THE FUNCTION OF SOX2 AND SOX3 IN EARLY OTIC DEVELOPMENT AND PATTERNING

An Undergraduate Research Scholars Thesis

by

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Submitted to the Undergraduate Research Scholars program
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Bruce Riley

May 2016

Major: Molecular and Cell Biology

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ABSTRACT

The Function of Sox2 and Sox3 in Early Otic Development and Patterning

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Sox genes encode transcription factors that function as part of a complex gene regulatory network governing development of the otic placode, the precursor of the inner ear. Sox genes have been implicated in maintaining pluripotency and in promoting differentiation depending on expression level or availability of other co-expressed transcription factors. Further, these genes may function in establishing sensory neural patterning within the ear. This study aims to examine which of these roles sox2 and sox3 perform within the developing inner ear. We will use mutant knockout lines to observe what happens when the genes are not functioning and heat shock activated transgenes to observe the effects of too much gene function. This will give a greater understanding of how these genes fit within the regulatory network governing otic placode formation, how they are used to balance pluripotency and differentiation during development, and which functions they perform in the process of sensory neural patterning. Elucidating these functions could lead to better understanding of causes of human deafness and inform efforts to develop effective treatments.

DEDICATION

I would like to dedicate this work to my parents for their continual love and support.

ACKNOWLEDGMENTS

I would like to thank Yunzi Gou for contributing a portion of the sox3 mutant data. I would also like to thank Husniye Kantarci for her help in teaching me the required methods. Finally I would like to thank Jinbai Gou, Jennifer Dong, and Andrea Gerberding for their aid and support in creating this work.

NOMENCLATURE

FGF	Fibroblast Growth Factor
HB	Hindbrain
HFP	Hours Post Fertilization
Hs:Sox2	Tg(hsp701:sox2)
Hs:Sox3	Tg(hsp701:sox3)
NGN1	Neurogenin 1
NRD	NeuroD
OP	Otic Placode
SAG	Stato-acoustic ganglion

CHAPTER I

INTRODUCTION

The cells of the otic placode begin to be specified in response to *fgf* signaling from the hindbrain and subotic mesoderm between 9 and 10 hours post fertilization (Liu *et al*. 2003; Phillips *et al*. 2001). These cells will form a morphologically visible patch of thickened epithelium, called the otic placode (Fig. 1). The otic placode will undergo further morphological changes in order to form the inner ear, which mediates hearing and balance (Reviewed by Whitfield *et al*. 2002). The two major cell types which form within the ear are sensory and neural cells. The hair cells, a sensory cell type, have cilia on their surface that allow the cell to sense vibrations and thus mediate hearing. The neural cell type includes the cells that form neurons of the stato-acoustic ganglion which connects the ear to the brain (Vemaraju *et al*. 2012).

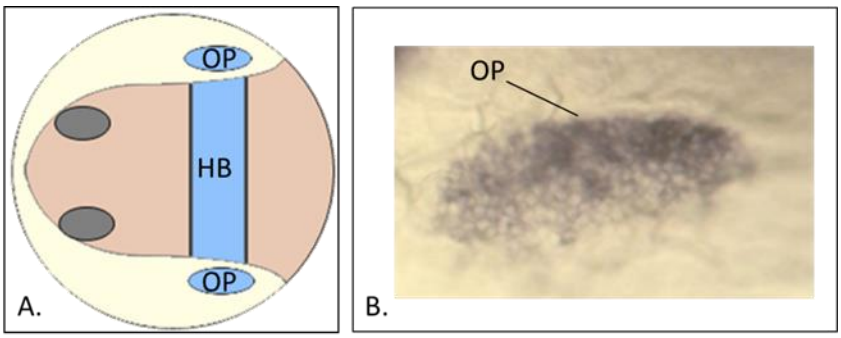

Figure 1: The appearance and location of the otic placode. **A)** A diagram looking down on top of an embryo, with the future head of the embryo facing left and future tail facing right. The otic placode, labeled OP, forms just to the side of the hindbrain, labeled HB. **B)** The otic placode is shown via staining for an early otic marker.

However, in order for the otic placode to form, the cells have to be able to respond to *fgf* signaling. This competence is provided by two sets of genes which form the beginning of two

genetic pathways functioning in otic induction and patterning of sensory versus neural cell types. One pathway begins with *foxi1* which allows cells to respond to *fgf* signaling and turn on downstream genes that specify neural cell fates. *Sox3* is believed to function in the *foxi1* pathway. The other pathway begins with *dlx3b/4b*. These genes also give competence to respond to *fgf*, and work with *fgf* in order to turn on genes which specify sensory cell fates. *Sox2* is believed to function in the *dlx3b/4b* pathway (Hans *et al*. 2004; Hans *et al*. 2013; Solomon *et al*. 2004).

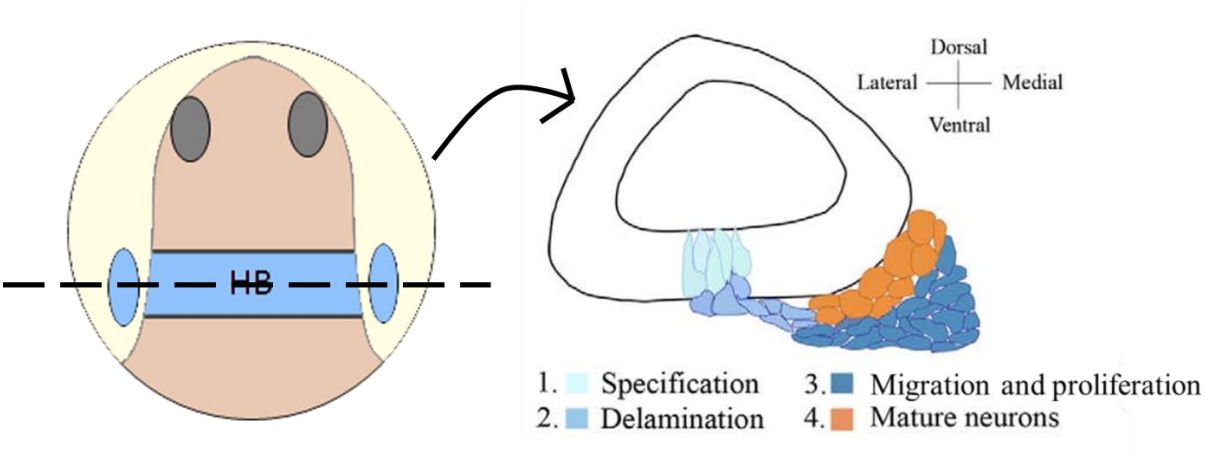

Figure 2: Neural specification within the ear. When the embryo is sectioned along the plane indicated by the dashed line, the otic vesicle appears as shown. The cells are initially specified as neural cells in the floor of the ear. They will leave the ear in a process called delamination. Next the cells migrate and proliferate before reaching their final location and forming mature neurons.

Neural cell fates are specified in the floor of the otic vesicle and undergo a complex developmental process before reaching the mature state. Initially these cells turn on expression of *ngn1*. Soon after this some number of these cells undergo an epithelial to mesenchymal transition and exit the floor of the vesicle. This process is termed delamination. After delamination the neuroblasts lose expression of *ngn1* and turn on expression of *neuroD*. These

will migrate and proliferate before becoming mature neurons of the SAG and extending projections to the brain and ear (Kantarci *et al.* 2015).

Sensory cell types, hair cells and support cells, form sensory epithelia in the floor of the ear. Initially, one large pro-sensory domain forms in the placode and expresses *atoh1b*. Later in development this will split into two separate patches corresponding to the utricle and saccule. The utricle, at the anterior of the ear, is important for vestibular function. The saccule, at the posterior of the ear, mediates hearing. The first two hair cells that form in the utricle and in the saccule are called tether cells and continue to express *atoh1b*. Hair cells will continue to proliferate within each patch of sensory epithelia as time progresses. These new hair cells, as well as the original tether cells, express *atoh1a* (Millimaki *et al.* 2007; Sweet *et al.* 2011).

Sox2 and Sox3 are members of the SoxB1 family of transcription factors (Okuda *et al*. 2010). Previous studies have shown that both *sox2*/3 have the ability to inhibit cell differentiation and maintain cell pluripotency (Bylund *et al*. 2003; Dee *et al*. 2008; Millimaki *et al*. 2010). Also, both *sox2/3* later play a role in specifying sensory and neural fate or giving cells the ability to adopt sensory and neural fates (Dee *et al.* 2008; Sweet *et al*. 2011). However, these factors may also inhibit subsequent stages of differentiation and help maintain some pluripotency (Millimaki *et al*. 2010). We hypothesize that whether these genes inhibit or promote differentiation depends on expression level and interactions with other genes.

The two major methods used to examine the function of a gene are removing and adding gene function. The function of a gene can be removed by using a null mutation, or by injecting a

morpholino oligomer to cause a knockdown of gene function. This allows you to study what a gene is required for. Excess gene function can be provided by using a heat shock activated transgene which drives high levels of mRNA transcription when embryos are placed in warmer than normal water. Ultimately this process should lead to production of high levels of protein. This allows observation of what a gene is capable of doing when expressed at high levels.

This rational was used to examine how *sox2* and *sox3* fit into the otic gene regulatory network, as well as how they regulate pluripotency, differentiation, and sensory-neural patterning. The results of this project should aid in understanding how the balance between pluripotency and specification is regulated during early development. Studying these processes in zebrafish is potentially quite useful, as zebrafish retain the ability to replace damaged hair cells and neurons, which mediate hearing and balance. Humans lose this regenerative ability early in development, and because of this, damage to the hair cells or neurons leads to irreversible deafness (Ozeki *et al.* 2007). Thus, finding differences between human and zebrafish development may help determine a way to allow hair cell regeneration in humans.

CHAPTER II

METHODS

Strains and developmental conditions

The wild type strain was derived from the AB line (Eugene, OR). *Sox2* overexpression was carried out using the Tg(hsp701:sox2) transgenic line and *Sox3* overexpression was carried out using the Tg(hsp701:sox3) transgenic line (Millimaki *et al*. 2010). The *brn3c:GFP* line used has been previously described (Xiao *et al.* 2005). All embryos were allowed to develop at 28.5^{o}C in fish water containing methylene blue, unless otherwise specified. Embryos were staged based on standard protocols (Kimmel *et al*. 1995).

Morpholinos

Translation blocking morpholino oligomers were obtained from Gene Tools Inc. and were used to block gene function. All morpholinos were injected at the one cell stage. The morpholino sequences used in this study have been previously described and tested for efficacy and specificity. To knockdown *foxi1,* embryos were injected with 5ng *foxi1* morpholino (5'-TAATCCGCTCTCCCTCCAGAAACAT-3') along with 5ng *P53* morpholino (5'-GCGCCATTGCTTTGCAAGAATTG-3') in order to inhibit non-specific cell death (Robu *et al*. 2007; Solomon *et al*. 2003).

In-situ hybridization

In-situ hybridization was carried out at 67^{o}C as previously described (Phillips *et al.* 2001). Following staining, embryos were cleared in methanol and washed through a glycerol dilution

series moving through water, 30% glycerol, 50% glycerol, and into 75% glycerol. Alternately, embryos were cleared in methanol and washed into PBS solution.

Cell transplantation

Donor embryos were injected with a dextran lineage tracer (dextran conjugated to rhodamine B, 10,000 MW) at the 1-2 cell stage. Labeled cells were transplanted from 3-4.5 hpf donors into non-labeled hosts of the same stage. Embryos were allowed to develop at 28.5ºC until heat shock. Transplanted cells were identified in hosts by rhodamine fluorescence.

Gene overexpression

A heat shock inducible transgene was used to overexpress *sox2* or *sox3*. Either heterozygous or homozygous transgenic embryos or embryos containing transgenic cells were heat shocked at 39ºC for one hour unless otherwise specified in the text. After heat shock all embryos were incubated at 33ºC until fixation in MEMFA (0.1 M Mops at pH 7.4, 2mM EGTA 1 mM $MgSO_4$, 3.7% formaldehyde). Fixation was allowed to progress for 2 hours at room temperature or overnight at 4ºC prior to staining.

Cryosectioning

Fixed embryos were washed into PBS and allowed to equilibrate for 5 minutes. The embryos were then washed into 20% W/V sucrose solution and stored at 4ºC overnight. After this, the embryos were washed into 30% W/V sucrose solution and allowed to equilibrate for approximately 15 minutes before placement in tissue freezing media (Electron Microscopy Sciences). This was frozen at -20ºC until solid and mounted in a Leica CM1860 cryostat.

Sections were cut into 10 µm slices, mounted on HistoBond slides (VWR), and allowed to dry overnight. A waterproof marker was used to draw around the edge of the slide, the slide was washed 2x in PBS, and a layer of 30% glycerol and cover slip were applied.

Statistical analysis

All statistical analysis were performed using a two sample t-test assuming unequal variances in Microsoft Excel. Statistically significant results are reported in the text along with the relevant P-value.

CHAPTER III

RESULTS

Characterizing the roles of *sox2/3* in otic induction

Previous experiments involving loss of function of *sox2/3* are contradictory. In recent experiments using morpholino, we have seen a very mild phenotype in both the single and double knockdown. However, in the past a more severe phenotype was seen in the double knockdown. Also, while there are many cases in which morpholinos are known to give the correct phenotype, recent studies have shown that some morpholinos may give a different phenotype than the null mutation (Nasevicius and Ekker 2000; Kok *et al*. 2015). Due to these results, we began by studying the phenotype of *sox2* and *sox3* mutants (Fig. 3).

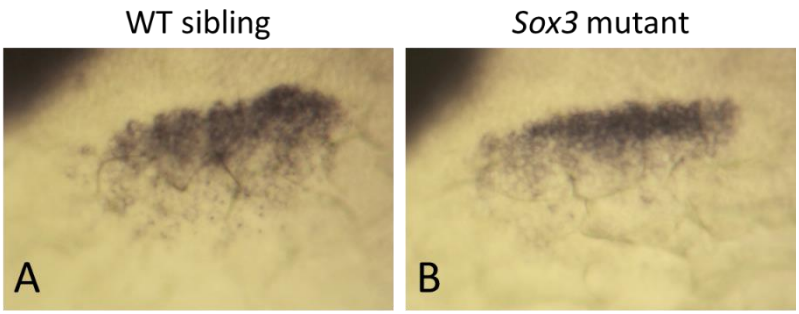

Figure 3: The phenotype of *sox3* mutants is mild. In both panels the otic placode at 12hpf is labeled with a marker gene shown in dark purple. **A)** A normal sized otic placode in a WT sibling embryo. **B)** The otic placode in a homozygous *sox3* mutant is slightly smaller.

Sox3 mutants show a very mild phenotype at 12hpf. The two dimensional area of the otic placode, as labeled using *pax2a* as an otic marker, shows a slight reduction (Fig. 3). This is similar to the data gathered using morpholino. Thus, the *sox3* morpholino seems to replicate the

phenotype of the *sox3* mutant in the early otic placode. The *sox2* mutants do not appear to have a deficit in placode size at 12hpf.

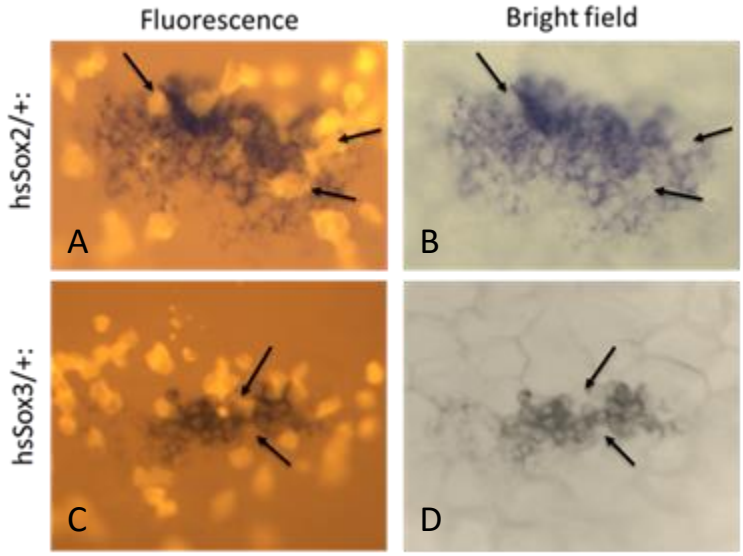

Figure 4: Early overexpression of *sox2/3* impairs otic differentiation. In all panels transgenic cells are labeled via fluorescence, the otic placode is labeled via expression of an otic marker gene shown in dark blue. **A)** The otic placode showing fluorescent cells carrying the hs:Sox2 transgene. **B)** The same otic placode showing loss of expression of an otic marker in cells overexpressing *sox2* (arrows). **C)** An otic placode showing fluorescent cells carrying the hs:Sox3 transgene. **D)** Expression of the otic marker is lost in cells overexpressing *sox3* (arrows).

We also studied the effects of overexpression of *sox2/3*. Early global overexpression of either *sox2* or *sox3* has a devastating effect on the development of many different organ systems. This includes the hindbrain, which is a crucial signaling center involved in the formation of the otic placode. Thus, otic phenotypes caused by global overexpression of these genes can't be interpreted due to loss of the hindbrain signaling center. In order to avoid this issue, a technique called cell transplantation is used. Cells carrying the heat shock transgene are labeled and transferred into a normal embryo, allowing overexpression in only certain cells within an otherwise normal background. Results using this technique showed that high levels of either *sox2*

or *sox3* early in development impair otic differentiation, as shown by the loss of otic markers in cells carrying the transgene (Fig. 4).

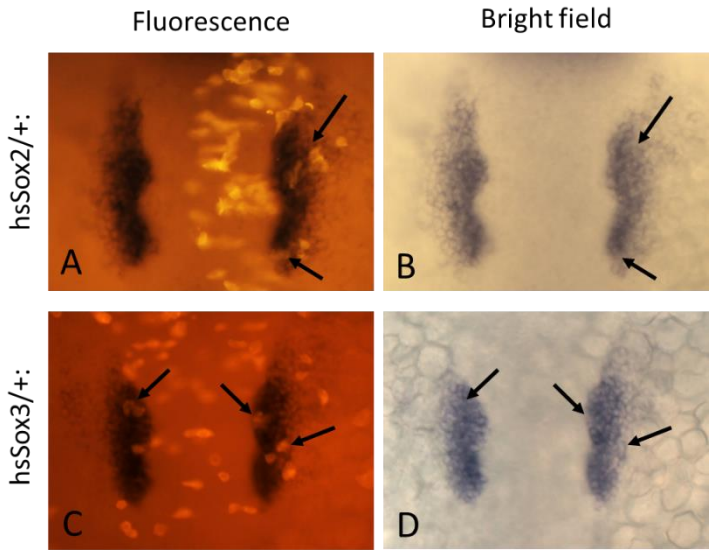

Figure 5: Later overexpression of *sox2/3* fails to reverse otic differentiation. In all panels transgenic cells are labeled via fluorescence, the otic placode is labeled via expression of an otic marker gene shown in dark blue. **A)** The otic placode showing fluorescent cells carrying the hs:Sox2 transgene. **B)** The same otic placode does not show loss of expression of an otic marker in cells overexpressing *sox2* (arrows). **C)** An otic placode showing fluorescent cells carrying the hs:Sox3 transgene. **D)** Expression of the otic marker is not lost in cells overexpressing *sox3* (arrows).

If *sox2* or *sox3* are overexpressed in isolated cells at later timepoints this loss of otic marker expression does not occur. By 12hpf overexpression of either *sox2* or *sox3* fails to cause loss of expression of *pax2a* within the otic placode in cell transplant experiments (Fig. 5). The severity of the phenotype resulting from global overexpression is also less severe if the overexpression occurs at 12hpf or later. Thus, neither *sox2* nor *sox3* are capable of reversing otic specification after it has been stably established.

Initial investigation of *foxi1*

Previous investigations have shown that loss of *foxi1* causes profound deficits in otic development (Padanand *et al.* 2011). *Foxi1* is known to serve as an upstream activator of *sox3* and is hypothesized to also affect *sox2* expression. Here these relationships, as well as their implications on sensory-neural development, are examined in more depth.

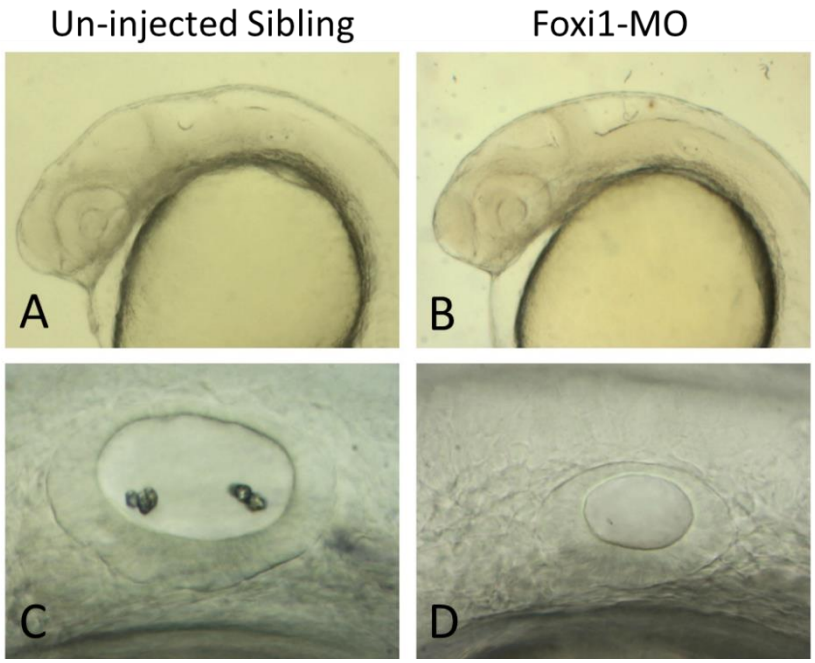

Figure 6: Loss of *foxi1* causes a reduction in otic vesicle size but does not cause other gross morphological deficits. All images are of live embryos at 24hpf. Otic vesicle sizes were calculated by outlining the ear in Adobe Photoshop and comparing the areas in pixels. **A)** An un-injected embryo showing normal morphology. **B)** The overall morphology of the *foxi1* morphant is also normal. **C)** A normal sized otic vesicle in an un-injected sibling. **D)** In *foxi1* morphants the otic vesicle is approximately 50% smaller.

Loss of *foxi1* via morpholino knockdown does not appear to cause gross morphological deficits. Major structures such as the eye and overall brain structure appear normal. The overall axis length also appears normal (Fig. 6 and data not shown). Taken together these data suggest that

the morpholino does not cause non-specific toxicity and that the phenotype is otic specific. This is consistent with previous studies showing that the phenotype of the *foxi1* morphants appears identicle to *foxi1* mutants (Solomon *et al.* 2003; Hans *et al.* 2013). The size of the otic vesicle in *foxi1* morphants is reduced to about 50% of normal. In approximately one third of injected embryos the otoliths, clumps of calcium carbonate and protein that form over each sensory domain, were not visible by 24hpf.

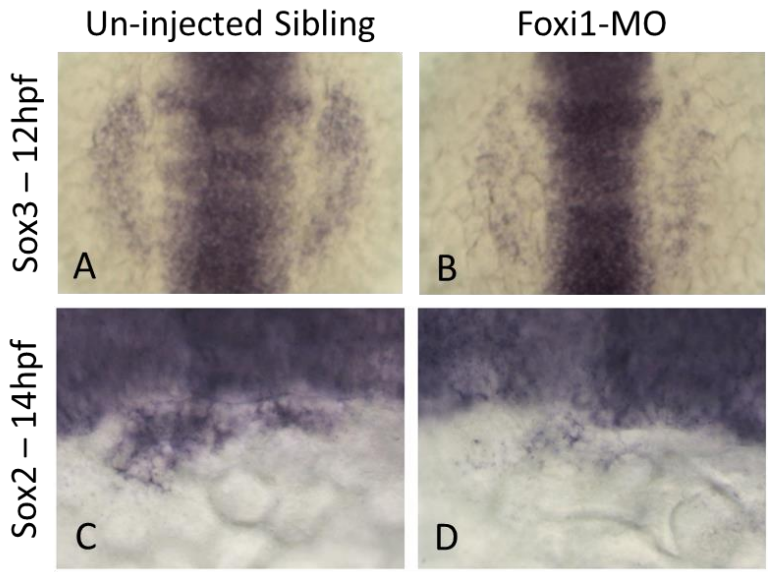

Figure 7: Loss of *foxi1* results in significant deficits in *sox2* and *sox3* expression. **A)** Normal *sox3* expression at 12hpf. **B)** The level of *sox3* expression is significantly decreased in *foxi1* morphants. **C)** Normal *sox2* expression at 14hpf. **D)** The level of *sox2* expression is also significantly decreased in *foxi1* morphants.

The expression levels of both *sox2* and *sox3* are strongly decreased in *foxi1* morphants. At 12hpf *sox3* is normally expressed in an arc, with only a low level of expression in the otic placode itself. *Sox3* is more highly expressed lateral to the otic placode at this stage. In *foxi1* morphants the expression level of *sox3* in both the otic placode and tissue lateral to it appears severely

reduced. Similarly, the level of *sox2* expression is also reduced in *foxi1* morphants. The size of the *sox2* labeled domain also appears to be reduced (Fig. 7).

The effects of *foxi1* on neural development

Previous studies have shown that *sox3* plays an important role in development of neural cell types within the otic placode and vesicle. The decreased expression levels of *sox3*, as well as the deficits in the overall size of the otic placode and vesicle, led to the hypothesis that development of both neural and sensory cell types would also be affected.

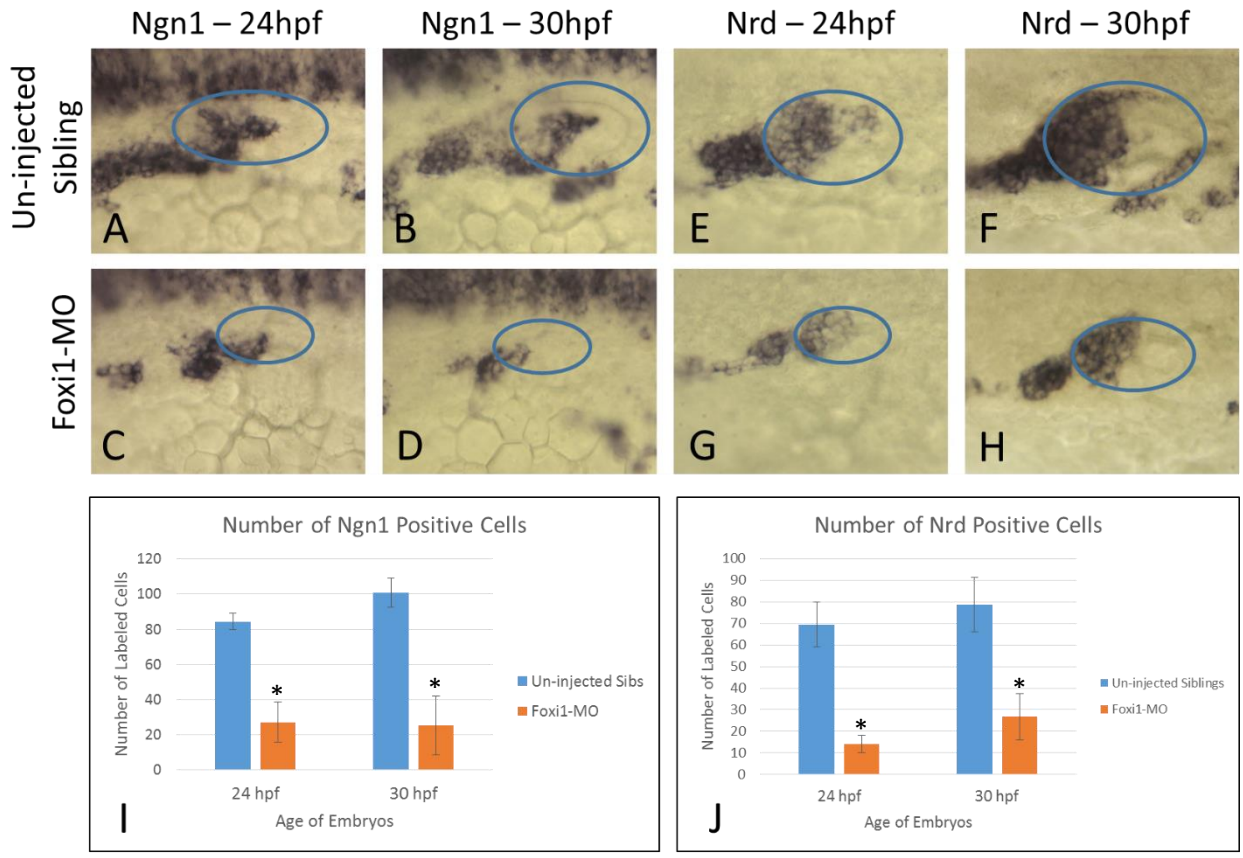

Figure 8: *Foxi1* morphants show significant decreases in the domain and number of neural cells. Otic vesicles are outlined in blue. **A, B)** Normal expression of *ngn1* at 24hpf and 30hpf, respectively. **C, D)** The domain of *ngn1* expression is decreased in *foxi1* morphants at 24hpf and

30hpf. **E, F)** Normal expression of *nrd* at 24hpf and 30hpf. **G, H)** The domain of *nrd* is also decreased in *foxi1* morphants at 24hpf and 30hpf. **I)** A bar graph showing decreases in the number of *ngn1* positive cells in *foxi1* morphants ($P < 0.0001$). **J)** A bar graph showing decreases in the number of *nrd* positive cells in *foxi1* morphants ($P < 0.0001$).

Examination of *ngn1* and *nrd* expression in *foxi1* morphants revealed significant decreases in the domain and number of neural cells. The morphants show deficits in neuroblasts in the floor of the otic vesicle, as labeled by *ngn1*, that persist at least through 30hpf (Fig. 8). Upon quantitation, a statistically significant decrease in the number of neuroblasts was seen at both 24 hpf and 30 hpf ($p < 0.0001$ and $n \geq 3$). In general the morphants have approximately 70-75% fewer *ngn1* positive cells than their un-injected siblings. Despite the decrease in cell number, the pattern and localization of *ngn1* expression in *foxi1* morphants was normal (Fig. 8).

Similarly, *foxi1* morphants have a significantly smaller domain of delaminated and migrating neuroblasts as shown by *nrd* expression when compared to their un-injected siblings. This decrease is evident at both the 24hpf and the 30hpf timepoint. Quantitation of *nrd* labeled cells revealed that the difference in cell number is statistically significant at both timepoints ($p < 0.0001$ and $n \geq 7$). The *foxi1* morphants have approximately 80% fewer *nrd* positive cells at 24hpf and 65% fewer at 30hpf as compared to their un-injected siblings. The pattern and localization of *nrd* expression in *foxi1* morphants was normal despite the decrease in cell number (Fig. 8).

The effects of *foxi1* on sensory development

Previous studies have shown that loss of *foxi1* causes a severe decrease in the expression of *atoh1b*. *Atoh1b* expression is important for sensory development and can be used as an indicator of how much sensory epithelium has formed (Millimaki *et al.* 2007; Hans *et al.* 2013). Thus, loss of *foxi1* is expected to cause deficiencies in sensory cell types. These deficits are examined in more detail below.

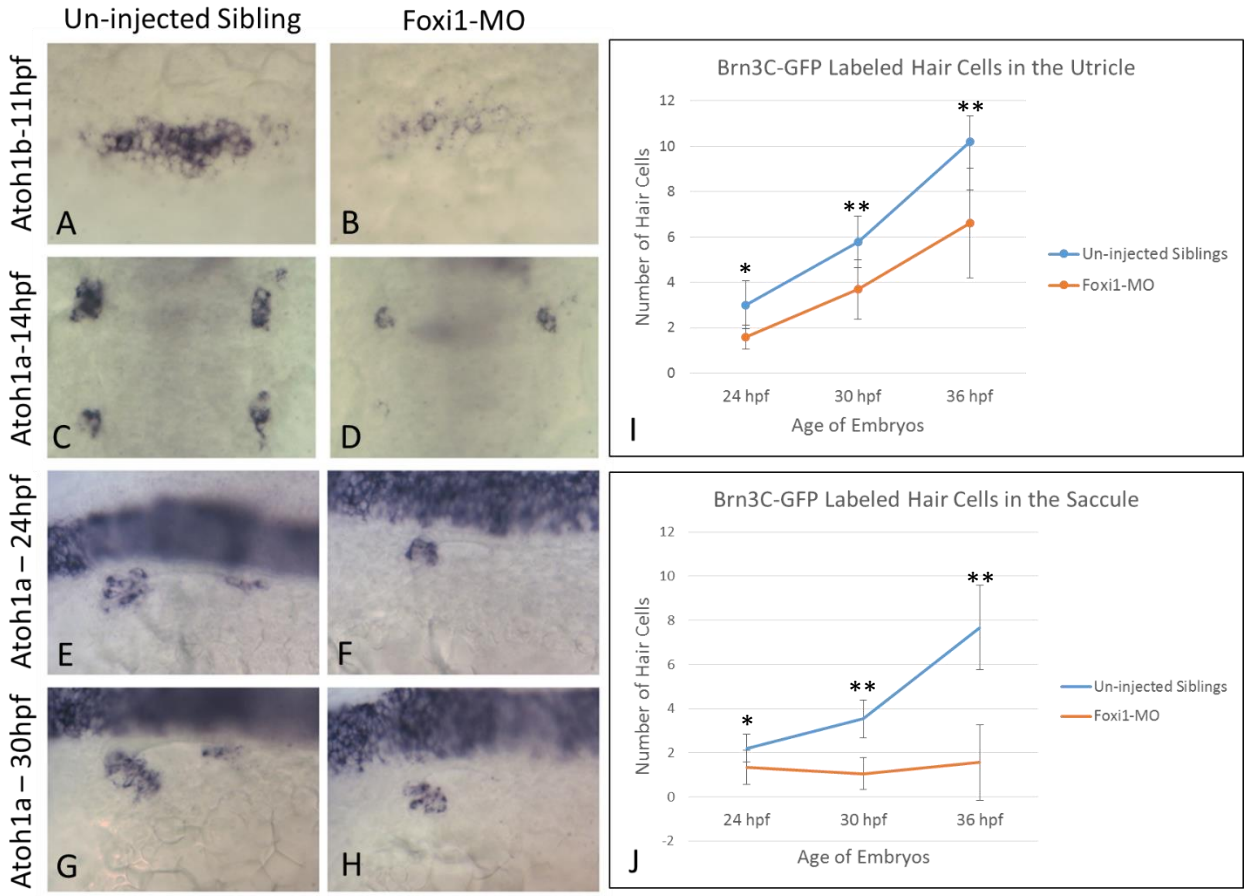

Figure 9: Loss of *foxi1* causes significant deficits in sensory cell types. **A)** Normal expression of *atoh1b* at 11hpf. **B)** *Foxi1* morphants show a drastic decrease in *atoh1b* expression. **C)** Normal expression of *atoh1a* at 14hpf is separated into two patches in each placode. **D)** In *foxi1* morphants the anterior patch of *atoh1a* is decreased and the posterior patch is either decreased or

missing. **E, G)** Normal *atoh1a* expression is divided into two domains at 24hpf and 30hpf. **F, H)** In *foxi1* morphants the anterior patch of *atoh1a* expression is reduced and the posterior patch is either severely reduced or missing at both 24hpf and 30hpf. **I, J)** Line graphs showing the number of hair cells present in the utricle and saccule over time as counted by Brn3c:GFP labeling. * indicates $p < 0.01$, ** indicates $p < 0.0001$.

As expected, a significant deficit in sensory cell formation was observed in the *foxi1* morphants. This deficit could be observed very early in development, as shown by *atoh1b* expression at 11hpf. As development proceeds, the pro-sensory domain splits into one anterior patch and one posterior patch. These correspond to the utricle and saccule, respectively. *Atoh1a* expression at 14hpf, 24hpf, and 30hpf shows significant deficits in the morphants. The utricle shows decreased expression and a smaller expression domain. The saccule shows more severe deficits, either showing severely decreased expression or a complete loss of expression. These deficits persist at least until 36 hpf (Fig. 9).

Quantitation was performed using the *Brn3c:GFP* transgenic line which expresses green fluorescent protein in hair cells and allows those cells to be easily counted. Upon quantitation, significant decreases in hair cell number were observed at every timepoint in both the utricle and saccule in *foxi1* morphants. A normal embryo forms 2 hair cells called tether cells in both the utricle and saccule by 24hpf. In the utricle, *foxi1* morphants formed 1.58 ± 0.51 tether cells by 24hpf. This decrease in cell number was statistically significant ($p < 0.01$ and $n \geq 12$). Statistically significant deficits persisted through the 30 hpf and 36 hpf timepoint ($p < 0.0001$ and $n \geq 15$ for both). Within the saccule, the phenotype was more variable and the deficits tended to be more severe. Most morphant embryos did form at least one tether cell by 24hpf, however tether cells were missing in some embryos. The decrease in cell number observed at this

timepoint was also statistically significant ($p < 0.01$ and $n \geq 12$). Again, statistically significant deficits persisted until at least 36 hpf ($p < 0.0001$ and $n \geq 15$) (Fig. 9).

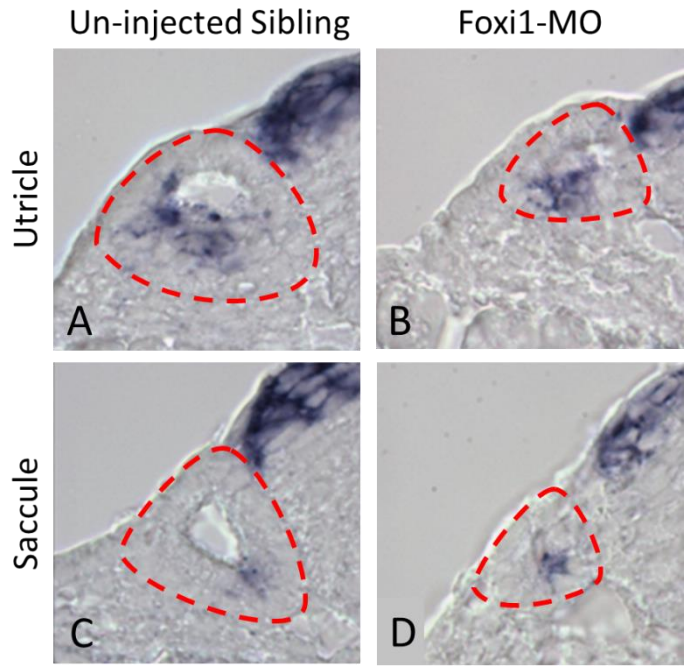

Figure 10: *Atoh1a* expression pattern is normal in *foxi1* morphants. All sections are of 24hpf embryos and show the otic vesicle outlined in red. A) The utricle is located towards the lateral side of the otic floor as marked by *atoh1a* expression. B) The utricle forms in the proper location in *foxi1* morphants. C) The saccule is located on the medial side of the otic floor, further to the posterior part of the otic vesicle. D) The saccule forms in the correct location when it is present in *foxi1* morphants.

Despite the drastic decrease in the number of hair cells present in *foxi1* morphants, the patterning of the otic vesicle is normal. The utricle forms towards the anterior on the floor of the otic vesicle in both *foxi1* morphants and their un-injected siblings, as seen by *atoh1a* expression. The saccule normally forms towards the posterior of the otic vesicle on the medial side of the otic

floor. When a saccule is present in *foxi1* morphants, it is in the correct location within the vesicle (Fig. 10).

CHAPTER IV

CONCLUSION

Sox3 likely functions to allow proliferative expansion in the early otic placode

In early otic development *sox3* is expressed at high levels within the otic placode. Later on it is downregulated within the placode and upregulated lateral to it; however, even at this stage a low level of *sox3* expression is maintained within the otic domain (Padanand *et al.* 2011). Here we have shown that *sox3* is capable of inhibiting differentiation if it is expressed at high levels (Fig. 4). In a normal embryo, the high levels of expression during early development may function to prevent the ear from differentiating too early. This gives time for the otic cells to divide and allows the placode to reach the proper size. In *sox3* mutants, the size of the otic placode is somewhat reduced (Fig. 3). This could be explained by the placodal cells differentiating too quickly, and consequently undergoing fewer cell divisions.

Sox2 is also capable of inhibiting otic differentiation when expressed at high levels during early stages (Fig. 4). However, loss of *sox2* does not cause a significant decrease in the size of the otic placode. This is likely because *sox2* is not expressed at high levels in the otic placode until 14hpf, after most of the processes which specify placode size have occurred. Since *sox2* is not expressed at high levels during early stages, it is probably not responsible for proliferative expansion of the placode under normal circumstances.

Later in development, neither *sox* gene can reverse otic differentiation (Fig. 5). This is expected because at this stage the placodal cells are much more strongly committed to an otic fate. It is

also consistent with previous studies showing that *sox2* and *sox3* will aid in specifying sensory and neural cell types, respectively (Dee *et al.* 2008; Sweet *et al*. 2011). Possible co-factors which influence the specification of sensory vs. neural cell fate are currently under investigation. Thus, early in development *sox3* functions to inhibit differentiation of the otic placode while later both *sox* genes in concert with other factors promote the specification of different cell types.

Foxi1 is upstream of both _sox2_ and _sox3_ in genetic signaling pathways

Previous studies have shown that expression of *sox3* in the otic placode and surrounding lateral regions requires *foxi1* activity (Sun *et al.* 2007). Thus, *foxi1* morphants should not have any *sox3* expression outside of the neural tube. However, in this experiment *sox3* is still expressed at low levels following *foxi1*-MO injection (Fig. 7). Therefore the knockdown of *foxi1* in this study is incomplete, meaning that some *foxi1* gene activity is still present in the injected embryos.Thus the phenotypes observed here may be less severe than in the *foxi1* mutants. However, in most regards the phenotypes appear very similar.

The observed reduction in the domain and expression level of *sox2* is consistent with previously published data showing that the size of the sensory domain is reduced when *foxi1* is absent (Millimaki *et al.* 2007; Hans *et al.* 2013). The data obtained here do not distinguish whether or not *foxi1* has direct activity on *sox2*. Previous studies have shown that loss of *foxi1* reduces the size of the sensory domain by causing a decrease in the amount of the *atoh1b* and *atoh1a* (Millimaki *et al.* 2007; Hans *et al.* 2013). This reduction in *atoh1* was replicated here and is the more likely explanation for the decrease in *sox2* expression.

Knockdown of *foxi1* causes reduction but not loss of sensory and neural domains

The size of the sensory and neural domains, as well as the numbers of sensory and neural cells show significant decreases in *foxi1* morphants. However, each cell type was still present and the patterning of the sensory and neural domains was normal in the morphants (Fig. 8-10). Further, loss of *foxi1* did not appear to affect delamination or migration of neuroblasts. Neuroblasts continue to express *ngn1* for a short time after they delaminate. These recently delaminated neuroblasts were observed in both the morphants and their un-injected siblings indicating that loss of *foxi1* does not prevent delamination. Similarly, the localization of the delaminated neuroblasts was correct. This indicates that the neuroblasts are capable of migrating to the correct location, albeit in reduced numbers. Therefore, these data support that loss of *foxi1* primarily decreases the number of neuroblasts initially specified on the floor of the ear.

The size of the sensory domain was also reduced. In many cases only one sensory patch, corresponding to the presumptive utricle, was formed. Whether or not the saccule formed was strongly correlated with the size of the otic vesicle. The smaller the vesicle, the less likely it was for the saccule to form. This is consistent with previously published data (Hans *et al.* 2013). Hair cells within the morphant utricle appeared to divide at a normal rate. However, in many cases the morphant saccule either failed to form at all by 36 hpf or only formed tether cells. In some cases hair cell division did occur in the *foxi1* morphant saccule but these occurred more slowly than in the un-injected siblings.

Future directions

We will continue to investigate how *sox2* and *sox3* fit into the genetic regulatory networks governing early otic development and sensory-neural patterning. The experiments involving *foxi1*-MO will be repeated in order to obtain a full knockdown of *foxi1* function. Also, a similar set of experiments will be performed examining the results of *dlx3b* and *dlx4b* loss of function in order to further examine how *sox2* and *sox3* fit into this genetic regulatory pathway. Finally, *sox2* and *sox3* mutants will be further analyzed for other placodal markers in order to more fully characterize these genes' role in early otic development.

REFERENCES

Bylund, M., Andersson, E., Novitch, B.G., and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. Nature neuroscience 6, 1162-1168.

Dee, C.T., Hirst, C.S., Shih, Y.H., Tripathi, V.B., Patient, R.K., and Scotting, P.J. (2008). Sox3 regulates both neural fate and differentiation in the zebrafish ectoderm. Developmental biology 320, 289-301.

Hans, S., Irmscher, A., and Brand, M. (2013). Zebrafish Foxi1 provides a neuronal ground state during inner ear induction preceding the Dlx3b/4b-regulated sensory lineage. Development 140, 1936-1945.

Hans, S., Liu, D., and Westerfield, M. (2004). Pax8 and Pax2a function synergistically in otic specification, downstream of the Foxi1 and Dlx3b transcription factors. Development 131, 5091-5102.

Kantarci, H., Edlund, R. K., Groves, A. K., & Riley, B. B. (2015). Tfap2a promotes specification and maturation of neurons in the inner ear through modulation of Bmp, Fgf and notch signaling. PLoS Genetics, 11(3), e1005037. doi: 10.1371/journal.pgen.1005037

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 203, 253-310.

Kok, F.O., Shin, M., Ni, C.W., Gupta, A., Grosse, A.S., van Impel, A., Kirchmaier, B.C., Peterson-Maduro, J., Kourkoulis, G., Male, I., et al. (2015). Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. Developmental cell 32, 97-108.

Liu, D., Chu, H., Maves, L., Yan, Y.L., Morcos, P.A., Postlethwait, J.H., and Westerfield, M. (2003). Fgf3 and Fgf8 dependent and independent transcription factors are required for otic placode specification. Development 130, 2213-2224.

Millimaki, B.B., Sweet, E.M., and Riley, B.B. (2010). Sox2 is required for maintenance and regeneration, but not initial development, of hair cells in the zebrafish inner ear. Developmental biology 338, 262-269.

Millimaki, B.B., Sweet, E.M., Dhason, M.S., and Riley, B.B. (2007). Zebrafish atoh1 genes: classic proneural activity in the inner ear and regulation by Fgf and Notch. *Development, 134*(2), 295-305.

Nasevicius, A., and Ekker, S.C. (2000). Effective targeted gene 'knockdown' in zebrafish. Nature genetics 26, 216-220.

Okuda, Y., Ogura, E., Kondoh, H., and Kamachi, Y. (2010). B1 SOX coordinate cell specification with patterning and morphogenesis in the early zebrafish embryo. PLoS genetics 6, e1000936.

Ozeki, H., Oshima, K., Senn, P., Kurihara, H., and Kaga, K. (2007). Development and regeneration of hair cells. Acta oto-laryngologica Supplementum, 38-44.

Padanad, M.S., and Riley, B.B. (2011). Pax2/8 proteins coordinate sequential induction of otic and epibranchial placodes through differential regulation of foxi1, sox3 and fgf24. Developmental biology 351, 90-98.

Phillips, B.T., Bolding, K., and Riley, B.B. (2001). Zebrafish fgf3 and fgf8 encode redundant functions required for otic placode induction. Developmental biology 235, 351-365.

Robu, M.E., Larson, J.D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A., and Ekker, S.C. (2007). p53 activation by knockdown technologies. PLoS genetics 3, e78.

Shruti Vemaraju, H.K., Mahesh S. Padanad, Bruce B. Riley (2012). A Spatial and Temporal Gradient of Fgf Differentially Regulates Distinct Stages of Neural Development in the Zebrafish Inner Ear. PLOS Genetics 8, e1003068.

Solomon, K.S., Kudoh, T., Dawid, I.B., and Fritz, A. (2003). Zebrafish foxi1 mediates otic placode formation and jaw development. Development 130, 929-940.

Solomon, K.S., Kwak, S.J., and Fritz, A. (2004). Genetic interactions underlying otic placode induction and formation. Developmental dynamics : an official publication of the American Association of Anatomists 230, 419-433.

Sun, S.-K., Dee, C.T., Tripathi, V.B., Rengifo, A., Hirst, C.S., and Scotting, P.J. (2007). Epibranchial and otic placodes are induced by a common Fgf signal, but their subsequent development is independent. Dev Biol, 303(2), 675-686.

Sweet, E.M., Vemaraju, S., and Riley, B.B. (2011). Sox2 and Fgf interact with Atoh1 to promote sensory competence throughout the zebrafish inner ear. Developmental biology 358, 113-121.

Vemaraju, S., Kantarci, H., Padanad, M.S., and Riley, B.B. (2012). A spatial and temporal gradient of Fgf differentially regulates distinct stages of neural development in the zebrafish inner ear. PLoS Genet 8, e1003068.

Whitfield, T.T., Riley, B.B., Chiang, M.Y., and Phillips, B. (2002). Development of the zebrafish inner ear. Developmental dynamics : an official publication of the American Association of Anatomists 223, 427-458.

Xiao, T., Roeser, T., Staub, W., and Baier, H. (2005). A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. *Development, 132*(13), 2955-2967.