

Distinct population of hair cell progenitors can be isolated from the postnatal mouse cochlea using side population analysis

Abbreviated title: cochlear SP stem/progenitor cells

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ABSTRACT

In mammals, the permanence of hearing loss is mostly due to the incapacity of the cochlea to replace lost mechano-receptor cells, i.e. hair cells (HCs). The generation of new HCs from a renewable source of progenitors is a principal requirement for developing a cell therapy within this sensory organ. A subset of stem cells, termed 'side population' (SP) has been identified in several tissues of mammals. The ATP-binding cassette transporter Abcg2/Bcrp1 contributes to the specification of the SP phenotype and is proposed as a universal marker for stem/progenitor cells. A defining character of these SP cells is a high efflux capacity for Hoechst dye.

Here, we demonstrate that Abcg2 transporter is expressed with two other stem/progenitor cell markers (i.e. Nestin and Musashi1) in distinct and overlapping domains of the supporting cells within the postnatal cochlea.

We have developed and describe a fluorescence activated cell sorter (FACS) technique that enables the purification of a discrete subpopulation of SP-supporting cells from the early postnatal mouse cochlea based on their ability to exclude Hoechst dye.

These FACS isolated cells can divide and express markers of stem/progenitor cells such as Abcg2 a determinant of the SP phenotype and Musashi1, a neural stem/progenitor cell marker. These markers can differentiate cells expressing markers of HCs and supporting cells *in vitro*. Our observation that these SP cells are capable of differentiating into hair cell-like cells implies a possible use for such cells, i.e. the replacement of lost auditory HCs within damaged cochlea.

INTRODUCTION

Stem/progenitor cells are capable of self-renewal and multilineage differentiation (1-2). While diversification of cell types is largely complete at or shortly after birth, many tissues in the adult undergo self-renewal and, accordingly, establish a population of progenitor cells for maintenance and regeneration (1, 3). Little is known concerning the stem/progenitor cells of mammalian sensory hair cells (HCs) and the cellular interactions that control their specification and differentiation. In birds and other non-mammalian vertebrates, lost HCs are regenerated by mitotic stimulation of quiescent supporting cells and functional hearing is recovered following a trauma (4, 5). In contrast, no regeneration is observed following the destruction of mature HCs in the mammalian cochlea. This lack of a documented cochlear progenitor cell population supports the paradigm that the mammalian cochlea is not capable of regeneration. The pool of terminally differentiated cochlear HCs is established before birth (6) and any insult resulting in their loss is an irreversible process resulting in deafness. Recent studies challenge these notions regarding cochlear repair in the mammals and suggest that the mammalian cochlea is capable of limited regeneration presumably through the activation and transdifferentiation of quiescent progenitor cells, probably the supporting cells (7, 8). Very recently, Segil and his co-workers (9) have reported the purification of HC progenitors from the postnatal mouse cochlea using p27^{kip1}-GFP transgenic mice and FACS. However, the purification of progenitor cells from the postnatal mouse cochlea through the application of universal epithelial stem/progenitor cells markers such as Abcg2 has not as yet been reported.

Abcg2, a half-transporter also called Bcrp1, is a member of the ATP-binding cassette (ABC) family of cell-surface transporter proteins. Unlike other ABC half-transporters, localized to intracellular membranes, Abcg2 is expressed exclusively in the plasma membrane (10) and considered as universal marker for stem cells (11, 12). Recently, Abcg2 was found in the primitive stem cells of several different tissues (13, 14) which supports the idea that it plays a significant role in maintaining stem cells in an undifferentiated state (15). Abcg2 is highly expressed in a specific population of cells, i.e., the so-called SP (side population) subset, which can be isolated by their ability to efflux of Hoechst 3342 dye (15-17). Therefore, in addition to its important use as a determinant of the SP phenotype, the Abcg2 gene is an attractive candidate marker for use in isolating stem/progenitor cells, including neural and otic progenitor cells. In this study, we localize, purify and culture Abcg2-

dependent SP cells from the postnatal mouse cochlea. This SP population includes many supporting cells also express Musashi1 (a putative marker of stem/progenitor cells) and display features that are consistent with the SP-progenitor cell phenotype.

MATERIALS AND METHODS

Mouse strains and Immunohistochemistry

Postnatal day-3 (P3) Swiss mice (Janvier, France) were used for this study in accordance with the INSERM guidelines. The nestin-EGFP transgenic mice were described previously (18) used to study the expression of nestin. Cochleae were removed from Swiss and nestin-EGFP transgenic P3 mice after deep Nembutal anesthesia. Cochleae were embedded in 4% agarose and cut into 50- μ m sections in PBS, using a vibratome. Free-floating cochlear sections were pre-incubated in a blocking solution and transferred to a solution containing primary antibodies. Sections were then incubated with Texas-Red conjugated or FITC secondary antibodies diluted 1/200 in PBS. Cover slipped slides were analyzed by Bio-Rad MRC 1024 scanning confocal microscope. Primary antibodies used for this study: polyclonal anti-Abcg2 (19), monoclonal anti-Musashi1 (20), monoclonal anti-Calbindin (Sigma-Aldrich), monoclonal anti-p27^{Kip1} (Neomarkers), polyclonal anti-Myosin VIIA (Affinity BioReagent) and polyclonal anti-Fimbrin (P. Matsudaira, Whitehead Institute, MA). Nestin was directly detected in transgenic mice expressing GFP under the control of Nestin promoter. For some specimens, counterstaining was performed with TRIC-conjugated phalloidin (Sigma) to visualize filamentous actin. Control sections were incubated without primary antibody. No specific immunofluorescence labeling was detected in any of these controls.

RT-PCR analysis

We extracted RNA from both cochlea of P3 and adult mice by using RNeasy kit (Qiagen). For reverse transcription with M-MLV reverse transcriptase (Invitrogen), we used 1 μ g total RNA treated with RNase-free DNase (Roche). PCR primers pairs were selected to discriminate between cDNA and genomic DNA by using individual primers specific for different exons, when possible. We used the following primers pairs (forward, reverse, cDNA product length in bp): Abcg2, (5'GCTGTGGAGCTGTTTCGTAGTGG3', 5'GCTAAAGTACTGAAGCCAGGAC3', 387 bp); Myosin VIIa (5'CTCCCTCTACATCGCTCTGTTTCG3', 5'AAGCACCTGCTCCTGCTCGTCCACG3', 628 bp);

Musashi1 (5'TACGCCAGCCGGAGTTACA3', 5'AAGCCGTGAGAGGGATAGCT3', 119 bp); p27^{Kip1} (5'CTGGAGCGGATGGACGCCAGAC3', 5'CGTCTGCTCCACAGTGCCAGC3', 525 bp); Gapdh (5'AACGGGAAGCCCATCACCC3', 5'CAGCCTTGGCAGCACCAG3', 442 bp). The data presented are representative of at least three independent experiments. The identities of the PCR products were confirmed by sequencing. Control PCR reaction lacking primers or cDNA did not produce product. FACS sorted SP and NSP cell dissociates were also subjected to RT-PCR analysis for the presence of Abcg2 and Musashi-1 mRNAs.

Flow cytometry

Twenty P3 mice were killed for the isolation of cochlear tissue. Stria vascularis and the spiral ganglia were both removed in PBS (Ca²⁺, Mg²⁺ free). Cochlear epithelia isolates were obtained by incubating the dissected cochleae with 0.5 mg/ml thermolysin (Sigma) at 37°C for 20 min. After stopping the reaction at 4°C, cochlear epithelia were subsequently digested with a mixture containing 1500 units/ml collagenase and 10 units/ml dispase for 30 min at 37°C, followed by several minutes of trituration. Tissues dissociates were filtered through 40 µm filter to remove cell debris. The yield was approximately 3-4 x 10⁵ viable cells from 40 dissected cochleae. These cochlear dissociates were re-suspended in DMEM-F12, and incubated with 5 µg/ml Hoechst 33342 (Sigma) for 90 min at 37°C under a slow swirling motion. In experiments to inhibit Abcg2 transporter, 50 µM verapamil (Sigma) was added 5 min before the addition of the Hoechst dye. Cells were spun down, resuspended in DMEM-F12 and 2 µg/ml propidium iodide (Roche). Cell sorting was performed using a FACSVantageTM (BD Biosciences) equipped with a 488 nm Laser. A live gate was defined on the FACS using Hoechst red and blue axes to exclude dead cells and debris. After 10⁵ events were collected within the live gates, the SP and non-SP (NSP) cells were defined as Hoechst-low and bright cells, respectively. SP and NSP cell fractions were sorted, centrifuged and used for RT-PCR analysis, immunolabeling or cultured.

Cell culture

FACS sorted cells were plated in 5 ml of medium onto poly-HEME (Sigma) coated T25 culture flasks to prevent cell attachment. The culture medium of this 'suspension' culture consisted of DMEM-F12 supplemented with N2 (Gibco), 20 ng/ml EGF and 10 ng/ml FGF2 (Preprotech). Five days in

'suspension' culture conditions generated aggregates/spheres that were then transferred in 24-wells plates coated with poly-D-lysine, i.e. 'adherent' condition. The medium for culture in the adherent condition remained the same except that the growth factors (i.e. EGF and FGF2) were omitted. The p27^{Kip1} and Myosin VIIA immunopositive cells were blind-counted in each sphere, 20 spheres were counted from each experimental condition i.e. SP and NSP cultures. The results were expressed as the mean number of labeled cells per sphere \pm standard deviation (n=4). Statistical significance was determined using a Student's *t*-test.

In three culture experiments, free floating SP and NSP primary spheres were dissociated to single cells by digestion with 0,25 % trypsin/0,53 mM EDTA (Invitrogen) at 37°C for 15 min and re-suspended in a 50:50 mixture of fresh and conditioned culture media that consisted of DMEM-F12 supplemented with N2, EGF and FGF2.

BrdU pulse

To label cells in S phase, BrdU (10 μ M) was added to the culture medium 3 days after the initiation of plating in 'suspension'. After 4 days i.e., 3 days in the presence of BrdU, plates containing BrdU-labeled cultures were examined for fluorescence.

RESULTS

Expression and tissue distribution of Abcg2, an SP phenotype marker

The expression of Abcg2 mRNA was investigated by RT-PCR in isolated cochlear epithelia from P3 and adult mice (Fig. 2). These reactions generated a single amplified product of the expected size (387 bp). RT-PCR analysis was also used to determine the presence of other inner ear cell type markers within the P3 and adult cochleae. We determined the expression of mRNA of Myosin VIIA: a hair cell marker, p27^{Kip1}: a marker expressed in the supporting cells and Musashi1: a marker for stem/progenitor cells (Fig. 2). Validity of all RT-PCR products was confirmed by sequence analysis (not shown).

We then carried out immunohistochemistry studies to determine the spatial expression of Abcg2 in the P3 cochlea using a rabbit polyclonal-Abcg2 antibody 405. This antiserum was raised against an 18-mer peptide of the ATP binding region of Abcg2. This sequence is located just proximal to the N-terminal and has 100 % identity only with Abcg2 (19, 21).

Abcg2 immunoreactivity was observed in many supporting cells (Figs. 3,4). All of the HCs were devoid of immunoreaction product, but Abcg2 immunolabel was significant in the three rows of Deiters cells (D) below the outer hair cells (OHCs), and Hensen's cells located laterally to the OHC area as well as in border and inner phalangeal cells, that surround the inner hair cells (IHCs) (Figs. 3B,3E,4 A). The IHC is flask shaped and is surrounded by supporting cells such as inner border cells (bc), inner phalangeal cells (ipc) and pillar cells (op and ip) (Fig. 1). A dense immunolabel for Abcg2 was present over the fibers of the spiral ganglion within the osseous spiral lamina projecting towards the area of the IHCs (Figs. 3B,4A,4D).

The lack of Abcg2 labeling from the HCs was confirmed by double immunostaining the cochlea for the presence of calbindin protein, a hair cell marker. Double labeling with Abcg2 and calbindin indicated that calbindin immunoreactivity is confined to the upper layer of cells of the organ of Corti that contains the HCs, whereas the Abcg2 expression was restricted to the basal layer located below the HCs that contains the supporting cells (i.e. Deiters, inner phalangeal, and border cells) (Figs. 3D,3F). The pattern of expression of p27^{Kip1}, the nuclear marker of supporting cells (Fig. 3G) allowed for recognition of the spatial location of the Abcg2 transporter to the plasma membrane of the supporting cells (i.e., Deiters' cells) (Fig. 3 I).

To gain insight on the spatial distribution of stem/progenitor markers within the postnatal mammalian cochlea, we determined the expression of Abcg2 in combination with that of Nestin and Musashi1. Our results indicate that the Abcg2 is expressed in overlapping domains with those of Nestin and Musashi1. The pattern of expression of Abcg2 and Nestin was studied in the P3 cochlea from transgenic mice expressing GFP under the control of the nestin promoter.

Analysis of promoter activity showed nestin expression in the border cells (bc), inner phalangeal cells (ipc) that surround the IHCs and the nerve fibers projecting toward the organ of Corti (Fig. 4B).

Nestin expression colocalizes with Abcg2 at the level of border cells below the IHCs and the spiral ganglion fibers (Fig. 4C) and was not present in the HCs.

Musashi1 was found in many supporting cells, e.g. pillar and Deiters' cells (Fig. 4E), Kolliker's organ cells located medial to the organ of Corti were also immunopositive for the presence of Musashi1 that colocalizes with Abcg2 in the Deiters's cells (Fig. 4F).

Isolation and culture of cochlear SP population

Cell dissociates from P3 mouse cochlear sensory epithelia were resuspended in 5 µg/ml Hoechst 33342 dye followed by FACS analysis using the dual Hoechst dye emission profile. FACS analysis revealed a large cell population with high Hoechst fluorescence (NSP) and a minor cell population with relatively lower Hoechst fluorescence (SP) (Fig. 5A). The emergence of the cochlear SP fraction was extremely sensitive to both dye concentration and the length of incubation in the dye. The proportion of the cochlear SP cells was around 0.53% of the total cells in the suspension dissociated from P3 cochlear epithelia.

To test the specificity of the SP subpopulation of cochlear cells that selectively eliminate the Hoechst dye, staining was performed in the presence of verapamil on an aliquot of dissociated cochlear cells. The efflux of Hoechst dye from stem cells has been shown to be verapamil sensitive and involves the Abcg2 transporter (15-17). Hoechst dye staining of cochlear cell dissociates in the presence of verapamil eliminated the fraction of SP cells within the cell suspension (Insert, Fig. 5A).

RT-PCR analysis showed a strong correlation between the presence of Abcg2 transcripts and the SP phenotype when the expression of Abcg2 was compared between SP and NSP sorted fractions. The expression of Abcg2 mRNA was evident in the SP fraction of cells while no signal was observed in the RT-PCR products from the NSP fraction (Fig. 5B).

Moreover, immunostaining confirmed the expression of Abcg2 protein in the SP cell population. The Abcg2 is distributed in punctuate clusters localized around the plasma membrane of the sorted SP cells (Fig. 5C). The NSP cells did not immunostain for the presence of Abcg2 (not shown).

Cochlear SP cells are capable of proliferation and differentiation

The FACS sorted SP cells were examined for aggregates/spheres formation by employing the *in vitro* procedure previously adapted for the inner ear tissue (22). After 7 days in 'suspension' culture condition, SP cells seeded at low density in the presence of EGF/FGF2 proliferated and formed substantial spherical shaped floating colonies, i.e. SP-spheres (Fig. 5D). To determine whether the SP-sphere was generated by mitosis, we used BrdU labeling. Representative BrdU labeled SP-spheres originating from cochlear SP cells are presented in (Figs. 5E, 5F). In a typical cochlear dissociate composed of approximately 2500 plated cells, SP fraction cells generated 20.25 ± 3.30 spheres as compared to the NSP cells which generated 4 ± 2.16 spheres ($P < 0.001$ four independent experiments) (Fig. 5G). After 3 days of continuous BrdU exposure, immunocytochemistry

revealed up to $20.18 \% \pm 6.89$ of cells that incorporated BrdU ($n=2$) of the total plated fraction of SP cells had replicated DNA as compared to the NSP plated fraction cultured under the same *in vitro* conditions which revealed only $3 \% \pm 1.8$ (Fig. 5H). This data indicate the major proliferative compartment of cells within the SP-spheres as compared to NSP-derived spheres.

To check whether SP-sphere-forming cells are capable of self-renewal, a requisite characteristic of stem cells, we dissociated SP-spheres and cultured the resulting individual cells at low density. We generate 2 ± 0.5 SP-secondary spheres ($n=3$) that were not expandable through additional passages probably due to their limited capability of self-renewal. Conversely, cells dissociated from NSP-spheres and maintained in the same *in vitro* conditions as the SP dissociated cells were not able to generate any secondary spheres after 10 to 15 days in culture (not shown).

We next examined whether cells included in expanded SP-spheres have the ability to develop antigenic properties of organ of Corti cells, i.e. express HC and SC epitopes. Spheres grown for 7 days in 'suspension' condition were plated on a poly-L-lysine substratum in serum-free medium in the absence of growth factors for 5 days (Fig. 6). Under these 'adherent' differentiating conditions, several SP cells within the sphere cell colony expressed the HC markers, myosin VIIA and fimbrin (Figs. 6B,6F). The SP cells with the strongest immunoreactivity for myosin VIIA expressed a more differentiated morphology, i.e. polarized nuclei and elongated cells bodies (Fig. 6F).

Other SP cells within the sphere expressed a supporting cell marker $p27^{Kip1}$ (Figs. 6C,6G), a protein that is initially expressed in hair and supporting cell progenitors and then downregulated in HCs but persists in mature supporting cells (23). In this culture paradigm, the amount of HCs and supporting cells generated was higher after 5 days of differentiation in the SP cell fraction as compared to the NSP fraction ($P < 0.001$). We obtained 5.10 ± 1.66 HCs and 14.70 ± 3.19 supporting cells per SP-sphere while only 0.90 ± 0.87 HCs and 2.30 ± 1.88 supporting cells were generated per NSP-sphere (Fig. 6I). To distinguish between incorporation in the SP-spheres of HCs that may have been present in the initial cell suspension and the formation of new HCs, the initial cell suspension was cultured in the presence of EGF/FGF2 for 5 days in 'suspension' culture followed by 4 days in "adherent" culture in the presence of BrdU. In this culture paradigm, we observed a subset of differentiated cells that exhibited overlapping nuclear immunoreactivity for BrdU incorporation and the early hair cell marker Math1 (Fig. 7) indicating that this subset of progenitors had divided, exited the cell cycle, and differentiated into Math1 positive immunolabeled cells.

DISCUSSION

The high incidence of hearing loss in humans (24, 25), combined with the lack of HC regeneration in mammals (26, 27), has fueled an interest in the possibility of identifying stem/progenitor cells that may be manipulated to participate in regenerative events. In mammals, auditory HCs are produced only during embryonic development (6) and do not regenerate if lost during postnatal life, therefore a loss of HCs will result in deafness (28). While stem/progenitor cells may exist in adult mammalian vestibular end organs (29), stem/progenitor cells have not been isolated from the postnatal cochlea.

SP cells were initially identified as a Hoechst dye low retention subpopulation isolated from bone marrow using flow cytometry (16). The ability of SP cells to efflux Hoechst dye is dependent on Abcg2 (15). Previous studies have shown that Abcg2 expression alone defines the SP phenotype and is a conserved feature of stem cells isolated from a wide variety of sources (11).

Here we report data, providing evidence that a subpopulation of supporting cells within the P3 mouse cochlea represent SP-progenitor cells that can be purified by FACS based on their ability to eliminate Hoechst 33342 dye. This cochlear SP cell population retains the ability to divide and progress in differentiation toward generating HC and supporting-like cells *in vitro*.

The Abcg2 expression is observed in many supporting cell types, including Deiters cells, inner phalangeal cells, border cells, and Hensen cells. Musashi1, another stem cell marker, colocalized with Abcg2 at the level of Deiters cells. The expression of a combination of these universal stem cell markers, along with p27^{Kip1}, an inhibitor of cell cycle progression, may help in specifying a subpopulation of the supporting cells as potential progenitor cells in the early postnatal cochlea.

Importantly, HCs do not express Abcg2 which makes their elimination possible by FACS purification.

To test whether Abcg2 expression identifies a subpopulation of supporting cells with SP progenitor features, we used FACS to separate them based on their ability to exclude Hoechst dye. A small but distinct fraction of SP cells obtained from four experiments was calculated to be 0.53 % of the total dissociated cells. This is a reasonably abundant value compared with that previously reported for other organs (17). However, this proportion of FACS sorted SP-cells was significantly lower than the outcome of the purification of supporting cells (19-22 %) obtained from p27^{Kip1}-GFP transgenic mice (9). This could be explained in part by the widespread expression of p27^{Kip1} protein in all supporting

cell types within the cochlear epithelium as compared to the Abcg2 which is not expressed in the pillar cells.

The supporting cells with an SP phenotype represent progenitor cells is supported by the fact that these cells can re-enter cell cycle, express Abcg2 and Musashi1, two conserved markers of stem/progenitor cells (11, 15, 30). Other studies have also reported Musashi1 expression in the supporting cells of both young and adult mammalian cochleae (31, 32).

A subset of purified supporting cells with an SP phenotype are able to proliferate and to form free-floating spheres *in vitro* when maintained in a non-adhesive environment with EGF/FGF2. This combination of growth factors promotes proliferation in our culture conditions, in accord with previous observations that implicated these factors in increasing the number of spheres derived from utricular epithelium (29), and with the expression of both EGF and FGF2 receptors in the supporting cells of P3 cochlea (33, 34). However, SP-supporting cells display only a limited capacity for self renewal (i.e. 2 passages) compared to the vestibular epithelium that contains stem cells capable of propagating for up to 10 passages (29). This may support the difference of the regenerative capabilities *in vivo* between the supporting cells within the mammalian cochlear epithelium that are unable to divide after HC loss and supporting cells from the vestibular epithelium that retain a limited ability to divide (35, 36).

We tested whether SP-spheres grown in 'suspension' culture can differentiate under 'adherent' culture conditions. Four days post-plating, a subset of SP-supporting cells were observed to trans-differentiate into cells that express epitope markers for HCs (Myosin VIIA, Fimbrin, Math1).

Cells that expressed high levels of Myosin VIIA (a marker for HC maturation) were more elongated with an eccentric located nucleus suggestive of a more mature cell morphology.

HC-like cells generated in our cultures derived from FACS SP-supporting cells since incorporation of mature preexisting HCs in the SP-spheres is ruled out because HCs do not express the abcg2 transporter which is responsible for the efflux of Hoechst dye.

In addition, a subset of new trans-differentiating HCs in the SP-spheres that expressed early marker for HC differentiation such as Math1 also incorporated BrdU. The presence of BrdU⁺ and BrdU⁻ cells within the same SP-sphere indicated that isolated SP-supporting cells can trans-differentiate into HCs through a combination of mitotic and non-mitotic mechanisms.

Consistent with this notion are the results of White et al., 2006 (9) showing that purified neonatal cochlear supporting cells from p27^{KIP1}-GFP transgenic mice can also divide and transdifferentiate into HCs by both mitotic and non-mitotic mechanisms.

Our findings suggest that Abcg2-positive SP-supporting cells have the potential to become HCs after injury *in vivo*, but that inhibitory signals or physical barriers inhibit cell proliferation, and that the dissociation of the cochlear tissue may act to release such inhibitory processes, presumably due to the destruction of cell-cell interaction and/or stimulation by the presence of exogenous EGF/FGF2 in the culture medium. Chen and Segil, 1999 (23) have reported that p27^{KIP1} continues to be expressed in the supporting cells of the postnatal cochlea and contributes to their quiescent state. The co-expression of Abcg2 and Musashi1 in Deiters cells seems similar to that of Notch1 (37-38). Notch1 is expressed by all the cells in cochlear epithelium at E14-E16, then disappearing from HCs but remaining in supporting cells. In contrast, Jagged2, one of the Notch1 ligands, and Numb are specifically expressed in HCs, but not in supporting cells (37, 39). Musashi1 gene encodes an RNA binding protein, initially identified in *Drosophila* where it was required for early asymmetric divisions in sensory organ precursors (40). Musashi1 is believed to influence the Notch signaling by suppressing Numb mRNA (20). These data suggest that during HC development, Musashi1 may act as an activator of Notch signaling through translational repression of Numb, and may contribute with Abcg2 to SP phenotype of the supporting cells.

Recent studies on HC development have brought new paths about potential regeneration of new mammalian HCs. Several genes are shown to be critical during HC differentiation (41, 42). In particular, a bHLH transcription factor, Atoh1, is necessary for HC differentiation (43-46). For example, when LER cells (i.e. Hensen's cells) that are shown to express Abcg2 are induced to express Atoh1, they become HCs (47, 48), which provides direct support for the notion that Hensen's cells may have progenitor cell properties. Furthermore, when adenovirus expressing Atoh1 was delivered to the guinea-pig cochlea, a trans-differentiation of supporting cells that remain in the injured cochlea is observed i.e., Deiters's and Hensen's (7-8). This trans-differentiation of existing supporting cells to new HCs after Atoh1 transduction also supports the hypothesis that Abcg2⁺ supporting cells in the P3 cochlea are putative HC progenitors.

Our report provides a method for the purification of a population of SP-supporting cells from the early postnatal mammalian cochlea through their expression of *Abcg2* that can differentiate *in vitro* into hair cells.

This protocol of isolating and culturing a subpopulation of supporting cells complement the recent study of purification of supporting cells using p27/GFP transgenic mouse line and flow cytometry (9) and could be the foundation for further studies to address fundamental questions relating to the molecular and cellular biology of this stem/progenitor cell population in the postnatal mammalian cochlea. An increase in the understanding of the signals that regulate this cell population may stimulate novel biotechnology strategies to promote cochlear regeneration in patients with sensorineural deafness.

REFERENCES

- 1 Fuchs E, Segre JA. Stem cells: a new lease on life. *Cell* 2000;100:143–155.
- 2 Alvarez-Buylla A, Lim DA. For the long run: maintaining germinal niches in the adult brain. *Neuron* 2004;41:683–686.
- 3 Hawke TJ, Garry DJ. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol* 2001;91:534–551.
- 4 Corwin J, Cotanche D. Regeneration of sensory hair cells after acoustic trauma. *Science* 1988;240:1772–1774.
- 5 Ryals BM, Rubel EW. Hair cell regeneration after acoustic trauma in adult Coturnix quail. *Science* 1988;240:1774–1776.
- 6 Ruben RJ. Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. *Acta Oto-Laryngol* 1967;(Suppl. 220): 1–44.
- 7 Kawamoto K, Ishimoto S, Minoda R, Brough D, Raphael Y. Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. *J Neurosci* 2003;23:4395–4400.
- 8 Izumikawa M, Minoda R, Kawamoto K, Abrashkin KA, Swiderski DL, Dolan DF, Brough DE, Raphael Y. Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. *Nat Med* 2005;11:271–276.
- 9 White PM, Doetzlhofer A, Lee YS, Groves AK, Segil N. Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. *Nature* 2006;441:984–987.
- 10 Rocchi E, Khodjakov A, Volk EL, Yang CH, Litman T, Bates SE, Schneider E. The product of the ABC half-transporter gene ABCG2 (BCRP/MXR/ABCP) is expressed in the plasma membrane. *Biochem Biophys Res Commun* 2000;271:42–46.
- 11 Bunting KD. ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* 2002;20:11–20.
- 12 Alison MR. Tissue-based stem cells: ABC transporter proteins take centre stage. *J Pathol* 2003;200:547–550.

- 13 Bhattacharya S, Jackson JD, Das AV, Thoreson WB, Kuszynski C, James J, Joshi S, Ahmad I. Direct identification and enrichment of retinal stem cells/progenitors by Hoechst dye efflux assay. *Invest Ophthalmol Vis Sci* 2003;44:2764–2773.
- 14 Martin CM, Meeson AP, Robertson SM, Hawke TJ, Richardson JA., Bates S, Goetsch SC, Gallardo TD, Garry DJ. Persistent expression of the ATP-binding cassette transporter, *Abcg2*, identifies cardiac SP cells in the developing and adult heart. *Dev Biol* 2004;265: 262–275.
- 15 Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. The ABC transporter *Bcrp1/ABCG2* is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001;9:1028–1034.
- 16 Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996;183:1797–1806.
- 17 Goodell MA, McKinney-Freeman S, Camargo FD. Isolation and characterization of side population cells. *Methods Mol Biol* 2005;290:343–352.
- 18 Kawaguchi A, Miyata T, Sawamoto K, Takashita N, Murayama A, Akamatsu W, Ogawa M, Okabe M, Tano Y, Goldman SA, Okano H (2001) Nestin-EGFP transgenic mice: visualization of the self-renewal and multipotency of CNS stem cells. *Mol Cell Neurosci* 17:259-273.
- 19 Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, Miyake K, Resau JH, Bates SE. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). *J Cell Sci* 2000;113:2011–2021.
- 20 Kaneko Y, Sakakibara S, Imai T, Suzuki A, Nakamura Y, Sawamoto K, Ogawa Y, Toyama Y, Miyata T, Okano H. Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Dev Neurosci* 2000;22:139–153.
- 21 Litman T, Jensen U, Hansen A, Covitz KM, Zhan Z, Fetsch P, Abati A, Hansen PR, Horn T, Skovsgaard T, Bates SE. Use of peptide antibodies to probe for the mitoxantrone resistance-associated protein MXR/BCRP/ABCP/ABCG2. *Biochim Biophys Acta* 2002;1565:6–16.
- 22 Malgrange B, Belachew S, Thiry ., Nguyen L, Rogister B, Alvarez ML, Rigo JM, Van De Water TR, Moonen G, Lefebvre PP. Proliferative generation of mammalian auditory hair cells in culture. *Mech Dev* 2002;112:79–88.

- 23 Chen P, Segil N. P27^{Kip1} links cell proliferation to morphogenesis in the developing organ of Corti. *Development* 1999;126:1581–1590.
- 24 Moscicki K, Elkins EF, Baum HM, McNamara PM. Hearing loss in the elderly: an epidemiologic study of the Framingham Heart Study cohort. *Ear Hear* 1985;6:184–190.
- 25 Gates GA., Cooper JCJ. Kannel WB Miller NJ. Hearing in the elderly: the Framingham cohort, 1983–1985. *Ear Hear* 1990;11:247–256.
- 26 Chardin S, Romand R. Regeneration and mammalian auditory hair cells. *Science* 1995;267:707-709.
- 27 Rubel EW, Dew LA., Roberson DW. Mammalian vestibular hair cell regeneration. *Science* 1995;267:701–703.
- 28 Corwin J, Oberholtzer JC. Fish n'chicks: model recipes for hair cell regeneration? *Neuron* 1997;19:951–954.
- 29 Li H, Liu H, Heller S. Pluripotent stem cells from the adult mouse inner ear. *Nat Med* 2003;9:1293–1299.
- 30 Sakakibara S, Nakamura Y, Yoshida T, Shibata S, Koike M, Takano H, Ueda S, Uchiyama Y, Noda T, Okano H. RNA-binding protein Musashi family: roles for CNS stem cells and a subpopulation of ependymal cells revealed by targeted disruption and antisense ablation. *Proc Natl Acad Sci USA* 2002;99:15194–15199.
- 31 Sakaguchi H, Yaoi T, Suzuki T, Okano H, Hisa Y, Fushiki S. Spatiotemporal patterns of Musashi1 expression during inner ear development. *Neuroreport* 2004;15:997–1001.
- 32 Murata J, Murayama A, Horii A, Doi K, Harada T, Okano H, Kubo T. Expression of Musashi1, a neural RNA-binding protein, in the cochlea of young adult mice. *Neurosci Lett* 2004;16:201–204.
- 33 Malgrange B, Rogister B, Lefebvre P P, Mazy-Servais C, Welcher A.A., Bonnet C, Hsu RY, Rigo JM, Van De Water TR, Moonen G. Expression of growth factors and their receptors in the postnatal rat cochlea. *Neurochem Res* 1998;8:1133–1138.
- 34 Zine A, Nyffeler M, de Ribaupierre F. Spatial expression patterns of epidermal growth factor receptor gene transcripts in the postnatal mammalian cochlea. *Hear Res* 2000;141:19–27.
- 35 Forge A, Li L, Corwin JT, Nevill G. Ultrastructural evidence for hair cell regeneration in the mammalian inner ear. *Science* 1993;259:1616–1619.

- 36 Warchol ME, Lambert PR, Goldstein BJ, Forge A, Corwin JT. Regenerative proliferation in inner ear sensory epithelia from adult guinea pigs and humans. *Science* 1993;259:1619–1622.
- 37 Lanford PJ, Lan Y, Jiang RL, Lindsell C, Weinmaster G, Gridley T, Kelley MW. Notch signalling pathway mediates hair cell development in mammalian cochlea. *Nat Genet* 1999;21:289–292.
- 38 Zine A, Van de Water TR, de Ribaupierre F. Notch signaling regulates the pattern of auditory hair cell differentiation in mammals. *Development* 2000;127: 3373–3383.
- 39 Eddison M, Le Roux I, Lewis J. Notch signaling in the development of the inner ear: lessons from *Drosophila*. *Proc Natl Acad Sci USA* 2000;97:11692–11699.
- 40 Sakakibara S, Okano H. Expression of neural RNA-binding proteins in the postnatal CNS: implications of their roles in neuronal and glial cell development. *J Neurosci* 1997;17:8300–8312.
- 41 Gao WQ. Hair cell development in higher vertebrates. *Curr Top Dev Biol* 2003;57:293-319.
- 42 Zine, A. Molecular mechanisms that regulate auditory hair-cell differentiation in the mammalian Cochlea. *Molecular Neurobiolol* 2003;27:223–238.
- 43 Bermingham NA, Hassan BA, Price SD, Vollrath MA Ben Arie N, Eatock, RA Bellen HJ, Lysakowski A, Zoghbi HY. *Math1*: an essential gene for the generation of inner ear hair cells. *Science* 1999;284:1837–1841.
- 44 Zheng JL, Gao WQ. Overexpression of *Math1* induces robust production of extra hair cells in postnatal rat inner ears. *Nat Neurosci* 2000;3:580–586.
- 45 Chen P, Johnson J, Zoghbi H, Segil N. The role of *Math1* in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. *Development* 2002;129:2495–2505.
- 46 Woods C, Montcouquiol M, Kelley MW. *Math1* regulates development of the sensory epithelium in the mammalian cochlea. *Nat Neurosci* 2004;7:1310–1318.
- 47 Shou J, Zheng J, Gao W-Q. Robust generation of new hair cells in the mature mammalian inner ear by adenoviral expression of *Hath1*. *Mol Cell Neurosci* 2003;23:169–179.
- 48 Zhai S, Shi L, Wang B.E., Zheng G, Song W, Hu Y, Gao WQ. Isolation and culture of hair cell progenitors from postnatal rat cochleae. *J Neurobiol* 2005;65:282–293.

FIGURES CAPTION

Figure 1. Schematic diagram depicting a section through the organ of Corti of P3 mouse. Sensory HCs as well as the non-sensory supporting cell types are indicated. Deiters' cells (d) surround each OHC separating them from each other and the nuclei of these supporting cells are located in a layer underneath the nuclei of the OHC. Inner phalangeal cells (ipc) and border cells (bc) surround each IHC. The inner (ip) and outer (op) pillar cells separate the IHCs from the OHCs. Hensen's (h) cells are external and distal to the OHC area.

Figure 2. RT-PCR analysis of the expression of *Abcg2*, hair cell (myosin VIIA), supporting cell ($p27^{Kip1}$) and stem/progenitor (*Musashi1*) markers in the sensory epithelia dissected from P3 neonate and adult cochleae. Ubiquitously expressed *Gapdh* is a reference gene.

Figure 3. *Abcg2* is expressed in the supporting cells of the organ of Corti. Phalloidin staining (A) was used to identify the circumferences of HCs and supporting cells, including Deiters's cells (D) and Hensen's cells (H). HCs are specifically labeled for calbindin (D) but lack *Abcg2* immunoreactivity. A strong $p27^{Kip1}$ label is restricted to the supporting cell types within the organ of Corti (G). *Abcg2* immunoreactivity (B,E and H) localizes to the supporting cells in both OHC and IHC areas. The reaction product is distinctively visible in the 3 rows of Deiters's cells (arrows, B, E, H) below the OHCs and in the border and inner phalangeal cells surrounding the IHCs (Large arrows, B, E, H). *Abcg2* is also detected in Hensen's cells (arrowhead, B) located laterally to the organ of Corti and at the level of the fibers within the spiral lamina (open arrowheads, B) The pattern of $p27^{Kip1}$ expression is nuclear allowing the distinction of the spatial location of the *Abcg2* to the plasma membrane. Scale bars = 20 μm (A-C); 30 μm (D-I).

Figure 4. *Abcg2*, Nestin and *Musashi1* are detectable in the supporting cells within the organ of Corti. *Abcg2* immunoreaction is predominantly visible in the 3 rows of Deiters's cells (arrows, A) and border cells surrounding the IHC (Large arrow, A). *Abcg2* is also detected in Hensen's cells (filled arrowhead, A) and at the level of the fibers within the spiral lamina (open arrowheads, A). Analysis of promoter

activity showed nestin expression in the border cells (large arrow, B), inner phalangeal cells (arrow, B) and fibers projecting toward the organ of Corti (arrowheads, B). Nestin colocalizes with Abcg2 at the level of border cells below the IHCs and the spiral fibers (C). Both Abcg2 and nestin are absent from the HCs. Musashi1 (E) is localized in both the nuclei of Deiters's cells (arrows,) as well as their phalangeal processes projecting toward the reticular lamina (arrowheads) and pillar cells (filled arrowhead). Kolliker's organ (OK) located at the medial aspect of the organ of Corti showed a diffuse pattern of immunoreactivity for Musashi1. Scale bars = 50 μm (A-C); 30 μm (D-F).

Figure 5. A) Isolation of an SP cell population from P3 cochlea using flow cytometry. SP cells were isolated from P3 cochlear epithelium dissociates using dual-wavelength FACS and Hoechst 33342 dye. Note that the SP cells are located within the gated regions. An SP cell profile is present in P3 cochlear tissue dissociates and accounts for approximately 0,53 % of the total cells analyzed. Hoechst dye exclusion from SP fraction cells is verapamil sensitive (inset, A). (B) RT-PCR on SP and NSP cell fractions conducted on RNA from these FACS-sorted cells. SP fraction revealed Abcg2 expression but not present in the NSP while Musashi1 was weakly detected in both SP and NSP cell fractions. (C) The restricted expression of Abcg2 to cochlear SP cells was confirmed when the FACS sorted SP cells were immunolabeled with an Abcg2 antibody. Note that Abcg2 (red) membrane labeling is apparent in all DAPI (blue)-stained nuclei, supporting the role of Abcg2 as the SP cell transporter responsible for Hoechst dye exclusion. The NSP fraction did not immunolabel for the presence of Abcg2 (not shown). (D-F) Abcg2 FACS sorted cochlear cells proliferate in response to EGF/FGF2. (D) An example of spheres that formed from cochlear FACS-SP collected cells cultured for a 7-days in 'suspension' condition. (E, F) Another SP-sphere formed after 7 days in 'suspension' culture showing BrdU positive cells (red). G) Quantification of spheres derived from SP and NSP cell fractions in culture supplemented with EGF/FGF2 after 7 days (mean value, \pm SD, n=3). (H) Percentage of BrdU (+) cells in the SP and NSP-spheres cultured 3 days in the presence of BrdU in 'suspension' with EGF/FGF2 containing medium.

Figure 6. FACS-derived SP-spheres generate cells that express HC and supporting cell markers under differentiation conditions (A-H). SP-spheres in 'suspension' culture give rise to cells that immunolabel for the presence of HC (myosin VIIA, and fimbrin) and supporting cell (p27^{Kip1}) markers

after 4 days (A-D) and 6 days (E-H) *in vitro*. Merge images (D, H) with fluorescence microscopy show non-overlapping expression of HC markers (myosin VIIA and fimbrin) and the supporting cell marker (p27^{Kip1}) within differentiated SP-sphere derived cells. (I) Quantification of the number of cells expressing HC (myosin VIIA) and supporting cell (p27^{Kip1}) markers in SP and NSP derived-spheres after 6 days *in vitro*. The individual bars visualize the fraction of immunopositive cells counted from 20 spheres. Shown are mean values with SD determined from three independent culture experiments for each dataset. Scale bars = 25 μm (A-D), 10 μm (E-H).

Figure 7. Purified SP-supporting cell progenitors divide and trans-differentiate into BrdU (+) HCs after 5 days in 'suspension' culture condition in the presence of EGF/FGF2 followed by 4 days under 'adherent' differentiation condition in the presence of BrdU. Nuclear immunoreactivity for the early HC marker Math1 (C, green) was detected in a subset of the cells immunostained with BrdU (B, red). Arrows indicate double labelled differentiating HCs. A) Dapi, B) anti-BrdU, C) anti-Math1 and D) Merge. Scale bar = 25 μm .

Figure 1

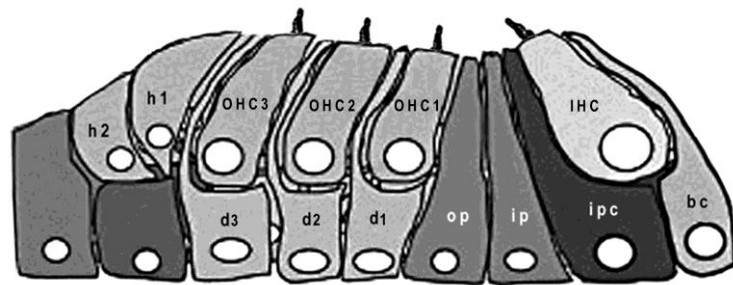


Figure 2

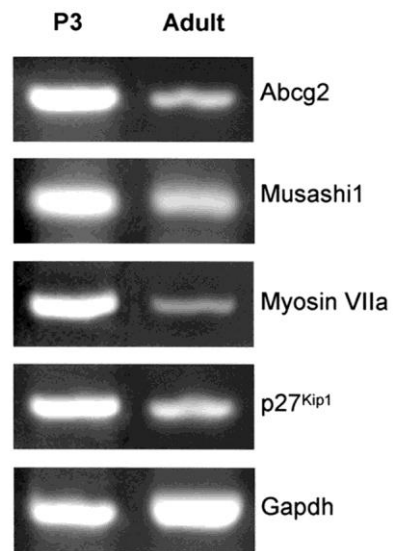


Figure 3

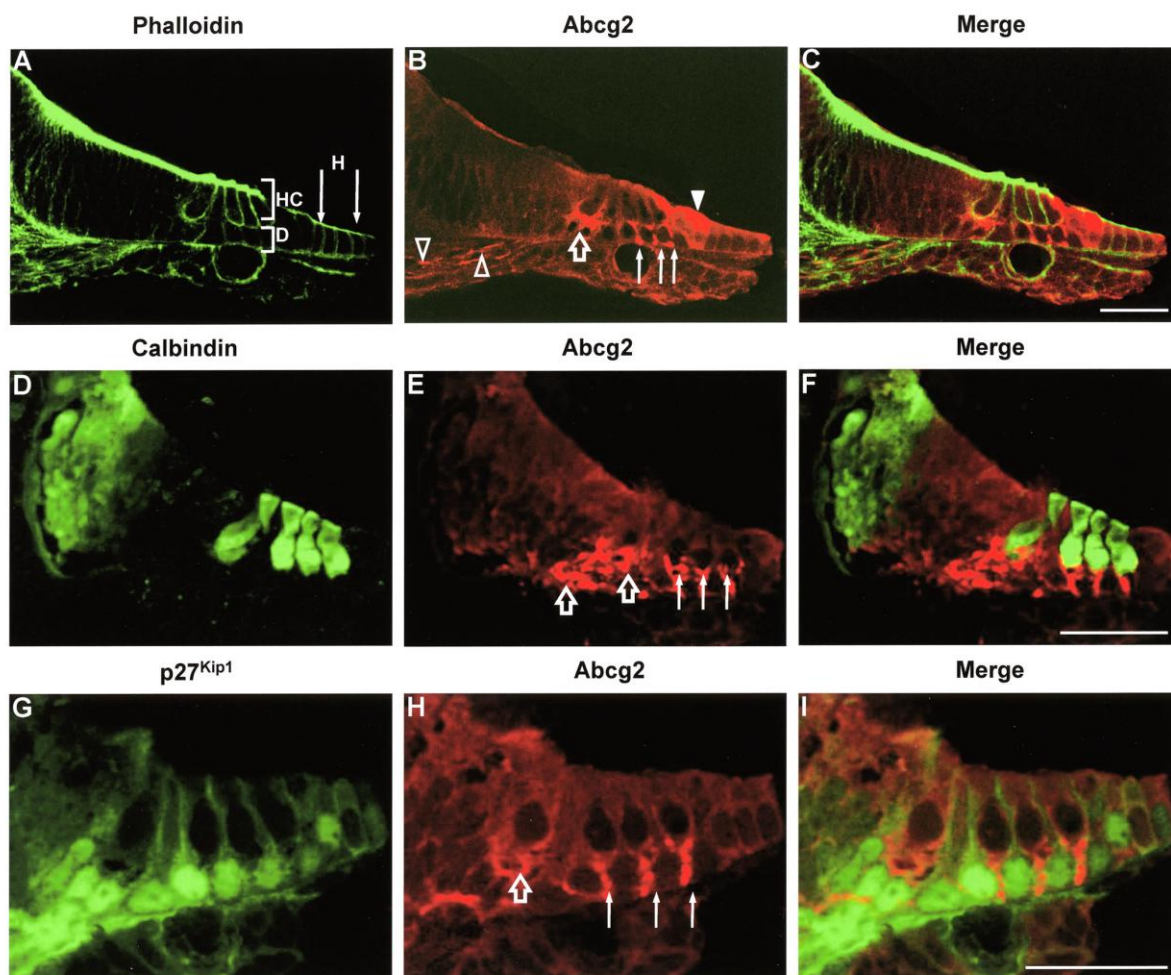


Figure 4

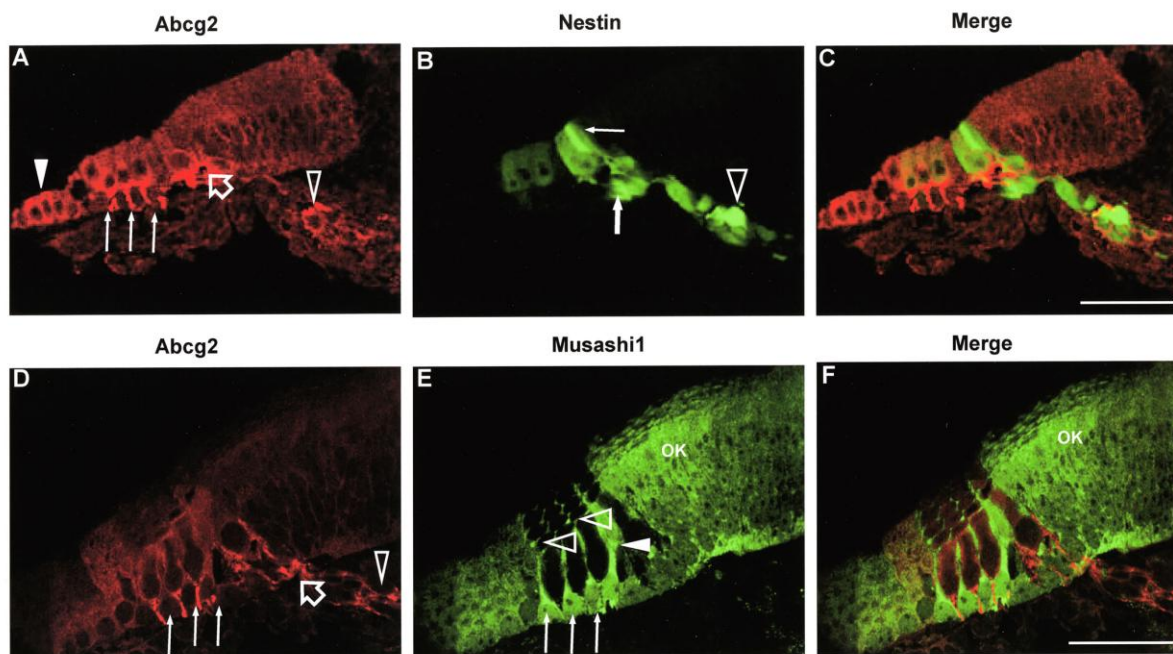


Figure 5

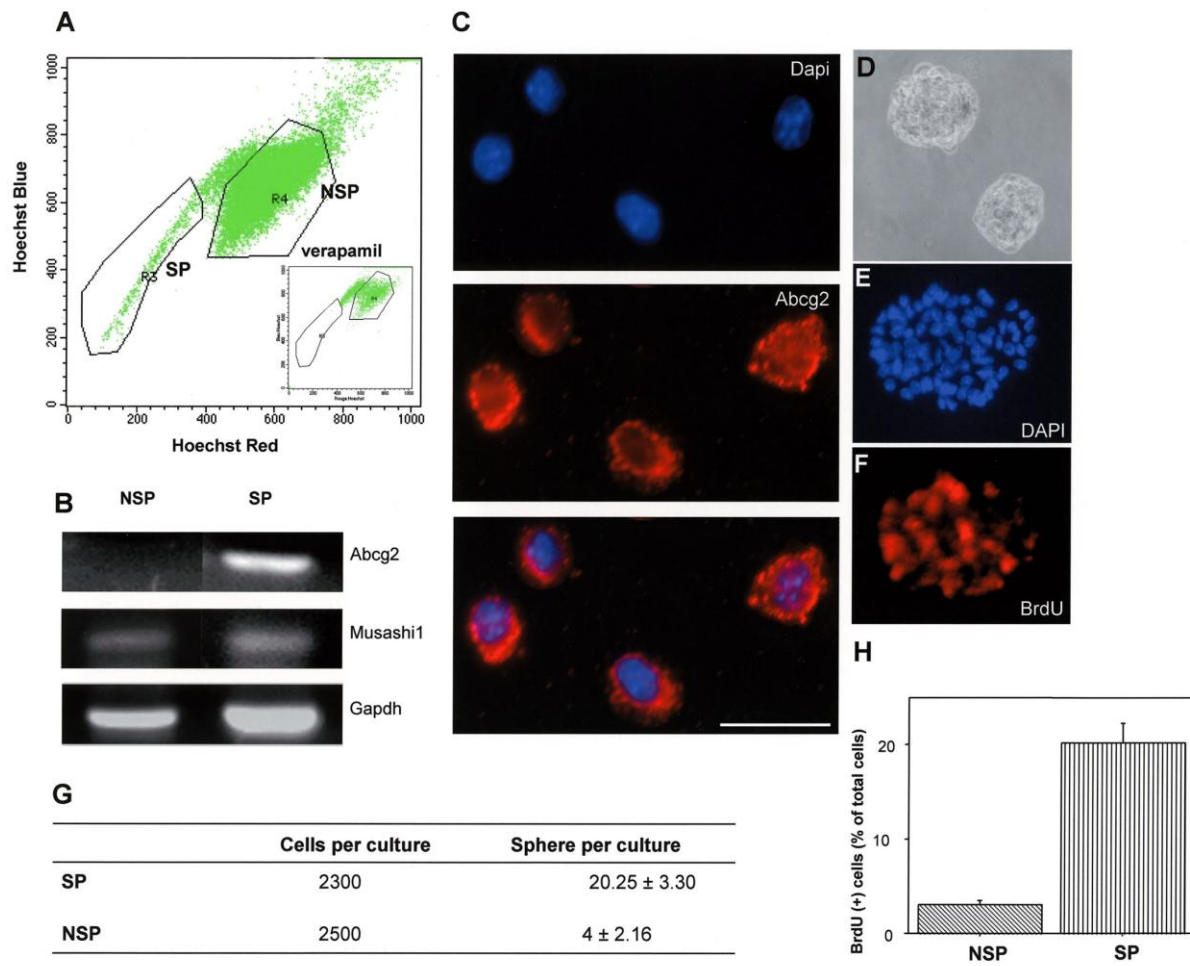


Figure 6

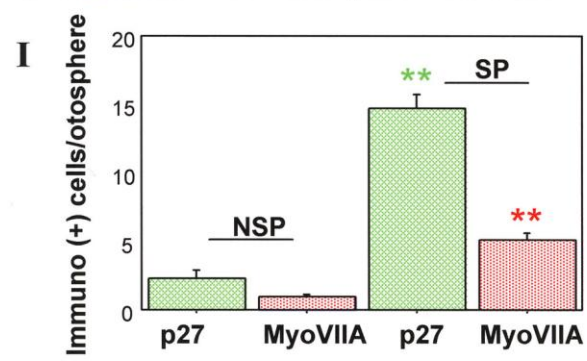
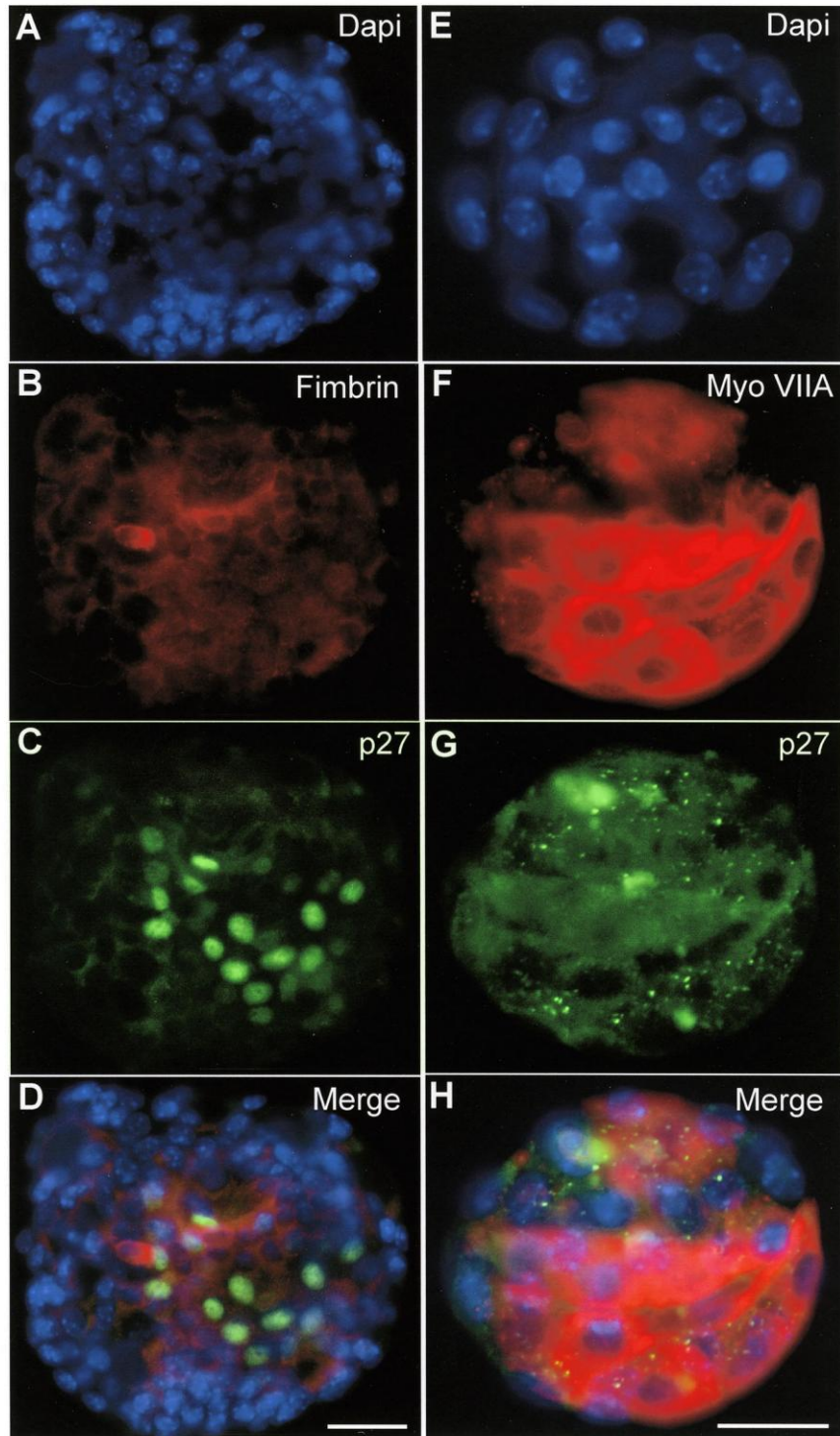


Figure 7

