Generation of Inner Ear Sensory Neurons Using Blastocyst Complementation in a Neurog1-Deficient Mouse

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Short report

Keywords: Inner ear, Spiral Ganglion Neurons, Neurogenin1, Blastocyst Complementation, Stem Cells, Regenerative Medicine

DOI: https://doi.org/10.21203/rs.3.rs-57441/v1

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Abstract

This research is the first to produce induced pluripotent stem cell-derived inner ear sensory neurons in the Neurog1+/− heterozygote mouse using blastocyst complementation. Additionally, this approach corrected non-sensory deficits associated with Neurog1 heterozygosity, indicating that complementation is specific to endogenous Neurog1 function. This work validates the use of blastocyst complementation as a tool to create novel insight into the function of developmental genes and highlights blastocyst complementation as a potential platform for generating inner ear cell types that can be transplanted into damaged inner ears to improve hearing.

Introduction

Hearing loss is the most common neurosensory deficit. Approximately 488 million individuals world-wide and 15% of Americans have some degree of hearing loss (1). Hearing depends on the mechano-sensory hair cells (HCs) and their innervating neurons, the spiral ganglion neurons (SGNs), which are responsible for transmitting auditory information from the HCs in the organ of Corti to the cochlear nucleus in the brainstem. Mammalian HCs and SGNs do not regenerate after damage, which results in sensorineural hearing loss (SNHL) (2). In addition, auditory neuropathy with relative preservation of hair cells is a substantial cause of deafness (3, 4). Cochlear implants are the only established therapy for severe to profound hearing loss; however, they require a viable SGN population for their success and efficacy (5).

Using exogenous stem cells to replace lost inner ear neurons is a potential strategy if stem cell derived neurons can form central and peripheral connections, form synapses on hair cells and cochlear nucleus neurons, and re-establish functional and tonotopic circuits (6). While early attempts to target cochlear tissues using stem cells largely produced unremarkable results (6, 7), two promising in vivo studies have shown that stem cells can survive and supplement SGNs within a cochlea and even partially restore hearing function (8, 9). However, there are two potential weakness in the previous studies: 1) Since the transplanted stem cells were differentiated in vitro to form otic progenitors and established markers of otic specific neurons were not used, these cells may not be equivalent to SGNs formed in vivo, which may explain the observed limited functional recovery; and 2) using allogenic stem cells requires that the transplant recipient may need to undergo long-term immunosuppression to prevent rejection of the transplanted progenitors. We address these potential limitations by adopting the technique of blastocyst complementation (BC) to generate inner ear neurons from induced pluripotent stem cells (iPSCs).

BC is a technique in which inactivation of a key gene for the development of a specific lineage creates a vacant niche (organogenesis disabled phenotype) that can be complemented by the progeny of wild type pluripotent stem cells injected into embryos at the blastocyst stage of development. Resulting chimeras from BC have successfully generated entire, functional organs such as the pancreas, kidney, eye, and lung, derived from the progeny of donor stem cells, by complementing the genes (PDX1, SALL1, MITF, and FGFR2, respectively) necessary for their formation (10–14).
Ma and colleagues (15, 16) developed a Neurogenin1 (Neurog1)−/− knockout mouse that lacked an otic ganglion at E10.5, which translated into P0 inner ears that lacked afferent, efferent, and autonomic nerve fibers in the Neurog1−/− null. In preliminary experiments using the Neurog1−/− mutant mouse, we produced the first histological analysis of E18.5 Neurog1−/− null inner ears (17), which confirmed the findings of Ma and colleagues. With the goal of generating stem cell-derived inner ear neurons, the Neurog1−/− knockout animal was the optimal choice for creating a vacant niche to generate inner ear neurons by blastocyst complementation.

**Methods**

All methods performed in this manuscript were in accordance with all policies of the University of Minnesota Institute of Animal Care Use Committee (IACUC), which approved the use and housing of these animals according to accepted principles of laboratory animal care (National Research Council 2003).

**Mice**

The Neurog1tm1And/J mouse strain was used (15, 16) (Jax #017306) in which the coding exon for Neurog1 was replaced with a non-functional GFP cassette, abolishing gene function (JAX # 017306). Mice were housed in a specific pathogen free (SPF), Research Animal Resource (RAR) (AAALAC approved) facility and plastic cages that were steam cleaned and autoclaved 3 times per week. The mouse colony was maintained by crossing Neurog1+/+ wild-type mice with Neurog1+/− heterozygous mice. Experimental mice were generated through heterozygous matings (Neurog1+/− X Neurog1+/−), which produced the following combinations of genotype: Neurog1+/+ wild-type, Neurog1+/− heterozygous, Neurog1−/− null mice. Neurog1 null mice die 24 hours after birth due to their inability to suckle, and therefore, they were only generated for blastocyst complementation or embryonic harvest. Toe clips were collected at one week of age, which were subsequently hydrolyzed for genotyping analysis. Genotyping was performed by Polymerase Chain Reaction (PCR) using the following primers: WT Forward (ACCACTAGGCCTTTGTAAGG), Mutant Forward (ATAGACCGAGGGCAGCTTCA), and Common (CGCTTCCTCGTGCTTTACGGTAT). This reaction yields a 198-bp wild type band and a 500-bp mutant band.

**Blastocyst Complementation**

Mouse x mouse blastocyst complementation was performed by injecting GFP-labeled mouse iPSCs, (the derivation of these cells were previously described by Greder and colleagues (18)) into Neurog1 deficient blastocysts. Injected blastocysts were transferred to pseudopregnant female surrogates, where they were allowed to develop until the time of natural birth. To produce mutant blastocysts an in vitro approach was used. To do this, the egg donors (Neurog1+/− heterozygous female mice) were superovulated (19) at 3-4 weeks of age, by giving CARD HyperOva (Cosmo Bio, Cat. No. KYD-010-EX-X5) 0.1 ml/mouse, i.p. at 17:30 pm (mouse room light:dark cycle: 6:00 – 20:00), followed by hCG (Sigma-Aldrich Cat. No. C 1063), 5 IU/mouse 47 - 48 hours later. Fresh sperm from a Neurog1 heterozygous (+/-) male was used for IVF.
Fertilized eggs were cultured in home-made modified Human Tubal Fluid (mHTF, a.k.a. high calcium HTF) medium until the blastocysts were formed in ~ 72 hours after IVF and ready for microinjection of iPSC. Each blastocyst was injected with 10 – 15 iPSC. After blastocyst injection, mouse blastocysts were transferred into the uteri of pseudopregnant surrogate mice.

**Inner ear harvesting from the embryo, cryosectioning, and immunohistochemistry**

For tissue harvesting, P1 mice were euthanized using CO₂ according to RAR guidelines, followed by decapitation. Heads were bisected with the intention of using one inner ear for cryosectioning and immunohistochemistry (IHC) and the other processed for imaging using sTSLIM. Bisected heads were fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), and were rinsed with PBS twice for fifteen minutes each before undergoing decalcification in 10% Ethylenediaminetetraacetic acid (EDTA) for three days. Inner ears to be analyzed by cryosectioning and IHC were cryoprotected through overnight incubations in ascending concentrations of sucrose up to 30%, and then embedded in tissue freezing medium (TFM) (General Data, TFM-5) and snap frozen on dry ice. Tissue was sectioned on a cryostat at a thickness of 16mm and directly mounted to slides. Slides were allowed to dry on the slide warmer for a minimum of one hour prior to beginning staining or storing at -20°C. Sectioned tissue was always stained within three days of sectioning. If previously frozen, tissue was rewarmed to room temp by placing on the slide warmer for a minimum of a half hour. Prior to performing immunohistochemistry, epitopes were exposed by performing antigen retrieval. Briefly, slides were placed in Copeland jars filled with boiling sodium citrate buffer (pH adjusted to 6.0 with 1N HCL) and incubated in a steamer for 20 minutes. Slides were allowed to cool to room temperature for at least an hour before continuing the staining protocol. After antigen retrieval, the slides were dried and the sections were outlined with a hydrophobic barrier pen (Super-HT Pap Pen, Polysciences #24230-1). Tissue was rinsed twice (ten minutes each rinse) with PBS and the rinsed in PBS twice (fifteen minutes each rinse) with 0.1% Triton X-100 (Sigma-Aldrich, X100). Non-specific binding was blocked against using 10% normal horse serum (ThermoFisher, Cat# 16050122) in 0.1% PBST for one hour at room temperature. Primary antibodies, diluted in the blocking solution, were applied and allowed to incubate in the 4°C overnight. All antibodies are listed in the below table. The following day, the tissue was rinsed with four 15-minute washes in 0.1% PBST, then sections were incubated in secondary antibodies, diluted in the blocking solution, for two hours at room temperature. The tissue was then rinsed in PBST with two ten-minute washes, then counterstained with 4’,6-diamidino-2-phenylindole (DAPI, ThermoFisher, Cat #: D1306) at a concentration of 1:5000 for five minutes. Lastly tissue was rinsed for ten minutes in PBS, and then coverslips were mounted to the slide after applying mounting medium with anti-fade agent (EMS, Cat#17985-11). Slides were sealed using with CoverGrip™ Coverslip Sealant.

**1° Antibodies**
### Antibodies

| Antibody                          | Vendor                | Cat#   | Dilution |
|-----------------------------------|-----------------------|--------|----------|
| rabbit polyclonal a-MYO6          | Proteus Biosciences Inc | 25-6791 | 1:500   |
| chicken a-GFP                     | Abcam Inc.            | ab13970 | 1:1000  |
| mouse monoclonal a-Tuj1          | Biolegend             | MMS-435P | 1:1000 |

### 2° Antibodies

| Antibody               | Vendor                        | Cat#      | Dilution |
|------------------------|-------------------------------|-----------|----------|
| 488 donkey a-chicken   | Jackson ImmunoResearch Alexa Fluor | 703-545-155 | 1:1000  |
| 555 donkey a-rabbit    | Invitrogen Alexa Fluor        | Ab150062 | 1:1000  |
| 647 donkey a-mouse     | Invitrogen Alexa Fluor        | A-31571  | 1:1000  |

### Microscopy and Image processing

All immunohistochemical imaging was performed on a Leica inverted light microscope. Images were exported as raw LIF files and processed in FIJI (Fiji Is Just ImageJ). The resolution of each image was adjusted to 300 dots per inch (DPI) in Photoshop and all resulting JPEGs were assembled using Adobe Illustrator.

### sTSLIM macro light-sheet microscope

In 2008 we developed a high-resolution microtome/microscope called scanning Thin Sheet Laser Image Microscope (sTSLIM) that can image whole cochleas, nondestructively (20-24). sTSLIM optically sections and digitizes all cochlear tissues to allows for a complete quantitative assessment of normal and pathological structures. The Santi laboratory has used sTSLIM to characterize mouse cochlear development (25), analyze normal spiral ganglion cell number in the mouse (26), and illustrate alterations in cochlear structures in two knock-out mouse models (ATOH1 and N-Myc) (27, 28). Since light scatter and absorption are the greatest limiting factors in resolution, we have performed both tissue engineering (improved transparency and accessibility to antibody labeling through decellularization) and optical engineering (scanned light-sheet, Bessel beam illumination, structured illumination, confocal line detection, and radial sectioning) to improve imaging of large specimens such as the mouse cochlea with portions of the brain attached. Microscope performance will be tested on a regular basis using 150 nm gold fluorescent beads to ensure that the point spread function of the microscope is stable and optimal for reproducible imaging.

### Tissue preparation for sTSLIM
Inner ears processed for sTSLIM were fixed and decalcified (described above) and then underwent a dehydration series in ascending concentrations of ethanol (30%, 50%, 70%, 100% EtOH) and then lightly stained by Rhodamine-B isothiocyanate (5mg/mL in 100%) for 1 hour. After rinsing in 100% EtOH, inner ears were cleared to transparency with BABB (benzyl alcohol: benzyl benzoate 1:2), and specimens were mounted to a specimen rod and placed in a BABB-filled chamber for imaging by sTSLIM.

**sTSLIM imaging**

sTSLIM optically sections non-destructively by moving a thin light sheet in the X- and Z-axes. A z-stack of well-aligned 2D optical sections of the inner ear was automatically imaged with x-axis scanning across the width of the specimen and with a z-step size of 5 µm. At this thickness the whole mouse cochlea contained ~300 images that take ~30 minutes to produce. Images were adjusted for brightness, contrast, and either unsharp masking or deconvolution using ImageJ (NIH). The z-stack was then loaded into the Amira 3D program and structures of interest were manually segmented, by drawing along their borders in different colors to prepare 3D reconstructions and volume calculations. Supplemental Fig.1 shows an example of a 2D optical section through the cochlea and Supplemental Movie. 1 shows segmented inner ears rotating horizontally.

**Results**

**Donor GFP-labeled stem cells create chimeric spiral ganglion and vestibular neurons in the Neurog1+/− heterozygote**

*Neurog1*-deficient blastocysts were established by performing *in vitro* fertilization (IFV) with zygotes extracted from *Neurog1*+/− heterozygous dams and fresh sperm from *Neurog1*+/− heterozygous male mice. Blastocysts were injected with GFP-labeled mouse induced pluripotent stem cells (iPSCs) at approximately embryonic day (E)3.5 and subsequently were transferred into surrogate pseudopregnant dams (Fig. 1A). Upon analysis at post-natal day (P)1, robust and specific stem cell incorporation of GFP-labeled cells derived from the donor stem cells was seen in the SGN of a *Neurog1*+/− heterozygote (Fig. 1).

In the complemented *Neurog1*+/−, GFP-labeled iPSCs contributed to the SGN and descending neuronal processes in the cochlea with minimal donor stem cell-derived cell incorporation in other tissue (Fig. 1D,E arrowheads). The specificity of labeling in the *Neurog1*+/− was unmistakable, given the lack of GFP labeling observed in the wild type SGN (Fig. 1C,F, arrows). GFP-labeled donor iPSCs also contributed to the cell bodies of Scarpa’s ganglion (Fig. 1G), in addition to vestibular neurons innervating the cristae ampullaris (Fig. 1H,I). Since *Neurog1* is known to be required for the formation of both cochlear and vestibular neurons (16), these results indicate that the integration pattern of cells derived from the wild type donor cells recapitulates that expected from cells wild type endogenous gene function and that *Neurog1* haplodeciency creates a vacant niche that can be filled by cells derived from exogenous stem cells to produce SGNs using BC.
Complementation of *Neurog1*-deficiency is distinct from general chimerism

A chimera is defined as a composite animal comprised of two genetically distinct cell populations (29). Performing BC in wild type animals will result in random chimerization throughout the developing embryo (30, 31). When performing BC in knockout blastocysts, in addition to exogenous donor-derived cells target a vacant developmental niche, random chimerism can also be observed in non-targeted cell types.

Two examples of complemented *Neurog1*+/− heterozygotes were highly chimeric, as a high degree of specific donor derived cell integration into the SGN was observed (Fig. 2B-D'), in addition to general chimerism, which was evident from the widespread GFP expression in non-sensory otic cell types (Fig. 2B-D'). This general chimerism is similar to that observed in the *Neurog1*+/+ control which also displayed the incorporation of GFP-expressing cells in non-sensory cells. Notably, the *Neurog1*+/− heterozygote SGNs, in some regions, appeared to be derived nearly entirely from donor iPSCs, whereas the *Neurog1*+/+ wild type SGNs were consistently negative for GFP expression (Figs. 1C, 2A,A').

The extent of donor-derived cell chimerism in the *Neurog1*+/+ wild type in comparison to the complemented *Neurog1*+/− heterozygotes was assessed further by looking at the respective level of GFP expression in the temporal lobe of the brain. This analysis clearly showed the incorporation of exogenous iPSC-derived cells in the *Neurog1*+/+ brain at a comparable level to the complemented *Neurog1*+/− heterozygotes (Fig. 2E,F). Therefore, chimeras successfully formed regardless of genotype, but in the absence of a *Neurog1*-deficient niche, no GFP-expressing cells derived from the exogenous donor iPSCs contributed to the formation of the SGN in wild type inner ears (Figs. 1F, 2A'). These results support our hypothesis by demonstrating that BC can specifically generate SGNs using donor stem cells.

We did not see complementation in the two *Neurog1*−/− mutant embryos that we obtained. However, both nulls showed few to no GFP-expressing cells engrafted throughout the whole embryo, suggesting that the lack of complementation was due to low chimerism. With an increased sample size, we expect to obtain highly chimeric *Neurog1*−/− mutants in which all of the SGNs are derived from donor stem cell progeny. Therefore, it is anticipated that, following Medel's law, 75% of blastocysts (heterozygous and null) obtained from BC will give rise to chimeric inner ears.

Donor-derived stem cells extensively contribute to the complemented *Neurog1*+/− vestibule

In the highly chimeric complemented *Neurog1*+/− inner ears, the contribution of donor-derived GFP-labeled cells appeared to increase in sections through the more basal cochlea in a trend that was dramatically more evident in the vestibule. In fact, it appeared that the majority of cells in the vestibule were derived from donor iPSCs, given the extensive presence of GFP in all vestibular cell types. Specifically, GFP entirely co-expressed with the neuronal marker class III beta-tubulin (TUJ1) in the neurites innervating the vestibular sensory organs (Fig 3A-B', arrow head and dotted lines), in addition to many Myosin 6 (MYO6)-expressing vestibular hair cells (Fig 3A-B, red) and nonsensory cells (Fig 3A'-B', small arrows). While the degree of donor cell contribution to the complemented *Neurog1*+/− vestibule was striking, the biological
significance of this was lacking, until a clear heterozygote effect in non-complemented Neurog1\textsuperscript{+/-} inner ears was detected.

**Blastocyst complementation rescues Neurog1\textsuperscript{+/-} inner ear malformations**

Non-complemented Neurog1\textsuperscript{+/-} heterozygote inner ears were observed to have inner ear morphological non-sensory malformations, which included inner ears reduced in size by approximately 60% of the wildtype control (Fig 4A, Supplemental Movie 1). Three-dimensional reconstructions revealed overt malformations particularly in the vestibule, in which the anterior and lateral ampullae and the saccule were notably reduced in size (Fig. 4A-D, Supplemental Movie 1). This finding was confirmed via cryosections, which displayed that the non-complemented Neurog1\textsuperscript{+/-} vestibule sometimes had an abnormally orientated saccule, utricle, and anterior ampullae. Specifically, unlike the typical orthogonal arrangement of the utricular and saccular maculae observed in the wild type vestibule, the non-complemented Neurog1\textsuperscript{+/-} sensory maculae (denoted by MYO6-expressing hair cells) were oriented in parallel (Fig. 4E,F; red, small arrows). Additionally, the utricular maculae and anterior crista ampullaris were unusually close to one another (Fig. 4F, arrowheads). Moreover, in some cases, the lateral ampulla appeared connected to the lateral semi circular canal (Fig. 4I, arrowheads). Together these observations suggest that a reduction in non-sensory cell formation and/or a failure of sensory organ separation occurred with the reduction of Neurog1 gene dose.

Strikingly, two Neurog1\textsuperscript{+/-} heterozygotes completely lacked a vestibule (not shown). Cochlear and vestibular HCs developed normally in the Neurog1\textsuperscript{+/-} heterozygote (as has been reported for the Neurog1\textsuperscript{-/-} mutant) despite impaired non-sensory formation. However, sensory development potentially occurred at the expense of non-sensory development, as ectopic HCs were sometimes seen in non-sensory regions in the lateral semi circular canal (Fig. 4I').

Importantly, non-sensory defects observed in the Neurog1\textsuperscript{+/-} heterozygotes were rescued in complemented Neurog1\textsuperscript{+/-} samples (Fig. 4A'-D,G,J). Given the extensive contribution of GFP to nonsensory tissue in the complemented Neurog1\textsuperscript{+/-} heterozygous vestibular sensory organs (Fig. 3), these results suggest that widespread incorporation of cells derived from the donor iPSCs to the vestibule in the complemented Neurog1\textsuperscript{+/-} heterozygote is not random, but rather reflects the recovery of a previously unappreciated biological function of Neurog1 in inner ear morphogenesis. This finding demonstrates the use of BC as a tool to elucidate novel gene function and to confirm or disprove concepts regarding the development of neurobiological systems.

**Discussion**

Presented here for, the first time, is the intraspecies complementation of Neurog1-deficient mouse blastocysts to generate a chimeric population of donor iPSC-derived SGNs. This is a major advancement in the field of regenerative medicine for hearing disorders. The complexity of the intersecting developmental pathways involved in lineage specification of inner ear sensory cells is a difficult task to
recapitulate in vitro (6). By contrast, the developing embryo initiates these processes reliably and seemingly effortlessly to produce what are presumed to be authentic spiral ganglion neurons. The approach of using BC to target Neurog1-deficiency is also advantageous, as Neurog1 is well-characterized to specify inner ear neuroblasts during the otocyst stage, a developmental stage when the future inner ear is which is a simple structure on a cellular level (32–34). Therefore, it would be a straightforward workflow to 1.) use blastocyst complementation to target a Neurog1-deficient niche in order to generate chimeric otocysts, that can 2.) be surgically extracted, 3.) GFP-labeled neuroblasts derived from donor cells isolated by flow cytometry, and 4.) could be transplanted to inner ears with damaged neuronal innervation. The prospect of isolating and transplanting autologous BC-derived cells has been successfully demonstrated when pancreatic islet cells were extracted from mouse-rat chimeras and transplanted into a chemically-induced mouse diabetes model and normalized host blood glucose levels for over a year (35). Moreover, developing the therapeutic application for using BC to create inner ear neurosensory cells is simpler than the goal of using BC for the generation of entire organs, which may require the nullification of several genes, (such as those required for development of the vasculature system (36)) in order to prevent immune rejection.

Unlike other studies using BC, we demonstrated that simply being haplodeficient for a master fate determining gene is sufficient to generate BC derived tissue, which adds nuance to the complementation field. This unexpected finding led to the analysis of heterozygous inner ears with and without complementation, and generated data which suggests that Neurog1 is dose-dependently required for non-sensory development while largely preserving neurosensory development. This may suggest that Neurog1 is involved in a binary decision to either adopt a non-sensory or neurosensory fate through an interaction with Notch signaling, a notion consistent with previous studies (15, 32, 33, 37). Importantly, the observed non-sensory defects in the Neurog1+/− were rescued in successfully complemented chimeric samples (Fig. 4). This supports the idea that our complementation results cannot be attributable to general chimerism, but rather are specific to an endogenous role for Neurog1 in inner ear morphogenesis, in addition to neurogenesis.

Here we establish, using intraspecies mouse chimeras, that BC is a platform that can be used for generating inner ear cell types. The generation of human induced pluripotent cells (hiPSCs) has opened the door for generating tissues that are either autologous or from closely related genetic backgrounds as the recipient (38). Therefore, this work sets the stage for interspecies complementation with the goal of generating human inner ear tissues using swine as biological incubators, which could lead to improved research models and drug screening methodologies and ultimately transplantation to deaf patients to improve hearing.

**Declarations**

*Ethics approval and consent to participate*

Not applicable.
Consent for publication

Not applicable.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declare no financial or competing interests.

Funding

Research reported in this publication was supported by the National Institute on Deafness and Other Communication Disorders of the National Institutes of Health under award number F31DC015153 (Predoctoral Fellowship, ARS) and the National Institute on Aging of the National Institute of Health under award number 2T32AG029796-11 (Postdoctoral Fellowship, ARS), and a private donation from Bridget Sperl and John McCormick supported the development of the \textit{Neurog1$^{-/-}$} mice and the acquisition of resources (PAS and WCL). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Acknowledgements

We would like to acknowledge individuals from the University of Rochester, in Rochester NY, Dr. Amy Kiernan, Dr. Patricia White, Dr. J. Christopher Holt, and Dr. Lin Gan, who’s expert mentorship and training of A.R.S supported the development of this project. Additionally, we would like to acknowledge members of the Stem Cell Institute at the University of Minnesota for advice and support.

Abbreviations

\begin{itemize}
  \item AA: anterior ampulla
  \item \textbf{ATOH1}: atonal homolog 1
  \item \textbf{ASCC}: anterior semicircular canal
  \item \textbf{BABB}: benzyl alcohol: benzyl benzoate
  \item \textbf{BC}: blastocyst complementation
  \item \textbf{E}: embryonic day
\end{itemize}
EDTA: ethylenediaminetetraacetic acid
DAPI: 4',6-diamidino-2-phenylindole
DPI: dot per inch (which reflects pixels per inch)
FIJI: Fiji Is Just ImageJ
GFP: green fluorescent protein
HC: mechano-sensory inner ear hair cell
hiPSCs: human induced pluripotent cells
iPSCs: induced pluripotent stem cell
IACUC: institute of animal care use committee
IHC: immunohistochemistry
Neurog1: neurogenin1
LA: lateral ampulla
LSCC: lateral semicircular canal
MYO6: unconventional myosin VI
P: postnatal day
PCR: polymerase chain reaction
PA: posterior ampulla
PBS: phosphate buffered saline
PBST: phosphate buffered saline with 0.1% Triton X-100
PFA: paraformaldehyde
PSCC: posterior semicircular canal
RAR: research animal resources
RC: Rosenthal’s canal with SGNs
SAC: saccule
SGN: spiral ganglion neurons
SPF: specific pathogen free
SNHL: sensorineural hearing loss
sTSLIM: scanning thin sheet laser image microscope
TFM: tissue freezing medium
TUJ1: class III beta-tubulin
Ut: utricle
VG: vestibular ganglion

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**Figures**
Figure 1

Donor GFP-labeled stem cells create chimeric spiral ganglion and vestibular neurons in the Neurog1 heterozygote (A) Schematic of experimental methods for BC. Neurog1+/- female mice were superoverulated with hormones. Zygotes were extracted and fertilized by in vitro fertilization (IVF) with Neurog1+/- male sperm and allowed to develop until blastocyst stage (~E3.5), at which point all blastocysts were injected with GFP-labeled iPSCs. Complemented blastocysts were transferred to a
pseudopregnant surrogate. Pregnancies were to taken to birth at which point embryos were harvested and complementation in the inner ear was assessed. (B) Diagram of the inner ear indicates the vestibular (balance) portion and cochlear (auditory) portion. Low magnification of the midmodiolar region of the cochlea in (C) the Neurog1+/+ wild type inner ear shows the absence of integration of GFP-labeled cells into the SGN (arrows) in contrast to (D) with specific complementation in the SGN in a Neurog1 +/- heterozygote (arrowheads). High magnification images of the basal cochlear turn show the (E) presence of GFP-labeled cells in the Neurog1 +/- heterozygous SGN (arrowhead) and absence in (F) the Neurog1+/+ wild type SGN (arrow). Specific complementation was seen in the Neurog1+/+ heterozygous vestibule, (G) in Scarpa’s ganglion adjacent to the utricle (UT) and saccule (Sac), (H) neurites (arrowhead) innervating the posterior ampulla, and (I) neurites innervating the anterior and lateral ampulla (arrowheads). AA: Anterior Ampulla, LA: Lateral Ampulla, PA: Posterior Ampulla, Ut: Utricle, SAC: Saccule, RC: Rosenthal’s Canal with SGNs. All scale bars are 100 μm. Scale bar in D also corresponds to C.

Figure 2

Complementation of Neurog1-deficiency is distinct from general chimerism. (A,A’) High magnification of the basal turn in a chimeric Neurog1+/+ cochlea does not have the donor-derived cell engraftment in the spiral ganglia, (solid outline) compared to (B-D’) examples from two separate chimeric and complemented Neurog1+/- cochleas in which donor iPSC-derived progeny extensively contribute to the
SGN (dotted outline). Chimerism was seen in both the (E) Neurog1+/+ brain and (F) the Neurog1+/- brain, indicating that while both embryos successfully formed chimeras, donor cells did not specifically contribute to the SGN in the absence of a vacant niche. All scale bars are 100μm. Scale bar in A’ also corresponds to A-B’, scale bar in F also corresponds to E. DAPI: 4,6-diamidino-2-phenylindole, TUJ1: class III beta-tubulin antibody, GFP: green fluorescent protein antibody against endogenous fluorescence, MYO6: Unconventional Myosin VI antibody.

**Complemented Neurog1+/- vestibule, example 111810**

**Figure 3**

Donor cells extensively contribute to the complemented Neurog1+/- vestibule. High magnification of the (A,A’) posterior ampulla and (B,B’) utricle in a complemented Neurog1+/- vestibule shows that GFP-labeled donor cells contribute to the near entirely of both structures, as GFP co-expresses with TUJ1-labeled vestibular nerves in the vestibular ganglia (VG) (dotted outlines), some MYO6-labeled vestibular hair cells (red) and non-sensory vestibular tissue (white arrows) (C) Inner ear diagram showing vestibule with anterior ampulla (AA), lateral ampulla (LA), posterior ampulla (PA), saccule (SAC) and utricle (UT). VG: vestibular ganglion. All scales bars are 100μm.
Figure 4

The Neurog1+/- heterozygous vestibule showed non-sensory defects that were rescued by stem cell complementation. Three-dimensional reconstructions of a (A) Neurog1+/+ wild type inner ear compared to (B) a Neurog1+/- inner ear, that is markedly smaller, which is particularly noticeable in the anterior (AA) and lateral ampullae (LA) (dotted boxes). (C) Higher magnification images of the dotted boxes from A,B, and a comparable region from a complemented Neurog1+/- heterozygote, shows a rescue of vestibular morphogenesis (A',B',C). (D) Quantification of the saccule volume in a Neurog1+/+ (n=2), non-complemented Neurog1+/- (n=3), and a complemented Neurog1+/- heterozygote (n=1) shows the rescue of the non-complemented Neurog1+/- utricular size with complementation. (E) Cross-section through the saccule (SAC) and utricle (UT) in a Neurog1+/+ control shows the orthogonal orientation of the sensory maculae (small arrows). By contrast, (F) the Neurog1+/- heterozygous vestibule had a smaller saccule (SAC) and the utricle (UT) and anterior ampulla (AA) were unusually close in orientation (arrowheads). Hair cells in each sensory region were orientated in the same direction (small arrows, red). (F') Ectopic
hair cells were occasionally seen in the vestibular ganglia adjacent to the vestibule. These deficits were rescued in (G) complemented Neurog1+/− heterozygote samples. Similarly, compared to (H) the anterior (AA) and lateral ampullae (LA) in a Neurog1+/+ control, (I) the non-complemented Neurog1+/− heterozygous anterior ampullae (AA) was smaller and appeared connected to the lateral semi-circular canal (LSCC, arrowheads). (I′) Ectopic hair cells were sometimes seen in the semicircular canals (box). These defects were rescued in (J) complemented Neurog1+/− samples. All scale bars are 100µm. Scale bar in B also corresponds to A; scale bar in G corresponds to E and F; scale bar in J corresponds to H & I.

**Supplementary Files**

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