

The Differentiation of Stem/Progenitor Cells Derived from the Cochlear Sensory Epithelium into Hair Cells Requires a Specific Spatial Supporting Structure of p27kip1-Positive Cells

Xianren Wang

Sun Yat-sen University First Affiliated Hospital

Xuemei Zhang

Sun Yat-sen University First Affiliated Hospital

Di Jiang

Sun Yat-sen University First Affiliated Hospital

Danqing Liu

Sun Yat-sen University First Affiliated Hospital

Hongyan Jiang (✉ jhongy@mail.sysu.edu.cn)

Hainan Provincial People's Hospital

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Abstract

Background: Cochlear sensory epithelium-derived progenitor cells initially give rise to compact solid/round spheres. These compact solid/round spheres then gradually convert into irregular and partially hollow spheres, which then ultimately transform into large hollow spheres. The purpose of this study was to observe the differentiation of cochlear sensory epithelium-derived progenitor cells into spheres, and determine factors necessary for their development into hair cells.

Methods: Cochlear epithelial sheets from postnatal day 1 C57BL/6 mice were dissociated and sphere cells were cultured. The morphological changes of the spheres were observed, and the different types of sphere cells were examined for their ability to differentiate into hair cell-like cells.

Results: Solid spheres formed first, and then gradually transformed into hollow spheres over approximately 260 hours. Adherent culture and Transwell culture assays, and immunohistochemistry staining revealed that neither solid nor hollow sphere cells alone could differentiate into mature hair cells. Solid sphere cells, however, were able to differentiate into mature hair cells when co-cultured with p27kip1-positive hollow sphere cells. Direct contact of the cells was necessary for the differentiation of the solid sphere cells into mature hair cell-like cells.

Conclusions: Cochlear sensory epithelium-derived progenitor cells require specific conditions to differentiate into mature hair cells.

Background

In the mammalian auditory sense organ (the cochlea), the hair cells are organized in a single row of “inner” hair cells and 3 rows of “outer” hair cells, while the supporting cells assume a variety of specialized morphologies [1–3]. The inner hair cells are the primary sensory receptors, while the outer hair cells act to amplify sound, in part through regulation of cochlear stiffness [1–3]. The inner hair cells are surrounded by specialized support cells, the inner phalangeal cells [1–3]. Pillar cells line the space between the inner and outer hair cells (the tunnel of Corti), and they provide rigidity and structure to the epithelium [1–3]. Finally, Deiters' cells support the outer hair cells, and each cell contains a process that reaches up around the outer hair cell and contacts its apical surface [1–3]. The hair cells and supporting cells arise from the same progenitor cells, and it has been shown that Notch signaling is important for the differentiation of the individual types of cells [4–6].

Hearing impairment is one of the most common sensory defects, and is mainly due to loss of hair cells [1]. Furthermore, hearing impairment can significantly reduce a person's quality of life [7]. In mature vertebrates, hair cell regeneration is uncommon and damage therefore leads to permanent auditory deficits [1–3]. However, studies have identified stem/progenitor hair cells in the neonatal and adult inner ear [8–12]. Furthermore, studies have indicated that progenitor cells can be induced to differentiate into hair cells [8,13–19], and the restoration of hair cells can restore auditory function [1,2,20–22].

In recent years, a technique to isolate stem/progenitor cells from the inner ear's sensory epithelium has been developed [1,8,10,13,19]. The method is based on the neurosphere assay used to isolate multipotent and self-renewing stem cells from the mammalian central nervous system [23–25]. Sphere-forming otic stem/progenitor cells display a distinct capacity to divide in nonadherent culture conditions, which results in the formation of clonal floating colonies (spheres) [9,26,27]. A number of studies have isolated and characterized cochlear cell-derived spheres, and a unique pattern of development has been observed [28–31]. Cochlear sensory epithelium-derived progenitor cells initially give rise to compact solid/round spheres. These compact solid/round spheres then gradually convert into irregular and partially hollow spheres, which then ultimately transform into large hollow spheres. However, the interactions between the different types of sphere cells, and the mechanisms of their ultimate differentiation into hair cells, has not been fully explored.

The purpose of the current study was to dynamically observe the differentiation of cochlear sensory epithelium-derived progenitor cells into spheres, and determine factors necessary for their development into hair cells using a murine model.

Methods

Animals and culture of isolated inner ear cells

Newborn postnatal day 1 (P1) C57BL/6 mice were killed, and careful dissection was performed to remove the otic bulla in order to visualize the otic capsule. After excision of the cartilaginous otic capsule, the membranous labyrinth was exposed, and the cochlear duct was excised. The cochlear epithelial sheet was micro-dissected from Reissner's membrane, the spiral ligament, and the stria vascularis.

Two cochlear epithelial sheets were transferred into Eppendorf tubes and incubated with Dispase II for 30 min at 37°C, and then trypsin with 0.05% trypsin/DNase I solution (10 µg/ml DNase I) for 10 min at 37°C. Dissociated cells were passed through a 70-µm cell strainer. Next, to promote proliferation, cells were plated in Petri dishes in serum-free DMEM/F12 high-glucose supplemented media with N2/B27 solution and growth factors (20 µg/ml epidermal growth factor, EGF; 50 ng/ml insulin-like growth factor, IGF; 10 ng/ml fibroblast growth factor-2, FGF-2; Peprotech, Rocky Hill, NJ). Experiments were conducted with solid spheres harvested after 3–4 days *in vitro* (DIV), with transitional spheres collected after 4–6 DIV, and with hollow spheres collected after 6–7 DIV, unless otherwise indicated.

Animal care and euthanization was conducted according to methods approved by the University of Sun Yat-sen Animal Care and Use Committee, following the Guidelines for the Care and Use of Laboratory Animals set forth by the National Institutes of Health.

Cell differentiation

To study cell differentiation, spheres were collected and transferred into plastic 4-well tissue culture plates (Greiner 35/10 mm 4-well tissue culture dishes) using an inverted microscope and a

micromanipulator-guided system (TransferMan NK 2 Micromanipulator; CellTram Oil Microinjector; Eppendorf North America, Westbury, NY, USA) equipped with pulled glass microcapillaries. Tissue culture plates were coated with fibronectin (20 μ l/ml; Sigma) for 2 h, and washed with sterile PBS directly before use.

Sphere cells were analyzed immediately after attachment to determine the uptake of 5-bromo-2'-deoxyuridine (BrdU) during sphere formation, the total cell number by visualizing nuclei with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen), and to determine the expression of unique cell markers.

For differentiation, the attached sphere-derived cells were maintained in a humidified incubator in a 5% CO₂ atmosphere at 37°C, in differentiation medium consisting of DMEM-F12 supplemented with N2/B27 solution, and ampicillin. Eighty percent of the medium was replaced every 3 days. The differentiated cells were analyzed by immunocytochemistry 7 days after plating. To study the stereotypic pattern of sphere cell differentiation, mouse embryonic fibroblast (MEF) culture cells were used as a supporting layer for the culture of hollow sphere-derived cells and solid sphere-derived cells. In addition, in a co-culture system (Corning; catalogue number 3401), hollow sphere cells were placed into Transwell inserts and cultured solid sphere cells that were adherent were placed in the plate well.

MEFs and MEF feeder cells were isolated, cultured, and immortalized of as previously described [32].

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS (pH 7.2) for 15 min at room temperature, then permeabilized in 0.1% Triton X-100 in PBS for 10 min at 4°C. Nonspecific binding sites were blocked with 2% bovine serum albumin (BSA; w/v) and 5% (v/v) heat-inactivated goat serum in 0.05% Triton X-100 in PBS (PBT-1) for 30 min at room temperature. The cells were then incubated overnight at 4°C with primary antibodies diluted in PBT-1: nestin (1:100; Santa Cruz Biotechnology, Inc., CA), myosin VII (1:100; Santa Cruz Biotechnology), Math1 (1:100; BioVision, Mountain View, CA), and Tbx1 (1:200; Invitrogen, Zymed Laboratories, CA). The following day, unbound antibodies were removed by three 15-min PBT-1 washes and one 15-min wash with PBT-1 lacking BSA or goat serum (PBT-2). Cells were then incubated overnight at room temperature with fluorophore-conjugated secondary antibodies in PBT-2 (1:200). After three 15-min washes with PBT-2, DAPI was used to visualize cell nuclei. For some specimens, counterstaining was performed with rhodamin-conjugated phalloidin (Invitrogen) to visualize filamentous actin. Control groups consisted of spheres incubated without any primary antibody. In all experiments, no specific immunofluorescence labeling was detected in control spheres incubated without primary antibody.

Results

Different sphere morphologies arise from neonatal cochlear sensory epithelium cell suspensions

It has been reported that stem/progenitor cells derived from the cochlear sensory epithelium give rise to spheres with distinct morphologies and features under appropriate culture conditions [9,11]. We dissected the sensory epithelia of newborn (P1) mice (Model Animal Research Center, Nanjing University) (Fig. 1), enzymatically and mechanically separated the cells, and plated the resulting single cell suspension on to non-adherent culture dishes. We then dynamically observed the cellular changes through an Olympus confocal microscope. Small spheres formed 24 h after culture, and solid spheres were detected at 3 DIV. The solid spheres then gradually became transparent on one side and gradually enlarged. On day 5 *in vitro*, the spheres were semi-hollow, and on day 7 *in vitro* enlarged, completely hollow spheres had formed.

At different time points (Fig. 1), 10 microscopic fields were randomly selected and the number of spheres in the 10 fields was counted. The proportion of solid spheres to all spheres was calculated. The ratio of solid spheres to all spheres was highest at 24 h of culture, and only 9.8% of spheres were hollow at this time point. During the first 144 h of culture, the number of spheres approximately tripled, while the ratio remained unchanged. After 144 h, the solid sphere ratio decreased and the number of hollow spheres gradually increased until at 8 DIV transitional spheres accounted for 40% of the total spheres. At 8 days, hollow spheres accounted for 20% of all spheres, and at day 9 the number and proportion of hollow spheres exceeded that of transitional and solid spheres. The overall number of all types of spheres combined was greatest on day 7

Solid sphere cells, but not hollow sphere cells, are progenitor cells

To investigate if the distinct sphere types that form from cochlear sensory epithelium-derived progenitor cells have different cell markers, we examine hollow sphere cells and solid sphere cells with immunofluorescence staining, and found that distinct sphere types express different stem cell markers. Nestin and abcg2, 2 important stem cell markers [19,29], were extensively expressed by solid sphere cells, but not expressed by hollow sphere cells. When sphere cell cultures that were 66 hours old were incubated with BrdU for an additional 6 h we found that almost all sox2-positive cells had incorporated BrdU. However, when hollow sphere cell cultures that were 162 hours old were incubated with BrdU for an additional 6 h hollow sphere cells did not express sox2 or uptake BrdU (Fig. 2). The hollow sphere cells, however, expressed p27kip1. This indicates that while the hollow sphere cells do not have stem cell characteristics, they have supporting cell characteristics because of their expression of p27kip1.

Distinct sphere cell types interact to promote the development of mature hair cell-like cells

The differential expression of the otic progenitor cell markers nestin and abcg2 by distinct sphere cell types led us to explore whether the different types of sphere cells vary in their capacity to generate mature inner ear cell types *in vitro*. We collected spheres cells of each type and cultured them in fibronectin-coated dishes for 2 weeks in serum-free medium with defined medium supplements in the absence of added growth factors. These culture conditions are sufficient to induce sphere cell attachment and spontaneous differentiation into mature cell types [12,19]. To identify differentiated cells, we used antibody specific for the early hair cell marker myosin VIIa [8]. Both solid and hollow sphere cells

differentiated into myosin VIIa-positive cells. We also observed that all myosin VIIa-positive cells aggregated together to form a spatial 3-dimensional (3D) structure.

These results promoted us to consider if solid and hollow sphere cells co-cultured in adhesion culture conditions could increase the number of myosin VIIa-positive cells. Thus, 7 day *in vitro* hollow sphere cells and 3 day *in vitro* solid sphere cells were co-cultured in adhesion culture medium for 7 days, and then examined by immunofluorescent staining. The results showed that the number of myosin VIIa immunofluorescent-positive cells was greater in areas where the cells had aggregated. The results indicated the distinct sphere cell types could interact to promote the development of mature hair cell-like cells, and that the 3D structure was important for their development.

Progenitor cell differentiation requires contact between solid and hollow sphere cells

The above findings led us to explore what is the most important factor impacting sphere cell differentiation. To explore if factors secreted by the cells impacted cell differentiation, we performed a Transwell culture assay. Three day *in vitro* solid sphere cells were cultured in the bottom of the Transwell chamber, and 7 day *in vitro* hollow sphere cells were cultured in the upper Transwell chamber. The co-culture was continued for 7 days. We found that only solid sphere cells expressed the mature hair cell marker myosin VIIa (Fig. 3). There was no difference between the Transwell culture and individual culture results. These results, together with the finding that co-culture of progenitor cells (with direct cell contact) resulted in differentiation to mature cells suggested that secretory factors may not impact otic progenitor cell differentiation.

Based on the above findings, we explored if the supporting structure impacted progenitor cells differentiation. Three day *in vitro* solid sphere cells were implanted on a supporting layer of MEFs and cultured for 7 days in a differentiation medium. The solid sphere cells did not form a distinct spatial structure, and only a few cells expressed myosin VIIa. Additionally, there was no difference in the number of myosin VIIa-positive cells between this culture method and the prior solid sphere culture. The results indicated that otic progenitor cell differentiation requires a unique supporting structure.

To explore the mechanism by which a supporting structure promotes cell differentiation, we compared the difference using hollow sphere cells as the supporting layer and using MEFs as the supporting layer. Cell cycle inhibitor p27kip1 is expressed in many cochlear supporting cell types [15,33]. As such, we detected p27kip1 expression in the 2 different supporting layers, and found only hollow sphere cells expressed p27kip1. Next, transitional sphere cells were cultured and differentiated for 7 days, and then the co-immunostaining was performed for myosin VIIa and p27kip1 (Fig. 4). Interesting, all myosin VIIa-positive cells were surround by p27kip1-positive cells. The same phenomenon was observed in the solid sphere cells/hollow sphere cells co-culture system (Fig. 5).

To further identify the function of p27kip1 in otic progenitor cell development to mature hair cells, the p27kip1 gene was knocked-down in hollow sphere cells by electrode transfection with adeno-associated virus (AAV) siRNA27. Immunofluorescence and PCR methods showed that p27kip1 knockdown was >

80%. When p27kip1 knockdown hollow sphere cells were used as the supporting layer during culture, solid sphere cells rarely differentiated into myosin VIIA-positive cells. On the other hand, when p27kip1-positive hollow sphere cells were used as the supporting layer, solid sphere cells differentiated into myosin VIIA-positive cells.

Discussion

In the present, we found that new born mice Cochlear sensory epithelium-derived stem/progenitor cells could give rise to solid sphere cells which gradually transformed into hollow sphere cells. Individually, the solid and hollow sphere cells have limited capability to differentiate into mature hair cells under adhesion culture conditions. However, the capability of solid sphere cells to differentiate into mature hair cells is robust when co-cultured with hollow sphere cells. Furthermore, directly contact with p27kip1-positive supporting cells is one of the most important factors for the differentiation into mature hair cell-like cells.

Sensorineural defects and vestibular dysfunction are the most common causes of hearing loss, and stem cell-based methods for treating hearing loss are being developed. Attempts to develop hair cells using embryonic and newborn mice cochlear epithelial cells have resulted in phenotypic conversion of stem cells into inner ear hair cell-like cells [2,3]. However, the number of hair cell-like cells produced is limited, regardless of if the cells are differentiated from embryonic cochlear epithelial cells or newborn cochlear epithelial cells [2,3]. An insufficient number of differentiated cells affects the results of stem cell-based therapeutic strategies.

Studies have described sphere formation from cultured neonatal cochlear or vestibular cells, and among the reports there is substantial variation of sphere morphology[9,28,30]. We dynamically observed changes in cultured cells, and found that initially solid sphere cells formed exclusively, and then gradually transformed into hollow sphere cells with the number of hollow sphere cells exceeding the number of solid sphere cells after about 7 days of culture. Furthermore, solid sphere cells, but not hollow sphere cells, maintained the ability for differentiation. Other studies have also suggested solid, but not hollow, sphere cells could differentiate into mature hair cell-like cells under adherent culture conditions [22,27,30]. Unique to our study, however, is the finding that the interaction between solid and hollow sphere cells is necessary for differentiation into mature hair cell-like cells.

The cell cycle inhibitor p27kip1 is expressed in many cochlear supporting cell types[15,33]. We found that the presence of p27kip1-positive hollow sphere cells as a supporting layer was necessary for the differentiation of solid sphere cells differentiated into myosin VIIA-positive cells. During development, the cell cycle is regulated by cyclin-dependent kinases and corresponding cyclins, that are positively acting [33]. Regulation of cyclin-dependent kinases/cyclins occurs by a number of different methods, one of which is by cyclin-dependent kinase inhibitor proteins, one of which is p27kip1 [33]. Loss of p27kip1 has been shown to be associated with a various tissue-specific defects as a result of a delay of the end of the cell cycle [33]. Lee et al. [33] showed that p27kip1 is critical for the development of precursors which ultimately produce hair cells and supporting cells of the organ of Corti. In another study, White et al. [15]

showed that supporting cell proliferative capacity that is age-dependent is related to downregulation of p27kip1. Interestingly, Doetzlhofer et al.[14] showed that both growth factor and supporting periotic mesenchyme cells were required for inner hair cell differentiation.

We found that only hollow spheres cells expressed p27kip1 and active-Notch1. The Notch signaling pathway is important for the differentiation and growth of many different tissues [34]. For example, down-regulation of Notch1 results in apoptosis and inhibition of cell growth of ovarian cancer cells [34]. Aguirre et al. [35] reported that the interaction of the Notch and the epidermal growth factor receptor (EGFR) pathways regulates neural stem cell number and the capacity for self-renewal. With respect to hearing, studies have reported that Notch signaling is important for the development of the inner ear, including the generation of hair and supporting cells [5,6,36].

In addition to the aforementioned markers, we also examine sphere cells for the expression of sox2. Sox2 is critical for the pluripotency, maintenance, and self-renewal of embryonic stem cells, as well as for the generation of inducible pluripotent stem cells [37]. It is also important for tissue homeostasis and development in adults. In a recent study, Waldhaus et al.[38] reported that the “stemness” of the organ of Corti is related to the sequence-specific methylation of the otic sox2 enhancers NOP1 and NOP2. Interestingly, other study has reported that Hedgehog signaling

Conclusions

Cochlear sensory epithelium-derived stem/progenitor cells give rise to solid sphere cells which gradually transform into hollow sphere cells under adherent culture conditions. Individually, neither solid nor hollow sphere cells can differentiate into mature hair cells. However, the capability of solid sphere cells to differentiate into mature hair cells is robust when co-cultured with p27kip1-positive hollow sphere cells, and direct contact of the cells is one of the most important factors for the differentiation into mature hair cell-like cells. These findings may aid in the development of stem cell-based strategies for treating hearing loss.

Abbreviations

DIV: days in vitro; MEF: mouse embryonic fibroblast; BSA: bovine serum albumin; AAV: adeno-associated virus

Declarations

Ethics approval and consent to participate

Animal care and euthanization was conducted according to methods approved by the University of Sun Yat-sen Animal Care and Use Committee, following the Guidelines for the Care and Use of Laboratory Animals set forth by the National Institutes of Health.

Consent for publication

Not applicable.

Availability of data and materials

All the data and material have been presented in the main paper.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

We declare that all the listed authors have participated actively in the study and all meet the requirements of the authorship. Drs. XW and HJ designed the study and wrote the protocol, Drs. XW and XZ acquired the manuscript, Drs. XW, XZ and DJ and DL analyze the data, Drs. XW and XZ wrote the first draft of the manuscript and mainly revised the manuscript. All authors approved the final version of the manuscript.

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Figures

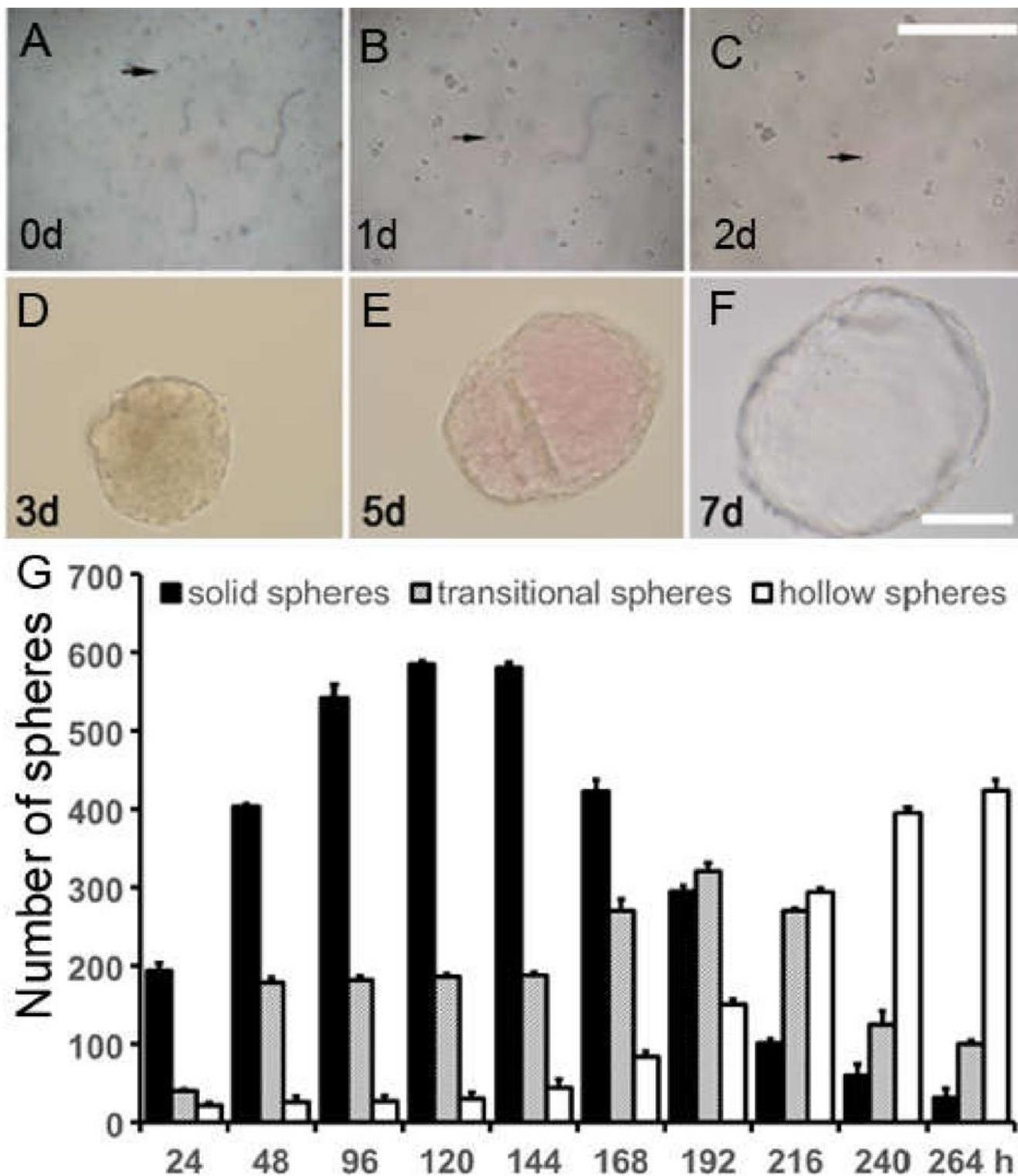


Figure 1

Changes in the morphology and number of sphere cells over time from cultured early postnatal murine cochlear sensory epithelial cells. A-F) Dynamic observed formation of solid spheres, to transitional cells, to hollow sphere cells. G) Numbers of different types of sphere cells at different time points (n = 4).

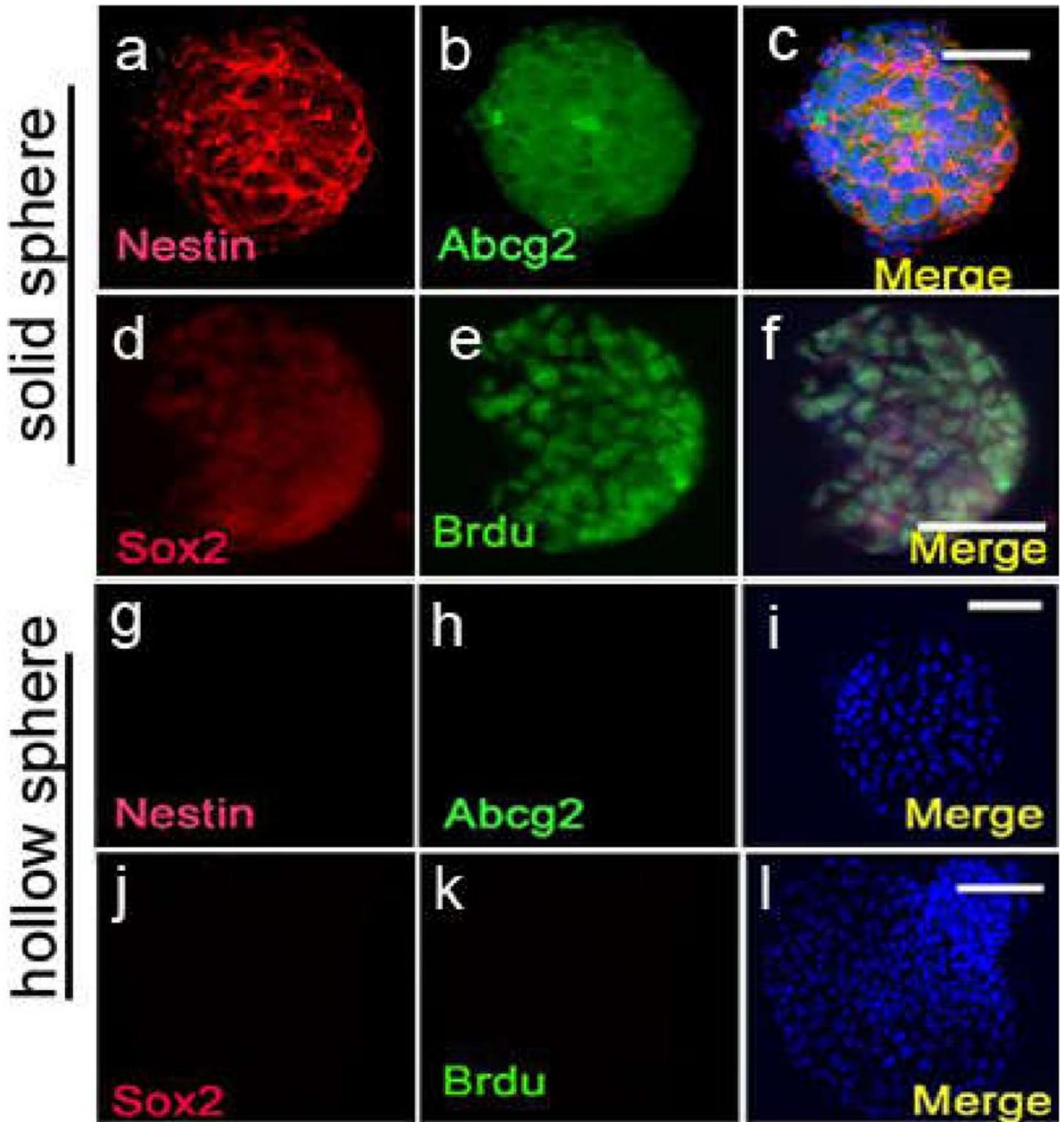


Figure 2

Expression of stem cell markers by solid sphere cells, and their proliferative ability. a-f) Solid sphere cell expression of nestin, abcg2, and sox2. BrdU staining showed the cells have proliferation ability. g-l)

Hollow sphere cells did not express stem cell marker. Bar = 100 μ m.

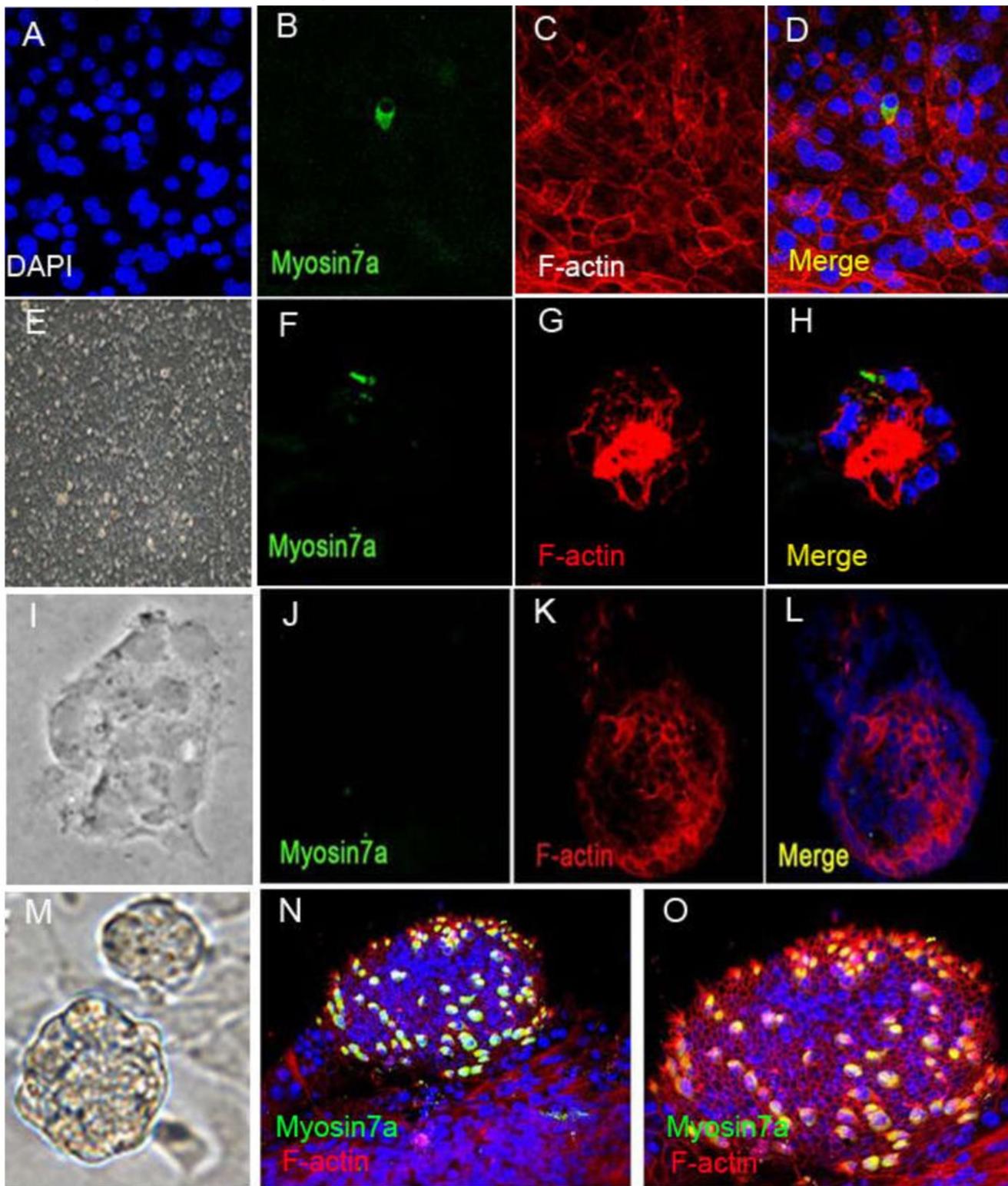


Figure 3

Differentiation of solid sphere cells into hair cell-like cells requires direct contact with hollow sphere cells and the formation of a 3-dimensional (3D) supporting structure. A-D) Twenty solid sphere cells were collected and differentiated under adhesion culture conditions. Differentiation was random and scarce, and myosin VIIa-positive cells were rare. M-O) There were a large number of myosin VIIa-positive cells

observed when 20 solid sphere cells and 20 hollow sphere cells were co-cultured under adhesion culture conditions. I-L) When 20 solid sphere cells and 20 hollow sphere cells were cultured in separate Transwell chambers, few myosin VIIa-positive cells were observed in either chamber. E-H) Solid sphere cells cultured on a supporting layer of mouse embryonic fibroblasts (MEFs) produced only rare myosin VIIa-positive cells.

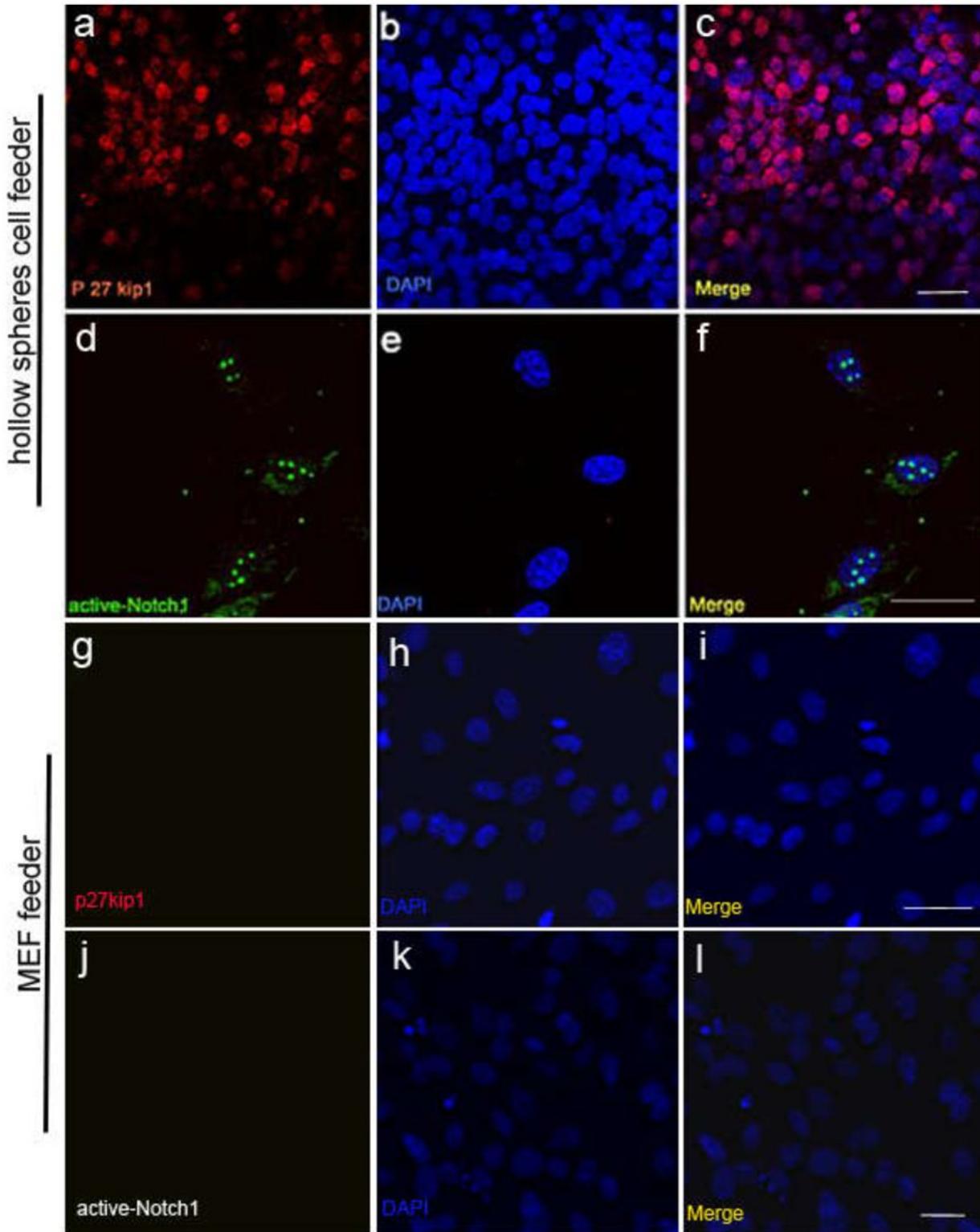


Figure 4

Hollow spheres cells and mouse embryonic fibroblasts (MEFs) were adhesion cultured and immunostained for p27kip1 and active-Notch1. Only hollow spheres cells expressed p27kip1 (a-c) and active-Notch1 (d-f). MEFs did not express not expression p27kip1 (g-i) or active-Notch1 (j-l). Bar = 20 μ m.

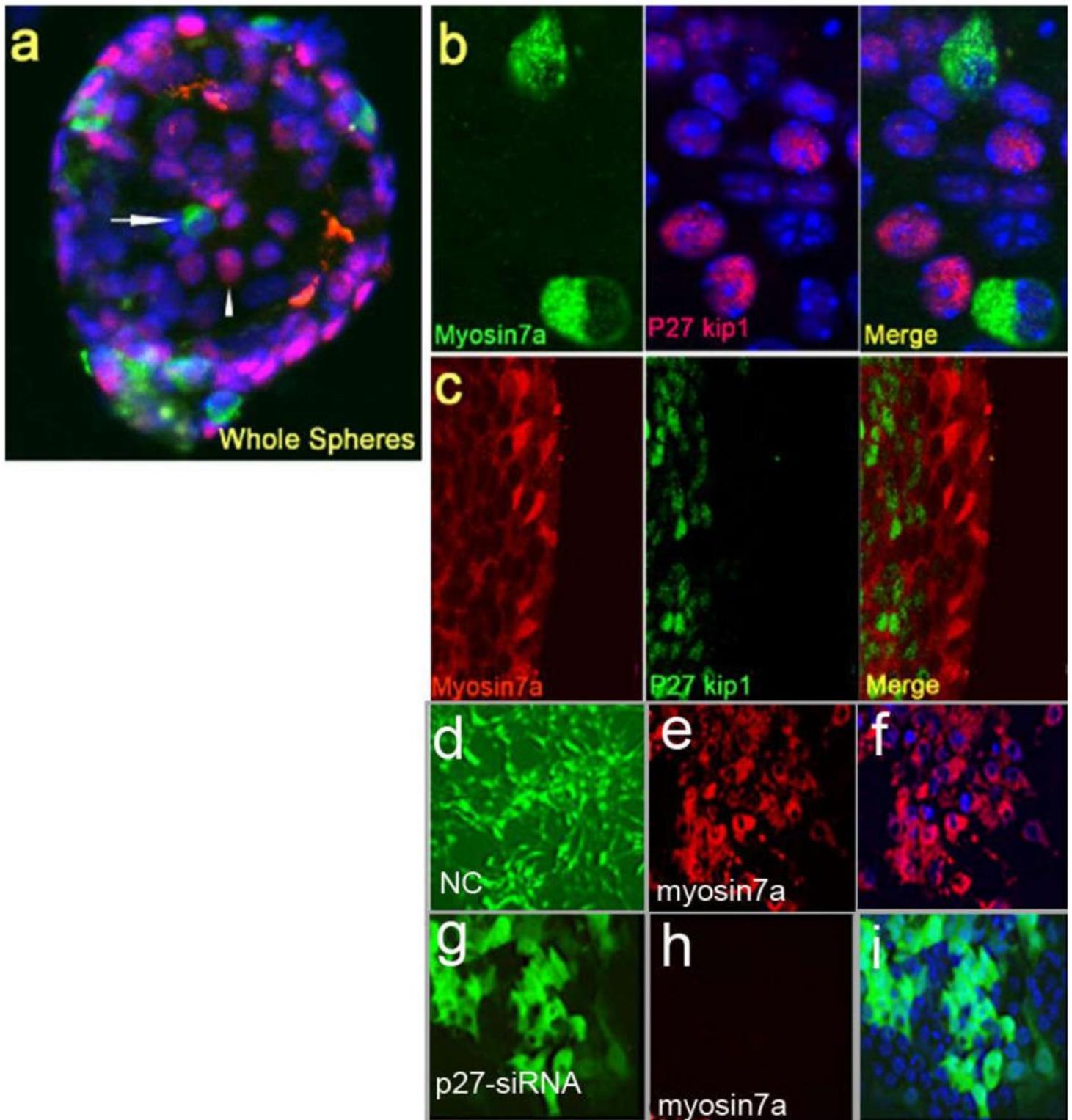


Figure 5

p27kip1-Positive cells are required for solid sphere cell differentiation. a-b) Transitional sphere cells were co-immunostained for p27kip1 and myosin VIIa. Myosin VIIa-positive cells were accompanied by 1 or 2

p27kip1-positive cells. c) Myosin VIIa-positive cells surrounded by several p27kip1-positive cells. d-i) Transfection of hollow sphere cells with adeno-associated virus (AAV) siRNA27 to knockdown the p27kip1 gene and co-culture with solid sphere cells. g-i) Solid sphere cell differentiation was not observed in the knockdown group. d-f) A large number of myosin VIIa-positive cells was noted in the control group (hollow sphere cells without p27kip1 knockdown).