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Characterization of a Unique Cell Population Marked by Transgene Expression in the Adult Cochlea of Nestin-CreER^{T2}/tdTomato-Reporter Mice

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Abstract

Hair cells in the adult mammalian cochlea cannot spontaneously regenerate after damage resulting in the permanency of hearing loss. Stem cells have been found to be present in the cochlea of young rodents; however, there has been little evidence for their existence into adulthood. We used nestin-CreER^{T2}/tdTomato-reporter mice to trace the lineage of putative nestin-expressing cells and their progeny in the cochleae of adult mice. Nestin, an intermediate filament found in neural progenitor cells during early development and adulthood, is regarded as a multi-potent and neural stem cell marker. Other investigators have reported its presence in postnatal and young adult rodents; however, there are discrepancies amongst these reports. Using lineage tracing, we documented a robust population of tdTomato-expressing cells and evaluated these cells at a series of adult time points. Upon activation of the nestin promoter, tdTomato was observed just below and medial to the inner hair cell layer. All cells co-localized with the stem cell and cochlear-supporting-cell marker Sox2 as well as the supporting cell and Schwann cell marker Sox10; however, they did not co-localize with the Schwann cell marker Krox20, spiral ganglion marker NF200, or GFAP-expressing supporting cell marker. The cellular identity of this unique population of tdTomato-expressing cells in the adult cochlea of nestin-CreER^{T2}/tdTomato mice remains

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Conflict of Interest

The authors declare no potential conflict of interest.

Author Contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: CC, WX, XZ, SG. Acquisition of data: CC, WX. Analysis and interpretation of data: CC, WX, PT, SG. Drafting of the manuscript: CC. Critical revision of the manuscript for important intellectual content: CC, WX, PT, XZ, SG. Statistical analysis: CC. Obtained funding: XY, SG. Administrative, technical, and material support: PT, XY. Study supervision: CC, SG.

unclear however these cells may represent a type of supporting cell on the neural aspect of the inner hair cell layer.

Keywords

inner ear; supporting cell; regeneration; stem cell; mouse; AB_10015251; AB_10013382; AB_2286684; AB_2195374; AB_306298; AB_10064079; AB_149792; AB_396354; AB_2251134; AB_2314882

Introduction

Many organs in the adult mammal maintain a population of progenitor cells for general maintenance and regeneration (Fuchs and Segre, 2000). The adult mammalian cochlea lacks the regenerative capacity to replace damaged or lost hair cells resulting in the permanent nature of most cases of sensorineural hearing loss. Non-mammalian vertebrates, in contrast, have the ability to replace lost hair cells through direct trans-differentiation of supporting cells or through mitotic division of supporting cells with subsequent differentiation into one hair cell and one supporting cell (Corwin and Cotanche, 1988; Ryals and Rubel, 1988).

The vestibular epithelia in mammals demonstrates some capacity for hair cell regeneration; however, the amount of functional restoration after regeneration of the sensory epithelia remains unclear (Forge et al., 1993; Walsh et al., 2000; Warchol et al., 1993). To explore reasons for cochlear and vestibular restorative differences, Oshima and colleagues collected cells from multiple areas of the inner ear at different postnatal time points to assess their ability to form spheres *in vitro*, a characteristic of stem cells (Oshima et al., 2007). Sphere-forming populations of putative stem cells were found in the utricular macula, saccular macula, ampullary crista, organ of Corti, spiral ganglion, and stria vascularis at early postnatal time points however during the second and third post natal weeks, there was a significant decrease in the sphere-forming ability of isolates from the auditory sensory epithelium as compared to the vestibular epithelium. This observation is consistent with reports of expression of the adult stem cell marker Lgr5 in some supporting cells of the organ of Corti from embryonic and neonatal mice (Chai et al., 2011). Cells expressing Lgr5 are proposed to have the capacity to generate hair cells in the developing inner ear (Shi et al., 2012) although their expression appears to diminish rapidly in the postnatal cochlea (Chai et al., 2011; Shi et al., 2012). In related work, Cox et al. (2014) have reported that neonatal mice have the capacity to regenerate hair cells spontaneously *in vivo* after hair cell ablation. New hair cells were derived by cell proliferation and direct trans-differentiation of the Lgr5-positive population of cochlear supporting cells. Although hair cell regeneration appeared to be restricted to the first postnatal week, this report provided further insight into the competency of the mammalian cochlea for some level of regeneration. In aggregate, these findings suggest that tissue-specific stem cells reside in the rodent cochlea but lose their ability to self-renew in early adolescence, in general agreement with the known absence of regenerative potential in the adult cochlea.

A number of investigators have evaluated nestin expression in the organ of Corti (Carricondo et al., 2010; Kojima et al., 2004; Lopez et al., 2004; Lou et al., 2007; Malgrange et al., 2002;

Smeti et al., 2011; Watanabe et al., 2012). This neural and stem cell marker is a type VI-intermediate filament protein widely used to identify cells with stem cell characteristics in developing and adult tissues (Lendahl et al., 1990). It is expressed in numerous proliferating tissues and is thought to be a reliable marker of mitotically active cells (Kachinsky et al., 1995; Lendahl et al., 1990; Sejersen and Lendahl, 1993; Suzuki et al., 2010). During murine embryonic development, nestin expression is observed in proliferating central nervous system (CNS) regions starting on embryonic day (E)7.75 (Dahlstrand et al., 1995). Expression decreases significantly after birth with the majority of nestin expression in proliferating neural tissues restricted to the subventricular zone of the lateral ventricles and dentate gyrus of the hippocampus (Alvarez-Buylla et al., 2002; Cameron and McKay, 2001; Fukuda et al., 2003; Gage, 2002; Lagace et al., 2007). Upregulation of nestin-expressing cells occurs following injury to the CNS (Sahin Kaya et al., 1999) and retina (Xue et al., 2006) suggesting that nestin expression may mark cells with stem- or progenitor-like qualities. Several studies have documented nestin expression in the cochlea of developing and postnatal rodents. In all cases, nestin expression was observed in the early postnatal cochlea and down-regulated in the mature cochlea. In some of these reports, nestin-expressing cells in the postnatal rodent cochlea were multipotent tissue-specific stem cells. Interestingly, the persistence of nestin expression during maturation, as well as the localization of nestin-expressing cells within the auditory sensory epithelium (the organ of Corti) has varied between reports (Carricondo et al., 2010; Kojima et al., 2004; Lopez et al., 2004; Lou et al., 2007; Malgrange et al., 2002; Smeti et al., 2011; Watanabe et al., 2012).

In this study, we examined the temporal and spatial organization of putative nestin-expressing cells and their progeny in the adult cochlea using nestin-CreERT²/tdTomato-reporter mice. We identified a robust, unique population of tdTomato-expressing cells on the neural aspect of the inner hair cell layer. In addition, we found tdTomato-positive cells co-localized with the stem- and supporting-cell marker Sox2 and did not express proteins indicative of a Schwann, neural or glial cell fate. Our characterization of putative nestin-expressing cells and their progeny provides insight into the possible persistence of a population of stem cell-like cells in the adult mammalian cochlea.

Materials and Methods

Animal Use

Animal subject protection—All experiments were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Madison. Animals were handled and cared for in compliance with the Animal Welfare Act and National Institutes of Health Guidelines.

Nestin-CreERT²/tdTomato-reporter mice were used to identify putative stem cells and their progeny in the adult mouse cochlea. The sample included 15 C57BL/6 mice ranging from ~8–26 weeks of age, 13 of which were used for quantitative analysis (Table 1).

Generation of mice expressing CreERT², and subsequent tdTomato, under nestin regulation was previously described (Lagace et al., 2007; Madisen et al., 2010). In brief, nestin-CreERT² mice and Rosa-tdTomato (Ai14) reporter mice were bred to create transgenic

offspring (Fig. 1A). CreER^{T2} is expressed under the control of a nestin promoter and remains in the cytoplasm of nestin-expressing cells. The expression of nestin-CreER^{T2} is inducible by means of tamoxifen (TMX) injection, which activates the estrogen receptor fused to Cre (CreER^{T2}), allowing it to enter the nucleus, recombine the LoxP sites to remove a stop codon, and express tdTomato in tissues expressing Cre-recombinase (Fig. 1B). Genotyping for Cre and Rosa-tdTomato (Ai14) reporter mice was performed as described previously (Guo et al., 2011).

In our experiment, 15 adult mice were given 5 injections of tamoxifen over 5 consecutive days (180 mg kg⁻¹ intraperitoneal injection, 30 mg ml⁻¹ in 10% EtOH mixed with sunflower oil) (Guo et al., 2011; Lagace et al., 2007). Cochlear tissue was harvested 1, 7, 14, 35, or 56 days post-tamoxifen injection (Fig. 1C; Table 1). Experimental cochleae were analyzed to ensure appropriate morphological appearance and expression of tdTomato. An adult control nestin-CreER^{T2}/tdTomato-reporter mouse confirmed no tdTomato was expressed in the cochlea in the absence of tamoxifen.

Tissue Preparation

Mice were sacrificed by intraperitoneal injection of sodium pentobarbital and then transcardially perfused with saline, followed by 4% paraformaldehyde (Guo et al., 2011). Temporal bones containing the inner ear were removed and fixed in 4% paraformaldehyde for 24 hours, after which they were moved to a 4% solution of ethylenediaminetetraacetic acid (EDTA) for 3 days to decalcify the bone. Whole mount microdissections of the organ of Corti from the temporal bone were then performed and processed for immunohistochemistry.

Immunohistochemistry—Cochlear tissue sections were incubated at 4 °C for 30 minutes in a solution containing 0.1% Saponin (Sigma 47036) and 0.1% Tween 20 (Sigma P7949) in PBS, followed by incubation for 1 hour in a blocking solution consisting of 10% normal donkey serum, 0.2% Triton X-100 in 1xTBS (adapted from Hume et al. (2003)). Tissue sections were then exposed to primary antibodies diluted in the blocking solution overnight. Primary antibodies used in this study include: polyclonal Myosin VIIa, Glial fibrillary acidic protein (GFAP), Sox2, Sox10, neurofilament-200 (NF200), Krox20 and nestin (See Table 2 for details regarding all antibodies). The following day, samples were washed 3 times in PBS for 5 minutes, then exposed to fluorescent labeled secondary antibodies and DAPI (Sigma-Aldrich, 1:1000) in the blocking solution overnight. Secondary antibodies used include: donkey anti-goat Alexa 488 (Invitrogen, 1:500) and donkey anti-rabbit Alexa 647 (Invitrogen, 1:500). The following day, samples were washed 3 times in PBS for 5 minutes and mounted with Vectashield-mounting medium for analysis.

Antibody Characterization

Rabbit polyclonal Myosin VIIa was generated from amino acids 877-1075 of human Myosin VIIa and specifically labels cochlear hair cells and their stereocilia (Hasson et al., 1995) (Proteus Biosciences Cat#25-6790, RRID: AB_10015251). This antibody was previously characterized by Burns et al. (2013), and found to recognize 230–250 kDa bands in immunoblots of temporal bone tissue from rats, which is consistent with the expected

molecular weight of Myosin VIIa. The Myosin VIIa antibody used in this study showed a similar staining pattern in the cochlea that has previously been identified as cochlear hair cells (Burns et al., 2013).

Rabbit polyclonal GFAP was isolated from the cow spinal cord and is a marker for glial cells (Dako Cat#70334, RRID: AB_10013382). This antibody was previously characterized and found to have appropriate band size and expression pattern consistent with previous reports (Fuentes-Santamaria et al., 2013; Yamanaka et al., 2011). The GFAP antibody used in this study localized to the border cell and inner phalangeal area (under the inner hair cell row), and showed a similar expression pattern compared to other studies evaluating GFAP in the inner ear (Rio et al., 2002; Smeti et al., 2011).

Goat polyclonal Sox2 antibody was generated from amino acids 277-293 of human Sox2 and labels all cells in the nascent sensory epithelium during early development, and is later down-regulated to supporting cells of the inner ear (Hume et al., 2007; Jones and Warchol, 2009; Millimaki et al., 2010; Oesterle et al., 2008) (Santa Cruz Cat#sc-17320, RRID: AB_2286684). This antibody was previously characterized and found to have a band size and expression pattern consistent with previous reports (Jones and Warchol, 2009). The Sox2 antibody used in this study showed a similar staining pattern in the cochlea that has previously been identified as supporting cells (Hume et al., 2007).

Goat polyclonal Sox10 antibody was generated from amino acids 1-20 of human Sox10 and labels neural crest cells during early development, and glia cells in later development and throughout adulthood (Kuhlbrodt et al., 1998; Pusch et al., 1998; Southard-Smith et al., 1998) (Santa Cruz Cat#sc-17342, RRID: AB_2195374). In the inner ear, this antibody labels all cells in the otic vesicle epithelium during early development and is later down-regulated to the supporting cell layer (Breuskin et al., 2009). This antibody was previously characterized and found to have the appropriate band size and specificity (Commo et al., 2004; Davies, 2007; Watanabe et al., 2000). The Sox10 antibody used in this study showed a similar staining pattern in the cochlea that has previously been identified as supporting cells (Wakaoka et al., 2013).

Rabbit monoclonal Ki67 was obtained from Thermo Scientific (Cat# (SP6) RM-9106, RRID: AB_149792). This antibody labels proliferating cells during late G1, S, M, and G2 stages of the cell cycle (Taupin, 2007). This antibody was previously characterized and found to co-localize with BrdU-positive cells (Garcia-Ovejero et al. (2013)). In mouse cochlear tissue, Ki67 was not observed in the organ of Corti nor spiral ganglion. This was not unexpected given that terminal mitosis in the mouse otocyst occurs during development between E13.5 and E16 (Ruben, 1967).

Rabbit polyclonal NF200 was obtained from Abcam (Cat#Ab8135, RRID: AB_306298). This antibody recognized a single band of about 200 kDa on mouse brain lysates and cochlear tissues in western blot analysis. The NF200 antibody used in this study stained a comparable area of the cochlea previously identified as spiral ganglion neurons (Jyothi et al., 2010; Provenzano et al., 2011).

Rabbit polyclonal Krox20 was obtained from Covance (Cat#PRB-236P, RRID: AB_10064079). This antibody labels Schwann cells and co-expressed the supporting and Schwann cell marker Sox10. The Krox20 antibody used in this study localized to the spiral ganglion area reported to contain Schwann cells and showed a similar expression pattern compared to other studies evaluating Schwann cells in the inner ear (Knipper et al., 1998; Provenzano et al., 2011).

In an effort to confirm that cre was faithfully expressed with nestin, we immunostained for nestin. A number of methods and antibodies were employed in this endeavor including antigen retrieval, increasing the incubation times, and varying the permeabilization methods. Despite these efforts we were unable to successfully identify nestin expression in the mouse cochlea. The antibodies we employed were obtained from BD Pharmaglin (Cat#556390, RRID: AB_396354), Millipore (Cat#MAB5326, RRID: AB_2251134), and Aves labs (Cat#NES, RRID: AB_2314882) and were characterized by (Naritsuka et al., 2009), (Farahani et al., 2011) and (White et al., 2010) respectively. These antibodies were reported to successfully label nestin-expressing progenitor cells in other tissues; however, as above they did not label nestin-expressing cells in adult mouse cochlear tissue.

Imaging and Analysis

Whole mount samples were imaged using a Nikon A1R-A1 confocal microscope with 60x and 10x objectives and a Lightsheet Z.1 by Carl Zeiss Microscopy GmbH with a 5x/0.16 EC Plan-NEOFLUAR objective. Images were exported into tiff-format, after which they were adjusted for brightness, contrast and sharpness using FUJII ImageJ software (Schindelin et al., 2012). Figure panels were produced using Adobe Illustrator™ software.

For quantification of tdTomato-positive cells, two to three 60x images were taken on the Nikon A1R-A1 confocal microscope of each region of the cochlea (apex, mid, base) for a total of 6–9 images per cochlea (12–18 high powered images per animal). TdTomato-positive cells were counted in each area of the cochlea and entered into a table for quantitative analysis (Table 1). TdTomato-positive cell counts from each high powered field were then averaged for each animal. Animals from the same time period after tamoxifen injection were averaged for statistical analysis for comparing counts at each time point. Statistical analysis of experimental data using a one-way analysis of variance (ANOVA) and post-hoc tests using Tukey HSD were performed using R statistical software (Team, 2008) and IBM SPSS™ software. For all analyses, differences were considered significant if $P < 0.05$.

Results

TdTomato-expressing cells are observed in a consistent location and distribution in the cochlea of nestin-CreER^{T2}/tdTomato mice

We used a transgenic line of nestin-CreER^{T2} mice with an inducible form of Cre recombinase, which allowed for Cre-mediated recombination only in nestin-expressing cells upon tamoxifen injection (Lagace et al., 2007). The specificity and efficiency of transgene expression in nestin-expressing cells in this mouse line has been validated (Gao et al., 2009;

Guo et al., 2011; Lagace et al., 2007; Sun et al., 2014). We crossed nestin-CreER^{T2} mice with the tdTomato-reporter line (Madisen et al., 2010) so that, upon tamoxifen injection, tdTomato expression represents the nestin-expressing cells or their progenies that have Cre-recombination. The cochleae obtained from adult nestin-CreER^{T2}/tdTomato-reporter mice appeared morphologically normal, with tdTomato observed on the neural (medial) aspect of the inner hair cell layer (Fig. 2). TdTomato-positive cells were observed throughout the cochlea for all time points examined (Fig. 3B–D). An adult negative control nestin-CreER^{T2}/tdTomato-reporter mouse confirmed the absence of tdTomato-positive cells in the organ of Corti in the absence of tamoxifen (Fig. 3AF). Staining with the hair cell marker Myosin VIIa and the supporting cell marker Sox2 revealed three rows of outer hair cells and one row of inner hair cells along with a normal complement of supporting cells at each time point examined (Fig. 3I, N, S, X, AC, AH). As expected, tdTomato-positive cells appeared in the spiral ganglion. In addition, a distinct population of cells expressing tdTomato consistently appeared adjacent to the organ of Corti medial (on the neural aspect) to the inner hair cell layer (Fig. 3G, L, Q, V, AA), which were of interest in this study.

High magnification cross-sectional evaluation revealed the tdTomato-positive cells to be consistently located throughout the cochlea on the neural side (medial) and beneath the inner hair cell layer (closer to Rosenthal's canal) (Fig. 4C, D). There did not appear to be any tdTomato-positive cells residing completely within the organ of Corti; however, apical protrusions of tdTomato-positive cells appeared to approach the inner most Sox2 expressing cell population, presumably the border cell population of supporting cells. In addition, the position and morphology of these cells was similar at each time point analyzed. A majority of these cells appeared oval at the base with an upward protrusion through the basal lamina towards the inner hair cell layer (Fig. 3G, L, Q, V, AA; Fig. 4C, D). Occasionally we observed that the protrusion at the apical end appeared to widen or flair to some extent (Fig. 5J). This type of morphology was seen in each region of the cochlea (apex, mid, base) and was observed in a seemingly random pattern across all time points.

We next sought to determine if the number of tdTomato-positive cells increased over time. The number of tdTomato-positive cells in each high powered field (apex, mid and base) was counted for each ear of 13 mice and averaged across time points. The mean number of tdTomato-positive cells increased considerably from day 1 after the last tamoxifen injection to day 7, after which there was a slight increase in the average number of cells per region which peaked at day 35 (Fig. 6A). Statistical analysis of experimental data using a one-way analysis of variance (ANOVA) revealed $P < 0.006$. Post-hoc tests using Tukey HSD revealed no significant difference for the number of tdTomato-positive cells between days 1 and 7 post tamoxifen injection, and no significant difference between days 7 and 56 (Fig. 6A, B). There was a small but significant increase in the number of tdTomato-positive cells between day 1 and days 14 to 56. To determine if the observed increase in the number of tdTomato-positive cells was due to cell proliferation, we stained cochlear tissues with antibodies to Ki67. We found no evidence of Ki67 positive cells in any locations in the cochlea or spiral ganglion.

Characterization of tdTomato-positive cells

We did not observe co-expression of Myosin VIIa, a hair cell marker, in tdTomato-positive cells in any specimen. We therefore sought to identify if the tdTomato-positive cells in our experimental animals co-expressed supporting cell markers. Antibodies to a panel of supporting cell markers were tested for reactivity. We found reliable staining for the stem cell and supporting cell marker Sox2 which appeared to co-localize with all tdTomato-positive cells (Fig. 3I, N, S, X, AC; Fig. 4B, C, D). Additionally, the Schwann cell and supporting cell marker Sox10 co-localized with all tdTomato-positive cells (Fig. 5G). The expression of Sox2 and Sox10 appeared to have some level of variability amongst the population of tdTomato-positive cells. In general, weaker Sox10 expression was observed in Sox10 cells that co-localized tdTomato compared to cells expressing Sox10 alone. In addition, weak to strong expression of Sox2 was observed in cells that co-localized tdTomato. Although the expression levels of Sox2 and Sox10 cells that co-localized with tdTomato varied, tdTomato-positive cells consistently co-expressed these supporting cell markers on some level.

To determine if the population of tdTomato-positive cells might be Schwann cells, spiral ganglion neurons, or glia, we stained for Krox20, NF200 and GFAP respectively. All antibodies utilized in this study were previously characterized except for Krox20 and NF200. Krox20 is expressed in Schwann cells (Kipanyula et al., 2013; Provenzano et al., 2011) and NF200 is expressed in spiral ganglion neurons (Jyothi et al., 2010; Provenzano et al., 2011). Immunohistochemical analysis of adult mouse cochlear tissue found the Krox20 antibody co-expressed with the supporting and Schwann cell marker Sox10 (Fig. 5A). Not all cells co-localized Krox20 and Sox10, suggesting Krox20 is a more specific Schwann cell marker. In addition, the NF200 antibody recognized a single band of about 200 kDa in adult mouse brain lysates and cochlear tissue upon western blot analysis (Fig. 5B).

TdTomato cells did not co-localize with the Schwann cell marker Krox20 (Fig. 5L:). Krox20 localized to the area surrounding the spiral ganglion in a comparable region of the cochlea reported to contain Schwann cells (Knipper et al., 1998; Provenzano et al., 2011) (Fig. 5I). In addition, tdTomato-positive cells did not co-localize with NF200, which labels spiral ganglion neurons in the inner ear (Fig. 5Q). NF200 localized to the area just under the inner hair cell layer, and found to have a staining pattern in the cochlea consistent with reports of its expression in spiral ganglion neurons (Jyothi et al., 2010; Provenzano et al., 2011) (Fig. 5N). Last, tdTomato-positive cells did not co-localize with the glia cell marker GFAP (Fig. 5V). GFAP is reported to be expressed by inner border cells and inner phalangeal cells in the organ of Corti (Rio et al., 2002; Smeti et al., 2011). GFAP expression localized to a region comparable to inner border cells and inner phalangeal cells, with tdTomato-positive cells located below and medial to these cells (Fig. 5U; Fig. 7).

We stained for relevant antibodies to nestin antigens in an effort to confirm cre-positive cells in the cochlea are nestin-positive, thus demonstrating tdTomato-expressing cells are indeed progeny of nestin-expressing cells. A number of methods and antibodies were employed in this endeavor; however, we were unsuccessful in these attempts to verify the fidelity of cre for nestin expression in our mouse model. Other investigators have encountered this challenge when staining mouse cochlear tissue for nestin (Watanabe et al., 2012).

Discussion

In this study, we examined tdTomato-positive cells in the organ of Corti of adult transgenic mice expressing an inducible form of CreER^{T2} under the control of a nestin promoter. In this model tamoxifen-mediated activation of Cre-recombinase leads to tdTomato fluorescence in nestin-expressing cells. Harvesting tissue shortly after tamoxifen activation can be used to evaluate ongoing expression of nestin. Harvesting tissue at later time points after nestin promoter activation allows for lineage tracing as has been demonstrated in the subventricular and subgranular zones of the brain in mice (Lagace et al., 2007). We used this technique to evaluate nestin expression in the adult murine inner ear and characterize the fate of nestin-expressing cells and their descendants.

A number of earlier studies have described nestin-expressing cells in the murine inner ear however their persistence during maturation and their cochlear localization has varied between reports (Lopez et al., 2004; Smeti et al., 2011; Watanabe et al., 2012). Using nestin-GFP transgenic mice, Lopez et al. (2004) observed nestin-GFP expression in border cells, inner phalangeal cells, Deiters' cells, Satellite cells and in the greater epithelial ridge in postnatal day (P)5 cochleae. A mild GFP signal was also seen in outer hair cells although inner hair cells were GFP-negative. In P60 adult mice, nestin-GFP was down-regulated and localized to only a few Deiters' cells under the outer hair cells. Smeti and colleagues (2011), in contrast, found nestin-GFP expression to be restricted to supporting cells near the inner hair cell area, particularly the border and inner phalangeal cells on P3 with mild expression of nestin in a small number of inner hair cells and outer hair cells. In 8-week-old mice, nestin-GFP expression was restricted to spiral ganglion cells and nerve fibers projecting towards the inner hair cells. Most recently, Watanabe et al. (2012) have reported that nestin expression is restricted to supporting cells lateral to the outer hair cell region using nestin β -gal mice, an observation more consistent with that made by Lopez et al. (2004). The number of β -gal positive cells decreased as the cochlea matured, with nestin-expressing cells becoming confined to the apical region of the cochlea by 3 weeks of age and minimally detectable in the apex by P90. Research evaluating nestin expression in the inner ear of rats has been more congruent (Carricondo et al., 2010; Kojima et al., 2004; Lou et al., 2007; Malgrange et al., 2002). All four studies on the topic in rats utilized immunostaining to evaluate the spatial and temporal expression of nestin in the organ of Corti and found nestin expression restricted to a region surrounding the basilar membrane and external edge of the spiral limbus (below the inner hair cell layer) in postnatal and adult animals (Carricondo et al., 2010; Kojima et al., 2004; Lou et al., 2007; Malgrange et al., 2002). In aggregate, in both mouse and rat studies it is clear that nestin expression is consistently found in the developing and young rodent cochlea; however, the localization and persistence of nestin-expressing cells in the adult rodent inner ear is less clear.

By using lineage tracing, we could document tdTomato-fluorescence in cells in adult mice ~ 8–26 weeks of age. The tdTomato-expressing cells were located beneath the inner hair cell layer at a level closer to Rosenthal's canal and also medial to the inner hair cell layer in a region similar to that described in other studies (Carricondo et al., 2010; Kojima et al., 2004; Lou et al., 2007; Malgrange et al., 2002; Smeti et al., 2011). Importantly, the localization and morphology of these cells was consistent at each time point we examined with oval cell

bodies having an upward protrusion through the basal lamina and into the organ of Corti in a vast majority of these cells.

We observed an increase in the number of tdTomato-positive cells following tamoxifen administration, with a significant difference in numbers of tdTomato-positive cells between day 1 and days 14 to 56. The increased numbers of tdTomato-positive cells during these intervals may reflect either delayed labeling or potentially some level of proliferation of this cell population over time. However we did not observe adjacent pairs of tdTomato-positive cells at later time points, which one might expect if this cell population was actively proliferating. Furthermore, we did not observe any tdTomato-positive cells that co-expressed Ki67. It is possible that proliferation of these tdTomato-positive cells are a rare event in healthy animals and the cell cycle window is too short to capture by Ki67 staining. It is also possible that the increased numbers of tdTomato positive cells over time was due to delayed labeling after tamoxifen administration.

It is unclear if the tdTomato-positive cells we have observed in the cochlea possess stem/progenitor characteristics. Nestin expression designates a progenitor fate in the central nervous system and other organ systems. While it appears the tdTomato-positive cells we have identified do not actively proliferate in the cochlea, these cells may represent a quiescent stem cell population. In adult tissues, stem cells may remain in a quiescent state until there is a need for proliferation such as in the event of injury. It is also possible that nestin-positive cells are not stem cells, but rather committed progenitors that are destined to become one particular differentiated cell type. Additional studies incorporating neonatal time points will help determine whether this cell population can give rise to differentiated cell types in the cochlea (such as hair and/or supporting cells) or proliferate in response to injury are ongoing. These studies will address more definitively whether nestin expression correlates with a stem or progenitor fate in the cochlea.

The location of tdTomato-positive cells medial to the inner hair cells suggests either a supporting or neuronal cell fate. We found that the tdTomato-positive cells also express both Sox2 and Sox10, although expression of the latter is more variable and less robust. These transcription factors are expressed in both supporting cells in the mature organ of Corti (Millimaki et al., 2010; Oesterle et al., 2008; Watanabe et al., 2000) and in the spiral ganglion (Neves et al., 2007; Wakaoka et al., 2013). Consequently, based on Sox2 and Sox10 expression alone, we could not establish whether tdTomato-positive cells are supporting cells at the medial aspect of the organ of Corti or alternatively peripherally located elements of the spiral ganglion cell population.

To attempt to resolve this question, we used additional antibodies to assess expression of Krox20, GFAP and NF200. We found that the tdTomato-positive cells do not co-express GFAP, a marker for glia cells including non-myelinating Schwann cells. GFAP expression in the organ of Corti has also been found in supporting cells around the inner and outer hair cell area, including inner phalangeal cells, border cells and Deiters' cells after P15 (Rio et al., 2002; Smeti et al., 2011). Lack of co-expression of tdTomato and GFAP suggests that the tdTomato-positive cells are neither border cells nor inner phalangeal cells, the two most likely supporting cells found in this area. These cells also did not express NF200, a marker

for spiral ganglion neurons, or Krox20, a Schwann cell marker (Garbay et al., 2000; Hansen et al., 2001). These observations imply that the tdTomato-positive cell population we identified is not a subpopulation of spiral ganglion neurons, Schwann cells or GFAP-expressing supporting cells. Future development of new and better cell type-specific markers will allow us to clarify whether these cells belong to a unique cell population distinctive from Schwann or supporting cell types.

It is possible that the cre expression in the mouse line that we employed may not faithfully report nestin expression at the time of tamoxifen administration. A number of findings would support that this is not the case. First, the fidelity of cre expression in cells expressing nestin has been evaluated (Sun et al., 2014) and the nestin cre mouse line that we used in this study (K line) was found to be superior to others (lines H & 4) in limiting ectopic expression of the reporter. Furthermore, we did not find any evidence of a leak in the promoter activity in our negative control animals. In addition, we found tdTomato expression to occur in the mouse cochlea in a similar area as reported by other investigators using a different, constitutive transgenic nestin reporter line (Smeti et al., 2011). Collectively these findings provide support for the fidelity of the reporter line that we used for nestin expression in the mouse inner ear.

In summary, while evidence suggests that the putative stem cell population in the mammalian inner ear diminishes in mature mammals, our results raise the possibility that cells expressing a marker of a stem cell fate persist into adulthood. By using a lineage tracing technique, we were able to define the spatial and temporal expression of nestin in mice of similar or older ages as compared to earlier reports (Lopez et al., 2004; Smeti et al., 2011; Watanabe et al., 2012). We could also show that tdTomato-positive cells have supporting cell characteristics based on co-expression of Sox2 and Sox10 however the subtype of supporting cells remains unclear. Further investigations are needed to compare nestin-CreER^{T2}/tdTomato-reporter mice to nestin-GFP mice. In addition, the proliferative capabilities and potency of this cell population should be evaluated. Advancing our understanding of these cells and their function may facilitate approaches to target this cell population as a treatment for hearing loss.

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Figure 1.

Generation of transgenic mice and activation of tdTomato following tamoxifen (TMX) administration. **A:** Nestin-CreERT² mice and Rosa-tdTomato (Ai14) reporter mice were bred to create transgenic offspring. **B:** CreERT² is expressed under the control of the nestin promoter. The expression of nestin-CreERT² is inducible by means of tamoxifen (TMX) injection, which activates the estrogen receptor fused to Cre (CreERT²), allowing it to enter the nucleus, recombine the LoxP sites to remove a stop codon, and express tdTomato in tissues expressing Cre-recombinase. **C:** Timeline of experiment. Mice were given 5 injections of tamoxifen (TMX) on 5 subsequent days, after which their tissue was harvested 1, 7, 14, 35 or 56 days following the last TMX injection.

Figure 2.

Cochlea from an adult nestin-CreER^{T2}/tdTomato-reporter mouse 56 days post tamoxifen injection. Low power macroscopic imaging analysis showed a pattern of tdTomato expression on the neural aspect of Sox2-positive supporting cells. An apical-basal gradient was not observed in tdTomato-positive cells. Image was acquired on a Lightsheet Z.1 by Carl Zeiss Microscopy GmbH with a 5x/0.16 EC Plan-NEOFLUAR objective. Scale bar: 500µm.

Figure 3.

TdTomato-positive cells are found throughout the adult mammalian inner ear for all time points examined. **A:** Orientation of the organ of Corti. **B–D:** TdTomato-positive cells are observed throughout the cochlea in the apex (B), mid (C), and base (D) 56 days after the last tamoxifen injection. Images were acquired using a Nikon A1R-A1 confocal microscope. **E–AH:** High-power images showing tdTomato-positive cells in the normal mammalian inner ear 1 day, 7 days, 14 days, 35 days, and 56 days after the last tamoxifen injection (G, L, Q, V, AA respectively). The morphology of the tdTomato-positive cells appeared consistent, having oval cells bodies with extensions protruding upward towards the inner hair cell layer; however, some tdTomato-positive cells appeared to have slightly different morphologies with elongated oval bodies and/or widening protrusions near the apical end. Cochlear hair cells, labeled with Myosin VIIa, reveal a normal configuration with three rows of outer hair cells and one row of inner hair cells (H, M, R, W, AB, AG). TdTomato co-localizes with the stem cell and supporting cell marker Sox2 (I, N, S, X, AC). An adult control nestin-CreER^{T2}/tdTomato-reporter mouse confirmed no tdTomato was expressed in the cochlea in the absence of tamoxifen (AF). Images were acquired using a Nikon A1R-A1 confocal microscope. Scale bar: 500 μ m in B–D; 50 μ m in E–AH.

Figure 4.

Cellular organization of tdTomato-positive cells in the normal mammalian inner ear. **A:** Orientation of the organ of Corti. **B–C:** Cross sectional view of a cochlea 35 days after the last tamoxifen injection reveals one row of inner hair cells and three rows of outer hair cells along with a normal complement of cells labeled with the stem cell and supporting cell marker Sox2. **D:** Apical region of the cochlea 7 days after the last tamoxifen injection. TdTomato-positive cells are located below and medial to the inner hair cell layer and co-localize with the stem cell and supporting cell marker Sox2. Scale bar: 10 μ m in B–D.

Figure 5.

Characterization of tdTomato-positive cells. **A:** In adult murine cochlear tissue, cells expressing Krox20 co-expressed with the Schwann cell and supporting cell marker Sox10 (arrows indicate double-layered cells with overlap shown in white). A smaller percentage of Sox10-positive cells expressed Krox20, suggesting Krox20 is a more precise Schwann cell marker **B:** NF200 antibody recognized a single band of about 200 kDa using adult murine brain lysates and cochlear tissues. **C–G:** 1 day after the last tamoxifen injection. The supporting cell and Schwann cell marker Sox10 co-localized with all tdTomato-positive cells. **H–L:** 14 days after the last tamoxifen injection. Krox20, a Schwann cell marker, did not co-localize with tdTomato-positive cells. **M–Q:** 14 days after the last tamoxifen injection. The spiral ganglion cell marker NF200 did not co-localize with tdTomato-positive cells. **R–V:** 7 days after the last tamoxifen injection. GFAP (expressed in border and inner phalangeal cell sub-populations of supporting cells) did not co-localize with tdTomato-positive cells. Images were acquired using a Nikon A1R-A1 confocal microscope. Scale bar: 10 μ m in A; 50 μ m in panels F, K, P, U; and 10 μ m in panels G, L, Q, V.

Figure 6.

Statistical analysis. **A:** Box plot displaying the averages and spread of data (number of cells per high powered field) for each time point (Day). The mean number of tdTomato-positive cells increased from day 1 after the last tamoxifen injection to day 35, after which there was a slight decrease at day 56. **B:** Post-hoc tests using Tukey HSD comparing each time point (Day) to a comparison time point (Comparison Day) and the significance. There was no significant difference for the number of tdTomato-positive cells between days 1 and 7 post tamoxifen injection, and no significant difference between days 7 and 56. The only statistically significant difference seen was between day 1 and days 14 to 56. For all analyses, differences were considered significant if $P < 0.05$.

Figure 7.

The cellular organization of the organ of Corti consists of several supporting cells including: Deiters', Hensen's, pillar, border, and inner phalangeal cells, in addition to inner and outer mechanosensory hair cells. TdTomato-positive cells appeared under and medial to the inner hair cell layer in the neural aspect.

Table 1

Sample Information

Specimen information used for quantitative analysis include sample number (Mouse), sex, date of birth (DOB), date of first injection (DOI), number of days tissue collected after the last tamoxifen injection (ND), age in weeks at tissue harvest (ATH), and average number of cells per region for that sample (Cells/Region/Sample).

Mouse	Sex	DOB	DOI	ND	ATH	Cells/Region/Sample
A1	M	2/3/12	3/25/12	1	8.1	14.5
A2	M	2/3/12	3/25/12	1	8.1	14.83
7	F	12/9/11	4/7/12	7	18.9	20.07
8	M	12/17/11	4/7/12	7	17.7	23.17
10	F	12/9/11	4/7/12	14	19.9	24.56
11	F	12/17/11	4/7/12	14	18.7	21.06
12	M	12/17/11	4/7/12	14	18.7	30.32
13	F	12/9/11	4/7/12	35	22.9	28.27
14	M	12/31/11	4/7/12	35	19.7	25.39
15	F	12/17/11	4/7/12	35	21.7	29.37
16	F	12/9/11	4/7/12	56	25.9	24.89
17	M	12/9/11	4/7/12	56	25.9	26.00
18	M	12/9/11	4/7/12	56	25.9	25.83

Table 2

Primary antibodies used in this study

Complete list of antibodies, labeling, immunogens, manufacturers, species and dilutions.

Antibody	Labeling	Immunogen	Manufacturer	Cat. No.	Species	Dilution	RRID
Anti-Myosin VIIA	Cochlear Hair Cells	Amino acids 877-1075 of human Myosin VIIA	Proteus Biosciences	25-6790	Rabbit Polyclonal	1:100	AB_10015251
Anti-GFAP	Border Cells and Inner Phalangeal Cells (Supporting Cell Subtypes)	GFAP isolated from cow spinal cord	Dako (Denmark)	70334	Rabbit Polyclonal	1:1000	AB_10013382
Anti-Sox2	Supporting Cells and Stem Cells	Amino acids 277-293 of human Sox2	Santa Cruz	sc-17320	Goat Polyclonal	1:100	AB_2286684
Anti-Sox10	Supporting Cells and Schwann Cells	Amino acids 1-20 of human Sox10	Santa Cruz	sc-17342	Goat Polyclonal	1:100	AB_2195374
Anti-NF200	Spiral Ganglion Neurons	Recombinant fragment (Cow)	Abcam	Ab8135	Rabbit Polyclonal	1:1000	AB_306298
Anti-Krox20	Schwann Cells	Amino acids 8-95 of Krox20 fused to GST	Covance	PRB-236P	Rabbit Polyclonal	1:100	AB_10064079
Anti-Ki67	Proliferating Cells	Human Ki67 C-terminus	Thermo Scientific	RM-9106-S1	Rabbit Monoclonal	1:250	AB_149792
Anti-Nestin	Nestin/Progenitor Cells	Rat (E15) spinal cord extracts	BD_PharmaGin	556309	Mouse Monoclonal	1:100, 1:250, 1:500	AB_396354
Anti-Nestin	Nestin/Progenitor Cells	Full-length human recombinant nestin	Millipore	MAB5326	Mouse Monoclonal	1:100, 1:250, 1:500	AB_2251134
Anti-Nestin	Nestin/Progenitor Cells	EKE DQR FPR SPE EDQ Q; EKE ROE SLK SPE DED QQ; EYE EGP ERE QHQ ESL RS	Aves Labs	Cat#NES	Chicken Polyclonal	1:100, 1:250, 1:500	AB_2314882