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Crossing the Border

A novel mouse line using Fgf10-cre ^{ERT2}to drive *Gata3* overexpression in nonsensory transcriptional context of the organ of Corti.

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✵ Abstract:

Hearing loss can occur in a variety of ways, and in every case, they have equally devasting effects. While hearing loss and the people who suffer from it shall not be discriminated against, attempting to offer options for treatments nevertheless should be of priority. As such, there has been much progress in the field of genetically based forms of treatment for sensorineural hearing loss. A gene that has been almost always implicated in the conversation of regeneration, Hair Cell and Spiral Ganglia development, and inner ear development, in general, is the Zing Finger transcription factors*Gata3*. Furthermore, since the Organ of Corti is a complex organization of cells, we found a perfect opportunity to document the effect of *Gata3* in cells of the GER that do not usually express high levels of *Gata3*. Thus, we were interested in documenting the effects of *Gata3* overexpression on the genetic profile of these cells. To do this we decided to overexpress gata3 in the cells of the GER. We utilized the tamoxifen-inducible Cre recombinase transgenic mouse line *Fgf10*-Cre^{ERT2} to characterize *Ffg10* expression in the GER (*Fgf10*-Cre^{ERT2}*:tdTomato*), to then have certainty in our method of inducing overexpression of Gata3 (*Fgf10*-Cre^{ERT2}*:Gata3^{OE})* in the cells of the GER. In doing this, we found no significant evidence that Gata3 overexpression causes any drastic morphologic or cell fate changes in the OC. This grants us some confidence. That if Gata3 ever be used as a treatment option in the future; for either sensorineural hearing loss treatment, HDR (Gata3 haploinsufficiency), or preemptive protection against expected otic trauma, it should not create any abnormal changes in the OC.

✵ Introduction:

- **Hearing loss and the layout.**

Sound perception is for many people, an extremely intricate piece of a subject's experience of the world around them. Hearing is associated with the pinnae, the ear canal, and the eardrum as a regular visit to the ear, nose, and throat doctor might have already revealed; however, the full story behind hearing is far more complex than any little look in the mirror should reveal. Loss of hearing, of any kind, is an extremely common affliction in the world, however, due to the complexity and the variety of different ways hearing loss can occur, in combination with the familiarity bred ignorance we have over this sense, hearing loss and hearing disorders have long been overlooked. In the United States, 15% of the population over eighteen years of age indicate some form of hearing impairment, while 13% percent of the population indicates hearing loss in both ears (Lin et al., 2011; Blackwell & Lucas, 2012). Sources of hearing loss include congenital hearing loss, which is a result of a genetic disorder, ototoxic drugs, often due to the overuse of antibiotics or use of chemotherapeutic drugs, such as cisplatin, noise-induced trauma, and age-related hearing loss (Prell et al., 2019; Hodge et al., 2021). As a result of the multitude of ways one can lose their hearing, treatment option

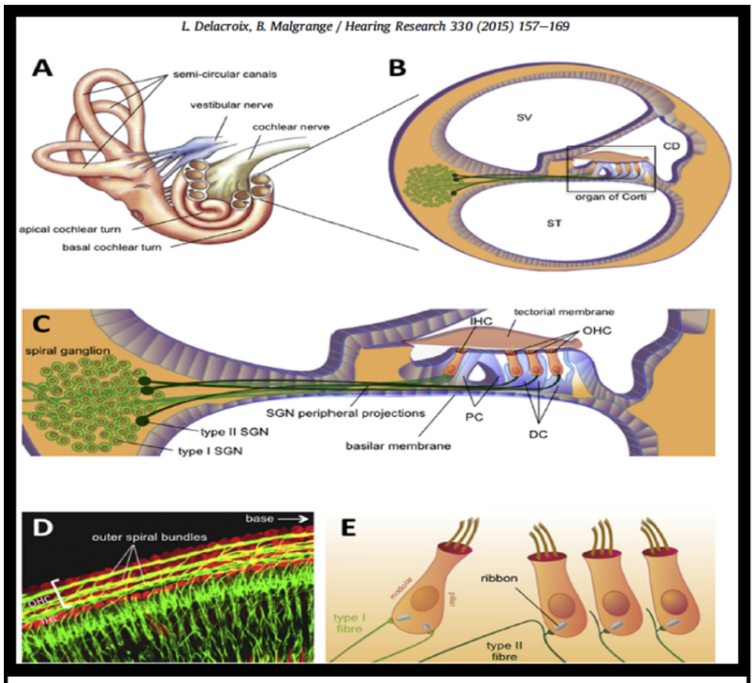

Figure (1) adapted from (Delacroix and Melgrange 2015). (A) Whole cochlea innervated by both vestibular and cochlear ganglion neurons. (B) Cross section of Cochlea, (SV) Scala Vestibuli, (ST) Scala Tympani, (OC) organ of Corti in square panel zoomed in on next panel. (C) cross section of OC with innervating spiral Ganglion neurons projecting towards the IHC and OHC, (PC) Pillar Cells. (D) Immunohistochemistry of projecting neurons (green fibers) and HC (red dots), type IIs in yellow. (E) IHC and OHC innervated by Type I and Type II neurons respectively.

development has been slow, and insufficient in many cases. Apparatuses, such as hearing aids and cochlear implants are some of the most recognizable. Reasons for the inefficiency of these methods can range from the necessity of the presence of the same sensory cells which are already damaged, or because they are simply unpleasant to wear and use.

Sound transduction is a multifaced process, with various checkpoints which allow for a variety of complications to occur. Hearing begins once sound waves are funneled into, essentially captured, by the auricle. The ear canal then allows the sound waves to travel and vibrate the tympanic membrane. Once the tympanic membrane is struck, it causes the vibration of the attached ossicles (from malleus to incus, to stapes), in the middle ear, which faithfully vibrates at the frequency of the sound waves that entered the ear canal. Transduction continues once these sound waves are transduced from the ossicles to the inner ear, by vibrating the oval window, which touches the sound transducing organ, the cochlea.

The inner ear and its labyrinth anatomy consist of six sensory organs that should be familiar to anyone investigating the functional anatomy of the organ. The detection of gravity, balance, linear and angular acceleration, and of course, sound, rely on the relationships between populations of ectoderm-derived cells that interact within the inner ear. There are six organs responsible for the perception of the five senses stated above and are distributed amongst two sensory domains, cochlear and vestibular. The first three vestibular organs are the three semicircular canals and their respective cristae organized in an orthogonal axis to each other, allowing for the perception of angular acceleration and balance. Next, are the other two vestibular organs responsible for the perception of gravity and linear acceleration, the saccule, and utricle. Finally, the cochlea exerts its role as the complex organ responsible for auditory function.

The cochlea is composed of three major delineated spaces: the Scala vestibuli, Scala tympani, and the cochlear duct. The oval window, once vibrated, moves the fluid termed perilymph in the Scala vestibuli back and forth, maintaining the initial frequency. This fluid is then able to vibrate a crucial structure of the cochlear duct called the basilar membrane. This structure is of great importance because it is able to selectively vibrate in response to different frequencies, which is termed mechanical tunning. This then creates a frequency gradient along the length of the cochlea. Higher frequencies, perceived as higher pitch, travel the smallest distance, due to the impedance of the fluid in the cochlea, and the basilar membrane vibrates to these frequencies at the basal region (closest to the oval window). Lower frequencies, perceived as lower pitch,
have longer wavelengths that travel further, and as such the basilar mirbane vibrates with selectivity to these frequencies in the apex of the cochlea. This allows for the concept of a spatial-frequency colocalization along the length of the cochlea, which allows for selective sensorineural transduction towards the central nervous system (CNS), otherwise known as the tonotopic map.

The perception of sound is attributed to the function of two crucial sensory cell populations, hair cells (HC) and spiral ganglion neurons (SGN). Within the cochlear duct (CD) between the greater epithelial ridge (GER) and the lesser epithelial ridge (LER), we can find a patch of tissue where the mechanosensory cells are found (Fig. 1) (Ohyama et al., 2010). The organ of Corti (OC), is home to several types of specific cells, which house two main populations: supporting cells (SC) and HCs (Kelly et al., 2009, Groves and Fekete 2012). The OC is the specialized epithelia in the CD and contains three rows of outer hair cells (OHC) and one row of inner hair cells (IHC) (Fig. 1) (Dabdoub et al., 2008; Basch et al., 2016). The IHCs are the cells that get directly stimulated by the moving fluid in the CD caused by the vibrating tectorial membrane, which is connected to the basilar membrane. On the other hand, OHCs offer a modulatory and tunning function by contracting and increasing IHC stimulation or protecting the same from excessively loud sounds (Puligilla et al ., 2010).

This population is followed by its opposing neuronal population, which innervates and allows for the final step in auditory transduction for central processing. The specific pattern of innervation in the OC is of utmost importance. The IHCs and OHCs respectively have their own specific innervating populations of SGNs. The two main types of SGNs are: Type I SGNs, which account for 95% of SGNs, and Type II SGNs, which account for 5% of SGNs (Benoudiba et al., 2013; Nishimura et al., 2016). Extending SGNs obey the border exertion set by the OC and the two nonsensory regions. Type I SGNs solely innervate IHCs along the more medial region of the OC, presenting multiple points of synapsing on these cells. Type IIs, on the other hand, innervate OHCs and extend past the tunnel of Corti, making a right “base oriented” turn to innervate and synapse with multiple OHCs along the three rows (Fig. 1). The multiple synapsing abilities of Type II SGNs further their role as a modulatory pathway for sound perception, and “loudness” (Fritzsch et al ., 2010; Weisz et al., 2009). The SGNs innervate the cochlea (Liu et al., 2000; Ma et al., 1998), and along with the vestibular neurons, will coalesce into the VIIIth cranial nerve, which then extends to the hindbrain (Bouchard et al., 2012). Upon reaching the hindbrain, the vestibular afferents project to the vestibular nucleus in the hindbrain, while cochlear afferents project towards the cochlear nucleus. Within the cochlear nucleus, the tonotopic organization, base to apex pattern, is preserved in multiple subdivisions, which offers our perception of frequency discrimination (Pickels 2015; Koundakjian et al., 2007). These physiological variations between HCs and SGNs, their organization, and the particular molecular interactions they have, are the microscopic tools that enable the auditory abilities of the inner ear.

- **Early otic development and crucial genes and signaling cascades.**

FGF signaling in general can work one of two ways, it can operate through the general receptor tyrosine kinase (RTK), or through the JAK-STAT pathway. FGF10, which is part of the keratinocyte growth factor superfamily or the FGF7

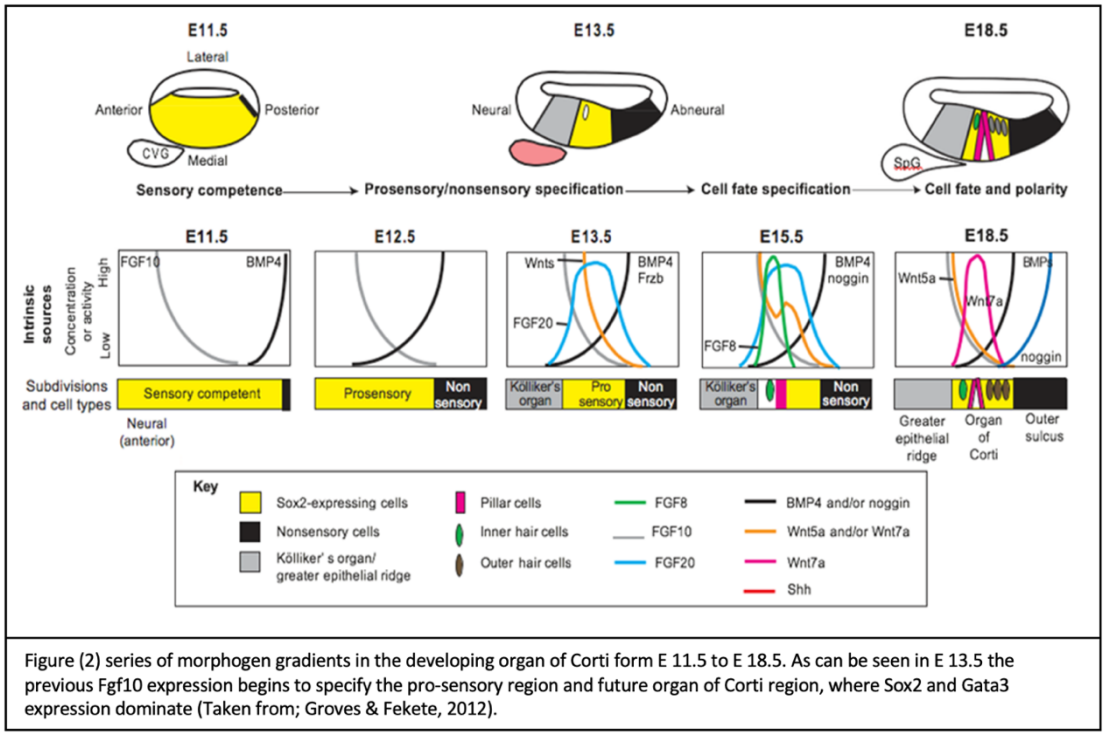

Figure (2) series of morphogen gradients in the developing organ of Corti form E 11.5 to E 18.5. As can be seen in E 13.5 the previous Fgf10 expression begins to specify the pro-sensory region and future organ of Corti region, where Sox2 and Gata3 expression dominate (Taken from; Groves & Fekete, 2012).

superfamily, all function through the RTK pathway. The RTK pathway functions primarily by allowing dimerization of two tyrosine-containing transmembrane proteins that will lead to phosphorylation of GAP proteins, which then proceed to activate the Ras G protein, which through binding to GTP allows for activation of proceeding downstream messengers. These second messengers eventually reach transcription factors within the nucleus that upon phosphorylation allow for the transcription of many target genes.

Inner ear development begins like every other sensory organ. Morphogen gradients that are generated in early gastrulation determine pre-placodal regions along the ectoderm, thus leading to specification of future sensory organs. After this pre-placodal sensory competent tissue had become committed to developing into its respective sensory tissue, specific genetic profiles begin to be expressed. In the case of the inner ear, many signaling pathways have been identified as being crucial for its initial development, and two target genes, *Fgf10* and *Gata3*, are expressed early on and continue to have an effect throughout development at different stages. One of the earliest signals of otic placode development are the precursor genes, *Fgf3* and *Fgf10* as they are expressed in the otic placode, just preceding otic invagination around E 7.5 (Wright & Mansour, 2003). Analysis of FGF signaling is difficult primarily due to the redundancy mentioned above. *Fgf2*, while it known to induce primordial otic fate genes, such as *Pax2*, is however not capable of promoting complete otic development, which demonstrated that a combination of FGFs signaling in a time-specific fashion is necessary (Martin & Groves 2006). As such, it has been postulated that the observed early *Fgf3* and *Fgf10* are possible inducers of the otic invagination process, as individual knockout of both of these yields' faulty otic development (Ohyama et al., 2007). FGF signaling is also heavily involved in the axiation process of the otic cup and signaling arrives from the hindbrain region adjacent to the developing Stato Acoustic Ganglion (SAG) in chicks (Schneider-Maunoury & Pujades, 2007).

Another crucial gene and one that could possibly be involved in the cascade of FGF signaling in the pre-placodal and cranial placodes is *Gata3*. The zinc finger domain transcription factor GATA3 is encoded by the *Gata3* gene, and for its purposes in the inner ear, its splicing pattern seems to be hinging on the fifth exon cleaved from the pre-mRNA. *Gata3* is expressed in a variety of tissues, however, it is very well known for its effects in renal development and immune cell differentiation, however it is also crucially involved in otic development. *Gata3* expression is observed early on in otic development and is visible throughout the otic vesicle after invagination in the earliest time points such as E8.5 and E9.5 (Luo et al., 2013). It has been previously shown, in accordance with this heavy expression, that *Gata3* is crucial for the proper development of the inner ear, and at different times it performs different functions throughout otic development. *Gata3*, along with *Sox2*, is one of the earliest sensory epithelia specifying genes, and they are both present at the earliest stages of invagination. While *Sox2* seems to have, a role in early stages of proliferative, *Gata3* is not so simple. From an early stage, *Gata3* has patterns of expression that appear to prime future sensory epithelia regions and has restricted expression just two days after invagination E10.5 (Karis et al., 2001).

The general trend that is observed in the earlier days of otocyst invagination and general domain specification is mainly spatiotemporal. Probably because of the severe redundancy that FGF signaling has in early development, and the incredible number of genes that are controlled by GATA3 it is hard to pin what is the specific connection between these two, most likely because there is not just one. Because of this parallel expression, the relationship between *Gata3* and *Fgf10*, and their respective phenotypes in null models have revealed extensive thinking as to why look further into this relationship at later and more specific stages.

- **FGF10 and GATA3 role in Sensory epithelia and organ of Corti development.**

A crucial characteristic of FGF signaling in the OC is in its specification of sensory and nonsensory cells in the prosensory region, a precursor area of competency for OC development. Specifically, FGF signaling is heavily involved in OC patterning by being presented in gradients. *Fgf20* is seen to be

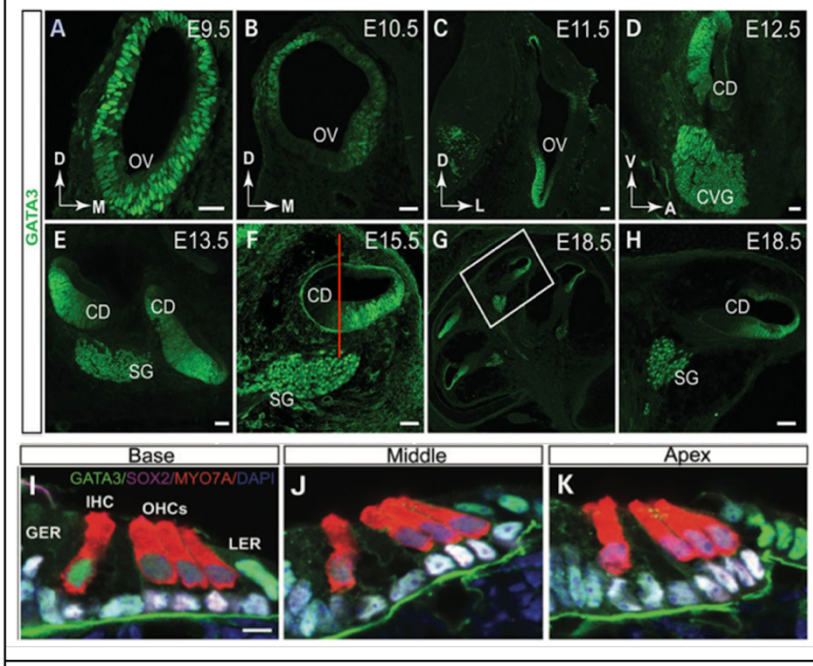

Figure (3) depicts the expression pattern of GATA3 (as its protein presence) in the developing otocyst and subsequent organ of Corti. In panel (B) the first indicates of Gata3 polarization is seen, FGF10 expression follows suit (not shown). Panel (F) demonstrates where the border imposition role of GATA3 begins. Panels (I, J, K) demonstrate GATA3's presence in HC, borders, and not of surrounding nonsensory regions (Taken form; Luo et al., 2013).

expressed in very high levels most specifically in the putative prosensory epithelia around E12 day of otic development, just after *Sox2* expression and development and proliferation of the sensory precursor cells (Martin & Groves 2006; Kiernan et al., 2005; Munnamalai & Fekete 2016) (Fig. 2). Furthermore, *Fgf10* is also involved in specifying two crucial regions of the OC and the morphology of the cochlear duct as well. *Fgf10* is known to influence *Bmp4* expression in the LER, as its expression is seen to be necessary for the specification of the *Bmp4* competent region (Ohyama et al., 2010; Urness et al., 2015). *Fgf10* is also necessary for the development of the Reisner's membrane and the general morphology of the vestibular system (Urness et al., 2015). However, in the cochlea, its strongest expression profile is apparent in the GER, in opposition to the OC on the neural side, where it remains until postnatal days.

Gata3 is one of the primary genes seen to be expressed in the OC, and the neuronal progenitor of the pre-delaminated cochlear vestibular ganglion (CVG) (Karis et al., 2001; Lawoko-Keralli et al., 2003) (Fig. 3). However, its role in OC function is most crucially seen in its apparent role in determining where sensory epithelia will develop. GATA3 staining demonstrates that its protein is most strongly seen from E12 until E14 to be restricted to the prosensory and future OC region, which is the period when the HC and SC progenitor populations are begin specified, identified by SOX2, EYA1/SIX1 expression in the same region (Luo et al., 2013, Kiernan et al., 2005, Ahmed et al., 2011). Aside from this apparent role in progenitor delineation, *Gata3* also appears to be a regulator of HC differentiation and monitoring which cells can become HCs and will mature into functional ones. There has been a significant investigation in the very tight regulation and specification between IHCs, phalangeal cells, pillar cells, and GER border cells, all of which elucidate the complexity of border specification. Specifically, it has been shown how this is regulated by *Hes/Hey* factors being active or not, of which themselves are regulated by *Gata3*, which has generated suspicion of this being a direct role of this gene (Basch et al., 2015; Luo et al., 2013).

In two studies, which eliminated *Gata3* at two different times, it was observed that by deleting *Gata3* at E8, during initial inner ear development, its deletion had great morphologic effects which either directly or by proxy caused the absence of any sensory tissue in the cochlea. The cochlea was severely atrophied and demonstrated little to no development at all, and logically, there was also no present sensory epithelia (Duncan et al., 2011). Deleting *Gata3* just a day later at E9.5, after some sensory specification has already taken place, *Gata3* conditional knockouts (CKO) also demonstrated severe morphological defects, however, these were less prominent than the earlier null mutants. Most specifically, however, there was a very sparse development of sensory epithelia and some HCs. The OC presented almost no organization, HCs were few, and also has significant development malfunctions, primarily observed by maldeveloped stereocilia.

The role of FGF signaling and most specifically of *Fgf10* expression in the inner ear has been in large part due to the data collected from null models. One null model, which eliminated *Fgf10* prior to any otic development shows a number of developmental problems. Most notable, is the presumable role FGF signaling has on the morphologic development of the vestibular system, as *Fgf10*$^{-/-}$ mice demonstrated complete loss of the posterior semicircular canal, as well as the atrophied cochlea. Furthermore, OC development demonstrated great defects as well. Null *Fgf10* models demonstrated a dose-dependence since *Fgf10*$^{+/-}$ mice demonstrated graded effects, such as the loss of a proper Reissner's membrane, shortened cochlear ducts, and small OC. However, there was the presence of HCs (Urness et al., 2015). It is interesting to note that *Fgf10* has a transient expression around the OC, as it begins to be expressed in the LER, supposedly priming future BMP4, and then progresses counterclockwise through Reisner's membrane and finally to the GER where it ends in the GER (Urness et al., 2015; Groves and Fekete, 2012). Thus, *Fgf10* seems to have a great morphologic impact by specifying nonsensory regions of the OC, which ultimately leads to interest in what is its role within nonsensory cells of the OC that are just adjacent to the OC, where *Gata3* is presently active, also taking into consideration that *Gata3* regulates *Fgf10* (Luo et al., 2013; Lilleväli et al., 2007).

The interaction of *Gata3* and *Fgf10* in the development of the sensory epithelia and neurons in the inner ear has been long suspected. Studies have shown that *Gata3* does indeed directly regulate *Fgf10* levels of mRNA expression

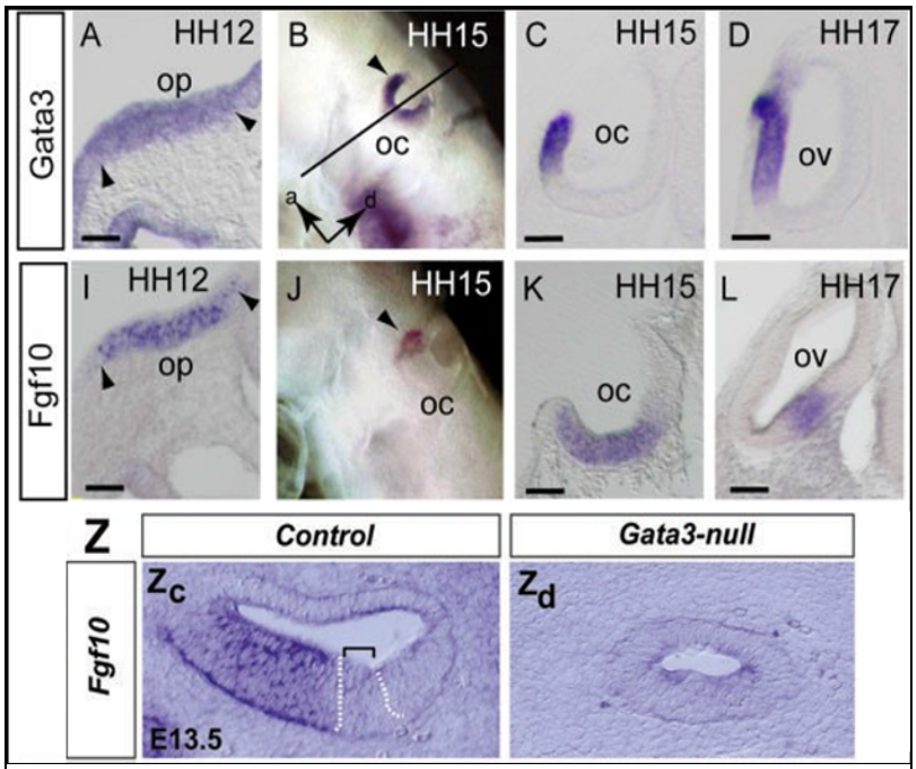

Figure (4) demonstrates the effects of loss of GATA3 on the organ of Corti. In the *Gata3* and *Fgf10* panels, the expression of *Gata3* and Fgf10 can be seen to always be close in proximity and in apposition sine placode induction. In panels Za and Zc, a WT and normal organ of Corti can be seen. In panels Zb and Zd, loss of morphology, *Fgf10* expression and proper border are apparent (Taken from; Luo et al., 2013; Lilleväli et al., 2007)

through in-situ hybridization and through qPCR analysis (Luo et al., 2013; Duncan et al., 2013). Notably, there is a specific effect on the nonsensory cells of the OC, both the GER and the LER cells, since *Gata3* knockdown also provokes a decrease in the *Bmp4* expression levels (Fig. 4). Furthermore, this interaction is made more interesting because *Gata3* and FGF signaling has been shown to be crucially involved in the specification of IHCs and the cells of the GER. It is not known what the actual mechanism for the border specification is. However, *Gata3* is crucially involved since it has a direct effect on GER-specific genes, such as *Fgf10*, and on OC genes for lateral inhibition, such as *Jag1* and *Hes/Hey* factors (Basch et al., 2016; Luo et al., 2013). Furthermore, *Gata3* has been shown to be necessary to keep cells from entering stages of HC development by controlling the expression of the mitotic factor gene *p27^{Kip1}*, which is thought to be necessary for determining what cells become in the OC (Walters et al., 2017).

Finally, the interplay between *Gata3* and *Fgf10* is also shown to be actively present within neurons. The same regulation of *Gata3* over *Fgf10* expression has been observed in neurons in the CVG, with *Gata3* known leading once more to a concomitant decrease in *FGf10* expression (Lilleväli et al., 2007). While a direct regulation of the gene of the ligand is evidence enough, *Gata3* knockdown also demonstrates a downregulation in one of the two main targets for such ligand, *FGFR1b*, which could serve as a possible explanation for why neuronal pathfinding and HC synaptogenesis is erroneous in *Gata3* knockdown (Appler et al., 2013).

- **The interest of investigation and plan of approach.**

With these relationships in mind, it is why the tamoxifen-inducible *Fgf10*-cre^{ERT2}: *Gata3^{OE}* line is of such interest. Although much of the developmental cues and interaction between these two genes, this study was nonetheless interested in investigating the effect that *Gata3* would have on postnatal time points. In adult time points, the OC and all of its cell types have been specified, differentiated, and matured. However, *Gata3* was still shown that in late embryonic time points and early postnatal time points if manipulated could allow for the presence of ectopic HC courses (Walters et al., 2017). Thus, given the various roles of *Gata3* and its influence on the crucial, equally multitasking, *Fgf10* gene, we set out to investigate this interplay. Our line utilizes Cre-LoxP technology to enable overexpression of *Gata3* in a cell-specific and time-specific manner. We first set out to characterize *Fgf10*-cre^{ERT2} expression in the mature cochlea, using the *Fgf10*-cre^{ERT2}: tdTomato line, with an injection of pups at P14 with tamoxifen (6 mg / 40 kg). This allowed us to localize and characterize where we would overexpress *Gata3* within the over-expresser model and determine the effects of *Gata3* overexpression in the tissues that correspondingly we identified with tdTomato recombination. We would like to know if the relationships outlined throughout embryonic development, and overexpression of *Gata3* would have any drastic effects on an adult and differentiated OC.

❋ Methods:

- **Transgenic Mouse lines.**

The Fgf10-cre^{ERT2} mouse line was gifted to the Duncan lab by Dr. Bellusci (Agha et al., 2021). This mouse line was characterized using the following primers: forward (ATTTGCCTGCATTACCGGTC), reverse (ATCAACGTTTTGTTTTCGGA). The Rosa26 locus TdTomato transgene mouse room was acquired from Jackson Laboratory. The following were used for identification: forward (AAGGGAGCTGCAGTGGAGTA) and reverse (CCGAAAATCTGTGGGAAGTC). The Gata3OE mouse line was gifted to the Duncan lab by Dr. Bouchard (Nguyen et al., 2013). The following primers were used: forward (AAAGTCGCTCTGAGTTGTTAT), reverse Mutant (GCGAAGAGTTTGTCCTCAACC), and reverse WT (GGAGCGGGAGAAATGGATATG).

- **Mouse Collections and Preparations.**

Mice were injected with 6 mg per 40 kg tamoxifen (Sigma, cat. T5648) pup body weight to induce the modified estrogen reception on the Cre-recombinase protein at P14. Pups were then collected at P21 by euthanization using a lethal 500 mg/kg dose of Avertin (2,2,2- tribromoethanol; Sigma cat. T48402). Pups were fixed using 4% paraformaldehyde (PFA) via transcardial perfusion using a peristaltic pump. All animal breeding and euthanasia were conducted following the procedures approved by Western Michigan University's Institutional Animal Care and Usage Committee (IACUC) #21-01-11. Before completing animal studies, researchers concluded the Collaborative Institutional Training Initiative (CITI) for animal research.

- **Immunohistochemistry**

Pups were dissected in PBS under a microscope, and the cochlea was then placed into a 96-well plate, where the cochleae remained in PBS for upwards of 1 hour. After removal, ears were prepared by being washed with 0.05% Tween20/PBS 5 times with each wash consisting of 5 minutes on a rocker. Ears were then blocked for 30 minutes on a rocker in a blocking solution, composed of 2.5 (5%) mL of donkey solution, 47.5 mL of PBS, 0.5 g of Bovine Serum (BSA), and 50 μL of TritonX-100. The ears were then washed with several exchanges of 0.05% Tween20/PBS. Ears were then placed into a combination of diluted primary antibody solutions. For *Fgf10*-cre^{ERT2}: tdTomato as well as *Fgf10*-cre^{ERT2}: *Gata3*^{OE} samples the following primary antibodies were added; Rabbit MyoVIIa (Proteus Biosciences) were diluted in Blocking solution 1:500, Chick NF200 (Aves©) diluted in Blocking solution 1:200, DAPI (Hoechst dye) diluted 1:2000, and Phalloidin 488 diluted 1:1000 in blocking solution all were added to individual ears, and Phalloidin 488 diluted 1:1000 in blocking solution. Ears were then incubated for at least two days at 4˚C on a rotating platform. Following the incubation, period ears were then removed from the primary antibody solution and rinsed with one rapid exchange of 0.05% Tween20/PBS. Then, four washes of 30 minutes of the same 0.05% Tween20/PBS were carried out on a rocking plate. Following this, secondary antibody solutions were added in equal amounts to each ear. All secondary antibody solutions were spun in a centrifuge for 10 minutes at 14000 rpm before addition into wells containing ears. Samples were then incubated at 4˚C on a rotating platform overnight. Following second incubation, samples were washed for 30 minutes three times with PBS. Following washes, samples were transferred onto glass slides arranged with hair cells facing upwards, and coverslips were added after samples were immersed in glycerol.

- **Imaging and image editing.**

Samples were taken on a C2 Nikon confocal that had previously undergone immunohistochemistry. Images were analyzed, and Z stacks were compiled using the Fiji-ImageJ editing software. Images were finalized and edited for observation using Corel Draw; the images generated here are seen in the results sections. All images are representative of – replicates.

Results:

- **Characterization of the *Fgf10*-cre^{ERT2}: tdTomato.**

Prior to overexpressing *Gata3*, we had to assure ourselves that the *Fgf10*-cre^{ERT2} method of transgenic engineering would confidently work in overexpressing *Gata3* in the nonsensory cells of the GER. For this, we utilized the tamoxifen-inducible (ERT2) Cre-recombinase protein. By knowing that Cre-recombinase would only be expressed under the *Fgf10* promoter. Under normal conditions, it would not be translocated to the nucleus. Upon activation by tamoxifen, which binds to the modified estrogen receptor on the Cre-

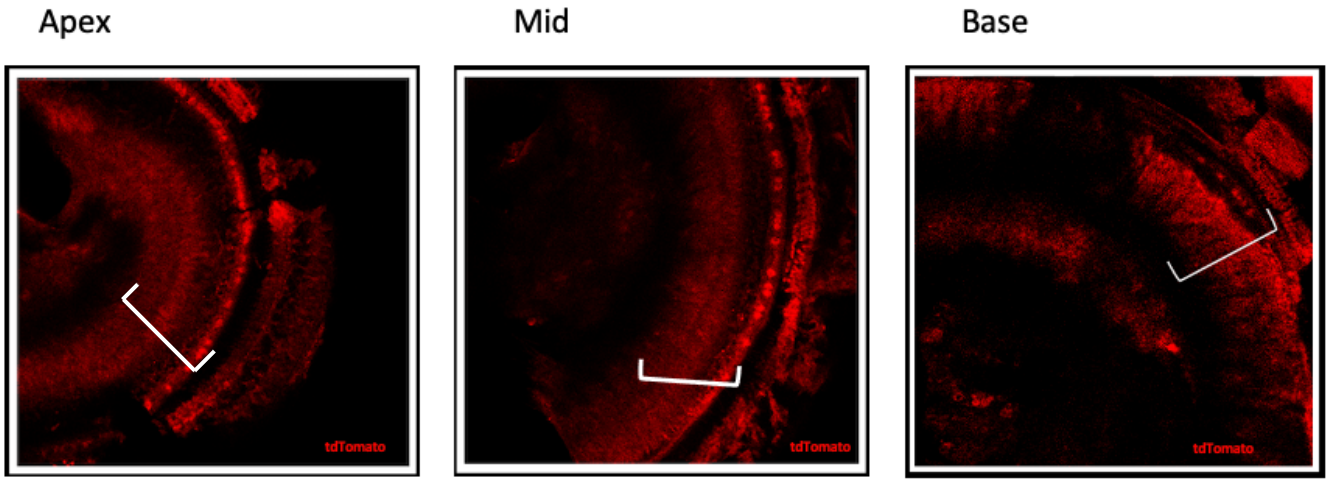

Figure (5) Panels labeled Apex, Mid and Base show cochlea section of a P14 mouse ear. Red staining is endogenous tdTomato expression cause by recombination of the gene allowing for fluorescent protein in regions correspondent to *Fgf10* expression. Cells of the GER are particularly stained and are indicated by the whit brackets in all three panels.

Recombinase protein, allowing for it to carry out genetic recombination. At the Rosa26 locus, a stop codon would be removed, allowing for the expression of the endogenous fluorescence of tdTomato which the mice possessed (Fig. 5).

After injection of the mice at P14, we collected mice at P21, where we prepared the samples for analysis. We observed sufficient recombination carried out by cre-Recombinase in the GER all along the length of the cochlea (Figure 6, white brackets). The border cells are specifically the most significant stained, and a decreasing gradient through the GER. Due to the observed fluorescence, we felt confident that utilizing the *Fgf10*-cre^{ERT2} mouse line would allow for overexpression of *Gata3,* only in the GER.

- ***Fgf10*-cr^{ERT2}: *Gata3*^{OE} provokes no observable change in OC organization, morphology, or cells types.**

We set out to, by the same mechanism of inducible Cre lines, overexpress *Gata3* in the GER. Upon overexpression, no observable phenotypes can be seen. HCs can be seen as being stained red, and in all three figures, control, heterozygous and homozygous, the HC rows (one IHC and three OHC) can be seen as present. *Gata3* overexpression at this stage also does not cause any IHC duplets, as previous data has shown to be the case in embryonic timepoints. Furthermore, overexpression of *Gata3* does lead to any drastic morphological phenotypes, as seen to be the case in *Gata3* null models. While null models dealt with the loss of *Gata3*, its role as a border maintainer gene, especially with its interactions with *Fgf10* was reason to believe overexpression could cause some morphologic, or border phenotypes (Fig 6). It is interesting to note that at this age it seems like this dynamic interaction at the border has reached a point of stagnation and seems solidified. Phalloidin reveals that the pillar cells, seen in between both HC rows, maintain the proper organization as well, and taken with the observed conserved order of the OC, it seems reasonable to postulate that *Gata3* overexpression does not have a significant role in border exertion, the morphology of OC, or HC fates in GER cells.

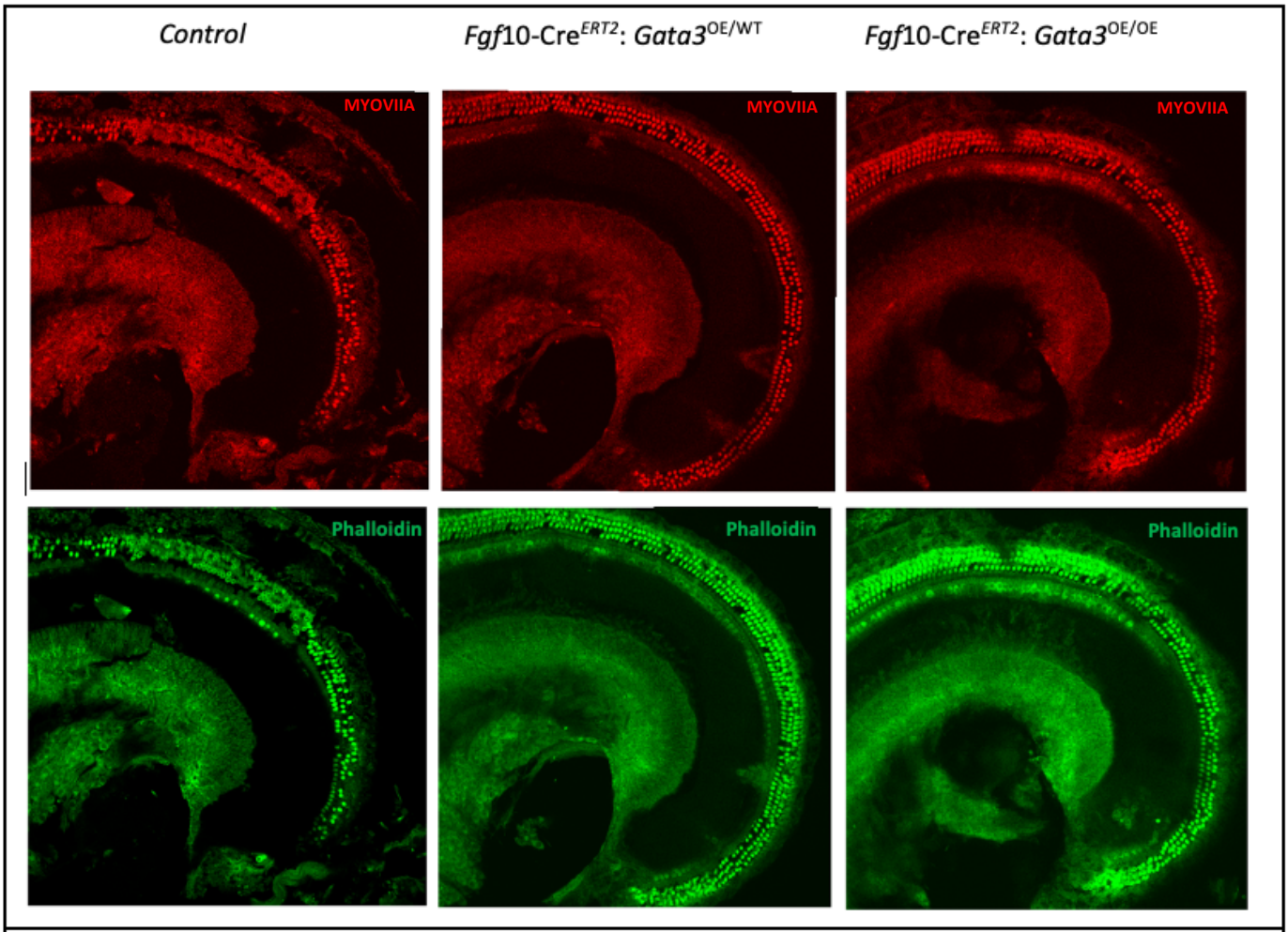

Figure (6), Demonstrates the effects, or lack thereof, of the overexpression of *Gata3* in cochleae of age P14. The cochlea was collected at age P21 and stained with MYO7A (red) and Phalloidin (Green). In all three cochleae, peripherin stains reveal the proper organ of the Corti structure by demonstrating the presence of all three rows of OHC, the presence of organized pillar cells, and single rows of IHC. MYO7A indicates that these rows of cells are indeed HCs and that no duplets were observed.

❋ Conclusion:

Most of the knowledge discussed previously has all been carried out in embryonic time points, with its focus to address the questions of congenital hearing loss, whereas postnatal studies typically focus on applying their findings to other forms of hearing loss, although not restricted to such.

It has been shown that congenital deficiencies in *Gata3* lead to misexpression of *Fgf10*, thus causing large-scale morphology problems in inner ear development. Focusing mainly on the border of the GER and the OC, *Gata3's* involved here, and subsequentially its influence over *Fgf*10 is crucial to establish which cells become HCs and which cells become nonsensory. Clearly, there is an intriguing mechanism at play here (Basch et al., 2016). It has been shown and hypothesized by many that these cells, phalangeal cells, pillar cells, GER cells, and IHCs have a very specific way of acquiring this fate, and given that GATA3 is seen in high levels in HCs at these stages, it is not impossible to imagine that its expression pattern here is crucial for the specification of which cells become which. Previous studies have demonstrated that transdifferentiating of HCs can occur by manipulating crosstalk between GER cells, IHCs, and inner pillar cells (McGovern et al., 2019); however, no studies have shown such a relationship this late in embryonic time points. At postnatal stages, the OC has mainly been considered static in terms of cell fate after some studies have pointed out that early in the postnatal days, and late embryonic days, *Gata3* seems to still play a role in the quiescence of supporting cells and GER by regulating mitotic factors *p27^{Kip1}*, and that if *Gata3* levels are reduced, along with loss of HCs, ectopic HCs can be identified (Xu et al., 2021, Walters et al., 2017).

Many different forms of hearing loss can occur, but they seem to target HCs, specifically noise-induced, ototoxicity, and age-related hearing loss can happen in a multitude of ways. However, typically the end result is the loss HCs or SGNs. Loss of *Gata3* has also been linked with deafness, primarily through HDR syndrome. HDR syndrome involves the haploinsufficiency of *Gata3*. The subsequent loss of one or more alleles of *Gata3* can cause congenital renal disease, sensorineural hearing loss, and hypoparathyroidism. As such *Gata3* has been linked with congenital hearing loss. Given the background of HCs, their differentiation, and the role of *Gata3* on border exertion, it has been postulated that *Gata3* overexpression could provide some help in HC regeneration, or at least protection. *Gata3,* along with other transcription factors has been demonstrated to allow for the expression of HC differentiation marker *Pou4f3*, which has led to much excitement over *Gata3* as being a new path for possible genetic therapies (Masuda et al., 2012). However, when it comes to rescuing HCs, the result has not been so promising. HCs show some form of regeneration in the vestibular system (Burns & Stone 2016), where *Gata3* expression seems alike in regulating HC differentiation however in the cochlea, HCs have presented any identification of a possible regenerative function.

However, *Gata3* nonetheless has been strongly linked to maintaining HCs, particularly for the data surrounding its role in upregulating *Pou4f3* in GER cells, and loss of *Gata3* has shown decreases in HC gene *Atoh1* and a decrease in HC critical Transcription factor *Eya1* (Duncan et al., 2013; Masuda et al., 2013). As such, the role of *Gata3* has been more strongly linked to being one of HC fate maintenance, rather than initiating the developmental cascade, possibly improving key transcription factors and regulatory site interactions. For this reason, many therapy options have surrounding possibly granting "extra copies" of *Gata3* as possible protection for expected HC trauma or for HDR syndrome patients. However, there is worry about utilizing *Gata3* as such because its regulatory functions are not entirely known, specifically surrounding HC fates, OC morphology, and cochlear duct morphology. And reluctance may be held in not knowing if *Gata3* requires a sort of homeostatic level for popper OC function, or if at this stage, only loss of *Gata3* can affect hearing.

Our study answers this question by knowing that at least overexpression of *Gata3* at this stage does cause any adverse reaction. Morphologically speaking, and most predictable the cochlea seems normal, as expected. Next, there were no observable ectopic hair cells in the GER or the OC. This is interesting because *Gata3* was one of the transcription factors needed for strong *Pou4f3* upregulation in the GER cells, hover it seems that at this stage, the GER cells have been specified sufficiently that they maintain the original epithelial fate. Finally, the OC and its border with the GER seems unaffected as well. It would however still be necessary to investigate what would happen if *Gata3* were to be lost abruptly, at this stage in life, the early postnatal days, if this would have any effects. It is also necessary to investigate and quantify through qPCR if the *Gata3* transcripts in the GER were elevated, since the possibility of these cells simply not even expressing the extra copies of *Gata3* also exists.

However, our data is reassuring as it allows us to know that "too much" *Gata3* is not a concern with perfectly healthy OCs. Thus, many treatments options that have been supposing transgenic methods of treating or alleviating hearing loss do not have to worry about it affecting the morphology, cell fate, or interfering with the extremely specific cell organization in the OC. Methods such as AAV injection could be used to supply patients with extra copies of *Gata3* if necessary, and our data would suggest that if this dose is given to an already normal level of *Gata3*, targeting cells of the GER at least, the OC should remain the same, while *Gata3* could offer its protective functions.

Taking all of this into account, this study demonstrates evidence that overexpression of *Gata3* through *Fgf10*-cre^{ERT2} recombination does not cause any aberrant or observable phenotypes, however more quantitative approaches are still necessary, as well as more investigation into the role of *Gata3*, both embryonically as well as postnatally if it ever were to be considered as a possible avenue for hearing loss or hearing disorder therapy.

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❋ References:

1) Lin, F. R., Niparko, J. K., & Ferrucci, L. (2011). Hearing loss prevalence in the United States. *Archives of internal medicine*, *171*(20), 1851–1852. https://doi.org/10.1001/archinternmed.2011.506.

2) Blackwell DL, Lucas JW, Clarke TC. Summary health statistics for U.S. adults: National Health Interview Survey, 2012 (PDF). National Center for Health Statistics. Vital Health Stat 10(260). 2014.

3) Le Prell, C. G., Hammill, T. L., & Murphy, W. J. (2019). Noise-induced hearing loss and its prevention: Integration of data from animal models and human clinical trials. *The Journal of the Acoustical Society of America*, *146*(5), 4051. https://doi.org/10.1121/1.5132951.

4) Hodge, S. E., Lopez, I. A., Ishiyama, G., & Ishiyama, A. (2021). Cisplatin ototoxicity histopathology. *Laryngoscope investigative otolaryngology*, *6*(4), 852–856. https://doi.org/10.1002/lio2.608.

5) Ohyama T, Basch ML, Mishina Y, Lyons KM, Segil N, Groves AK. BMP signaling is necessary for patterning the sensory and nonsensory regions of the developing mammalian cochlea. *J Neurosci*. 2010;30(45):15044-15051. doi:10.1523/JNEUROSCI.3547-10.2010

6) Kelley MW, Driver EC, Puligilla C. Regulation of cell fate and patterning in the developing mammalian cochlea. *Curr Opin Otolaryngol Head Neck Surg*. 2009;17(5):381-387. doi:10.1097/MOO.0b013e3283303347

7) Groves AK, Fekete DM. Shaping sound in space: the regulation of inner ear patterning [published correction appears in Development. 2012 Feb;139(4):826]. *Development*. 2012;139(2):245-257. doi:10.1242/dev.067074

8) Dabdoub A, Puligilla C, Jones JM, et al. Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. *Proc Natl Acad Sci U S A*. 2008;105(47):18396-18401. doi:10.1073/pnas.0808175105

9) Basch ML, Brown RM 2nd, Jen HI, et al. Fine-tuning of Notch signaling sets the boundary of the organ of Corti and establishes sensory cell fates. Elife. 2016;5:e19921. Published 2016 Dec 14. doi:10.7554/eLife.19921

10) Puligilla C, Dabdoub A, Brenowitz SD, Kelley MW. Sox2 induces neuronal formation in the developing mammalian cochlea. J Neurosci. 2010;30(2):714-722. doi:10.1523/JNEUROSCI.3852-09.2010

11) Appler JM, Goodrich LV. Connecting the ear to the brain: Molecular mechanisms of auditory circuit assembly. Prog Neurobiol. 2011;93(4):488-508. doi:10.1016/j.pneurobio.2011.01.004

12) Nishimura K, Noda T, Dabdoub A. Dynamic Expression of Sox2, Gata3, and Prox1 during Primary Auditory Neuron Development in the Mammalian Cochlea. PLoS One. 2017;12(1):e0170568. Published 2017 Jan 24. doi:10.1371/journal.pone.0170568

13) Fritzsch B, Dillard M, Lavado A, Harvey NL, Jahan I. Canal cristae growth and fiber extension to the outer hair cells of the mouse ear require Prox1 activity. PLoS One. 2010;5(2):e9377. Published 2010 Feb 23. doi:10.1371/journal.pone.0009377

14) Weisz C, Glowatzki E, Fuchs P. The postsynaptic function of type II cochlear afferents. Nature. 2009;461(7267):1126-1129. doi:10.1038/nature08487

15) Liu M, Pereira FA, Price SD, et al. Essential role of BETA2/NeuroD1 in development of the vestibular and auditory systems. *Genes Dev*. 2000;14(22):2839-2854. doi:10.1101/gad.840500

16) Ma Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ. neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron*. 1998;20(3):469-482. doi:10.1016/s0896-6273(00)80988-5

17) Bouchard M, de Caprona D, Busslinger M, Xu P, Fritzsch B. Pax2 and Pax8 cooperate in mouse inner ear morphogenesis and innervation. BMC Dev Biol. 2010;10:89. Published 2010 Aug 20. doi:10.1186/1471-213X-10-89

18) Pickles JO. Auditory pathways: anatomy and physiology. Handb Clin Neurol. 2015;129:3-25. doi:10.1016/B978-0-444-62630-1.00001-9

19) Koundakjian EJ, Appler JL, Goodrich LV. Auditory neurons make stereotyped wiring decisions before maturation of their targets. J Neurosci. 2007;27(51):14078-14088. doi:10.1523/JNEUROSCI.3765-07.2007

20) Wright TJ, Mansour SL. Fgf3 and Fgf10 are required for mouse otic placode induction. Development. 2003;130(15):3379-3390. doi:10.1242/dev.00555

21) Martin K, Groves AK. Competence of cranial ectoderm to respond to Fgf signaling suggests a two-step model of otic placode induction. Development. 2006;133(5):877-887. doi:10.1242/dev.02267

22) Ohyama T, Groves AK, Martin K. The first steps towards hearing: mechanisms of otic placode induction. Int J Dev Biol. 2007;51(6-7):463-472. doi:10.1387/ijdb.072320to

23) Ohyama T, Groves AK, Martin K. The first steps towards hearing: mechanisms of otic placode induction. Int J Dev Biol. 2007;51(6-7):463-472. doi:10.1387/ijdb.072320to

24) Luo XJ, Deng M, Xie X, et al. GATA3 controls the specification of prosensory domain and neuronal survival in the mouse cochlea. Hum Mol Genet. 2013;22(18):3609-3623. doi:10.1093/hmg/ddt212

25) Karis A, Pata I, van Doorninck JH, et al. Transcription factor GATA-3 alters pathway selection of olivocochlear neurons and affects morphogenesis of the ear. J Comp Neurol. 2001;429(4):615-630. doi:10.1002/1096-9861(20010122)429:4<615::aid-cne8>3.0.co;2-f

26) Kiernan AE, Pelling AL, Leung KK, et al. Sox2 is required for sensory organ development in the mammalian inner ear. Nature. 2005;434(7036):1031-1035. doi:10.1038/nature03487

27) Munnamalai V, Fekete DM. Notch-Wnt-Bmp crosstalk regulates radial patterning in the mouse cochlea in a spatiotemporal manner. Development. 2016;143(21):4003-4015. doi:10.1242/dev.139469

28) Urness LD, Wang X, Shibata S, Ohyama T, Mansour SL. Fgf10 is required for specification of non-sensory regions of the cochlear epithelium. Dev Biol. 2015;400(1):59-71. doi:10.1016/j.ydbio.2015.01.015

29) Ahmed M, Wong EY, Sun J, Xu J, Wang F, Xu PX. Eya1-Six1 interaction is sufficient to induce hair cell fate in the cochlea by activating Atoh1 expression in cooperation with Sox2. Dev Cell. 2012;22(2):377-390. doi:10.1016/j.devcel.2011.12.006

30) Duncan JS, Lim KC, Engel JD, Fritzsch B. Limited inner ear morphogenesis and neurosensory development are possible in the absence of GATA3. Int J Dev Biol. 2011;55(3):297-303. doi:10.1387/ijdb.103178jd

31) Lilleväli, K., Haugas, M., Pituello, F., & Salminen, M. (2007). Comparative analysis of Gata3 and Gata2 expression during chicken inner ear development. Developmental dynamics: an official publication of the American Association of Anatomists, 236(1), 306–313. https://doi.org/10.1002/dvdy.21011

32) Walters BJ, Coak E, Dearman J, et al. In Vivo Interplay between p27Kip1, GATA3, ATOH1, and POU4F3 Converts Non-sensory Cells to Hair Cells in Adult Mice. Cell Rep. 2017;19(2):307-320. doi:10.1016/j.celrep.2017.03.044

33) Appler JM, Lu CC, Druckenbrod NR, Yu WM, Koundakjian EJ, Goodrich LV. Gata3 is a critical regulator of cochlear wiring. J Neurosci. 2013;33(8):3679-3691. doi:10.1523/JNEUROSCI.4703-12.2013

34) McGovern, M. M., Randle, M. R., Cuppini, C. L., Graves, K. A., & Cox, B. C. (2019). Multiple supporting cell subtypes are capable of spontaneous hair cell regeneration in the neonatal mouse cochlea. Development (Cambridge, England), 146(4), dev171009. https://doi.org/10.1242/dev.171009

35) Xu, J., Yu, D., Dong, X., Xie, X., Xu, M., Guo, L., Huang, L., Tang, Q., & Gan, L. (2021). GATA3 maintains the quiescent state of cochlear supporting cells by regulating p27kip1. Scientific reports, 11(1), 15779. https://doi.org/10.1038/s41598-021-95427-3

36) Masuda, M., Pak, K., Chavez, E., & Ryan, A. F. (2012). TFE2 and GATA3 enhance induction of POU4F3 and myosin VIIa positive cells in nonsensory cochlear epithelium by ATOH1. Developmental biology, 372(1), 68–80. https://doi.org/10.1016/j.ydbio.2012.09.002

37) Burns, J. C., & Stone, J. S. (2017). Development and regeneration of vestibular hair cells in mammals. Seminars in cell & developmental biology, 65, 96–105. https://doi.org/10.1016/j.semcdb.2016.11.001