L sound exposure chamber. Fish were exposed for 40 hours at 24.5-25°C, and then they were separated into four groups of 20 fish each. Following the termination of sound exposure, three groups of sound exposed fish were injected with either phosphate buffer, salmon growth hormone (20  $\mu$ g salmon GH/gram body mass; Dr. Shunsuke Moriyama for his kind donation of salmon GH) or growth hormone antagonist (40  $\mu$ g GH antagonist/gram body mass; Pro-Spec-Tany Technogen Ltd., Israel) and they



were marked B1, G1 and A1 respectively (Table 1). They were then moved to three separate tanks and allowed to recover for one day. The fourth group of sound exposed fish were injected with phosphate buffer, and allowed to recover for 2 days, and marked as B2 (Table 1)). They were placed in the sound exposure chamber for the same time and temperature as other treatment groups with the sound generator turned off.

*Isolation of mRNA from zebrafish inner ear:*

Fish were sacrificed one at a time with an overdose of Tricaine methane sulfonate (MS-222), their heads were removed, and the pair of ears (sacculae, lagena, utricle and semi-circular canals) from each fish were immediately dissected out while being completely submerged in RNA*later*® (Ambion, Austin, TX), as preliminary work indicated that either the small size of the sacculae, or the length of time needed to separate it from the inner ear, resulted in low RNA yield. Ears were then placed in sterile microfuge tubes filled with lysis buffer provided in the mirVana miRNA isolation kit (Life Technologies). One to two hours were required to dissect all the fish in one group. Although each fish was dissected quickly, the ears were not contaminated with surrounding tissue other than perhaps residual parts of the auditory nerve. Once all the ears for a sample were collected, the tissue was pooled and homogenized with a Kontes Pellet Pestle Microgrinder and sterile disposable pestles (Kontes, Vineland, NJ), then processed for RNA isolation using the mirVana miRNA isolation kit (Life Technologies). RNA quality was checked with the aid of an Agilent 2100

Bioanalyzer (Agilent, Wilmington, DE). For this project, sharp ribosomal RNA bands were evident with an RNA integrity number greater than 7.0. A total of 100 ng RNA per sample was sent to Cofactor Genomics, MO. Ovation® RNA-Seq System V2 kit (Nugen Inc., San Carlos, CA) was used to amplify the RNA to amounts sufficient for sequencing library construction. The cDNA was then used to construct sequencing libraries using the Illumina TruSeq Kit.

*Enrichment of transcriptome RNA and cDNA synthesis:*

The whole transcriptome (mRNA) was extracted from total RNA by removing small and large rRNA using RiboMinus Bacterial Kit (Invitrogen, Carlsbad, CA). In brief, total RNA was hybridized to rRNA specific biotin labeled probes at 70 °C for 5 minutes. The rRNA probe complexes were then removed by streptavidin-coated magnetic beads and the remaining free transcriptome RNA was concentrated by ethanol precipitation. After enrichment, the RNA was fragmented by incubation with fragmentation buffer included in the Illumina kit (Illumina, San Diego, CA) for 5 minutes at 94 °C. Fragmented RNA was enriched by ethanol precipitation. First strand cDNA was synthesized by priming the fragmented RNA using random hexamer and then followed by reverse transcription by Superscript II (Invitrogen, Carlsbad, CA). The second strand was synthesized by incubating in second strand buffer, RNase Out and dNTP provided in the Illumina kit on ice for 5 min. The reaction mix was then treated with DNA Pol I and RNase H at 16° C for 2.5 h.

*Library preparation and sequencing:*

The cDNA synthesized from mRNA was used for preparing the RNA-Seq library. The double stranded DNA was treated with a mix of T4 polymerase, Klenow large fragment and T4 polynucleotide kinase to create blunt-ended DNA which subsequently added a single A-base at the 3' end using Taq polymerase and dATP. These A-tailed DNA were ligated to paired end adaptors using T4 ligase provided by the Illumina RNA-Seq kit. Size selection of adapter-ligated DNA was performed using 4-12% polyacrylamide gel electrophoresis. These size selected DNA libraries were amplified using in-gel PCR using the Phusion High-Fidelity system (New England Biolabs). In this way, the mRNA library for sound-exposed zebrafish injected with either phosphate buffer at one and two days, salmon growth hormone or growth hormone antagonist at one day following sound exposure were prepared.

*Primary processing and mapping of RNA-Seq reads:*

RNA-Seq reads were obtained using Illumina Pipeline version SCS 2.8.0 paired with OLB 1.8.0. The sequence files were generated in FASTQ format and were uploaded into the Galaxy tool available at galaxy.psu.edu. The reads were pre-processed using FASTQ Groomer using Galaxy available at galaxy.psu.edu (Pennsylvania State University). The quality of reads was checked and mapped by TopHat program using Galaxy. The latest build of UCSC (University of California Santa Cruz) *Danio rerio* Zv9/danRer7 was used as a reference genome for the read mapping. The mapping resulted in the generation of splice junctions

and accepted hits. Potential exons were identified from the accepted hits file and this was used for all subsequent analysis. Default parameters were used for TopHat.

*Transcript abundance estimation using Cufflink:*

The mapped reads were processed by Cufflink using Galaxy. Cufflink assembles transcripts from the Tophat aligned RNA-Seq reads, estimates their abundance, and reports a parsimonious set of transcriptome assembly in RNA-Seq samples (Trapnell *et al.*, 2012). UCSC *Danio rerio* Zv9/danRer7 build was used as reference annotation to Cufflink. The Cufflink tool makes use of normalized RNA-Seq fragment counts to measure the relative abundances of transcripts. The unit of measurement is Fragments Per Kilobase of exon per Million fragments Mapped (FPKM).

*Tracking differential expression using Cuffdiff:*

Once all the samples were assembled using Cufflink, they were merged together using Cuffmerge with the reference annotation. Cuffmerge is a meta-assembler that takes all Cufflink assembled files and merges them into a parsimonious transcriptome dataset. Cuffmerge helps to reconstruct a complete gene that might be lacking by Cufflink due to low expression level or low sequencing depth. Cuffdiff was used to estimate the relative abundances of genes and transcripts, differential usage of splice junctions, promoters along with Cuffmerge file as a reference (Trapnell *et al.*, 2012). Differential expression of

transcripts was examined using Cuffdiff with default parameters but with a false discovery rate of 0.10. Six pairwise comparisons were made as following to identify the differentially expressed genes and transcripts: buffer-injected day1/control (B1/C), buffer-injected day 2/control (B2/C), buffer-injected day 2/buffer-injected day 1 (B2/B1), growth hormone/buffer-injected day 1 (G1/B1), growth hormone/buffer-injected day 2 (G1/B2) and antagonist vs growth hormone (A1/G1). For example, B1/C comparison provided information on differentially regulated genes in buffer-injected day 1 (B1) condition compared to control (C) condition (Table 1). The files containing information about differential transcripts in each condition were filtered based on whether they were significant or not. Cuffdiff was used to compare differentially expressed transcripts among the five samples.

*Functional analysis using Ingenuity Pathway Analysis:*

The statistically significant transcripts in all conditions were filtered from non-significant ones. All the raw expression values were added with a constant of 0.15 before log transformation to account for any extreme values of transcripts that were not expressed under any sample conditions. After scaling and finding the log ratios of two samples in a pairwise comparison, the files were uploaded into the Ingenuity Pathway Analysis (Redwood City, CA) tool for functional analysis. The top ten up-and down-regulated canonical pathways and transcripts, were selected for further examination.

## Results

### *Analysis of RNA-Seq data:*

The total number of reads generated by all five samples ranged between 27,239,458 and 30,855,367 with a median of 28,100,529 (Table 2). The sequence files were converted from Fastq format into Sanger Fastq format using Galaxy. The average length of the all reads was 59 bases. The quality of each base throughout the total read length for all reads in all five samples was checked for good quality before further processing. No trimming was done on any reads as the average median of mean base quality was found to be high (between 37.5 and 38, Fig 1) in all the samples although minor variation existed across experimental conditions. After determining the quality of reads, they were mapped to UCSC build danRer7 zebrafish genome by the Tophat program using default parameters in Galaxy. While some fraction of the reads was discarded in the mapping process, because they did not pass the default parameters of Tophat, a majority of the reads were mapped to the reference dataset. The maximum and minimum percentages of the mapped reads were 80.15% for control and 67.79% for B2 sample respectively (Table 2). The mapped reads were input to Cuffdiff and six different pairwise comparisons (B1/C, B2/C, B2/B1, G1/B1, G1/B2, A1/G1) were made as explained in the Methods section to identify the differentially expressed genes and transcripts.

The Cuffdiff tool measures the relative abundance of genes and transcripts based on Fragments Per Kilobase of exon per Million fragments Mapped (FPKM). These FPKM values are normalized measures and they were

added a constant 0.15 and log-base 10 transformed to account for any zeros during heatmap generation (Fig 2).

*Differentially expressed genes and transcripts:*

The differentially expressed genes and transcripts were identified using Cuffdiff tool and were tested for their statistical significance. Cuffdiff analysis also performs multiple testing on genes using Benjamini-Hochberg correction and the resulting output files contain information about gene IDs, expression measure in FPKM, log fold change, p value, q value and significance. The log fold change were converted into fold change and used for further analysis. We found some overlap between the genes that were significantly expressed across all pairwise conditions. Two three-set Venn diagrams between B1/C, B2/C, B2/B1 and G1/B1, G1/B2, A1/G1 were produced (Figs. 3 & 4).

*Functional analysis using IPA:*

The differentially expressed transcripts were input into Ingenuity Pathway Analysis (IPA) for functional analysis. IPA takes only the annotated transcripts for processing, thus the unannotated transcripts were excluded in further analysis. The top 15 canonical pathways for all pairwise comparisons are given in Tables 3 and 4. Some of the important pathways that were significant at B1 and B2 are calcium signaling, ILK (Integrin-linked kinase) signaling, tight junction signaling, actin cytoskeleton signaling and antigen presentation pathways (Table 3). The NRF2-mediated oxidative stress pathway was significantly regulated in B1/C and

in B2/B1. In G1/B1 pairwise comparison, FXR/RXR activation, iNOS signaling, ERK5 signaling, ILK and acute phase response signaling were significantly regulated. When G1 is compared to B2 (G1/B2), four different Rho involved pathways were significantly regulated: regulation of actin-based motility by Rho, signaling by Rho family GTPases, RhoA signaling and RhoGDI signaling. Other significant canonical pathways included mitotic roles of polo-like kinase, actin cytoskeleton signaling, and cdc 42 signaling. In A1/G1 comparison, I found significant regulation of Rho family GTPases signaling, actin regulation by Rho, tight junction signaling, GH and IGF-1 signaling and others.

The top ten up- and down-regulated transcripts in all six pairwise comparisons are given in Tables 5-10. The pairwise comparisons B1/C and B2/C showed significant up-regulation of GTF2F2 (general transcription factor IIF), HBZ (hemoglobin Z), NDRG1 (N-myc downstream regulated 1) and C10orf32 (chromosome 10 open reading frame 32) while they both showed down-regulation of DCLK2 (double cortin-like kinase 2), TNNI2 (troponin I type 2) and UPK1A (uroplakin 1A) (Tables 5 and 6). FGFR2 (Fibroblast growth receptor 2) was up-regulated in B1/C but it is down regulated in B2/C. A comparison of G1 with B1 (G1/B1) showed up-regulation of different solute carrier family molecules such as SLC6A12, SLC5A1, SLC12A3 and cell adhesion molecule CDH17 (cadherin 17), and transmembrane protein TMEM27 (Table 8). A pattern of strong down-regulation for transcripts GTF2F2, HBZ and BASP1 (brain abundant membrane attached signal protein 1) was found in G1/B1 which was strongly up-regulated in B1/C, suggesting that GH injection regulates these transcripts. G1



compared to B2 showed up-regulation of SNX12 (sorting nexin 12), FGFR2, POMC (proopiomelanocortin) and RABIF (RAB interacting factor) while GTF2F2, HBZ were down-regulated. FGFR2 also showed significant down-regulation in the A1/G1 comparison.

I compared this current NGS results with a previous microarray study from the Smith lab (Schuck *et al.*, 2011) to examine similarities in transcripts that were significantly regulated. When comparing the top 10 up-regulated transcripts from the microarray experiment to our current NGS data, I found GH1 (growth hormone), POMC (proopiomelanocortin), CGA (glycoprotein hormones-alpha peptide) to be significantly up-regulated in B1/C but they were down-regulated in A1/G1 comparison. In G1/B1 comparison, we found up-regulation of GH1 and POMC but not CGA. When comparing the top 10 down-regulated transcripts between the microarray and our NGS data, we found zgc: 66286 and ATP2A2 (Ca<sup>++</sup> transporting ATPase) were down-regulated in B1/C but they were up-regulated in G1/B1 comparison. We did not find any significant differential regulation of zgc: 66286 and ATP2A2 in A1/G1 comparison. Interestingly, we did not find any significant regulation of GH1, POMC, CGA, zgc: 66286 or ATP2A2 in B2/C but we found ATP2A1 to be down-regulated in both B2/C and A1/G1 comparisons.

When analyzing the other significantly regulated molecules across the six pairwise comparisons, I found that a number of the transcripts fell into a pattern of up-regulation in the GH treatment while they were down-regulated in the antagonist treatment (Table 11). Transcripts such as GH1, POMC, SLC6A12,

CYP7A1, TMEM27, CDH17 and FGFR2 were up-regulated in G1/B1 and G1/B2 but they were down-regulated in A1/G1 comparison. GTF2F2, Fos, FosB, JUN/JUNB/JUND were down-regulated in G1/B1 but up-regulated in B1/C and A1/G1.

A number of myosin-related molecules were differentially regulated across the pairwise comparisons, including myosin (MYO), myosin heavy chain (MYH) and myosin light chain (MYL) molecules. In A1/G1, I found the following myosin molecules significantly regulated: MYH1 (-2.4 fold), MYH11 (+1.5 fold), MYL1 (-2.7 fold), MYL6 (-1.7 fold), MYL9 (+1.6 fold) and MYL10 (-2.1 fold). In G1/B1, I found the differential regulation of MYH1 (+4.7 fold), MYH7 (+2.5 fold), MYL3 (-2.9 fold), MYL10 (+2.6 fold), MYO1F (+1.6 fold) and MYO1G (+2 fold). When B1 was compared to control, molecules such as MYH1 (+2.8 fold), MYH7 (-3 fold), MYL2 (-3.5 fold), MYL3 (-2.8 fold), MYL10 (-2 fold) and MYL6B (-54 fold) were expressed significantly.

Many family members of solute carrier molecules were significantly regulated but only the SLC6A family was regulated in all pairwise comparisons. SLC2A, SLC5A, SLC22A were regulated in all pairwise comparisons except in B1/C. SLC 37A and SLC 43A members were specifically regulated in A1/G1 but not in the other comparisons. Other solute carriers that were regulated in G1/B1 include SLC3A, SLC12A, SLC13A, SLC16A, SLC20A, SLC26A and SLC47A. A majority of the solute carriers such as SLC12A, SLC13A, SLC22A, SLC2A, SLC30A, SLC37A, SLC43A, SLC47A, SLC5A, SLC6A and SLC7A were down-regulated in A1/G1.

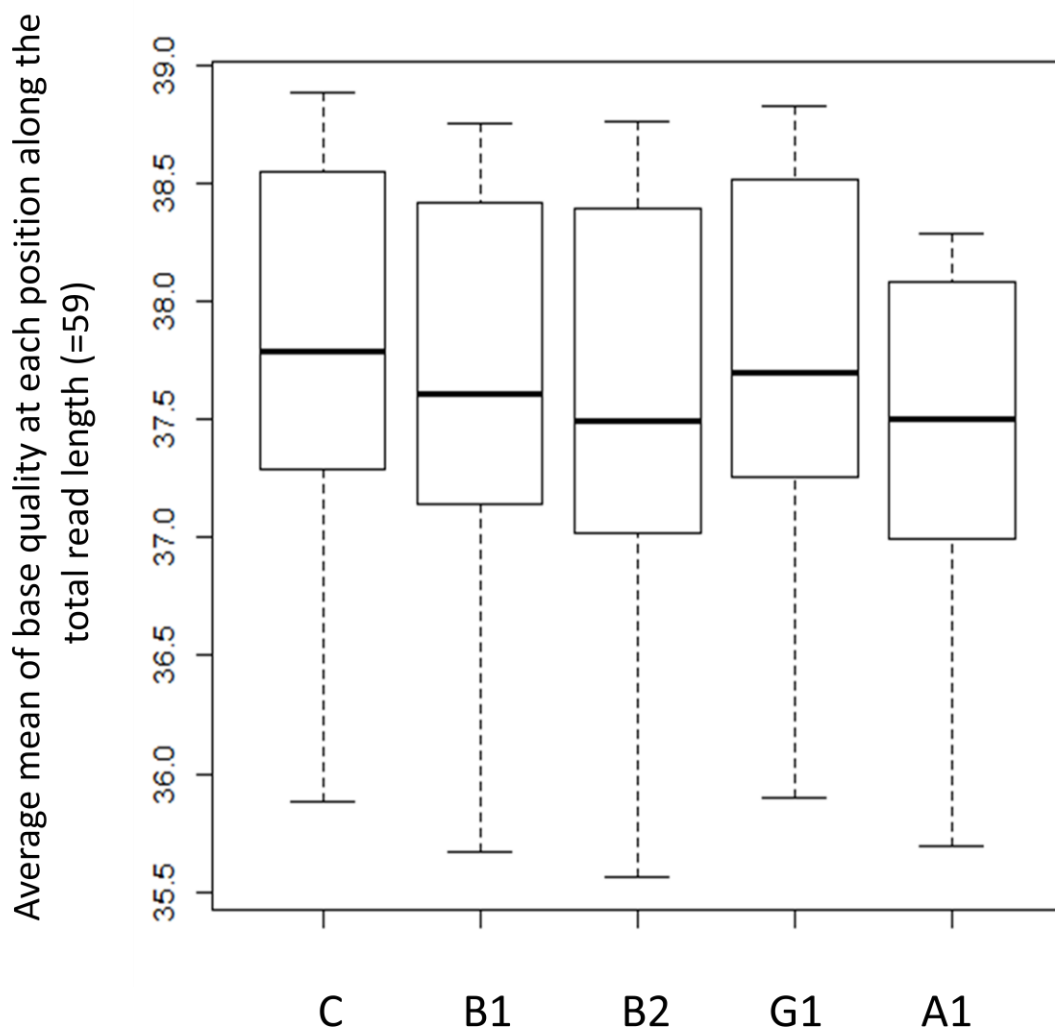


Figure 1. Boxplot showing the average mean of base quality at each position along the total read length in all samples (C=control, B1=buffer-injected day 1, B2=buffer-injected day 2, G1=GH-injected day 1, A1=antagonist-injected day 1). The whiskers extend 1.5 times the interquartile range from the box.

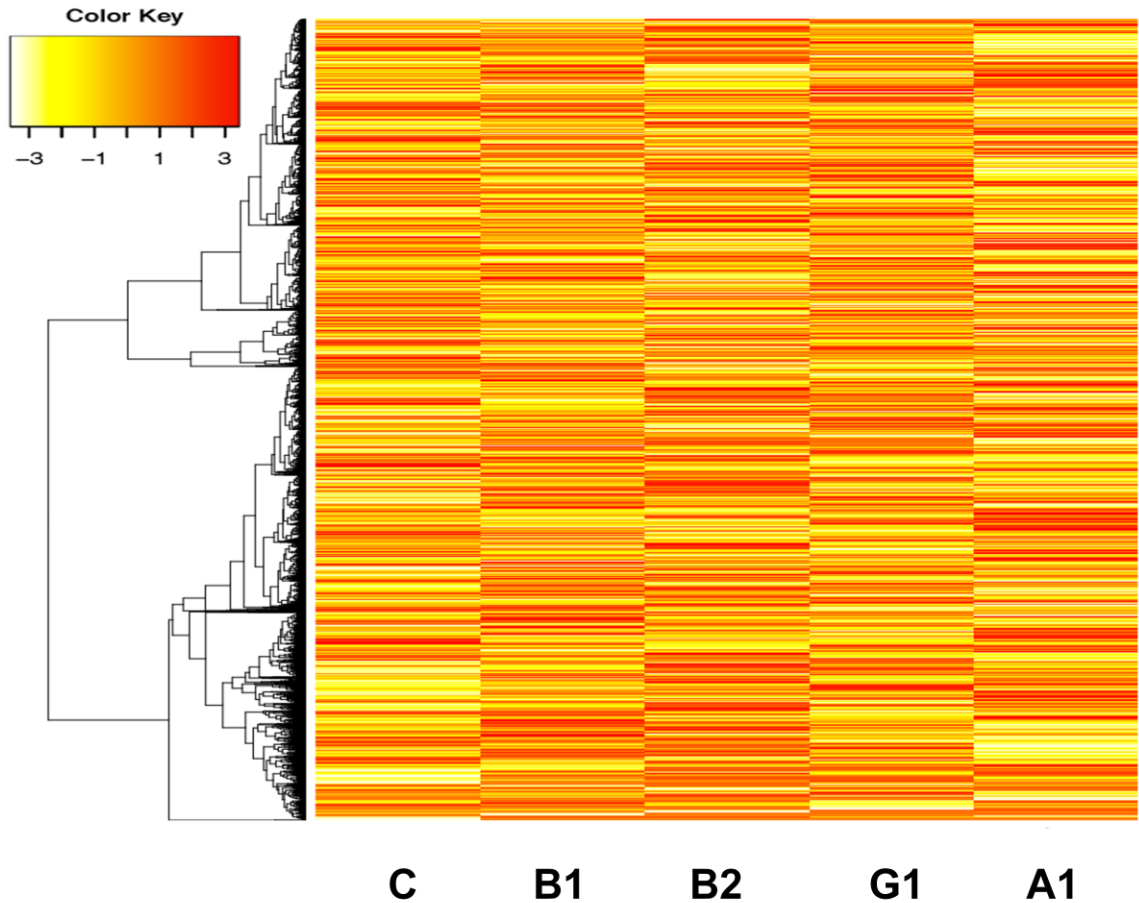


Figure 2. Heatmap generated using the program R showing the transcript expression (log-base 10 (FPKM)) across all five experimental conditions (C=control, B1=buffer-injected day 1, B2=buffer-injected day 2, G1=GH-injected day 1, A1=antagonist-injected day 1). The red color indicates a high transcript expression and a yellow color indicates a low expression (color key). The transcripts were clustered based on Euclidean distances.

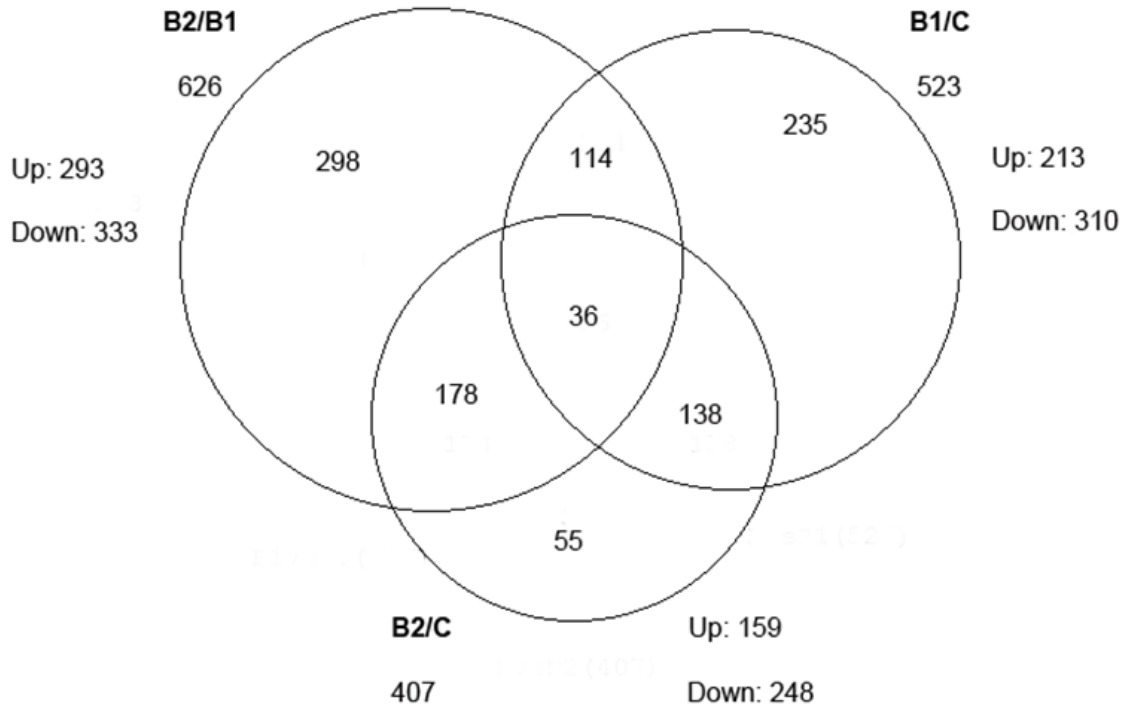


Figure 3. Venn diagram showing differentially expressed transcripts that are unique to and common between buffer-injected day 1 compared to control (B1/C), buffer-injected day 2 compared to control (B2/C) and buffer-injected day 2 compared to buffer-injected day 1 (B2/B1) conditions. The numbers outside the circles show the total number of transcripts that are differentially regulated in that comparison along with the total number of up- and down-regulated transcripts.

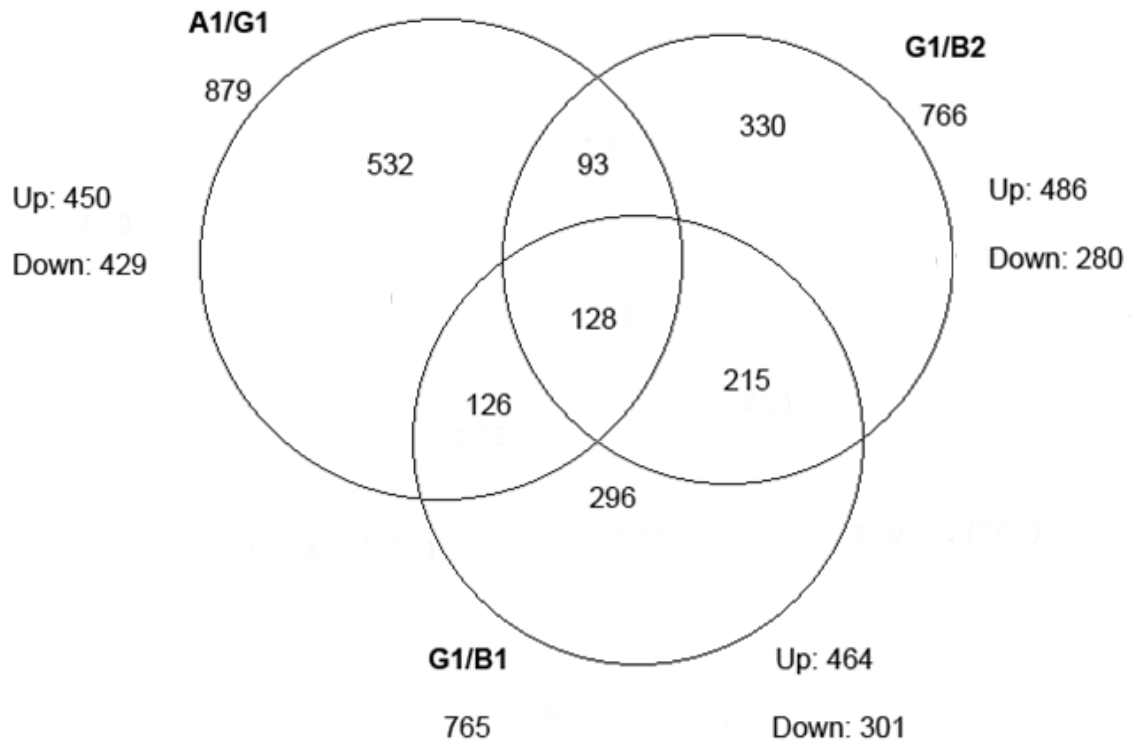


Figure 4. Venn diagram showing differentially expressed transcripts that are unique to and common between GH-injected day 1 compared to buffer-injected day 1 (G1/B1), GH-injected day 1 compared to buffer-injected day 2 (G1/B2) and antagonist-injected day 1 compared to GH-injected day 1 (A1/G1) conditions. The numbers outside the circles show the total number of transcripts that are differentially regulated in that comparison along with the total number of up- and down-regulated transcripts.

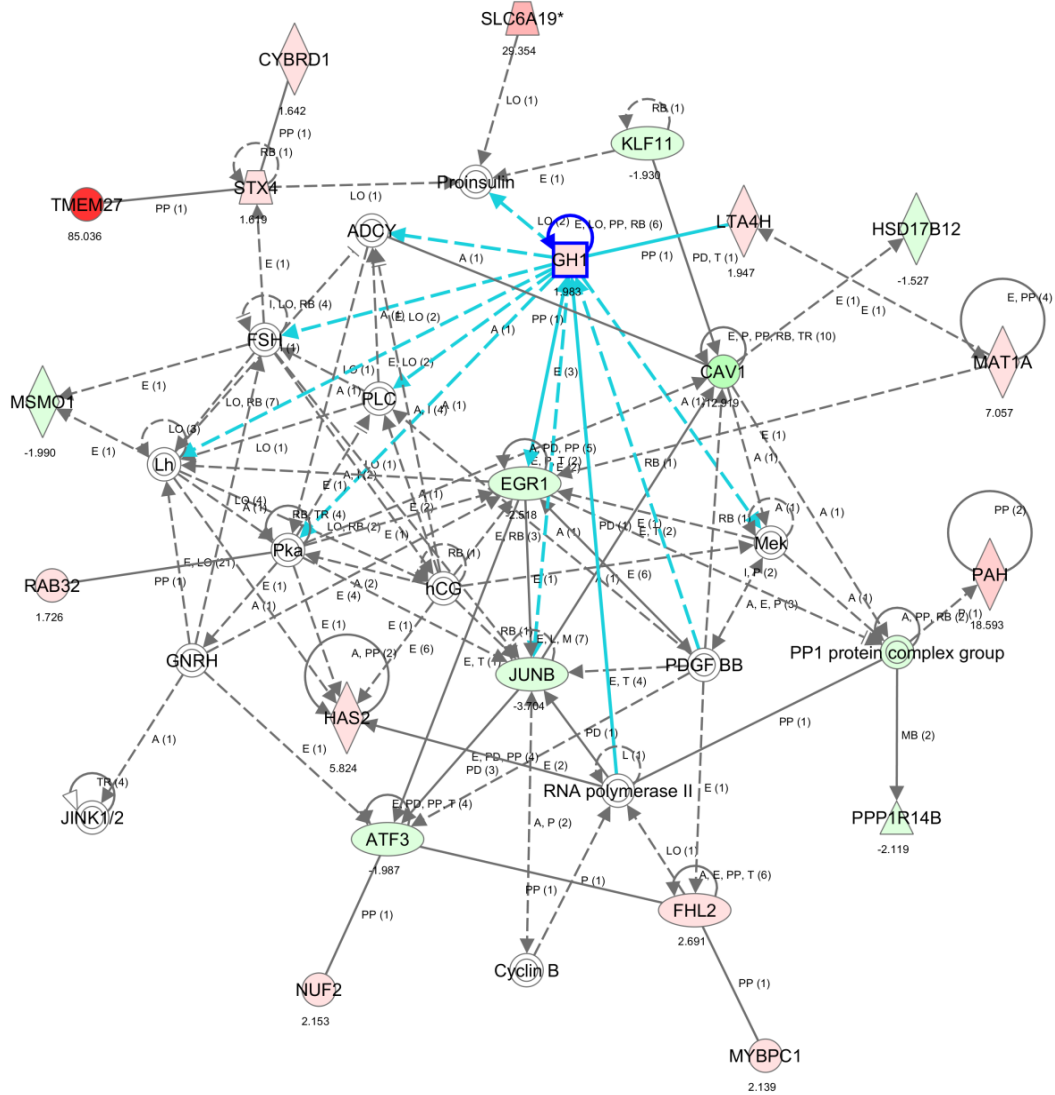


Figure 5. Cell growth and proliferation network in GH-injected compared to buffer-injected day 1 (G1/B1) generated using Ingenuity Pathway Analysis. Red indicates up-regulation and green indicates down-regulation. The signed number below the molecules indicates the fold change of regulation. The solid lines show a direct connection and a dashed line shows an indirect connection between molecules. An arrowed connection indicates that a molecule acts on the other and a non-arrowed line indicates that they are binding partners.

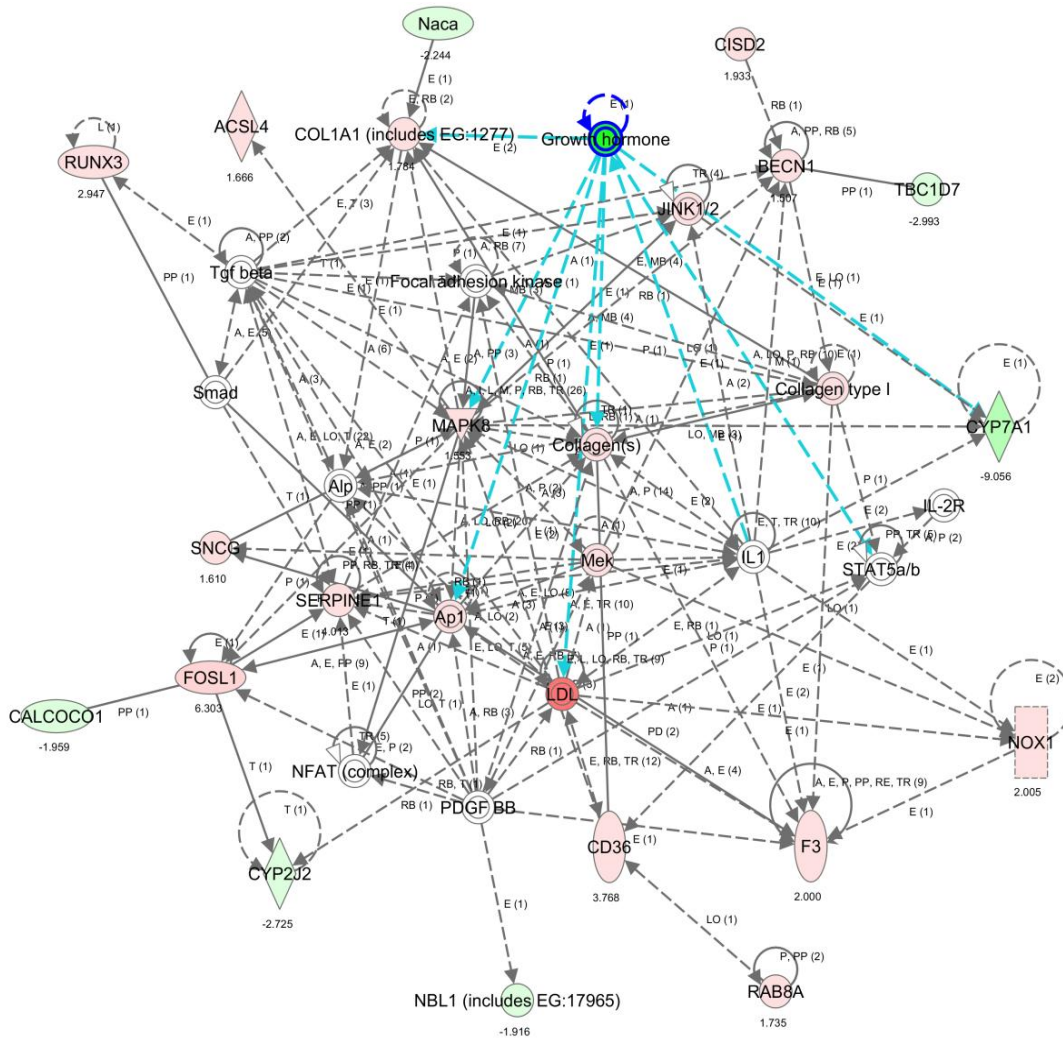


Figure 6. Cell death network in antagonist-injected compared to GH-injected (A1/G1) generated using Ingenuity Pathway Analysis. Red indicates up-regulation and green indicates down-regulation. The signed number below the molecules indicates the fold change of regulation. The solid lines show a direct connection and a dashed line shows an indirect connection between molecules. An arrowed connection indicates that a molecule acts on the other and a non-arrowed line indicates that they are binding partners.



Table 1: Experimental design. Experimental group abbreviations that will be used throughout the paper are defined in terms of sound exposure, injection, and the recovery period following sound exposure.

Experimental group abbreviation	Experimental groups	Sound exposure (40 hours at 150 Hz tone at 179 dB re 1 $\mu$ Pa RMS)	Injection	Recovery period following sound exposure and injection
C	Control	None	None	N.A.
B1	Buffer-injected day 1	Yes	Phosphate buffer	1 day
B2	Buffer-injected day 2	Yes	Phosphate buffer	2 days
G1	GH-injected day 1	Yes	Growth hormone (GH)	1 day
A1	GH antagonist-injected day 1	Yes	GH antagonist	1 day

Table 2: Percentages of mapped and unmapped RNA-Seq reads to UCSC zebrafish danRer 7 build by Tophat v1.5.0 for all five experimental conditions.

	Control (C)	Buffer-injected day 1 (B1)	Buffer-injected day 2 (B2)	GH-injected day 1 (G1)	GH antagonist-injected day 1 (A1)
Total reads	28046918	30855367	29943980	28100529	27239458
% of reads mapped	80.15	79.58	67.79	69.91	75.26
% unmapped reads	19.85	20.42	32.21	30.09	24.74

Table 3. Top 20 significant canonical pathways in B1/C, B2/C and B2/B1 obtained from Ingenuity Pathway Analysis based on differentially regulated transcripts. Pathways are sorted by statistical significance (top=most significant).

<b>Buffer-injected day 1 compared to control (B1/C)</b>	<b>Buffer-injected day 2 compared to control (B2/C)</b>	<b>Buffer-injected day 2 compared to buffer-injected day 1 (B2/B1)</b>
Calcium signaling	Calcium signaling	Calcium signaling
ILK signaling	Tight Junction signaling	Tight Junction signaling
G alpha 12/13 signaling	ILK signaling	ILK signaling
14-3-3-mediated signaling	Signaling by Rho Family GTPases	RhoA signaling
PAK signaling	Antigen Presentation Pathway	Actin Cytoskeleton signaling
Axonal Guidance	Cellular Effects of Sildenafil	Antigen Presentation Pathway
Sertoli Cell-Sertoli Cell Junction signaling	VEGF signaling	NRF2-mediated Oxidative Stress Response
Tight Junction signaling	MODY signaling	EIF2 signaling
CXCR4 signaling	RhoGDI signaling	Regulation of Actin-based Motility by Rho
Cdc42 signaling	Pentose Phosphate Pathway	PI3K Signaling in B Lymphocytes
Extrinsic Prothrombin Activation	RhoA signaling	Hepatic Fibrosis/Hepatic Stellate Cell Activation
Coagulation System	Regulation of Actin-based Motility by Rho	Protein Kinase A signaling
Hepatic Fibrosis/Hepatic Stellate Cell Activation	Cdc42 signaling	VEGF signaling
Cardiomyocyte Differentiation via BMP Receptors	Agrin Interactions at Neuromuscular Junction	Cellular Effects of Sildenafil
NRF2-mediated Oxidative Stress Response	Hepatic Fibrosis/Hepatic Stellate Cell Activation	Fc gamma Receptor-mediated Phagocytosis in Macrophages and Monocytes
Cellular Effects of Sildenafil	Actin Cytoskeleton signaling	Acute Phase Response signaling
Actin Cytoskeleton signaling	Glycolysis/Gluconeogenesis	Mechanisms of Viral Exit from Host Cell
Germ Cell-Sertoli Cell Junction signaling	Protein Kinase A signaling	Airway Pathology in Chronic Obstructive Pulmonary Disease
Thrombin signaling	Fructose and Mannose Metabolism	G alpha 12/13 signaling
Signaling by Rho Family GTPases	G alpha 12/13 signaling	TR/RXR Activation

Table 4. Top 20 significant canonical pathways in G1/B1, G1/B2 and A1/G1 obtained from Ingenuity Pathway Analysis based on differentially regulated transcripts. Pathways are sorted by statistical significance (top=most significant).

<b>GH-injected day 1 compared to buffer-injected day 1 (G1/B1)</b>	<b>GH-injected day 1 compared to buffer-injected day 2 (G1/B2)</b>	<b>Antagonist-injected day 1 compared to GH-injected day 1 (A1/G1)</b>
Tight Junction signaling	Intrinsic Prothrombin Activation Pathway	Calcium signaling
Calcium signaling	Mitotic Roles of Polo-Like Kinase	Signaling by Rho Family GTPases
Antigen Presentation Pathway	Regulation of Actin-based Motility by Rho	Acute Phase Response signaling
MODY signaling	Signaling by Rho Family GTPases	Regulation of Actin-based Motility by Rho
Pyrimidine Metabolism	Actin Cytoskeleton signaling	RhoGDI signaling
ILK signaling	RhoA signaling	Tight Junction signaling
Signaling by Rho Family GTPases	Extrinsic Prothrombin Activation Pathway	Glycolysis/Gluconeogenesis
FXR/RXR Activation	ILK signaling	Cellular Effects of Sildenafil
Acute Phase Response signaling	Coagulation System	G alpha 12/13 signaling
Nicotinate and Nicotinamide Metabolism	RhoGDI signaling	Growth Hormone signaling
ERK5 signaling	Calcium signaling	ILK signaling
Intrinsic Prothrombin Activation Pathway	Cdc42 signaling	Coagulation System
One carbon pool by Folate	Circadian Rhythm	RhoA signaling
iNOS signaling	Acute Phase Response signaling	G Beta Gamma signaling
Production of NO and ROS in macrophages	Atherosclerosis	IGF-1 signaling
Extrinsic prothrombin activation pathway	Fc gamma Receptor-mediated Phagocytosis in Macrophages and Monocytes	MODY signaling
PXR/RXR activation	Integrin signaling	Actin Cytoskeleton signaling
Histidine metabolism	Cellular Effects of Sildenafil	Cdc42 signaling
Coagulation System	LXR/RXR Activation	Sertoli Cell-Sertoli Cell Junction signaling
Pentose Phosphate Pathway	Glioma Invasiveness	PAK signaling

Table 5: Top 10 up- and down-regulated molecules in B1/C (buffer-injected day 1 compared to control).

Gene	Gene ID	Entrez Gene Name	GO Function	Fold Change	P-value
GTF2F2	NM_001103133	general transcription factor IIF, polypeptide 2	transcription regulator	108.918	2.23E-12
HBZ	NM_001033093	hemoglobin, zeta	transporter	66.568	4.07E-06
NDRG1	NM_213348	N-myc downstream regulated 1	kinase	62.074	1.71E-11
BASP1	NM_001202454	brain abundant membrane attached signal protein 1	transcription regulator	41.379	2.94E-10
C10orf32	NM_001039988	chromosome 10 open reading frame 32	other	20.923	0.0004556
FGFR2	NM_001243004	fibroblast growth factor receptor 2	kinase	18.255	2.31E-09
PGD	NM_213453	phosphogluconate dehydrogenase	enzyme	14.931	3.49E-05
SCAMP2	NM_201194	secretory carrier membrane protein 2	transporter	13.247	0.0003283
H2AFV	NM_001201563	H2A histone family, member V	other	11.47	0.0039926
RTP3	NM_001128720	receptor (chemosensory) transporter protein 3	other	11.13	1.07E-05
PITX2	NM_130975	paired-like homeodomain 2	transcription regulator	-14.764	4.77E-06
PRSS21	NM_001083582	protease, serine, 21 (testisin)	peptidase	-15.17	0
CNBP	NM_199749	CCHC-type zinc finger, nucleic acid binding protein	transcription regulator	-16.41	0.0005084
DCLK2	NM_001145789	Double cortin-like kinase 2	kinase	-19.871	2.22E-08
RABIF	NM_001168213	RAB interacting factor	transporter	-21.334	0.0014708
MBNL2	NM_001099998	muscleblind-like splicing regulator 2	other	-24.686	4.48E-09
TNNI2	NM_001136492	troponin I type 2 (skeletal, fast)	enzyme	-25.081	7.55E-05
BVES	NM_001257164	blood vessel epicardial substance	other	-48.612	3.70E-08
UPK1A	NM_001040242	uroplakin 1A	other	-48.976	6.05E-11
MYL6B	NM_001089511	myosin, light chain 6B, alkali	other	-54.693	1.56E-06

Table 6: Top 10 up- and down-regulated molecules in B2/C (buffer-injected day 2 compared to control).

<b>Symbol</b>	<b>Gene ID</b>	<b>Entrez Gene Name</b>	<b>GO Function</b>	<b>Fold Change</b>	<b>P Value</b>
NDRG1	NM_213348	N-myc downstream regulated 1	kinase	180.883	2.54E-11
CDH17	NM_194422	cadherin 17, LI cadherin (liver-intestine)	transporter	168.384	0
HBZ	NM_001033093	hemoglobin, zeta	transporter	154.963	3.10E-06
UFC1	NM_001003650	ubiquitin-fold modifier conjugating enzyme 1	enzyme	70.929	1.88E-05
GTF2F2	NM_001103133	general transcription factor IIF, polypeptide 2	transcription regulator	55.288	6.28E-06
UROC1	NM_001135129	urocanase domain containing 1	enzyme	37.927	0
C10orf32	NM_001039988	chromosome 10 open reading frame 32	other	27.906	0.0017546
SLC12A3	NM_001045080	solute carrier family 12	transporter	25.739	6.75E-14
FBP1	NM_213132	fructose-1,6-bisphosphatase 1	phosphatase	17.099	6.69E-06
HNF1B	NM_131880	HNF1 homeobox B	transcription regulator	14.651	3.00E-09
ABP1	NM_001077598	amiloride binding protein 1	enzyme	-14.82	1.09E-06
DCLK2	NM_001145789	doublecortin-like kinase 2	kinase	-19.755	3.78E-06
TNNI2	NM_001007365	troponin I type 2 (skeletal, fast)	enzyme	-20.337	0
8-Mar	NM_001161435	membrane-associated ring finger (C3HC4) 8	enzyme	-22.048	0.0023873
MAL	NM_001077463	mal, T-cell differentiation protein	transporter	-23.075	0.0029527
UPK1A	NM_001040242	uroplakin 1A	other	-24.96	9.36E-08
CRYZ	NM_001099976	crystallin, zeta (quinone reductase)	enzyme	-34.265	6.55E-06
MGMT	NM_001256246	O-6-methylguanine-DNA methyltransferase	enzyme	-44.178	1.04E-05
FGFR2	NM_001243004	fibroblast growth factor receptor 2	kinase	-83.48	6.00E-15
SNX12	NM_001145894	sorting nexin 12	transporter	-262.807	0

Table 7: Top 10 up- and down-regulated molecules in B2/B1 (buffer-injected day 2 compared to buffer-injected day 1)

<b>Symbol</b>	<b>Gene ID</b>	<b>Entrez Gene Name</b>	<b>GO Function</b>	<b>Fold Change</b>	<b>P Value</b>
CDH17	NM_194422	cadherin 17, LI cadherin (liver-intestine)	transporter	176.485	0
BVES	NM_001257164	blood vessel epicardial substance	other	60.515	3.24E-11
SLC12A3	NM_001045080	solute carrier family 12	transporter	35.254	2.60E-11
HNF4A	NM_194368	hepatocyte nuclear factor 4, alpha	transcription regulator	32.881	4.82E-08
UROC1	NM_001135129	urocanase domain containing 1	enzyme	32.716	0
MBNL2	NM_001099998	Muscle blind-like splicing regulator 2	other	29.376	3.53E-10
TRAM2	NM_213071	translocation associated membrane protein 2	other	25.109	0.0038039
HOXB8	NM_131120	homeobox B8	transcription regulator	19.22	4.90E-05
CNBP	NM_199749	CCHC-type zinc finger, nucleic acid binding protein	transcription regulator	17.533	0.0003372
PDZK1	NM_001128670	PDZ domain containing 1	transporter	16.621	2.01E-05
MX1	NM_001128672	myxovirus (influenza virus) resistance 1	enzyme	-13.196	8.10E-08
RTP3	NM_001128720	receptor (chemosensory) transporter protein 3	other	-13.962	4.19E-05
TNNI2	NM_001007365	troponin I type 2 (skeletal, fast)	enzyme	-14.047	0
CRYZ	NM_001099976	crystallin, zeta (quinone reductase)	enzyme	-14.203	0.0001604
PGD	NM_213453	phosphogluconate dehydrogenase	enzyme	-14.673	2.73E-05
FGFR2	NM_001243004	fibroblast growth factor receptor 2	kinase	-17.91	8.45E-10
FKBP3	NM_001004519	FK506 binding protein 3, 25kDa	enzyme	-23.387	9.54E-05
MGMT	NM_001256246	O-6-methylguanine-DNA methyltransferase	enzyme	-30.029	1.49E-06
BASP1	NM_001202454	brain abundant membrane attached signal protein 1	transcription regulator	-40.195	1.07E-10
SNX12	NM_001145894	sorting nexin 12	transporter	-80.473	3.26E-12

Table 8: Top 10 up- and down-regulated molecules in G1/B1 (GH-injected day 1 compared to buffer-injected day 1)

<b>Symbol</b>	<b>Gene ID</b>	<b>Entrez Gene Name</b>	<b>GO Function</b>	<b>Fold Change</b>	<b>P Value</b>
CDH17	NM_194422	cadherin 17, LI cadherin (liver-intestine)	transporter	327.578	0
TMEM27	NM_001139458	transmembrane protein 27	other	85.036	0
SLC6A12	NM_001080077	solute carrier family 6, member 12	transporter	80.151	0
STAR	NM_131663	steroidogenic acute regulatory protein	transporter	75.496	6.21E-11
DDC	NM_213342	dopa decarboxylase	enzyme	61.797	0
RABIF	NM_001168213	RAB interacting factor	transporter	59.106	4.55E-06
SLC5A1	NM_200681	solute carrier family 5, member 1	transporter	48.379	9.98E-12
BVES	NM_001257164	blood vessel epicardial substance	other	43.495	1.66E-07
HNF4A	NM_194368	hepatocyte nuclear factor 4, alpha	transcription regulator	43.056	1.88E-08
SLC12A3	NM_001045080	solute carrier family 12, member 3	transporter	41.087	6.35E-12
KLHL9	NM_001099229	kelch-like 9 (Drosophila)	other	-14.475	1.88E-07
FOSB	NM_001007312	FBJ murine osteosarcoma viral oncogene homolog B	transcription regulator	-19.33	8.04E-09
RTP3	NM_001128720	receptor (chemosensory) transporter protein 3	other	-21.567	0.0002772
MBNL2	NM_001161669	muscleblind-like splicing regulator 2	other	-21.627	3.57E-08
MGMT	NM_001256246	O-6-methylguanine-DNA methyltransferase	enzyme	-30.051	3.84E-06
IRX1	NM_207185	iroquois homeobox 1	transcription regulator	-30.417	1.26E-09
CLDND1	NM_001161597	claudin domain containing 1	other	-35.854	1.08E-07
BASP1	NM_001202454	brain abundant membrane attached signal protein 1	transcription regulator	-41.336	3.53E-10
HBZ	NM_001033093	hemoglobin, zeta	transporter	-66.311	6.25E-06
GTF2F2	NM_001103133	general transcription factor IIF, polypeptide 2, 30kDa	transcription regulator	-107.565	2.89E-12



Table 9: Top 10 up- and down-regulated molecules in G1/B2 (GH-injected day 1 compared to buffer-injected day 2)

<b>Symbol</b>	<b>Gene ID</b>	<b>Entrez Gene Name</b>	<b>GO Function</b>	<b>Fold Change</b>	<b>P value</b>
SLC6A12	NM_001080077	solute carrier family 6, member 12	transporter	725.92	3.76E-24
SNX12	NM_001145894	sorting nexin 12	transporter	226.45	0
FGFR2	NM_001243004	fibroblast growth factor receptor 2	kinase	83.322	1.33E-15
DDC	NM_213342	dopa decarboxylase	enzyme	80.746	0
RABIF	NM_001168213	RAB interacting factor	transporter	58.728	1.55E-06
PGD	NM_213453	phosphogluconate dehydrogenase	enzyme	35.474	4.84E-08
POMC	NM_181438	proopiomelanocortin	other	31.732	5.11E-14
H2AFV	NM_001201563	H2A histone family, member V	other	21.029	0.0005781
MYEF2	NM_001037423	myelin expression factor 2	transcription regulator	19.809	0
CYP7A1	NM_201173	cytochrome P450, family 7, subfamily A, polypeptide 1	enzyme	19.662	9.52E-05
IRX1	NM_207185	iroquois homeobox 1	transcription regulator	-13.862	5.57E-05
CNBP	NM_199749	CCHC-type zinc finger, nucleic acid binding protein	transcription regulator	-17.657	0.0002539
RHCG	NM_001089577	Rh family, C glycoprotein	transporter	-21.655	1.88E-07
TULP4	NM_001044838	tubby like protein 4	transcription regulator	-21.84	0.0070638
RNF8	NM_205553	ring finger protein 8, E3 ubiquitin protein ligase	enzyme	-22.286	6.04E-08
CAV1	NM_001024162	caveolin 1, caveolae protein, 22kDa	other	-23.296	4.41E-05
MBNL2	NM_001161669	muscleblind-like splicing regulator 2	other	-39.388	6.29E-11
GTF2F2	NM_001103133	general transcription factor IIF, polypeptide 2, 30kDa	transcription regulator	-55.076	3.18E-08
UFC1	NM_001003650	ubiquitin-fold modifier conjugating enzyme 1	enzyme	-70.633	1.25E-07
HBZ	NM_001033093	hemoglobin, zeta	transporter	-154.373	1.12E-08

Table 10: Top 10 up- and down-regulated molecules in A1/G1 (GH antagonist-injected day 1 compared to GH-injected day 1)

Symbol	Gene ID	Entrez Gene Name	GO Function	Fold Change	P Value
NAPB	NM_001024651	N-ethylmaleimide-sensitive factor attachment protein, beta	transporter	62.817	8.51E-10
MBNL2	NM_001161669	muscleblind-like splicing regulator 2	other	54.925	7.85E-13
HRSP12	NM_001002576	heat-responsive protein 12	other	31.629	0.0005054
CELA2A	NM_199886	chymotrypsin-like elastase family, member 2A	peptidase	30.823	9.47E-08
CLDND1	NM_001161597	claudin domain containing 1	other	26.631	7.27E-06
MGMT	NM_001256246	O-6-methylguanine-DNA methyltransferase	enzyme	26.554	2.44E-06
CAV1	NM_001024162	caveolin 1, caveolae protein, 22kDa	other	26.237	3.55E-05
CELA1	NM_001003737	chymotrypsin-like elastase family, member 1	peptidase	22.18	1.11E-10
CHIA	NM_213213	chitinase, acidic	enzyme	21.7	4.97E-05
SERPINA1	NM_001013259	serpin peptidase inhibitor, clade A, member 1	other	20.715	5.64E-07
RFNG	NM_001001830	RFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	Enzyme	-8.785	2.13E-10
CYP7A1	NM_201173	cytochrome P450, family 7, subfamily A, polypeptide 1	enzyme	-9.056	2.27E-06
JMJD5	NM_001002663	jumonji domain containing 5	other	-10.9	2.42E-05
MYEF2	NM_001037423	myelin expression factor 2	transcription regulator	-13.67	0
CGA	NM_205687	glycoprotein hormones, alpha polypeptide	other	-14.093	0.0004521
H2AFV	NM_001201563	H2A histone family, member V	other	-21.35	0.000576
SLC6A12	NM_001080077	solute carrier family 6, member 12	transporter	-28.287	0
POMC	NM_181438	proopiomelanocortin	other	-48.099	1.24E-10
GH1	NM_001020492	growth hormone 1	cytokine	-86.524	2.87E-11
HMGN3	NM_001243176	high mobility group nucleosomal binding domain 3	other	-594.464	3.00E-19

Table 11: Differential regulation of transcripts across six pairwise comparisons showing opposite patterns. Red designates molecules that were up-regulation and green shows those that were down-regulated.

<b>B1/C</b>	<b>B2/C</b>	<b>B2/B1</b>	<b>G1/B1</b>	<b>G1/B2</b>	<b>A1/G1</b>
GTF2F2	GTF2F2		GTF2F2	GTF2F2	-
GH1	-	GH1	GH1	GH1	GH1
-	-	-	POMC	POMC	POMC
SLC6A12	SLC6A12	SLC6A12	SLC6A12	SLC6A12	SLC6A12
-	-	-	CYP7A1	CYP7A1	CYP7A1
-	TMEM27	TMEM27	TMEM27	TMEM27	TMEM27
-	HNF4A	HNF4A	HNF4A	-	HNF4A
FOS	FOS	FOS	FOSB	FOS	FOS
JUN/JUNB/ JUND	JNK	JUN/JUNB/ JUND	JUN/JUNB/ JUND	-	JUN/JUNB/ JUND
BASP1	-	BASP1	BASP1	-	-
-	CDH17	CDH17	CDH17	CDH17	CDH17
FGFR2	FGFR2	FGFR2	FGFR2	FGFR2	FGFR2
DCLK2	DCLK2	DCLK2	-	DCLK2	DCLK2

## Discussion

The current study is the first to examine the effects of growth hormone (GH) on gene expression in the zebrafish inner ear and the second to analyze the hair cell transcriptome of the zebrafish inner ear following sound exposure (Schuck *et al.*, 2011). Hundreds of differentially expressed transcripts were identified in all six pairwise comparisons. I will focus my discussion here on a few highly expressed categories of genes: solute carriers, growth factors, cell adhesion molecules and transcription factors. Cell proliferation network and cell death networks are also discussed.

### *Role of GH in the zebrafish inner ear:*

GH is a neuroendocrine hormone that is involved in numerous functions inside a cell. It is involved in nutritional regulation (Perez-Sanchez *et al.*, 1995), growth regulation, energy homeostasis and immune functions (Perez-Sanchez 2000; Calduch-Giner *et al.*, 1997). Apart from these normal functions, the effect of GH in hair cell regeneration has recently been recognized in the zebrafish inner ear (Schuck *et al.*, 2011). Our previous microarray experiment found significant up-regulation of GH in the inner ear organ two days following sound exposure (Schuck *et al.*, 2011). Another study by Sun *et al.*, (2011) found that GH treatment protected zebrafish inner ear hair cells from sound-induced cell death and it also significantly promoted hair cell regeneration. In support of these previous observations, a significant up-regulation of GH in our current next generation sequencing study was found in both B1/C and G1/B1 comparisons,

but the expression of GH was not observed when B2 was compared to control. Growth hormone receptor was also upregulated (1.9 fold) in GH treatment compared to B1.

*Role of GH in cell growth and proliferation network:*

Cell growth and proliferation is one of the important cellular functions regulated by GH in the G1 treatment compared to the B1 treatment (Fig 5). It is interesting to note that the transcription factors Atf3 (Frisina *et al.*, 2009) and Jun-B (Yogev & Shaulian, 2010), which are involved in apoptosis, are down-regulated following GH injection. This indicates that GH may induce a protective effect upon the zebrafish inner ear. In the A1/G1 comparison, transcription factors Atf3 and Jun-B were up-regulated to 2.3 and 2.9 fold, respectively. Jun-B was down-regulated in the microarray study two days post-sound exposure and was correlated with increased cell death (Schuck *et al.*, 2011). The immediate early gene and transcription factor, early growth response 1 (EGR1) was down-regulated in the cell proliferation network (Fig 5). Several immediate early genes were found to have increased expression following noise exposure in rat cochlea and they initiate genetic cascades as part of pathological signals (Lomax *et al.*, 2001). Atf3 is also found to be one of the downstream target molecules of EGR1. This suggests that the down-regulation of EGR1 also down-regulates Atf3 and may protect hair cells from undergoing apoptosis in GH-injected conditions.

*Cytoprotective effects of GH in the zebrafish inner ear:*

Brain abundant signal protein 1 (BASP1) is one of the top 10 molecules expressed in our B1/C comparison and is expressed in mammary glands, testis, kidney and lymphoid tissues. BASP1 promotes apoptosis when it is over-expressed in cultured renal tubular cells (Sanchez-Nino *et al.*, 2010). BASP1 was significantly up-regulated in B1/C while it was found to be down-regulated in B2/C and G1/B1. Its over-expression one day following acoustic trauma indicates that it might promote apoptosis while injection of GH potentially protects hair cells from undergoing cell death by decreasing the expression levels of BASP1. The comparison B2/B1 showed down-regulation of BASP1 and this decreased expression in B2 treatment could be due to the normal recovery process. This also supports the observation of decreased apoptosis in the zebrafish inner ear two days following sound exposure (Sun *et al.*, 2011).

A previous study found that cytochrome P450, family 2 (CYP2S1) is differentially regulated in all three mouse inner ear organelles (i.e., saccule, utricle and cochlea). It is specifically expressed in the ear tissues and not in non-ear tissues (Yoon *et al.*, 2011). Another cytochrome P450, family 26 (CYP26A1) molecule is differentially regulated in the mouse cochlea (Sajan *et al.*, 2007). Over-expression of CYP26A1 in HeLa (human cervical cancer cell line) cells showed antiapoptotic effects against apoptotic agents such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), oxidative stress, heat shock, genotoxic agents and  $\gamma$ -irradiation by metabolizing retinoic acid. Microarray analysis showed that overexpression of CYP26A1 in HeLa cells

increased apoptosis-inhibitory genes and decreased apoptosis-inducing genes CYP26A1 also had a slight cell proliferation effect (Osanai & Petkovich, 2005). Interestingly, I found the differential regulation of CYP2J28 (+2 fold) in the A1/G1 comparison, and CYP24A1 (+3.2 fold), CYP19A1 (-3.5 fold), CYP7A1 (+30.9 fold), and CYP46A1 (+3.3 fold) in the G1/B1 comparison. In the B1/C comparison, I found the differential regulation of CYP24A1 (-1.9 fold), CYP2J2 (-2.5 fold) and CYP46A1 (-2 fold). In comparing the cytoprotective effects of CYP26A1 with other cytochrome molecules expressed in G1/B1, I propose that these molecules might also offer protection against apoptosis in zebrafish hair cells but their exact roles during hair cell regeneration still need to be established.

*Regulation of cell death network in GH antagonist treatment:*

Growth hormone injection decreased apoptosis in the zebrafish ear following acoustic trauma (Sun *et al.*, 2011). This current study discovered some of the genes that are involved in this process. Analysis of the cell death network in the A1/G1 pairwise comparison showed that GH is strongly down-regulated in the presence of antagonist and is involved in cell death pathways (Fig 6). A decrease in levels of GH in the antagonist treatment is observed along with increased expression levels of the Ap-1 transcription factor that forms homo- or hetero-dimers between the Fos and Jun families. Ap-1 is found to be induced in rat organ of Corti explants following gentamycin application (Albinger-Hegy *et al.*, 2006) and in organ of Corti in guinea pigs following noise-induced damage

(Nagashima *et al.*, 2005). In the A1/G1 comparison, the Fos and Jun families were found to be up-regulated FosB (+7.3 fold), Fos (+2.5 fold), FosL1 (+6.3 fold) and JunB (+2.9 fold). This pattern of increased expression of FosB (+7.5 fold), Fos (+4.4 fold), FosL1 (+5.8 fold) and JunB (+2.9 fold) was also evident in B1/C suggesting that the intense sound exposure activated these transcription factors. While these transcription factors were increased in B1/C and A1/G1, they were strongly down-regulated in G1/B1 with FosB (-19.3 fold), Fos (-9.9 fold), FosL1 (-6.2 fold) and JunB (-3.7 fold). This suggests that growth hormone protects the cells from undergoing cell death that would have been caused by the accumulation of stress and reactive oxygen species due to acoustic overstimulation. In contrast, the GH antagonist induces cell death in the zebrafish inner ear by increasing the expression of Ap-1 transcription factor components. JNK1/2 was also found to be up-regulated in the cell death network in the GH antagonist treatment (Fig 6). Activation of JNK signaling cascades have been shown to induce apoptosis in response to stressful stimuli in cochlear hair cell cultures and these JNKs in turn regulate c-JUN, a component of AP-1 (Pirvola *et al.*, 2000).

Caspases play an important role in mammalian apoptosis and in inducing inflammation (Tadros *et al.*, 2008). In our A1/G1 comparison, I found a significant up-regulation of CASP1 (+2.9 fold) and significant down-regulation of CASP1 (-2 fold) in G1/B1. This indicates that the GH treatment has the potential to decrease apoptotic pathways in the zebrafish inner ear.



*Role of growth factors in the zebrafish inner ear:*

Growth factors and cytokines have a potent mitogenic role in cell proliferation. Here I found a significant up-regulation of prolactin (PRL; 38 fold) in G1/B1 and a down-regulation of PRL (-38.3 fold) in A1/G1. This shows that GH has a positive effect on PRL expression. Administration of GH and PRL increased proliferation of leukocytes in rainbow trout (Yada *et al.*, 2004) and in Chum salmon (Sakai *et al.*, 1996). Over-expression of GH and PRL might help in the proliferation of leukocytes at the injured site of the zebrafish inner ear. It is evident that leukocytes and macrophages reside in the sensory epithelia of the undamaged inner ear. These immunocytes help to engulf the damaged cells and secrete cytokines and growth factors that are necessary for the hair cells to regenerate (Matsui *et al.*, 2005).

Fibroblast growth factor (FGF) is implicated in many cellular functions such as cell differentiation, proliferation, survival and motility. FGF family members such as FGF 2, FGF 3, FGF 8, FGF 10 and FGF 19 are involved in otic neurogenesis. FGF2 is expressed in the otic placode and otic vesicle of mouse and chicken (Sanchez-Calderon *et al.*, 2007). FGF2 is also found to promote the activity of brain derived neurotrophic factor (BDNF) by up-regulating its high affinity TrkB receptor in cultured mouse auditory neurons (Sanchez-Calderon *et al.*, 2007). Although I did not find the expression of FGF2, a significant up-regulation of its receptor FGFR2 18 fold in B1/C, 15 fold in G1/B1 and down-regulated 4.8 fold in A1/G1 was found. Fibroblast growth factor receptor-like 1 (FGFRL1) is also up-regulated in G1/B1 but is down-regulated in A/G1.

I found an up-regulation of insulin-like growth factor binding protein 1 (IGFBP1) to 3 fold and IGFBP7 to 2.1 fold in G1/B1 comparison. GH up-regulates the expression of IGF-1 and IGFBP3 in the small intestine of albino rats (Ersoy *et al.*, 2009). IGFBP3 mRNA is also found to be highly expressed in the otic vesicles and pharyngeal arches of zebrafish. Although the temporal expression pattern of IGFBP3 differs between these two tissues, otic vesicles showed persistent expression of IGFBP3. Knockdown of IGFBP3 showed defects in hair cell and semicircular canal differentiation (Li *et al.*, 2005). The high affinity IGF binding proteins modulate biological responses in cells by binding IGF molecules and they are capable of regulating IGF-independent actions (Oesterle *et al.*, 1997, Li *et al.*, 2005). Up-regulation of IGFBP1 and IGFBP7 in our data suggests that GH regulates the action of IGF through these IGFBPs.

*Role of transmembrane proteins in the zebrafish inner ear:*

To maintain ion homeostasis and for conduction of signals in hair cells, many ion transporters are present that transport sodium, calcium, potassium and chloride ions in and out of the cell (MacArthur *et al.*, 2011). A major class of transcript molecules that were regulated in our current study is the solute carrier (SLC) molecules. SLC26 is in the family of solute carriers whose function is either to transport chloride-iodide ions, chloride-bicarbonate exchangers, sulfate transporters or in case of SLC26A5, they function as motors (Weber *et al.*, 2003). Prestin (SLC26A5), another solute carrier molecule, is involved in mechanical

amplification in mammalian cochlea while pendrin (SLC26A4) is found in the developing cochlea (Weber *et al.*, 2003). Other members of the SLC26 family, SLC26A1 and SLC26A6, were found to be up-regulated in G1/B1 and these members might serve an important function in the development of hair cells. SLC6A12, a Betaine/ $\gamma$ -aminobutyric acid (GABA) transporter, is the top up-regulated solute transporter in G1/B1 but it is down-regulated in A1/G1.

Interestingly, a recent study has found that SLC6A12 is regulated by Janus-activated kinase 2 (JAK2) (Hosseinzadeh *et al.*, 2012), Binding of GH to its receptor activates JAK2, a receptor associated with intracellular tyrosine protein kinase activity. This initiates a series of protein phosphorylation cascades and activation of transcription factors such as STAT1, STAT3, STAT5a and 5b among others (Woelfle *et al.*, 2003). Significant up-regulation of SLC6A12 in G1/B1 suggests that injection of GH might play an important role in up-regulating SLC6A12 through activation of JAK2. SLC12A3, a member of SLC12 solute carrier family that transports sodium/chloride, was up-regulated 41 fold in G1/B1, but down-regulated 5 fold in A1/G1. Another member of the SLC12 family, SLC12A2, is expressed in epithelial and non-epithelial cells of mammals. Disruption of this molecule causes an inner ear dysfunction (Hebert *et al.*, 2004; Friauf *et al.*, 2011). Although some SLC molecules have established roles in ear and other tissues, the role of remaining solute carriers in relation to hair cell regeneration is still lacking.

Transmembrane protein 27 (TMEM27) is an amino acid transporter and is also called collectrin. TMEM27 is one of the top 10 up-regulated molecules in

G1/B1 (85 fold) and down-regulated 2 fold in A1/G1. It is found to be a downstream target of hepatocyte nuclear factor 1 $\alpha$  (Malakauskas *et al.*, 2009) and reported to have a role in cell growth (Zhang *et al.*, 2004).

Uroplakin 1A, one of the most highly negatively regulated molecules in B1/C, is present in the ureter of mice. The absence of Uroplakin is correlated to the decreased expression of lysosomal integral membrane protein-2 (LIMP-2) in ureter that causes a deafness phenotype in mice (Gamp *et al.*, 2003; Hughes *et al.*, 2006). Examination of the cochlea of LIMP-2 deficient mice showed histological observations such as atrophy of stria vascularis, gradual reduction and finally loss of both outer and inner ear hair cells and severe reduction in the neurons of spiral ganglion (Gamp *et al.*, 2003). The down-regulation of Uroplakin in the inner ear tissue of zebrafish in B1/C may have resulted in the deficiency of LIMP-2 and its associated deafness phenotype. The expression levels of Uroplakin increases in B2/C, and this suggests that this gene may be important for the process of recovering from acoustic trauma.

#### *Role of cell adhesion molecules in the zebrafish inner ear:*

Tip links which are made of Cadherin 23 (CDH23) and Protocadherin 15, are essential features of hair cells which connect stereocilia together and aid in opening or closing the ion channels depending upon whether hair cells are excited or inhibited (Sakaguchi *et al.*, 2009). Mutations in CDH23 also cause deaf-blindness in humans (Sengupta *et al.*, 2009) and are involved in causing Usher 1D syndrome (Reiners *et al.*, 2006). In our study both CDH17 (cadherin

17) and CDH23 are up-regulated in G1/B1, and CDH17 was the most highly upregulated molecule. In correlation to this observation, tight junction signaling was the most significant canonical pathway in G1/B1 comparison as cadherins are present in tight junctions. CDH23 is down-regulated in B1/C, suggesting that the tip link function is also regulated as part of hair cell damage. Fibronectin1, a component of extracellular matrix, is found to enhance the proliferation of inner ear sensory epithelial cells in cell culture systems (Warchol, 2002). In our data, I found an increase in the expression of fibronectin 1 in B1/C which may be necessary for hair cell proliferation after acoustic trauma.

Claudins are transmembrane proteins that are involved in maintaining tight junctions. It also establishes a barrier that controls the flow of molecules between epithelial cells (MacArthur *et al.*, 2011). There are at least 10 claudin proteins expressed in the cochlear inner ear of mammals and claudin 11 and 14 are associated with hearing loss (Elkouby-Naor *et al.*, 2008). Defects in claudin proteins would impact the integrity of epithelial cells which causes leakage of molecules from the cell (MacArthur *et al.*, 2011). In murine models, claudin 3 and 4 are found to be up-regulated during inflammation (MacArthur *et al.*, 2011). Here I report an up-regulation of claudin 3 (+2.6 fold) in G1/B1 and a down-regulation of claudin 3 (-3.2 fold) in B1/C. In addition to claudin 3, I also found claudin 8 (-8.9 fold) in B1/C and claudin 15 (+12.9 fold) in G1/B1. Claudin 8 is found to be expressed in the normal utricle of mouse inner ear (Sajan *et al.*, 2007) and I found it to be reduced in the zebrafish inner ear following sound exposure

(current study). This might result in loss of epithelial integrity in the zebrafish inner ear following acoustic trauma.

*Differential regulation of transcription factors in the zebrafish inner ear:*

The most highly regulated transcription factor in G1/B1 was hepatocyte nuclear factor alpha (HNF4A). HNF4A is found to be one of the important transcription factors that regulate axolotl limb regeneration, and is found to be interconnected with four other transcription factors: c-Myc, SP-1, ESR-1 (estrogen receptor-1) and p-53 (cellular tumor antigen) and the many targets of HNF4A, ESR-1 and p-53 overlap during limb regeneration (Jhamb *et al.*, 2011). The strong expression of HNF4A in our current study suggests that it might have a similar effect of regeneration in zebrafish hair cells through activation of its target genes. HNF4A also has a role in inflammation and it produces pro-inflammatory cytokines such as interleukins and tumor necrosis factors under stress (Wang *et al.*, 2011). These cytokines in turn activate acute phase response signaling and these acute phase proteins help to achieve homeostasis (Wang *et al.*, 2011). In our current data it is evident that this signaling event is one of the top 15 canonical signaling pathways in the GH (G1) treatment (Fig 6). In the GH antagonist (A1) treatment, HNF4A is down-regulated and this signaling event is also down-regulated under antagonist conditions.

GTF2F2, a general transcription factor II F, was the most highly up-regulated molecule in B1/C while it was the most highly down-regulated molecule in G1/B1. This strong up- and down-regulation of GTF2F2 was found to occur

with a significant up- and down-regulation of Fos and Jun family transcription factors. Interestingly, general transcription factor 2F (TFIIF) and TFIIE-34 were found to bind the dimerized form of Fos-Jun transcription factors (Martin *et al.*, 1996). In light of this finding, our data suggest that an up-regulation of GTF2F2 might bind to the up-regulated Fos and Jun homo- or hetero-dimers and promote apoptosis in the inner ear following one day post-acoustic trauma while the reverse is true in G1/B1 comparison.

Early growth response 1 (EGR-1) is a nuclear receptor and a transcription factor that responds to pathological conditions. It showed an increased expression following noise exposure in rat cochlea (Lomax *et al.*, 2001), but EGR-1 showed a decreased expression (-2.5 fold) in G1/B1 suggesting that GH suppresses the negative effects of EGR1, potentially protecting hair cells.

In humans, mutations in Sox10 causes sensorineural deafness that causes Waardenburg syndrome type IV. Sox 2 promotes survival of cochlear progenitors during otocyst formation in mice (Breuskin *et al.*, 2009). I found an increase in Sox10 (+1.5 fold) in G1/B1 suggesting that it might have a role in survival of supporting progenitor cells in zebrafish.

Growth factor independent -1 (GFI-1) is a transcription factor which is shown to cause decreased apoptosis, increased levels of cell proliferation and decreased levels of cell cycle inhibitors (Wallis *et al.*, 2002). This transcription factor is also required in hair cells for proper differentiation and maintenance (Wallis *et al.*, 2002). Consistent with this, I found decreased expression of GFI-1 (-1.6 fold) in the A1/G1 comparison. This shows that the GH antagonist

negatively regulates GFI-1 and inhibits the maintenance and differentiation of hair cells in zebrafish.



## Conclusions and Future Directions

There was increased expression of GH in the inner ears of zebrafish one day following sound exposure in buffer-injected controls and in GH-treated zebrafish. I also found the regulation of genes that are required for proper functioning of hair cells such as cadherin 23, solute carrier molecules, growth promoting factors such as GH, prolactin and the receptors required for binding of growth factors, transcription factors and cytoskeleton molecules to be significantly differentially regulated in the GH-injected condition. Cell proliferation network in G1/B1 showed significant up-regulation of GH and down-regulation of apoptotic transcription factors while an opposite pattern is evident in the cell death network in A1/G1 comparison suggesting potential involvement of GH in the zebrafish inner ear.

This current study focused on the genes that were regulated one day following sound exposure. Future studies at further time points will be needed to identify the change in gene expression patterns specifically related to cell death and cell growth pathways. It is known that growth factors play a major role in hair cell proliferation, so it will be interesting to study the how various cocktails of growth promoting factors such as growth hormone, insulin growth factor-1, prolactin, brain derived neurotrophic factor, neurotrophin-3, will effect hair cell regeneration using cell culture techniques using zebrafish as a model system.

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