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Genetic Control of Inner Ear Development in Xenopus Laevis

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GENETIC CONTROL OF INNER EAR DEVELOPMENT IN XENOPUS LAEVIS

by

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Abstract:

Inner ear functions (such as hearing and balance) rely on a highly-ordered arrangement of mechanosensory hair cells. To reconstitute hair cells using defined treatments, more needs to be known about what controls their development. *Pax2 and Pax8* are transcription factors known to regulate vertebrate organogenesis, including that of the inner ear. Depletion of *Pax2* and *Pax8* (separately or in combination) has been researched in mice and zebrafish. The results show a range of vestibular and cochlear defects. To understand more, we have characterized depletion of *Pax2*/*Pax8* genes in frogs (*Xenopus laevis*), which have been microinjected with antisense morpholino oligos (MOs) against *Pax2* and *Pax8* at the 8-cell stage. We have found that depletion of these gene products, in combination, can block formation of an otic vesicle and surrounding sensory neural pathways unilaterally in frogs. Our results suggest that expression of *Pax2*/*Pax8* genes are essential for proper ear morphogenesis. Furthermore, we found that this depletion results in a reduction of *Sox9* expression from the early embryo. With this information, a combination of transcription factors necessary for hair cell development is closer to being determined.

Introduction:

In vertebrates, the inner ear is the organ responsible for hearing and vestibular function (balance and angular acceleration). The functional units of the inner ear are mechanosensory hair cells. When endolymphatic fluid pushes against hair cells, signals are transduced and propagated to the central nervous system.

Development of the ear begins with formation of the otic placode, a thickening of the outer epithelium, which invaginates, pinching off, to form the otic vesicle. The otic vesicle develops into the inner ear through a process of organized signaling pathways, transcription factors, transmembrane proteins, and extracellular matrix components.[1] A key factor in differentiating

the otic placode from other developing placodes is the expression of *Pax2* and *Pax8* transcription factors.[2] Past studies have shown that *Pax 2,5,* and *8* genes are vertebrae orthologs.[3] Along with ear development, *Pax 2,5, and 8* contribute to formation of brain, kidney, eyes, and other organs in vertebrates.[2] *Pax8* is one of the earliest genes activated in the otic placode of frogs, zebrafish, mice, and humans. In later stages, *Pax8* is believed to be co-expressed with *Pax2.*[1] Multiple studies in *Pax2* null mice suggest that expression of *Pax2* is necessary for cochlear development, but range its effects on other inner ear structures including mechanosensory hair cells.[4,5] Because the cochlea evolved in mammals,[6] the expression of *Pax2* in zebrafish indicates the conserved nature of the gene.[1] Instead, in *Pax8* null mice, no defects in sensorineural development or inner ear formation were detected. Further studies show that in combination *Pax2*/*Pax8* null mice have reduced otic vesicle size (in comparison to wild type) and are unable to sustain neurosensory function. [2] The objective of these experiments is to determine the necessary factors for proper inner ear development. By using antisense morpholino oligos (MOs), expression of specific genes (such as *Pax2* and *Pax8*) can be inhibited on a translational level. As a result, the phenotypic effects provide insight into what roles each gene plays in inner ear morphogenesis. This knowledge helps to create a set of defined transcription factors that could induce hair cell development from stem cells.

Frogs *(Xenopus laevis)* were chosen as the model organism because of their highly conserved embryonic development. Frog embryogenesis occurs quickly (relative to mice and humans) and has predictable cellular divisions that allow for accurate and detailed fate maps.[7]

Materials and Methods:

Xenopus embryo preparation

Female frogs were injected with human chorionic gonadotropin (hCG 1000 U; Sigma) to stimulate ovulation. Their eggs were fertilized *in vitro* using a sperm suspension and cultured in 0.1XMMR (1XMMR:0.1 M NaCl, 1.8 mM KCL, 2.0mM $CaCl_2$, 1.0 mM $MgCl_2$, 15.0mM HEPES,

pH 7.6). After the second cleavage, the jelly coat was removed using cysteine (2% in 0.1 x MMR).[8]

Embryo microinjections

Antisense morpholino oligonucleotides were injected into eight-cell stage embryos in Ficoll (2% Ficoll in 0.5 x MMR). The dorsal and ventral cells on one side (future right side) of the animal pole were injected with MO doses totaling: 5ng, 10ng, 15ng, or 20ng (one-half dose per blastomere). Antisense MOs complementary to *Pax2* and *Pax8* were purchased from Gene Tools: MoPax2 5′-GGTCTGCCTTGCAGTGCATATCCAT-3′ and MoPax8 5′-CAGAGCAGCTCCTTGTAGCCAATGT-3′. X. laevis L and X. laevis S are frog homeologs who's sequences are complementary. The complementary sequence for the injected *Pax 2* is MoPax2.2 5′-TCTGCCTTGCAGTGCATATCCATG-3′. MoPax8 is complementary to MoPax8.B 5′-CTAGTCCCTGGTAAATCCACAGTGC-3′.[9] Embryos were raised until stage 42, when the otic vesicle is fully developed in wild type tadpoles.[10] They were then anesthetized using MS-222 (Ethyl 3-aminobenzoate methanesulfonate salt 1g/L), and imaged *in vivo*.

Immunofluorescence

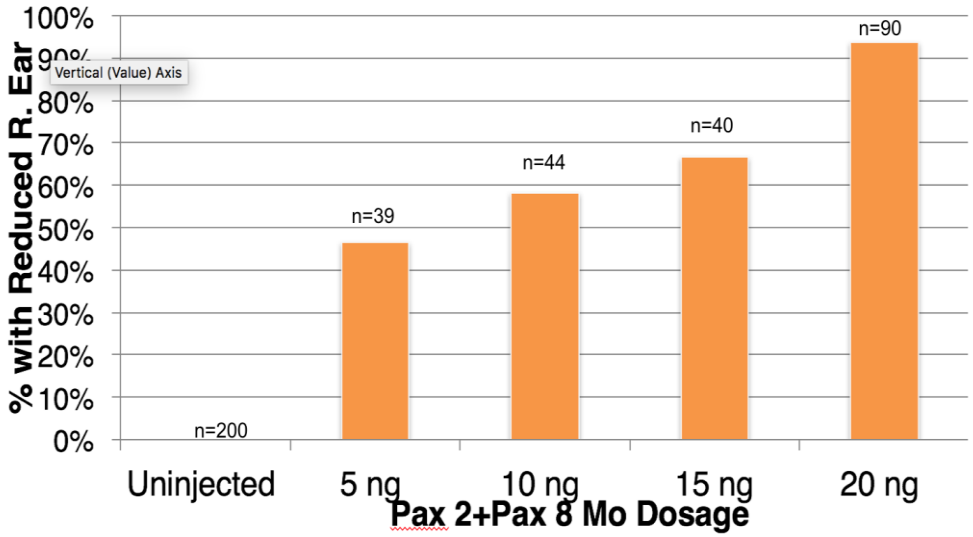
Stage 42 embryos were dehydrated in 70% ethanol overnight and rehydrated and blocked with 0.05% normal goat serum in PBS containing 0.01% Triton-X-100 for 1 hour at room temperature. The embryos were then washed with PBS, and incubated at 4°C for 48 hours in antibodies recognizing Acetylated Tubulin (Cell Signaling Technology) and MyoVI (Proteus Biosciences) in dilutions of 1:800 and 1:400. After incubation, the embryos were washed 3X 10min. in PBS and placed in species-specific secondary antibodies (Alexa), which were diluted in PBS 1:500. 1µL of Hoechst stain was added before overnight incubation in 4°C on shaker.

Stained embryos were washed in PBS (6 X 15 min.), mounted in glycerol, and imaged by Leica TCS SP5 confocal microscope.

Whole mount in situ hybridization

Whole mount in situ hybridization was carried out as described previously (Kerr et al., 2008)[11]. Anti-dioxigenin-AP antibody (Roche Applied Science) concentrated to 1:2500 was used. BM Purple (Roche Applied Science) identified hybridization upon color change. Reaction was stopped with Bouin's fixative then thoroughly washed with 70% ETOH/10mM Tris pH 8.0 before bleaching, as described (Sive et al., 2000).[12] DNA templates for digoxigenin-labeled RNA probes were linearized using restriction enzymes transcribed using compatible polymerases (Promega) as specified[11] to target *Sox9* mRNA (Alissas paper). Final probe concentration was 1 µg/ml diluted into hybridization buffer. Whole embryos imaged.

Figure 1: Percent Tadpoles with Otic Vesicle Defects in Injected Side



Results:

Microinjections of *Pax2*/*Pax8* morpholinos resulted in a range of phenotypic effects localized to the injected (right) side of the tadpole. Overall, resulting defects observed on the injected side include: complete absence of an otic vesicle, decreased otic vesicle size, eye deformation, and curved body. With the most common defect being decreased or absent otic vesicle. Percentage of tadpoles with developmental defects was dose dependent. Tadpoles that showed visible otic

vesicle defects were tallied depending on dose shown in Fig. 1. 93% of tadpoles injected with 20 ng (n=90) had improper otic vesicle development restricted to the injected side.

Uninjected tadpoles (Fig 2. A.) served as a control. At stage 42, control embryos had symmetrical eye placement, ear development (arrows), and head shape. In contrast, most embryos injected with *Pax2*/*Pax8* MO (Fig 2. B.) lack an otic vesicle, utricle, and saccule on the injected side. No visible defects to eye placement, ear development, and head shape were seen on uninjected side in Fig 2. B.

Figure 2: Otic Vesicle Development in Control vs. *Pax2/Pax8* MO

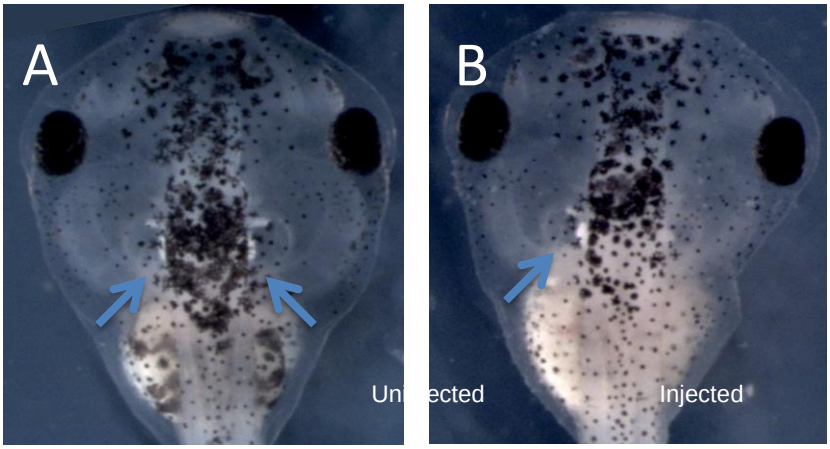
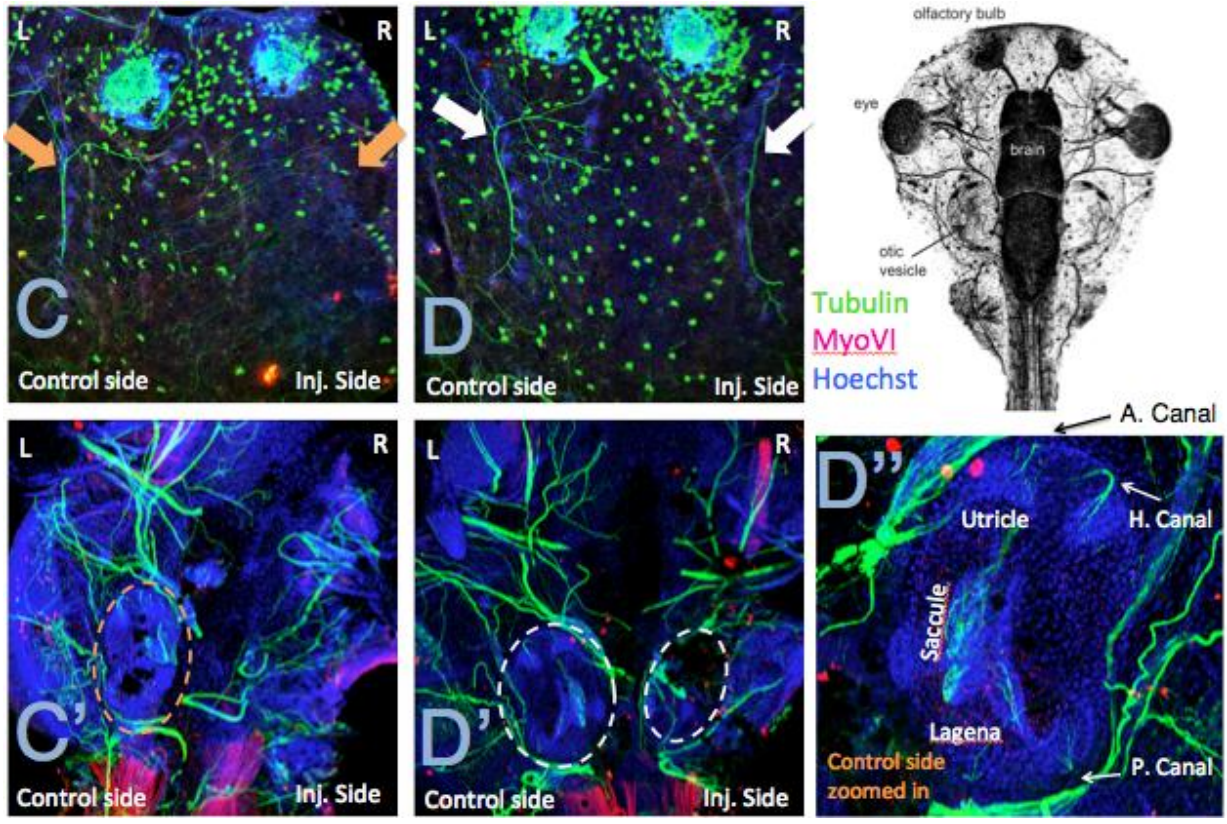
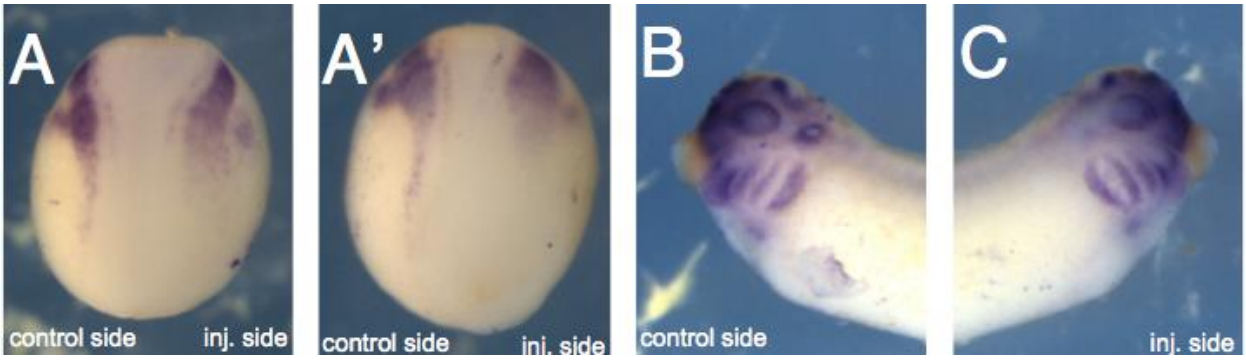


Figure 3: Immunofluorescence of Ear and Associated Neural Networks in Stage 42 Tadpoles



Skins of tadpoles (stage 42; Fig.3 C, D) were stained for tubulin (green), which is enriched in neural cells. Neural development in sides injected with *Pax2/Pax8* MO ranged from decreased (Fig. 3. D.) to nonexistent (Fig. 3. C.), as highlighted in green, while on the control side (Fig. 3. C,D), neural network works developed normally. Intracranial sections were imaged (stage 42; Fig.3 C',D') and show otic vesicles in blue (outlined by circles). On the inj. side otic vesicles were underdeveloped (Fig. 3. D') or not present (Fig. 3. C'). Zoomed in, Fig. 3. D' points out anatomical structures of the inner ear, synonymous to those in humans, on the control (uninjected) side.

Figure 4: Whole Mount In Situ Hybridization of *Sox9* in Injected Embryos



Embryos were fixed at the neural stage (stage 16; Fig.4 A, A') or the tailbud stage (stage 30; B, C). and in situ hybridization was performed against *sox9* mRNA (blue staining) as a marker of the otic vesicle. *Sox9* is reduced on the injected side at both stages (arrows). Dorsal views of two examples are shown in A and A'; left and right sides of the same embryo are shown in B and C, respectively. Overall, these data show

Discussion:

Effects of Pax2 and Pax8 depletion on otic vesicle development

In other model vertebra (mice and chicken), independent knockdown of Pax2 and Pax8 result in a range of otic defects, but are insufficient to completely inhibit otic vesicle formation.[2,5]

We have shown for the first-time phenotypic defects in frogs that result from depletion of *Pax2*/*Pax8* transcription factors. These data suggest that without expression of *Pax2*/*Pax8,* invagination of the otic placode in frogs is blocked. This is characterized by a decrease in otic vesicle size or complete absence of otic vesicle in tadpoles injected with MOs at the early embryo stage. Observed otic defects are in line with previous experiments in zebrafish.[1] In mice, *Pax2*/*Pax8* null mutants form small otic vesicles in comparison to their wildtype counterparts.[2] However, none lack otic vesicles. Pointing to an evolutionary change in the interaction between *Pax2* and *Pax8*, most likely related to cochlear development.

In addition, inner ear structures which develop downstream to otic placode induction, such as the utricle and saccule, are prevented from forming. This is shown in Fig 2. B where the utricle and saccule are absent on the injected side.

Our double knockdown experiment suggests *Pax2*/*Pax8* expression is critical for ear development starting at the otic placode.

***Pax2* and *Pax8* regulate neural network formation**

Previous work has identified alterations in the organization of sensory hair cells and neural pathways in *Pax2/Pax8* null mice.[2] In stage 42 tadpoles with reduced otic vesicles, our tracing of the neural marker tubulin and other immunofluorescent structures were similar to previous descriptions.[1,2] Concerning the skins (Fig.3 C, D), there was a reduction in branching of neurons (green), as well as abnormal neuromast (blue pockets) size and placement along the lateral lines. Specific effects on hair cells are inconclusive due to a flaw in the equipment tracing the hair cell marker MyoVI. But previous studies in zebrafish concur with our hypothesis that quantity of hair cells would be severely depleted on the side of tadpoles injected with *Pax2*/*Pax8* MOs.[5]

Most interesting is the complete knockdown of neural network development seen in tadpoles without otic vesicles. This suggests that construction of neural pathways associated with the inner ear rely on successful thickening, invagination, and pinching off, of the otic placode.

***Pax2*/*Pax8* antisense morpholino oligos can affect embryonic development unilaterally**

In frogs, each cleavage furrow prevents embryonic determinants from mixing between the cells. This allows morpholinos to remain localized, thus only affecting target gene expression in the injected cells. Due to highly detailed fate mapping in frogs, we could determine which cells would eventually become the future otic region.[7]

To evaluate the effects of *Pax2*/*Pax8* depletion on other transcription factors, whole mount *in situ* hybridization was used to target *Sox9* mRNA. The reduction of *Sox9* mRNA in both the neural and tailbud stages (in the otic region), shows that *Sox9* is dependent on upstream events which involve *Pax2*/*Pax8* expression.

Additional phenotypic defects, which include delineated eye, bent body, and deformed kidney, are due to the various regulatory roles *Pax2* and *Pax8* have during embryogenesis.[13] All defects appeared only on injected sides. These results agree with our hypothesis that expression of target genes was only effected in the injected cells.

Conclusion

Through these experiments, we have begun determining a minimal set of transcription factors necessary for ear development. Future research will focus on rescuing *Pax2/Pax8* knockdown frogs by overexpression of related mRNAs. Results will help determine a set of genes, specific to the inner ear, necessary for organogenesis.

In previous experiments, once organ-specific genes (i.e. in the pancreas and kidney) were identified, treatments on animal cap cells induced differentiation of diverse cell types with complex interactions analogous to those of the target organ.[13] This research has established an *in vitro* induction system for 22 different organs and tissues, not including the ear.

In humans, hairs cells are generated in the first trimester of embryonic development, and are not renewed over one's life span. Degeneration of cochlear hair cells leads to permanent hearing loss.[14] Determining an approach for hair cell generation provides insight into cell-based therapies for restoration of sensorineural hearing loss.

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