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Inner Ear Hair Cells Produced In Vitro via a Mesenchymal-To-Epithelial Transition

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Abstract

Sensory hair cell loss is a major contributor to disabling hearing and balance deficits that affect >250 million people worldwide. Sound exposures, infections, drug toxicity, genetic disorders, and aging all can cause hair cell loss and lead to permanent sensory deficits. Progress toward treatments for these deficits has been limited, in part because hair cells have only been obtainable via microdissection of the anatomically complex internal ear. Attempts to produce hair cells in vitro have resulted in reports of some success, but have required transplantation into embryonic ears or co-culturing with other tissues. Here we show that avian inner ear cells can be cultured and passaged for months, frozen, and expanded to large numbers without other tissues. At any point from passage 6 up to at least passage 23, these cultures can be fully dissociated and then aggregated in suspension to induce a mesenchymal-to-epithelial transition that reliably yields new polarized sensory epithelia. Those epithelia develop numerous hair cells that are crowned by hair bundles, comprised of a single kinocilium and an asymmetric array of stereocilia. These hair cells exhibit rapid permeance to FM1-43, a dye that passes through open mechanotransducing channels. Since a vial of frozen cells can now provide the capacity to produce bona fide hair cells completely in vitro, these discoveries should open new avenues of research that may ultimately contribute to better treatments for hearing loss and other inner ear disorders.

(233 words)

Introduction

Sensory hair cells, which are named for mechanotranducing hair bundles that extend into the endolymph of the internal ear, are essential for hearing and balance. When hair cells are lost through trauma, toxicity, infection, genetic disorders, or aging that can lead to impaired hearing or disruption of vestibular reflexes that normally stabilize gaze, posture, and locomotion (*1, 2*). Such deficits affect humans and other mammals permanently, but birds, fish, and amphibians are able to regenerate hair cells and recover sensory functions within weeks. In those species, supporting cells, which reside between neighboring hair cells, respond to the loss by dedifferentiating, dividing, and giving rise to progeny that can differentiate as self-renewing supporting cells and replacement hair cells (3-6).

Although the deficits caused by hair cell loss remain clinically irreversible, supporting cells in the balance organs of humans and rodents appear to hold the potential for regeneration, since some are able to respond to trauma by dividing (7). The incidence of vestibular supporting cell division is low in adult mammals (7, 8), but the majority of supporting cells in the balance organs of young mammals enter S-phase and proliferate when cultured with appropriate pharmacological agents (9-13).

In mammals, vestibular supporting cell responses to mitogens decline dramatically during the first postnatal weeks and that decline appears to be responsible for the mammalian ear's unique vulnerability to permanent hair cell loss (13). In vitro pharmacological treatments that help to restore proliferation in mature mammalian vestibular epithelia have recently been identified (13-15), but the achievement of effective regeneration in mammalian ears is likely to depend in part on discovering how hair cell differentiation is controlled.

The progeny of supporting cell divisions and the progeny of a recently discovered stem cell are important sources of newly differentiated hair cells in non-mammalian regeneration (16, 17), and there is evidence that supporting cells sometimes convert directly into hair cells without an immediately preceding cell division (18). The basic HLH gene Atoh1 is required for the embryonic differentiation of hair cells, and forced expression of Atoh1 induces the formation of ectopic hair cells in organ culture (19-21). In fact, functional auditory recovery in adult guinea pigs has been attributed to viral delivery of Atoh1 (22), but much remains to be learned about how cells are guided to differentiate as hair cells.

A number of cell types have been investigated for their potential to begin differentiating toward a hair cell phenotype. Cultures of conditionally immortalized cells (23, 24), primary cells dissected from the ears of mice (25, 26), murine ES cells (27), and olfactory precursor cells (28) all have been found to contain some cells that express hair cell proteins. However, the formation of hair bundles, characteristic hair cell bodies, and other more convincing indications of hair cell differentiation are reported to require transplantation of cultured cells into the ears of embryos (27) or co-culturing with other tissues from the developing head (26).

Since avian vestibular organs produce hair cells throughout life (29-31), we investigated the differentiation of hair cells by culturing cells from the utricular sensory epithelium of chicken embryos, greatly expanding their numbers, freezing, thawing, and passaging them during several weeks and months of culture. Then we experimented with advanced-passage cells taken from these cultures at various times and discovered that a mesenchymal-to-epithelial transition (MET) can lead to the differentiation of bona fide hair cells completely in vitro.

Results

Production of pure utricular cell cultures

To avoid potential contamination by inadvertent inclusion of cells from the non-sensory epithelium and the underlying stroma, we followed three procedural steps when isolating pure sheets of hair cell epithelium, and only cultured the cells from the central part of the sensory epithelium (see Methods). We pooled those cells from 16 ears, and used trypsin and gentle trituration to produce partially-dissociated primary sensory epithelium cultures (Fig. 1A). On different days we repeated all of the steps to establish four independently-derived lines of cells for use in parallel testing to make certain that the results could be consistently replicated.

To expand the number of proliferating cells and eliminate pre-existing, post-mitotic hair cells, we passaged the cells for several weeks and monitored the progressive loss of hair cells. The primary cultures (designated P0) contained many solitary cells, but also small epithelial islands comprised of pre-existing hair cells and supporting cells, which were visibly linked by epithelial junctions. The supporting cells that grew in isolation as individual cells and those that were near the edges of the epithelial islands were the first to flatten and spread as thin polygonal shaped cells (13-15), followed in turn by the remaining supporting cells within those islands. In contrast to the supporting cells, the hair cells remained essentially cylindrical and were readily identifiable by virtue of their circular apical surfaces and hair bundles. Each week we passaged the cells, counting and progressively expanding the cultures into larger wells, then into flasks in subsequent passages, identified here as P1 through P23 (Fig S1). We froze all of the cells at passage 5 through passage 7 and periodically thawed and cultured cells from those stored vials,

continuously passaging some cells from each of the four original cultures and freezing samples at later passages.

By the end of the first three passages, the cells in the epithelial islands had become completely disaggregated, >95% grew as solitary cells (Fig. 1B, S1), with the remaining cells growing in small clumps that lacked epithelial junctions (Fig. S1). After 3 to 4 weeks in 2-D culture, pre-existing hair cells and the hair cell markers myosin VIIa and calretinin were no longer detectable in the cultures (Fig. 1C, D). These cultured cell populations doubled every 118 ± 18 hrs (mean ± standard deviation) during at least 19 passages in which cell numbers were quantified. The cells in these cultures incorporated Bromo-deoxy-uridine (BrdU) as they entered S-phase and divided to produce millions of cells more than were in the primary cultures (Fig. 1E-G, S1).

An epithelial-to-mesenchymal transition (EMT) occurred in the 2-D cultures.

The disaggregation of the epithelial islands coincided with the progressive loss of epithelial junctions and the loss of E- and N-cadherin expression (Fig. 2A, B). Like other dissociated epithelial cells these transitioned to a mesenchymal phenotype and grew as solitary cells when cultured on a solid substrate (32). F-actin was organized in stress fibers in these cells (Fig. 1D, 2), and they expressed the mesenchymal intermediate filament, vimentin (Fig. 2C), and the mesenchymal transcription factor, slug (Fig. 2D). Vimentin and slug were not expressed in the hair cell epithelium in vivo (Fig. 2C, D).

Suspension culture led to a mesenchymal-to-epithelial transition (MET).

Reasoning that differentiation and the establishment of cell polarity would depend on the formation of epithelial junctions, we attempted to induce junction formation by growing cells to 100% confluence, but that failed to produce any detectable signs of E-cadherin, N-

cadherin, myosin VIIa, or calretinin expression, and the F-actin did not form cortical bands (Fig. 2). We suspected that adhesion to the rigid 2-D substrate was preventing epithelial junction formation by supplying signals that overwhelmingly promoted cell spreading and the retention of the mesenchymal phenotype (Fig. 3A). To test this hypothesis, we dissociated cells that had been passaged at least 6 times (~ 6 weeks), and cultured them in suspension with gentle agitation in serum-free medium containing N2, B27, and FGF2. Later, we achieved similar, but more consistent results by suspending the cells in hanging drops of that medium. At the start, all these suspension cultures contained well over 95% individual, fully dissociated cells (Fig. 3B). Within 8 hours, both suspension methods resulted in the progressive aggregation of the cells into small clusters (Fig. 3C). After 1-2 days, 85-95% of the suspensions contained larger aggregates that formed when the small clusters adhered to each other and coalesced (Fig. 3D-F). By 4-6 days, >50% of the now spherical aggregates developed lumens (Fig. 3G), and by day 8, these hollow spheres averaged 238 ± 51 μm in diameter and their irregular surfaces had become smooth as they passed through changes that resembled embryonic compaction (Fig. 3H, I).

The aggregation of the cells in suspension culture led to a decline in the rate of proliferation from that measured in parallel 2-D cultures (Fig. 1E-G), and it induced the mesenchymal-to-epithelial transition we sought (Fig. 2A-D, 3A-I). After the solitary cells aggregated in suspension they formed cell-cell adhesions, and developed the epithelial junctions required for apical-basal polarity and the development of asymmetric distributions of proteins into apical and basolateral domains (33). After 6-8 days, the cells in these spherical aggregates expressed E- and N-cadherin (Fig. 2A, B) and no longer expressed detectable levels of vimentin and slug (Fig. 2C, D). Their actin filaments

became organized in cortical bands juxtaposed to apical epithelial junctions that were readily identifiable in light and electron microscopy (Fig. 2, 3H, I, 4A, G), and laminin was expressed at the inner, basal surface of the spherical epithelia (Fig. 3J).

Hair cells differentiation followed the mesenchymal-to-epithelial transition.

As early as the sixth day after suspension, some of the spheres began to develop cells that expressed the hair cell markers myosinVIIa (Fig. 3I, 4A, G, S2), calretinin, parvalbumin 3, and otoferlin (HCS-1, Fig. S2). Two days later, clearly recognizable hair cell bundles crowned the apical surfaces of 24 ± 19 cells/sphere (mean ± standard deviation, representing 3% ± 2% of the cells in each sphere), which also expressed pairs of the hair cell markers. Four days later, the spheres averaged 113 ± 44 hair cells (mean ± standard deviation, or 15% ± 6% of the cells in each sphere) with those characteristics (Fig. 1E, n = 6 spheres/time point). In each case, the hair bundles and apical surfaces of the hair cells projected outward toward the medium surrounding the sphere, rather than into the sphere's lumen (Fig. 3I, 4B, C). Confocal microscopy (Fig. 3I) and both scanning (Fig. 4D) and transmission electronic microscopy (Fig. 4E, F) showed individual hair cells and regions that contained small groups of hair cells that alternated with the other epithelial cells in mosaic patterns (Fig. 3I, 4A, G). The supporting cell marker SCA was expressed in other epithelial cells of the spheres (Fig 3K, L) (34).

The hair bundles that developed on these polarized hair cells were comprised of actin-filled stereocilia and a single eccentrically positioned kinocilium that labeled with anti-acetylated tubulin (Fig. 4 F, H). They also labeled with HCA, an antibody to a 275 kDa antigen specific to hair cell bundles (Fig. 4I) (35). Hair cell bundles developed in spheres made from cell cultures maintained to at least passage 23.

FM1-43 permeates the hair cells rapidly and selectively.

By virtue of its rapid permeation through mechanosensory transduction channels that are in the open state (36, 37), the cationic, styryl pyridinium dye N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridiniumdibromide (FM1-43) provides a visual assay for ion channel function in hair cells and has been used to identify the onset of transduction channel function in developing hair cells (38, 39). To test for the possibility that the hair cells might express functional mechanotransduction channels, we briefly incubated spheres produced from the 15th passage of one cell line and others from the 19th passage of another line with a fixable form of FM1-43. Ten seconds of incubation in 5 μM FM1-43 that was followed by 5 minutes of thorough rinsing in DMEM/F12 labeled just the hair cells in those spheres (Fig. 4J-L) and incubation in 1 mM gentamicin blocked the permeation of FM1-43 reversibly. Using DIC microscopy after a brief exposure to FM1-43, we identified 25 hair bundles in optical sections of 5 living spheres. All 25 of those hair cells were labeled by FM1-43 (Fig 4J-L). While dye permeation experiments cannot take the place of electrophysiological investigations for establishing the functional status of the hair cells, the evidence from these FM1-43 permeation experiments is consistent with the possibility that the new hair cells contain open and potentially functional mechanotransduction channels.

Discussion

We have isolated and investigated cells from the utricular hair cell epithelium of E14 chicken embryos. In 2-D culture these cells grew for months, with ~5-day population doublings. By the end of the third passage, the cells had lost their original epithelial characteristics as they completed an EMT. After the EMT, fully dissociated cell

suspensions were readily produced by trypsinization, and samples taken from passages 6 through 23 were aggregated under suspension culture conditions, which eliminated opportunities for adhesion to a substrate and thereby promoted cell-to-cell adhesion. Within days, small aggregates formed, adhered to each other, and coalesced as larger aggregates whose cells underwent a MET that reliably yielded hollow spheres of polarized epithelium. By six days after the change to suspension culture, the spheres began to develop hair cells that grew in number over the subsequent days. These cells expressed multiple hair cell proteins, developed normal hair cell shapes, and most significantly developed bona fide hair bundles and exhibited rapid permeance to a dye that enters open mechanotransduction channels.

The results of this study differ in several important ways from prior attempts to produce hair cells in vitro. The hair cells formed in our experiments were produced entirely in vitro from homogeneous, passaged lines of cells that were frozen, thawed, and greatly expanded during weeks and months of culture. The formation of these hair cells did not require transplantation into the ears of embryos (27) or co-culturing with other tissues from the head (26). These hair cells also were crowned by bona fide hair bundles, as confirmed by immunocytochemistry and ultrastructural examination. Our experiments also differed in regard to the procedures that we used.

The spheres described here were produced by methods that are distinctly different from those described in two prior investigations that sought to produce hair cells via suspension culture (25, 40). The cells that we used to produce spheres were from completely dissociated suspensions of cells that originated from cultures that had completed 8 to >20 population doublings while passaged on progressively larger 2-D

substrates for 6 to more than 20 weeks after the primary cultures were established. Rigorous procedural controls for distinguishing between pre-existing hair cells and the newly generated hair cells sought in this form of investigation are important, so we did not produce spheres directly from freshly dissected and dissociated sensory epithelia, to avoid the potential for carrying fragments of pre-existing hair cell epithelia over into such cultures where they would form spheres by adhesive resealing.

We confirmed that the epithelial junctions between the cells disappeared prior to Passage 6. After that, when grown on 2-D substrates new junctions failed to develop even at sites where the individual cells, which now exhibited mesenchymal phenotypes, eventually grew into contact with each other. In each of the experiments, other cells from the sampled suspension were returned to culture flasks for continuing expansion. Those cells and their progeny expressed mesenchymal phenotypes while they grew on the 2-D substrates of the flasks. When those cells and cells from passages to at least P23 were sampled and tested for their capacity to undergo a MET and form sensory epithelia in suspension they reliably produce sensory epithelia and hair cells.

These results have revealed that it is possible to produce bona fide hair cells completely in vitro without the need for transplantation of cells into embryos or co-culturing with other tissues from the developing head. They also have shown that supporting cells from the avian vestibular epithelium transition to a mesenchymal phenotype when passaged in cultures that provide a 2-D substrate for adhesion. Under those conditions the supporting cells quickly spread, changed from columnar to squamous, proliferated, progressively lost epithelial junctions, lost apical-basal polarity, and exhibited a mesenchymal phenotype. When those cells were fully dissociated and

then cultured in suspension so as to prevent adhesion to a substrate, cell-to-cell adhesion was promoted. The aggregated cells then transitioned from a relatively homogeneous mesenchymal phenotype to an epithelial phenotype and they formed spheres of sensory epithelia that developed hair cells which alternated in a mosaic pattern with cells that expressed a supporting cell marker.

It appears likely that a mesenchymal-to-epithelial transition may be fundamental to the in vitro generation of neuroepithelia from other organs, independent of whether the 2-D cultures are from mammalian or non-mammalian sources. The presence of a mesenchymal feeder layer has been shown to influence the differentiation of hair cell characteristics in mammalian experiments (26, 41), and we cannot rule out the possibility of species differences in hair cell differentiation requirements. Yet, our experiments raise the possibility that such mesenchymal co-culturing may have stimulated differentiation by virtue of the promotion of a mesenchymal-to-epithelial transition. In fact, we expect that cells cultured from the ears of embryonic mammals may be induced to generate hair cells by methods similar to those we have described above. In preliminary experiments we have observed that immortalized cells from the ears of wild type and H-2K^b-tsA58 transgenic mice will form spheres when re-aggregated after 2-D culture, but we have not observed hair bundle formation as yet.

In summary, lines of epithelial cells that undergo an epithelial-to-mesenchymal transition can be expanded in culture for months, will survive cryogenic storage, and can be used to produce hair cells through culture procedures that promote a mesenchymal-to-epithelial transition. The production of bona fide hair cells that develop hair bundles and normal characteristics while completely in vitro, without the need for specialized and

time-consuming microdissections of animal ears, and also without cell transplantation into embryonic ears or co-culturing with other tissues, should accelerate research and allow more effective screening of drug candidates for the treatment of hearing impairment, tinnitus, motion sickness, and other highly prevalent hearing and balance disorders.

Methods (Detailed methods are found in the Supporting Materials.)

Generation of the advanced-passage cell cultures

Generation of the primary cultures: We dissected utricles from embryonic day 14 white leghorn chicken embryos and used microdissection, followed by thermolysin digestion, followed by a second microdissection to isolate the pure central part of the hair cell epithelia. We pooled the pure sensory epithelium from 16 utricles and dissociated them in 0.05% trypsin-EDTA for 10 minutes at 37 ^{0}C followed by gentle trituation. After centrifugation, we produced a suspension of single cells with a few cell clumps. We plated ~8000 cells/cm^2 in 24-well plates in DMEM/F-12 with 10% FBS at 37^{0}C in a 5% CO_2 atmosphere. Four independent primary (passage 0) cell cultures were generated from 64 ears following these procedures.

Cell expansion: Cells were passaged when they reached ~70% confluence. We gradually expanded the cells into larger wells and flasks during subsequent passages up to passage 23.

Sphere generation

We used two methods to culture cells in liquid suspension beginning at passage 6. First, we cultured 500 µl suspensions of ~32,000 cells/ml in 24-well plates with gentle agitation in a serum-free medium containing DMEM/F-12, B27, N2 with FGF_2, and heparan sulfate

proteoglycan. More recently, we have cultured suspensions of 4000 cells in 20 μl hanging drops.

Proliferation assays

The expansion of cultures passaged in serum-containing medium was evaluated by counting cells using a hemocytometer. We determined the incidence of S-phase entry in serum-free cultures by adding 3 μg/ml BrdU, and compared the incidence of S-phase entry in 2-D cultures and suspension cultures. BrdU incorporation was detected using anti-BrdU immunocytochemistry and unlabeled DNA was counterstained with DAPI. The BrdU-positive and DAPI-stained nuclei were counted using MetaMorph software or Volocity software after complete 3-D reconstruction from the confocal images of the spheres.

Assays for mesenchymal and epithelial phenotypes

To assay for and localize cells that had adopted a mesenchymal cell phenotype, we used antibodies against the mesenchymal intermediate filament, vimentin, and the mesenchymal zinc finger transcriptional factor, *slug*, and fluorescent phalloidin labeling of F-actin organized in stress fibers. To assay for cells that had adopted an epithelial phenotype, we used antibodies against E-cadherin and N-cadherin proteins localized to adherens junctions and fluorescent phalloidin labeling of F-actin organized in cortical bands juxtaposed to epithelial cell-cell junctions.

Hair cell differentiation assay

We used the following hair cell markers in immunocytochemical assays for hair cell differentiation: anti-myosin VIIa, anti-calretinin, anti-parvalbumin3, and anti-HCS-1/otoferlin.

Hair cell bundle identification

We used scanning electron microscopy, transmission electron microscopy, immunocytochemical labeling of the kinocilium (anti-acetylated tubulin) and HCA (antibody specific to a 275 kDa antigen of the avian hair bundle), and fluorescent phalloidin-labeling of stereocilia F-actin to identify the newly formed hair bundles.

Assay for potential mechanotransduction channel function

We used FM1-43FX permeation and gentamicin block and recovery to investigate the potential for mechanotransduction channel function in the newly generated hair cells.

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Figure legends

Figure 1. Advanced-passage cultures from pure hair cell epithelia.

(A) A schematic diagram showing how inner ear hair cells were produced in vitro. (B) Cells cultured from E14 chicken embryo hair cell epithelia changed from columnar to flattened polygonal shapes as illustrated in this scanning electron micrograph of a cell from passage 3. (C-D) By passage 3, pre-existing hair cells and the hair cell markers myosin VIIa and calretinin were no longer detectable in the 2-D cultures and phalloidin-labeled F-actin became organized in stress fibers in the cultured cells. (E-G) Cells in 2-D cultures proliferated and continued to incorporate BrdU during 24-hr labeling periods that preceded fixations at different stages (E, solid dark line in G), but the incidence of proliferation decreased after cells were suspended and formed spheres (F, dashed dark line in G, mean ± standard deviation). Hair cells differentiated as early as the sixth day after suspension and increased in number for at least 6 days more (blue line in G). Scale bars: 20 µm.

Figure 2. A mesenchymal-to-epithelial transition leads to hair cell differentiation.

(a-c in A-D) The utricle sensory epithelium fixed in situ. (d-f in A-D) Fixed 2-D cell cultures. (g-j in A-D) spheres of sensory epithelium produced by dissociation and re-aggregation of cells from 2-D cultures under conditions that prevented adhesion to a substrate. Phalloidin labeled F-actin that was in cortical bands and hair bundles and the hair cells expressed myosin VIIa in the epithelia of the utricle and the spheres (in a and g in A, B, and D), while F-actin was organized as stress fibers and there was no expression of

myosin VIIa in the 2-D cultures (d in A-D). Likewise, E- and N-cadherin where expressed in the epithelia (b and h, in A-B) but absent from the cells cultured on 2-D substrates. The sensory epithelia also expressed the hair cell marker calretinin (b and h in C), but not the mesenchymal intermediate filament, vimentin, or the mesenchymal transcriptional factor, slug (b and h in D). Both vimentin (e in C) and slug (e in D) were expressed in the cells cultured and passaged in 2-D culture, but those mesenchymal proteins were not detectable in the epithelia that formed via re-aggregation (h in C and D). Scale bars: 10 µm.

Figure 3. Aggregation of late-passage cells led to formation of hollow smooth-surfaced spheres with apical structures pointing outward. (A) Advanced-passage cells growing in 2-D culture. (B) A fully dissociated sample of cells ten minutes after it was trypsinized from such a 2-D culture and placed in suspension. Cells progressively adhered to each other and progressively formed larger aggregates at 8, 16, and 32 hours after dissociation from the 2-D cultures and suspension in the serum-free medium. (F) The aggregates formed smooth spheres by the 8th day after suspension. (G) An optical section showing the hollow lumen of such a sphere and (H) a scanning electron micrograph showing the characteristic smooth surface of a sphere 8 days after suspension of the cells from an advanced-passage 2-D culture. (I) Phalloidin labeled F-actin in hair bundles projecting outward from cells that are also labeled by anti-myosinVIIa. F-actin juxtaposed to epithelial junctions is also visible in this 3-D reconstruction from confocal microscopy. (J) Laminin (arrows) is expressed in patches along the inner, basal epithelial surface of the sphere shown and was found in similar patches in 4 other spheres. Note the hair bundle extending from the sphere's outer surface (double arrow). Other cells in the epithelia of the spheres express the supporting

cell marker, SCA (in K and the merged image in L). Scale bar: 20 µm in A-F; 50 µm in G; 30 µm in H, I; 10 µm in J-L.

Figure 4. Bona fide hair cells form sensory hair bundles that project outward from the spheres. (A) Hair bundles on the apical surfaces of myosinVIIa-positive hair cells surrounded by epithelial junctions. (Arrowheads and arrows in B-C) Hair bundles at the surface of a living sphere visualized by DIC microscopy. (D) Another hair bundle visualized by scanning electron microscopy. The spheres in B-D were produced from cells that had been cultured through 19 passages. (E) Longitudinal and (F) transverse sections through hair bundles at the surfaces of spheres viewed by transmission EM. Note the characteristic eccentrically positioned kinocilium (arrow in F, which was observed in a fortuitous transverse section through one of the 18 hair bundles examined in the 5 spheres that were processed for transmission EM). (G) Phalloidin-labeled stereocilia on the surface of a myosin VIIa-positive hair cell and its neighbor, while F-actin outlined the surrounding columnar cells of the sphere's wall. (H) Anti-acetylated tubulin labeled a single kinocilium extending above the phalloidin labeled hair bundles on at least eight of the 21 myosin VIIa-positive hair cells observed in limited examinations of the 5 spheres immunostained with that antibody. (I) Hair bundle double labeled with phalloidin and antibody to the 275 kDa hair cell antigen (HCA). (J-L) In optical assays for open mechanotransduction channels, FM1-43 permeated hair cells after 10 second exposures to the dye that were followed by thorough rinsing, but no other cells in the spheres were labeled. The spheres and hair cells in this figure were all derived from cultures that had been frozen at passage 5, then thawed and continuously cultured until passage 19, when they were dissociated and re-aggregated in suspension. Scale bar: 10 µm in A, C, G-L; 50 µm in B; 1 µm in D-F.