




THESIS APPROVED BY


8/09/2022  
Date

  
\_\_\_\_\_  
Jian Zuo, Ph.D., Chair

  
\_\_\_\_\_  
Garrett Soukup, Ph.D.

  
\_\_\_\_\_  
Peter Steyger, Ph.D.

  
\_\_\_\_\_  
Jeffrey North, Ph.D.

  
\_\_\_\_\_  
Mardell A. Wilson, Ed.D., RDN  
Provost

COMBINATION OF COMPOUNDS A2CE AND C18 FOR COCHLEAR HAIR CELL  
REGENERATION IN ADULT MICE

---

By  
Fred Millan V

---

A THESIS

Submitted to the Faculty of the Graduate School of the Creighton University in Partial  
Fulfillment of the Requirements for the Degree of Master of Science in the Department of  
Biomedical Science.

---

Omaha, NE  
(August 08, 2022)



## **Abstract:**

Hearing loss is a permanent disability that affects a significant percentage of the population, with many interventions relying on secondary medical devices such as cochlear implants, but regeneration of hearing has been an important avenue to explore to restore normal hearing function. Hair cells are a specialized cell type in the inner ear that are crucial for transducing sound into electrical signals that our neurons can interpret. They are post-mitotic, which means once they die or are damaged, they do not regenerate. Previous studies have utilized genetic manipulations in mice to upregulate pro-hair cell fates in a subpopulation of cells that contain stem-cell like markers, called supporting cells, which should push these cells to transdifferentiate and replace lost hair cells in the inner ear. However, due to the nature of these studies and their use of transgenic mouse lines, they are not very translatable to clinical trials as-is.

Here, using a combination of compounds that either upregulate a gene of interest or suppress a gene that inhibits regeneration, we aim to produce the first drug-induced regeneration of hair cells in adult mice. In our cocktail we have two drugs Alsterpaullone, 2-Cyanoethyl (A2CE) and proprietary drug Compound 18 (C18). C18 is an up regulator of the gene POU4F3, which has been shown to be sufficient in producing hair cells from supporting cells in transgenic mice. A2CE is a transcriptional inhibitor of p27kip1, which has been shown to have a non-canonical role in suppressing a cofactor for Atoh1, which is an upstream of POU4F3 and is an important developmental gene in hair cell development. We gave transtympanic injections of our drug cocktails to either FVB or Sox2 CreER; Tdtomato mice and collected their cochlea 4 weeks

following injections. We found that following injections of our cocktail into the ear, regeneration of immature hair cells expressing both supporting cell markers and hair cell markers were found. We saw high amounts of variance among samples, which significantly impacts the efficacy of our drug cocktail, and needs to be addressed in future studies. From our Sox2 CreER; Tdtomato samples we confirmed that our population of converted hair cells arise from the Sox2 expressing supporting cells, which is consistent with previous genetic models. Future studies should include pharmacokinetic studies focusing on drug distribution through the round window and the inner ear, which we hypothesize may contribute to the high amount of variance amongst the samples.

## Acknowledgements:

René Vielman Quevedo (MD) for his mentorship and support throughout my master's program, none of this would be possible without it.

Jian Zuo, PhD, for allowing me the opportunity to work in his lab as a master's student and giving me the chance to work on this project.

Zhenhang Xu (MD) and Sarath Vijaykumar (PhD) for their continually guidance and assistance with this project throughout my master's program.

My thesis committee members Jian Zuo, Jeffrey North (PhD), Peter Styger (PhD) and Garrett Soukup (PhD) for their support and instruction throughout my master's program.

Joseph R. Frank for his preliminary work on Compound 18 and assistance with transitioning into this project.

June Li for her patient assistance with any questions and help I needed.

Kind thanks to all other members of the Zuo lab, who assisted me every single day no matter the task and without who I wouldn't have gotten this far.

Special thanks to Abyss K. Wilson, who assisted with editing and proofreading this paper.

This work has been supported by the NIH R01DC015010, 1P20GM139762, State of Nebraska Stem cell Subaward (3782), DoD W81XWH-18-1-0442, and ONR N00014-18-1-2507.

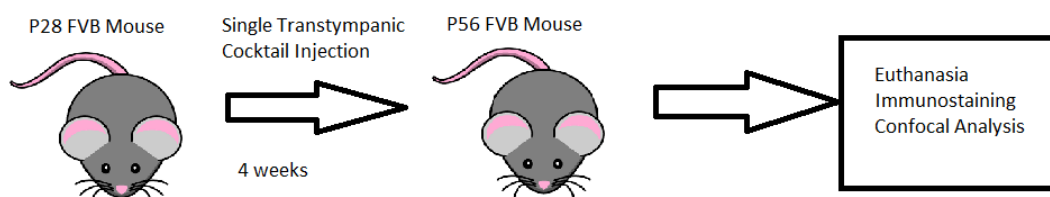
## Table of Contents:

I. Abstract.....	i
II. Acknowledgments.....	iii
III. Figures and Tables.....	v
IV. Introduction.....	1
V. Experimental Methods.....	15
VI. Results.....	22
VII. Discussion.....	26
VIII. Conclusions.....	35
IX. References.....	36

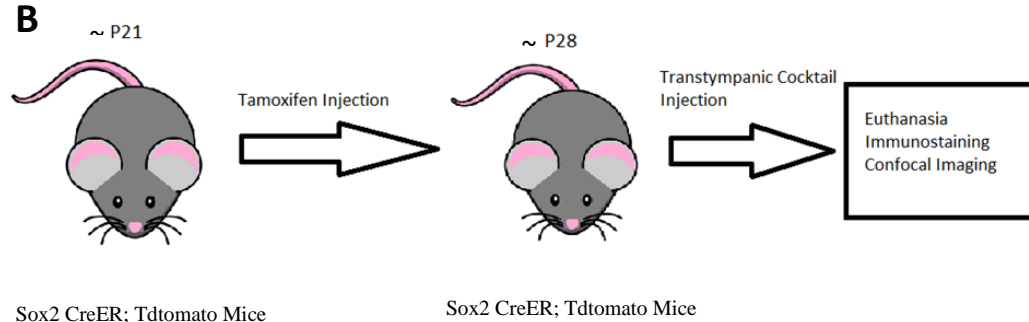
## Figures:

### Figure

#### A



#### B



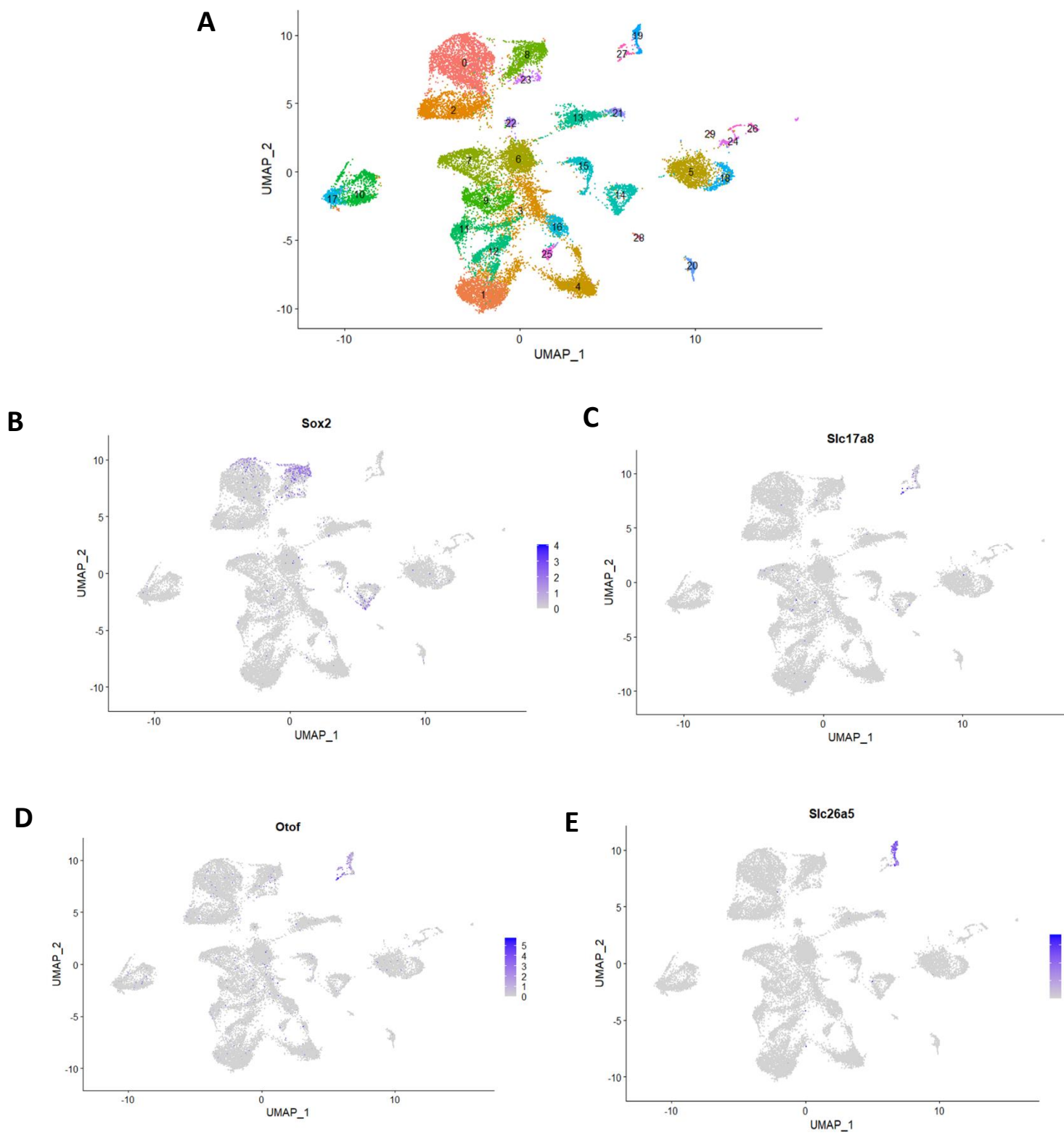
### Figure 1. Schematic design of our two Injected mice cohorts; FVB and Sox2 CreER;

**Tdtomato.** (A) P28 FVB mice were given a single transtympanic injections of our drug cocktail and then monitored for 4 weeks before harvesting temporal bones for whole mount

immunofluorescence. (B) Sox2 CreER; Tdtomato mice were given a single tamoxifen injection (250mg/kg body weight) around ~P21, with some mice slightly older due to breeding constraints.

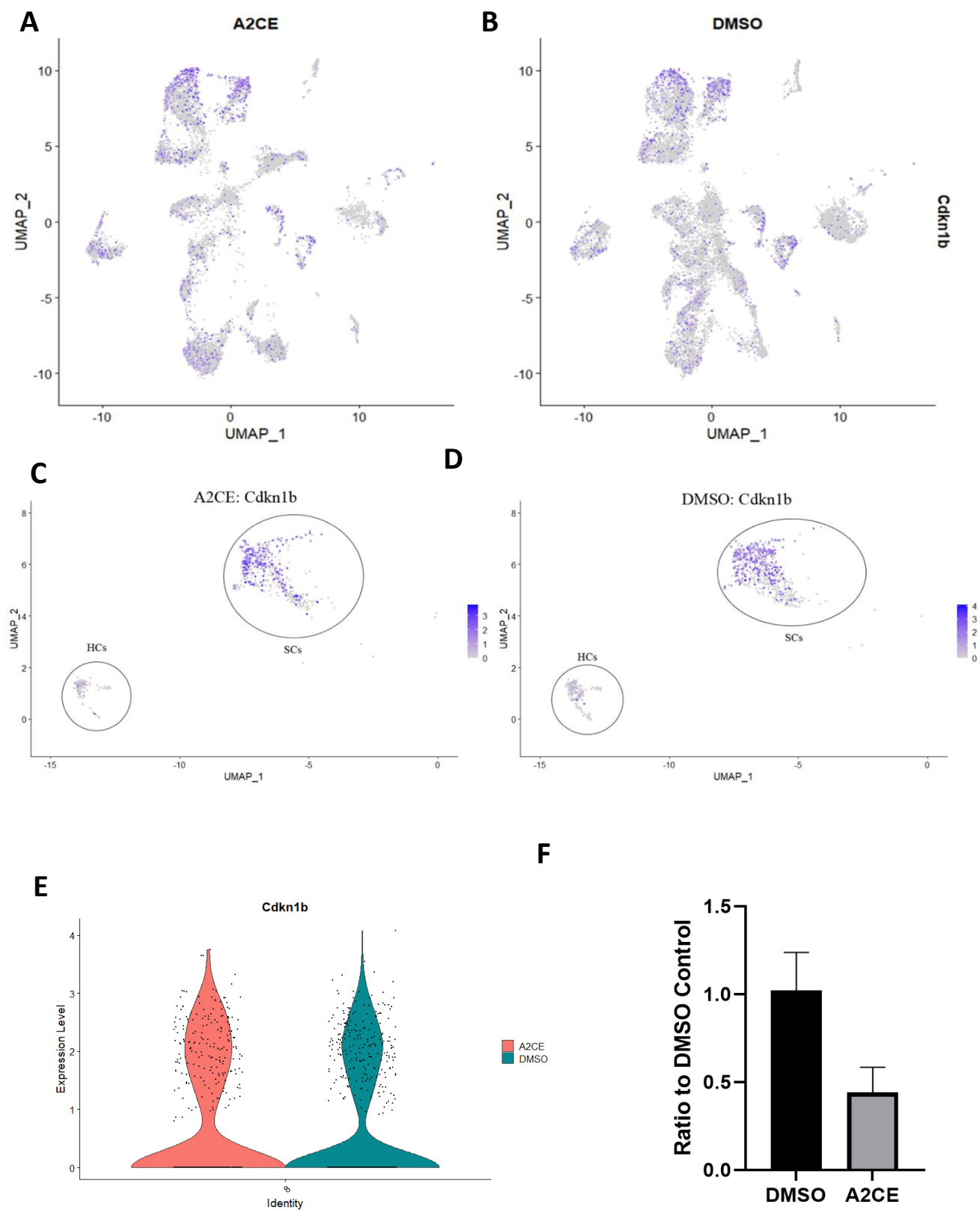
One week later, Sox2 CreER; Tdtomato mice were then given transtympanic injections of our drug cocktail, and consistent with our FVB experiments, were harvested four weeks later for immunostaining and confocal analysis.

Figure 2.



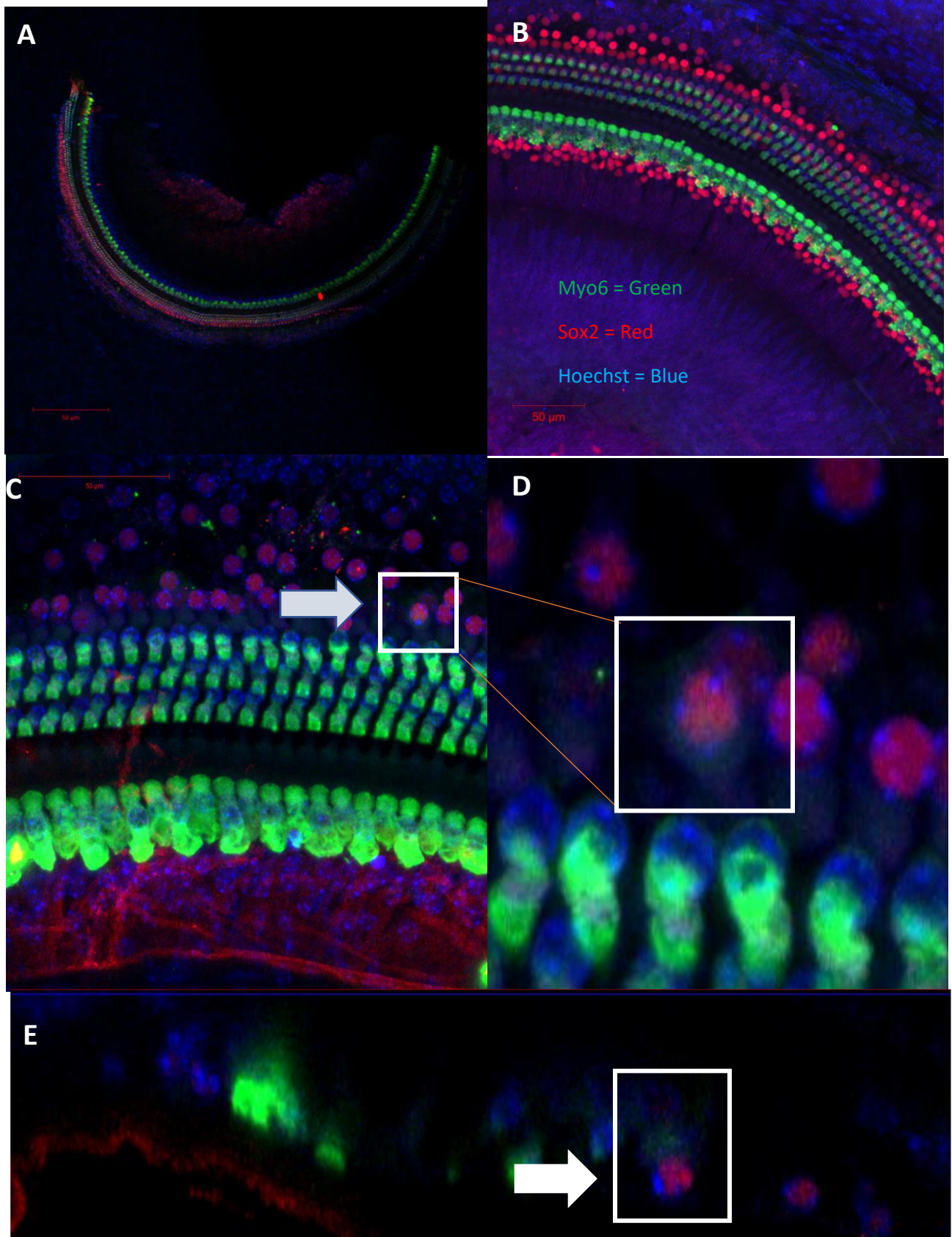
**Figure 2. Single Cell RNA-Sequencing of inner ear of A2CE transtympanic injected or DMSO vehicle control for 14 P60 C57 mice 4 hours post injections.** (A) UMAP of both treatment groups, PCA analysis was utilized to cluster and separate sorted cells. (B-E) Representative Feature maps of a few genes used for cluster biomarkers for relevant cell populations. HC's and SC's clusters were identified using cluster biomarkers of known differentially expressed HC and SC genes; Myo6, Myo7a, VGLUT3, and Otoferlin were used for IHC identification. Myo6, Myo7a, Prestin and Oncomodulin were used in OHC identification and Sox2, Gjb2, Tubb3 were used as biomarkers for relevant SC clusters.

Figure 3.

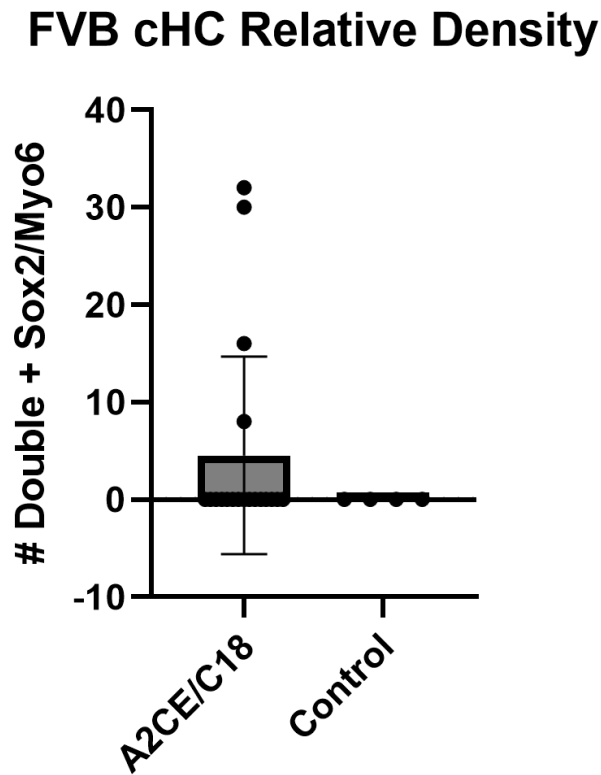


**Figure 3. p27 Expression across A2CE and DMSO treated cells. (A-B) UMAP of single cell RNA-seq, separated by treatment. (C-D) Separated HC's and SC's clusters were separated and re-clustered for further analysis. We observe 29 IHC's, 118 OHC's and 568 SC's in A2CE treated mice and 59 IHC's, 118 OHC's and 683 SCs in our DMSO control mice using cells with 35%< mitochondria counts. (E) Violin plot showing the largest SC cluster relative Cdkn1b expression. We see relatively similar levels of expression between A2CE and DMSO, which is recapitulated in Log2 fold change levels; we see a Log2 fold change of 1.44 for A2CE, and 1.16 for DMSO. DMSO is slightly lower but not significantly. (F) RT-PCR at 24-hour time point. 4-hour time point not shown, but no significant reduction was observed.**

Figure 4.

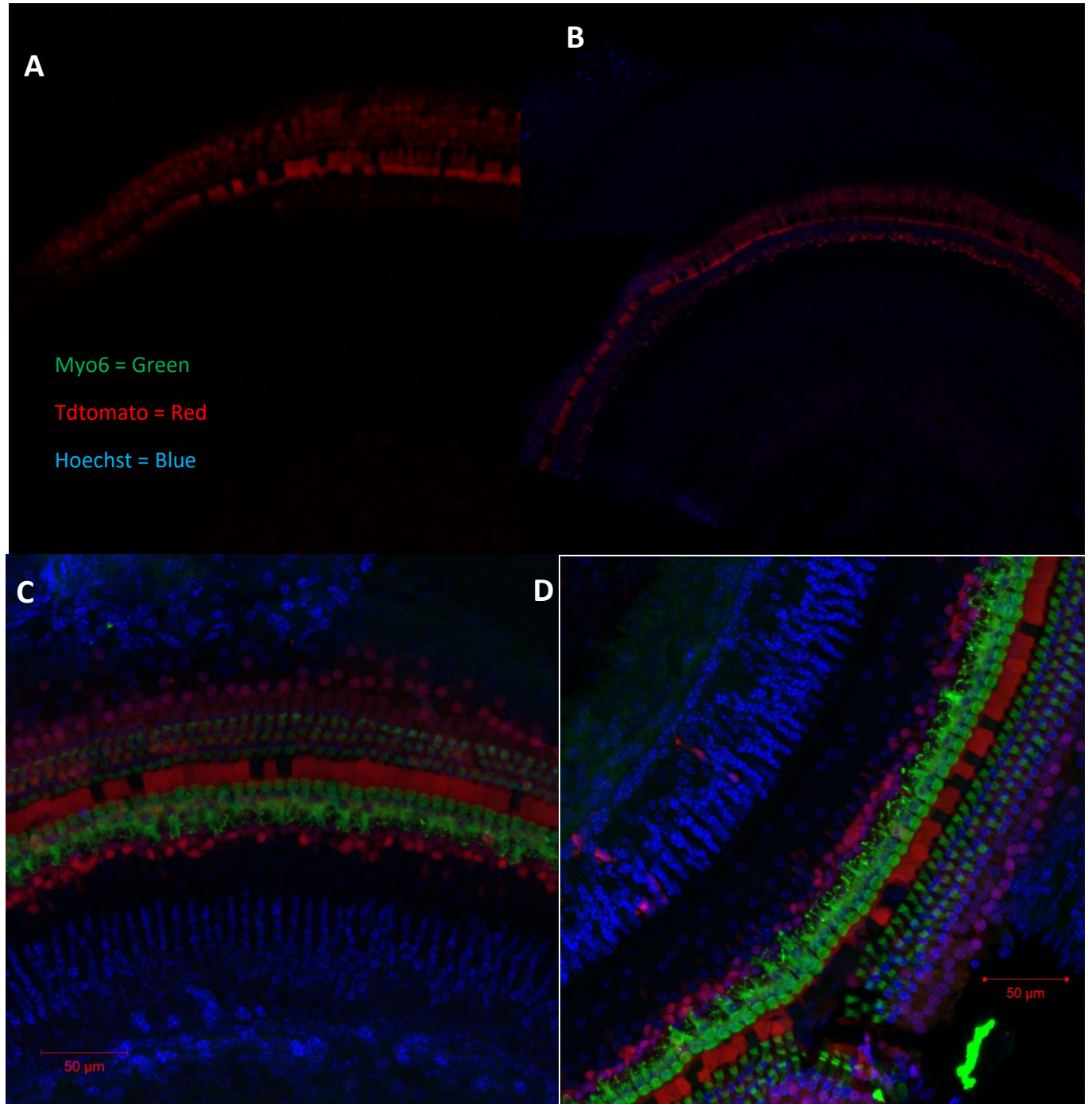


**Figure 4. FVB Cocktail Injections Representative Images.** (A-B) representative images of Control images at 10X and 20X magnification respectively, showing specific staining of OHC's and IHC's with Myo6 (green) and supporting cells with Sox2 (red). (C-D) Representative images showing a cHC expressing both Myo6 and Sox2. (E) Orthogonal cross section showing cell migrating parallel to 3<sup>rd</sup> row OHC's. Antibody staining for cHC's showed a consistently weaker Myo6 signal than endogenous hair cells, but still above background. Control samples show consistent staining between groups, with no reported double positive cells in any untreated samples.

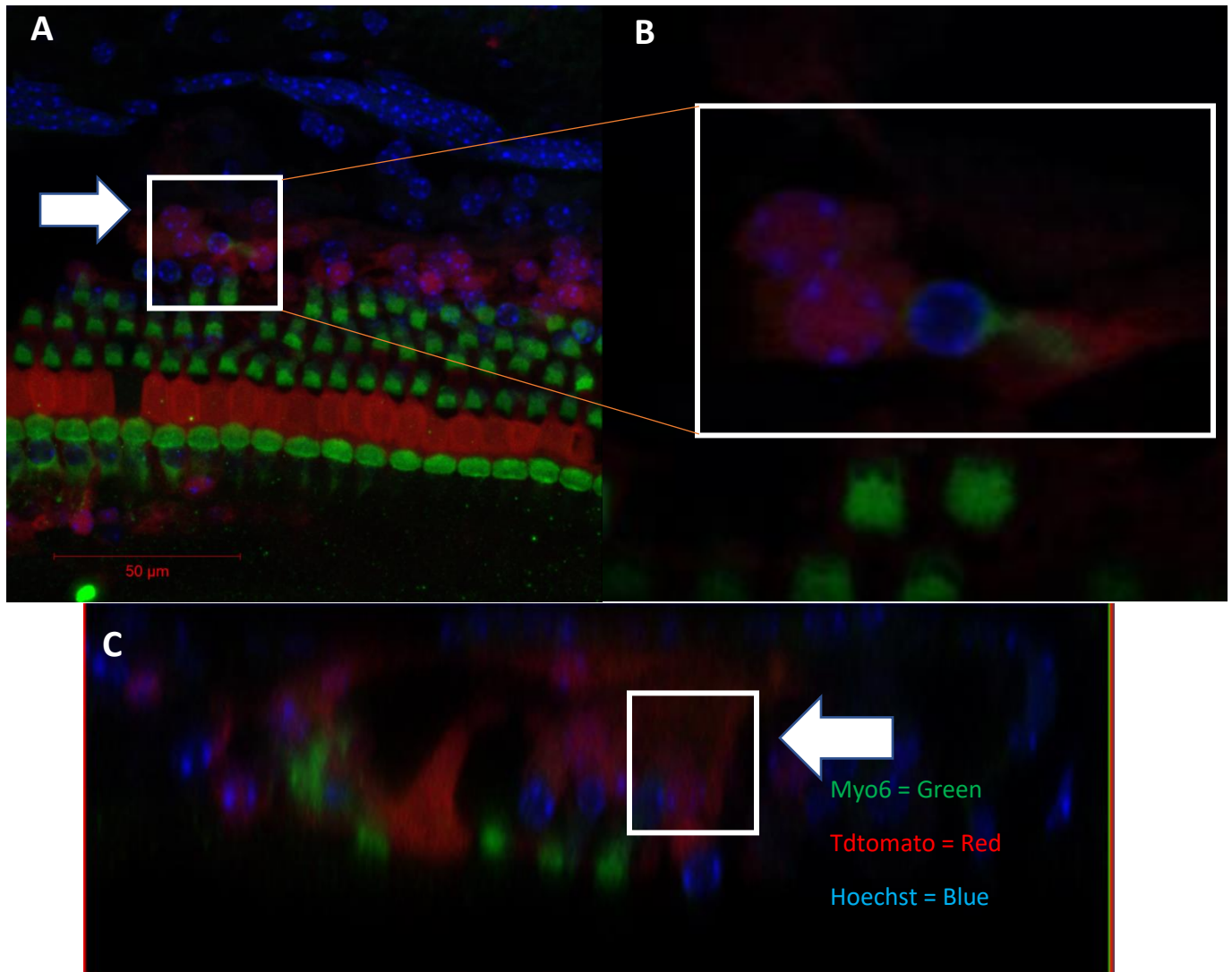
**Figure 5.**

**Figure 5. FVB cHC Relative Density.** Of the 19 total mice across three separate cohorts of n=5, n=8 and n=6 respectively, 4 mice were seen to have some regeneration. The remaining 15 samples showed no distinct converted hair cells in any collected images. Later cohorts had reduced cHC numbers than the first-round cohort, who had significantly more conversion. Subsequent investigations of our 15 samples with more careful analysis and advanced software will be conducted later.

Figure 6.



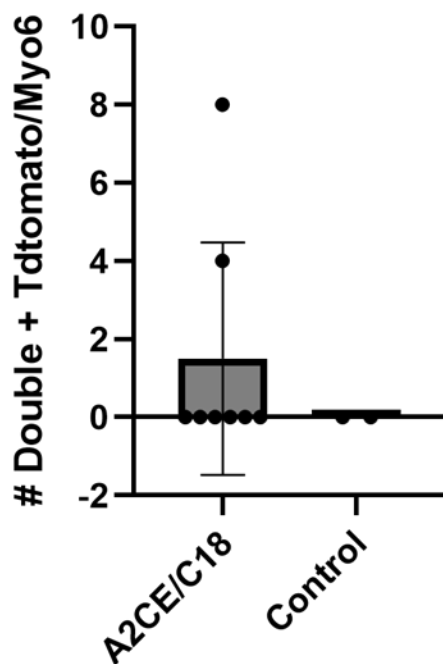
**Figure 6. Characterization of Sox2 CreER; Tdtomato Mouse Phenotype.** (A-B) Control images (10X) showing Tdtomato expression is localized to supporting cells only, no hair cells or other cell types were reported to express Tdtomato following tamoxifen injections. (C) Control image showing Myo6 staining is specific to hair cells, with no Tdtomato expressing supporting cells containing any Myo6 immunofluorescence. (D) Drug treated sample showing us the stains are consistent across treatment types, and validates our cHC's in our experimental cohorts

**Figure 7.**

**Figure 7. Sox2 CreER; Tdtomato Cocktail Injections Representative Images.** (A) representative image showing a converted hair cells expressing both Tdtomato (red) and Myo6 (green) with the three rows of outer cells directly parallel to it. (B) Close up of the same section shows the converted hair cell cytoplasm expressing by both markers, with its nucleus on the same plane as it's cell body. (C) Orthogonal cross section showing us the same cell with the Tdtomato stain partially obscuring the Myo6 that is clearly seen top down. All converted hairs cells observed had arose from Sox2 expressing supporting cells, proving that it's this population that is most susceptible to conversion.

Figure 8.

## Sox2 CreER cHC Relative Density



**Figure 8. Sox2 CreER; Tdtomato cHC Relative Densities.** Of the 8 Sox2 CreER; Tdtomato mice (n=3 male, and n=5 female), we saw conversion in 2 of the samples (1 males and 1 female). For our control samples, right ears were taken from 2 male and 2 female mice from our cohort and scanned to compare to and validate our experimental samples. We observe a similar number of converted hair cells as the 2<sup>nd</sup> and 3<sup>rd</sup> FVB cohorts, which were less than the 1<sup>st</sup> cohort conducted 5 months earlier. Subsequent analysis of our Sox2 CreER; Tdtomato mice with more careful analysis and more accurate software will be conducted later.

## **Introduction:**

According to the World Health Organization predicts by 2050 that one in ten people will have disabling hearing loss.<sup>1</sup> This number is much higher for those experiencing low to moderate hearing loss, affecting as high as 20% or 1.5 billion individuals, with that number projected to reach 2.5 billion by 2030.<sup>1</sup> Hearing loss is a permanent disability, with human's incapable of restoring their hearing following damage, age, disease, or heredity. A multitude of factors affect a person's individual experience with hearing loss, with certain populations like veterans especially vulnerable due to the nature of their environment contributing to damage or stress to the inner ear.<sup>1,2</sup> Certain hereditary conditions, usually autosomal recessive and nonsyndromic disorders, can also contribute to hearing loss.<sup>2</sup>

Hearing loss is associated with a variety of non-medical related factors. It's been reported in elderly populations that hearing loss is associated with loneliness, isolation, dependence, and communication disorders.<sup>3</sup> Similar instances have been reported that in veteran populations, who in addition to being at risk for mental illness, have extremely high rates of hearing loss due to factors relating to serving such as noise induced hearing loss or traumatic injuries.<sup>4</sup> Hearing loss's effects can be mitigated by interventions, speech therapy, and a variety of other factors, but ultimately these interventions are limited by the scope and severity of the patient's hearing loss.<sup>5,6</sup> Interventions such as hearing aids, cochlear implants, and Bone-anchored hearing aids are all common treatments for hearing loss but fail to fully restore hearing.<sup>6</sup> Many of these common interventions to this disability fail in scope of full restoration of communication, and many individuals have reported difficulties even after therapeutic intervention.<sup>5,6,7</sup>

Complete hearing regeneration is a developing field, with functional recovery still a long way off. To date, there are no treatments to restore hearing loss completely following damage, and current interventions rely on supplementing or implanting new devices to restore some function. Previous publications have used transgenic animal models to elicit specific genetic manipulations of pathways of interest to regenerate immature hair cells. These new hair cells lack the function of hair cells, they have unorganized stereocilia bundles, and lack mature innervation and position.<sup>9,10,11,14,18</sup> A combination of genetic changes have been shown to be successful in producing higher levels of conversion, and in specific papers, have been able to express prestin (a mature hair cell marker) and disordered hair cell bundles.<sup>28</sup> We hypothesize that our combination of compounds A2CE and C18 are sufficient in producing conversion of supporting cells to hair cells in adult mice. Manipulations of one factor failed to produce functional recovery, and it seems that the path of complete regeneration relies on utilizing a combination of manipulations to mimic the complex regeneration of hair cells seen in neonates and non-mammalian species.

### **Anatomy of Ear: Outer, Middle, Inner:**

The mammalian ear serves two primary functions, sensory hearing, and postural equilibrium, which includes coordination of head and eye movements. The temporal bones are overlaid by the sides of the head, known as the temples, and house the structures of the ears. It's made up of three separate distinct parts; the outer ear, the middle ear, and the inner ear. The outer ear is made up of a visible outer portion known as the auricle, or pinna, and the external auditory canal

(or tube) that connects to the tympanic membrane known as the eardrum.<sup>8</sup> The middle ear is a narrow air-filled cavity in the temporal bone, housing the ossicles, canals, and hearing organs, as well as protecting the middle and inner ear structures, which are more delicate. The middle ear is spanned by three tiny bones known as the malleus (hammer), incus (anvil), and stapes (stirrup) which are all collectively known as the auditory ossicles.<sup>7,8</sup> These bones take sound waves collected from the tympanic membrane and conduct them into the inner ear via the ossicles.

The inner ear is made up of two parts: the cochlea and the vestibular systems. The vestibular system is a sensory system responsible for motion, head position, and spatial orientation, which in turn affects dexterity and equilibrium. Without it, it would be difficult to tell up from down. The cochlea is a hollow, spiral-shaped bone found in the inner ear that is responsible for conducting sound waves collected from the outer and middle ear and uses sensory hair cells to transduce sound waves into electrical signals that are transmitted to spiral ganglion neurons and then to the brain where the signals can be interpreted.<sup>7,8</sup>

The outer ear is made up of the Pinna or auricle, the skin and cartilage that makes up the outermost part of the ear, and the external auditory canal or tube which connects the outer ear to the middle and inner ear. The function of the outer ear is to collect sound waves and guide them to the tympanic membrane. The shape of the outer ear is shaped to aid in picking up sound, and for humans the configuration of the outer ear selectively boosts the sound pressure 30- to 100-fold for frequencies around 3 kHz.<sup>7,8</sup>

The middle ear is separated from the outer ear by a thin membrane known as the tympanic membrane, or the more colloquial named, the eardrum. The tympanic membrane both separates the outer ear from the middle ear, but also vibrates in response to noise stimulus and transmits this noise to the ossicles, known as oscillations. The auditory ossicles are made up of three bones: the malleus, incus, and stapes. The malleus is the largest bone and connects directly to the eardrum, which transmits the oscillations to the incus via the handle of the malleus. The incus has a main “body” and two arms, which connect to the middle ear posterior wall and to the stapes. The stapes is the smallest bone in the body and connects the ossicles to the oval window and is known for its stirrup-like appearance.<sup>7,8</sup>

The inner ear is made up of two parts; the bony labyrinth which contains the cochlea, vestibule and three semicircular canals, and the membranous labyrinth which contains the cochlear duct, semicircular ducts, utricle, and the saccule. In addition, there are two openings that connect the middle ear to the inner ear; the oval window that lies between the middle ear and the vestibule connected to the ossicles via the stapes. The round window separates the middle ear from the Scala tympani and is the target for local administration of drugs into the inner ear and the organ of Corti. The membranous labyrinth contains the balance portions of the inner ear, which contains the saccule, the utricle, and semicircular canals, all of which help contribute to orientation and our sense of balance.

The cochlea itself houses the cochlear ducts of the membranous labyrinth and is most known for its distinct shape that spirals up the modiolus in an anterolateral position. The spiral lamina

extends from the modiolus and holds the structure to the cochlear duct. The duct itself is flanked by the Scala vestibuli and the Scala tympani above and below it. The vestibulocochlear nerve (CN VIII) is responsible for innervating inner ear, splitting into the vestibular and cochlear nerves. The organ of Corti is situated on the Scala media of the cochlea and contains three rows of outer hair cells and inner hairs flanked by a number of supporting cells subtypes. These hair cells contain stereocilia bundles, which are stimulated in response to noise stimulus and allow for their tuning capabilities. The outer hair cells help contribute as acoustical amplifiers, which help transmit sound waves to the inner hair cells. Inner hair cells are innervated by multiple nerves, while a single nerve connects to multiple outer hair cells. This innervation helps the inner hair cells transmit sound into electrical signals, which are transmitted to the brain. Supporting cells, such as pillars and Deiter cells, help in development, survival, phagocytosis, death, and regeneration in the inner ear. They perform similar functions to glial cells in other parts of the nervous system.<sup>49,50</sup>

Zooming out anatomically for a moment, an important aspect of the mammalian ear is the blood labyrinth barrier. The blood labyrinth barrier refers to membranous barrier vasculature and the inner ear fluids by either endolymph or perilymph, depending on which area of the ear you are in. Due to the usual ionic fluid gradient of the inner ear, this barrier is crucial for keeping homeostasis and preventing pathogens from entering this closed space. This barrier unfortunately complicates delivery of systemic drugs, so in order to deliver drugs effectively to the inner ear we must either overcome this barrier or deliver the drugs locally.<sup>51,52</sup>

**Regeneration: Mammal vs. Non-Mammal:**

While adult or mature mammals lack regeneration of hearing, many other species of animals do have the ability to restore hearing after damage which are important to understand and study in order to develop a framework for mammalian regeneration. Avians, like chickens, retain the ability to restore hearing following damage. Regeneration of any tissue is typically associated with rapid proliferation of progenitor stem-like cells, which are grown by the organism and directly differentiate into the lost cell type, such as regeneration of lost tissue from damage.<sup>20,61</sup> However, studies into the mechanism of avian sensory epithelium have revealed an additional mechanism of regeneration. Rather than rapid proliferation of stem cells that eventually terminally differentiate into cells as needed, an endogenous population of non-sensory supporting cells in the inner ear were found to transdifferentiate directly into new hair cells.<sup>20,52,61</sup> Supporting cells are a population of cells that help facilitate normal function of the sensory epithelium by providing non-sensory support to endogenous hair cells, hence their name. This is interesting for a variety of reasons.

Normal differentiation of cell types arises from a population of pluripotent stem cells which first proliferate then begin differentiating into whatever subtype of cell is needed for the organism for development or normal function. Previously, it was thought that supporting cells, in response to damage to hair cells, re-entered the cell cycle where they would proliferate and split into either hair cells or supporting cells to maintain the integrity of the sensory epithelium.<sup>20</sup> However, a secondary mechanism was discovered in avians called direct transdifferentiation. Rather than re-enter the cell cycle, the supporting cells were observed to change their gene expression into hair cells without ever entering the cell cycle and reverting to a more stem-like cell.<sup>20</sup> These two

mechanisms were found to have distinct spatial and temporal paradigms and are differentially regulated from each other.<sup>20,61</sup> This is interesting in the scope of mammalian regeneration for a few reasons. First, this presents two differing methodologies for regeneration in the mammalian sensory epithelium. Both paradigms of regeneration are distinct in how they restore lost hair cells, so when designing therapeutics, it's crucial to employ one of the mechanisms in order to best maximize success of treatment. Second, because the avian system utilizes a combination of repair pathways rather than just one, it points to the possibility that both systems are necessary in some way for the ability of avians to restore damaged hearing, so subsequent studies should explore both pathways in some regard for the best chance at full mammalian restoration.

### **Regeneration Theory: Proliferation vs. Direct Transdifferentiation?**

There are two schools of thought for regeneration strategies of hair cells in the inner ear:

Proliferation or Direct Transdifferentiation. Proliferation is the traditional model thought to be the underlying mechanism for regeneration of hair cells in non-mammal species, though the discovery of an additional mechanism via Transdifferentiation brings an interesting question of what mechanism should be utilized in successful regeneration of mammalian sensory hair cells? Both mechanisms are viable options, however, here we'll take a closer look at the reasoning for employing direct transdifferentiation for our drug-induced hair cell regeneration, rather than proliferation.

No present regeneration is seen in sensory hair cells in mammals following loss from noise or damage, however that is not true for all stages of development. Neonatal mice have been shown

to have spontaneous regeneration of hair cells following damage, and this regeneration is lost following maturation, although many newly generated hair cells were eventually lost.<sup>14</sup> Before this discovery, it was assumed mammals lack endogenous repair pathways for hair cells once they differentiate and become permanently post-mitotic, that this damage is permanent and the path to functional recovery relied on creating a brand new mechanism for regeneration from stem-like cells or implantation of new populations given the limited available pool in the inner ear.<sup>13,14</sup> However, the discovery of an endogenous developmental regeneration pathway means that rather than trying to retrofit a new mechanism with a higher chance at failure, we can maximize our chances by taking advantage of the endogenous mechanism. If mammals already have spontaneous regeneration of hair cells as neonates, we should use this as a model for deciding which of the regeneration theories. Fate mapping from the same study revealed that all newly formed hair cells had arisen from nearby supporting cell populations.<sup>14</sup> Knowing that the spontaneous regeneration is facilitated via direct transdifferentiation of supporting cells, a population of supporting cells exists in the inner ear that express stem cell-like markers that should make them more likely to transdifferentiate, and transgenic models of mice have proved manipulations of key pro-hair cell fate factors can push supporting cells towards hair cell fate, means that this method of regeneration will most likely produce the first drug-induced hair cell regeneration in adult mice. Of the supporting cell populations, Lgr5 expressing 3rd row Deiter cells are the cell type we are most concerned with. Lgr5 is a stem cell marker, and highly expressed in the third row of Deiter cells at adult ages, and due to expression of other stem cell markers like Sox2, makes this cell population the most progenitor-like of all available cells.<sup>12</sup> Stem cell markers, such as the Yamanaka factors, have been shown to be the master regulators of hair cell fate.<sup>54</sup> Expression of these markers gives stem cells their pluripotency, so finding cells

with similar markers increases the likelihood that our manipulations will influence the cell to change its transcriptome and transdifferentiate.

### **Pathways to Mammalian Regeneration: POU4F3, Atoh1, p27 and Lgr5 expressing 3rd row Deiter cells**

Previous studies into hair cell regeneration have identified a host of relevant pathways of interest that are crucial for normal hair cell development and are clinically relevant to our studies.<sup>9,10,11,18,28,42,43,44</sup> Atoh1 is a basic pro-neural helix-loop-helix transcription factor which is required for normal development of hair cells in mice and fish. This transcription factor has a co-factor, GATA3, which is important in regulation and expression of Atoh1.<sup>9</sup> Atoh1 expression represents the pro-sensory region in the development of cochlea. Its role as a master regulator for sensory hair cells and in regeneration makes this gene and its related pathways crucial for direct transdifferentiation.<sup>11</sup> Atoh1 is first expressed in neonatal mice around E 12.5, and eventually guides the sensory domain of cochlea development until about E 17.5, with its expression required for hair cell maturation including stereocilia bundles.<sup>16</sup> Most notably, this transcription factor was found to be sufficient in converting endogenous supporting cells into hair cells.<sup>42</sup> Hair cells converted via Atoh1 in adult mice were sparse and immature, lacking important genes such as prestin and functional recovery of hearing.<sup>9,10,11</sup> Neonatal mice have some capacity for spontaneous regeneration of hair cells following damage, but this regeneration is lost after one week of age. This observation in neonatal mice is interesting because of the mechanism of regeneration, with researchers finding through fate-mapping that the newly regenerated hair cells arise from neighboring supporting cells.<sup>14</sup>

POU4F3, or POU class 4 homeobox 3, is a POU-domain family of transcription factors that has important roles in control of cell identity in several systems, including the retina and the inner ear.<sup>9,21</sup> Loss of this gene's function has also been associated with a variety of non-syndromic sensorineural deafness. POU4F3 has been shown to be a pioneer factor of Atoh1, meaning it allows Atoh1 to access more of its enhancer network in developmentally closed chromatin.<sup>21</sup> In addition, previous studies have shown that POU4f3 is sufficient in producing converted hair cells from genetically manipulated overexpression in supporting cells.<sup>9</sup> POU4F3 is expressed in mature hair cells, unlike Atoh1, and its persistent expression in mature hair cells makes it a potential biomarker for identifying hair cell populations in RNA sequencing or immunochemistry. Due to this gene's expression in mature cells, and its expression alone can produce converted hair cells, it makes this a strong target for therapeutic treatment.

P27kip1 (p27) is a member of the universal cyclin-dependent kinase inhibitor (CDKI) family that canonically regulates the cell cycle and proliferation. It is known that p27 regulates quiescence in HCs and SCs, with a negative association with regeneration in other tissues.<sup>19,21,23</sup> P27 was found to enhance regeneration of converted hair cells via Atoh1 overexpression from supporting cell populations when knocked out in transgenic mice.<sup>9</sup> While the role of p27 is traditionally understood in the cell cycle, when canonical downstream targets Rb1, p107 and p130 were inhibited, they saw no change in the level of conversion seen with the p27 knockout, meaning a new non-canonical function of p27 was discovered to affect co-factor GATA3 and its inhibition enhanced the regenerative effects of Atoh1 and POU4f3 overexpression.

### **LGR5 Expressing Supporting cells: 3rd row Deiter Cells**

Of all supporting cell types, we hypothesize that Lgr5 expressing 3<sup>rd</sup> row Deiter cells are the most susceptible to direct transdifferentiation into converted sensory hair cells. Lgr5 is a stem cell marker, the encoded protein is a receptor for R-spondins and is involved in the canonical Wnt signaling pathway.<sup>12</sup> Lgr5 plays a role in the formation and maintenance of adult intestinal stem cells during postembryonic development and is lethal in mouse knockouts. Lgr5 is expressed in mice at E15.5 with other prosensory markers, nascent hair cells, and supporting cells express this marker at 18.5 before being downregulated in all cell types following maturation, except for 3rd row Deiter cells.<sup>12</sup> Lgr5's role in development of sensory cell types hints at its role in influencing progenitor sensory cells and its expression in 3rd row Deiter cells means this cell type might retain enough pluripotency to be available for conversion.

### **Drug Selection and Pharmacokinetics of Drug Delivery:**

The inner ear is a spatially complex system, with large fluid-filled extracellular spaces known as Scalae, each with multiple interfaces with other Scalae and with outside compartments, not to mention the change of medium between the inner and middle ear. The middle ear is an air-filled space while the inner ear is a fluid filled space, which affects our methods of delivery. Because the inner ear lacks the constant “flow” that most other body fluids have, it makes delivery of drugs slow and usually via passive diffusion.<sup>8</sup> Diffusion of drugs is heavily dependent on its physical characteristics and can change the rate of diffusion depending on permeability.<sup>24,25</sup> On top of this, the blood labyrinth barrier prevents systemic access to this space for delivery of

drugs, meaning all our drugs need to be delivered locally in the inner ear for regenerative therapeutic effect. For our experiments, we have elected to utilize transtympanic injections of our drug cocktail.

Transtympanic injections involve delivery of the drug through the eardrum, where the fluid permeates the middle ear space, and diffuses across the round window. Due to the possibility of leakage through the eustachian tube, injections were only given in the left ears similar to those done in humans, and mice were left to recover with their ears up to encourage maximal round window coverage and diffusion. In addition, due to the semipermeable membrane diffusion rate of the round window and in order to deliver enough drug to have our desired effect, our injections are limited to one ear at a time in mice. Active processes for the round window have been assumed for large molecules and other complex proteins but have not been confirmed so far.<sup>24,33</sup> Simultaneous applications of substances via the round window have been reported to affect round window permeability, although the effect of our cocktail specifically needs to be analyzed in a future study.<sup>55</sup>

Knowing the importance of manipulations of pathways to push supporting cells towards conversation leads to questions about translatability of therapies and studies that have shown regeneration of hair cells. Most manipulations, presently, have been conducted in transgenic mouse lines. These mice lines are great animal models for study, but ultimately one weakness of them is their lack of clinical translation. While the discovery of key pathways of interest are

crucial first steps in developing regenerative hearing therapies, genetic manipulations are not techniques that are widely approved or available to patients. Development of small molecules and other drugs that regulate these pathways is critical for clinical applications for human restoration of hearing.

p27kip1 (p27) is a gene of interest we seek to target with our drug cocktail. Its negative association with proliferation and regeneration of converted hair cells means that by inhibiting this gene we can enhance the regenerative effect of the other drugs in our cocktail. Previous publications have conducted high-throughput drug screens of clinically relevant drugs to find drugs and small molecules with the best therapeutic effect, with one top drug in particular shown to produce the most potent inhibition of p27 (IC<sub>50</sub>=200 nM).<sup>56</sup> Due to the intrinsically disordered nature of the protein, direct inhibition of the protein is extremely difficult, so screening for transcriptional inhibition was much more viable.<sup>56</sup> Preliminary experiments have shown that the transcriptional inhibition of p27 via A2CE (100 μM) was roughly equivalent to a complete knock out of p27, and when combined with Atoh1 overexpression in supporting cells, produced converted hair cells. Here, we aim to combine the A2CE with our other drug in the cocktail, C18, to reproduce the converted hair cell phenotype using only our cocktail in adult wildtype mice.

Compound 18 is a drug that directly upregulates POU4F3 expression in affected cells. POU4F3 is a downstream target of Atoh1, and due to its function as a pioneer gene, helps contribute to the enhancer network of Atoh1 and should therefore make the cells more susceptible to direct

transdifferentiation. We chose to pursue drugs that target POU4F3 for several reasons. First, POU4F3 is expressed in endogenous mature hair cells. Meaning, the chance of unintended side effects of expressing the developmental gene Atoh1 is reduced, such as cell death. Second, POU4F3 was found to be sufficient to produce converted hair cells alone, and the regenerative effect of p27 inhibition was more pronounced in POU4F3 overexpression than Atoh1.<sup>9</sup>

Preliminary data on C18 suggest that it produced a significant increase in POU4F3 expression following transtympanic injections. And following injections, the mice were observed to have regeneration of converted hair cells, albeit at very low levels. We hope by introducing an inhibitory effect on p27 that we will substantially increase the level of regeneration.

## **Experimental Methods:**

### **Preliminary Data: Single cell RNA-Sequencing + RT-PCR:**

Prior to drug-induced hair cell regeneration in adult mice, we conducted preliminary experiments to test efficacy of A2CE through two experiments, RT-PCR and Single Cell RNA-Sequencing. C18 testing was completed independently through a different researcher. The Single Cell RNA-sequencing dissection and sample collection for analysis was completed by separate individuals, with data analysis and filtering conducted afterwards by me. RT-PCR was conducted to validate the therapeutic time point for transcriptional inhibition of A2CE.

Adult C57 mice (P28) of both sexes were given 5mM A2CE via transtympanic injections to the left ear. After waiting 24 hours (n=5), mice were euthanized and the inner ear organs of Corti were collected and harvested for RT-PCR. p27 mRNA was normalized to a housekeeping gene. Basilar membranes were isolated at 4 hours (n=15) for single cell RNA-seq and all steps for the cDNA library were performed following 10x Genomics protocols, with Chromium Next GEM Single Cell 3' Reagents Kit v3.1. Illumina Nextseq and raw counts were processed using Cell Ranger (v6.0.1). Single-cell data was analyzed in R studio and clustered with Seurat (v4.0.4).

### **Animal Models:**

We used two different strains of animals in our experiment; FVB and Sox2 CreER; Tdtomato. FVB is a laboratory albino mice strain, named after its susceptibility to Friend leukemia virus B. These mice are defined by their homozygous retinal deterioration allele of the PDE6B, which

results in blindness by weaning age. Their large litter sizes make generation of mouse lines ideal, and their oocytes have pronounced pronuclei which makes them ideal for any transgenic research.<sup>30,48</sup>

30 mice (males n=14, females n=16) total were tested, with 3 rounds of FVB mice (n=22, equal amounts of male and female mice) and one round (n=8) of Sox2 CreER; Tdtomato (male n=3, female n=5). All mice ages were around ~P28, with some Sox2 CreER; Tdtomato mice slightly varied in ages due to breeding, but all mice were less than 2 months in age. Mice were housed in Creighton University Animal Research Facility, and animal care has been handled by Animal Research Facility staff. Mice were maintained in same sex cages, in addition to soft bedding and nesting materials for animal comfort. Food and water were unrestricted, with a consistent 70 degrees temperature and 12-hour day/night cycles. All procedures and housing conditions were approved by Creighton institutional IACUC.

### **Transtympanic Injections:**

Drug cocktail was prepared from stock solution into working solutions prior to each round of injection, to minimize potential for drug deterioration. Each working solution was diluted to 100uM A2CE + 500 uM of Compound 18, all dissolved in 40% DMSO. Mice were given intraperitoneal injections of (Drug) at (concentration). Mice were carefully observed until anesthetized and transferred to sterile surgery space on their right sides with left ears facing up. Mice eardrums were exposed using forceps, before a small plastic stabilizer was inserted to prevent ear flaps from inhibiting visualization. Two small incisions were made in the tympanic

membrane, one made inferior and dorsal for the injections and another more superior but anterior to give the normally air-filled space an exhaust port to escape from. Hamilton needles were loaded with 5 uL of drug cocktail and inserted into the injection incision where the fluid collects in the middle ear. Once the drug has been completely injected, or the middle ear space is completely filled and escaping out the exhaust incision, the Hamilton needle is removed, and the mouse is returned to the cage. The mice are placed with their left ear facing up in recovery to maximize round window diffusion, and the cage is heated for comfort and recovery. Injections were identical for all FVB cohorts and Sox2 CreER; Tdtomato mice.

#### **Tamoxifen injections:**

Stock tamoxifen was taken from the freezer and dissolved in corn oil at a concentration of 15 mg/mL at 37-degree Fahrenheit using an automatic roller for an hour. Tamoxifen is light sensitive and was covered in aluminum foil to prevent decay. Once the sample was adequately dissolved in solution, it was filtered, sterilized, and allotted to a separate tube for use. Sox2 CreER; Tdtomato mice were then given intraperitoneal injections of tamoxifen at 250 mg/kg dissolved in corn oil, with each mouse weighed before injections to determine how much tamoxifen is delivered to each animal. Injections were given one-week prior to transtympanic injections, to allow for Cre-Lox recombination to elicit phenotype in Sox2 expressing supporting cells. Previous studies have utilized several tamoxifen injections prior to experiments, but a specific study using Sox2 CreER; Tdtomato found a single 250 kg/mg injection sufficient to express phenotype.<sup>31</sup> Animals were returned to the animal room following injections, and cages were changed 7 days following injections, per SASP protocol.

**Whole Mount Immunofluorescence:**

4 weeks following injections, mice cohorts were collected and euthanized per approved IACUC protocol. Temporal bones were exposed by bisecting the cranium medially and removing the brain without damaging the sides of the skull where the temporal bones rest. Cranial nerves were scraped away and using tweezers we carefully pulled the otic capsule from the cochlea, making sure to keep forceps on the posterior semicircular canal as we do so. Once the otic capsule separates from the cochlea, we use our thumb and forceps to gently pry the temporal bones from the skull, making sure not to damage the overall structure. Extracted temporal bones were then fixed in 500-1000 uL of 4% Formaldehyde diluted in 10mM PBS (pH=7.4) and incubated overnight (~14 hours). Some studies have suggested making an incision in the apical of cochlea, but it is unnecessary for proper fixation.<sup>57</sup> Following fixation, temporal bones were then decalcified with 2 mL of 120 mM ethylenediaminetetraacetic acid (EDTA) placed on an end-over-end rotator at 4rpm at room temperature. Because our samples are adult aged, EDTA was changed daily for at least 3 days, with some samples requiring an additional day of EDTA to completely decalcify. For samples longer than 3 days, 1% PFA was added to prevent decay. Each sample was tested for decalcification before being transferred to PBS for dissection.

In a silicone elastomer dish, cochlea were carefully held down by their vestibular system with forceps and using scissors insert one blade into the oval window and make several small cuts along the spiral ligament. This exposes the basal turn of the organ of Corti and separates the middle and apical sections of the cochlea from the basal turn which is still connected to the

vestibular system. Putting aside the top part of the cochlea, separate the exposed basal turn from the rest of the vestibular system using scissors. We made sure to cut away spiral ganglion nerve fibers from modiolus to remove some tension from the basal turn. We cut away the spiral ligament from both above and below the organ of Corti, in addition to removing any remaining Reissner's membrane that may be covering the sensory epithelium. Finally, we reduced the thickness of the spiral ganglion axons as much as possible to let the sample lie flat when mounting later.

For the middle and apical cuts, we took the top part of the cochlea isolated earlier and placed it apical down. We placed the scissors in the Scala media of the sample where the basal turn used to rest, and carefully cut along the spiral ligament of the cochlea. We then placed our scissors along the area we just cut we just made and outside the bony labyrinth (at a 90-degree angle from the apical tip) before making a single cut to separate the two turns. Dissections were then conducted similar to basal turn, reducing lateral wall coverage from sensory epithelium where hair cells rest, and removing any leftover Reissner's membrane and spiral ganglion axons. Samples were then placed in 1 mL PCR tubes in PBS.

Once the turns were isolated and dissected, we moved onto immunofluorescence. For the FVB samples we stained for 3 antibodies; Sox2 with Alexa 568 (Conc. 1:200), Myo6 with Alexa 647 (Conc. 1:500) and either DAPI or Hoechst stain (Conc. 1:800) for nucleic DNA. For Sox2 CreER; Tdtomato mice we utilized the endogenous Tdtomato signal in Sox2 expressing supporting cells, Myo6 with Alexa 647 (Conc.1500), and Hoechst (Conc. 1:800). Samples were

first placed blocking/permeabilization solution (1% Triton X-100, 1% bovine serum albumin (BSA), and 10% normal goat serum (NGS) diluted in 10 mM PBS pH 7.4) for 2 hours at room temperature. After waiting the eclipsed time, samples were replaced with our primary antibody solution (0.1% Triton X-100, 1% BSA, and 5% NGS diluted in 10 mM PBS pH 7.4) overnight (minimum 14 hours) at 4 degrees Celsius. After a minimum 14 hours, samples were washed 3 times for 10 minutes in PBS to remove leftover primary antibody from the sample. We replaced the PBS wash with a secondary antibody solution (0.1% Triton X-100, 1% BSA, and 5% NGS diluted in 10 mM PBS pH 7.4) and incubated for 2-3 hours at room temperature, making sure to cover samples from light. We repeated the PBS for 10 minutes three times, before placing samples in ~100  $\mu$ l per well of Hoechst 33342 (diluted 1:500 in 10 mM PBS pH 7.4) to label nuclei for 10-15 minutes. We repeated the same wash in PBS as the previous antibody solutions and maintained a dark environment to prevent light bleaching of antibody labels.

Once samples were labeled, they were mounted on slides. Pertinent information was included for future reference, ~50 microliters of mounting media was placed on the slide with the sample transferred carefully into the solution. Stereo microscope was used to make sure sample orientation and position was correct, and double check to make sure parts of the lateral wall or spiral ganglion neurons aren't covering the sensory epithelium or any bubbles were near the sample. The coverslip was glued on the corners, and gently placed over the sample making sure the sample doesn't twist or misorient. Once the sample was covered correctly, clear nail polish was used on the edges to seal the slide and maintain long term integrity of the sample.

**Sample Analysis (Confocal):**

Samples were imaged using either Carl Zeiss LSM700 or an LSM710 point scanning confocal microscope. Slides of antibody treated samples were analyzed and scanned using ZEN software packages (Carl Zeiss), with additional analysis conducted using IMARIS proprietary software. Settings between samples were reused on the software to keep data analysis between scans of samples as consistent as possible. Antibodies and dilutions used in immunostaining are available in Table 1. Immunopositive cells were manually counted from images of whole mount samples using ZEN Black software packages, with successful conversion of a supporting cell defined by expression of a Sox2 + Myo6 immunolabel for FVB mice and Myo6 + Tdtomato expression in Sox2 CreER; Tdtomato mice. Cells expressing both markers were manually evaluated ornithologically to confirm the signal, in addition to 3D reconstructions using IMARIS software when appropriate. Converted hair cell counts were obtained from three representative images (average: 160 nanometers  $\pm$  3 nanometers) from each of the three cochlea cuts; basal, middle apical. Each image was separated by roughly equal distances along the axis from basal to apical. Densities of positive converted hair cells per micrometer were then extrapolated from total cochlear length assuming an average of 6mm for each mouse cochlea. Some samples lacked all three cuts of the cochlea due to dissection issues and extrapolated densities were adjusted for loss of cochlear length.

## **Results:**

### **Single Cell RNA-sequencing + RT-PCR:**

In vivo transtympanic administration of 5 mM A2CE at the 24-hour time point showed significant reduction of p27 in RT-PCR analysis of cochlear sensory epithelia. Single Cell RNA-sequencing at the 4-hour time point following injections resulted in clear clustering of the supporting cell (SC) and hair cell (HC) populations (IHC: 189 cells, OHC: 468 cells) with sensory epithelium populations revealed using differentially expressed genes. A total number of 26118 samples were collected across both treatments, with 19,201 genes detected, after manual filtering of raw counts based on nCount and nFeature with mitochondria percentage  $35\% <$ . No significant reduction was detected in p27kip1 between the vehicle (15% DMSO) control or A2CE injected drugs in any relevant cell populations. RT-PCR data showed that transcriptional inhibition of p27 by A2CE takes at least 24 hours for the effect to be significant, and the single cell data at the 4-hour time point corroborates this.

### **FVB Cocktail Injections:**

Of the 22 FVB mice that were analyzed, 3 were excluded due to complications unrelated to Cocktail injections. Mice cohorts had an equal number of male and female mice. Of the 19 total mice across three separate cohorts of n=5, n=8 and n=6 respectively, 4 mice were seen to have some regeneration, with the remainder observing no conversion. For each of the treatment types, 4 right sided cochlea were scanned using the confocal microscope to validate and compare to experimental samples. In those samples, a majority of our mice positive for converted hair cell regeneration came from our first cohort of FVB, which were injected at a different time than the

other two cohorts. Migrating converted hair cells appear to move toward the same plane as the hair cells, into the area directly next to the 3rd row OHC's. We observed a relative density of converted hair density of 33, 16, 30 and 8 respectively. We observe a stark difference in densities between the first and the subsequent cohorts, suggesting some change in factors are affecting our conversion rate. Fluorescence between endogenous hair cells and converted hair cells is noteworthy, with much less expression levels seen in our converted samples, which could be due to their immaturity. Contralateral ears were used as negative controls due to presence of blood labyrinth barrier significantly limiting possibility of cross contamination. No distinct differences in sex emerged in response to drug treatment, with each of our cohorts having roughly equal male and female mice. We observed both male and female response to drug treatment, in addition to both male and female mice of our 15 samples without any reported conversion.

For our first cohort of mice, we observed on average 1 converted hair cell per cut for those that were positive, with a few samples expressing more than others. The 3rd round cohort produced no noticeable conversion of 3rd row Deiter cells, which contributes to the huge variance seen in our samples. Some samples did not have all three turns of the cochlea available for analysis due to mounting and dissection issues, particularly the early cohorts. While the efficacy of the drug itself was less than desired, the number of converted hair cells in our samples that were positive had decent results. Our supporting cell conversion mostly took place in basal and middle turns, with little to no conversion detected in the apical turn of cochlea. Some cuts from our samples were lost due to dissection issues, so some samples only had one or two total cuts to analyze which could contribute to results. No significant supporting cell damage or loss was observed in our samples, unless they were from clear mounting or dissection damage.

Analysis of this data set was conducted on a strict timetable, without much room for careful analysis of each section of the cochlea. Subsequent analysis of the data will be conducted with more precise and advanced imaging software and senior researchers, who will be able to look over and evaluate the data later, and provide more careful analysis at a later date. Additional factors such nucleus size and position, which were unable to be collected due to time issues, will be included in this subsequent analysis and should provide more evidence to the efficacy of the drugs.

### **Sox2 CreER; Tdtomato Cocktail Injections:**

Of the 8 Sox2 CreER; Tdtomato mice (n=3 male, and n=5 female), we saw conversion in 2 of the samples (1 males and 1 female). For our control samples, right ears were taken from 2 male and 2 female mice from our cohort and scanned to compare to and validate our experimental samples. Our relative converted hair cell density for our samples were 8 and 4 respectively, extrapolated from a 6mm cochlear length. We observed a majority of conversion in the middle and basal turns, with little to no conversion in apical turns of the cochlea. Due to basal turn dissections, some turns were unable to be analyzed due to 3rd row Deiter loss and may have affected our outcomes. No unintended cell death or loss was observed in any samples between cocktail or non-injected controls in relevant cell populations. Similar to FVB samples, 2 contralateral ears were tested alongside drug treated samples with reported no unusual or unintended staining of hair cells with Tdtomato or supporting cells with Myo6.

All Sox2 expressing cells following tamoxifen injections were Tdtomato positive, with no reported expression of Tdtomato in other cell types. Myo6 staining was weaker for converted hair cells than endogenous hair cells, but above background. No converted hair cells were observed to be in migrating towards the hair cell layer without Tdtomato stain and Myo6, if any non-Sox2 expressing cells had converted and migrated towards the OHC rows, they would have expressed only Myo6 and Hoechst nuclear staining. Similar to other studies, migrating converted hair cells appear to move toward the same plane as the hair cells, so if any cells were converting, they would appear as a “4th row” of Myo6 expressing cells lacking the Tdtomato marker.

Analysis of the Sox2 CreER; Tdtomato mice were subject to the same time constraints as the FVB cohorts, and as such will be carefully analyzed independently in subsequent experiments related to A2CE and C18 drug cocktail. Additional factors such as nucleus size and position will be collected as well as utilizing more precise imaging software which will allow for faster and more precise identification of converted hair cells.

## Discussion:

Our hypothesis that our drug cocktail containing a combination of A2CE and C18 delivered locally into the inner ear would produce converted hair cells regeneration in adult mice, while it did succeed in producing converted hair cells, is hindered by the high amount of variance. While some our results were positive, the efficacy of the drug itself still needs to be addressed with many samples of our cohorts producing no conversion of hair cells. Of our 19 analyzed FVB samples, 4 samples produced converted hair cells. Of our Sox2 CreER; Tdtomato, we saw conversion of supporting cells to hair cells in 2 of our 8 samples. Relative converted hair cell densities were utilized due to the technical hurdles of confocal analysis. It's time restrictive to analysis each section of the 6 mm cochlear length one 160 micrometer image per section. Instead analyzed 9 images equidistant along the cochlea dissections and extrapolate a relative density in order to account for any sections of cochlear that do have conversion, but we missed in data collection. In addition, due to time constraints, analysis of the data set was limited in scope and scale. Subsequent analysis of the data set to more carefully evaluate the samples still needs to be conducted, specifically utilizing more factors such as nucleus size and more advanced visualization confocal software, which may reveal many more conversion of hair cells in our samples. The imaging software used for these samples used ZEN Black, but other imaging software's such as IMARIS allow for more precise visualization and 3D imaging of sensory epithelium, which allows us to visualize and identify cHC's much easier and straightforward.

Why did we see a high amount of variance across samples? A variety of factors need to be evaluated when explaining the data. One explanation for the high amount of variance seen in our

samples is drug distribution through the inner ear. Our experiments utilized transtympanic injections through the tympanic membrane into the middle ear, which has been reported effective for a variety of different compounds, but not our two drugs specifically.<sup>24,25,33</sup> Due to the presence of the blood labyrinth barrier, local delivery of drugs to the inner ear is difficult. Middle ear injections of drugs rely on diffusion across the round window membrane, which has been hypothesized to have active mechanisms, but mainly relies on simple passive diffusion for the majority of transmission. The eustachian tube is an opening that connects the middle ear with the nasal-sinus cavity, and its potential factor that can explain our variance. Because our injections are delivered into the middle ear, the possibility of drainage into the eustachian tube and subsequent loss of drug cocktail could help explain the high amount of variance.<sup>24</sup> Avertin is a systemic anesthetic that is given via intraperitoneal injections in mice prior to injections. Avertin allows us to keep an animal anesthetized during the entire procedure and lets us use gravity to aid in diffusion across the round window. Mice respond differently to dosage of this drug, with some mice able to recover relatively quickly with others taking longer to recover.<sup>58</sup> This recovery time could explain the variance among samples, if a mice recovered too quickly from Avertin, they could inadvertently increase the amount of drug lost to the eustachian tube by getting up and moving around. Mice who were more likely to recover slowly therefore should have had more time for our drug cocktail to permeate the inner ear. We observed this phenomenon amongst our mice, with some mice in our cohort recovering relatively quickly, and others take much longer than their littermates. Unfortunately, we lacked the foresight to track mice recovery times, and subsequent experiments should document mice recovery times to address this variable.

Another factor to consider when delivering drugs across a semipermeable membrane, such as the round window, is drug permeability. Drug permeability is determined by a number of factors including physicochemical properties (such as lipid solubility), drug formulation, and the route of administration.<sup>24,25</sup> It's also affected by the physicochemical properties of the membrane itself. In addition, several studies have shown that round window membrane permeability is affected by simultaneous applications of other substances in addition to the drug properties of each compound separately.<sup>24,55</sup> Which means, that even if characterization of a drug is established before this simultaneous application, by adding the drug to a cocktail, the properties of the substance in this new solution could complicate its efficacy. Both drugs in our drug cocktail were tested separately, with A2CE being combined with Atoh1-HA overexpression and C18 tested alone, both in adult mice. Combining both drugs to a single dilution in DMSO could affect its efficacy of its constituents. Preliminary data utilized 15% DMSO when testing A2CE alone in adult mice, with C18 using 40% DMSO due to solubility issues. These solubility issues in C18 carried over into the drug cocktail concentrations as well. DMSO is a solvent commonly used in drug formulation because it is aprotic, relatively inert, nontoxic, and stable at high temperatures.<sup>35</sup> Even though it has also been reported to increase absorption through dermal or oral routes, drug permeability is heavily affected by minute changes in drug formulation and properties and could be an avenue to help explain our results.<sup>49,59</sup> This change of DMSO concentration could affect the therapeutic efficacy of A2CE, which may have unresolved issues in a higher concentration of solvent. This problem is exacerbated by our delivery method. Due to reliance on passive diffusion across the semipermeable round window membrane, any changes to the properties of the drugs will affect their penetration into the inner ear. This drug penetration problem is a significant barrier to overcome when relying on local drug delivery to the inner ear,

and when developing experiments in future studies, should be made a priority in understanding and improving this therapeutic treatment.

Another factor that could have influenced our results was the time of injections for our cohorts. Our initial cohort (n=6) of FVB mice were injected in the fall, in October, while the additional cohorts (n=6 and n=8 respectively) were injected in the spring (March). Mice have been reported to have differential responses to the changing of the seasons, with reported changes in behavior and physiology. One explanation for our results might be due to time of year, with specifically the FVB strain responding much differently to the drugs under disparate seasonal conditions.<sup>30,60,61</sup>

Finally, let's explore apoptosis's role in the lack of conversion seen in our samples. Several studies have been successful in producing immature hair cells following genetic manipulations of key pathways, Walters<sup>9</sup> specifically described seeing converted hair cells undergoing clear apoptosis and cell death. Due to the nature of the immature converted hair cell morphology, the possibility of loss of converted hair cells following manipulations is very possible. For our timeline, we chose to allow 4 weeks following injections in order to mimic the regeneration scheme of other non-mammals, such as avians which require 3 weeks or longer for functional recovery. One interpretation of our data may be catastrophic loss of converted hair cells some time before our 4-week timepoint. Supporting cells that begin migrating towards the hair cell layer and expressing hair cells markers may begin experiencing apoptosis, and by the time we go to collect our samples for whole mount analysis, miss the window of opportunity to determine

whether we saw conversion. If this interpretation is correct, then our immature converted hair cells may be unable to function normally, and we need to explore other options to help stop this loss of converted hair cells. Due to the complex nature of adult regeneration, the possibility of the opposite may also be true, because we're retrofitting a novel regeneration pathway it may take longer for supporting cells to successfully convert. And in order to produce better results we may need to extend the time we allow for regeneration to take place.

Why did our drug fail to produce significant conversion of hair cells? When evaluating our results, it's crucial to identify potential shortcomings that could have contributed to a negative result for our drug cocktail. One factor that could affect our results is drug quality. For our experiments, each cohort of mice were given injections of our drug cocktail from working solutions made from a frozen stock solution. Each preparation was made the day before injections, with nearly identical conditions for each preparation. However, one potential issue could be degradation of the stock solution. The stock solution was acquired several years ago, and each time a working solution was prepared the possibility of degradation of stock increased with each subsequent removal from the fridge. By the time we made more working solutions for subsequent cohorts in the spring, the drugs quality could have been degraded and affected the therapeutic effect of the cocktail. The reduce number of reported hair cells in later cohorts supporting this conclusion, and even in the samples that did produce conversion we observed a lower number of cHC's on average.

In addition, one explanation for the lack of positive results could be the efficacy of C18 itself. This drug has been hypothesized as enhancing POU4F3 expression enough to enact changes in vulnerable supporting cells, however the further examination of the preliminary data reveals a limited regenerative effect. Of the total of 4 adult FVB mice injected with C18, we only see 7 converted hair cells total, with less than one converted hair cell per cut of cochlea. Meaning, the desired drug efficacy of C18 may be too low for consistent clinical use. In fact, the initial preliminary data may be affected by the same amount of variance across samples we're seeing in our cohorts. Due to the low number of samples tested in C18 alone, it may be best to look for derivatives of the drug to see if changes in drug structure could enhance the therapeutic effect and reduce variance across samples.

Future directions for this study would include a variety of experiments. Firstly, our results suffer from a lack of time available to conduct a perfect analysis of every section of cochlea samples. Subsequent experiments using this experiment should carefully comb through sections of our cochlea using more advanced imaging software, which should make visualization of the entire sensory epithelium much more streamline. IMARIS utilizing 3D reconstructions with our fluorescent antibodies, which makes visualization of cHC's in a 3D section much easier than what's available in ZEN Black. Doing so might reveal much more conversion of supporting cells than reported in this thesis and affect the overall efficacy of the drug cocktail.

Second, the high amount of variance needs to be addressed for this drug cocktail. There are a multitude of factors that could be influencing our results, but highest priority would most likely

be drug analysis and permeability. Round window membrane permeability remains a critical aspect of local delivery of drugs, and due to the nature of transtympanic injections into the middle ear, analyzing how well our drug penetrates the inner ear is crucial for determining its efficacy. Perilymph sampling or Liquid chromatography–mass spectrometry (LC-MS) could help determine how far our drug is penetrating the sample, if at all, and to which section of the cochlea. A potential study may focus on changing the medium of delivery rather than the drug cocktail itself to increase its efficacy, such as utilizing polymers, nanoparticles, or more invasive techniques like intracochlear injections.<sup>24,25</sup> These alternative delivery routes all have their benefits and risks and need to be validated individually to determine effectiveness.

In addition to optimizing delivery routes, it's important to explore addition of drugs or small molecules to our cocktail to enhance its regeneration. *Ikzf2* is a gene of interest that has been successful in expressing prestin, a mature hair cell gene required for normal stereocilia bundles and hearing, in converted hair cells, which is an improvement from previous hair cell regeneration papers.<sup>28</sup> Exploring drugs that interact with this gene of interest may be critical in producing more mature hair cells, with the path of regeneration relying on manipulation of a series of factors rather than just one or two. Another potential avenue to increase our conversion would be manipulation of the Wnt pathway, which has been experimentally shown to enhance proliferation of cell types, which may be needed to replace any supporting cells lost to transdifferentiation.<sup>12</sup>

Finally, another worthwhile future direction for this study might be single-cell RNA-sequencing of drug treated mice. Single-cell RNA seq allows us to take a sample of our sensory epithelium, separate out each individual cell from their heterogeneous populations, and get the transcriptome of that cell. For our purposes, it would help reveal changes in cell types of interest following our drugs. This snapshot of changes informs us of the level of change of the genes of interest, in addition to any other pathway changes we may not have been aware of. With this technique we could identify converted hair cells from all cell populations and look exactly at its transcriptome to determine a multitude of information. We could compare its transcriptome to endogenous hair cells and determine the similarities and differences, which could inform us of what genetic changes we need to manipulate to push it towards native hair cell state. In addition, we could see the effect on non-target cell types to determine any negative off-target effects, normal hearing function requires a variety of cell types including supporting cells and if we accidentally kill off a significant portion of supporting cells regenerating hair cells then we still lack functional hearing and our experimental approach needs to be addressed. Our 4 hour single cell analysis and RT-PCR of A2CE revealed that transcriptional inhibition of p27kip1 takes at least 24 hours to take effect, so subsequent studies of our drug cocktail will need to take this into account when exploring transcriptome changes through this technology.

A significant limitation of our study is the lack of mature hair cells in our samples. While our converted hair cells are able to overcome most endogenous mechanisms of differentiation for our supporting cells, our cells clearly lack important features necessary for functional recovery. In addition to maturity, our drug cocktail lacks consistent efficacy, with the large amount of variance across samples not making the drug reliable for desired therapeutic effect as is. Our

converted hair cells lack mature bundle morphology, seen in endogenous hair cells, and expression of hair cells genes is at a lower level than seen in IHC and OHC rows. Our regeneration is also at a low level, and with significant damage associated with noise-induced hearing loss or other damage, would be inefficient in replacing the necessary amount of cells for normal hearing. Full functional recovery, such as expression of mature marker prestin, requires input of additional factors in conjunction with POU4F3 and p27 inhibition in order to achieve complete success. Whether those inputs need to be gene regulation or transcription factor manipulation or a combination of both remains to be seen.

## Conclusions:

While our combination of compounds A2CE and C18 for drug-induced cochlear hair cell regeneration was successful in producing some conversion of supporting cells in adult mice, it is hindered by its efficacy. Our preliminary data characterized our small molecule A2CE efficacy with single-cell RNA-sequencing and RT-PCR, which confirmed the effect on its target p27 and when this transcriptional inhibition took place. We found that our conversion suffered from a significant amount of variance amongst our cohorts, which needs to be addressed in subsequent experiments. We also showed that our converted hair cells arise from the Sox2 expressing supporting cells, which was the hypothesized subpopulation our new hair cells came from. We provided a framework for subsequent drug-based regeneration therapies, which can build upon our work and optimize their strategies to hopefully overcome and eventually produce functional hearing recovery.

## References:

1. World Health Organization. (2021, April 21). Deafness and hearing loss. World Health Organization. Retrieved from <https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss>
2. Cunningham, L. L., & Tucci, D. L. (2017). Hearing Loss in Adults. *The New England journal of medicine*, 377(25), 2465–2473. <https://doi.org/10.1056/NEJMra1616601>
3. Ciorba, A., Bianchini, C., Pelucchi, S., & Pastore, A. (2012). The impact of hearing loss on the quality of life of elderly adults. *Clinical interventions in aging*, 7, 159–163. <https://doi.org/10.2147/CIA.S26059>
4. United States Department of Veterans Affairs . (2021, August 30). VA research on Hearing Loss. United States Department of Veterans Affairs Office of Research & Development. Retrieved from <https://www.research.va.gov/topics/hearing.cfm>
5. U.S Food and Drug AdministrationU. (2017, November 11). Cochlear Implants: A Different Kind of 'Hearing'. U.S Food and Drug Administration. Retrieved from <https://www.fda.gov/consumers/consumer-updates/cochlear-implants-different-kind-hearing#:~:text=Cochlear%20implants%20don't%20restore,inclusing%20when%20using%20a%20telephone.>
6. Center For Disease Control and Prevention. (2021, June 21). Hearing Loss Treatment and Intervention Services. Center For Disease Control and Prevention. Retrieved from <https://www.cdc.gov/ncbddd/hearingloss/treatment.html>
7. Purves, Dale, George J. Augustine, David Fitzpatrick, William C. Hall, Anthony-Samuel LaMantia, James O. McNamara, and Leonard E. White (2008). "Chapter 13". *Neuroscience*. 4th ed. Sinauer Associates. p. 317. ISBN 978-0-87893-697-7.

8. Sundar, P. S., Chowdhury, C., & Kamarthi, S. (2021). Evaluation of Human Ear Anatomy and Functionality by Axiomatic Design. *Biomimetics* (Basel, Switzerland), 6(2), 31. <https://doi.org/10.3390/biomimetics6020031>
9. Walters, B. J., Coak, E., Dearman, J., Bailey, G., Yamashita, T., Kuo, B., & Zuo, J. (2017). In Vivo Interplay between p27Kip1, GATA3, ATOH1, and POU4F3 Converts Non-sensory Cells to Hair Cells in Adult Mice. In *Cell Reports* (Vol. 19, Issue 2, pp. 307–320). Elsevier BV. <https://doi.org/10.1016/j.celrep.2017.03.044>
10. Hicks, K. L., Wisner, S. R., Cox, B. C., & Stone, J. S. (2020). Atoh1 is required in supporting cells for regeneration of vestibular hair cells in adult mice. *Hearing research*, 385, 107838. <https://doi.org/10.1016/j.heares.2019.107838>
11. Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A., & Zoghbi, H. Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* (New York, N.Y.), 284(5421), 1837–1841. <https://doi.org/10.1126/science.284.5421.1837>
12. Chai, Renjie & Xia, Anping & Wang, Tian & Jan, Taha & Hayashi, Toshinori & Bermingham-McDonogh, Olivia & Cheng, Alan. (2011). Dynamic Expression of Lgr5, a Wnt Target Gene, in the Developing and Mature Mouse Cochlea. *Journal of the Association for Research in Otolaryngology : JARO*. 12. 455-69. 10.1007/s10162-011-0267-2.
13. He, Z., Ding, Y., Mu, Y., Xu, X., Kong, W., Chai, R., & Chen, X. (2021). Stem Cell-Based Therapies in Hearing Loss. In *Frontiers in Cell and Developmental Biology* (Vol. 9). Frontiers Media SA. <https://doi.org/10.3389/fcell.2021.730042>

14. Cox, B. C., Chai, R., Lenoir, A., Liu, Z., Zhang, L., Nguyen, D. H., Chalasani, K., Steigelman, K. A., Fang, J., Rubel, E. W., Cheng, A. G., & Zuo, J. (2014). Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. *Development (Cambridge, England)*, 141(4), 816–829. <https://doi.org/10.1242/dev.103036>
15. Waqas, M., Us-Salam, I., Bibi, Z., Wang, Y., Li, H., Zhu, Z., & He, S. (2020). Stem Cell-Based Therapeutic Approaches to Restore Sensorineural Hearing Loss in Mammals. *Neural plasticity*, 2020, 8829660. <https://doi.org/10.1155/2020/8829660>
16. Stojanova, Z. P., Kwan, T., & Segil, N. (2015). Epigenetic regulation of *Atoh1* guides hair cell development in the mammalian cochlea. *Development (Cambridge, England)*, 142(20), 3529–3536. <https://doi.org/10.1242/dev.126763>
17. Cox, B. C., Chai, R., Lenoir, A., Liu, Z., Zhang, L., Nguyen, D.-H., Chalasani, K., Steigelman, K. A., Fang, J., Cheng, A. G., & Zuo, J. (2014). Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. In *Development (Vol. 141, Issue 4, pp. 816–829)*. The Company of Biologists. <https://doi.org/10.1242/dev.103036>
18. Costa, A., Sanchez-Guardado, L., Juniat, S., Gale, J. E., Daudet, N., & Henrique, D. (2015). Generation of sensory hair cells by genetic programming with a combination of transcription factors. In *Development (Vol. 142, Issue 11, pp. 1948–1959)*. The Company of Biologists. <https://doi.org/10.1242/dev.119149>
19. Chien, W.-M., Rabin, S., Macias, E., Miliani de Marval, P. L., Garrison, K., Orthel, J., Rodriguez-Puebla, M., & Fero, M. L. (2006). Genetic mosaics reveal both cell-autonomous and cell-nonautonomous function of murine p27 Kip1. In *Proceedings of the National Academy of Sciences (Vol. 103, Issue 11, pp. 4122–4127)*. Proceedings of the National Academy of Sciences. <https://doi.org/10.1073/pnas.0509514103>

20. Rubel, E. W., Oesterle, E. C., & Weisleder, P. (1991). Hair cell regeneration in the avian inner ear. *Ciba Foundation symposium*, 160, 77–102.  
<https://doi.org/10.1002/9780470514122.ch5>
21. Yu, H. V., Tao, L., Llamas, J., Wang, X., Nguyen, J. D., Trecek, T., & Segil, N. (2021). POU4F3 pioneer activity enables ATOH1 to drive diverse mechanoreceptor differentiation through a feed-forward epigenetic mechanism. In *Proceedings of the National Academy of Sciences* (Vol. 118, Issue 29). *Proceedings of the National Academy of Sciences*. <https://doi.org/10.1073/pnas.2105137118>
22. Chen, P., & Segil, N. (1999). p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. In *Development* (Vol. 126, Issue 8, pp. 1581–1590). The Company of Biologists. <https://doi.org/10.1242/dev.126.8.1581>
23. Georgia, S., & Bhushan, A. (2006). p27 Regulates the Transition of  $\beta$ -Cells From Quiescence to Proliferation. In *Diabetes* (Vol. 55, Issue 11, pp. 2950–2956). American Diabetes Association. <https://doi.org/10.2337/db06-0249>
24. Sawamura, S., Ogata, G., Asai, K., Razvina, O., Ota, T., Zhang, Q., Madhurantakam, S., Akiyama, K., Ino, D., Kanzaki, S., Saiki, T., Matsumoto, Y., Moriyama, M., Saijo, Y., Horii, A., Einaga, Y., & Hibino, H. (2021). Analysis of Pharmacokinetics in the Cochlea of the Inner Ear. In *Frontiers in Pharmacology* (Vol. 12). Frontiers Media SA.  
<https://doi.org/10.3389/fphar.2021.633505>
25. Salt, A. N., & Plontke, S. K. (2005). Local inner-ear drug delivery and pharmacokinetics. *Drug discovery today*, 10(19), 1299–1306. [https://doi.org/10.1016/S1359-6446\(05\)03574-9](https://doi.org/10.1016/S1359-6446(05)03574-9)

26. Ciorba, A., Bianchini, C., Pelucchi, S., & Pastore, A. (2012). The impact of hearing loss on the quality of life of elderly adults. *Clinical interventions in aging*, 7, 159–163.  
<https://doi.org/10.2147/CIA.S26059>
27. Suhong Sun, Shuting Li, Zhengnan Luo, Minhui Ren, Shunji He, Guangqin Wang, Zhiyong Liu (2021) Dual expression of *Atoh1* and *Ikzf2* promotes transformation of adult cochlear supporting cells into outer hair cells *eLife* 10:e66547.  
<https://doi.org/10.7554/eLife.66547>
28. Goycoolea M. V. (2001). Clinical aspects of round window membrane permeability under normal and pathological conditions. *Acta oto-laryngologica*, 121(4), 437–447.  
<https://doi.org/10.1080/000164801300366552>
29. Taketo, M., Schroeder, A. C., Mobraaten, L. E., Gunning, K. B., Hanten, G., Fox, R. R., Roderick, T. H., Stewart, C. L., Lilly, F., & Hansen, C. T. (1991). FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proceedings of the National Academy of Sciences of the United States of America*, 88(6), 2065–2069.  
<https://doi.org/10.1073/pnas.88.6.2065>
30. Walters, B. J. et al. (2015). Sox2-CreER mice are useful for fate mapping of mature, but not neonatal, cochlear supporting cells in hair cell regeneration studies. *Sci. Rep.* 5, 11621; doi: 10.1038/srep11621
31. Okano T. (2014). Immune system of the inner ear as a novel therapeutic target for sensorineural hearing loss. *Frontiers in pharmacology*, 5, 205.  
<https://doi.org/10.3389/fphar.2014.00205>
32. Kanzaki, S., Fujioka, M., Yasuda, A., Shibata, S., Nakamura, M., Okano, H. J., Ogawa, K., & Okano, H. (2012). Novel In Vivo Imaging Analysis of an Inner Ear Drug Delivery

System in Mice: Comparison of Inner Ear Drug Concentrations over Time after Transtympanic and Systemic Injections. In D. Vavvas (Ed.), PLoS ONE (Vol. 7, Issue 12, p. e48480). Public Library of Science (PLoS).

<https://doi.org/10.1371/journal.pone.0048480>

33. Murillo-Cuesta, S., Vallecillo, N., Cediell, R., Celaya, A. M., Lassaletta, L., Varela-Nieto, I., & Contreras, J. (2017). A Comparative Study of Drug Delivery Methods Targeted to the Mouse Inner Ear: Bullostomy Versus Transtympanic Injection. *Journal of visualized experiments : JoVE*, (121), 54951. <https://doi.org/10.3791/54951>
34. Capriotti, K., & Capriotti, J. A. (2012). Dimethyl sulfoxide: history, chemistry, and clinical utility in dermatology. *The Journal of clinical and aesthetic dermatology*, 5(9), 24–26.
35. Zucker, I. in *Circannual rhythms Mammals in Handbook of Behavioral Neurobiology*] (2001) *Handbook of Behavioral Neurobiology Vol. Vol 12* 509–529 .
36. Ferguson, S. A., & Maier, K. L. (2013). A review of seasonal/circannual effects of laboratory rodent behavior. In *Physiology & Behavior* (Vol. 119, pp. 130–136). Elsevier BV. <https://doi.org/10.1016/j.physbeh.2013.06.007>
37. Ni, W., Lin, C., Guo, L., Wu, J., Chen, Y., Chai, R., Li, W., & Li, H. (2016). Extensive Supporting Cell Proliferation and Mitotic Hair Cell Generation by In Vivo Genetic Reprogramming in the Neonatal Mouse Cochlea. In *The Journal of Neuroscience* (Vol. 36, Issue 33, pp. 8734–8745). Society for Neuroscience.  
<https://doi.org/10.1523/jneurosci.0060-16.2016>

38. Piu, F., & Bishop, K. M. (2019). Local Drug Delivery for the Treatment of Neurotology Disorders. In *Frontiers in Cellular Neuroscience* (Vol. 13). Frontiers Media SA.  
<https://doi.org/10.3389/fncel.2019.00238>
39. Chessum, L., Matern, M. S., Kelly, M. C., Johnson, S. L., Ogawa, Y., Milon, B., McMurray, M., Driver, E. C., Parker, A., Song, Y., Codner, G., Esapa, C. T., Prescott, J., Trent, G., Wells, S., Dragich, A. K., Frolenkov, G. I., Kelley, M. W., Marcotti, W., Brown, S., ... Hertzano, R. (2018). Helios is a key transcriptional regulator of outer hair cell maturation. *Nature*, 563(7733), 696–700. <https://doi.org/10.1038/s41586-018-0728-4>
40. Kelly MC, Chang Q, Pan A, Lin X, Chen P. (2012) Atoh1 directs the formation of sensory mosaics and induces cell proliferation in the postnatal mammalian cochlea in vivo. *J Neurosci.*;32:6699–6710.
41. Liu Z, Dearman JA, Cox BC, Walters BJ, Zhang L, Ayrault O, et al. (2012) Age-dependent in vivo conversion of mouse cochlear pillar and Deiters' cells to immature hair cells by Atoh1 ectopic expression. *J Neurosci.*;32:6600–6610.
42. Liu Z, Fang J, Dearman J, Zhang L, Zuo J. (2014) In vivo generation of immature inner hair cells in neonatal mouse cochleae by ectopic Atoh1 expression. *PLoS One.*;9:e89377.
43. Shou J, Zheng JL, Gao WQ. (2003) Robust generation of new hair cells in the mature mammalian inner ear by adenoviral expression of Hath1. *Mol Cell Neuroscience.*;23:169–179.
44. Yang J, Cong N, Han Z, Huang Y, Chi F. (2013) Ectopic hair cell-like cell induction by Math1 mainly involves direct transdifferentiation in neonatal mammalian cochlea. *Neurosci Lett.*;549:7–11.

45. Chandrasekhar SS, et al. (2000) Dexamethasone pharmacokinetics in the inner ear: comparison of route of administration and use of facilitating agents. *Otolaryngol Head Neck Surg.*;122:521–528.
46. Selivanova O, et al. (2003) [The effects of Streptolysin-O and sodium hyaluronate on the permeability of the round window membrane in guinea pigs--an electrophysiologic study] *Laryngorhinootologie.*;82:235–239.
47. Ho MK, Li X, Wang J, Ohmen JD, Friedman RA. (2013) FVB/NJ mice demonstrate a youthful sensitivity to noise-induced hearing loss and provide a useful genetic model for the study of neural hearing loss. *Audiol Neurotol Extra.*;4(1):1-11.  
doi:10.1159/000357770
48. Malgrange, B; Van de Water, T.R; Nguyen, L; Moonen, G; Lefebvre, P.P (2002). "Epithelial supporting cells can differentiate into outer hair cells and Deiters' cells in the cultured organ of Corti". *Cellular and Molecular Life Sciences.* **59** (10): 1744–1757. doi:[10.1007/pl00012502](https://doi.org/10.1007/pl00012502)
49. Géléoc GS, Holt JR (2003). "Auditory amplification: outer hair cells pres the issue". *Trends Neurosci.* 26 (3): 115–7. doi:10.1016/S0166-2236(03)00030-4. PMC 2724262. PMID 12591210.
50. Ishiyama, G., Lopez, I.A., Ishiyama, P. et al. (2017) The blood labyrinthine barrier in the human normal and Meniere's disease macula utricule. *Sci Rep* 7, 253  
<https://doi.org/10.1038/s41598-017-00330-5>
51. Juhn, S. K., Ryback, L. P. & Prado, S. (1981). Nature of blood-labyrinth barrier in experimental conditions. *Ann Otol Rhinol Laryngol.* 90, 135–52

52. Bhave, S. A., Stone, J. S., Rubel, E. W., and Coltrera, M. D. (1995). Cell cycle progression in gentamicin-damaged avian cochleas. *J. Neurosci.* 15, 4618–4628. doi: 10.1523/JNEUROSCI.15-06-04618.1995
53. Guerin, D. J., Kha, C. X., & Tseng, K. A.-S. (2021). From cell death to regeneration: Rebuilding after injury. *Frontiers in Cell and Developmental Biology*, 9. <https://doi.org/10.3389/fcell.2021.655048>
54. Liu, X., Huang, J., Chen, T., Wang, Y., Xin, S., Li, J., Pei, G., & Kang, J. (2008). Yamanaka factors critically regulate the developmental signaling network in mouse embryonic stem cells. In *Cell Research* (Vol. 18, Issue 12, pp. 1177–1189). Springer Science and Business Media LLC. <https://doi.org/10.1038/cr.2008.309>
55. Plontke, S. K., & Salt, A. N. (2006). Simulation of application strategies for local drug delivery to the inner ear. *ORL; journal for oto-rhino-laryngology and its related specialties*, 68(6), 386–392. <https://doi.org/10.1159/000095284>
56. Walters, B. J., Lin, W., Diao, S., Brimble, M., Iconaru, L. I., Dearman, J., Goktug, A., Chen, T., & Zuo, J. (2014). High-Throughput Screening Reveals Alsterpaullone, 2-Cyanoethyl as a Potent p27Kip1 Transcriptional Inhibitor. In Y. Wang (Ed.), *PLoS ONE* (Vol. 9, Issue 3, p. e91173). Public Library of Science (PLoS). <https://doi.org/10.1371/journal.pone.0091173>
57. Montgomery, S. C., & Cox, B. C. (2016). Whole Mount Dissection and Immunofluorescence of the Adult Mouse Cochlea. *Journal of visualized experiments : JoVE*, (107), 53561. <https://doi.org/10.3791/53561>

58. Navarro, K. L., Huss, M., Smith, J. C., Sharp, P., Marx, J. O., & Pacharinsak, C. (2021). Mouse Anesthesia: The Art and Science. In *ILAR Journal* (Vol. 62, Issues 1–2, pp. 238–273). Oxford University Press (OUP). <https://doi.org/10.1093/ilar/ilab016>
59. Capriotti, K., & Capriotti, J. A. (2012). Dimethyl sulfoxide: history, chemistry, and clinical utility in dermatology. *The Journal of clinical and aesthetic dermatology*, 5(9), 24–26.
60. Meyer, L., Caston, J., & Mensah-Nyagan, A. G. (2006). Seasonal variation of the impact of a stressful procedure on open field behavior and blood corticosterone in laboratory mice. *Behavioral brain research*, 167(2), 342–348.  
<https://doi.org/10.1016/j.bbr.2005.09.023>
61. Helm, B., Ben-Shlomo, R., Sheriff, M. J., Hut, R. A., Foster, R., Barnes, B. M., & Dominoni, D. (2013). Annual rhythms that underlie phenology: biological time-keeping meets environmental change. *Proceedings. Biological sciences*, 280(1765), 20130016.  
<https://doi.org/10.1098/rspb.2013.0016>
62. Stone, J. S., & Rubel, E. W. (2000). Cellular studies of auditory hair cell regeneration in birds. In *Proceedings of the National Academy of Sciences* (Vol. 97, Issue 22, pp. 11714–11721). *Proceedings of the National Academy of Sciences*.  
<https://doi.org/10.1073/pnas.97.22.11714>